Antimicrobial Properties of Wound Dressings

Louise Ellen Freeman-Parry

A thesis submitted in partial fulfilment of the requirements

of Sheffield Hallam University

for the Degree of Doctor of Philosophy

May 2020

Candidate Declaration

I hereby declare that:

1. I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my research degree.

2. None of the material contained in the thesis has been used in any other submission for an academic award.

3. I am aware of and understand the University's policy on plagiarism and certify that this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged.

   The development of the antimicrobial material was undertaken in collaboration with colleagues in the Materials Engineering Research Institute, SHU. The preparation of different formulations and the analysis of the composition of the final composite has been acknowledged and all diagrams attributed to colleagues in MERI in the relevant chapters. The involvement of external partners in the scale up of the material, its sterilisation and the development of potential secondary dressings has been acknowledged.

   I have played a major role in the development of the material and undertaken the antimicrobial testing of the materials. Contributions made by other students to certain experiments have been undertaken under my advice and direction. All contributions have been explicitly referenced in the text and figure legends.

4 The work undertaken towards the thesis has been conducted in accordance with the SHU Principles of Integrity in Research and the SHU Research Ethics Policy.

5 The word count of the thesis is 58000.

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l) Abstract

Wound infections are a very common healthcare problem and can lead to significant mortality and morbidity. In the UK there are four classes of antimicrobial dressings, honey, silver, iodine and biguanides, available to treat infected wounds. The evidence to support the efficacy of these materials is often scarce, as they are classed as medical devices and as such are not required to undertake the rigorous clinical evaluations expected for medicinal products. This research was designed to investigate the antimicrobial properties of wound dressings and to determine if bacterial wound pathogens are more susceptible to a particular class of antimicrobial wound dressing. The primary aim of the research was to develop a novel wound dressing which could deliver a controlled release of an antimicrobial agent over a sustained period of time.

The *in vitro* antimicrobial activity of 20 antimicrobial dressings were tested against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa,* bacteria commonly found in chronic wounds. Whilst most of the dressings demonstrated a degree of antimicrobial efficacy against all of the bacteria, their duration of antimicrobial action was limited. Only the silver dressings were shown to be effective for more than 48 hours.

A new material was created by the addition of clay platelets to a composite containing polyvinyl alcohol [PVOH] and polyvinylpyrrolidine [PVP] containing 6wt% iodine. The material was effective against the test bacteria for at least 72 hours and was also able to eradicate *in- vitro* biofilms containing *S. aureus* and *E. coli*. The novel material formed a self-stratifying film and both sides of the material demonstrated different release characteristics. One side of the material was still effective against *S. aureus* after eight days continuous use. The new material has been shown to be significantly different to any of the commercial dressings tested. The unique two side release profile, absorbent wicking properties and the colour change indicator make this material different to any of the dressings currently available in the UK and provides a novel material for the treatment of infected wounds.

II) Dedications

There are a number of people to whom I dedicate this thesis:

Firstly, my grandfather, Gwilym Orin Freeman-Parry Snr. who developed a venous leg ulcer which was treated with daily salt water baths and eventually became infected with what was described as "gas gangrene" and required a below knee amputation. He was convinced that if he had treated his wound with Germoline which his father had done when he had developed a leg ulcer, that he could have saved his limb. My grandfather encouraged me to become a pharmacist and his experience developed my interest in wound care and the use of antiseptics.

Secondly my dear late father, Gwilym Orin Freeman-Parry Jnr. who instilled in me the love of science and in particular his subject Biology and who supported me through my A levels and pharmacy studies at University.

2018 was a particularly sad year with the loss of two very important people.

Professor Chris Breen, who developed our novel wound dressing sadly passed on 12th February. Having worked with Chris from the start of my PhD I have learned so much from him, not only about materials science, but about scientific research in general. He was an inspirational scientist and mentor and is dearly missed.

Also, my beloved mother, Judith Ann Freeman-Parry, died on February 14th having just been diagnosed with a venous leg ulcer which was infected with *P. aeruginosa*. Unfortunately, the management of her ulcer, despite all the available modern treatments was not much better than my grandfather's treatment 30 years earlier. One of the last things she said to me was " Have you still not finished your PhD?". I promised her the weekend before she died that it would be submitted, unfortunately now she was not able to see the completed work.

These four people, in different ways, have motivated me to complete this thesis but I could not have done this without the support and encouragement of my family. So, I also dedicate this thesis to my beloved husband, Roger William Brookes, my wonderful children, Robert William Emyr Wynn Brookes and

Kathryn Louise Angharad Brookes and my dear brother, Robert William Freeman-Parry.

Finally, this thesis is also dedicated to all the patients I helped to manage throughout my career as a hospital pharmacist and my hope is that some of the work undertaken during this research will in some way help to alleviate the suffering of patients with infected wounds.

III) Acknowledgements

First, I would like to thank my Director of Studies, Dr Keith Miller, for taking on the poison chalice of supervising me, a work colleague. Without Keith's enthusiasm for this research and his continuous encouragement, support and guidance this thesis would never have been written. Also, my second Supervisor, Professor Peter Strong has been a constant source of wisdom and has guided me through this challenging adventure. I am indebted to both Keith and Peter for being inspirational supervisors.

I would also like to thank Professor Nicola Woodroofe who listened to my ideas for this research and gave me the opportunity to undertake my PhD in the Biomolecular Sciences Research Centre.

This research would never have occurred had it not been for a conversation with Professor Neil Bricklebank about iodine dressings. Neil's interest in iodine chemistry led to the initial discussions with Professor Chris Breen in the Materials Engineering Research Institute and the collaboration which ultimately produced the CROWD material. Neil has given me such a wonderful insight into the world of research, and I wish to thank him for taking on my ideas and guiding the project to such a successful conclusion.

I have to say a huge thank you to Professor Chris Breen and Dr Francis Clegg the material scientists in the IN-CROWD team. Sadly, we lost Chris in 2018 but his memory will live on with the development of this work. Chris was an incredible research scientist and provided me with an excellent role model. I really miss our team meetings and will particularly remember him for his kindness and amazing sense of humour. I am also indebted to Francis for

preparing the CROWD material and all his help and advice over the past ten years. In particular, thank you for patiently explaining the intricacies of materials science. I also need to thank Dr Marriane Labet and Dr Lukáš Petra, Post-Doctoral researchers in MERI for their involvement in making the test dressing samples and Dr David Hogg at Rejuvetech for manufacturing the final material.

Dr Patrick Harrison deserves a special thank you, not only for his technical support during the project, but for also for being a motivational fellow PhD student who has encouraged me to keep on writing.

A part of this research has been the investigation into the commercialisation of the material, and I have to thank, Steve Cotton, Managing Director (Brightwake) for his interest in this project and for having our material commercially sterilised and for making the prototype dressings. I would also like to thank James Pink (NSF International) for introducing me to Steve and for sharing his extensive knowledge of medical device legislation.

I would also like to acknowledge Dr Joey Sheppard (University of Sheffield) and Dr Amanda Harvey (BMRC) for sharing their expertise in tissue engineering. Thank you to Dr Lucy Crookes and Dr Florian Wulfert for their statistical advice.

There are numerous people who have contributed to my research in various ways. I would like to thank all my colleagues in the Department of Biosciences and Chemistry for their continued support throughout the 10 years of my studies.

Finally, I would like to thank my family for looking after me, particularly during the last few months of writing; I could not have done this without you.

IV) Candidates Statement: Louise Ellen Freeman-Parry 2021

This thesis was developed from an interest in wound management which started during my career as a hospital pharmacist. The research was designed to try to identify if there was a difference in the antimicrobial efficacy of different wound dressings that are available to in the UK to treat infected wounds. The aim of the research was to develop a novel wound dressing which could deliver a controlled release of an antimicrobial agent over a sustained period of time.

The creation of the CROWD material involved collaboration between scientists in the Biomolecular Sciences Research Centre (BMRC) and Materials Engineering Research Institute (MERI). The prototype materials were developed by Professor Chris Breen and Dr Francis Clegg (MERI). Antimicrobial testing was undertaken by Louise Freeman-Parry.

Other material science contributions -

Dr Marianne Labet (Post Doctoral Researcher, MERI) Sample preparation, Chapter 4.

Dr Francis Clegg (MERI) XRF Analysis of Iodine release, Chapter 4

Dr Lukáš Petra (Post Doctoral Researcher, MERI) Sample preparation and the effect of hydration on the CROWD composite, Chapter 6.

Dr David Hogg (Rejuvetech) Large scale preparation of the material, Chapter 6.

Mr Steve Cotton (Brightwake) sterilisation of the material and preparation of prototype dressings, Chapter 6.

The following experiments were conducted under the supervision of and according to protocols devised by Louise Freeman-Parry.

Aneesah Mahmood (BSc Biomedical Science student, SHU 2018) Biofilm testing, Chapter 5.

Dr Patrick Harrison (Post Doctoral Researcher, BMRC) technical support for cytotoxicity testing, Chapter 5 and antimicrobial testing, Chapter 6.

Statistical advice was taken from Dr Florian Wulfert.

V) Presentations, Publications and Grants Awarded

Presentation of Research

Date	Venue	Presentation
16.12.2010	BMRC Christmas poster session, SHU.	PhD poster presentation – Antimicrobial properties of wound dressings
12.01.2011	EfL Annual conference Sheffield institute of Arts Gallery, SHU.	CROWD Poster presentation
22.02.2011	BMRC Bioscience Forum, SHU.	First year PhD Oral presentation – Antimicrobial properties of wound dressings
05.05.2011	Health Services Research and Pharmacy Practice Conference. The University of East Anglia.	PhD Oral presentation – PhD Forum – Abstract 46
30.06.2011	Faculty of Health and Wellbeing (HWB) Research Day, SHU	Crowd Oral presentation
20.07.2011	EfL meeting Manchester Metropolitan University	CROWD Oral presentation
26.10.2011	EfL Research Café Showroom Cinema Sheffield	CROWD Oral presentation
16.12.2011	BMRC and MERI Winter Poster Event. SHU	CROWD Poster presentation
09.07.2012	BMRC PhD Research conference, SHU	CROWD Oral presentation
Sep 2012	Mumbai, Delhi and Pune, India	Lecture Tour – Antimicrobial wound Dressings
07.11.2012	BMRC Biosciences Forum, SHU.	PhD Oral presentation
18.09.2013	BMRC Biosciences Forum, SHU.	Transfer Report and VIVA
28.11.2013	Antibiotic Resistance Mechanism Conference, Birmingham	PhD poster presentation – Antimicrobial Properties of Wound Dressings
17.12.2013	BMRC and MERI Winter Poster Event, SHU.	CROWD Poster presentation
13.01.2014	Proof of Concept Funding, SHU.	IN-CROWD Oral presentation
19.03.2014	SHU Imagine Funding	OCIB Oral presentation
24.06.2014	HWB Faculty Research Day, SHU	IN-CROWD Oral presentation
27.11.2014	Antibiotic Resistance Mechanism Conference, Birmingham	CROWD Poster presentation
04.02.2015	Department of Biosciences and	Open lecture for all students and staff in department CROWD Research lecture

	Chemistry Research Seminar, SHU	
05.03.2015	SHU Commercialisation Oversight Working Group	Further technical development of IN-CROWD presentation
21.05.2015	SCI Fest, SHU	CROWD Poster presentation
17.06.2015	HWB Faculty Research Day, SHU.	CROWD Oral presentation
15.03.2016	Vietnam National University HCMC – University of sciences	"Fighting resistant Bacteria – Bursting Biofilm Bubbles" lecture.
16.03.2016	Ton Duc Thang University HCME Vietnam	"Fighting resistant Bacteria – Bursting Biofilm Bubbles" lecture.
17.03.2016	VNU University of Sciences, Ha Noi Vietnam	"Fighting resistant Bacteria – Bursting Biofilm Bubbles" lecture.
12.05.2016	European Wound Management Association Conference, Bremen, Germany	CROWD eposter and Oral presentation
13.07.2016	BMRC Biosciences SHU	RF2 talk
03.08.2016	Mercia Technologies RIO, SHU	The IN-CROWD Project presentation
07.09.2016	Microbiology Team Meeting, SHU	PhD Update Talk
21.02.2017	University of Ghana School of Health Sciences	Antimicrobial Wound dressings lecture
13.03.2017	HUTECH University Ho Chi Minh Vietnam	Antimicrobial Wound dressings lecture

Publications

Fowler Davis S, Cholerton R, Freeman-Parry L, Tsoneva J (2021) The Contribution of Pharmacists and Pharmacy Technicians to Person-Centred Care within a Medicine's Optimisation in Care Homes Service: A Qualitative Evaluation. *Pharmacy,* 9(1), 34; https://doi.org/10.3390/pharmacy9010034

Lewis E E L, Barrett MR, Freeman-Parry L, Bojar RA, Clench MR (2018) Examination of the skin barrier repair/wound healing process using a living skin equivalent (LSE) model and matrix-assisted laser desorption-ionization-mass spectrometry imaging (MALDI-MSI). *International Journal of Cosmetic Science,* 40 (2): 148-156.

Bradshaw CE (2011) An *in vitro* comparison of the antimicrobial activity of honey, iodine, and silver wound dressings. *Bioscience Horizons,* 4(1): 61-70.

(Undergraduate research project undertaken under the supervision of L Freeman-Parry)

Bath P, Dean J, Freeman-Parry L, Gandhi LT, Hutchinson A, Karnon J, Karsh BT, McIntosh A, Oakley J, Pratt P, Tappenden PT, Thomas N (2008) Modelling the expected net benefits of interventions to reduce the burden of medication errors. *J Health Serv Res Policy*, 13(2):85-91.

Publications in Preparation

1. An *in vitro* comparison of the duration of action of antimicrobial wound dressings.

2. An *in vitro* investigation of the antimicrobial properties of a novel slow release iodine containing clay composite material.

3. Development of a novel clay composite designed to deliver a slow release of antimicrobial iodine.

Grants Awarded

Engineering and Physical Sciences Research Council (EPSRC) Engineering for Life Grant (EfL) (2011-2012) £ 18,500

Project Title: CROWD: Controlled Release from Open Wound Dressings.

Outcome: Creation of a slow-release iodine containing antimicrobial material.

Sheffield Hallam Imagine Funding (2013- 2014) £20,000

Project Title: OCIB: Overcoming Connectivity in Biofilms.

Outcome: Development of a Biofilm model for testing antimicrobial wound dressings.

Sheffield Hallam University Proof of Concept Funding (2014 – 2015) £15,000

Project Title: IN-CROWD- Iodine Nanocomposites for Controlled Release of Open Wound Dressings.

Outcome: Upscale of manufacture of the material and work with a potential commercial partner to sterilise the material and investigate alternative formulations.

Medical Research Council (MRC) Confidence in Concept Round 4 Grant (2016 – 2017) £43,157

Project Title: IN-CROWD Cytotoxicity Screening and Efficacy Testing.

Outcome: Direct cytotoxicity testing of the new material compared to commercial dressings on a skin model in collaboration with University of Sheffield.

Patent Application Filed 13.3.2015: A Novel Antimicrobial Material

International Patent Application: No PCT/GB2016/050658

Sheffield Hallam University

Inventors: Chris Breen, Neil Bricklebank, Francis Clegg, Louise Freeman-Parry, Marianne Labet, Keith Miller.

Vl) Table of Contents

lX) Abbreviations

Acyl-HLS – Acylhomoserine Lactone

AMP - Antimicrobial Peptides

ANOVA - Analysis of Variance

ATCC - American Type Culture Collection

ATP – Adenosine Triphosphate

AWD- Antimicrobial Wound Dressing

AWM - Applied Wound Management

BBWC - Biofilm Based Wound Care

BC - Benzethonium Chloride

BMRC – Biomolecular Sciences Research Centre

BNF - British National Formulary

BRMS - Biological Response Modifiers

CDFF - Constant Depth Film Fermenter

CFU - Colony Forming Units

CMC - Carboxymethylcellulose

CNS – Coagulase –negative *Staphylococci*

CPS- Capsule Polysaccharide

CRE – Carbapenin Resistant *Enterobacteriacea*

CROWD - Controlled Release from Open Wound Dressing

CWPB - Chronic Wound Pathogenic Biofilms

DHA - Dihydroxyacetone

DMEM - Dulbecco's Modified Eagle's Medium

Da – Daltons

DNA – Deoxyribo Nucleic Acid

DVT - Deep Vein Thrombosis

EC_{50} – Half maximum Effective Concentration

EDTA - Ethylenediaminetetraacetic acid

EMRSA - Epidemic methicillin-resistant *Staphylococcus aureus*

EMT- Epithelial Mesenchymal Transmission

EPS - Extracellular Polymeric Substance

ESBL – Extended Spectrum Beta-Lactamase

EWMA - European Wound Management Association

FCS - Foetal Calf Serum

FD - Foam Dressing

FEP - Fluorinated Ethylene Propylene

FGF - Fibroblast Growth Factor

FHC - Fluid-Handling Capacity

HAI – Hospital Acquired Infection

HPA – Health Protection Agency

HFD - Hydrofibers

Hr - Hour

IWII - International Wound Infection Institute

LPS - Lipopolysaccharide

LSE - Living Skin Equivalent

MALD-MSI - Matrix-Assisted Laser Desorption-Mass Spectrometry Imaging

MBC - Minimum Bacterial Concentration

MBEC - Minimum Biofilm Eliminated Concentration

MDE – Multidrug-Resistant Enterobacteriacea

MDR – Multi Drug Resistant

MDT - Maggot Debridement Therapy

MERI - Materials Research Engineering Institute

MGO - Methyl glyoxal

MH - Manuka Honey

MIC - Minimum Inhibitory Concentration

Mm – milli metre

mM – milli Molar

MSSA - Methicillin - susceptible *Staphylococcus aureus*

MRSA - Methicillin - resistant *Staphylococcus aureus*

NaCL – Sodium Chloride

NICE - National Institute for Clinical Excellence

Nm – Nano metre

NPA - Non-Peroxide Activity

NPWT- Negative Pressure Wound Therapy

PBP – Penicillin Binding Protein

PCR – Polymerase Chain Reaction

PBS - Phosphate Buffered Saline

PDGF – Platelet Derived Growth Factor

PE - Phosphatidylethanolamine

PEG - Polyethylene Glycol

PHE - Public Health England

PHMB - Polyhexamethylene biguanide

PG - Phosphatidylglycerol

PMN - Polymorphonuclear Neutrophils

PTFE - Polytetrafluoroethylene

PVA - Polyvinyl Acetate

PVL – Panton-Valentine leukocidin

PVOH – Polyvinyl Alcohol

PVP – Poly (vinyl pyrrolidone)

PVP-I - Povidone Iodine

RCT - Randomized Controlled Trial

RNA – Ribo Nucleic Acid

rRNA – Ribosomal RNA

ROS – Reactive Oxygen Species

S - Standard Formulation

SD - Sulfadiazine

SEM – Scanning Electron Microscope

SHU - Sheffield Hallam University

SNK - Student -Newman- Keuls

SSD - Silver Sulfadiazine

SSI - Surgical Site Infection

SWF - Simulated Wound Fluid

TE - Tissue Engineering

TEM – Transmission Electron Microscopy

TGA – Thermogravimetric Analysis

TGF-β – Transforming Growth Factor Beta

THIN - The Health Improvement Network

TLC - Technology Lipido-Colloid

UK - United Kingdom

UMF - Unique Manuka Factor

UV - Ultraviolet

VAC - Vacuum-Assisted Closure

VLU - Venous Leg Ulcer

VRE - Vancomycin-resistant *enterococci*

WIC - Wound Infection Continuum

WHO - World Health Organisation

XRF – X-Ray Fluorescence

ZOI - Zone of Inhibition

α – alpha

β – beta

ɣ - gamma

Chapter 1: Introduction

1.1. Wounds, Wound infection and Wound Management

Wound infections are a common healthcare problem and can lead to significant mortality and morbidity. A wound is a breach in the epidermis or dermis due to trauma or physiological change which activates the repair process. Wounds can occur as part of a disease process or have an accidental or intentional aetiology. A wound will trigger a coordinated physiological response to provide haemostasis and start the healing processes of inflammation, proliferation and remodelling (Young and McNaught 2011).

Wounds can occur due to any damage to the skin and/or underlying tissues caused by an accident, act of violence, surgery or an underlying pathology. Wounds in which the skin or mucous membrane is broken are described as "open" and those that remain intact as "closed" (Chhabra et al. 2017). Wounds can be broadly divided into two main categories either acute or chronic.

Acute wounds heal within a predictable time frame. Wounds that fail to heal within six weeks of the initial injury are described as chronic and often have prolonged pathological inflammation (Zhao et al. 2016).

Chronic wounds usually occur because of an underlying pathophysiological abnormality. The most common chronic wounds include venous leg ulcers, diabetic foot ulcers and pressure ulcers which account for over 70% of chronic wounds in the UK (Hardwicke et al. 2008).

1.1.1. Pressure ulcers (PU)

Pressure ulcers affect patients who have reduced mobility, such as those confined to wheelchairs or hospital beds and can develop in a matter of hours in vulnerable patients. Pressure ulcers usually occur on bony prominences, in areas such as the buttocks, elbows and heels where the weight of the body produces external pressure and at a critical pressure above 33 mmHg arterial occlusion results in local tissue hypoxia, cell dysfunction and death (Agrawal and Chauhan 2012). Pressure ulcers are notoriously hard to manage and dress due to their locations and the depth of the wound cavities. The large areas of dead, necrotic tissue are a nutrient rich environment for microbial growth and

because of the reduced blood supply to the area the body's host defences are reduced making the patient very susceptible to clinical infection.

Ulcers affecting the lower limbs can have very different aetiologies and a differential diagnosis is crucial to ensure appropriate treatment.

1.1.2. Venous Leg Ulcers (VLU)

The most common type of leg ulcers are venous which account for 60-80% of cases (Singer 2017). The estimated prevalence in the UK is between 0.1-0.3% and this increases with age rising to 20 per 1000 in people over 80 years of age (SIGN 2010). The lifetime risk of developing a VLU is 1% (Lim 2018).

Venous ulcers occur in patients with venous valve incompetence, deep vein thrombosis (DVT) or calf muscle insufficiency which leads to venous stasis and sustained venous hypertension (Lim 2018; Wounds UK 2019). Venous ulceration develops due to a complex cascade of cellular and humeral events intiated by venous hypertension (O'Donnell and Passman 2014). This results in an increase in capillary permeability which leads to extravasation of fibrinogen from capillaries. Within the tissues soluble fibrinogen is converted into insoluble fibrin which forms a cuff around the blood vessels blocking the diffusion of nutrients. This has the effect of reducing the oxygen supply to the affected area leading to tissue ischaemia and ultimately tissue necrosis (Vasudevan 2014; Burnand et al. 1982). Venous hypertension has also been shown to activate white blood cells (Thomas, Nash and Dormandy 1988). The white blood cells adhere to the venous endothelium and migrate into and through the vein wall (Comerota and Lurie 2015). Coleridge and colleagues in 1988 suggested that white cell trapping occludes the capillaries and also leads to ischaemia of the skin.

Venous leg ulcers classically affect the skin around the medial malleolus at the ankle and then spread up the lower leg (Fonder et al. 2008; NICE 2019). Compression therapy is the first-line treatment for venous leg ulcers.

It increases venous and lymphatic return, which improves microcirculation thus reducing inflammation and oedema (Alavi et al. 2016; Franks et al. 2016). Between 30 – 75% of venous leg ulcers will heal after six months of compression therapy (Lim et al. 2018). Before starting compression therapy, the

ankle brachial pressure index (ABPI) should be measured to exclude arterial insufficiency. The ABPI measures the ratio of systolic blood pressure at the ankle to the arm with a normal value being 1. NICE (Oct 2020) advises that compression may be safely applied to most patients with an ABPI above 0.8 but should generally be avoided in patients with an ABPI between 0.5 and 0.8 and is contraindicated in patients with an ABPI less than 0.5.

1.1.3. Arterial Ulcers (AU)

Whilst most leg ulcers are due to venous disease a significant number, around 22% of patients have arterial insufficiency (Broderick, Pagnamenta and Forster 2020). Arterial ulcers manifest as a consequence of arterial insufficiency often caused by atherosclerosis, or less commonly thromboemboli or radiation damage (Bonham 2003). They occur in patients with conditions such as peripheral vascular disease or diabetes mellitus where the arteries are narrowed by atherosclerosis or occlusion. The ulcers develop when narrowing of the arterial lumen reduces perfusion to the affected areas leading to ischaemia and tissue hypoxia and ultimately cell death (Zhao et al. 2016). Arterial ulcers normally occur distally on bony prominances and treatment often requires restoration of the peripheral flow with angioplasty or reconstructive surgery (Grey, Harding and Enoch 2006). Compression therapy for arterial ulcers would be detrimental as it would further worsen the blood supply and can cause necrosis or even lead to amputation (Forster and Pagnamenta 2015).

1.1.4. Diabetic Foot Ulcers (DFU)

The number of people developing diabetes mellitus has increased dramatically over the last thirty years with an estimated 422 million adults worldwide living with the disease in 2014 (WHO 2016). Amongst these patients the incidence of diabetic foot ulcers has been reported to be between 4% and 10% (WUWHS 2016) with each diabetic patient having a one in four risk of developing a DFU during their lifetime (Armstrong et al. 2017).

DFU are complex chronic wounds which are associated with diabetic changes such as neuropathy and vascular disease (Wounds International 2013) and they have a major impact on the morbidity, mortality and quality of the patient's life.

There are three common classifications of DFU according to their aetiology (incidence in brackets); neuropathic (35%), ischaemic (15%) and neuroischaemic (50%) (Wounds International, Best Practice Guidelines 2013). DFUs are prone to infection which often leads to hospitalisation (Skrepnek et al. 2017) and not uncommonly amputation of the affected limb (WUWHS, 2016). Wu et al. (2007) estimated that around 50% of DFU develop an infection and of these 20% will lead to amputation.

One of the challenges in the management of DFUs is that due to arterial insufficiency and loss of sensation many diabetic patients do not demonstrate the classic signs of wound infection such as redness, heat, pain and swelling. In 2004 Edmonds et al. recommended that patients with DFU should be assessed for further signs of infection such exudate, malodour, wound undermining and friable granulation tissue. More recently a WIfI (Wound, Ischaemia, foot, infection) guide has been validated and adopted by the Society for Vascular Surgery (Mills et al. 2014) for use in both diabetic and non – diabetic patients to help identify the major factors that impact on the risk of amputation; tissue loss, ischaemia and infection.

1.2. Prevalence and Economic Burden of Wounds

Due to the diverse nature of wounds and the fact that patients are managed in a variety of different health care settings it is often difficult to determine the exact incidence of different types of wound within a large healthcare community.

A retrospective cohort analysis of records of patients in the UK was undertaken in 2015 by Guest at al. using The Health Improvement Network (THIN) data base to estimate the prevalence of wounds managed by the National Health Service (NHS) in 2012/2013. In the one-year study period it was estimated that the NHS had treated 2.2 million adult patients with a wound, which equated to about 4.5 % of the adult population of the UK at that time. The annual cost of managing the wounds and their associated co-morbidities was calculated to be £5.3 billion. Wound care products accounted for about 14% of the total cost (Table 1.1) (Guest et al. 2015; Guest et al. 2017). The figures in Table 1.1. illustrate that chronic wounds; leg ulcers (34%), pressure ulcers (7%) diabetic foot ulcers (8%) and open wounds (11%) accounted for 60% of the wounds

treated at a total cost of £3,432.9 million (65% of total expenditure) (Adapted from Guest et al. 2015 and Guest et al. 2017).

Table 1.1: Annual cost (£ million) of NHS resource to managing 2.2 million wounds and the associated comorbidities stratified by wound type.

Wound Type	Annual Number of wounds	Total Cost	Wound Care Products
Abscess	160,000 (7%)	£289.51	£42.18 (14.6%)
Burn	87,000 (4%)	£89.56	£9.63 (10.8%)
Diabetic foot Ulcer	169,000 (8%)	£554.14	£60.72 (10.96%)
Leg Ulcer (arterial)	9,000 (<1%)	£46.45	£7.19 (15.5%)
Leg Ulcer (mixed)	24,000 (1%)	£113.69	£25.63 (22.54%)
Leg Ulcer (unspecified)	420,000 (19%)	£836.62	£144.60 (17.28%)
Leg Ulcer (venous)	278,000 (13%)	£941.13	£168.08 (17.86%)
Open wound	240,000 (11%)	£409.73	£122.24 (29.83%)
Pressure Ulcer	153,000 (7%)	£531.14	£52.45 (9.87%)
Surgical wound	253,000 (11%)	£982.90	£55.26 (5.6%)
Trauma	158,000(7%)	£159.25	£10.02 (6.40%)
Unspecified	271,000(12%)	£363.62	£44.41 (12.21%)
Total	2,222,000 (100%)	£5317.72	£742.70 (13.97%)

A survey undertaken in primary care in the UK in 2013 (Dowsett et al. 2014) found that out of the 1174 wounds being treated 36.1% were lower limb wounds which included leg ulcers and diabetic foot ulcers in primary care. The same study determined that the incidence of pressure ulcers was 18.1% which was the same as the incidence reported by Ousey at al. (2013) in their survey of wound care in English trusts.

The prevalence of chronic wounds is predicted to rise because of the aging population and increased incidence of Type 2 diabetes. In February 2019 Diabetes.org.uk published new statistics stating that the number of people with diabetes in the UK had reached 4.7 million and that this number was expected to rise to 5.5 million by 2030 (Diabetes.org. 2019).

Also, the UK population is predicted to increase from 66.4 million, the estimated figure in 2018, to more than 70.1 million by 2029 (Office of National Statistics 2019). The number of people in the UK aged 65 and over increased in the 20-year period from 1998 when around one in six people were 65 years and over (15.9%), to one in every five people in 2018 (18.3%). This number is projected

to reach around one in every four people (24.2%) by 2038 (Office of National Statistics 2019).

As more people live longer there is likely to be a significant increase in the number of patients with chronic wounds and a corresponding rise in the cost of care (Posnett and Franks 2008).

In 2017 Guest et al. predicted that the annual incidence of acute, chronic and unspecified wounds was increasing at rates of 9%, 12% and 13% respectively, which suggests that future demands for wound care services will continue to rise and create a huge financial burden on increasingly scarce health care resources. Wounds are an international problem and access to treatment may be problematic, particularly in developing countries, where the supply of affordable dressings may be limited.

1.3. Wound Healing

Any trauma to the skin that penetrates the dermis will result in bleeding. This triggers the complex wound healing process which consists of four dynamic and overlapping phases: haemostasis, inflammation, proliferation and remodelling/maturation (Lindley, Stojadanovic and Pastar 2016).

1.3.1. Haemostasis (Day 1 following trauma)

Damaged blood vessels immediately constrict to reduce blood loss. The exposed sub endothelium, collagen and tissue factor activates platelet aggregation which results in degranulation and the formation of a fibrin-platelet clot to seal the damaged blood vessel (Gauglitz et al. 2011). Platelets have a key role in haemostasis and coagulation, are involved in assisting innate immunity and provide anti-infection activity by secreting a variety of biological response modifiers (BRMS) (Garraud, Hozzein and Badr 2017). They also support inflammation (Jenne and Kubes 2015) and the wound healing processes by secreting growth factors which stimulate the production of blood cells in bone marrow and chemokines which attract innate immune cells (Rossaint and Zarbock 2015) and cytokines involved in wound repair (Garraud, Hamzeh-Cognasse and Cognasse 2012).

1.3.2. Inflammation (Day 1-20)

About 10 minutes after the initial vasoconstriction, histamine released from mast cells causes vasodilation and creates pores in blood vessels leading to leakage of proteins and white blood cells into the damaged tissue (Noli and Miolo 2001).

White blood cells and platelets produce a variety of inflammatory cytokines such as interleukins (IL), tumour necrosis factor (THF)-α, platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β which attract other cells to the wounded area (Zhao et al. 2016). PDGF initiates the chemotaxis of neutrophils, monocytes, smooth muscle cells and fibroblasts (Diegelmann and Evans 2004).

The first white cells to reach the wound by emigration through the vessel walls are the Polymorphonuclear neutrophils (PMNs) (de Oliveira, Rosowski and Huttenlocher 2016). Neutrophils perform a variety of roles including the production of free radicals via the myeloperoxidase pathway to kill bacteria which they then phagocytose (Goldman 2004). They break down non-viable tissue by secreting proteolytic enzymes such as proteases and also matrix metalloproteinase (MMP) -2 and -9 which degrades collagen (Xue and Jackson 2013).

Around three days post injury; monocytes migrate into the wound and mature into pro-inflammatory macrophages (“M1” macrophages) which phagocytose bacteria, remove foreign debris, damaged matrix and bacteria – filled neutrophils (Diegelmann and Evans 2004). The collection of dead cells and fluid on the wound surface is described as slough.

As macrophages clear apoptotic cells, they undergo a phenotypic transition to reparative “M2” macrophages which promote anti-inflammatory effects and the migration and proliferation of fibroblasts, keratinocytes and endothelial cells to rebuild the dermis, epidermis and vasculature (Krzyszczyk et al. 2018). This initiates the transition to the proliferative phase of healing.

Normally the inflammatory phase resolves after one or two weeks (Nagaraja et al. 2014) however in the presence of external noxious stimulus causing tissue

damage, such as infection, inflammation can become prolonged and lead to a non-healing, chronic wound (Zhao et al. 2016).

1.3.3. Proliferation (Day 3 – 40)

During normal healing in this phase fibroblasts in the wound proliferate, migrate into the fibrin clot and produce new collagen and matrix proteins to produce granulation tissue. As inflammation reduces, reconstruction of the dermis starts, with endothelial cells and fibroblasts accumulating in the wound to synthesise granulation tissue to replace the original fibrin clot (Li, Chen and Kirsner 2007). Granulation tissue is made up of extracellular matrix (ECM) consisting of collagen, proteoglycans, hyaluronic acid and elastin produced by fibroblasts (Su et al. 2010). The collagen combines with the ground substance to create a scaffold for ongoing repair (Young and McNaught 2011).

Three to five days after tissue injury new blood vessels appear in the wound bed and form part of the granulation tissue (Tonnesen, Feng and Clark 2000). The angiogenesis in wounds is controlled by a number of factors including hypoxia, inflammation and the release of growth TGF-β, PDGF and FGF from platelets (Honnegowda et al. 2015). The new capillaries are fragile and permeable which lead to increased oedema in the area and the characteristic red colour of healthy granulation tissue.

When the wound cavity has been filled with granulation tissue, fibroblasts in response to cytokines such as TGF-β increase expression of α-smooth muscle actin (Darby, Skalli and Gabbiani 1990) and transform into a myofibroblast phenotype which develop pseudopodia to enable them to connect to fibronectin and collagen (Werner and Grose 2003). Myofibroblasts orientate linearly and stimulate smooth muscle cells to draw in the wound margin so reducing the wound size.

Re-epithelialisation occurs when keratinocytes and epithelial stem cells proliferate and migrate over the fibrin/ECM surface to produce a new layer of epidermal cells to cover the wound (Reinke and Sorg 2012). When the cells meet, they cease lateral proliferation due to contact inhibition but continue to regenerate radially to produce a multicellular layer to resurface the wound

(Yang and Weinberg 2008). Wounds with a very large surface area may require skin grafts to cover the defect (Braza and Fahrenkopf 2019).

1.3.4. Remodelling/Maturation (21 days – 2 years)

Maturation of the wound can take up to 2 years and is designed to strengthen the scar by remodelling and will at best reach about 70% of the tensile strength of unwounded skin (Stadelmann et al. 1998). During this phase fibroblasts upregulate the expression of stronger type I collagen and the original type III collagen is broken down by matrix metalloproteinases (MMPs) which control the degradation of the extracellular matrix (McCarty et al. 2012; Haukipuro et al. 1991).

This process is a tightly controlled balance between production of type I collagen and removal of type III collagen. Reorientation of type I collagen creates parallel bundles of collagen along tension lines which produces a higher tensile strength (Gurtner and Evans 2000). As the wound tissue matures, cellular activity in the wound area diminishes and the vascular network regresses (Gurtner et al. 2008). The cell rich granulation tissue is converted into a collagen filled, hypo cellular, avascular scar (Desmouliere, et al. 1995).

1.3.5. Factors Affecting Healing

Numerous factors can lead to impaired wound healing such as infection, increasing age, peripheral vascular disease, diabetes, immunocompromised conditions and various medications including glucocorticosteroids, non-steroidal anti-inflammatory drugs, antiplatelets, antineoplastics and vasoconstrictors such as β - blockers (Guo and DiPietro 2010).

Non - healing wounds have deviated from the normal path and tend to be stuck in one phase of the healing process, often the inflammatory phase (Frykberg and Banks 2015). To facilitate progression to the next stage of healing the underlying disease pathology needs to be managed and the conditions within the wound environment optimised to promote healing.

1.4. Wound Infection

Skin is a fundamental component of innate immunity and defends against infection by activation of humeral and cell-mediated immune responses (Hannigan and Grice 2013). As part of the defence against infection, skin cells produce antimicrobial peptides such a β- defensin-2 and cathelicidin (Nizet et al. (2001). Healthy skin is often colonized by microorganisms such as *Staphylococcus, Micrococcus, Corynebacterium, Propionibacterium, Acinetobacter* and *Dermabacter* (Percival et al. 2012). Any breach in the skin provides an opportunity for microorganisms to enter the damaged tissue.

Wound infection has been defined as "the invasion of a wound by proliferating microorganisms to a level that invokes a local and/or systemic response within the host" (IWII 2016: p6). Wound infection can occur in any type of wound and is one of the most frequent complications of non-healing wounds. Most chronic wounds involve extensive tissue damage, and the edges cannot be closed so the exposed subcutaneous tissue provides a moist, warm and nutritious environment for a wide variety of microorganisms to contaminate and colonise (Gardner and Frantz 2008).

Wound microorganisms are thought to originate from either endogenous sources such as skin flora, the environment; for example, entry through traumatic injury or evolve from endogenous sources such bacteria from the gastrointestinal or genitourinary tract (Bowler, Duerden and Armstrong 2001). Many acute wounds can heal despite the presence of microorganisms, and it has been suggested that healing can only occur when the bacterial load is $< 10^5$ Colony Forming Units (CFUs) per gram of tissue (Edwards and Harding 2004). In an open wound the presence of $> 1x10^5$ CFU of cultivable bacteria per gram of tissue (biopsies) has been used as an indication of clinical infection (Bowler 2003) and has been associated with tissue damage due to the release of inflammatory cytokines (Arya et al. 2014). However, many chronic wounds that have $<10^5$ CFU per gram of tissue do not show obvious signs of clinical infection but still fail to heal (Philips et al. 2008). Whilst the bioburden in the wound appears to influence healing, other factors such as the patient's immune system and other co-morbidities may also affect the healing rate (Tuttle 2015).

Failure to heal can also be affected by the type of microbial species present, their associated virulence factors (Lindsey, Oates and Bourdillon 2017) and polymicrobial diversity, where often four or more species are infecting the wound (Trengove et al. 1996).

In clinical practice the Wound Infection Continuum (Figure 1.1) has been used to describe the stages in the development of a wound infection and the impact the microorganisms have on the healing process (Schultz et al. 2003; Edwards and Harding 2004). The descriptions in the continuum were updated in 2016 to include all microorganisms, not just bacteria and to illustrate that microbial virulence as well as the number of microorganisms contributes to the development of wound infection (IWII 2016).

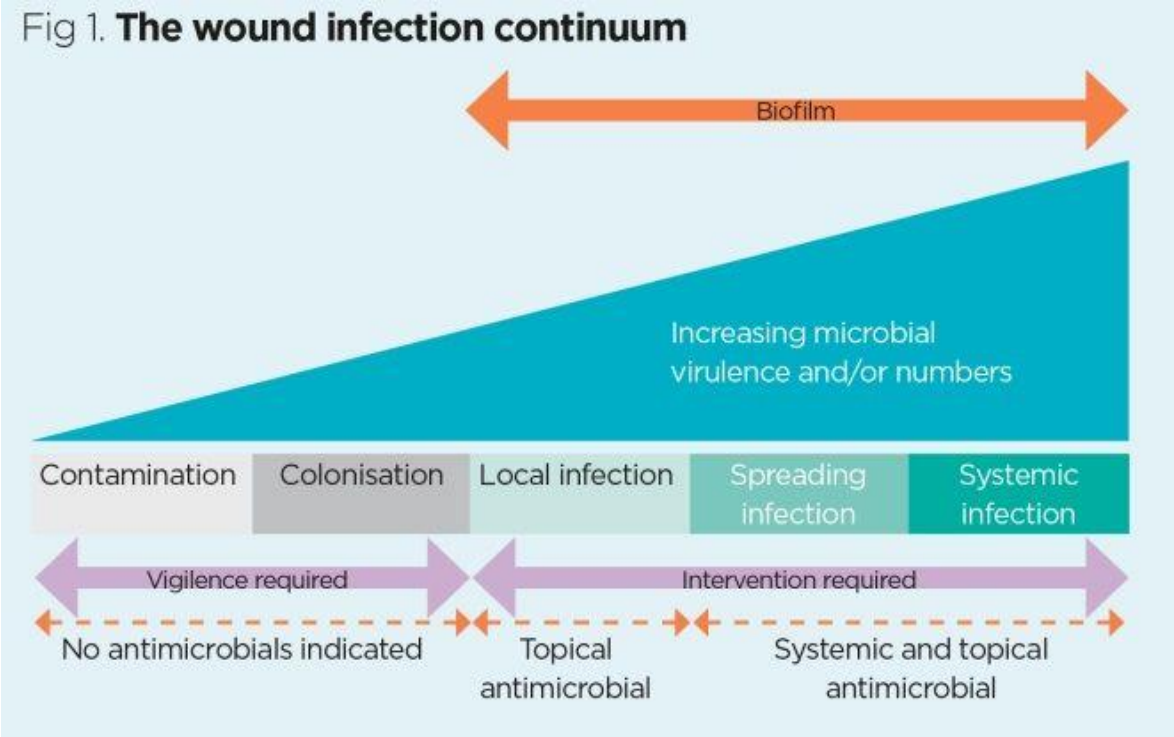


Figure 1.1: Wound Infection Continuum (International Wound Infection Institute, 2016) (Reproduced with permission from Omniamed.com)

1.4.1. Contamination

From the time of wounding all open wounds are contaminated by microorganisms but not all wounds are clinically infected (Bowler 2003). Contaminated wounds contain non-proliferating microorganisms which do not evoke a host reaction (Collier 2004). In most cases contaminated wounds do not require antimicrobial treatment.

1.4.2. Colonisation

In a colonised wound the growth and death of microorganisms in the wound is balanced and controlled by the host's immune system (Heinzelmann et al. 2002). The presence of colonising bacteria in a chronic wound is usually of no clinical significance as this level of bioburden does not interfere with wound healing or damage wound tissue (Leaper, Assadian and Edmiston 2015).

Colonised wounds do not usually require treatment with topical antimicrobials unless there are concerns about the patient's immune response or overall medical condition. In some circumstances topical antimicrobials may be used prophylactically, for example wounds with a history of recurrent infections, including some diabetic foot ulcers and wounds on lymphoedematous limbs (Gray et al. 2010).

1.4.3. Local Infection

Wound infection occurs when pathogenic microorganisms evade the host's immunological defences and enter the host tissues and multiply causing a host reaction (Collins et al. 2002). Local infection is contained in one location, system or structure. Initially there may be subtle signs of infection such as hyper granulation which is friable and has a tendency to bleed. Epithelial bridging and pocketing in the granulation tissue may occur and the wound may enlarge or break down. Other covert signs of infection include delayed healing, pain and malodour. As the host response to the microbial infection increases then the overt, classic, signs of infection develop. These include new or increasing pain and swelling, spreading erythema, production of purulent exudates, increasing malodour and delayed wound healing (Gardner and Frantz 2008; Gardner, Frantz and Doebbeling 2001).

In locally infected wounds where there are no signs of the infection spreading, topical antimicrobials are recommended (EWMA 2006; IWII 2016). The aim of treatment is to remove infection from surrounding tissue and reduce the wound bioburden (Gray et al. 2010). Some clinicians recommend a more aggressive approach to treatment involving the use of topical antimicrobials with additional oral antibiotics (Kingsley 2005).

1.4.4. Spreading Infection

Spreading infection occurs when infective organisms spread from the wound and invade surrounding tissue (WUWHS 2008).

The spread of infection can involve deep tissue, muscle, fascia, organs and body cavities. Elbright (2005) suggested that infection in wounds can also present as cellulitis, abscesses, and may develop into necrotising fasciitis, osteomyelitis, sepsis and bacteraemia. The signs of spreading infection in the wound include extending induration and erythema, often with wound breakdown and dehiscence with or without satellite lesions (IWII 2016). The patient may develop crepitus, malaise, loss of appetite, inflammation of the lymph glands and lymphangitis.

Wounds exhibiting signs of spreading infection require aggressive treatment. This usually involves administering large doses of broad-spectrum antibiotics and the application of topical antimicrobial dressings (EWMA 2006; IWII 2016). Many chronic wounds develop because of an underlying physiological problem associated with poor arterial circulation. This means that when administering systemic antibiotics large doses are often required to ensure that an effective concentration of the antibiotic reaches the poorly perfused wound tissue. This often necessitates the use of intravenous injections / infusions rather than the oral administration of antibiotics (NICE 2019 Draft Guidance NG152). The effects of wound infection can range from delaying healing, causing disability and hospital admission to serious life-threatening septicaemia, sometimes leading to death.

1.4.5. Systemic Infection

Systemic infection occurs when microorganisms from the wound spread throughout the body via the vascular or lymphatic systems (Leaper et al. 2012; IWII 2016). This leads to a systemic inflammatory response, sepsis, septic shock, organ failure and sometimes death. Patients require aggressive therapy with intravenous antibiotics and in extreme cases life support.

1.5. Wound Infection and Biofilms

1.5.1. Biofilms

Microorganisms in wounds can exist in different phenotypic forms; either in a fast growing, free living, planktonic state or as attached sessile state in which the microorganisms attach to each other to create aggregated communities of slower growing cells, which can develop into a biofilm (Percival, McCarty and Lipsky 2012).

Biofilms have classically been defined as a community of bacteria attached to a surface, which are encapsulated in a self-produced extracellular matrix (ECM) composed of polysaccharides, proteins and glycoproteins referred to as extracellular polymeric substance (EPS) (Bjarnsholt et al. 2017). Within the biofilm the microorganisms create an environment conducive for their protection and are tolerant to antimicrobial agents (antibiotics and antimicrobials) (Grant and Hung 2013). One of the first reports which identified an increase in the presence of bacterial biofilm structures in chronic wounds compared to acute wounds was published in 2008 by James et al. Using light and scanning electron microscopy to examine wound biopsies from 66 patients they found biofilms in 60% of the 50 chronic wounds, compared with 6% (1 in 16) of the acute wounds. Since then, attention has focused on the ability of bacteria in chronic wounds to form and exist in biofilms and the impact this has on the wound healing process (Hill et al. 2010).

More recently, in 2017, Malone et al. undertook a systematic review and meta-analysis of published data to try to ascertain the prevalence of biofilms in chronic wounds. They identified only nine studies, involving 185 chronic

wounds, which met their eligibility criteria. The chronic/ non - healing wounds included in the review had been diagnosed as DFUs, VLUs, PU or non - healing surgical wounds (NHSWs) and had a biofilm confirmed by visualisation of aggregated bacteria within the wound bed. They found that the prevalence of biofilms in the chronic wounds assessed was 78.2% [Confidence interval 61.6-89, $p<0.002$]. Three out of the nine studies identified positive biofilm samples in no less than 60% of the samples while the six other studies observed 100% biofilm prevalence. These results suggest that biofilms are highly prevalent in human chronic non healing wounds (Malone et al. 2017).

In mature wounds the biofilms are usually polymicrobial consisting of several different species that cooperate synergistically to cause infection (Wolcott et al. 2013).

1.5.2. Biofilm Formation

Biofilms develop when planktonic bacteria explore the wound surface in a process described as twitching (Singh at al. 2002). They attach to the wound surface or co-aggregate with other microorganisms at surface interfaces through weak interactions via bacterial components (e.g. fimbriae, pili and flagella) and adhesion molecules (Nouraldin et al. 2016). *In vitro* studies have shown that planktonic bacteria, for example *Staphylococci, Streptococci, Pseudomonas* and *Escherichia coli*, can attach (become sessile) within minutes (Bester et al. 2010). Following successful attachment, complex signalling events alter the bacteria's genetic expression from the planktonic to biofilm phenotype via quorum sensing and sRNA based systems (Wu, Cheng and Cheng 2019).

This initial attachment is reversible and susceptible to antimicrobial treatment (Nouraldin et al. 2016). However, if the patient is immunocompromised, has poor nutrition or an underlying pathology such as diabetes then the inability of the host immune system to effectively control microbial growth may lead to biofilm development (Philips et al. 2010). The first microorganisms to attach to a surface have been described as pioneers and they initiate biofilm development by secreting EPS (Percival, McCarty, and Lipsky 2015).

The composition of the EPS differs between species but usually contains polysaccharides, alginate, extracellular DNA, proteins, glycoproteins, metal ions, and lipids (Bjarnsholt et al. 2008). The EPS surrounds the colony and acts as a protective barrier against the host immune response (Cutting and McGuire 2015). It also helps to promote microbial aggregation and may provide a source of nutrients for growth and proliferation. The microorganisms on the wound surface divide and develop into clusters or microcolonies. This leads to enhanced production of EPS which helps to embed the aggregating microorganisms deep within the biofilm (Percival, McCarty and Lipsky 2015).

Within the first hours of attachment up to one third of the microorganism's genome, around 800 dormant genes are expressed (Sauer et al. 2002). These new genes can enable the microorganism to develop tolerance to antibiotics (Stewart and Costerton 2001) and evade host adaptive and innate immunity (Vuong et al. 2004).

Other microorganisms are attracted and join the colony causing the formation of micro-colonies. The microcolonies are connected by water filled channels which allow nutrients in and enable removal of metabolic waste products (Davies 2003). Microorganisms within the biofilm communicate via a process known as quorum sensing (QS) which enables them to further develop the biofilms architecture. QS also facilitates the microorganism's ability to co-ordinate their growth rates, enzyme production, toxin production, antimicrobial resistance and bacterial virulence. QS is a density dependent form of cell-cell communication in which bacteria synthesize and react to signalling molecules (Zhao et al. 2012). Genes in Gram-negative bacteria cause the production of extracellular acylhomoserine lactone (acyl-HLSs) which diffuse through the wound to signal to other bacterial cells. The acyl-HLSs accumulate in proportion to the number of cells and give an indication of the density of the population (Waters and Bassler 2005). Gram-positive bacteria use peptide derivatives such as autoinducing peptides of around 5-17 amino acids as signalling molecules as an alternative to acyl-HLSs. Furanosyl diester, a signalling molecule known as autoinducer -2, is expressed and recognised by both Gram-positive and Gram-negative bacteria (Zhao 2012).

The biofilm grows and at a critical level the sensing signals activate genes that differentiate the biofilm into loosely packed pillar and mushroom shaped colonies with the bacteria embedded within the thick polymeric walls. The mature biofilm consists of about 10-20% cells and 80-90% matrix material (Flemming and Wingender 2010). By this stage the host defences are unable to eradicate the biofilm. To enable the biofilm to colonise new areas microbial cells break away from the mature biofilm by shedding, detachment or shearing and reseed on the wound surface and form new biofilms (Uppuluri and Lopez-Ribot 2016). Figure 1.2 shows the phases of the biofilm life cycle.

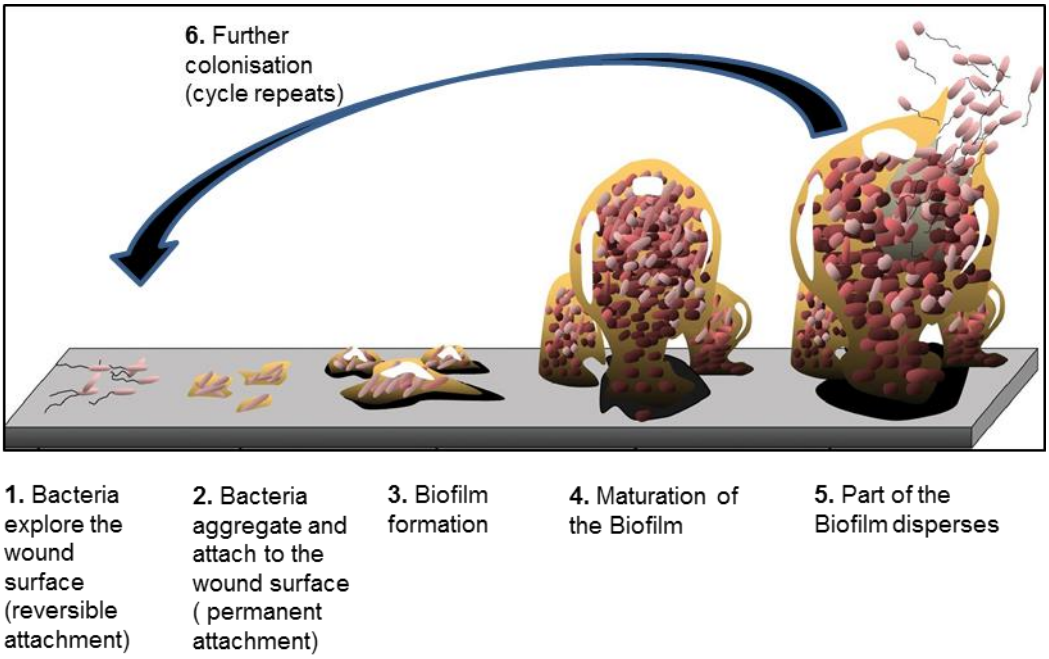


Figure 1.2: The Biofilm Life Cycle (Adapted from Monroe D (2007) Looking for Chinks in the Armor of Bacterial Biofilms. PLoS Biol 5(11): e307. https://doi.org/10.1371/journal.pbio.0050307).

1.5.3. Biofilm defences against host immunity and antimicrobials

The biofilm phase of growth offers numerous advantages over the planktonic growth state. Bacteria living in biofilms appear to be protected from environmental stresses, including attack from the host immune response. Within the biofilm community the close proximity of cells enables horizontal gene transfer and sharing of metabolic by-products (De Kievit 2009).

1.5.3.1. Biofilm defence against host immunity

There are a number of ways in which the biofilm mode of growth provides defence mechanisms against the host's immune system. The EPS matrix provides physical protection to the bacteria within the biofilm (Wu, Cheng and Cheng 2019). The matrix can interfere with phagocytic activity of the host inflammatory cells and inhibit the stimulation of the complement system (Seth et al. 2012). Early studies involving *Pseudomonas aeruginosa* demonstrated that the polymeric barrier is effective against reactive species such as hypochlorite and hydrogen peroxide which are produced by phagocytic cells (Simpson, Smith and Dean 1989). The alginate in the EPS of *Pseudomonas aeruginosa* has been shown to protect the bacteria against interferon g-mediated macrophage killing (Leidet al. 2005).

Biofilms of *P. aeruginosa* can respond to the presence of PMNs by upregulating the synthesis of a number of quorum sensing-controlled virulence determinants, including rhamnoilipids which surround the biofilm and on contact eliminate incoming PMNs (Jenson et al. 2007; Alhede et al. 2009) They also produce catalase that is able to break down hydrogen peroxide, a major reactive oxygen species (ROS) produced by PMNs, to oxygen and water so reducing the antimicrobial activity of the PMNs (Bjarnsholt et al. 2008).

P. aeruginosa also secretes other proteases, such as elastase, which inactivate parts of the complement system (Schultz and Miller 1974).

1.5.3.2. Biofilm defence against antimicrobials

The biofilm not only offers protection against natural antibodies, phagocytic inflammatory cells but can also develop tolerance to disinfectants, antiseptics and antibacterial substances (Koo et al. 2017). The dense negatively charged exopolymeric matrix wall of the biofilm provides a physical barrier limiting the diffusion of antimicrobial molecules (Rafii and Hart 2015). Diffusion of positively charged antimicrobials may be prevented by molecular binding or chemical interaction with the biofilm matrix (Lewis 2001). As mentioned, *Pseudomonas*

aeruginosa produces catalase which may be responsible for increased resistance to disinfectants containing hydrogen peroxide (Stewart et al. 2000).

Within the biofilm there are differences in the concentrations of nutrients and chemicals due to metabolic activity and protein synthesis which leads to population diversity within the biofilm with some cells having slow or no growth and others in the stationary phase (Werner et al. 2004). Bacteria in the biofilm demonstrating a decreased growth rate exist in a sessile state that is less susceptible to antibiotics which are typically designed to target rapidly dividing planktonic bacteria and their metabolic processes (Fleck 2006). In particular, in niches of the biofilm, local limitations of nutrients and oxygen may facilitate the growth of anaerobes (Nikolaev and Plakunov 2007).

There is also thought to be a subset of cells in biofilms, known as persister cells, which have reduced cellular activity which are dormant and are therefore tolerant to antimicrobial agents (Keren et al. 2004; Lewis 2007). Persister cells form between 0.1-10% of the biofilm population and are thought to be created by a non-hereditable phenotype switch rather than the result of antimicrobial pressure (Helaine and Kugelberg 2014). When antibiotic treatment is stopped the persister cells become metabolically active and start reforming the biofilm (Lewis 2012). Antibiotic resistance is enhanced within the biofilm community due to the close proximity of cells which enables horizontal gene transfer of resistant plasmids (Rahim et al. 2017). Bacteria in biofilms have been reported to be up to 500 times more resistant to antibiotics than their planktonic counterparts (Donlan and Costerton 2002). Studies which have determined the minimum biofilm eradication concentration (MBEC) of antibiotics found that standard oral doses of antibiotics which effectively kill planktonic bacteria, have little or no antimicrobial effect on biofilm bacteria *in vivo* (Phillips et al. 2008).

1.5.4. Biofilms and Non - healing Wounds

Open wounds are an ideal environment for the development of biofilms because collagen and extracellular matrix proteins such as fibronectin and fibrinogen are exposed in the damaged tissues so enabling attachment of bacteria. The moist wound surface with its adhesive, proteinaceous substrate and ready supply of nutrients facilitates biofilm development (Wolcott, Rhoads and Dowd 2008).

Many acute wounds can heal in the presence of bacterial colonisation (Bjarnsholt et al. 2017). However, development of a bacterial biofilm in an acute wound appears to stimulate chronic inflammation. This leads to an influx of inflammatory cells (neutrophils, macrophages and mast cells) which secrete proteases and release reactive oxygen species (ROS). The two major families of proteases found to be elevated in non-healing wounds are matrix metalloproteases (MMPs) and neutrophil elastase (NE) (Beidler et al. 2008; Liu et al. 2009; Rayment, Upton and Shooter 2008). Increased levels of proteases cause a number of adverse effects in the wound such as destroying extracellular matrix (ECM) proteins (Wysocki and Grinnell 1990; Herrick et al. 1992) and growth factors including platelet - derived growth factor (PDGF) (Pierce et al. 1995).

In normal wound healing tissue levels of inhibitor of metalloproteinases (TIMPs) increase to downregulate levels of proinflammatory cytokines that stimulate MMP production (Gill and Parks 2008). In non-healing wounds the levels of MMPs have been shown to increase but their associated TIMPs are reduced leading to prolonged activity of the MMPs (Trengove et al. 1999). Also found to be elevated in chronic wound fluid and biopsies are the levels of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-1-alpha (IL1-α) (Trengrove et al. 2000). Cytokines attract more inflammatory cells to the wound which secrete more MMPs and NE and release ROS which destroy proteins essential for healing which lead to the development of a chronic wound (Figure 1.3.).

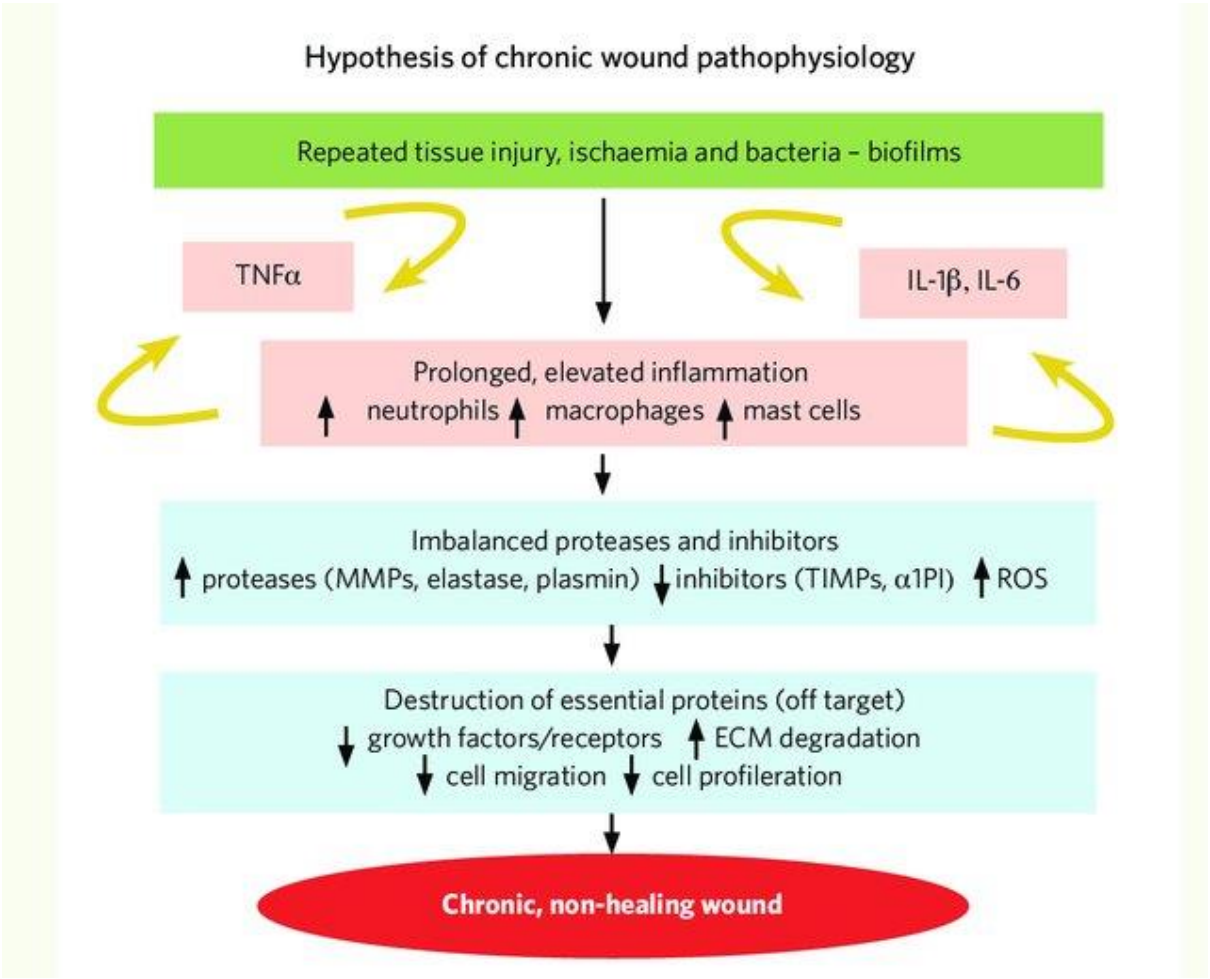


Figure 1.3: Hypothesis of chronic wound pathophysiology and biofilms (Mast and Schultz 1996) (Reproduced with permission from John Wiley & Sons).

Another factor thought to contribute to the delay in wound healing is the production of proteases by the bacteria contaminating the wound. A number of common wound pathogens including, *Stapylococcus spp*., *Streptococcus spp., Enterococcus spp.* and P*. aeruginosa* are known to produce proteases (Lindsay, Oates and Bourdillan 2017). Bacterial proteases can degrade tissue proteins and growth factors, impede the immune response and contribute to tissue degradation (Travis et al. 1995). Secreted bacterial proteases are also thought to play a role in enabling pathogens to disseminate into deeper layers of the skin possibly leading to the spread of infection (Koziel and Potempa (2013).

Chronic wound pathogenic biofilms are resistant and highly adaptable systems and it would appear that the ability of the host to control these multispecies

entities may decrease in accordance with the diversity of the wound's bacterial community (Boles et al. 2004). These pathogenic biofilms constitute a major concern in wound care because of their enhanced microbial activity and the diminished effectiveness of antimicrobial agents in their presence. It is therefore important that wound dressings containing antimicrobial agents are able to disrupt biofilms as well as killing planktonic bacteria (Percival et al. 2008).

1.6. Wound Pathogens

The incidence of wound infection varies depending on the site of the wound and the contaminating organism. Other factors such as the health of the patient, the effectiveness of their immune response and the virulence of the microorganism will determine if a clinical infection becomes established. The imbalance between pathological local factors and the integrity of host immune defences found in chronic wounds promotes colonization with both Gram-positive and Gram-negative bacteria (Serra et al. 2016; Kim et al. 2014). The bacteria commonly involved in wound infection are listed in Table 1.2.

Table 1.2: Common Bacterial Wound Pathogens

(Adapted from Edwards – Jones 2016)

Gram – positive Bacteria	Gram – negative Bacteria
S. aureus	*Escherichia coli*
Methicillin – Resistant *S. aureus* (MRSA)	*Pseudomonas aeruginosa*
Coagulase-negative *staphylococci*	*Klebsiella aerogenes*
Streptococcus pyogenes	
Other streptococci (Groups B, C, D, F & G)	
Enterococcus spp. including vancomycin-resistant (VRE) strains	
Anaerobic Gram – positive Bacteria	**Anaerobic Gram – negative Bacteria**
Clostridium perfringens	*Bacteroides* spp.
Peptostreptococcus spp.	*Prevotella* spp.

A study published in 2015 investigated the bacterial profiles of 217 chronic infected wounds of differing aetiologies including pressure ulcers, leg ulcers and diabetic foot ulcers isolated 28 different microbial species with polymicrobial infection identified in 59 (27.1%) of the samples. *S. aureus* (37%) was the most common pathogen identified followed by *P. aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%) and *Corynebacterium* spp. (5%) (Bessa et al. 2015). It would appear that the infecting pathogens vary depending on the type of wound and the anatomical position.

1.6.1. Infections in Pressure Ulcers

Pressure ulcers which develop on bony prominences are common in the sacral region where they are susceptible to faecal contamination (Therattil, Pastor and Granick 2013). Pressure ulcers have been shown to have varied flora including *S. aureus,* methicillin - resistant *S. aureus, P. aeruginosa, Enterobacteriaceae,*

Acinetobacter baumannii and multi - resistant Gram - negative bacilli (Braga et al. 2013). Deep pressure ulcers are at risk of developing underlying mixed aerobic/anaerobic osteomyelitis and bacteraemia (Landis 2008).

1.6.2. Infections in Leg Ulcers

Most leg ulcers are not clinically infected but are likely to be colonised (NICE Leg Ulcer Infection Guidance June 2019). An early study by Gjødsbøl et al. (2006) designed to investigate the bacterial profile of chronic venous leg ulcers (CVLU) identified *S. aureus* (in 93.5% of the ulcers), *Enterococcus faecalis* (71.7%), *P. aeruginosa* (52.2%), coagulase-negative *staphylococci* (45.7%), *Proteus* species (41.3%) and anaerobic bacteria (39.1%). They also found that 76% of the ulcers contained two or more, sometimes up to five different resident bacteria of which *S. aureus* and *P. aeruginosa* where the most common. An interesting finding in this study was that ulcers colonised with *P. aeruginosa* were significantly larger ($P<0.005$) than those that did not contain these bacteria which led the authors to suggest that the presence of *P. aeruginosa* in CVLUs appears to enlarge the size of the ulcer which may delay healing (Gjødsbøl et al. 2006).

A more recent study (Serra et al. 2014) designed to investigate the expression of MMPs in infected CVLU found the three most common bacteria in the 63 patients studied were *S. aureus* (38.09%), *Corynebacterium striatum* (19.05%) and *P. aeruginosa* (12.7%). They also identified higher levels of MMP-I and MMP-8 in patients with infected ulcers compared with uninfected ulcers. Similarly raised levels of interleukins, vascular endothelial growth factor and tumour necrosis factor alpha were found in patients with infected ulcers (Serra et al. 2014).

1.6.3. Infections in Diabetic Foot Ulcers

Diabetic foot infection (DFI) is a frequent and severe complication of Diabetes Mellitus (DM). It has been estimated that diabetic patients have a 25% chance of developing a foot ulcer in their lifetime (Singh, Armstrong and Lipsky 2005) and around 50% of DFU are clinically infected at presentation (Lavery at al. 2006; Prompers et al. 2008).

The prevalence and severity of the infection can be associated with both host and pathogen related factors. Diabetic patients have a decreased immune response including impaired macrophage function which results in delayed healing and increased susceptibility to infection (Ammons and Copié 2013; Serra et al. 2015). In an insensate, deformed diabetic foot, ulcers develop when a trauma breaches the protective skin. Bacteria colonise the subcutaneous tissues which may lead to infection which is often clinically unapparent (Fisher et al. 2010).

The classic signs and symptoms of diabetic foot infection are often diminished in patients with peripheral neuropathy or vasculopathy. In the absence of the common signs of infection clinicians are advised to examine the patient for secondary findings such as foul odour, friable or discoloured granulation tissue and rim undermining as indications of clinical infection (Cutting and White 2004).

In acute DFI aerobic, Gram-positive cocci are the predominant pathogen with *Staphylococci aureus* being the most commonly isolated pathogen (Roberts and Simon 2012; Lipsky et al. 2012). However, in chronic DFU infections are frequently polymicrobial and often include aerobic Gram - negative bacilli and obligate anaerobic bacteria (Uçkay et al. 2013).

In developed countries, especially those with hot and humid climates, *P. aeruginosa* is often the major cause of DFIs (Bansal et al. 2008). The microbial flora in wounds appears to change over time. In early acute wounds normal skin flora such as *Staphylococcus epidermidis*, other coagulase negative *Staphylococci* and *Corynebacterium* spp. predominate. Soon after *Staphylococcus aureus* and β-haemolytic *Streptococcus* spp. often appear, particularly in diabetic foot ulcers. After about four weeks facultative aerobic organisms such as *Proteus* spp., *E. coli* and *Klebsiella* spp. can colonise the wound (Dow et al. 1999). As wounds deteriorate and deeper tissues are affected, anaerobes become more common (Bowler 2001).

1.7. Wound Management

Whilst a clinical diagnosis to determine the type of wound and the underlying pathology is important, the management of chronic wounds depends mainly on their location, size and the stage of the healing process they are confined to.

1.7.1. Clinical Wound Classification

In order to determine an appropriate treatment, wounds are often classified by their colour. Table 1.3. illustrates the different colour classifications; **Black** for necrotic wounds, **yellow** for infected or sloughy wounds and **red** for healing wounds that are either granulating or re-epithelializing and outlines the different management strategies (Vowden and Vowden 2017).

Table 1.3: Colour Classification of Wounds and the treatment requirements.

Wound Colour	**Description**	**Management**
Black	Necrotic Tissue	Debridement
Yellow (Infection)	Inflamed, localised heat and swelling. Yellow or green slough. May have offensive odour	Swab to identify contaminating microorganism and treat appropriately
Yellow (Slough)	Slough is a natural by-product of the inflammatory process formed by the presence of dead cells in the wound exudate.	Physical removal of slough and debridement
Red (Granulation)	Cavity wounds	Cavity requires packing with an absorbent material until the cavity is filled with new granulation tissue.
Red (Re-epithelialisation)	New layer of epithelial cells	New fragile skin requires protection from the external environment.

1.8. Wound Bed Preparation

Necrotic non-viable tissue provides an environment for wound infection which can exacerbate the inflammatory response and impede wound healing (Wolcott, Kennedy and Dowd 2009). Where either slough or necrosis is present in a wound it should be removed as it can support the attachment and development of biofilms (Percival and Suliman 2015).

Debridement is defined as removal of dead devitalised tissue to enable the natural healing process to progress (EWMA 2013). Debriding the wound should enable it to become an acute wound and progress through the normal phases of wound healing (Attinger et al. 2006). There are numerous methods of debridement including surgical, autolytic, biological and mechanical (Sibbald et al. 2011).

Evidence suggests that a combination of methods is often required (Milne 2015). The choice of method often depends on the pathology and location of the wound and also the general health of the patient. Certain types of debridement require highly skilled healthcare professional input and may have to be performed in a hospital or community setting (Strohal et al. 2013).

1.8.1. Surgical Debridement

Surgical debridement is the most efficient way to remove dead tissue; although not without risk, as the tissue is usually heavily contaminated with bacteria which if exposed to blood may cause septicaemia. Patients often require general or local anaesthesia and antibiotic prophylaxis. Special caution is required especially with elderly patients and patients with diabetes, arterial disease and clotting disorders (Heath et al. 2019).

Necrotic tissue from diabetic foot ulcers is often removed by sharp debridement using a scalpel, scissors or curette. This procedure may be performed in an outpatient setting by experienced clinicians without a general anaesthetic. In some cases, a local anaesthetic is required but often the inability to feel pain due to peripheral neuropathy obviates the need for local anaesthesia. Caution is required in patients with ischaemic feet (WUWHS 2016) and those with large, ulcerated areas who should be referred to a vascular surgeon who is experienced in the management of foot infections (Gray et al. 2011).

Other slower, non-surgical options include autolytic debridement, maggot therapy, use of hydrotherapy, and negative pressure wound therapy.

1.8.2. Autolytic Debridement

Dressings such as hydrogels, hydrocolloids and honey that create a moist wound environment can be used to promote autolysis of necrotic tissue and facilitate debridement (Thomas 2006; Percival and Suleman 2015).

Autolytic debridement promotes the release of endogenous proteolytic enzymes and activates phagocytes. The enzymes break down necrotic and sloughy tissue which can then be ingested by macrophages. Autolytic debridement should not be used on bleeding wounds, fistula, in body cavities or highly exuding wounds. Use is contraindicated in infected wounds or wounds susceptible to anaerobic infection (EWMA 2013).

1.8.3. Biological Debridement - Maggot Debridement Therapy (MDT) - (Larval Therapy, Biotherapy or Biosurgery)

The ability of maggots to clean and disinfect wounds has been recognised for centuries (Pritchard and Nigam 2013).

Larvae of the common green bottle, *Lucilia sericata* are attracted to human wounds and feed on unhealthy or abnormal tissue without affecting healthy tissue. Maggot therapy has three key effects on the wound: debridement of necrotic tissue, antimicrobial action and promotion of granulation tissue (Dinman 2007).

Maggots enhance wound healing by mechanical debridement using “mouth hooks” and their rough bodies to scratch necrotic tissue (Gottrup and Jorgensen 2011). They also excrete salivary enzymes which contain collagenases and trypsin-like and chymotrypsin-like collagenase which break down necrotic tissue into a semi-liquid which they then ingest (Blake et al. 2007). The salivary enzymes are also able to breakdown bacterial biofilms which can then be ingested by the maggots (Brin et al. 2007).

The antimicrobial activity of maggots is thought to occur in the hind gut where bacteria are killed by the maggot digestive system (Mumcuoglu et al. 2001).

They are also believed to excrete waste products such as ammonia and secrete other antimicrobial agents that are bactericidal (Nigam et al. 2006).

Clinical studies have demonstrated that MDT has been effective in the treatment of wound infections caused by various common wound pathogens including *Staphylococcus aureus,* methicillin–resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Sun et al. 2014; Blueman and Bousfield 2012).

The third area of activity associated with MDT that is undergoing further research is their potential role in promoting wound healing by stimulating tissue regeneration. Early evidence suggested that the maggot secretions which contain calcium carbonate, urea and allantoin could stimulate granulation tissue production (Hunter et al. 2009).

In a more recent *in vitro* study investigating the effect of salivary gland extract from *Lucilia sericata* on human dermal fibroblast proliferation researchers observed increased cell metabolism and protein production which corresponded with the formation of microfibrillar net which was used for fibroblast migration and the production of extracellular matrix. This was thought to be due to specific proteinases in the saliva changing fibroblast movement within the extracellular matrix to enhance the remodelling process (Polakovičova et al. 2015).

There has also been research using genetic engineering to create transgenic larvae that can secrete human platelet derived growth factor (PDGF-BB) which may promote wound healing (Linger et al. 2016).

Currently medicinal maggots are commercially available from BioMonde® either as a BioBag® Dressing where the larvae are contained in a finely woven net pouch or “Free Range Larvae”. The free-range larvae are applied directly to the wound and retained with a sterile nylon net. Both treatments can be left on the wound for up to four days per application (Naik and Harding 2017).

1.8.4. Negative Pressure Wound Therapy (NPWT)

Negative Pressure Wound Therapy involves the placement of a foam dressing in the wound cavity which is attached to an evacuation tube which connects to a vacuum pump (Sandoz 2015). The negative pressure produced maintains a moist wound environment, removes interstitial fluid and wound exudate and is

able to rebalance the cytological disturbances in the wound bed (Streubel et al. 2012). The mechanical stress enhances granulation tissue formation (Schintler 2012). There is also evidence to suggest that NPWT promotes angiogenesis and enhances tissue perfusion (Moues et al. 2011).

Since 2012 there have been five Cochrane reviews of the use of NPWT in different wound types which concluded that there was no conclusive Randomised Control Trial (RCT) evidence of the effectiveness of NPWT in pressure ulcers, leg ulcers and partial thickness burn wounds. There was some evidence to suggest that NPWT was more effective at healing foot wounds in diabetic patients compared to moist wound dressings (Dumville et al. 2013). The evidence supporting the effectiveness of NPWT on Surgical Site Infections (SSI), wound dehiscence and time to complete healing was also unclear.

The European Wound Management Association (2013) advised that NPWT should not be used on clinically infected wounds or wounds with local ischaemia or active bleeding. It is also contra-indicated in wounds where vessels, tendons, muscles, joints or bones are exposed.

1.8.5. Hydro-Responsive Wound Dressings (HRWD™)

A recent development in wound bed preparation proposed for therapy of a variety of wounds is the use of hyper hydration (Rippon et al. 2016). This occurs when wounds are hydrated beyond what a normally acceptable level is using a dressing which provides a continuous rinsing and absorption mechanism that can cleanse and debride by softening and detaching necrotic tissue and slough (Rippon and Ousey 2015; Spruce et al. 2016). HydroClean®plus (Hartmann), the first Hydro-Responsive Wound Dressing, is a soft conformable pad containing superabsorbent polyacrylate (SAP) particles which are pre-activated with isotonic Ringer's solution and the antiseptic Polyhexanide (PHMB) (Hartmann 2020). The pads continuously release fluid into the wound which softens necrotic tissue and fibrin, and the SAP particles absorb bacteria and proteinaceous wound exudate (Ousey, Rogers and Rippon, 2016). *In vitro* studies have shown that the SAP particles can sequestrate and inactivate proteinases which are found in high levels in chronic wound exudate and are known to contribute to tissue breakdown (Eming et al. 2008). Once the

wound has been cleansed the HydroClean®plus is replaced with HydroTac® dressing which can be used for the granulation and epithelialisation stages of healing. HydroTac® has a hydrogel wound contact layer covered with foam and an outer air-permeable, waterproof and bacteria proof backing. The dressing is designed to deliver moisture using "AquaClear™ Gel Technology" to provide optimal hydration levels in the wound bed. The moist wound environment created should promote accumulation of growth factors which has been suggested can accelerate epithelial wound closure (Smola et al. 2016).

1.9. Treatment of Infected Wounds

Wound infections often delay wound healing, cause pain and distress to patients and may even result in death (Edward - Jones 2013). Diagnosis of wound infection requires clinical observation of the wound itself and laboratory testing to identify potential wound pathogens. Acute wound infections are usually characterised by the classic signs of infection; erythema, heat, pain and swelling whereas wound infections in immunocompromised patients and chronic wounds may be more difficult to identify (Leaper et al. 2012).

Validated scoring systems to help aid diagnosis of infection have not yet developed. In certain situations, such as an acute wound showing the classic signs of infection, a chronic wound with signs of spreading or systemic infection and an infected wound that has failed to respond to antimicrobial treatment it may be appropriate to undertake microbiological analysis of wound specimens (IWII 2016).

The most common testing method involves wound swabbing; either using a zigzagging approach over the wound surface or the Levine technique. The Levine technique which involves applying a wound swab over a $1cm^2$ area of the wound applying pressure and rotating the swab to collect exudate is thought to be more effective than the Z-swab technique (Angel et al. 2011). The wound swab cannot be used to diagnose infection but may indicate which organisms are present, their virulence and antibiotic resistance (PHE 2014). The swab will only identify organisms on the wound surface. A culture study involving chronic wounds found that *S. aureus* was living on the wound surfaces but *P.*

aeruginosa inhabited deeper tissues (Fazli et al. 2009). For this reason, it has been suggested that wound biopsies should be taken, however they are not routinely performed due to the cost of analysis and the discomfort to the patient (Copeland-Halperin et al. 2016).

The routine use of DNA sequencing techniques should enable clinicians to identify species of microorganisms in a wound specimen that are not easily identified by culture-based techniques (IWII 2016). A study investigating the microbiome of 52 non-healing diabetic foot ulcers which were not exhibiting signs of clinical infection found that the microbial load and the diversity of the potential pathogens was underrepresented when the wound swabs were processed by bacterial culture methods compared with sequencing of bacterial ribosomal genes (Gardner et al. 2007).

The aim of antimicrobial treatment is to reduce the levels of microorganisms within the wound to enable the patient's immunological defence to tackle the infection (O'Brien 2007). Biofilm Based Wound Care (BBWC) treatment strategies are based on tackling the different phases of the biofilm life cycle and preventing the re-formation of dispersed biofilms. This could involve preventative action such as interfering with microbial attachment, biofilm development or removal or disruption of an existing biofilm and prevention of reformation (Cooper, Bjarnsholt and Alhede 2014).

Breakdown of an established biofilm is critical to enable antimicrobial agents to eradicate the exposed microorganisms. Wounds should be vigorously cleansed using products designed to disrupt biofilms (Dowsett 2013).

Biofilms should then be debrided using the one of the methods described for necrotic wounds (Section 1.8). Debridement may not remove all the biofilm and any remaining bacteria could reform into a mature biofilm within days (Wolcott, Kennedy and Dowd 2009). Topical antimicrobial therapy in the form of an antiseptic (with a surfactant) or antimicrobial dressing is required to ensure that remaining planktonic bacteria are not able to reform a biofilm. The wound should be covered with an appropriate dressing to prevent recontamination and be reassessed regularly. Repeated debridement and antimicrobial application may be required until the wound is healing (Figure 1.4).

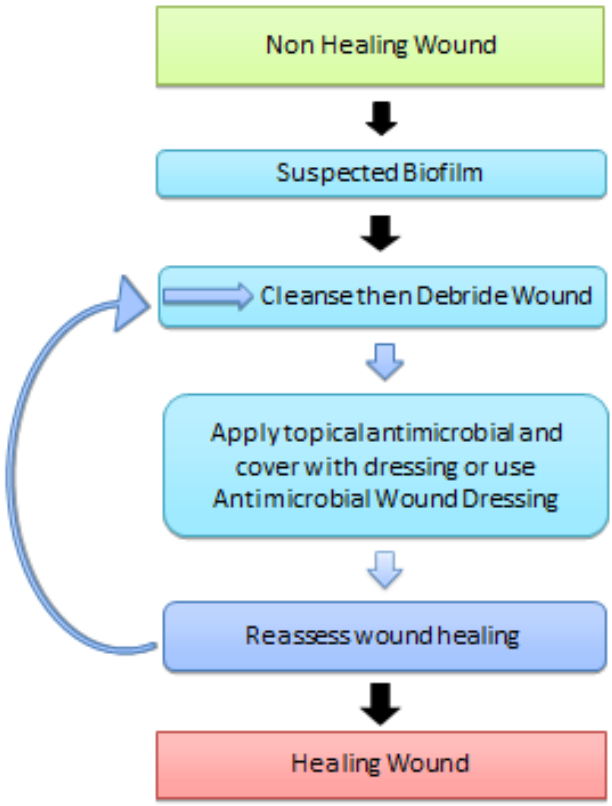


Figure 1.4: Principles of Wound Biofilm Management

(Adapted from Bjarnshalt et al. 2017).

1.9.1. Role of Antimicrobials in Wound Care

Antibiotics are naturally occurring or synthetic molecules which either kill or inhibit the growth of microorganisms. They usually act on one specific target, may have a broad spectrum of activity, are relatively non-toxic, but are susceptible to losing their effectiveness to bacterial resistance (Lipsky and Hoey 2009).

Since the discovery of penicillin by Alexander Fleming in the 1920's clinicians have been using antibiotics to prevent and treat systemic and localised wound infections. Indiscriminate use of antibiotics has led to the widespread development of resistance with the result that multi-resistant bacteria are becoming more common and leading to increased mortality rates (Fair and Tor 2014; Prestinaci, Pezzotti and Pantosti 2015).

A position paper on antimicrobial stewardship in wound care was published in 2016 (Lipsky et al. 2016 p 3026) which stated that “the rate of antibiotic resistance is directly related to the level of antibiotic use” and highlighted some of the problems associated with inappropriate use of antimicrobials in wound care. The authors identified that infection is often difficult to diagnose particularly in chronic wounds, there was a lack of guidelines for treatment of infection in wounds. To improve antibiotic prescribing in wound care local guidelines for management of wound infections are required and antimicrobial stewardship (AMS) programmes should be introduced. AMS involves a multiprofessional team who are responsible for patient’s care to ensure timely and optimal selection of antimicrobial agents that are given at the correct dose and for an appropriate duration, to achieve the best clinical outcome for the patient (Nathwani 2012).

It is recommended that antibiotics should only be prescribed for clinically infected wounds; the antimicrobial chosen should have the narrowest spectrum for the suspected or proven pathogen and the lowest risk of adverse effects for the patient and the community (Dryden et al. 2011).

The use of systemic antibiotics for prophylaxis is usually reserved for surgical or traumatic wounds where the level of microbial contamination is expected to be significant (Gottrup et al. 2005). Systemic antibiotics may also be indicated for prophylaxis when patients are undergoing surgical debridement (NICE Guideline NG125 (1.2.17) 2019).

In non - healing wounds the use of systemic antibiotics should be reserved for wounds diagnosed as being clinically infected (Wounds UK 2013).

Due to the aetiology of these wounds being associated with poor circulation large doses of systemic antibiotics may be required to reach therapeutic concentrations. Patients who are immunocompromised will require aggressive systemic antibiotic treatment, usually with a broad-spectrum antibiotic administered intravenously (EWMA 2006).

Whilst consensus exists on the role of systemic antibiotics in wound care the use of topical antibiotics is more controversial. The use of topical antibiotics which contain a low dose of the antibiotic may induce resistance (IWII 2016).

Most guidelines do not recommend the use of topical antibiotics because of the risk of resistance and also because they can cause delayed hypersensitivity and superinfection (Siddiqui and Bernstein 2010). A recent Wound Infection in Clinical Practice International Consensus document published by the International Wound Infection Institute in 2016 advised that due to global concern regarding antibiotic resistance use of topical antibiotics should only be considered in infected wounds under very specific circumstances (Wolcott 2015). An example is the use of silver sulphadiazine for the treatment of burns and wounds (International consensus Wound International 2012). Also, topical Metronidazle, which is effective against Gram - positive and Gram - negative bacteria, has been advocated as the treatment of choice for anaerobic infections (Löfmark, Edlund and Nord 2010). Metronidazole gel is also able to reduce odour, exudate and pain in malodorous wounds (Ousey 2018). Topical mupirocin is effective against Gram - positive organisms including methicillin-resistant *Staphylococcus aureus* (Dabiri, Damstetter and Phillips 2016).

1.9.2. Topical Antimicrobial Preparations

Whilst systemic antibiotics are often appropriate for the treatment of spreading infection in wounds (IWII 2016) for some localised infections topical antimicrobials have particular benefits in that they can achieve high antimicrobial levels at the wound surface; they may have less risk of systemic side effects and allow the use of agents that cannot be administered systemically (Lipsky and Hoey 2009).

Traditionally a wide range of topical antimicrobial preparations have been used in wound care containing antibiotics, antiseptics or disinfectants (Siddiqui and Bernstein 2010). Disinfectants are active against virtually all disease-causing microorganisms. They can be sporostatic but are not necessarily sporicidal (Mc Donnell and Russell 1999). Disinfectants are used to sterilise inanimate objects or surfaces and are usually toxic to tissues so are not used to treat wounds.

Antiseptics kill or inhibit growth of microorganisms in or on living tissue. They often have multiple microbial targets, a broad antimicrobial spectrum and residual anti-infective activity. Antiseptics can be used on intact skin and some

open wounds but may be toxic to host tissues e.g., fibroblasts, keratinocytes and possibly leukocytes (Lipsky and Hoey 2009). Due to the chemical nature of these materials many of them can cause adverse reactions on skin such as irritation, allergic contact dermatitis and immunological contact urticaria (Lachapelle 2012). If applied to large areas of exposed damaged tissue antiseptics may be absorbed into the blood stream and exhibit systemic toxicity (Morrison 1989).

The topical antiseptics used in the UK include alcohols, heavy metal derivatives e.g., silver compounds, quaternary ammonium compounds for example Biguanides such as chlorhexidine and polyhexanide, and halogen releasing agents including iodine compounds (McDonnell and Russell 1999).

Topical antimicrobial agents used in wound care can be divided into two categories:

1. Lotions with antimicrobial properties are used to irrigate and cleanse wounds. These agents usually have only brief contact time with the wound surface and are applied as irrigations, as packs or soaks. Examples include povidone iodine, biguanides, peroxides and less commonly hypochlorite, hexachlorophene, potassium permanganate and gentian violet (Norman et al. 2016).

2. Preparations designed to remain in contact with the wound surface for longer periods. These preparations include creams, ointments and impregnated wound dressings (O'Meara et al. 2001).

In recent years there has been increased interest in the use of more "natural" topical antimicrobial agents such as sugar paste and honey (Alam et al. 2014).

Non - antibiotic antimicrobials are widely used in wound care and may be particularly useful in chronic wounds with localised infection (Gottrup et al. 2013). Applying non-antibiotic topical agents after debriding wounds with secondary signs of localised infection may help suppress biofilm formation (Leaper, Assadian and Edmiston 2015; Cutting and White 2005).

The wound dressings market has been inundated with new advanced wound dressing materials designed to regulate the wound surface by retaining

moisture or absorbing exudate to protect the wound and surrounding tissue (Agency for Healthcare Research and Quality 2014). Many of these dressing materials have antimicrobial agents incorporated into them to create antimicrobial wound dressings (AWD) which are discussed in Sections 1.10 and 1.11.

1.10. Advanced Wound Dressings

A variety of materials have been used to treat wounds since ancient times. Sumerian cuneiform tablets (before 2000 BCE) describe the application of poultices made of mud, milk and plants to wounds (Ovington 2007). Ancient Egyptian papyruses (1550 to 1650 BCE) explained how to wash wounds, prepare and apply plasters of honey, plant fibres and animal fat and dress with bandages (Majno 1975). In recent years there have been major developments in wound dressing materials designed to enhance the wound healing process.

Traditional wound dressings such as lint, gauze, cotton wool and Gamgee have the disadvantage that they can stick to the wound surface and shed fibres making removal traumatic and delaying the wound healing process. Up until the 1960s it was believed that the best way to promote healing and reduce bacterial infection was to keep the wound surface dry and encourage scab formation. Research undertaken by Winter (1962) on superficial wounds on pig skin demonstrated greatly increased reepithelialisation rates in wounds that remained moist and did not form a scab. The concept of moist wound healing was confirmed in humans a year later by Hinman and Maibach (1963) and revolutionised the way wounds are treated. It was shown that when the scab is shed the stratum corneum is lost. This causes significant trans epidermal water loss. Keeping the wound moist, by preventing scab formation, was shown to help retain moisture, and facilitate different stages of the wound healing process including keratinocyte proliferation, migration and differentiation (Abdelrahman 2011).

This change in wound management led to the development of occlusive dressings designed to maintain a moist environment at the wound surface. Initially there were concerns that the moist environment under the occlusive

dressing would encourage microbial growth. However, a review of 100 studies comparing wound infection under occlusive and non-occlusive dressings by Hutchinson et al. (1990) showed that infection rates were 2.6% versus 7.1% ($P < 0.001$) in favour of occlusive dressings. Hutchinson also found that the bacteria colonising the wounds under occlusive dressings enhanced the phagocytic properties of the wound encouraging an antimicrobial environment (Hutchinson and Lawrence 1991). The occlusion also appeared to lower the pH at the wound surface creating an acidic environment which is hostile to bacteria.

Wound dressings that provide a moist wound environment can be classified in a variety of ways. Primarily, there are those that are suitable for direct application to the wound surface categorised as primary dressings, and then materials that are used to cover primary dressings and keep them in place, called secondary dressings. Some newer dressings have a combination of both, consisting of a primary dressing attached to a covering secondary dressing which often has an adhesive border to keep the dressing in place. These island dressings often contain an absorbent central region surrounded by an adhesive portion.

Dressings can also be defined by their function such as debriding, absorbing, occlusive or antibacterial (Purner and Babu 2000). A variety of materials are used to produce wound dressings, and the physical form of the dressing, for example (foam, film, gel, ointment) is important as it can affect the healing process. The key functions of advanced (interactive) dressings are to provide patient comfort and ensure that the wound remains moist, but not macerated, by absorbing wound exudate and slough. The dressing should minimise the danger of contamination from pathogens and foreign bodies and protect the wound from mechanical stress (Cascone and Lamberti 2020).

An ideal dressing should contribute to maintaining an appropriate temperature to promote blood flow to the wound, must be sterile, nontoxic and non-allergenic (Dhivya, Padma and Santhini 2015).

Another important function of a dressing is to maintain a high level of oxygen tension at the wound's surface as oxygen is an important element in reparative processes such as cell proliferation, synthesis of collagen and aids the generation of leukocytes (Tandara and Mustoe 2004).

There are currently seven different classes of advanced wound dressing which differ in their formulation, mechanism of action and their clinical indications. (Table 1.4)

Table 1.4: Advanced Wound Dressings

Dressing Type	Formulation	Mechanism	Indications
Hydrofibers (HFDs) 	Sodium carboxymethylcellulose textile fibres	Highly absorbent due to vertical wicking of wound exudate. (Abdelrahman 2011)	Heavily exuding wounds for up to 3 days.
Alginates 	Calcium and Sodium salts of alginic acid available as freeze-dried porous sheets (foams) and fibrous ribbons/ropes	In contact with wound exudate the fibers undergo an ion exchange to form sodium calcium alginate a soft non adherent hydrophilic gel. (Thomas 2000)	Moderate to heavily exuding wounds. (Agren 1996)
Hydrocolloids 	Film or sheets made from carboxymethylcellulose, gelatins and pectins secured on a polyurethane film or foam pad. (Seaman 2002)	Hydrocolloid layer absorbs wound exudate to form a soft gel which becomes more permeable to water and air as the gel forms. (Thomas 1997)	Light to moderately exuding sloughy or necrotic wounds for up to 7 days. Autolytic debridement. Not recommended for infected wounds
Foams (FDs) 	Hydrophilic polyurethane (PU) polyvinyl alcohol (PVA) or silicone foam.	PU and silicone foams unable to retain the fluid they absorb whereas PVA can retain about 30%. (White et al. 2012)	Moderately exuding wounds. Non adherent, provide thermal insulation and protection for up to 7 days.

Dressing Type	Formulation	Mechanism	Indications
Soft Polymers 	Soft Silicone polymer	Non adherent wound contact layer	Light to moderately exuding wounds.
Hydrogels 	1st Generation: 80% water with 2-3% gel forming polymer either carboxymethylcellulose, modified starch or sodium alginate combined with 20% propylene glycol. 2nd Generation: insoluble, swellable hydrophilic materials made from synthetic polymers such as poly methacrylates and polyvinylpyrrolidine.	Permeable to water vapour and oxygen and have three-dimensional, cross-linked polymers which give the gel structure which limits their fluid handling capacity. 2nd generation hydrogels are able to absorb and donate water according to the needs of the wound. (Moore 2006)	Moist wound healing, are non-adherent, cool the wound surface and reduce pain. Not recommended for infected wounds.
Vapour Permeable Films 	Semi-permeable films are made from polyurethane covered with hypoallergenic acrylic derivatives.	Allow passage of water vapour and oxygen but are impermeable to water and microorganisms. (Moshakis et al 1984)	Superficial low exudating wounds. Not suitable for treatment of deep, infected or heavily exuding wounds.

The physical form of the dressing can impact on the wound healing process. One of the important actions of these dressings, particularly in chronic wounds which produce large volumes of exudate, is the ability of the material to absorb fluid. Hydrofibers are highly absorbent as the textile fibers enhance the vertical wicking of wound exudate (Abdelrahman 2011). Alginates also have effective fluid handling capacity which ranges between 15-25g/100cm^2 (Thomas 2000). Hydrocolloids and soft polymers are less absorbent, and foams vary in their ability to retain the fluid they absorb with Polyvinyl Alcohol being the most absorbent as it can retain approximately 30% of the fluid it absorbs (White et al. 2012). Vapour permeable films allow the passage of water vapour and oxygen but are impermeable to water and microorganisms. They are suitable for the treatment of shallow, lightly exuding wounds but not deep, infected or heavily exuding wounds (Dhivya et al. 2015).

Hydrogels maintain a high moisture content at the wound site and allow gaseous exchange between the wound and external environment (Sharma, Parmar and Mehta 2018; Cascone and Lamberti 2020). Hydrogels are available as sheet dressings for shallow wounds and amorphous gels used to pack cavity wounds. In the sheet dressings the polymers are crosslinked and able to absorb and retain large volumes of wound exudate. However, the gel formulations have limited ability to absorb wound exudate so are generally used to donate liquid to dry sloughy wounds and to facilitate autolytic debridement of necrotic tissue. Due to the fixed structure of the hydrogels and their limited fluid handling capacity they should not be used on heavily exuding or infected wounds (Moore 2006; Morgan 2004; Jones and Milton 2000).

Occlusive dressings such hydrocolloids provide a barrier to the ingress of microorganisms. They are useful in the management of chronic wounds but are not usually recommended for use in clinically infected wounds unless they are used in conjuction with other treatments such as antibiotics. Like all occlusive dressings they should not be used in the presence of anaerobic bacterial infection (Ousey et al. 2012).

1.11. Antimicrobial Wound Dressings

A variety of antimicrobial wound dressings (AWD) have been developed by incorporating antimicrobial agents into a range of advanced dressing materials.

There are 4 classes of antimicrobial dressings listed in the British National Formulary; honey, iodine, silver and “other antimicrobials” which includes the biguanide antiseptics (BNF 2019).

1.11.1. Biguanides

In the UK there are currently two biguanide antiseptics; chlorhexidine and polyhexamethylene biguanide, which have been incorporated into a variety of antimicrobial wound dressings as shown in Table 1.5.

1.11.1.1. Chlorhexidine

Chlorhexidine has been used as a topical antiseptic solution since 1954 (Milstone et al. 2008). It is classified as a bisbiguanide because it has two cationic groups separated by a hydrophobic bridging structure (hexamethylene). Chlorhexidine gluconate is available in a variety aqueous and alcoholic liquid formulations ranging in concentrations from 0.05% - 4% (BNF 2019). Traditionally the lower strength 0.05% aqueous solution has been used for wound cleansing and the higher strengths used for preoperative skin preparation. It has been shown that at low concentrations chlorhexidine affects microbial membrane integrity but at higher concentrations is causes the cytoplasmic contents to precipitate causing cell death (McDonnell and Russell 1999). Even at the lower concentration of 0.05% Chlorhexidine solution has been shown to have broad spectrum of activity against Gram-positive and Gram-negative bacteria, facultative anaerobes and aerobes, yeast and some lipid enveloped viruses such as HIV (Harbison and Hammer 1989).

A wound dressing in the form of a paraffin gauze tulle impregnated with 0.5% chlorhexidine acetate ointment (Bactigras ™) is available and indicated for adjunctive treatment and prevention of skin loss lesions, including wounds, burns and ulcers (Smith - Nephew.com)

1.11.1.2. Polyhexamethylene biguanide (Polihexanide) (PHMB)

PHMB is a polycationic linear polymer with a hydrophobic backbone and numerous cationic groupings separated by six carbons: a hexamethylene chain (Gilbert and Moore 2005). This structure is very similar to many naturally occurring linear or α-helical antimicrobial peptides (AMPs) such as LL-37 (Heilborn et al. 2003). As mentioned previously AMPs are known to be important for innate immunity. They are produced by many cells within the wound bed such as keratinocytes and neutrophils and are thought to help protect the wound from infection (Sorensen et al. 2003). AMPs are positively charged and so can bind to bacterial cell membranes destroying their integrity and causing cell lysis (Moore and Gray 2007).

PHMB is thought to have a similar mechanism of action as it targets the outer and cytoplasmic membranes causing them to leak potassium ions and other cytosolic components resulting in cell death. It does not affect the neutral phospholipids in animal cell membranes but forms a strong association with acidic phosphatidylglycerol (PG) found in bacterial cell membranes (Ikeda et al.1984; Andriessen and Eberlein 2008).

By artificially depleting phosphorous in *E. coli* cultures Broxton et al. (1984) demonstrated that the reduced PG levels created in the test bacteria conferred resistance to PHMB. It has also been shown that PHMB binds to DNA and other nucleic acids causing them to precipitate out of solution damaging and inactivating the bacterial DNA (Allen et al. 2004).

PHMB has a broad spectrum of action and is effective against many of the bacteria commonly found in wounds including Gram-positive and Gram-negative bacteria, Staphylococcus *aureus* and methicillin - resistant *Staphylococcus aureus* (MRSA) and biofilms (Moore and Gray 2007; Wiegend et al. 2009).

Another advantage of PHMB demonstrated by Werthen et al. (2004) was that it was able to kill common ulcer-derived bacteria in the presence of human wound fluid. They were also able to show that PHMB could reverse the effect of elastase-expressing *P. aeruginosa* which is able to degrade wound fluid proteins and human skin during infection *ex vivo*.

In 2010, Cooper stated that there had been no reported cases of resistance to PHMB. It is thought that resistance is unlikely to develop because unlike monoatomic agents the polycationic PHMB is not affected by efflux pumps. When PHMB binds to the bacterial membrane it pulls acidic phospholipids reorganising the membrane and creating an acidic lipid domain. Due to the surface activity of the PHMB is does not enter the lipid domain that the efflux pumps require so it cannot be removed by the bacteria using this mechanism (Shah 2007).

PHMB formulations for wound treatment include gels and a variety of wound dressings as shown in Table 1.5.

Table 1.5: Biguanide Wound Dressings (Wound Care Handbook 2019-2020, BNF 2019)

Category	Dressing	Formulation	Supplier	Comments
Biguanides	Chlorhexidine Gauze Dressing BP	Fabric of leno weave, weft and warp threads of cotton or viscose containing chlorhexidine acetate 0.5 % ointment	Smith & Nephew Healthcare Ltd	Used to prevent infection in minor skin loss injuries
	Kendall AMD Antimicrobial Foam Dressing	NA semi occlusive polyurethane foam with polihexanide 0.5%	H & R Healthcare	Antibacterial barrier effective for up to 7 days. Not intended for use as a primary treatment for infection. Can be used in conjunction with prescribed therapies for treatment of infection.
	Prontosan Wound gel Dressing	Hydrogel containing betaine surfactant and polihexanide (0.1%)	B.Braun Medical Ltd	Used to cleanse, decontaminate and moisturise wounds.
	Suprasorb X + PHMB Dressings	Biosynthetic cellulose fibre dressing with polihexanide 0.3%	(L & R Medical UK Ltd	Used to treat critically colonised and infected wounds.
	Telfa AMD NA Wound Dressing	Absorbent pad with NA contact layer and non- woven backing with polihexanide 0.2%	H & R Healthcare	Used to prevent infection in dry to lightly exuding wounds

Most of the commercially available PHMB formulations are used to reduce the bioburden in wounds that are at risk of infection and are not intended as a primary treatment for infection. (Wound Care Handbook 2019-2020) Only Suprasorb® X + PHMB is currently licensed for the treatment of infected wounds (https://lohmannrauscher.co.uk/woundcare/suprasorb/suprasorb-x-phmb).

Suprasorb® X dressings are made of biosynthetic hydrobalance fibres which are created by *Acetobacterxylinium* during a cellulose fermentation process. The cellulose fibres produced are 200 times finer than cotton and are woven by the bacteria into a mesh which gives the material a large surface area, ability to absorb large amounts of wound fluid and a high tensile strength. The Suprasorb® X + PHMB dressing contains 0.3% PHMB. Wound exudate is absorbed into the dressing where the PHMB exerts its antimicrobial effect. The PHMB is also released into the wound fluid along a concentration gradient (Kingsley et al. 2009).

1.11.2. Iodine Dressings

Iodine was discovered by Bernard Coutois in 1811. Iodine occurs naturally as iodide salts in seaweeds, fish, shellfish and sea water and is essential for the production of thyroid hormones in man, which are required for maintenance of homeostasis (Cooper 2007).

Iodine is bactericidal, fungicidal, virucidal and sporicidal (McDonnell and Russell 1999). The broad spectrum of antimicrobial activity is due to the action of free iodine (I_2) which causes catastrophic changes to cell walls, membranes and cytoplasm and results in rapid microbial death (Cooper 2007).

Iodine rapidly penetrates microorganisms and attacks key proteins, nucleotides and fatty acids which cause cell death. Aqueous solutions of iodine are unstable and at least seven iodine species are present including: I_2, HOI, OI^-, H_2OI^+, I^{3-}, I^- and IO^{3-}. Only hydrated iodine (I_2) and hypoiodous acid (HOI) and iodine cation (H_2OI^+) have demonstrated antimicrobial properties. The pH of the solution influences the dynamic equilibrium, and the iodine concentration and

maximum antimicrobial activity occurs when the forms of iodine without antimicrobial activity are minimised (Gottardi 1999).

Lugol's iodine an aqueous solution containing iodine (5%) and potassium iodide (10%) has been used as a topical antimicrobial in wound care since it was developed in 1829 (Calissendorff and Falhammer 2017).

Aqueous and alcoholic solutions of iodine are widely used for skin preparation prior to surgery (Leaper and Durani, 2008). The solutions have however been associated with skin irritation, pain and excessive staining.

1.11.2.1. Povidone Iodine (PVP-I)

The problems associated with aqueous iodine solutions were overcome by the development of iodophores; povidone-iodine and poloxymer-iodine by complexing iodine and a solubilising agent or carrier which acts as a reservoir of the active "free" iodine (Gottardi 1991). Povidone iodine was first created in 1956 by Scelanski who combined iodine as a triiodide with polyvinyl-pyrrolidine (povidone), a non-ionic synthetic polymer which has no antimicrobial activity. The Iodine is rendered less toxic by incorporation with these surface-active agents and the polyvinyl-pyrrolidone iodine (PVP-I) complexes make it more soluble and less irritant and allergenic (Murdoch and Lagan 2013).

PVP-I (povidone-iodine) is available as aqueous and alcoholic solutions, sprays, creams, ointments and has been incorporated into tulle grass dressings; Inadine® (Leaper and Durani 2008).

In an aqueous environment, such as wound exudate, the PVP-I complex (iodophore) is diluted and slowly disperses to release free iodine, known as available iodine. The activity of the iodophore is related to the amount of iodine released (Cooper 2007), for example Betadine® 10% PVP-I aqueous solutions yield 1% available iodine. It has been reported that concentrations of PVP-I between 0.1 and 1% have a more rapid antimicrobial action than higher concentrations such as 10%. This was thought to be due the dilution weakening the bonds between the free molecular iodine and the polyvinyl-pyrrolidine causing the release of more free available iodine (Rackur 1985).

As the iodine is inactivated following the destruction of microorganisms further free iodine is released from the iodophore, maintaining equilibrium until the iodine reservoir is exhausted (Bigliardi et al. 2017).

The exact antimicrobial mechanism of action of iodine is not fully understood. There appear to be several bacterial structures and functions that iodine affects as shown in Figure 1.5. Early electron microscope and biochemical observations by Schreier et al. (1997) identified that iodine interacts with bacterial cell walls causing pores or solid - liquid interfaces which lead to the loss of cytosol. Iodine has been shown to react with C=C bonds in the unsaturated fatty acids in the bacterial lipid bilayer causing membrane disruption (Reimer et al. 1998). Iodine has also been found to inactivate substances in the cytoplasm and cause coagulation of nuclear material (Schreier et al. 1997).

Iodine binds to bacterial proteins which lead to their denaturation; this changes the structure and function of enzymes and structural proteins adversely affecting microbial function. Iodine oxidises S-H bonds in amino acids such as cysteine and prevents hydrogen bonding by reacting with N-H groups in arginine, histidine and lysine and the phenolic group of tyrosine (Cooper RA 2007).

Hydrogen bonding in nucleic acids is prevented by iodine binding to nucleotides such as adenine, cytosine and guanine. This prevents disulphide bridges being formed to link the double helix in the bacterium's DNA, affecting the formation of key proteins and enzymes (Gottardi 1985). No acquired resistance or cross resistance has been reported for iodine (Lachapelle, Castel and Casado 2013) and this is probably due to the multiple mechanisms of action of iodine (Sibbald, Leaper and Queen 2011).

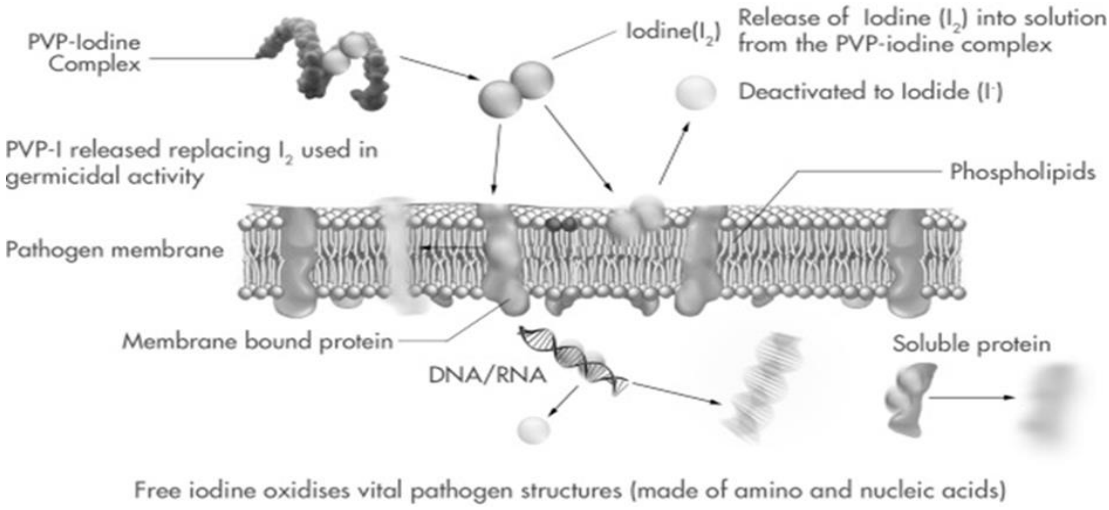


Figure 1.5: Mechanism of action of povidone iodine in equilibrium with free iodine. The active moiety is iodine, oxidising pathogen nucleotides and fatty/ amino acids and thus deactivates proteins as well as DNA/RNA (Bigliardi et al. 2017).

Iodine and iodophors have a broad spectrum of activity against Gram-positive and Gram-negative bacteria, tubercle bacilli, fungi, protozoa and viruses (Zamora 1986). Iodophors however have been shown to be less active than tinctures of iodine against certain spores and fungi and can be inactivated in the presence of organic matter such as blood or pus (Zamora et al.1985).

PVP-I is thought to alter redox potentials and contribute to a pro-oxidant effect in healing (Schmidt et al.1995). It has also been shown to be able to modify cellular mechanisms and enhance healing by activating monocytes, T lymphocytes and macrophages, enhancing pro-inflammatory cytokines (Burkes 1998). PVP-I may also inhibit excessive levels of proteases in chronic non-healing wounds (Eming et al. 2006). It was found to be less toxic than silver sulphadiazine or chlorhexidine in studies investigating the histological effects of antimicrobials on wound healing and demonstrated enhanced angiogenesis (Bennett et al. 2001).

1.11.2.2. Cadexomer Iodine

A different formulation iodophor dressing; Cadexomer iodine was introduced in 1981. Cadexomer iodine is a three-dimensional lattice of micro spherical, hydrophilic, biodegradable starch beads, 0.1 to 0.3mm in diameter which

contain 0.9% (w/w) iodine. The dextrin-epichlorohydrin beads absorb wound exudate and swell, the starch polymer separates allowing a slow release of free iodine. 1g of cadexomer can absorb 6ml of fluid and the slow release of iodine from the beads is designed to maintain iodine availability at 1ppm (Philips et al. 2015).

This ability to absorb large volumes of wound exudate is useful for treatment of heavily exuding wounds. Like other iodine formulations cadexomer iodine has a broad spectrum of antimicrobial activity and claims to have a sustained antimicrobial action which lasts up to 24 hours (Bianchi 2001).

Cadexomer iodine is available as an ointment and powder (Iodosorb®) and a paste layered between two sheets of gauze (Iodoflex®). shown in Figure 1.6.

Figure 1.6: Iodoflex®

Cadexomer iodine was shown to significantly reduce MRSA and total bacteria in colonised partial thickness wounds on the backs of pigs compared with cadexomer dressing (without iodine) and no treatment (Mertz et al. 1999).

A more recent study using an ex vivo skin explant biofilm model to compare the efficacy of five types of antimicrobial agents containing silver, iodine, PHMB, honey and ethanol found that a time release silver gel (Silvasorb® gel) and cadexomer iodine were most effective in reducing mature biofilm. The authors concluded that the results of this study indicated that the anti-biofilm activity was influenced by time of exposure, the number of applications, moisture levels and the sustained release dressing formulation (Philips et al. 2015).

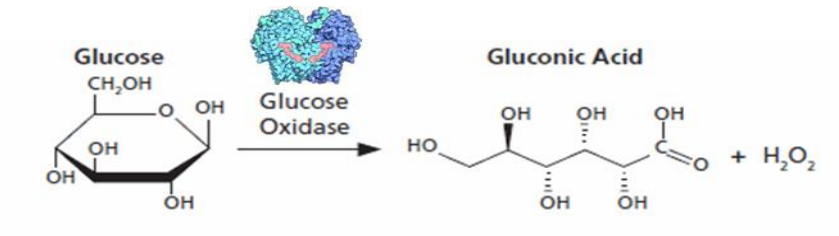
In 2010 a systematic review of randomised controlled trials (RCTs) which investigated the benefits and harm of iodine in wound care was undertaken by Vermeulen et al. They found only 27 RCTs had been published since 1976 which investigated the use of PVP-I or cadexomer iodine in acute and chronic

wounds, burns, pressure ulcers and skin grafts. Based on the evidence from the trials the authors concluded that iodine is an effective antiseptic and does not appear to impair wound healing (Vermeulen, Westerbos and Ubbink 2010).

A later review of the role of PVP-I and cadexomer iodine in the management of acute and chronic wounds, Murdoch and Lagan (2013), also concluded that the literature supports both these iodophors as being effective antimicrobial agents.

1.11.2.3. Hydrogel – Iodine composite dressings

Two novel composite hydrogel dressings; Oxyzyme® and Iodozyme® were awarded CE marks and launched in 2006 and 2007 as class III medical devices incorporating a medicinal substance (NICE 2014). The dressings consisted of two separately packaged hydrogels. The primary wound contact gel layer contained glucose and potassium iodide and the secondary gel layer contained the enzyme, glucose oxidase. Oxygen from the air was enzymatically captured by the glucose oxidase in the secondary layer and drawn into the hydrogel. The glucose oxidase enzyme then utilised the oxygen to oxidise the glucose in the primary hydrogel to produce gluconic acid and hydrogen peroxide (H_2O_2) (Figure1.7).



The hydrogen peroxide then oxidised the iodide in the primary hydrogel to produce antimicrobial iodine.

$$H_2O_2 + 2I^- \longrightarrow I_2 + 2HO^-$$

The hydrogen peroxide also underwent dissociation to generate oxygen that was released from the hydrogel into the wound.

$$2H_2O_2 \longrightarrow O_2 + 2H_2O$$

Figure 1.7: Chemical Reactions occurring within Iodozyme Dressings on exposure to air.

The amount of dissolved free oxygen produced beneath the two dressings over 24 hours is shown in Figure 1.8. As discussed previously, oxygen plays a key role in many wound healing processes; enabling metabolic support, matrix repair and facilitating an effective cellular response (Ivins et al. 2007). The dressings were described by Davis (2007) as acting like a molecular pump, transporting dissolved oxygen through the hydrogel layers from the external environment to the wound surface.

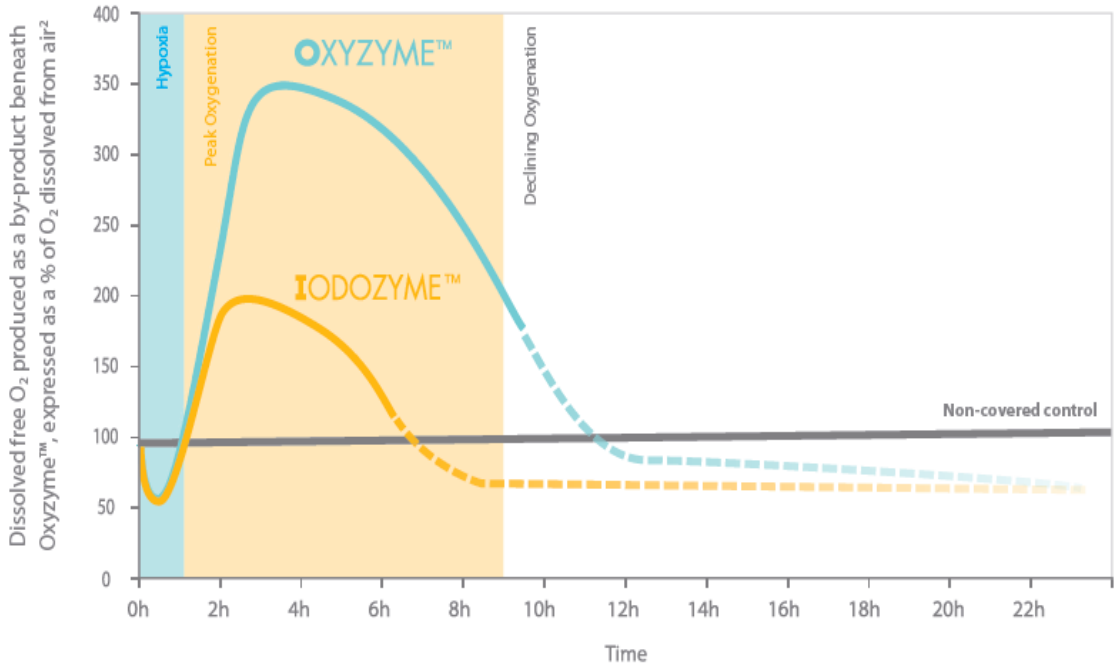


Figure 1.8: Dissolved free oxygen produced by Iodozyme and Oxyzyme over 24 hours (Archimed 2010a).

The level of iodine generated by the dressings is dependent upon the concentrations of glucose oxidase enzyme and potassium iodide, the pH, the surfactant concentration, and the dimensions of the hydrogel layers. Wood and colleagues (2010) stated that the peak level of iodine produced by Iodozyme was approximately 0.2%w/w.

Iodozyme produces levels of iodine about five times higher than those created by Oxyzyme over a 24-hour period as shown in Figure 1.9 (Wood et al. 2010). However, the levels of Iodine produced in Iodozyme are not as high as the levels used in the two other commercial iodine containing dressings; Inadine and Iodoflex, as illustrated in Figure 1.10 (Thorn et al. 2006).

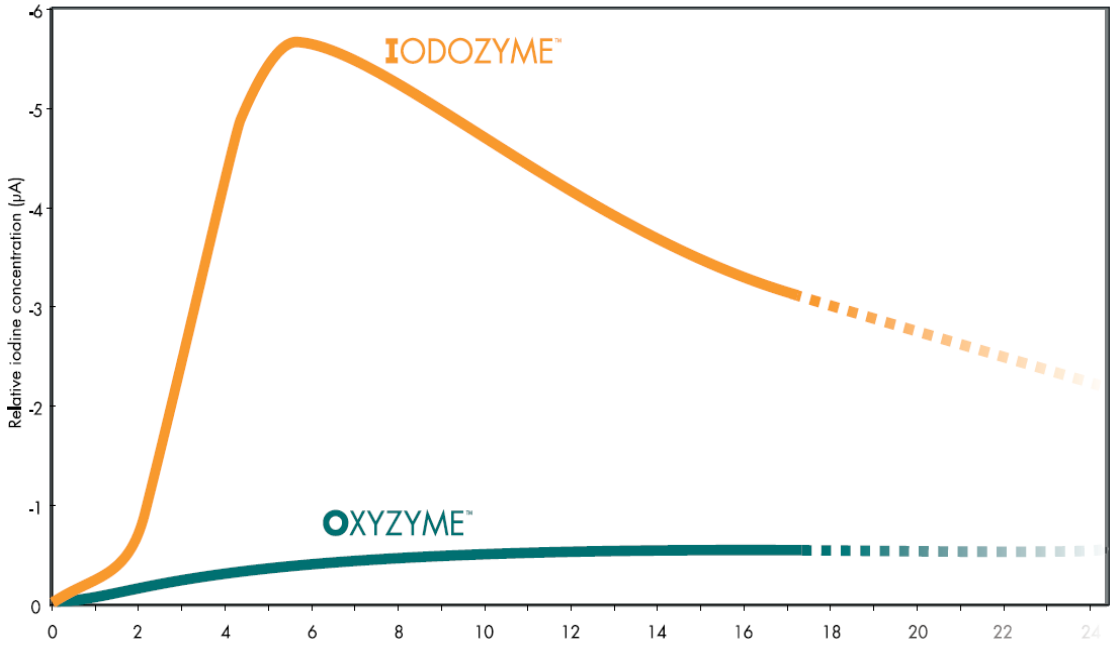


Figure 1.9: Relative Iodine produced by Iodozyme and Oxyzyme over 24 hours (Archimed 2010b).

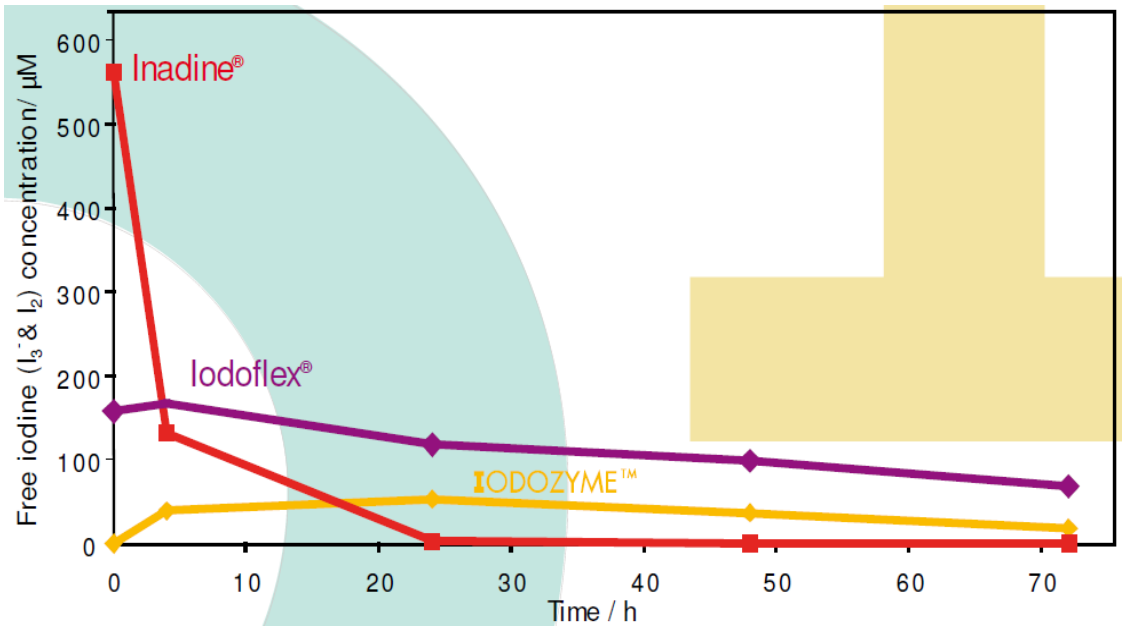


Figure 1.10: Iodine levels in Iodozyme in comparison to other iodine-containing dressings (Archimed 2010c).

An advantage of this system is that inactivated iodide can be reactivated by oxidation by hydrogen peroxide so the level of iodide in the dressing can be reduced (Cooper 2007). Oxyzyme was intended to be used on non-infected

wounds whereas Iodozyme was licenced for the treatment of infected superficial wounds (BNF 2015-2016).

There are very few clinical studies to support the efficacy of Iodozyme in treating chronic wounds (Wood et al. 2010). Marketing of both Oxyzyme and Iodozyme was discontinued in the UK in 2017.

1.11.2.4. Hyaluronate-iodine complex

Another innovative use of topical iodine involved the creation of a wound dressing which is a combination of Iodine and hyaluronan (HA) (Hyiodine®). HA is a glycosaminoglycan, which is a naturally occurring component of the extracellular matrix of the skin (Brown 2004). It is highly absorbent, able to take up 1000 to 3000 times its own weight in water which enables it to absorb large volumes of wound exudate (Jiang et al. 2007). It is thought to enhance the motility of cells by binding to CD44 receptors and the receptor for HA-mediated motility on lymphocytes, inflammatory cells and connective tissue (Anderson 2001). This action facilitates the migration of monocytes, macrophages and neutrophils to the wound centre promoting healing. Frankova et al. (2006) demonstrated that hyaluronate-iodine complex improved wound healing by increasing the production of IL-6 and TNF-α by lymphocytes. These proinflammatory mediators stimulate keratinocyte and fibroblast proliferation and migration.

The iodine complex provides the dressings antimicrobial activity, enabling it to be used for the treatment of infected wounds, and also helps stabilize the hyaluronic acid and prevents its degradation by bacteria. (https://www.hyiodine.com/about-hyiodine).

A study conducted by Brenes et al. (2011) on 14 patients was designed to validate the use of hyaluronate-iodine as a wound healing agent. They concluded that the combination dressing was safe and efficacious for healing a variety of wounds including traumatic, diabetic, and postoperative and stasis ulcers. The commercial Iodine containing dressings available in the UK during this study are listed with their clinical indications in Table 1.6.

Table 1.6: Commercial Iodine Dressings (Wound Care Handbook 2019-2020, BNF 2017 and 2019)

Dressing	Manufacturer	Formulation	% Iodine	Indications
Inadine	Systagenix Wound Management Ltd	Knitted viscose primary dressing impregnated with 10% povidone - iodine	1% w/w available iodine	Management and prevention of infection in ulcers, minor burns and minor traumatic skin injuries
Iodosorb	Smith & Nephew Healthcare Ltd	Cadexomer powder or ointment with cadexomer iodine	0.9% w/w	Treatment of chronic exuding wounds. Reduce bacteria on wound srurface
Iodoflex Paste	Smith & Nephew Healthcare Ltd	Cadexomer iodine dressing in a paste with gauze backing	0.9% w/w	Treatment of chronic exuding wounds. Reduce bacteria on wound surface
Iodozyme*	Crawford Healthcare Ltd	Hydrogel two compartment dressing containing glucose oxidase and iodide ions.	<0.04% iodide ions & glucose oxidase	Infected, superficial wounds with low to moderate levels of exudate
Oxyzyme*	Crawford Healthcare Ltd	Hydrogel two compartment dressing containing glucose oxidase and iodide ions.	<0.04% iodide ions & glucose oxidase	Non-infected, superficial wounds with low to moderate levels of exudate.
Hyiodine	H & R Healthcare Ltd	Viscous solution (gel) of hyaluronic acid(sodium hyaluronate 1.5%) iodine 0.1% and potassium iodide 0.15%		Fistulae and sinuses, cavity wounds, surgical dehiscence, infected wounds and chronic wounds.

* Iodozyme and Oxyzyme were withdrawn from the UK market in 2017

1.11.3. Silver Dressings

Silver has been used as an antimicrobial agent for centuries. It exists in two forms: as a neutral atom (47 electrons and 47 protons) described as “elemental” or “metallic” silver or as a positively charged atom (46 electrons and 47 protons)

known as “ionic” silver or “silver cation Ag^{+}”. Silver cations are potent antimicrobials (Barillo and Marx 2014).

Elemental silver is relatively unreactive but in the presence of oxygen or in aqueous fluids such as wound exudate the elemental silver oxidises to create silver oxide ($Ag^{+}{}_{2}O^{2-}$). When dissolved in fluids silver oxide dissociates to release antimicrobial cations, Ag^{+}. The availability of silver ions is dependent upon this dissociation and their solubility in wound fluid which is pH dependent. Wound fluid contains glucose, proteins, lactate, urea and electrolytes including chloride anions (CL^{-}). The concentration of chloride ions in wound fluid can influence the availability of Ag^{+} in solution (White and Cooper 2005). At low chloride concentrations (around 100mM) soluble silver binds to the bacteria cell surface and can exert its antimicrobial effects (Bragg and Rainnie 1974).

However, at moderate concentrations of chloride it binds with the Ag+ to form insoluble AgCl. At higher chloride concentrations, which can be found in wound exudate, the silver returns to solution as the bioavailable anion $AgCl^{2-}$ (Gupta, Maynes and Silver 1998). This ability of highly diluted heavy metals such as silver to inhibit microorganisms was first described as “oligodynamic” by von Nageli in 1893 (White and Cooper 2005).

Silver appears to have at least four different mechanisms of antimicrobial action. Bacterial membranes contain polymers with highly electronegative groups which can absorb metal cations (Zhang and Rock 2008). Binding of silver cations to tissue proteins causes structural changes in the bacterial cell membranes, causing them to leak or rupture and which prevents the passage of nutrients into the cell. The silver ions enter the cell and bind to proteins and interfere with enzyme function and inhibit energy production by inhibition of the respiratory chain involved in electron transport (Lemire, Harrison and Turner 2013). Also, inside the cell Ag^{+} binds and denatures bacterial DNA and RNA, inhibiting cell division and replication. The fourth action is the generation of reactive oxygen species that damage microbial cell components (Marx and Barillo 2014).

Concentrations as low as 10^{-9} to 10^{-6} mol/L silver cations have been shown to be effective against a wide range of microorganisms including Gram-positive

and Gram-negative bacteria, fungi, protozoa and viruses (Russell and Hugo 1994). Silver is also effective against many antibiotic – resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) (Percival, Bowler and Russell 2005).

In vitro studies investigating the effect of silver dressings on biofilms have suggested that the silver can reduce bacterial adhesion and disrupt the biofilm matrix (Chaw et al. 2005). Silver has been shown to kill bacteria within a biofilm matrix and may increase the susceptibility of bacteria to antibiotics (Percival et al. 2008; Thorn et al. 2009; Kostenko et al. 2010). There is also more recent evidence that silver is effective against biofilms of multidrug - resistant bacteria including MRSA and VRE (Kaqlan et al. 2017).

The aim of treatment of wounds with topical silver is to reduce wound bioburden, treat local infection and prevent systemic spread of infection (International Consensus 2012). A variety of different silver formulations have been incorporated into numerous carrier dressings such as alginates, hydrofibers and foams as shown in Table 1.7. One of the common formulations used in antimicrobial dressings is elemental silver which consists of silver metal or nanocrystalline silver, which are very small crystals between 10-100 nanometres in diameter (Wilkinson, White and Chipman 2011).

Other dressings contain organic compounds such as silver alginate and silver carboxymethylcellulose. The third type of silver used in dressing formulations are inorganic compounds such as silver oxide, sulphate, phosphate, chloride, silver-calcium-sodium phosphate, silver zirconium compound and silver sulfadiazine (International Consensus 2012; Edwards-Jones 2009).

Silver sulfadiazine (SSD) was created by a combination of sulfadiazine (SD), a sulphonamide antibiotic, with silver nitrate to form a complex silver salt. The polymeric structure contains six Ag^+ ions bound to six sulfadiazine molecules via the nitrogen of the sulfadiazine pyrimidine rings (Fox 1983).

SSD has been formulated as 1% SSD antimicrobial topical cream (Flamazine™) which has extensively been used in the topical treatment of burns (White and Cooper 2005). More recently SSD has been incorporated into

dressings such as tulles (UrgoTul SSD) and foams (Allevyn Ag) for the treatment of infected wounds (Table 1.7).

The silver component of a wound dressings may be coated on an external surface of the dressing (often elemental or nanocrystalline silver) or be incorporated within the body of the dressing. Within the dressing the silver may be coated on the dressing material or within the spaces in the dressing (either compound or elemental silver) or may be an integral part of the dressing material such as silver alginate fibres (International Consensus 2012).

Silver coated on the dressing wound contact layer will have an antimicrobial effect on the wound surface where it is in direct contact. However, many wound dressings are designed to absorb wound exudate and bacteria and so the silver has an antimicrobial effect within the dressing. The silver can also diffuse from the dressing into the wound to have a wider effect on the wound surface (Thomas and McCubbin 2003).

Dressings differ in the total amount of silver held within the silver “reservoir” and the mechanism of silver cation release. It has been shown that in the wound environment the amount of silver delivered to the wound does not directly correlate with the amount of silver contained in the dressing as the silver ions interact with components in the wound exudate such as proteins and chloride ions (Woo et al. 2008). The amount of silver metal or silver compound incorporated in the different dressings and the rate of release of Ag^+ ions and the duration and peak levels of silver release appear to influence the antimicrobial effect (Ovington 2004; Sood, Granick and Tomaselli 2014).

The presence of bacterial biofilms in chronic wounds reduces the antimicrobial efficacy as the silver cations tend to bind to the proteins of the extracellular matrix instead of the bacterial cell wall (Mertz 2003). Another potential concern relating to topical silver is the lack of specificity of the action of silver which may not only affect bacterial proteins but also those of the host leading to potential cytotoxicity. Consequently, in wounds with lower concentrations of bacteria the effect of silver on the wound tissue may be greater and could slow down healing (Innes et al. 2001). Silver is also known to accumulate in the skin and other

tissues producing an undesirable cosmetic effect known as argyria (Lansdown and Williams 2004).

A number of systematic reviews have been undertaken to try to evaluate the antimicrobial efficacy of silver dressings which produced limited and sometimes controversial conclusions. In 2007 a Cochrane review was undertaken by Vermeulen at al. which identified only three RCTs comparing silver dressings with foam or alginate dressings or best practice for treating infected wounds. Two RCTs, one which compared silver foam (Contreet®) with hydrocellular foam (Allevyn®) and the second comparing silver alginate (Silvercel®) with a plain alginate dressing (Algosteril®), found no differences between the dressings. However, a third study comparing silver foam (Contreet®) with a range of dressings; foams and alginates (53%), hydrocolloids (12%), gauze (3%), silver dressings (17%), other antimicrobial dressings (9%) or other active dressings (6%), reported statistically significantly faster healing rates with Contreet® but did not report complete healing. The authors concluded there was insufficient evidence to recommend the use of silver dressings in the treatment of infected or contaminated wounds.

A later Cochrane review in 2010 by Storm-Versloot identified 26 RCTs, 20 of the RCTs involved burn wounds, where silver dressings or SSD cream had been used to prevent wound infection. Most of the trials were small and considered to be of poor quality and the authors concluded there was insufficient evidence to support the use of silver dressings or creams as they did not appear to promote wound healing or prevent wound infection (Storm-Versloot et al. 2010).

The VULCAN trial compared silver dressings with non adherent dressings (in addition to standard compression) in the treatment of venous leg ulcers and found no significant differences in median time to complete healing or healing rates at 3, 6 and 12 months (Michaels et al. 2009). Whilst this was considered to be a well-designed RCT (NICE 2016) there has been some criticism of its relevance to clinical practice and the fact that some patients included did not necessarily have wounds that were infected or were at high risk of becoming infected (Gottrup and Apelqvist 2020). Despite the lack of definitive evidence supporting the efficacy of silver dressings they are currently in widespread

clinical use (NICE 2019). It has been suggested that a properly powered clinical trial is needed to justify the extra cost and compare the efficacy and safety of silver dressings against other established antimicrobials such as povidone-iodine (Ousey, Roberts and Leaper 2016).

The silver dressings available in the UK are listed in Table 1.7 grouped into different classifications depending upon their carrier dressing material.

Table 1.7: Commercial Silver Dressings (Wound Care Handbook 2019-2020, BNF 2019)

Dressing Type	Product	Manufacturer	Carrier Dressing	Indications
Alginates	Algisite Ag	Smith& Nephew Healthcare Ltd	Calcium Alginate impregnated with silver	Infected wounds or wounds at risk of infection. Can be left on wound up to 7 days
	Askina Calgitrol Ag	B.Braun Medical Ltd	Calcium Alginate and silver alginate with 10% of bonded water polyurethane foam backing	Infected or critically colonised wounds. Can be left on wounds up to 7 days
	Biatain Alginate Ag	Coloplast Ltd	Calcium alginate, CMC and ionic silver.	Moderate to heavily exuding infected wounds. Haemostatic. Wear time up to 7 days
	Melgisorb Ag	Molnycke Health Care Ltd	Alginate and Carboxymethylcellulose (CMC) fibre dressing with ionic silver	Moderate to heavily exuding partial and full thickness wounds. ? Infected
	Silvercel	KCl an Acelity Company	Hydro alginate, High G (guluronic acid) alginate, carboxymethylcellulose & silver coated nylon fibres.Releases silver ions into the wound	Moderate to heavily exuding and bleeding wounds. ? infected wounds
	Sorbsan Silver Flat	Aspen Medical Europe Ltd	Calcium Alginate impregnated with 1.5% silver fibres. High in mannuronic acid and low in guluronic acid.	Heavily exuding and infected wounds. Haemostasis in bleeding wounds.
	Sorbsan Silver Plus	Aspen Medical Europe Ltd	Sorbsan Silver bonded to a super absorbent viscose pad.	Heavily exuding and infected wounds
	Suprasorb A + Ag	Lohmann & Rauscher (UK) Ltd	Calcium Alginate dressing with silver	Infected heavily exuding wounds Can use for 5-7 days
	Tegaderm Alginate Ag	3M Health Care Ltd	High G (guluronic acid) calcium alginate and Carboxymethylcellulose with silver sodium hydrogen zirconium phosphate	Moderate to heavily exuding infected wounds. Can use for up to 14 days, silver ions protect the dressing from infection.
	UrgoSorb silver	Urgo Medical Ltd	Highly absorbent guluronic type calcium alginate fibres and carboxymethylcellulose dressing impregnated with 0.5% ionic silver.	Moderate to heavily exuding wounds.

Dressing Type	Product	Manufacturer	Carrier Dressing	Indications
Cream	Flamazine	Smith& Nephew Healthcare Ltd	Silver Sulfadiazine 1.0% w/w cream (10mg/g)	Prophylaxis and treatment of burns. Short term use in infected chronic wounds.
Soft Polymers	Allevyn Ag Gentle	Smith& Nephew Healthcare Ltd	Soft polymer wound contact dressing with SSD impregnated polyurethane foam layer.	Acute and chronic exuding wounds and fungating/malignant wounds
	Mepilex Ag	Molnycke Health Care Ltd	Soft silicone wound contact dressing with polyurethane foam film backing with silver.	Low to moderately exuding wounds. Infected wounds Antimicrobial activity up to 7 days
	Urgotul Silver	Urgo Medical Ltd	Non adherent soft polymer wound contact dressing with TLC – Ag Technology containing silver particles.	Non to low exuding wounds with signs of localised infection or critical colonisation Can be used for up to 7 days
Tulle	UrgoTul SSD	Urgo Medical Ltd	Polyester mesh impregnated with hydrocolloid, petroleum jelly and silver sulfadiazine particles.	Non to low exuding wounds, infected or at risk of infection. Can be used for up to 7 days.
Foams	Acticoat Moisture Control	Smith& Nephew Healthcare Ltd	3 layer polyurethane foam; wound contact layer with nanocrystalline silver, hydrocellular foam layer & waterproof outer layer	Antimicrobial barrier dressing for full and partial thickness wounds. Works for 7 days
	Allevyn Ag	Smith& Nephew Healthcare Ltd	Silver sulfadiazine impregnated polyurethane foam film	Infected, malignant and fungating wounds. Can be used for up to 7 days.
	Aquacel Ag Foam	ConvaTec Ltd	Hydrofiber (sodium carboxymethylcellulose) and polyurethane foam dressing with 1.2 % ionic silver	Infected wounds or wounds at risk of infection. Antimicrobial activity for up to 14 days?
	Biatain Ag Adhesive	Coloplast Ltd	Soft polyurethane foam film impregnated with patented Silver complex with a vapour permeable film backing	Moderate to highly exuding infected wounds. Wear time up to 7 days

	Product	Manufacturer	Carrier Dressing	Indications
	PolyMem Silver	Aspen Medical Europe Ltd	Polyurethane foam containing monocrystalline silver (0.2%/2000PPM) with a vapour-permeable film backing.	Low to moderately exuding infected and critically colonised wounds and wounds at risk of infection
	Polymem Silver WIC	Aspen Medical Europe Ltd	PolyMen Silver without film backing designed for cavity wounds.	Low to moderately exuding infected cavity wounds.
	UrgoCell Silver	Urgo Medical Ltd	NA polyurethane foam dressing containing silver with TLC Healing Matrix Wound contact layer	Low to moderately exuding exuding wounds with signs of infection or critical colonisation.
Hydrofiber/hydro colloids	Aquacel Ag	ConvaTec Ltd	Soft non-woven pad containing hydrocolloid fibres impregnated with 1.2% W/W ionic silver.	Infected wounds, shallow, cavity and sinus wounds.
	Aquacel Ag+ Extra	Convatec Ltd	2 layers of Aquacel Ag Hydrofiber (sodium carboxymethylecelluose) with 1.2% ionic silver. 9 x stronger and 50% more absorbent than Aquacel Ag	Moderate to highly exuding wounds that are infected or at risk of infection. Wounds where bacteria are causing chronicity.
	Physiotulle Ag	Coloplast Ltd	Knitted polyester net impregnated with CMC particles suspended in petrolatum and silver sulfadiazine	Wounds with critical colonisation or at risk of infection
	Urgoclean Ag	Urgo Medical Ltd	Non adherent poly – absorbent (polyacrylate) fibres with TLC – Ag healing matrix.	Exuding infected or at risk of infection wounds. Can leave in place up to 7 days.

1.11.4. Honey Dressings

Honey has been used in wound care since ancient times. Egyptian papyri dating from 2000BC describe honey being used to treat wounds (Zumla and Lulat 1989). Honey is created by bees reducing the water content of nectar and converting the sucrose to fructose using their salivary enzyme “invertase” (Cray 2010). Honey is a supersaturated solution containing about 17% water and 80% sugars, mainly fructose and glucose. It also contains proteins, enzymes; glucose oxidase and catalase and around 18 essential and non-essential amino acids (Sharp 2009).

Honeys of differing botanical provenance have varying levels of anti-bacterial and wound healing activity (Merckoll et al. 2009). Honey has been shown to have a broad spectrum of antibacterial activity and has been reported to inhibit more than 80 species of bacteria including multi- drug resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, groups A and C *Streptococci*, *Pseudomonas aeruginosa* and *Actinomyces* spp. (Maddocks et al. 2012). Honeys have a low pH between 2.3 and 4.5 which can inhibit the growth of most microorganisms (Molan 2001).

Initially the antimicrobial activity of honey was thought to due to the high osmolarity caused by the high sugar content (80% w/v) and low availability of water, which reduces the ability of bacteria to reproduce (Molan 1992). When used on heavily exuding chronic wounds the honey is diluted to an extent that the bacteria could potentially replicate. However, Sackett in 1919 observed that the antimicrobial activity of some honeys increased when the honey was diluted thus indicating that the honey had other mechanisms to reduce bacterial growth.

In 1963 White and colleagues demonstrated that the activity of honey in low concentrations is due to the production of hydrogen peroxide which results from glucose oxidase in honey coming into contact with catalase in wound exudate. However, the production of hydrogen peroxide by some honeys, such as Manuka honey produced from nectar of the Manuka tree (*Leptospermum scoparium*) is low. The antimicrobial activity of Manuka honey is not affected by

catalase and has been described as “non-peroxide activity” or NPA and is now known to be due to the Unique Manuka Factor identified as methyl glyoxal (MGO) (Adams et al. 2008; Mavric et al. 2008). MGO is created by the spontaneous dehydration of dihydroxyacetone (DHA) a phytochemical found in the nectar of flowers of *Leptospermum scoparium*, *Leptospermum polygalifolium* and some related *Leptospermum* species native to New Zealand and Australia (Adams et al. 2009; Williams et al. 2014; Norton et al. 2015). The MGO reacts with macromolecules such as DNA, RNA and proteins to exert its antimicrobial activity (Adams et al. 2008, Mavric et al. 2008; Majtan et al. 2014).

In 2010 Kwakman et al. were able to fully characterise the antibacterial activity of medical-grade honey sourced in the Netherlands. They identified that this honey contains an antimicrobial peptide, bee defensin–1 which had been previously isolated from royal jelly and that it made a significant contribution to antimicrobial activity.

Attempts have been made standardise the quality of manuka honeys by measuring the concentration of MGO and labelling products with their Unique Manuka Factor (UMF®) regulated by the UMF® Honey Association (New Zealand). The standards compare the NPA of the honey with phenol. A manuka honey with a UMF 10+ has equivalent NPA to 10% phenol solution and UMF 12+ a 12% phenol solution. UMF 12+ is equivalent to 355mg/kg of MGO and this is the quality of honey used in all the Medihoney Dressings (Cokcetin et al. 2016).

Medihoney was the first modern honey dressing, developed from Manuka honey in Australia and licensed in 1999. Since then, a number of formulations have been created from sterile honey without additives, honey in ointments and honey impregnated dressings in the form of gel sheets, alginate sheets, meshes and tulles.

A large variety of CE- marked sterile honey and honey-impregnated dressings are commercially available in the UK for the treatment of wounds. The honey dressings have been classified depending on their carrier dressing formulation in Table 1.8.

The commercial dressings contain honeys from different geographic locations such as Manuka honey from New Zealand and medical grade honey from the UK, Bulgaria and India. The term medical grade honey usually indicates that the honey has been filtered to a higher level than food grade honey and has been sterilised (Cooper and Jenkins 2009).

Studies have traditionally tested liquid honey against planktonic bacteria. More research is required to determine the efficacy of honey wound dressings, and the influence of the carrier dressing on single and multiple species biofilms. Microbial resistance to honey has never been reported (Kwakman et al. 2008). The *in vitro* activity of honey against antibiotic resistant bacteria and the reported success of honey used to treat chronic non – healing wounds make honey an interesting treatment option (Mandal and Mandal 2011).

Table 1.8: Commercial Honey Dressings (Wound Care Handbook 2019-2020, BNF 2019)

Form	Dressing	Formulation	Supplier	Indications
Honey gels	Activon Honey	100% medical –grade manuka honey	Advancis Medical	Infected wounds and cavities
	L-Mesitran Soft Ointment dressing	40% medical grade honey	Aspen Medical Europe Ltd	Burns, acute and chronic colonised wounds .
	MANUKApli Honey	100% leptospermum Medical Grade Manuka Honey	Manuka Medical Ltd	Chronic wounds, burns, surgical and trauma wounds.
	Medihoney Antibacterial Medical Honey	100% Standardised medical grade, Leptospermum sp. Honey.	Derma Sciences Europe Ltd	Superficial wounds and burns. Deep wounds, sinuses, wounds with necrosis and slough; infected wounds
	Medihoney Antibacterial Wound Gel	Standardised medical grade-honey Leptospermum sp. 80% with waxes and oils to increase viscosity and aid application. Single patient use tube	Derma Sciences Europe Ltd	Superficial wounds and burns, deep wounds, sinuses, wounds with necrosis and slough; infected wounds. More like ointment designed to stay in place.
	L - Mesitran Ointment	48% medical-grade honey, medical-grade hypoallergenic lanolin, sunflower oil, cod liver oil, Calendula officinalis, Aloe Barbadensis, vitamins C and E and zinc oxide.	Aspen Medical Europe Ltd	Chronic wounds, fungating wounds and colonised acute and post -operative wounds.

Form	Dressing	Formulation	Supplier	Indications
Honey Impregnated Dressings Tulle	Actilite Dressing	Knitted NA Viscose net dressing coated with 99% medical grade manuka honey and 1% manuka oil	Advancis Medical	Use on low to moderately exuding wounds. Granulating and epithelializing wounds.
	Activon Tulle	Knitted viscose mesh impregnated with 100% medical grade manuka honey	Advancis Medical	Eliminates wound odour and antibacterial. Chronic and infected wounds.
	L- Mesitran Tulle	Non – adherent polyethylene dressing impregnated with L- Mesitran Soft 40% ointment.	Aspen Medical Europe Ltd	
	Medihoney Antibacterial Honey Tulle	Non-adherent woven fabric impregnated with Medihoney Antibacterial Medical Honey.	Derma Sciences Europe Ltd	Acute and chronic, infected, malodourous Wounds and graft sites, burns and surgical wounds.
	MelMax	Inert Acetate wound contact layer impregnated with a metal –ion formulation and medical grade buckwheat honey 75% in ointment basis. Contains calcium, Rubidium, Potassium, Zinc and Citric acid.	Double Wing Medical	Claims to regulate MMP balance in the wound by pH modulation. . Enhances extracellular matrix formation, reduces production of reactive oxygen species and assists the healing process.
	Melladerm Plus Tulle	Knitted viscose impregnated with medical grade honey (Bulgarian, mountain flower) 45% in a basis containing polyethylene glycol. Produces H_2O_2 25g/g honey forup to 48hours.	Sanomed Manufacturing BV	Superficial wounds, abrasions, infected wounds, burns, leg ulcers, diabetic ulcers, sloughy and necrotic malodourous wounds

Form	Dressing	Formulation	Supplier	Indications
	Algivon	Absorbent, calcium alginate dressing impregnated with 100% medical grade manuka honey	Advancis Medical	Suitable for large cavity wounds and large areas of necrotic and sloughy tissue.
Alginates	Algivon Plus	Reinforced calcium alginate dressing impregnated with 100% medical grade manuka honey	Advancis Medical	Moderately exuding and infected wounds. Chronic wounds an burns
	Medihoney Gel Sheet	Non adherent Sodium alginate dressing and 100%Medihoney Antibacterial Medical Honey. (20% alginate & 80% honey)	Derma Sciences Europe Ltd	Acute and chronic, infected, malodourous Wounds and graft sites, burns and surgical wounds.
	Medihoney Antibacterial Honey Apinate Dressing	Non – adherent antibacterial honey dressing containing calcium alginate and Medihoney Antibacterial Honey.	Derma Sciences Europe Ltd	Acute and chronic, infected, malodourous Wounds and graft sites, burns and surgical wounds.
	L-Mesitran Border	Hydrogel, low adherent, semipermeable dressing contains 30% medical grade honey, an acrylic polymer gel and water with a polyurethane film backing with adhesive border.	Aspen Medical Europe Ltd	Can absorb up to 7 times it's weight in exudate. Acute and chronic and fungating wounds.
Hydrogel	L-Mesitran Net	Open –weave net dressing coated with hydro active honey-hydrogel; contains 20% medical-grade honey, acrylic polymer gel and water on a polyester mesh structure that allows the passage of exudate	Aspen Medical Europe Ltd	Low to heavy exuding acute, chronic or fungating wounds

1.11.5. Wound Dressing Prescribing

As described previously there are a large variety of wound dressings available with a wide range of physical characteristics such as adhesion, fluid handling properties and the ability to prevent or treat infection. The ability of clinicians to choose an appropriate wound dressing based on clinical evidence is hindered by the lack of robust evidence of their clinical or cost effectiveness. A NICE Evidence summary published in 2016 concluded there was little high-quality evidence in the form of randomised control trials or systematic reviews of controlled clinical trials to support the use of advanced or antimicrobial dressings (NICE 2016). Despite this, the use of these dressings is extensive and accounted for £92 million in prescription costs in primary care in England in the year to July 2018 (NICE 2019). Analysis of prescribing data from the NHS Business Service shown in Figures 1.11 and 1.12 illustrate that prescriptions for the antimicrobial dressings; honey, iodine and silver accounted for 3.1%, 11.8% and 8.7% respectively of the total number of prescriptions for wound dressings. However, in terms of prescription costs (Figure 1.12) the expenditure on silver dressings (17.7% = £16.2 million) was much higher than the expenditure on honey (1.8% = £1.7 million) and iodine (2.4% = £2.2 million) dressings. This data suggests that iodine dressings are the most widely prescribed type of antimicrobial dressing in primary care.

The data does not include dressings used in hospitals and private care settings so is an underestimate of the total use of wound dressings in England. It does however demonstrate the high cost of the use of these products in a healthcare community.

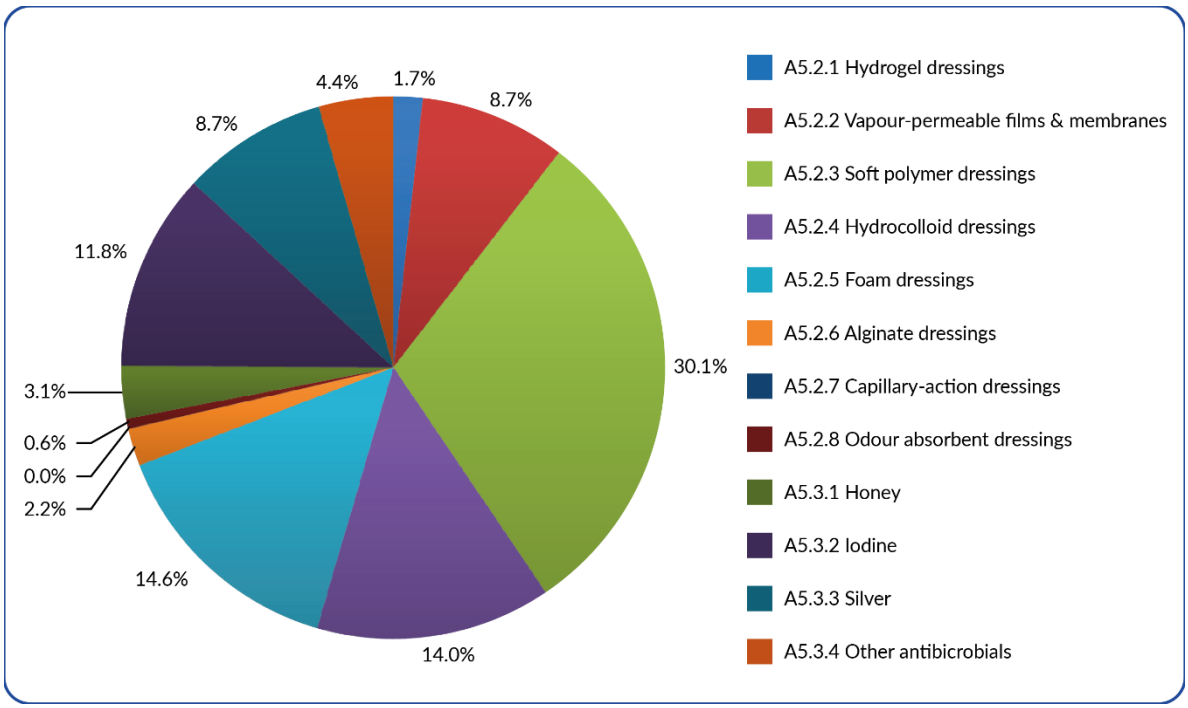


Figure 1.11: Annual prescribing volumes of advanced wound dressings in primary care in England. (August 2017- July 2018) © NICE [2019]. Prescribing data, metrics or supporting resources. Available from https://www.nice.org.uk/advice/ktt14/chapter/Prescribing-data-metrics-or-supporting-resources . All rights reserved.

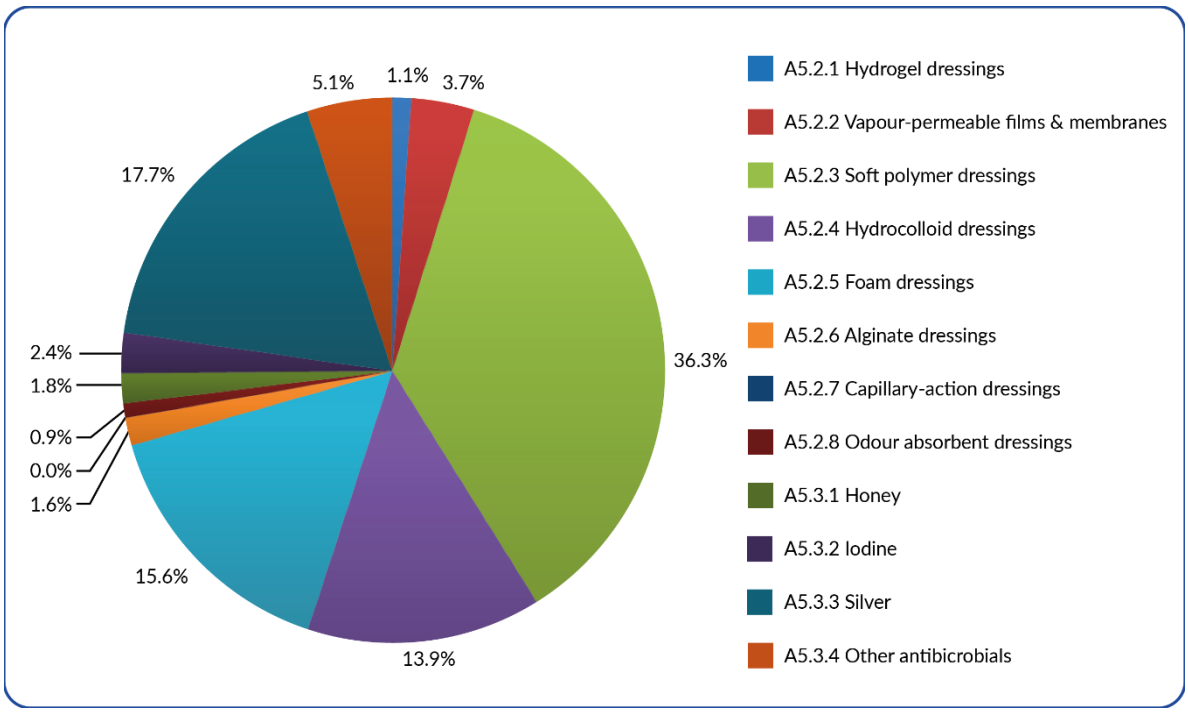


Figure 1.12: Percentage of the annual prescribing costs of the different classes of advanced wound dressings in primary care in England (August 2017- July 2018) © NICE [2019]. Prescribing data, metrics or supporting resources. Available from https://www.nice.org.uk/advice/ktt14/chapter/Prescribing-data-metrics-or-supporting-resources . All rights reserved.

The British National Formulary (BNF 2019) advises prescribers that for local wound infection a topical antimicrobial dressing can be used to reduce the level of bacteria at the wound surface but will not eliminate spreading infection. The BNF states that medical grade honey is antibacterial, that iodine dressings can be used to treat clinically infected wounds and silver dressings should only be used when clinical signs of infection are present. There is no guidance to aid the selection of an appropriate AWD if a particular bacterial infection is present in the wound and prescribers are advised to consider the amount of wound exudate when selecting a dressing. This suggests that there is still a gap in knowledge available to assist clinicians in the choice of the most effective AWD to treat a particular wound pathogen.

As mentioned in the discussion of the different classes of antimicrobial dressings, systematic reviews and meta – analyses have identified a paucity of good quality evidence to support the use of advanced or antimicrobial dressings particularly for the treatment of chronic wounds (NICE 2016).

Antimicrobial wound dressings (AWD) are usually classed as medical devices provided the antimicrobial agent is considered to provide an ancillary action on the wound (Centre for Evidence-based Purchasing 2008). Up until the introduction of the new European Medical Device Directive in 2017 (MDR 2017/745) medical devices had to conform to the essential requirement on safety and performance described in the Medical Device Directive (93/42/EEC). Clinical data was required to demonstrate satisfactory performance of the medical device and any adverse effects. Unlike medicinal/pharmaceutical products which require data from RCTs, wound dressings, classed as medical devices, have been licensed and marketed without having undergone rigorous clinical trials and therefore the clinical evidence supporting their effectiveness is often limited.

Traditionally dressings have undergone standard laboratory tests that are described in the British Pharmacopoeia to measure their physical properties such as absorbance, strength, moisture vapour permeability and conformability. However, these tests may not predict how well the dressings will perform in the

clinical situation (Centre for Evidence based Purchasing Buyer's 2008 guide: advanced wound dressings).

In vitro studies which are designed to establish the effectiveness of antimicrobial dressings are often difficult to compare as they use different experimental methodologies. In 2014 a draft British Standard "EN16756: Antimicrobial wound dressings - Requirements and test methods", was published for public consultation. Last accessed on 12th February 2020 it was still in draft form and was not an established British Standard (https://standards.globalspec.com/std/1688355/PREN%2016756).

As there are still no universally accepted standards for *in vitro* testing to compare the efficacy of the antimicrobial dressings this makes selection of dressings on the basis of scientific evidence difficult.

The idea for this research arose from this gap in knowledge regarding the lack of evidence supporting the efficacy of different classes of antimicrobial dressings and a discussion with the Sheffield wound care formulary team who wanted to be able to advise clinical colleagues about the most appropriate choice of AWD if a particular bacterium had been identified in a wound. Having personally developed numerous wound care formularies for a number of NHS Hospital Trusts it had always been a challenge to find independent, peer reviewed literature which investigated the clinical efficacy of the range of available AWD. Sensitivity testing against a range of antimicrobials may aid the selection of appropriate systemic antibiotic treatment but does not often assist the choice of AWD as very few manufacturers actually state which bacteria their dressings are effective against.

This lack of evidence led to the development of a pilot study to investigate the antimicrobial properties of a selection of antimicrobial dressings that were is common use in Sheffield (Bradshaw 2011). This intial research suggested that the species of bacteria present in the wound was an important consideration when choosing an antimicrobial dressing and also identified some interesting results particulary in relation to the effectiveness of iodine and silver containing dressings. The full details of the study are discussed in Chapter 3. This work has been widely cited in publications (44 citations as of March 2021) and clearly

demonstrated a gap in knowledge in relation to the antimicrobial efficacy of different antimicrobial wound dressings against common wound pathogens.

From these initial experiments which investigated the antimicrobial activity of the dressings over a 24-hour period it was decided to undertake a more comprehensive review of the commercial dressings available in the UK and also determine the duration of action of the dressings against common wound pathogens. The interesting results with the commercial silver and iodine dressings led to these antimicrobial agents being identified for further research to try to develop a novel antimicrobial material which could provide a sustained release of the antimicrobial over a prolonged period of time. The advantages of this were perceived to be that a sustained release of an antimicrobial could control the bioburden in the wound for a prolonged period and that the mechanism of release would provide a lower concentration of antimicrobial so minimising the risk of cytotoxicity to the wound surface. Another aim of the research was to develop an antimicrobial material which was easy to formulate and inexpensive to produce.

1.12. Thesis Aims and Objectives

Thesis Aims:

Investigate the antimicrobial properties of Commercial Antimicrobial Wound Dressings available in the UK to identify a gap in the market for a low cost, long-acting antimicrobial dressing.

Develop and evaluate a new type of dressing which can deliver a controlled release of an antimicrobial agent over a sustained period of time.

Objectives:

1. Compare the antimicrobial properties of a wide selection of Antimicrobial Wound Dressings available in the UK.

2. Establish the duration of antimicrobial action of individual antimicrobial wound dressings against a selection of common wound pathogens.

3. Identify the most appropriate antimicrobial agent for incorporation into a novel low-cost Antimicrobial Wound Dressing.

4. Formulate a new material that can deliver a controlled release of a selected antimicrobial over a sustained period of time.

5. Determine the duration of action of the new material against the same wound pathogens used to test the commercial antimicrobial wound dressings.

6. Assess the cytotoxicity of the new material as an *in vitro* proxy for safety.

Chapter 2: General Methods

2.1. Microbiological Studies

2.1.1. Organisms and Materials

2.1.1.1. Test Organisms

The following bacteria were used throughout this study: -
Laboratory strains of *Escherichia coli* JM109 and *Staphylococcus aureus* SH1000
An antibiotic susceptible clinical isolate from a burns patient of *Pseudomonas aeruginosa H085180216*
Antibiotic resistant strains of *S. aureus* used in later experiments were Epidemic methicillin-resistant *S. aureus* EMRSA-16 and *S. aureus* USA300.
All test bacteria were archived in glycerol in a - 80^{o}C freezer.

2.1.1.2. Test Medium

Experiments were performed using Mueller-Hinton broth (MH broth) and Mueller-Hinton agar (MH agar) (Oxoid, Basingstoke, UK)

MH broth was prepared by dissolving 11g of the powder in 500ml distilled water and MH agar 19g of the powder in 500ml distilled water. The media were sterilised in an autoclave at 121^{o}C for 15 minutes. The MH broth was allowed to cool at room temperature. The MH agar was cooled in a water bath at 50^{o}C and whilst still liquid 20ml was poured into individual Petri dishes. The MH agar plates were allowed to dry at room temperature and then stored in a fridge for up to 3 weeks before use.

2.1.2. Microbiological Experimental Protocols

All experiments had a minimum of 6 technical replicates, unless otherwise stated.

2.1.2.1. Bacterial Culture

A sample of the test bacteria stored in glycerol was removed from the – 80^{o}C freezer and a sterile inoculating loop used to extract a drop of the sample. This was then streaked onto a MH agar plate and then placed in a static incubator

for 24 hours at 37°C. Streak plates of prepared test bacteria were kept in a fridge at 4°C for a maximum of 4 weeks.

From the prepared streak plate, a single colony of the test bacteria was removed and added to 9ml of sterile MH broth. The inoculated test media was then incubated at 37°C in an orbital incubator at 100rpm for 24 hours.

100µl of overnight bacterial culture was added to 2.9ml sterile 1% phosphate buffered saline (Dulbecco's PBS, Gibco).

Circular Petri dishes containing a 5mm layer of MH agar were inoculated with 100µl of the ≈1 x 10^6CFU/ml broth culture of each test organism. The bacterial culture was then evenly distributed across the surface of the agar using a sterile L shaped plastic spreader and then allowed to dry.

2.1.2.2. *In vitro* Growth Inhibition Assay

Square 2 x 2cm samples were cut from the test dressings using a sterile scalpel. The squares were then sterilised by UV radiation for 15 minutes on both sides using a Mini-V/PCR (Telstar Life Solutions, Terrassa, Spain).

The 2 x 2cm test square was placed, using sterile tweezers, in the centre of the inoculated MH agar plate, the lid replaced, and the test plates were then incubated for 24 hours in a static incubator at 37°C.

An open MH agar plate was left in the laminar air flow cabinet for the duration of each experiment and then incubated for 24 hours at 37°C and then inspected to ensure there were no visible signs of microbial growth.

A control MH agar plate was produced for each test bacteria which was incubated without a test dressing to ensure that the bacterial culture produced a complete lawn of microbial growth.

After 24 hours incubation at 37°C the plates were examined for bacterial growth and the presence of a zone of inhibition of microbial growth both around the test dressings.

The Zone of Inhibition was determined by measuring the two diameters of the zone and calculating the surface area of an oval using the equation: -

Surface area $= \pi r_1 r_2$ where r_1 and r_2 are half the length of the two diameters d_1 and d_2. The surface area was measured in mm^2 (Figure 2.1).

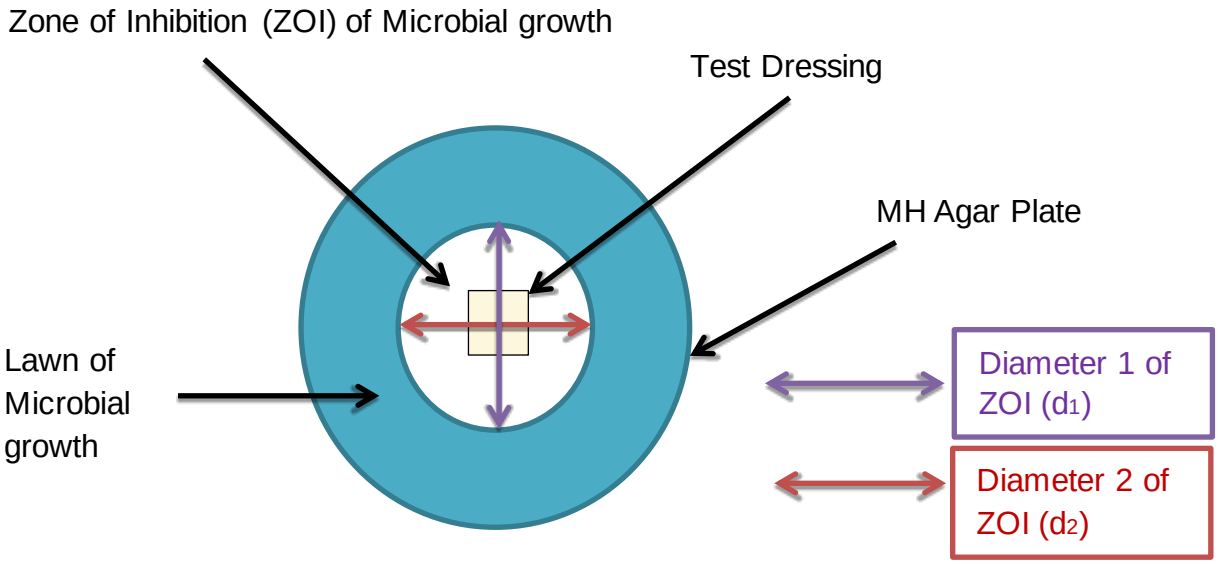


Figure 2.1: Measurement of the Zone of Inhibition of microbial growth.

2.1.2.3. Duration of Antimicrobial Activity – Passage Studies

Analysis of antimicrobial activity over a period of days was used to determine the duration of action of the test dressings. The samples were prepared as described in the growth inhibition assay. After 24 hours the ZOI was measured, and the used dressing sample transferred to a fresh agar plate inoculated with the same test organism. The samples were then incubated for a further 24 hours at 37°C, the ZOI was measured, and the dressing transferred to another fresh agar plate inoculated with the same organism. This was then incubated for a further 24 hours at 37°C and the ZOI measured giving a reading of the remaining antimicrobial activity after 72 hours used. Unless otherwise stated in the Methods Summary Section the experiment was repeated until the point the test dressing no longer produced a clear ZOI and was assumed to be no longer able to inhibit the growth of the microorganism.

2.1.2.4. Biofilm Model

Biofilms were created by inoculating 9ml of MH broth with 1 colony of the test bacteria. To the broth was added a 10 x 10mm piece of Nitrocellulose filter membrane (BioRad, California, USA). The filter membrane had been UV irradiated on both sides for 15 minutes. The broths were placed in an orbital incubator (100 rpm) at 37^{o}C for 24, 48 or 72 hours. After the allocated incubation time the filters were removed aseptically from the broth and placed in 9ml of sterile 1% Phosphate Buffered Saline (PBS) (Sigma Aldrich, St Louis, USA). The samples were then placed on a rocking platform set at 25rpm for 30 minutes to enable any planktonic bacteria to detach from the filter.

After 30 minutes the filters and attached biofilms were carefully removed from the PBS and placed in the centre of a circular Petri dish containing MH agar.

A 2 x 2 cm sample of the test dressing was placed over the biofilm and the Petri dish lid replaced and the samples incubated for 24hours at 37^{o}C. Controls were created by growing the biofilm as described but not covering it with a dressing. After 24 hours the dressing samples were removed and the filter plus any remaining biofilm placed in 9ml PBS 1%. These samples were then placed on a rocking platform set at 25rpm for 30 minutes. The filters were then carefully removed and placed in a plastic universal tube containing a fresh 9ml of sterile 1%PBS. The tubes were then placed in a sonicating water bath for five cycles of two minutes on and two minutes off to dislodge the bacteria whilst minimising the risk of cellular lysis as a consequence of sonification.

The bacterial suspension was then serially diluted for counting.

2.1.2.5. Bacterial Viability (LIVE/DEAD ™BacLight™)

Bacterial membrane integrity following treatment with antimicrobial dressings was determined using the LIVE/DEAD™ BacLight kit ™(Thermofisher Scientific, UK). A sample of the biofilm was removed using a sonicating water bath. The cells recovered from the biofilm were placed in 1ml sterile PBS 1% and the BacLight reagents were added as per the manufacturer's instructions. The treated biofilm was then placed on a microscope slide, a cover slip applied, and fluorescent images of the biofilm were produced using a confocal microscope (LSM 510 laser confocal scanning microscope, Zeiss, Oberkochen, Germany).

Bacterial cells with a compromised membrane that were dead or dying stained red whereas cells with an intact membrane stained green.

2.1.2.6. Minimum Inhibitory Concentration (MIC)

The MIC is the lowest concentration of an antimicrobial chemical found to inhibit the visible growth of a test microorganism after overnight incubation (Andrews 2001). In order to assess the MIC of test dressings a broth dilution MIC was developed based on the method described by Andrews 2001.

To determine the MIC of the antimicrobial dressings the antimicrobial chemicals had to be extracted from the dressing materials.

To do this the test dressings were cut using a 9mm sterile cork borer and each disc incubated at 37^{o}C in 1ml of MH broth for 24 hours. A total of 28 discs of each dressing were soaked, the discs absorbed the media and swelled and yielded a total of 23ml of eluent. The discs were removed, and the eluent produced by each dressing was then serially diluted with MH broth to 11 different concentrations from 100% to 50% then 25% down to 0.0976%. Povidone-Iodine Solution = Videne ® Antiseptic Solution (Ecolab, Northwick, UK.) a 10% (w/w) solution of PVP-I containing 1% (w/w) available iodine was also serially diluted with MH broth to produce 11 different concentrations.

180 µl of each of the different dilutions were placed in 200µl wells. To each dilution was added 20µl of the test bacteria (≈1 x10^{6} cfu/ml) and then incubated overnight at 37^{o}C. An end point assay scoring either growth or no growth was measured using the TeCan Infinate 200 (TeCan Group Ltd, Männedorf, Switzerland) at an optical density of 600nm.

2.2. Cell Biology

2.2.1. Cells and Materials

2.2.1.1. Cell Lines

HaCat keratinocytes (Cat Number T0020001) (AddexBio, San Diego, USA)

Normal Human Dermal Fibroblasts (NHDF-C39315) (Promocell, Heidelberg, Germany)

2.2.1.2. Reagents and Equipment

Dulbecco's modified Eagle's medium (DMEM) Glutmax (4500mg/L glucose) (Sigma Aldrich, St Louis, USA)

10% (V/V) Foetal Calf Serum (FCS) (Biowest Biosera, UK)

Penicillin 100iu/ml and streptomycin 100µg/ml (Sigma Aldrich, St Louis, USA).

Amphotericin B 0.625µg/ml (Sigma Aldrich, St Louis, USA)

Alamar Blue® Cell Viability (Invitrogen Molecular Probes, ThermoFisher Scientific, UK)

Povidone Iodine 10% Aqueous Solution (Videne®, Ecolab, Northwick, UK)

Peracetic acid (PAA) 0.1% (Sigma Aldrich, St Louis, USA)

Sodium Chloride (NaCl) 1M (Sigma Aldrich, St Louis, USA)

Clariostar Plate Reader (BMG LABTECH Ltd, Buckinghamshire, UK)

Cork borer (VWR, UK)

Thincerts (12 well, 0.4µm pore diameter, transparent) (Greiner BioOne (GmbH), Frickenhausen, Germany)

2.2.2. Cell Biology Experimental Protocols

2.2.2.1. Cell Culture

HaCat cells were cultured in DMEM Glutmax (4500mg/L glucose) supplemented with 10% v/v FCS and penicillin (100 IU/ml) and Streptomycin (100µg/ml) until a confluence of 80% was reached.

Normal Human Dermal Fibroblasts were cultured in DMEM Glutmax (4500mg/L glucose) supplemented with 10% v/v FCS, 2 $x10^{-3}$ M L-glutamine, 0.625µg/ml amphotericin B, penicillin (100 IU/ml) and Streptomycin (100µg/ml) until a confluence of 80% was reached.

2.2.2.2. Cytotoxicity testing of Dressings on Cell Cultures.

The test methodology was adapted from an indirect cytotoxicity experiment described by Yunoki et al. (2015) which was based on ISO 10993-5:2009 (Biological evaluation of medical devices – Part 5: Tests for *in vitro* cytotoxicity).

HaCat keratinocytes and Normal Human Dermal Fibroblasts were cultured separately in their respective culture media (as described in Section 2.2.2.1.) until a confluence of 80% was reached.

The cells were then seeded to six different cell densities between 1 x 10^4 and 6 x 10^4 in 96 well plates and allowed to adhere to the base of the well over a 24 hour period at 37 ^oC in 5% CO_2.

3.5g samples of each test dressing were submerged in 35ml of DMEM to produce a (10%w/v) solution and incubated at 37^oC for 24 hours. The dressing was removed from solution and the resultant eluent contained 100% of the antimicrobial eluted from the dressing. The extracted eluent was diluted with DMEM to give a range of different concentrations: 100% eluent, 80%, 60%, 40%, 20%, 10%,1% and 0% (100% DMEM). 200µl of each dilution was added to the six different HaCat cell densities which had been grown overnight. The samples were all tested in triplicate and incubated for 24 hours at 37^oC. After 24 hours the eluent was removed, and the cells rinsed with sterile PBS 1% to remove any excess iodine. The washed cells were then incubated with 10µl/ml (Carrier DMEM) of resazurin salt (2mg/ml-(DMEM Carrier) Alamar Blue® for 2 hours at 37^oC in the dark. A cell density of between 30-40K was found to be optimal for developing a consistent confluent monolayer of cells so the experiment was repeated using 3 x 10^4 cells per well.

Fluorescence was measured using a Clariostar Plate reader.

Povidone Iodine (PVP-I) 10% aqueous solution was used as a positive control.

2.2.2.3. Preparation of Tissue Engineered (TE) Skin Model

De-epidermised dermis (DED) was prepared at the University of Sheffield by Patrick Harrison using the method described by MacNeil, Shepherd and Smith (2011).

Briefly, donated human skin (Euroskin) was split using a Braithwaite knife to produce split thickness skin (STS) which consisted of the epidermis and part of the dermis. The STS was placed into 100ml sterile containers containing 1M Sodium Chloride (NaCl) solution and incubated for between 14 –18 hours at 37^{o}C until there was visible separation of the dermis from the epidermis. The dermis was then carefully detached from the epidermis taking care not to damage the basement membrane.

The de-epidermised dermis (DED) was sterilised by immersion in 0.1% peracetic acid (PAA) which had been adjusted to pH 7 (by the addition of Sodium Hydroxide) for 3 hours at room temperature. The DED was then removed and washed in sterile Phosphate Buffered Saline (PBS) 1% for 30 minutes. The washings were repeated a total of four times to ensure that any NaCl in the DED was removed. The washed DED was placed in fibroblast culture medium (Described in section 2.2.2.1.) and incubated at 37^{o}C in 5% CO_2 humidified atmosphere for 48 hours.

The DED was cut using a sterile cork borer (VWR, UK) into 12mm diameter discs.

The dermis discs were placed in the bottom of Thincerts (12 well, 0.4µm pore diameter, transparent) and 1ml of DMEM added to the bottom of the well. The discs were fed with 250 µl of fibroblasts at a cell density of 1 x 10^5 /well and 250 µl of HaCat keratinocytes at a cell density of 4 x 10^5/well. They were then incubated at 37^{o}C for 24 hours to allow the cells to integrate into the dermis.

After 24 hours the cell culture media (DMEM) was replaced. The cell culture media was then replaced every 48 hours for 17 days to allow the tissue to develop.

2.2.2.4. Cytoxicity testing of Dressings on a Tissue Engineered (TE) Skin Model

The iodine dressings were cut using a cork borer into 9mm diameter discs. The samples were then sterilised by UV irradiation for 15 minutes on both sides. The dressing discs were placed on top of the TE model in the holders and incubated at 37^{o}C for 24 hours.

After 24 hours the discs were removed, and the TE model was washed with sterile PBS 1% to remove excess iodine. The TE skin was then incubated with alamarBlue® reagent (ThermoFisher Scientific, UK) for 2 hours. After 2 hours incubation the fluorescence was read on the BMG Clariostar Plate reader and the results compared with a positive control (No PVP-I disc, just cells) and a negative iodine control (PVP-I disc but no cell dermis) and a PVP-I negative control (no cell dermis + no PVP-I disc) to determine if iodine was having any influence on the Alamar blue assay.

The test dressing results were compared with the positive control (no PVP-I disc) and the negative iodine control (DMEM + alamarBlue + PVP-I disc)

The average of 9 fluorescence readings were calculated for each test and the controls and the percentage tissue viability calculated using the following equation: -

$$\% \text{ Tissue Viability} = \frac{\text{Dressing sample (average)} - \text{Negative Iodine Control (average)}}{\text{Positive Control (average)}}$$

2.3. Material Formulations and Analysis

2.3.1. Reagents

Starch: octenylsuccinate modified waxy corn starch (Cargill, Surrey, UK)

This starch contains ca. 98% amylopectin and ca. 2% amylose.

PEG_{600}: Poly (ethylene glycol) (Sigma-Aldrich, Dorset, UK) = number average molecular weight = 600g.mol $^{-1}$

Sodium Clay (Sodium Cloisite) = Cloisite® NA $^{+}$ (Southern Clay products, Gonzales, Texas, US)

Silver Clay = Silver clay obtained from sodium clay by ion exchanged with silver nitrate (MERI) Silver content 0.5%

Laponite Clay = Laponite® RD (Rockwood Additives, Widnes, UK)

Potassium iodide, purity >99.5% (Fischer Scientific, Loughborough, UK)

KI_3 = Potassium triiodide (Sigma Aldrich, Dorset, UK)

Povidone-Iodine Solution = Videne ® Antiseptic Solution (Ecolab, Northwick, UK.)

= 10% (w/w) solution of PVP-I containing 1% (w/w) available iodine.

PVOH = Poly (vinyl alcohol) = Mowiol® (Sigma-Aldrich, Dorset, UK.)

Mowiol® 4-98 (M_w = 27 000 $g.mol^{-1}$, 98.4+/- 0.4% hydrolysed) (Mw 27KDa)

Mowiol®28-99 (M_w = 145 000 $g.mol^{-1}$, 99.4 +/- 0.4% hydrolysed) (Mw 145KDa)

2.3.2. Development of Antimicrobial Materials

2.3.2.1. Preparation of Starch/Clay Composites

A series of sodium-clay and silver exchanged clay composites were prepared in the Materials Research Engineering Institute (MERI) at Sheffield Hallam University (SHU) by Marianne Labet.

1. Sodium Clay & Starch
2. Silver Clay & Starch
3. Sodium Clay & Starch & Iodine
4. Silver Clay & Starch & Iodine

5g starch was placed into a two-neck round bottom flask with a condenser. To this was added 36g de-ionised water and the flask placed in a water bath on top of a heating plate set at 95^{o}C and stirred for two hours. The temperature was reduced to 60^{o}C and then 1g of PEG_{600} in 2g of de-ionised water was added

and the mixture left for 30 minutes before adding 1.66g of Cloisite®Na+. The mixture was then left stirring for a further 90 minutes. Then the appropriate quantity of silver clay was added and mixed for a further 30 minutes before the required volume of potassium triiodide solution was added.

Each formulation contained 5g starch, 36g water and 1g PEG_{600} with differing concentrations of either silver clay or iodine or both. 0.5g aliquots of each of the different formulations were applied to cover the surface of 2 x 2cm microscope cover slips. The samples were then dried at room temperature.

2.3.2.2. Preparation of Laponite/Povidone Iodine Composites

Various composites were created by mixing different amounts of Laponite powder with various weights of povidone iodine solution. For example, 0.2g of Laponite was combined with 19.8g of Povidone iodine solution to produce a 1wt% mixture. The amount of the Laponite in the mixtures prepared ranged from 1 wt% to 30wt%.

2.3.2.3. Preparation of PVOH/Povidone Iodine Composites

PVOH was placed in a two neck round bottom flask with a condenser and to this de-ionised water was added. The flask was heated to 95^{o}C as before, with stirring until the PVOH was fully dissolved. The solution was allowed to cool (with continuous stirring) to room temperature. PVP-I was added and stirred until homogenous. The higher molecular weight PVOH (Mowiol 28-99) was more viscous and required more water than the Mowiol 4-98 to dissolve all the polymer. To allow for the additional water 0.65g of the Mowiol 28-99 was added to the cover slip compared to 0.5g of the Mowiol 4-98 mixture. The samples were allowed to dry at room temperature.

A series of composites were created by mixing different amounts of PVP-I with PVOH to produce composites containing between 1-8.5 wt% PVP-I.

2.3.2.4. Preparation of PVOH/Povidone Iodine/ Clay Composites.

A variety of composites containing PVOH, Povidone iodine and a clay, either Cloisite® Na^{+} or Laponite were created to investigate the effect of the addition of clay on the release of iodine from the materials. The different formulations were created by MERI using the method described below.

PVOH (Mowiol 28-99) was added to distilled deionised water in a flask which was placed in a water bath at 95°C for 1 hour to dissolve the PVOH.

Cloisite® Na^+ was added to distilled deionised water in a separate flask, placed in a water bath at 95°C for 1 hour and mixed using a magnetic stirrer bar. When dissolved the temperature of the water bath was reduced to 60°C. The PVOH solution was added to the clay solution and mixed for a further hour at 60°C. After an hour the water bath was turned off and when the water temperature had dropped to 35°C the required volume of povidone iodine solution was added and the mixture stirred for a further hour.

60g of the suspension was then poured into 9cm circular Petri dishes and dried in a vacuum oven at 37°C over 7 days. The samples were then cut into 2 x 2cm squares for testing.

2.3.2.5. Large Scale Preparation of the preferred CROWD Material

Scale-up of production and manufacture of a prototype wound dressing was undertaken by Dr David Hogg from Rejuvetech Ltd and Dr Francis Clegg (MERI, SHU).

The preferred dressing formulation contained: -

12.5 wt% Cloisite® Na^+ + 35 wt% PVOH + 52.5 wt% PVP-I

PVOH (Mowiol® 28-99, Mw ~ 145,000, 99-99.8% degree of hydrolysis) (Aldrich) was fully dissolved in deionised water by heating at 95°C for 30 minutes. The solution was then cooled to 60°C.

Cloisite® Na^+ (BYK Additives) clay was dispersed in deionised water and using a magnetic stirrer bar was stirred continuously for 2 hours at 60°C.

The Clay suspension was added to the PVOH solution (at 60°C) and stirred using a saw-tooth blade, overhead mixer for 1 hour. This suspension was then cooled to 35°C.

PVP-I 10% w/w aqueous solution (Ecolab Videne® Antiseptic Solution) at room temperature was poured into the Clay/PVOH suspension and further mixed for 1 hour with the overhead saw-tooth mixer.

The final suspension was poured into a square Petri dish (24.5 x 24.5 cm) and dried in a vacuum oven in which the shelves were heated to 37°C. The sample experienced a cyclic vacuum in which it is held at 300 mbar for 1 hour, then ramped to atmospheric pressure over 15 minutes and held for 30 minutes before repeating the vacuum procedure. It took approximately 9 days for the sample to dry.

2.3.2.6. Determination of the composition of the preferred CROWD material

The method for separating the two layers of the CROWD material and analysing the constituents was developed by Chris Breen, Francis Clegg and Lukáš Petra in the Materials Engineering Research Institute (MERI), Sheffield Hallam University. The analysis was undertaken by Lukáš Petra (MERI) in 2015.

Samples of the preferred CROWD formulation (12.5wt% Cloisite®Na+/ 35wt% PVOH /52.5wt% PVP-I) were first separated into the two layers; Top (Dark Layer) and Bottom (Light Layer). The layers were cut into small pieces and dried in an oven at 90°C for 3 days. The mass of dried samples was then measured (Figure 2.2).

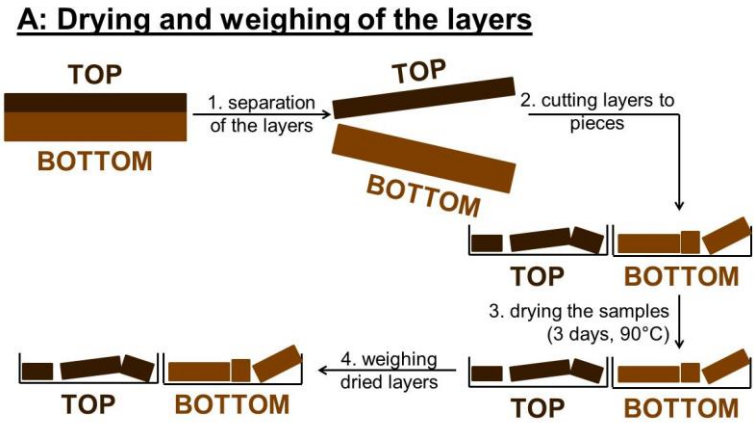


Figure 2.2: Separation of the two CROWD layers and determination of the dried mass (Petra 2015)

The next step was an extraction of Iodine (Figure 2.3). Dried samples of the layers were placed in two beakers and 100 ml of deionised water was added to each. The release of Iodine was observed immediately, and the films were kept

in a solution for 24 hours without stirring. After 24 hours the solution was poured into bottles and another 100ml deionised water was added to each beaker containing the washed samples. This procedure was repeated several times until the iodine was completely washed off, this process took 5 days. The solid films were then removed from the beakers and placed in Petri dishes and dried in an oven at 60°C for 24 hours. The prepared samples were labelled “Dried top solid without Iodine” and “Dried bottom solid without Iodine”. The solutions in the bottles were labelled “Top filtrate and Bottom filtrate”. These solutions were used to determine the Iodine content. The filtrates contained Iodine and PVP.

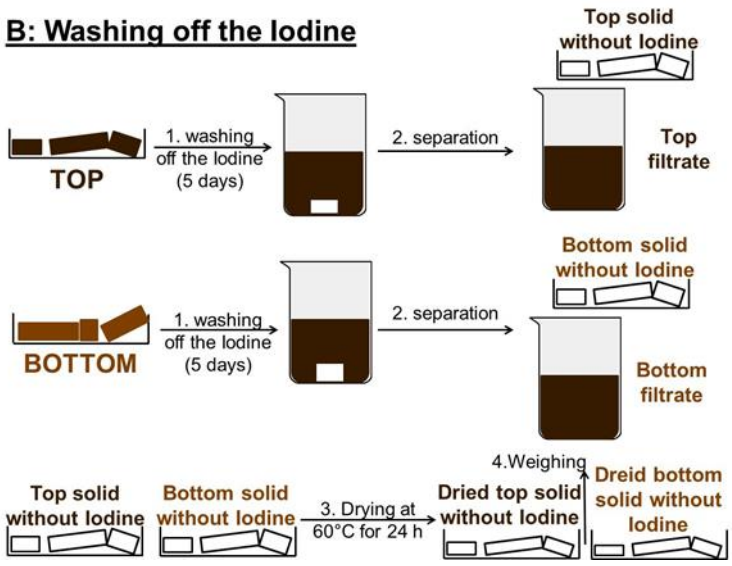


Figure 2.3: Extraction of Iodine from the two separated CROWD layers (Petra 2015)

The “Top filtrate” and “Bottom filtrate” were placed into two separate beakers. (Figure 2.4) and the samples were mixed for 2 hours at 90° C to drive the decomposition of the triiodide anion reaction. This results in the loss of a portion of the iodine (two thirds of it) as described in the equation. The filtrate contains both iodine and some dissolved polymer. Weighing the amount of dissolved polymer and iodine before heating and after heating allows the calculation of the amount of iodine present. Determination of the amount of dissolved polymer presnt was calculated from the solid recovered from the washing off step (i.e., the ‘top solid without iodine).

After 1 hour the solutions became colourless. This was believed to be caused by the decomposition of triiodide anion:

$(I_3)^-(aq) \rightarrow I_2(g) + I^-(aq)$

The colourless solutions were then poured into Petri dishes and dried in an oven at 60°C for 24 hours. The prepared films labelled “Top film” and “Bottom film” were then weighed.

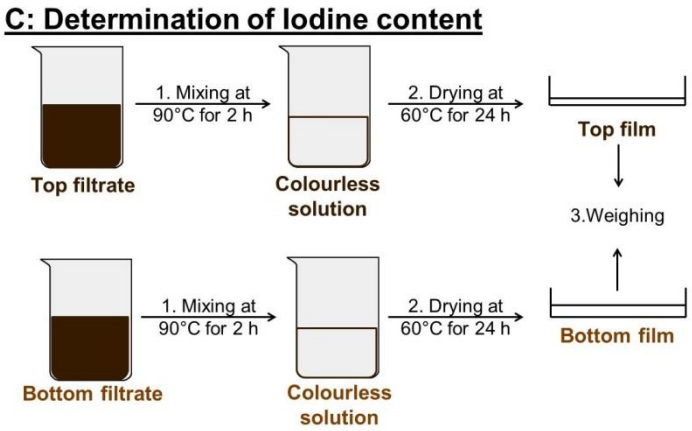


Figure 2.4: Determination of the Iodine content in each layer of the CROWD-composite (Petra 2015)

The final step in the process was the polymer extraction (Figure 2.5). The dried top (or bottom) solid without Iodine were placed into a beaker containing 120 ml of deionised water. The mixture was heated to 90°C mixed for 2 hours. After 2 hours the samples had dissolved and formed dispersions. After cooling to room temperature, the dispersions were centrifuged. The supernatants were poured into a Petri dish and put into an oven at 60°C for 24 hours. The homogenous colourless films that formed were labelled “bottom solution” and “top solution”. Solid residues were transferred from the tubes to Petri dishes by the addition of 30 ml deionised water onto the solid residues. The tubes were mixed in a shaker for 1 hour. Prepared dispersions were poured into Petri dishes and dried in an oven at 60°C for 24 hours. Formed films were labelled “top solid” and “bottom solid”. All samples were weighed and analysed by Thermogravimetric Analysis (TGA). The gravimetric analysis and TGA results were used to calculate the composition of the film.

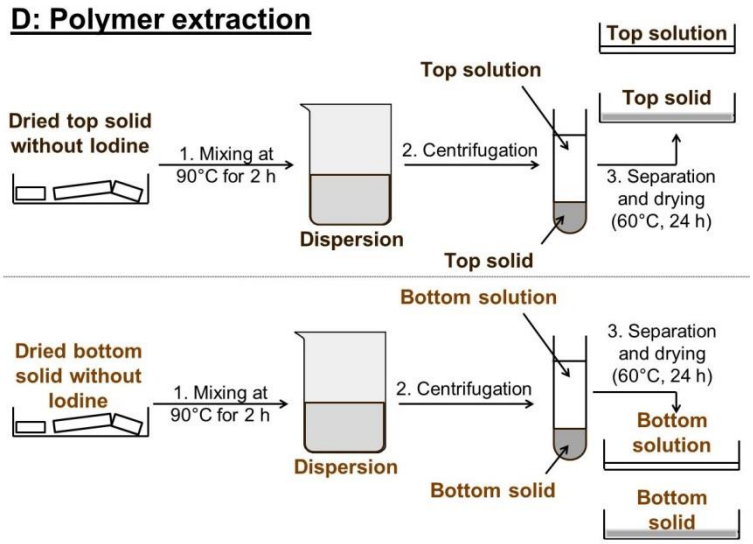


Figure 2.5: Extraction of the Polymer from the Top and Bottom Layers of the CROWD composite (Petra 2015)

2.3.2.7. X-Ray Fluorescence (XRF) Analysis of Iodine content of different CROWD Formulations

The XRF analysis was undertaken by Francis Clegg in the Materials Engineering Research Institute (MERI), Sheffield Hallam University.

XRF spectra were collected directly from 2x2cm dressing samples that had been allowed to dry at room temperature and humidity, using a PANalytical MagiX PRO XRF (PW2440) spectrometer (Malvern PANalytical Ltd, Malvern, UK) and a Rh anode X-ray source (Malvern PANalytical Ltd, Malvern, UK).

Data collection involved four different detectors (flow, sealed, scintillator and duplex) and 10 different mono-chromator crystals to produce 10 different energy ranges, which covers the range of elements from Fluorine to Americium. Data was analysed using the default settings within the 3-iq+ programme.

XRF data were analysed using SuperQ software (PW2450); Data collector (Measure and Analyse – Version 4.0R (4.1561.3)) and Data Analysis (IQ plus - Version 4.0R (4.1561.3)) (Malvern PANalytical Ltd, Malvern, UK) and quantification were obtained by utilising the raw data (counts per second).

Raw data were used since the calibrated methods were unable to provide reliable data for the samples containing no clay, which was because the dressings predominantly contain carbon and hydrogen, and these elements cannot be detected by the XRF technique.

Chapter 3: Antimicrobial Susceptibility Testing of Commercial Antimicrobial Wound Dressings

3.1. Introduction

The development of microbial resistance to antibiotics has led to renewed interest in the use of other topical antimicrobial agents for the treatment of infected wounds (Kramer et al. 2017).

As mentioned in Chapter 1 the British National Formulary (BNF 2019) advises prescribers that for local wound infection a topical antimicrobial dressing can be used to reduce the level of bacteria at the wound surface. However, there is no guidance to aid the selection of an appropriate AWD if a particular bacterial/microbial infection is present in the wound.

Prescribers are advised to consider the amount of exudate being produced by the wound when selecting an antimicrobial dressing and suggestions are given as to an appropriate choice based on the ability of the carrier dressing to adsorb exudate (Table 3.1.).

Table 3.1: Wound contact materials for Wounds with Signs of Infection (BNF 2019)

Low Exudate	Moderate Exudate	Heavy Exudate
Low adherence Tulle with honey Low adherence Tulle with iodine Low adherence Tulle with silver Hydrocolloid with silver Honey topical	Hydrocolloid-fibrous with silver Foam with silver Alginate with silver Honey-topical Cadexomer-iodine	Hydrocolloid–fibrous with silver Foam extra absorbent with silver Alginate with honey Alginate with silver

In 2016 (NICE) stated in their Evidence Summary on advanced wound dressings and antimicrobial dressings that “systematic reviews and meta-analyses have identified little good quality evidence from randomised controlled trials (RCTs) to support the use of advanced or antimicrobial dressings (Such as iodine, honey or silver dressings) for chronic wounds. As well as being few in number, many of the RCTs have significant limitations and the evidence is generally of low quality”. This research started in 2010 shortly after the publication of the Cochrane Review on silver dressings and the VULCAN study which had raised concerns about the use of silver dressings for the prevention and treatment of infection in wounds. The Sheffield wound care formularly group were investigating the available evidence to develop a formulary for clinical staff to support the choice of suitable antimicrobial dressings, not only for different wound types but also wounds infected with particular pathogens. Most of the antimicrobial dressings on the market had limited evidence regarding which pathogens they were effective against. So, a pilot research project was developed to compare a wide selection of antimicrobial dressings that were being used in Sheffield to determine whether or not there was a significant difference in their antimicrobial efficacy against common wound pathogens. The study (Bradshaw 2011) involved one honey, three iodine and 9 silver containing dressings which were being used in primary care and the hospitals in Sheffield. The research involved a 24 hour *in vitro* growth inhibition assay (Described in Section 2.1.2.2.) using 1 x 1 cm square samples of the dressings tested against *Staphylococcus aureus* (SH1000), *Escherichia coli* (JM109) and *Pseudomonas aeruginosa* (H085180216). The initial analysis showed that there were no significant differences in the action of honey, iodine or silver against the bacteria tested ($p = 0.342$). However further analysis identified that the activity of individual dressings varied significantly against each bacterial species. The cadexomer iodine dressing, Iodoflex, produced the largest ZOI against all three bacteria but interestingly the other dressings varied in their action against different species. The silver dressings: Urgotul SSD, Sorbsan Silver Flat, Aquacel Ag and the iodine containing dressings Iodozyme and Iodoflex produced large ZOI against *E. coli.* The three iodine dressings; Iodoflex, Iodozyme and Oxyzyme produced the largest ZOI against the Gram-positive, *S. aureus*. The dressings which produced the largest ZOI against *P.*

aeruginosa were Iodoflex and the absorbant silver dressings; Sorbsan Silver Flat, Urgosorb Silver and Aquacel Ag. It had been suggested at the time of the initial study (Cutting et al. 2009) that selection of wound dressings is influenced by the characteristics of the individual wound, the ability of the dressing to manage pain, fluid levels within the wound, sustain a release of an antimicrobial agent over time and to modulate inflammation (Cutting, White and Hoeskstra 2009). This intial research identified that the organisms tested had different degrees of sensitivity towards antimicrobials. It also suggested that the species of bacteria present in the wound may also be an important consideration when choosing an antimicrobial dressing. This work demonstrated a gap in knowledge in relation to the characteristics of different antimicrobial wound dressings against common wound pathogens.

One of the limitations of the initial study was that the samples were only tested for 24 hours and so the duration of the antimicrobial activity of the individual dressings had not been investigated. Antimicrobial dressings with a sustained duration of antimicrobial action could have numerous advantages in clinical practice to control the bioburden within the wound and reduce the frequency of dressing changes.

This larger study was developed to not only compare the antimicrobial activity of a wider range of different antimicrobial dressings but also to determine their duration of action and to identify if the antimicrobial effects were different against the three test bacteria and within each class of dressings.

The three test bacteria included in this study were three common wound pathogens found in chronic wounds: *Staphylococcus aureus* (SH1000) (Laboratory strain), *Escherichia coli* (JM109) (Laboratory strain) and *Pseudomonas aeruginosa* (H085180216) (Antibiotic susceptible clinical isolate from a burns patient).

Staphylococcus aureus is a facultative anaerobic, Gram - positive coccus capable of forming biofilms. It colonizes the skin and is one of the most common pathogens isolated from infected wounds (Bessa et al. 2015; Rahim et al. 2017; Serra et al. 2015). Many of the strains that cause wound infection produce extracellular enzymes that can break down host tissues and invade deeper

layers of the skin to form abscesses. Enzymes such as staphylokinase activate plasminogen causing break down of fibrin clots and promote bacterial dissemination. Other enzymes produced include collagenase that breaks down peptide bonds in collagen, coagulase which converts fibrinogen to fibrin, hyaluronidase which breaks down hyaluronic acid which is part of the extracellular matrix and lipases that catalyse the hydrolysis of triglycerides (Tam and Torres 2019). Certain strains produce various toxins such as haemolysins that can lyse red blood cells and enterotoxins which cause nausea and vomiting (Edwards-Jones 2016). The particular strain of *S. aureus* used in this study was SH1000 which was derived from lab strain 8325-4. The 8325-derived *S. aureus* strains were shown to have reduced activity of the alternative stress sigma factor, SigB. High hemolysin and extracellular protease expression observed in 8325-derived strains was linked to the low activity of SigB. To address this issue, an *rsbU*+ repaired derivative of 8325-4 was constructed (SH1000) that has increased SigB and can also form biofilms so is representative of a disease forming *S. aureus* (Bæk et al. 2013; O'Neill 2010).

Escherichia coli is a common Gram - negative bacteria found in the gastrointestinal tract that has been associated with various wound infections including pressure ulcers and surgical site infections (Chakraborty et al. 2017). The strain used in this study, JM109, is derived from a clinical isolate, is a known biofilm former and is commonly used in infection models.

Pseudomonas aeruginosa is an opportunistic, aerobic, Gram-negative bacilli. It produces a number of virulence factors including the enzymes elastase which cleaves collagen and elastin, phospholipase and lecithinase which can break down cell membranes and serine and cysteine proteinases which denature proteins. *P. aeruginosa* can also produce toxins such as cytotoxin which is toxic to most eukaryotic cells and pyocyanin a redox active phenazine which produces toxic oxygen species and causes the characteristic blue green colour (Edwards-Jones 2016). *P. aeruginosa* is known to cause biofilms. The strain used in this study was a clinical isolate from a burns patient.

The dressings included in the research were chosen to represent each of the four main classes of antimicrobial wound dressings (AWD) available in the UK; Antiseptics, Iodine, Silver and Honey. The criteria used for the selection of

dressings was that they were licensed for the treatment of wound infection and were available as a formulated dressing; tulle, alginate, hydrofibre, foam or soft polymer so could be cut into 2 x 2cm squares.

For each of the four classes of AWD a range of dressings made with different carrier materials e.g., alginates, foams were included to try to determine if the carrier dressings affected the size of the Zone of Inhibition (ZOI) and the duration of antimicrobial action.

Gel formulations such as honey ointments and amorphous hydrogels were not included as they could not maintain their form and were likely to spread across the agar plate and not produce clear Zone of Inhibition (ZOI). This meant that after 24 hours incubation the gel formulations could not be passaged.

3.2. Methods Summary

Passage studies, as described in Chapter 2, Section 2.1.2.3. were undertaken to assess the duration of antimicrobial activity of 2 x 2cm samples of the 20 commercial antimicrobial dressings listed in Table 3.2. The samples were passaged until they were no longer producing a clear ZOI.

Table 3.2: Commercial Antimicrobial Wound Dressings (AWD) included in the study

AWD Class	Commercial Dressing	Manufacturer
Biguanide	Chlorhexidine Tulle	Smith & Nephew healthcare Ltd
	Suprasorb X+PHMB	Lohmann &Rauscher (UK)
Iodine	Inadine Tulle	Systagenix Wound Management Ltd.
	Iodoflex	Smith & Nephew healthcare Ltd
	Iodozyme	Crawford Healthcare Ltd
Silver Alginates	Sorbsan Silver Flat	Aspen Medical Europe Ltd
	Urgosorb Ag	Urgo Medical Ltd
Silver Hydrofibers	Aquacel	ConvaTec Ltd
	Aquacel Ag+ Extra	Convatec Ltd
Silver Soft Polymers	Urgotul SSD	Urgo Medical Ltd
	Urgotul Ag	Urgo Medical Ltd
Silver Foams	Biatain Ag	Coloplast Ltd
	Allevyn Ag	Smith & Nephew healthcare Ltd
Honey Alginate Dressings	Medihoney Gel Sheet	Derma Sciences Europe Ltd
	Medihoney Apinate	Derma Sciences Europe Ltd
	Algivon	Advancis Medical
Honey Tulles	Activon	Advancis Medical
	Actilite	Advancis Medical
	Melladerm Plus	Sanomed Manufacturing BV
Honey Hydrogel	L- Mesitran Net	Aspen Medical Europe Ltd

3.3. Results

3.3.1. Biguanide Dressings – Passage Study Results

Passage studies were undertaken using 2 x 2cm samples of two biguanide dressings: Chlorhexidine Tulle (Bactigras®) and a Polihexanide (PHMB) dressing (Suprasorb®X + PHMB).

3.3.1.1. Chlorhexidine Tulle

After 24 hours the Chlorhexidine Tulle did not produce a ZOI against *S. aureus* or *P. aeruginosa* and there was evidence of microbial growth through the weave of the gauze. The tulle did however produce a small ZOI (Mean Surface Area (Mean) 460$\pm$ 82.46mm^2) against *E. coli* after 24 hours but after 48 hours there was complete microbial growth under the sample.

3.3.1.2. Polihexanide (PHMB)

The polihexanide containing dressing produced a large ZOI against *S. aureus* and *E. coli* for 48 hours but after 72 hours there was complete microbial growth under the dressing (Figure 3.1). The size of the ZOI produced against the two bacteria was very similar *S. aureus* (Mean 1316 ±116.4mm^2) and *E. coli* (Mean 1244 ± 63.37mm^2) reducing to Mean 973 ±114.0mm^2 for *S. aureus* and Mean 920± 47.99mm^2 for *E. coli* on the second day.

The ZOI against *P. aeruginosa* were smaller than those produced against the other two bacteria, but the duration of action was 3 days with the ZOI produced after 24 hours Mean 705±104.42mm^2, 48 hours Mean 690 ±160.36mm^2 and 72 hours Mean 730 ±135.06mm^2, Suprasorb® did not produce a ZOI at 96 hours.

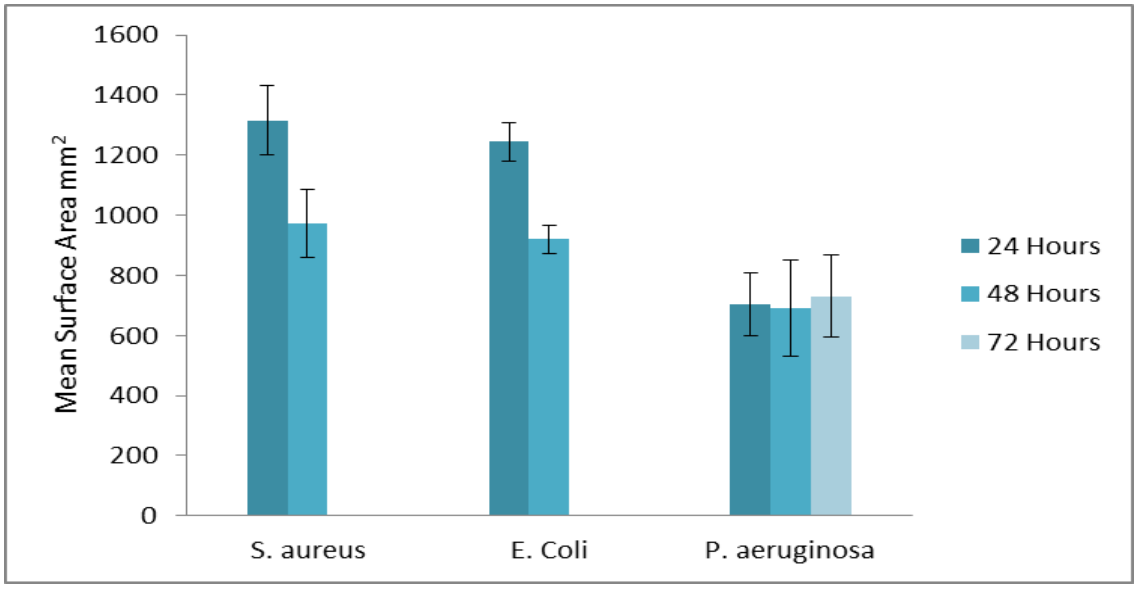


Figure 3.1: ZOI produced by Suprasorb®X + PHMB against the three test bacteria (Error Bar = Standard Deviation, n = 6)

3.3.2. Iodine Dressings – Passage Study Results

In these experiments Iodoflex, Iodozyme and Inadine were tested against the three bacteria. Oxyzyme was not included because it was not licensed for use in infected wounds. The Iodozyme has a separate top layer which was cut to 1 x 1cm and placed on top of the 2 x 2cm wound facing hydrogel layer (in accordance with the manufacturer's instructions).

The ZOI produced by the three iodine dressings against the three test microorganisms is shown in Figures 3.2, 3.3 and 3.4.

Iodoflex produced the largest ZOI against all three test microorganisms and was able to produce a clear ZOI after 48 hours repeated use. At 72 hours Iodoflex did not produce any ZOI. The ZOI produced by Iodoflex after 48 hours were much larger for *S. aureus* (Mean 3112±363.37mm^2) and *E. coli* (Mean 1861±96.05mm^2) than the ZOI produced in the first 24-hour period (Mean 731± 82.15mm^2) and (Mean 674±130.44mm^2) respectively. However, when Iodoflex was tested against *P. aeruginosa* the ZOI after 24 hours (Mean 1649±188.91mm^2) was much larger than the ZOI produced during the second 24-hour period (Mean 486± 84.81mm^2).

Inadine produced a ZOI for 48 hours against *S. aureus* and *E. coli* but only 24 hours against *P. aeruginosa*. After 24 hours the largest ZOI was against *S. aureus* (Mean 1118mm^2± 60.94mm^2) then *E. coli* (Mean 932±120.83mm^2) and smallest *P. aeruginosa* (Mean 410± 24.49mm^2). After 48 hours the ZOI were only slightly larger than the surface area of the sample 400mm^2 for both *S. aureus* and *E. coli.* There were no ZOI against *P. aeruginosa* at 48 hours. At 72 hours there was complete microbial growth of all three test bacteria.

Iodozyme produced a clear ZOI against the three test bacteria for the first 24 hours. The ZOI were smaller than those produced by Iodoflex and Inadine. After 48 hours there was still evidence of microbial inhibition but the size of the surface area of the ZOI was less than the original size of the dressing sample. On the second day the Iodozyme dressings had curled away from the surface of the agar and appeared to be inhibiting microbial growth where the material remained in contact with the agar. At 72 hours there was no inhibition of microbial growth.

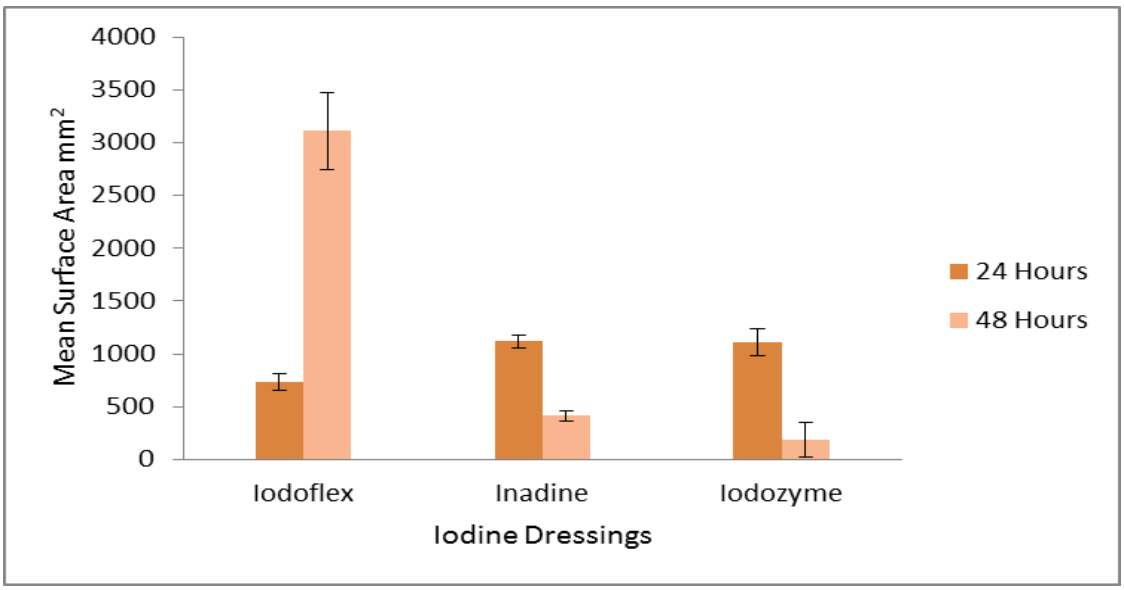


Figure 3.2: ZOI produced by Iodine Dressings against *S. aureus* (Error Bar = Standard Deviation, n = 6)

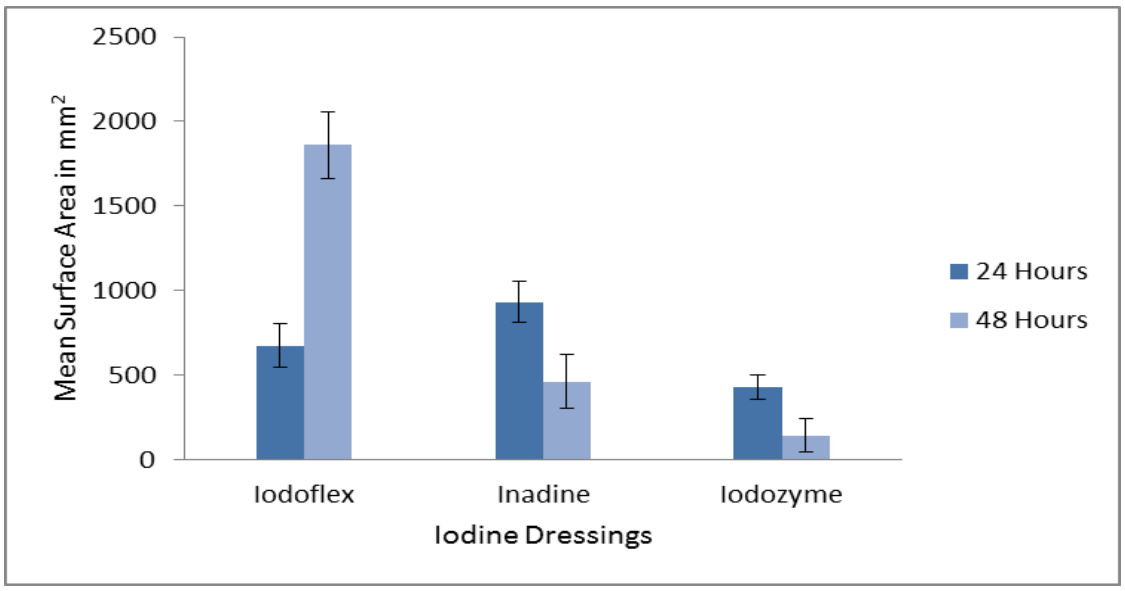


Figure 3.3: ZOI produced by Iodine Dressings against *E. coli* (Error Bar = Standard Deviation, n = 6)

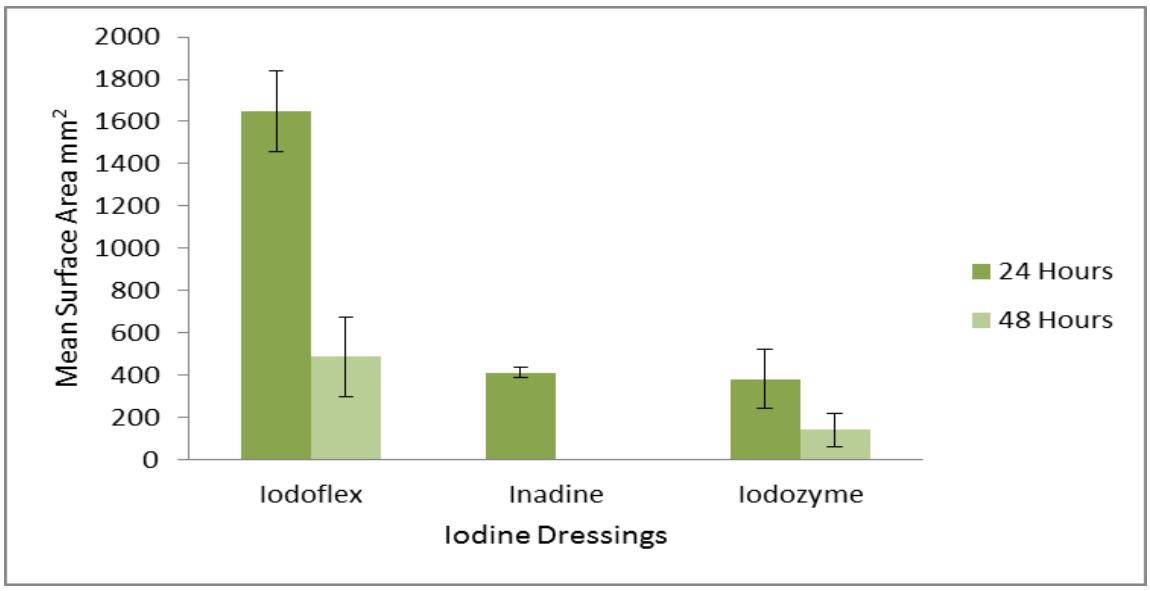


Figure 3.4: ZOI produced by Iodine Dressings against *P. aeruginosa* (Error Bar = Standard Deviation, n = 6)

3.3.3. Silver Dressings – Passage Study Results

The eight silver dressings tested are listed in Table 3.3 categorised based on their carrier primary dressing type.

Table 3.3: Silver Dressings tested classified by primary dressing type.

Alginates	Hydrofibers	Soft Polymers	Foams
Sorbsan Silver Flat	Aquacel Ag	Urgotul SSD	Biatain Ag
Urgosorb Ag	Aquacel Ag+ Extra	Urgotul Ag	Allevyn Ag

3.3.3.1. *S. aureus* - Results

The alginate dressings: Sorbsan Ag and Urgosorb Ag and the two hydrofiber dressings (Aquacel Ag and Aquacel Ag+ Extra) produced large clear ZOI against *S. aureus* for 96 hours (Figure 3.5). Aquacel Ag+ Extra produced the largest ZOI for the four days. All the dressings except Urgosorb Ag produced smaller ZOI after 48 hours than they produced in the first 24 hours.

The two soft polymer dressings; Urgotul SSD and Urgotul Silver only produced ZOI against *S. aureus* for 48 hours. Urgotul SSD produced a large ZOI (Mean 916$\pm$43.71mm^2) after 24 hours but the size reduced to Mean 419$\pm$67.38mm^2 after 48 hours whereas the ZOI produced by Urgotul Silver were very similar on

both days Mean 580± 35.9mm^2 after 24 hours and Mean 557±43.22mm^2 after 48 hours.

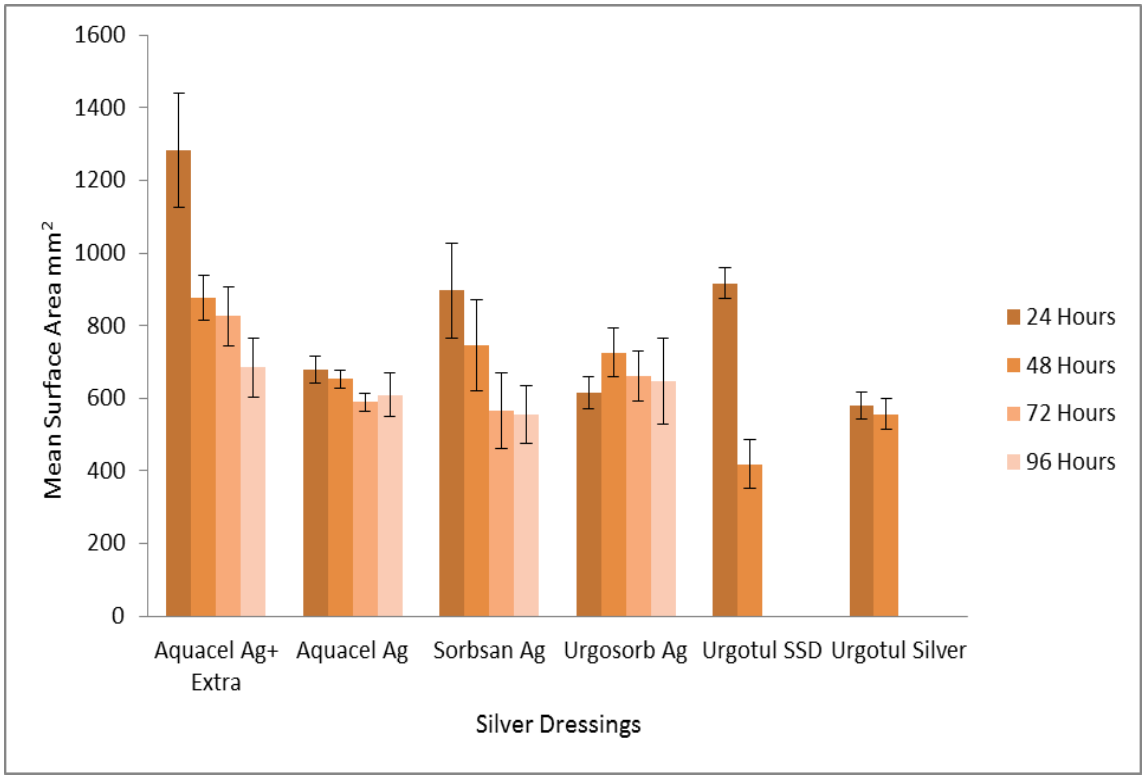


Figure 3.5: ZOI produced by Silver Dressings against *S. aureus* (Error bar = Standard Deviation, n = 6)

3.3.3.2. *E. coli* - Results

The two hydrofiber silver dressings, Aquacel Ag and Aquacel Ag$^+$ Extra were active against *E. coli* for 120 hours whereas the alginate dressings, Sorbsan Ag and Urgosorb Ag were effective for 96 hours. The two soft polymer dressings, Urgotul SSD and Urgotul Silver only produced ZOI for 48 hours (Figure 3.6). All the dressings, except the two soft polymers, produced larger ZOI after 48 hours than they had done in the first 24 hours. The ZOI produced by all dressings except Urgotul Silver were smaller than those produced against *S. aureus*. The largest ZOI was produced by Aquacel Ag+ Extra (Mean 742±29.75mm^2) which was smaller than the largest ZOI it produced against *S. aureus* (Mean 1284±157.19mm^2)

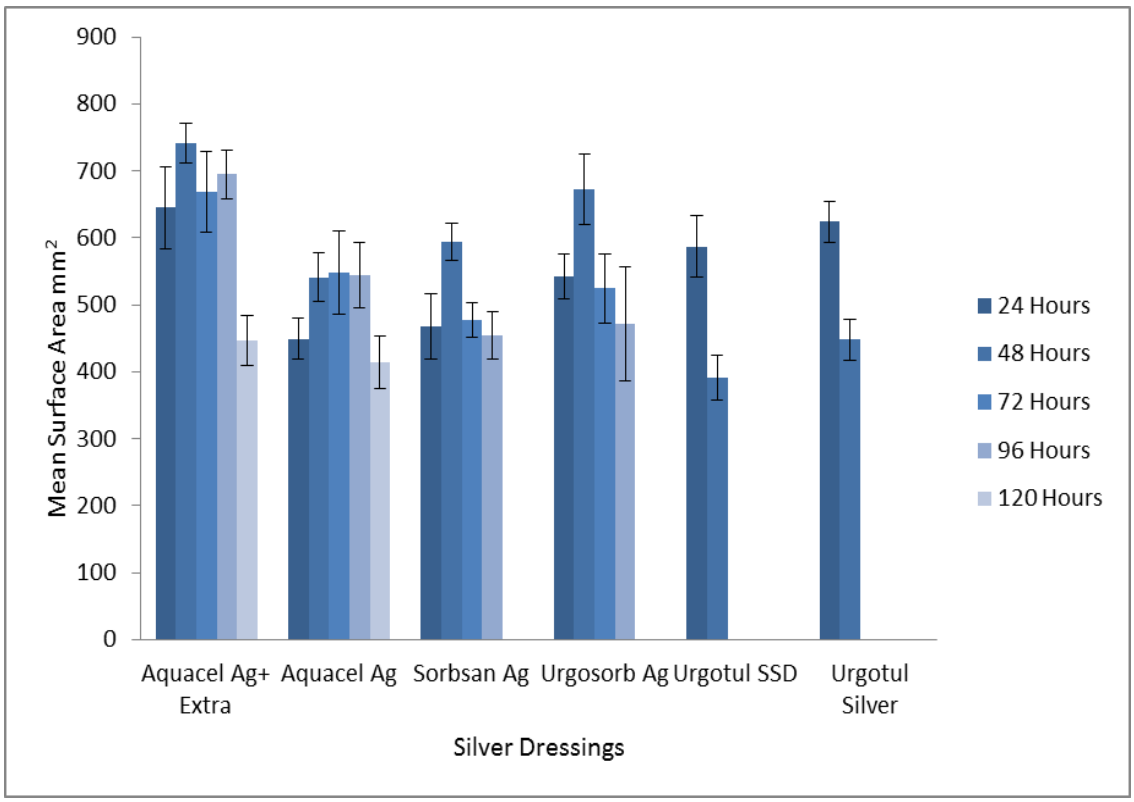


Figure 3.6: ZOI produced by Silver Dressings against *E. coli* (Error bar = Standard Deviation, n = 6)

3.3.3.3. *P. aeruginosa* - Results

All six silver dressings produced ZOI against *P. aeruginosa* for 96 hours (Figure 3.7). They all produced larger ZOI against *P. aeruginosa* than they had against the other two bacteria. The largest ZOI were produced by the hydrofibers, Aquacel Ag and Aquacel Ag⁺Extra and the alginates, Sorbsan Ag and Urgosorb Ag. Aquacel Ag⁺ Extra again produced the largest ZOI (Mean 1871±76.26mm²). All the dressings produced larger ZOI after 48 hours than they had in the first 24 hours. After 96 hours all the silver dressings were still producing large clear ZOI with a surface area > 500mm². At 120 hours they were no longer producing ZOI.

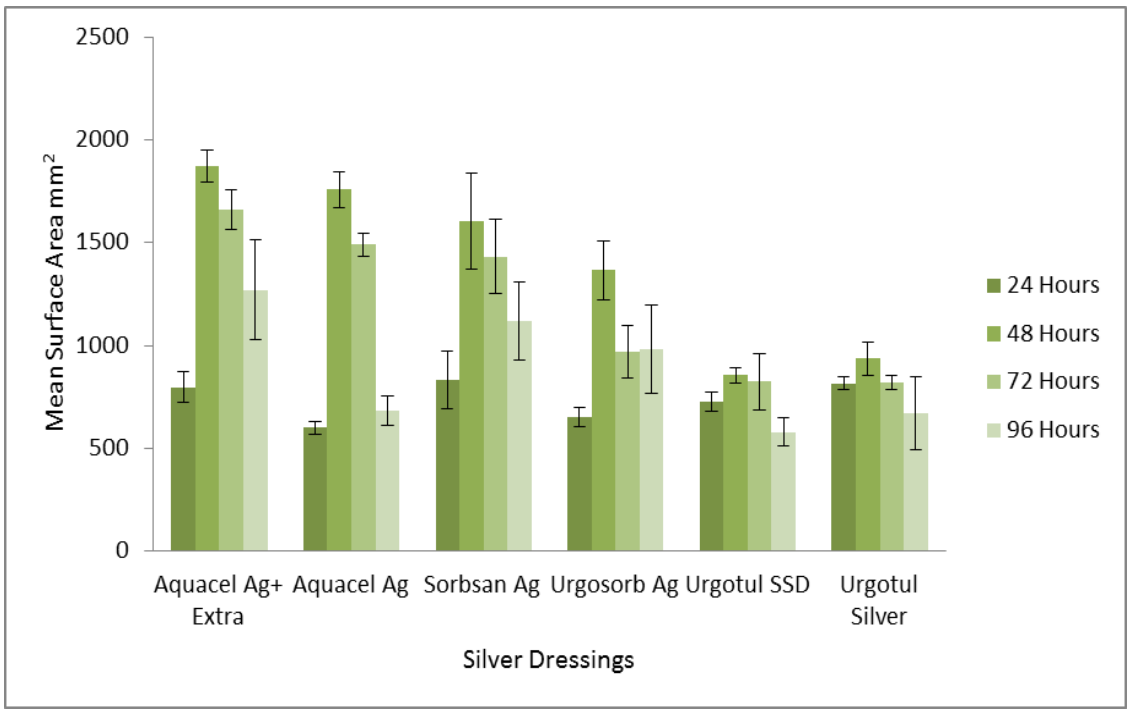


Figure 3.7: ZOI produced by Silver Dressings against *P. aeruginosa* (Error bar = Standard Deviation, n = 6)

3.3.3.4. Silver Foam Dressings – Passage Study Results

The samples of the two silver foam dressings; Allevyn Ag and Biatain Ag produced very different results to the other silver containing dressings so have been described separately.

The Biatain Ag dressing did not produce a clear ZOI against either *S. aureus* or *E. coli* but was effective against *P. aeruginosa* (Figure 3.8).

However, Allevyn Ag produced inconsistent results against *S. aureus* and *E. coli.* Against *S. aureus* only 5 out of the twelve Allevyn Ag samples produced a clear ZOI after 24 hours. One sample continued to produce a small ZOI for a total of 72 hours.

Only two out of twelve Allevyn Ag samples tested against *E. coli* produced a clear ZOI after 24 hours. These two samples were transferred to freshly seeded agar plates and continued to produce a very small ZOI for a further 7 days.

Both silver foams tested produced large ZOI against *P. aeruginosa* for 7 days. For the first 48 hours Allevyn Ag produced larger ZOI, at 72 hours they had very similar sized ZOI, there after Biatain Ag produced the larger ZOI (Figure 3.8).

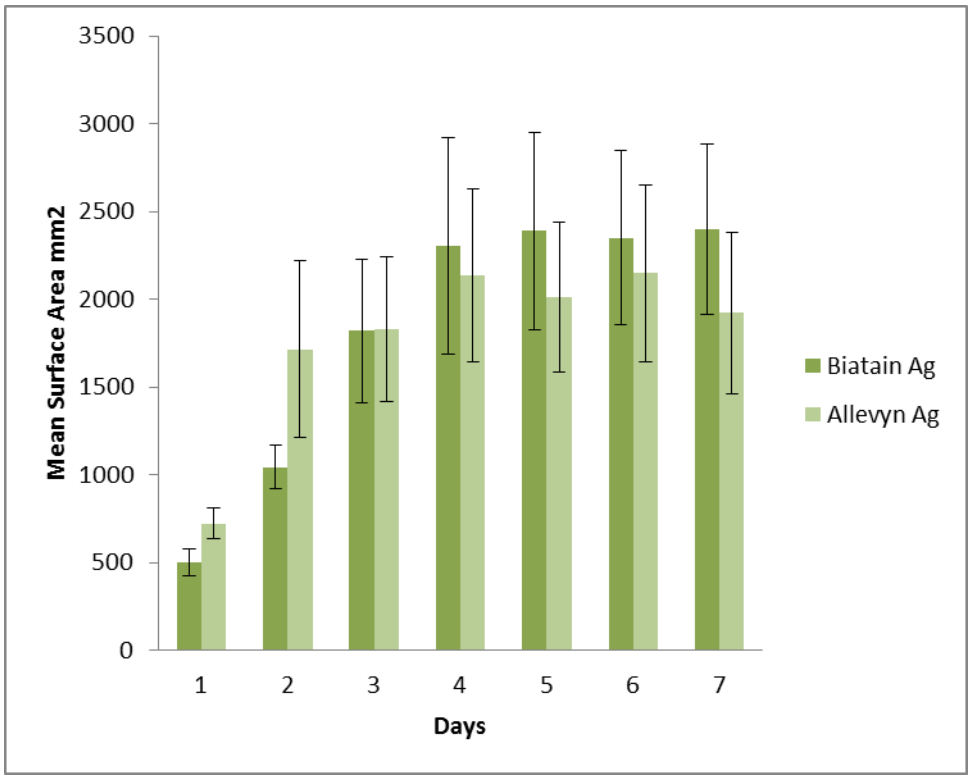


Figure 3.8: ZOI produced by the two Silver Foam Dressings against *P. aeruginosa* (Error bar = Standard Deviation, n = 6)

The duration of action of the silver dressings, classified by the carrier dressing is shown in Table 3.4.

Table 3.4: Duration of action of different classes of silver dressings against *S. aureus*, *E. coli* and *P. aeruginosa*.

	S. aureus	*E.coli*	*P. aeruginosa*
Alginates	96 hours	96 hours	96 hours
Hydrofibers	96 hours	120 hours	96 hours
Soft Polymers	48 hours	48 hours	96 hours
Foams	≤ 24 hours	≤ 24 hours	168 hours

3.3.4. Honey Dressing – Passage Study Results

The honey dressings included in the passage studies are listed in Table 3.5.

Table 3.5: Honey Dressings tested classified by carrier dressing type.

Dressing	Formulation
Medihoney Gel Sheet	Sodium Alginate 20% w/w with 80% w/w Manuka Honey
Medihoney Apinate	Calcium Alginate with 100% Leptospermum Manuka honey
Algivon	Calcium Alginate with 100% Manuka honey
Activon Tulle	Knitted Viscose with 100% Manuka Honey
Actilite Tulle	Knitted Viscose with 99% Manuka Honey and 1% Manuka oil
Melladerm Plus Tulle	Knitted Viscose with 45% medical grade Bulgarian mountain flower honey
L- Mesitran Net	Open weave mesh coated with 20% medical grade honey

The sodium alginate dressing, Medihoney Gel sheet melted after 24 hours incubation at 37^{o}C so could not be transferred so the ZOI could only be measured after 24 hours. Medihoney Gel Sheet produced a clear ZOI against each of the three test microorganisms.

L- Mesitran Net, the 20% medical grade honey tulle, curled into a tube after 24 hours incubation at 37^{o}C. None of the samples tested demonstrated any antimicrobial activity against any of the three test microorganisms after 24 hours. It was not possible to weigh down the dressings as any weight would have blocked the large open pores in the mesh and affected the integrity of the dressing.

Melladerm Plus Tulle, the 45% medical grade Bulgarian honey tulle, did not demonstrate any antimicrobial activity against any of the test microorganisms and there was evidence of microbial growth through the pores in the dressing.

Four of the Manuka Honey dressings; Medihoney Gel sheet, Medihoney Apinate, Algivon, and Activon Tulle produced clear ZOI against the three test microorganisms. The ZOI produced are shown in Figures 3.10, 3.11 and 3.12.

Actilite Tulle which contains 99% Manuka honey, and 1% Manuka oil was only effective against S. *aureus* and produced a very small ZOI similar to the size of the sample.

3.3.4.1. *S. aureus* – Results

After 24 hours all 5 Manuka honey dressings had produced a clear ZOI against *S. aureus* (Figure 3.10). On the second day only the MH honeys; Algivon and Activon Tulle were still demonstrating any signs of antimicrobial activity. They both produced a very small square shaped ZOI around the edge of the sample. By 72 hours there was complete microbial growth under the samples of both these dressings. The two tulle dressings Activon and Actilite produced two distinct ZOI. The photographs of the ZOI produced by Activon Tulle in Figure 3.9, show a clear ZOI around the Activon sample and then a larger area of reduced antimicrobial growth surrounded by an orange ring which presumably is the limit of the honey's diffusion across the agar. The clear ZOI was used in the calculation of the surface area of inhibition of microbial growth.

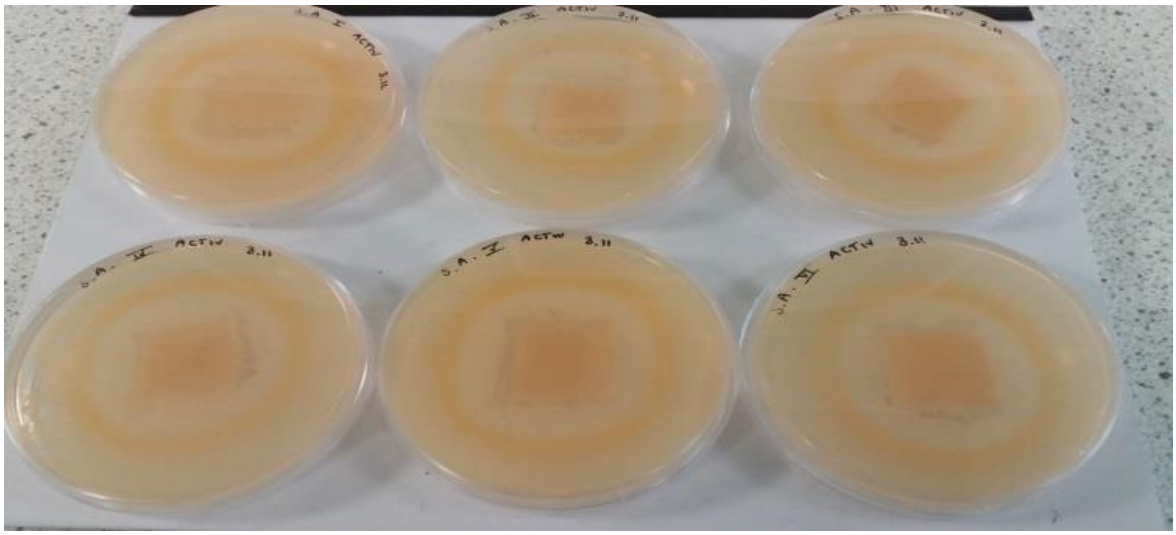


Figure 3.9: Two ZOI produced by Activon Tulle against *S. aureus*.

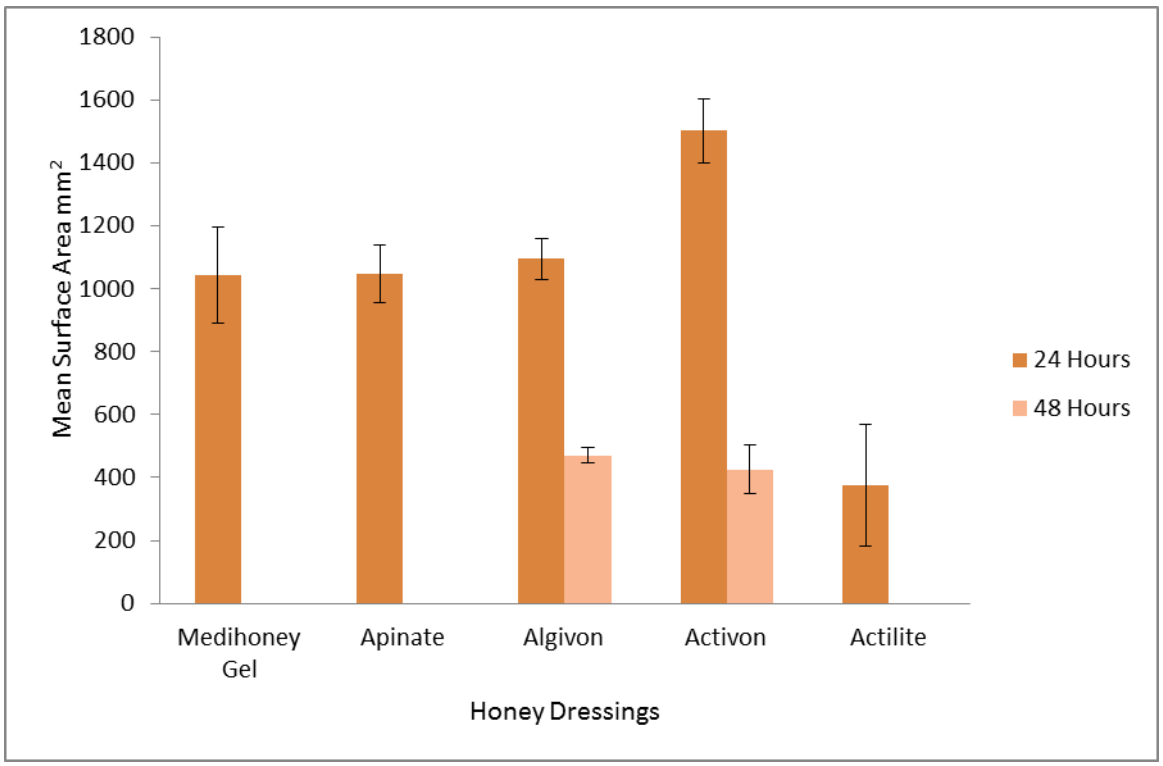


Figure 3.10: ZOI produced by Manuka Honey Dressings against *S. aureus* (Error bar = Standard Deviation, n = 6)

3.3.4.2. *E. coli* – Results

All four Manuka honey dressings except, Actilite Tulle, produced a clear ZOI after 24 hours (Figure 3.11). After 48 hours only the two calcium alginate MH honey dressings were still producing ZOI; Algivon (Mean 2686.92±168.46mm^2) and Medihoney Apinate (Mean 2218.67±100.82mm^2). The size of the ZOI produced by Algivon against *E. coli* was more than double the ZOI it had produced against *S. aureus,* Mean 1094±65.8mm^2. Interestingly both Algivon and Medihoney Apinate produced a larger ZOI after 48 hours than they did in the first 24 hours but did not produce a ZOI at 72 hours.

The two MH Honey Tulle dressings; Actilite and Activon produced two distinct ZOI as demonstrated previously in the *S. aureus* experiments. However, under the Actilite the agar looked clearer but there was not a clear ZOI but a larger circular area which showed evidence of reduced microbial growth. Actilite was not included in the comparison because it did not produce a clear ZOI. Activon Tulle produced a large clear ZOI (Mean 1222±133.52mm^2) plus an even larger

zone of reduced microbial growth (2926mm^2) after 24 hours but no ZOI at 48 hours.

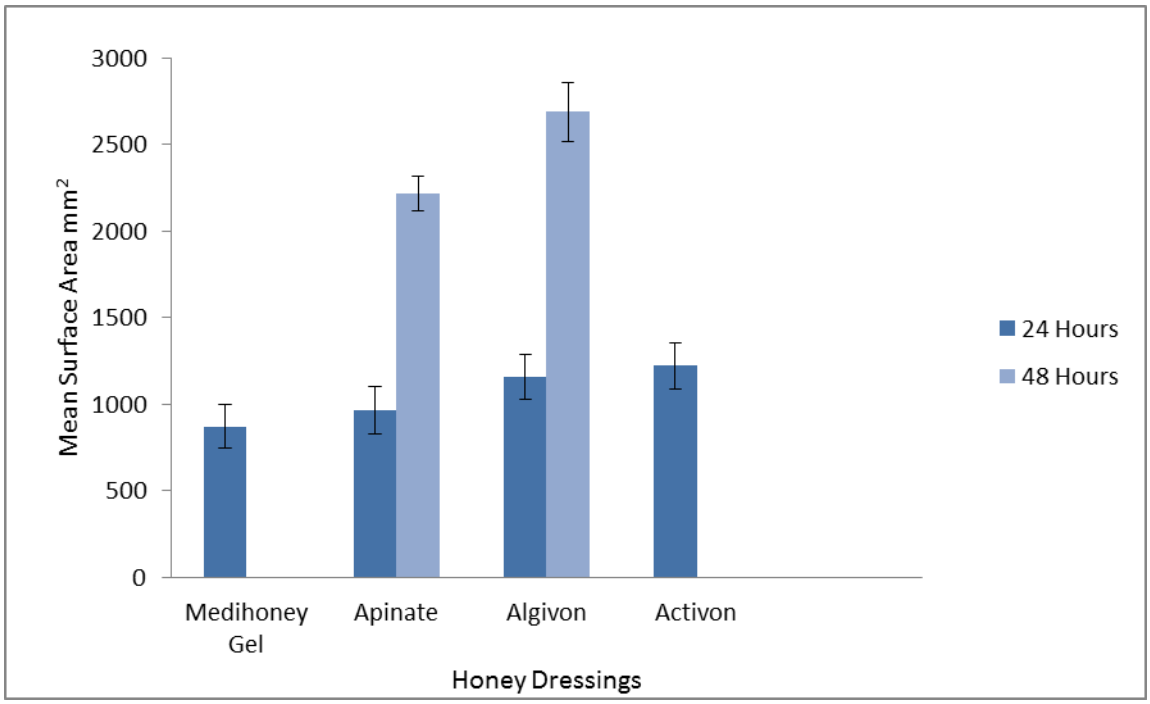


Figure 3.11: ZOI produced by Manuka Honey Dressings against *E. coli* (Error bar = Standard Deviation, n = 6)

3.3.4.3. *P. aeruginosa* – Results

Actilite Tulle did not produce a ZOI against *P. aeruginosa* and after 24 hours there was complete microbial growth through the weave of the Tulle. The other four Manuka Honeys produced clear ZOI after 24 hours but the size of the ZOI were smaller than the ZOI they produced against *S. aureus* and *E. coli*. At 48 hours there was complete microbial growth under all the test samples (Figure 3.12).

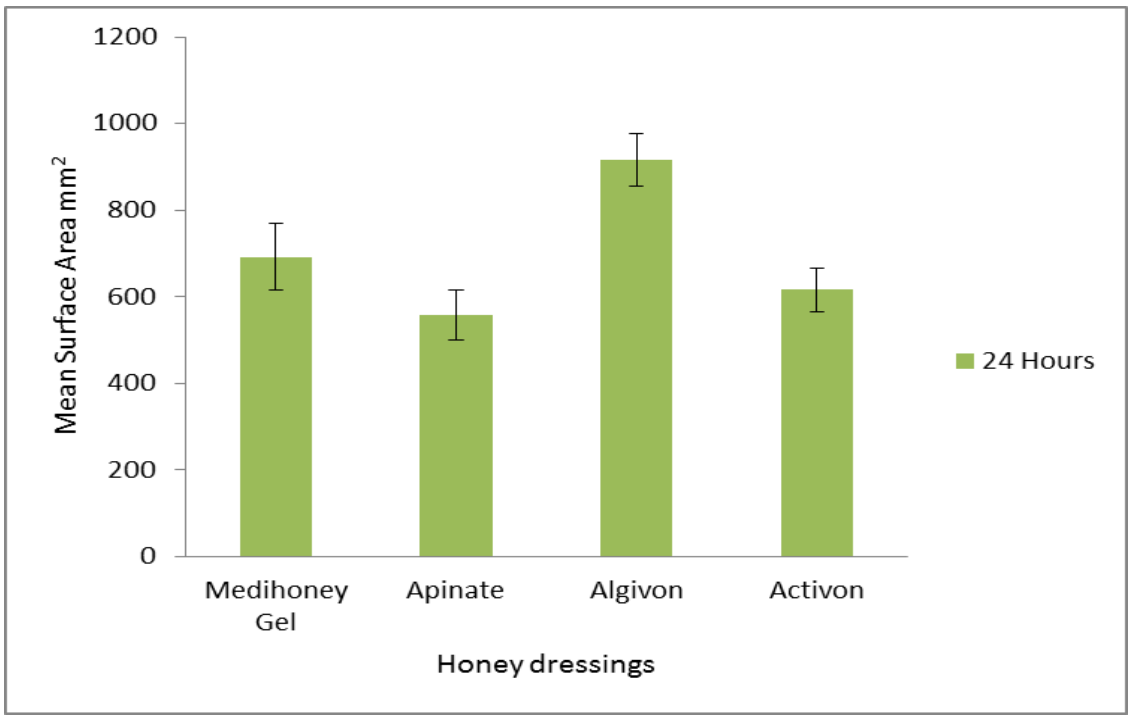


Figure 3.12: ZOI produced by Manuka Honey Dressings against *P. aeruginosa* (Error bar = Standard Deviation, n = 6)

3.3.5. Comparison of the activity of the different antimicrobial dressings against the three test bacteria.

3.3.5.1. *S. aureus*

After 24 hours all the dressings except the silver foam, Biatain Ag and the chlorhexidine tulle, Bactigras, produced clear ZOI against *S. aureus*. The largest ZOI were produced by Suprasorb X + PHMB, Activon Tulle and Aquacel Ag+ Extra. Interestingly most of the different classes of dressings produced very large ZOI against *S. aureus* on the first day. After 48 hours Iodoflex produced the largest ZOI of all the dressings, which was more than 4 times larger than the ZOI it had produced the previous day. Suprasorb and all the silver dressing also produced larger ZOI on the second day. However, after 48 hours all the honey dressings were no longer producing clear ZOI except Activon and Algivon Tulle which produced very small ZOI around the samples. The only dressings which were still producing a clear ZOI on day 3 and day 4 were the silver hydrofibers; Aquacel and Aquacel Ag+ Extra and the silver alginates; Sorbsan Ag and Urgosorb Ag.

3.3.5.2. *E. coli.*

After 24 hours all the dressings except Actilite tulle and the silver foam Biatain Ag, produced clear ZOI against *E. coli*. The largest ZOI were produced by Suprasorb X + PHMB and the MH honeys; Algivon and Activon Tulle. However, after 48 hours the cadexomer iodine, Iodoflex, produced the largest ZOI followed by Algivon and Apinate. Interestingly these three dressings produced much larger ZOI on the second day than they did in the first 24 hours. By day 3 only the silver hydrofiber dressings, Aquacel, Aquacel Ag+ Extra and the alginate silver dressings; Urgosorb Ag and Sorbsan Ag continued to produce a ZOI. The two silver hydrofiber dressings were the only dressings still producing a ZOI after 5 days continuous use.

3.3.5.3. *P. aeruginosa*

The results were very different for *P. aeruginosa*. All the dressings except Actilite Tulle produced clear ZOI against *P. aeruginosa* after 24 hours. Iodoflex produced the largest ZOI followed by Algivon and Sorbsan Ag. However, after 48 hours only the silver dressings and Iodoflex produced a clear ZOI. All the silver dressings continued to produce a ZOI until day 5. From day 5 to 7 only the two silver foams; Allevyn Ag and Biatain Ag were still producing ZOI.

3.4. Discussion

Most of the dressings were effective against all three bacteria for either 24 or 48 hours. Only the silver dressings and the polihexanide dressing, Suprasorb®X + PHMB demonstrated a prolonged duration of action greater than 48 hours. The silver hydrofibers and alginates were active against all three bacteria for 4 days. Interestingly, the two silver foam dressings which were not consistently effective against *S. aureus* and *E. coli* produced very large ZOI against *P. aeruginosa* for 7 days. This would suggest that the formulation of the material and not just the antimicrobial agent had an impact on the antimicrobial efficacy. The study included a variety of carrier dressings from each class of antimicrobial dressing to investigate this phenomenon.

3.4.1. Biguanides- Bactigras and Suprasorb®X + PHMB

The chlorhexidine gauze dressing (Bactigras) after 24 hours produced a small ZOI against *E. coli* but no ZOI against *P. aeruginosa* or *S. aureus*. Another study found that Bactigras produced a ZOI against *S. aureus,* a smaller ZOI against *E. coli* and was not effective against *P. aeruginosa (*Aramwit et al. 2010). In comparison to all the other dressings in our study Bactigras was one of the least effective. The poor performance could be related to the physical properties of the dressing. Bactigras is composed of chlorhexidine acetate in a hydrophobic white soft paraffin ointment on a gauze open weave fabric and so is poorly absorbent. In these experiments which involved overlaying the material on agar, which is a very moist environment, dressings with encapsulated antimicrobials that rely on moisture gradients to release their antimicrobials may perform better than dressings that do not rely on that type of mechanism. However, the polihexanide containing dressing, Suprasorb®X + PHMB was very effective and produced large ZOI against *S. aureus* and *E. coli* for 48 hours and smaller but consistant sized ZOI against *P. aeruginosa* for 72 hours. Suprasorb®X + PHMB is a biocellulose dressing incorporating 0.3% PHMB which is not chemically bound to the material and is designed to release the PHMB into the wound (Butcher 2012). The physical properties of this dressing, its ability to absorb exudate and release PHMB may have enhanced its antimicrobial efficacy.

3.4.2. Iodine Dressings

Within the class of Iodine dressings, Iodoflex produced the largest ZOI against *P. aeruginosa* in the first 24 hours and the largest ZOI against *E. coli* and *S. aureus* after 48 hours. It is not clear why the iodine was more rapidly released against *P. aeruginosa,* and this interesting phenomenon warrants further investigation. Cadexomer iodine beads absorb fluid and swell to form a gel allowing the slow release of iodine until equilibrium is reached. The release and diffusion of the antimicrobial into the surrounding agar could explain why these dressings produced such large ZOI. After 48 hours the beads had turned from

brown to white indicating the loss of iodine and this corresponded to the lack of antimicrobial efficacy after 2 days use.

The two other iodine dressings; Inadine and Iodozyme, were also effective against all three bacteria for 24 hours. They produced their largest ZOI against *S. aureus* but by the second day they were either not producing a ZOI or producing a very small ZOI against the three bacteria. The Inadine samples turned white after 24 hours suggesting that the iodine had been depleted. A recent study (Szweda, Gorczyca, and Tylingo 2018) compared a range of antimicrobial dressings, including Inadine, against *S. aureus*, *E. coli* and *P. aeruginosa* using a disc diffusion-like method and a time killing assay. They found that Inadine placed in a bacterial suspension (10^9-10^{10} CFU/ml) for 4 hours eliminated all of the test bacteria. However, the activity of Inadine was lower in the disc diffusion assay than other dressings and the authors concluded that the large molecular weight and the partially controlled release of povidone iodine from the polyethylene glycol base may hinder the diffusion of iodine into the agar.

The Iodozyme samples tended to swell and curl up after 24 hours and did not produce a clear ZOI on the second day but had areas of reduced microbial growth where the hydrogel remained in contact with the agar. The short duration of action of Iodozyme may be due to the glucose oxidase having been utilised and so H_2O_2 is no longer produced so the iodide in the wound contact hydrogel could not be oxidised into antimicrobial iodine.

Figure 1.10 (Page 54) illustrates the free iodine (I^{3-} and I_2) concentration/µM produced by the three Iodine dressings over a 70 hour period. It shows that the Inadine free iodine concentrations decrease to zero after 24 hours which would explain the lack of efficacy in the second day of the study. The Iodoflex maintained free iodine concentrations above 100/µM for 48 hours whereas Iodozyme concentrations were less than 50/µM after 48 hours which may explain the differences in the duration of antimicrobial efficacy seen in this study.

3.4.3. Silver Dressings

All the silver dressings, apart from the two foams, Allevyn Ag and Biatain Ag, were effective against all three bacteria.

3.4.3.1. Foams: Allevyn Ag and Biatain Ag

Out of the two foams, Allevyn Ag was the only one to demonstrate any activity against *S. aureus* or *E. coli* and this may be due to the difference in the type of silver contained in the foam. Allevyn Ag is a silver compound dressing with silver sulphadiazine dispersed throughout a polyurethane foam film. Allevyn Ag dressings have been shown to release on average 79 ppm of silver on day 1 reducing to 45 ppm of sliver on day 7. (1.24 to 0.72mg/g or 0.087 to 0.05mg/cm^2 silver released over a 7 day test period) (Daubney 2007). Biatain Ag is a soft polyurethane foam film impregnated with a patented silver complex homogeneously dispersed through out the foam matrix. The manufacturers Coloplast state that in the presence of wound exudate silver is continuously released into the wound bed for up to seven days (www.Coloplast.co.uk>biatain-ag-non-adhesive-en-gb).

Interestingly the silver foams produced particularly large ZOI against *P. aeruginosa* and maintained their antimicrobial efficacy for the seven days of the study and so demonstrated the longest duration of antimicrobial activity of all the commercial dressings tested. This could suggest that the silver dressings may be useful in the management of chronic wounds infected with *P. aeruginosa*. Studies have suggested that *P. aeruginosa* may be particularly sensitive to the antimicrobial actions of silver. A recent study investigating the effect of silver nanoparticles (AgNP) against multi-drug resistant *P. aeruginosa* suggested that one of the main mechanisms of cell death appeared to involve disturbance of the equilibrium between the cells oxidation and anti-oxidation processes and the failure to eliminate excess ROS (Liao et al. 2019).

As described previously, silver is incorporated into dressings as either elemental or compound silver, which is in the active, ionic form (Ag+). Silver dressings have been shown to vary in their release profile depending on the type of silver and the initial silver concentration. Dressings containing compound silver usually contain lower concentrations of silver than elemental

silver dressings. They also tend to release the silver over a short time whereas the elemental silver dressings act as a reservoir and produce a more sustained delivery of silver cations (Lindsay 2011).

Most of the dressings included in this study contained compound/ ionic silver. The exact form of the silver contained in Biatain Ag is unclear as it is described by the manufacturers as a “patented silver complex”.

As discussed in Chapter 1 the oligodynamic effect of silver is well recognised. It is believed silver ion release of between 10-40 parts per million (< 60ppm for particularly resistant bacteria) is necessary for antimicrobial activity in wounds. (Marx and Barillo 2014; Percival and McCarty 2015).

Publications reporting on aqueous silver concentrations do not often differentiate between active ionic silver (Ag+) and inactive silver (Ag^0) they usually measure total silver content. Parsons et al. (2005) concluded from their *in vitro* study of the antimicrobial, physical and chemical characteristics of silver dressings that there appears to be no correlation between total silver in solution and antimicrobial efficacy. They suggested that exposure to low levels of constantly replenished ionic silver over an extended period of time causes accumulation of silver ions within the bacterial cell and subsequent death. Therefore optimal antimicrobial activity would be expected from dressings that can produce and maintain high concentrations of ionic silver in the wound environment. This sustained delivery of ionic silver from dressings is difficult to assess by simple chemical measurements of silver content or silver release into solutions. The authors advocated using a simulated wound environment to measure the potential antimicrobial activity of silver dressings.

The results from this study illustrate differences in the antimicrobial activity between the silver dressings associated with the carrier dressing, type of silver and the mechanism of silver activation and release.

3.4.3.2. Hydrofibers: Aquacel Ag & Aquacel Ag+ Extra

The hydrofibers also exhibited a prolonged duration of action being effective against *P. aeruginosa* and *S. aureus* for 4 days and *E. coli* for 5 days. They produced larger ZOI after 48 hours against the two Gram - negative bacteria

than they had after 24 hours. The opposite effect was seen against *S. aureus* where the ZOI were largest after 24 hours.

Aquacel Ag consists of hydrocolloid (sodium carboxymethylcellulose) fibres impregnated with 1.2% w/w ionic silver with a silver content of 8.3mg/100cm^2 (Thomas and McCubbin 2003). The dressing absorbs moisture to form a gel, binding sodium ions and releasing silver ions. This enables the dressing to exert significant antimicrobial activity despite the relatively modest silver content (Aquacel Ag 1ppm Ag+) (Walker et al. 2006). Aquacel Ag+ Extra consistently produced larger ZOI than Aquacel Ag which is explained by the fact that is made up of two layers of Aquacel Ag. Aquacel Ag+ Extra was developed to include two additional ingredients; ethylenediaminetetraacetic acid (EDTA) and benzethonium chloride (BEC) designed to synergistically disrupt biofilms and enhance the delivery and efficacy of ionic silver against the exposed bacteria (Said et al. 2014). The BEC reduces the surface tension between the biofilm and wound bed. It also affects the biofilm matrix and facilitates the release and dispersal of the biofilm. It also enhances the absorption of biofilm into the dressings (Seth et al. 2014).

EDTA behaves as a metal chelator which is thought to disrupt biofilm matrix integrity and facilitate delivery of silver to bacteria. The biofilm matrix structure and viscosity is contributed to by the cross linking of multi-valent metal ions such as calcium and iron which bacteria obtain from wound exudate. EDTA chelates these ions and therefore disrupts and liquefies the biofilm matrix preventing its reconstruction (Banin, Brady and Greenberg 2006).

These mechanisms may also contribute to the dressing's efficacy against planktonic bacteria.

In a recent study Yukoni et al. (2015) compared the *in vitro* antimicrobial activity of 5 silver dressings, Biohesive Ag, Mepilex Ag, PolyMem Ag, Mepilex Ag and Aquacel Ag against *S. aureus* and *P. aeruginosa* using a disc diffusion assay. They found that Aquacel Ag and Algisite Ag (Calcium Alginate Ag+) showed the highest antimicrobial activity of the dressings tested.

Hydrofiber and alginate dressings have also been shown to absorb and sequester bacteria which should assist the reduction of the bioburden in colonised wounds (Newman et al. 2006).

3.4.3.3. Alginates: Sorbsan Silver & Urgosorb Silver

Sorbsan Silver flat contains calcium alginate impregnated with 1.5% silver fibres. The alginate is high in mannuronic acid and low in guluronic acid which means it will form a soft flexible gel in contact with wound exudate (Mani 2011).

However, Urgosorb Silver is made up of the highly absorbent guluronic type calcium alginate fibres and carboxymethyl cellulose dressing impregnated with 0.5% ionic silver. The guluronic acid forms a firmer gel which maintains its form (Wound Care Handbook 2019-2020). The calcium ions in the alginate fibres exchange with sodium ions in fluid to produce water soluble sodium alginate which enables the fibres to absorb large volumes of water. Alginate wound dressings are usually non-woven and when the fibres swell the spaces between the fibres close, trapping bacteria which have entered the dressing in the wound exudate (Qin 2005). Silver ions incorporated into the alginate are released and kill the trapped bacteria and bacteria on the wound surface.

In this study both silver alginate dressings produced ZOI against all three bacteria for four days. An earlier study compared Sorbsan Ag, Urgosorb Ag and Aquacel Ag against *P. aeruginosa* and *E. coli* and found all three dressings were effective against *P. aeruginosa* but only Sorbsan Ag was effective against *E. coli* (Qin 2005).

3.4.3.4. Soft polymers: Urgotul Ag and Urgotul SSD

The two soft polymers contain an antibacterial wound contact layer with Technology Lipido-Colloid (TLC) - Ag healing matrix (Urgo Med) but different silver formulations. UrgoTul Ag is a polyester mesh impregnated with hydrocolloid and petroleum jelly (UrgoTul Dressing) which contains 3.5% silver sulphate (White, Cowan and Glover 2015).

In contact with wound exudate the silver sulphate contained in the dressing is converted into Ag^{+} ions and SO_4^{2-}. The carboxymethylcellulose particles of the hydrocolloid hydrate and swell to form a surface lipido-colloid film. The Ag^{+} ions

join the lipido-colloid layer, become active and provide a reservoir capable of releasing a bactericidal concentration of silver while in contact with the wound surface (Urgomedical). Urgotul SSD is an UrgoTul dressing containing silver sulfadiazine particles 3.75%. Urgotul SSD in contact with wound exudate releases silver ions and sulphadiazine which stay within the lipido-colloidal gel and have a topical action (Urgomedical).

Both UrgoTul Ag and UrgoTul SSD were effective against *S. aureus* and *E. coli* for 48 hours but maintained their activity against *P. aeruginosa* for 4 days. A study comparing Urgotul SSD and Aquacel Ag also found they were both effective against *S. aureus*, *E. coli* and *P. aeruginosa* and produced their largest ZOI against *P. aeruginosa* (Aramwit et al. 2010). An earlier study (Parsons et al. 2005) which compared the *in vitro* antimicrobial activity of 7 silver containing dressings and found that Aquacel ®Ag, Acticoat™ Absorbent and Silvercel™ and Urgotul® SSD demonstrated the greatest antimicrobial activity against *S. aureus* and *P. aeruginosa*.

Interestingly another factor that may affect the susceptibility of *P. aeruginosa* to silver is the ability of the bacteria to produce pyocyanin. *P. aeruginosa* has been shown to achieve silver resistance by producing redox- active pyocyanin that reduces antimicrobial Ag^{+} to non toxic elemental nanoparticulate Ag^{o} (Muller 2018).

Thomas and McCubbin (2003) undertook an *in vitro* analysis of the antimicrobial properties of 10 silver containing dressings and concluded that the total silver content of the dressings was important but other factors also influenced the antimicrobial efficacy. Firstly, the location of the silver within the dressing was significant. They found that a dressing where the silver was applied as a surface coating tended to perform better than dressings where silver was dispersed throughout the material. The chemical and physical form of the silver (present as a metallic, bound or ionic state) also appeared to impact on the efficacy. The final factor was the ability of the dressing to absorb moisture to enable release of the antimicrobial into the aqueous environment.

3.4.4. Honey Dressings

In the honey study the two medical grade honey dressings; L Mesitran Net and Melladerm Plus Tulle were compared to Manuka honey dressings and shown to have no antimicrobial effect against the three test bacteria. This result is contrary to a previous study which demonstrated that Mesitran was effective against all three bacteria (Jenkins, Burton and Cooper 2008). However other studies have shown that Mesitran and Melladerm were only effective against *S. aureus* (Jenkins, Burton and Cooper 2011). One study demonstrated that Melladerm Plus was active against *S. aureus* even when diluted 30 times (Nestiones and Vandeputte 2012).

L Mesitran Net and Melladerm Plus Tulle contain 20% and 40% medical grade honey which is a much lower concentration of honey than any of the manuka honeys tested, which were all >80% w/v. This may account for the lack of efficacy. Melladerm Plus Tulle is claimed by the manufacturer (Sanomed Manufacturing BV) to produce hydrogen peroxide 25g/g of honey for 48 hours so there may be a reason why this was not effective in the agar diffusion assay.

All the Manuka honey dressings demonstrated some antimicrobial activity for 24 hours. However only the calcium alginate dressings; Algivon and Apinate were still active after 48 hours and this was only against *E. coli.* Medihoney Gel sheet which contains 20% sodium alginate and only 80% MH produced smaller ZOI than the other honeys against all three bacteria possibly due to the lower concentration of honey. Unlike the calcium alginate honeys which retained their structure, the Medihoney melted into the agar so could not be passaged. The ability of the calcium alginates to retain their form, absorb moisture and release their active antimicrobial agents may explain why they produced larger ZOI on the second day of use. What is not known and requires further investigation is why the longer duration of action was only seen with *E. coli.*

There was a marked difference in the efficacy between the two tulle dressings. Actilite Tulle (99% MH and 1% Manuka oil) was only active against *S. aureus* and produced a much smaller ZOI than the Activon Tulle (100% MH) which produced the largest ZOI of all the honeys against *S. aureus*. This would imply that the Manuka oil may be affecting the antimicrobial efficacy *in vitro*.

The two calcium alginate MH honeys; Algivon and Apinate produced their largest ZOI against *E. coli*, then *S. aureus* and their smallest ZOI against *P. aeruginosa*. This difference in susceptibility between bacteria was also found in a study which compared 13 different honeys against *E. coli* and *P. aeruginosa* using a well diffusion method and demonstrated that *E. coli* was more susceptible to honey than *P. aeruginosa* (Wilkinson and Cavanagh 2005). It has also been shown that high levels of hydrogen peroxide or MGO usually produce the most active honey (Molan 2008; Kwakman et al. 2011; Chen et al. 2012; Lu et al. 2013). The level of leptosperin, a glycoside of methyl sringate found exclusively in Leptospermum honey was also found to correlate with the potency of the honey and may modulate the antimicrobial activity (Kato et al. 2012).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of manuka honey which had an antibacterial potency of 18% (w/v) phenol has been determined against *P. aeruginosa* as 9.5% (w/v) and 12 % (w/v) (Henriques et al. 2011) and against *S. aureus*, 2.9% (w/v) and 4.5% (w/v) indicating that *S. aureus* was more susceptible (Henriques et al. 2010). The mechanisms of action of honey against these two bacteria have been shown to be different. Manuka honey appears to cause cell lysis in *P. aeruginosa* but against *S. aureus* it interferes with the cell cycle and prevents successful cell division (Kilty et al. 2011). Honey treatment has been reported to cause Gram-negative species *E. coli* and *P. aeruginosa* to have both abnormally shorter and longer cells (Lu et al. 2013).

Another interesting observation in the honey study was that the two Tulle dressings created a clear ZOI around the sample and then a larger second ZOI that was less dense than the lawn of bacteria outside the zone. This phenomenon was first reported in our earlier study which investigated antimicrobial efficacy after 24-hour exposure to different AWD (Bradshaw 2011). It is still unclear whether this is due to a bacteriostatic effect of the honey or whether the bacteria have developed some form of resistance. These two ZOI were only observed with *S. aureus* and *E. coli* and not *P. aeruginosa.* This could possibly be because the *P. aeruginosa* produces a dark green pigment

which would obscure the honey coloured ring which surrounded the second ZOI.

The inconsistency in the results between this study and the different *in vitro* studies discussed could be due to variations in experimental methodology. One of the adaptations to the ZOI method identified involved soaking the dressings with simulated wound fluid to simulate an exuding wound (Parson et al. 2005). Other differences such as in bacterial strains, inoculum concentration and incubation times may influence the results. In some studies, the inoculated plates are incubated prior to the exposure to the dressing rather than after exposure to the dressing which may affect bacterial growth patterns. Any change in experimental design may generate different results when evaluating the same dressing.

3.5. Conclusions

The results of this study demonstrate that most of the dressings tested, except the two Tulles containing chlorhexidine and iodine, and the two silver foams Allevyn Ag and Biatain Ag were effective against the three test bacteria for up to 48 hours continued use. The silver dressings however demonstrated a longer duration of action than the other classes of antimicrobial dressings being active for up to 4 days against the three bacteria. The two silver foams produced large ZOI against *P. aeruginosa* for the 7 day duration of the study which would suggest that the silver dressings could be particularly useful in the treatment of wounds infected with this particular strain of this pathogen.

The method used in this study enabled a wide range of dressings to be screened and provided an insight into the duration of antimicrobial activity against of a number of pathogens commonly found in infected chronic wounds.

Future work, using a wider variety of strains, would help determine whether these observations are applicable across multiple strains.

Chapter 4: Novel Antimicrobial Materials

4.1. Introduction

Following the initial research investigating the antimicrobial properties of commercial antimicrobial wound dressings a new project was initiated - CROWD (Controlled Release from Open Wound Dressings). The aim of the project was to create a new type of dressing which could deliver a controlled release of an antimicrobial agent over a sustained period of time. The objective was to develop a series of biocompatible materials containing either silver or iodine which were found to be the most antimicrobially active in the previous study (Chapter 3) and to subject them to the same susceptibility testing as the commercial dressings.

The intention was to develop an antimicrobial material that was effective against all the test bacteria but had a longer duration of action than the commercial dressings previously tested. The proposed advantages of a controlled release of an antimicrobial from the material were to deliver a low but effective concentration of antimicrobial to the wound surface to control the bioburden but also minimise the risk of cytotoxicity. Also being able to leave the dressing in contact with the wound for a number of days may enhance to healing process and also reduce the frequency of dressing changes and save clinical staff time.

4.2. Formulation of new Antimicrobial Materials

4.2.1. Introduction

In the initial experiments various combinations of polymers, clays and antimicrobial agents were mixed to create film dressings which were cast onto 2 x 2 cm glass microscope cover slips. All samples were sterilised by UV radiation for 15mins on each side using a Mini-V/PCR (Telstar Life Solutions, Terrassa, Spain) prior to testing.

In this chapter concentrations of formulation components are expressed as wt% calculated as g of component in 100g of formulation. Where formulations are dried in manufacture (e.g CROWD dressing) the concentration of antimicrobial

agent is expressed as g antimicrobial in 100g of final (dry) product. For consistency iodine concentrations are expressed as iodine irrespective of the iodine source (e.g., PVP-I)

4.2.2. Methods Summary

4.2.2.1. Preparation of Starch/Clay Composites

The first formulations created were a series of sodium-clay and silver exchanged clay composites prepared in the Materials Research Engineering Institute (MERI) at Sheffield Hallam University (SHU) by Marianne Labet. The methods used to prepare the Starch/Clay composites are described in Section 2.3.2.1.

Four different combinations of starch and clay were created with and without iodine: -

1. Sodium Clay & Starch
2. Sodium Clay & Starch & Iodine
3. Silver Clay & Starch
4. Silver Clay & Starch & Iodine

The composition of the formulations of the 9 samples created are listed in Table 4.1.

Table 4.1: Formulations of starch – based antimicrobial films (Labet 2011)

Batch	Starch (g)	Water (g)	PEG_{600} (g)	Clay Na (g)	Clay Ag (g)	M KI_3 soln. (g)	Antimicrobial content
F-0102	5.0	36.0	1.0	1.67	0	0	No antimicrobial
F-0105	5.0	36.0	1.0	1.66	0.0084	0	Ag Clay only
F-0109	5.0	36.0	1.0	1.65	0.0167	0	Ag Clay higher concentration
F-0112	5.0	36.0	1.0	1.67	0	1.3	Iodine 1.3g with no Ag Clay
F-0115	5.0	36.0	1.0	1.66	0.0084	1.3	Ag clay and Iodine 1.3g
F-0118	5.0	36.0	1.0	1.65	0.0167	1.3	Higher Ag Clay and Iodine 1.3g
F-0021	5.0	36.0	1.0	1.67	0	2.6	Iodine only 2.6 g
F-0024	5.0	36.0	1.0	1.66	0.0084	2.6	Ag Clay and 2.6g Iodine
F-0027	5.0	36.0	1.0	1.65	0.0167	2.6	Higher Ag Clay and 2.6g Iodine

Each formulation contained 5g starch, 36g water and 1g PEG_{600} with differing concentrations of either silver (Ag) clay or iodine or both. 0.5g aliquots of the different formulations were coated on to cover the surface of 2 x 2cm microscope cover slips. The samples were then dried at room temperature. These nine formulations were tested for antimicrobial activity using the *in vitro* growth inhibition assay described in Section 2.1.2.2.

4.2.2.2. Preparation of Laponite/Povidone – Iodine (PVP-I) composites

A second series of formulations were prepared using a synthetic clay, Laponite®RD and povidone-iodine solution as described in Section 2.3.2.2.

Composites were prepared by Marianne Labet in MERI using increasing amounts of Laponite combined with various volumes of povidone iodine (PVP-I) solution to produce composites containing between 1-30wt% Laponite (Table 4.2).

Table 4.2: Formulations of PVP-I/ Laponite Composites

Batch	Concentration of Laponite	Mass Laponite (g)	Mass Povidone-I Solution (g)
G-0001	1wt%	0.2	19.8
G-0002	2wt%	0.4	19.6
G-0003	5wt%	1.0	19.0
G-0004	10wt%	2.0	18.0
G-0005	30wt%	6.0	14.0
G-0006	10wt%	2.0	18.0
G-0007	12wt%	2.4	17.6
G-0008	**15wt%**	**3.0**	**17.0**
G-0009	20wt%	4.0	16.0
G-0010	13wt%	2.6	17.4
G-0011	14wt%	2.8	17.2

The first four samples produced, containing 1, 2, 5 and 10 wt% Laponite, split into two phases when allowed to settle (Figure 4.1). The bottom phase was a gel and the top phase was liquid. Increasing the amount of Laponite increased the thickness of the gel and at 15wt% the whole material was gelling. 0.5g of G-0008, the 15wt% gel was added to glass cover slips and allowed to dry. This sample underwent antimicrobial testing with the other 9 starch based samples.

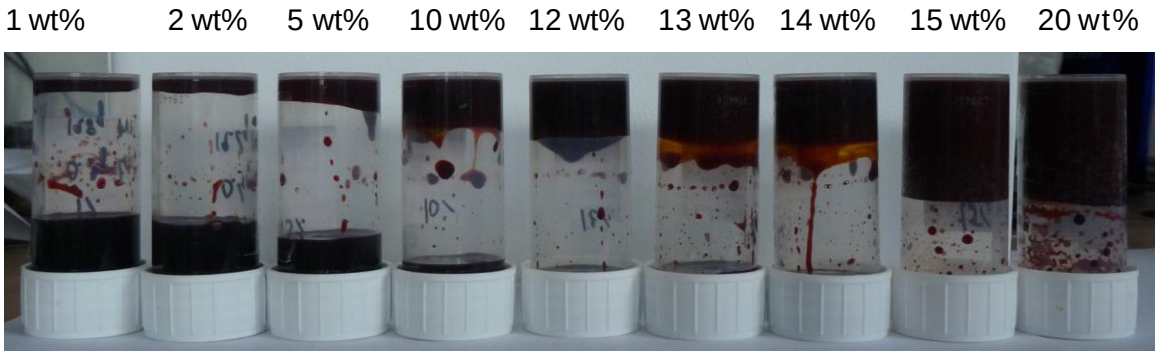


Figure 4.1: Formulations of PVP-I/Laponite Composites containing increasing concentrations of Laponite from 1 to 20 wt% (Labet 2011)

4.2.2.3. Preparation of PVOH/Povidone Iodine composites

A third series of composites were then created using a polymer, polyvinyl alcohol (PVOH) instead of starch or clay. The PVOH was combined with

different amounts of Povidone Iodine (PVP-I) 10%w/w aqueous solution containing 1% (w/w) available iodine. Two different molecular weight samples of PVOH were used in the formulation, Mowiol 4-98 and the higher molecular weight Mowiol 28-99. This was designed to determine whether the molecular weight of the PVOH influenced the antimicrobial activity of the material. The method used in the preparation of the composites is described in Section 2.3.2.3.

Three formulations were created as described in Table 4.3

Table 4.3: Formulations of the PVOH/PVP-I Composites

Batch	PVOH Type	Mass PVOH (g)	Mass Water (g)	Mass PVP-I (g)	Approx wt% Iodine
F-0029	Mowiol 4-98	7.0	35.0	7.0	1wt%
F-0032	Mowiol 28-99	7.0	45.0	7.0	1wt%
F-0035	Mowiol 4-98	7.0	35.0	14.0	2wt%

4.2.2.4. Antimicrobial Susceptibility testing of the Composite Samples

Two squares each measuring 2 x 2cm of the 13 newly created materials listed in Table 4.4. were tested for antimicrobial activity against three microorganisms: *Staphylococcus aureus (SH 1000)*, *Escherichia coli* (JM109) and *Pseudomonas aeruginosa (H085180216)*. These were the same strains that had been previously used to test the commercial antimicrobial dressings.

The *in vitro* growth inhibition assay methodology (Section 2.1.2.2.) applied to the commercial antimicrobial dressings was also used to test the novel composite materials.

Uncoated glass microscope slide coverslips were also subjected to the same *in vitro* growth inhibition assay tests to try to determine if the glass had any impact on the growth of the three test bacteria.

All test samples and the cover slips were UV irradiated for 15 minutes on each side prior to testing to ensure sterility.

Table 4.4: Samples tested for Antimicrobial Susceptibility

Sample	Batch	Formulation
1	F0102	Starch/ PEG/ Na-Clay
2	F0105	Starch/ PEG/ Na-Clay 99.5%/ Ag-Clay 0.5%
3	F0109	Starch/ PEG/ Na-Clay/ Ag-Clay 1%
4	F0112	Starch/ PEG/ Na-Clay/ KI_3 1%
5	F0115	Starch/ PEG/ Na-Clay 99.5%/ Ag-Clay 0.5%/ KI_3 1%
6	F0118	Starch/ PEG/ Na-Clay 99%/ Ag–Clay 1% / KI_3 1%
7	F0021	Starch/ PEG/ Na-Clay/ KI_3 2%
8	F0024	Starch/ PEG/ Na-Clay 99.5%/ Ag-Clay 0.5%/ KI_3 2%
9	F0027	Starch/ PEG/ Na-Clay 99% / Ag-Clay 1% / KI_3 2%
10	F0029	PVP-I/ PVOH Mowiol 4-98 / 1 wt% I_2
11	F0032	PVP-I/ PVOH Mowiol 28-99/ 1 wt% I_2
12	F0035	PVP-I/ PVOH Mowiol 4-98 / 2 wt% I_2
13	G0008	PVP-I/ Laponite (85% PVP-I/15% Laponite) 8.5 wt% I_2

PEG = Polyethylene Glycol

Na-Clay = Sodium Clay = Cloisite®Na^+

Ag-Clay = Silver Clay

Unfortunately, due to time constraints in the manufacturing process only six 2 x 2cm samples were made of each formulation so only two replicates of each sample were tested against the three different microorganisms. After 24 hours incubation at 37°C the agar plates were removed from the incubator and the surface area of the Zone of Inhibition (ZOI) of microbial growth was measured in mm^2 as described in Section 2.1.2.2.

Figures 4.2 and 4.3. show samples of the composite containing no iodine (Sample 1) and a sample containing 2wt% Iodine as PVP-I (Sample12).

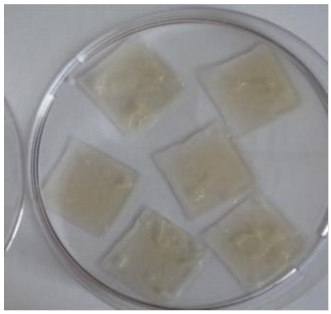


Figure 4.2: Sample 1 containing Starch/PEG/Na-Clay on a 2cm x 2cm microscope slide coverslip (Labet 2011).

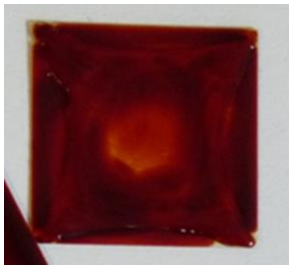


Figure 4.3: Sample 12 PVOH/PVP-I containing 2 wt% Iodine as PVP-I on a 2cm x 2cm microscope slide coverslip (Labet 2011).

4.2.3. Results

4.2.3.1. *S. aureus* – *In vitro* Growth Inhibition Assay Results

Samples 1-9 containing Starch/PEG/Na-Clay with either Ag-Clay, KI_3 or both did not produce a zone of inhibition of bacterial growth.

Samples 10-13 which contained different concentrations of iodine all produced clear ZOI of inhibition of bacterial growth as shown in Table 4.5.

Table 4.5: ZOI produced by Samples 10, 11, 12 and 13 against *S. aureus.*

Sample	Batch	Approx Iodine	Replicate 1 SA (mm^2)	Replicate 2 SA (mm^2)	Mean Surface Area (Standard Deviation)
10	F0029 Mowoil 4-98	1 wt%	No growth under dressing	707	N/A
11	F0032 Mowoil 28-99	1 wt%	397	491	$444 \pm 66.47 mm^2$
12	F0035 Mowoil 4-98	2 wt%	572	491	$531.5 \pm 57.28 mm^2$
13	Laponite 15 wt%	8.5 wt%	933	592	$762.5 \pm 241.12 mm^2$

4.2.3.2. *E. coli* – *In vitro* Growth Inhibition Assay Results

Samples 1-9 containing Starch/PEG/Na-Clay with either Ag-Clay, KI_3 or both did not produce a zone of inhibition of bacterial growth.

Sample 10 which contained the lower molecular weight PVOH (Mowiol 4-98) and 1wt% iodine also did not produce a zone of inhibition of bacterial growth.

Samples 11-13 which included the higher molecular weight PVOH (Mowiol 28-99) and 1wt% Iodine, the lower molecular weight PVOH (Mowiol 4-98) with 2wt% Iodine and the Laponite samples all produced small ZOI (Table 4.6)

Table 4.6: ZOI produced by samples 11, 12 and 13 against *E. coli.*

Sample	Batch	Approx Iodine	Replicate 1 SA (mm^2)	Replicate 2 SA (mm^2)	Mean Surface Area (Standard Deviation)
11	F0032 Mowoil 28-99	1 wt%	572	488	$530 \pm 59.4 mm^2$
12	F0035 Mowoil 4-98	2 wt%	510	612	$561 \pm 71.1 mm^2$
13	Laponite 15 wt%	8.5wt%	452	Coverslip totally detached left fragments which produced 5mm to 1cm ZOI around fragments	N/A

4.2.3.3. *P. aeruginosa – In vitro* Growth Inhibition Assay Results

Samples 1-9 containing Starch/PEG/Na-Clay with either Ag-Clay, KI_3 or both did not produce a zone of inhibition and the bacteria appeared to have grown into the dressing. This was shown by a green discolouration of the samples.

Samples 10-13 did not produce a ZOI. The PVOH samples all swelled and detached from the cover slip. However there appeared to be no bacterial growth under the samples (Table 4.7).

Table 4.7: ZOI produced by samples 10, 11, 12 and 13 against *P. aeruginosa.*

Sample	Batch	Approx Iodine	Replicate 1 SA (mm^2)	Replicate 2 SA (mm^2)
10	F0029 Mowoil 4-98	1 wt%	No growth under dressing*	No growth under dressing *
11	F0032 Mowoil 28-99	1 wt%	No growth under dressing*	No growth under dressing*
12	F0035 Mowoil 4-98	2 wt%	No growth under dressing*	No growth under dressing*
13	Laponite 15 wt%	8.5 wt%	Inhibition of growth under cover slip and ZOI 1mm around individual fragments	Sample detached from cover slip into small fragments. No growth where dressing fragments in contact with agar

* Samples swelled and some detached from cover slip. No bacterial growth was observed where the dressings were still in contact with the agar.

4.2.4. Discussion

Samples 1-9 which contained starch/PEG/Na-Clay with the addition of Ag-Clay, or KI_3 or both Ag-Clay and KI_3 did not appear to inhibit the growth of any of the test bacteria.

None of the formulations containing silver (Ag) clay appeared to have any antimicrobial activity against any of the three test bacteria. This was unexpected and one of the reasons could have been the aging of the silver containing clay used to prepare the composites. It was likely that the lack of antimicrobial activity of the starch/PEG/Clay materials was a function of the structure because the silver clay had demonstrated antimicrobial activity in the absence of KI_3 in previous studies (Breen 2012: Personal communication). It was also not known where in the material the silver ions were located relative to the clay, they could have been occupying cation exchange surface sites, possibly in addition to a range of silver species including Ag_2O, metallic silver thus producing very small amounts of active silver. Also, the silver ions could have been absorbed into the clay displacing the sodium which could lead to the silver ions reacting with I_3^- to form insoluble AgI (Breen 2012 Personal communication).

The lower molecular weight polymer, Mowiol 4-98 (1wt% iodine as PVP-I) (Sample 10) was able to reduce the growth of *S. aureus* but did not appear to

inhibit the growth of *E. coli*. However, the higher molecular weight PVOH, Mowiol 28-99, with the same concentration of iodine (1wt %), produced a clear ZOI against *E. coli*. It was therefore decided to use the higher molecular weight PVOH in future formulations.

Samples that contained PVP-I and PVOH or Laponite (Samples 11-13) all produced a clear ZOI against *S. aureus* and *E. coli*. However, these samples did not produce a clear ZOI against *P. aeruginosa* but did appear to reduce the growth of the bacteria under the sample.

The microscope cover slips without a dressing attached appeared to reduce microbial growth of all three test bacteria in the 2 x 2cm square area under the cover slip.

4.3. Determining the Optimum Antimicrobial Concentration

4.3.1 Introduction

The lack of efficacy of KI_3 and silver containing composites and the promising results produced by the dressings containing PVP-I led to the decision to create a series of dressings using the higher molecular weight PVOH, Mowiol 28-99 with differing concentrations of iodine. A variety of different amounts of PVP-I were added to the formulations in an attempt to determine the optimum antimicrobial concentration.

4.3.2. Methods Summary

4.3.2.1. Composites with increasing Iodine Concentration

The active antimicrobial agent incorporated into the new formulations was Povidone Iodine (Videne® 10%) aqueous solution.

Dressings were prepared using the two water soluble polymers; Poly (vinyl alcohol) [PVOH] (Mowiol 28-99) which provided cohesion to the material and Poly (vinyl pyrrolidone) [PVP] which stabilised the active ingredient, PVP-I.

Six samples were prepared containing differing amounts of PVP-I to produce composites containing iodine concentrations between 2 - 8.5 wt% Iodine (Table 4.8). The 2 x 2cm samples were UV sterilised as previously described. Six

replicates were tested against each of the three bacteria. The zones of inhibition of bacterial growth were measured after a 24 hour incubation period at 37°C.

Table 4.8: Formulations containing differing concentrations of Iodine

Sample	Formulation
14	PVP-I/ PVOH/ **2 wt%** Iodine
15	PVP-I/ PVOH/ **3 wt%** Iodine
16	PVP-I/ PVOH/ **8.5 wt%** Iodine
17	PVP-I/ PVOH/ **4.5 wt%** Iodine
18	PVP-I/ PVOH/ **6 wt%** Iodine
19	PVP-I/ PVOH/ **7.5 wt%** Iodine

4.3.3. Results

The effect of increasing the iodine concentration on the size of the ZOI produced against the three test bacteria is shown in Figure 4.4.

The figure includes the results for the PVP-I/PVOH (Mowiol 28-29)/1wt% iodine (Sample 11) listed in Tables 4.5, 4.6 and 4.7. The 1wt% and 2wt% iodine composites appeared to have reduced the growth of *P. aeruginosa* under the samples but did not produce a ZOI; however, they did produce clear ZOI against *S. aureus* and *E. coli*. With the 3wt% iodine composite the growth of *P. aeruginosa* was still visibly reduced under the sample but it was not until the concentration was increased to 4.5wt% iodine, that a clear zone of inhibition around the sample was produced. The size of the ZOI continued to increase as the wt% of the iodine in the composite increased.

The iodine produced the largest zones of inhibition against *S. aureus*. Again, increasing the concentration of iodine produced a corresponding increase in the mean surface area of the ZOI created. The effect was similar against *E. coli*, but not in a sequential manner. The 3wt% iodine sample produced a comparable ZOI with the 4.5wt% and the 6wt% samples produced similar ZOI to the 7.5wt%. All the ZOI against *E. coli* were smaller than those produced against *S. aureus* but larger than those against *P. aeruginosa* at the same iodine concentrations.

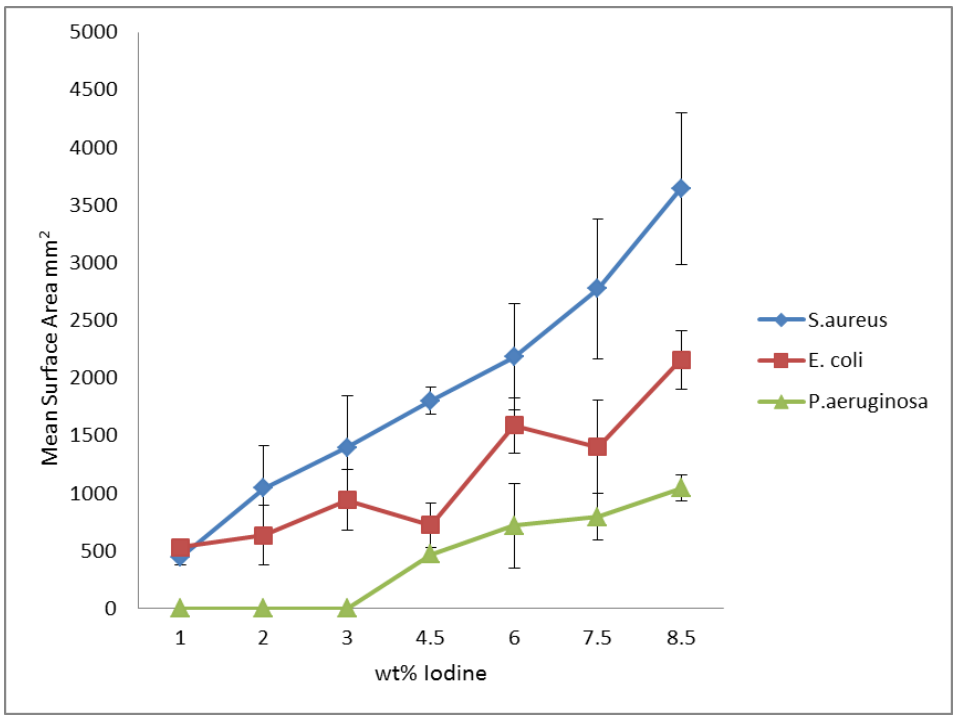


Figure 4.4: Effect of increasing iodine concentration on the zone of inhibition.
(Error bar = Standard Deviation, n = 6)

4.3.4. Discussion

The important finding from this experiment was that there was a clear trend in terms of increasing iodine concentrations producing larger ZOI. The ZOI were largest against *S. aureus*, then *E. coli* and smallest against *P. aeruginosa*.

The critical step is the availability of free iodine to inhibit the growth of microorganisms. As the concentration of free iodine is increased in the dressing materials the elution of iodine into the agar is increased and the inhibition of growth is more pronounced.

The formulation containing 6wt% iodine produced clear zones of inhibition against all three test bacteria after the 24 hour incubation period so this formulation was chosen for further study. As discussed, when reviewing the commercial dressings there were concerns about cytotoxicity with higher concentrations of iodine so it was considered important to try and keep the concentration of available iodine in the test dressing as low as possible.

4.4. Addition of Clay to prolong antimicrobial release.

4.4.1. Introduction

The commercial iodine containing dressings all demonstrated a short duration of antimicrobial action (48 hours maximum) against the test bacteria, as described in Chapter 3. One of the main aims of the CROWD project was to try to create a material that would deliver a controlled release of an antimicrobial agent over a prolonged period of time. This could then be used to reduce the iodine concentration at the wound surface which could reduce the risk of cytotoxicity and enable the dressing to remain on the surface for longer reducing the frequency of dressing changes.

The next series of experiments were designed to test the impact of the addition of clay platelets to the composite on the release of the antimicrobial agent from the material. In these experiments the concentration of iodine in the composite was fixed at 6wt% as PVP-I. Clay is a common name for a number of natural and synthetic fibrous or layered minerals such as kaolin, talc, smectite, hectorite, saponite, vermiculite, mica, attapulgite and sepiolite. The hypothesis was that the addition of clay platelets would impede the flow of iodine out of the film and slow the release profile (Figure 4.5).

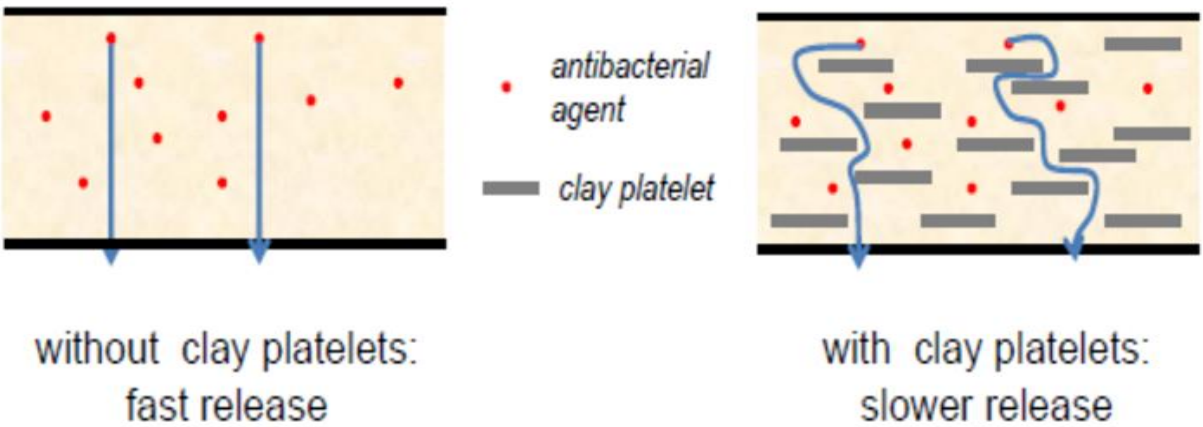


Figure 4.5: Proposed Mechanism of action of Clay Platelets (Breen 2012 Personal Communication)

Initially two formulations were created in MERI which contained 5wt% and 12.5wt% sodium clay. The clay used, Cloisite® Na^+, is bentonite which is an

impure 2:1 layered montmorillonite. Montmorillonite is a layered clay mineral of the smectite group with an aluminosilicate structure which is hydrophilic in character (Braya, Theng and Lagaly 2006).

4.4.2. Methods Summary

Samples containing PVOH/PVP-I (6wt% iodine) and the clay, Cloiste®Na^+ either 5wt% or 12.5wt% were prepared as described in Section 2.3.2.4.

4.4.2.1. Visible Iodine Release into a solvent

An experiment was conducted to investigate the release of iodine from the samples containing 5wt% and 12.5wt% Cloisite® Na^+. Square 2 x 2 cm samples of each of the materials were added to separate Petri dishes containing a fixed volume of water. The photographs in Figures 4.6 and 4.7 show the release of iodine into the solvent after 1 minute. The solvent in the 5 wt% clay dish was darker than in the 12.5wt% dish and iodine can clearly be seen flowing out of the sample (Figure 4.6).

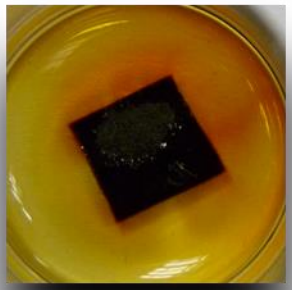


Figure 4.6: Sample of formulation PVOH/PVP-I/ Cloisite® Na^+ 5 wt% immersed in water for 1 minute

Figure 4.7: Sample of formulation PVOH/PVP-I/ Cloisite® Na^+ 12.5 wt% immersed in water for 1 minute

The samples were then left in the water for 16 hours before being transferred to a new Petri dish containing the same volume of fresh water. This process was repeated every 24 hours until the water was no longer discoloured suggesting the sample was no longer releasing iodine (Figure 4.8).

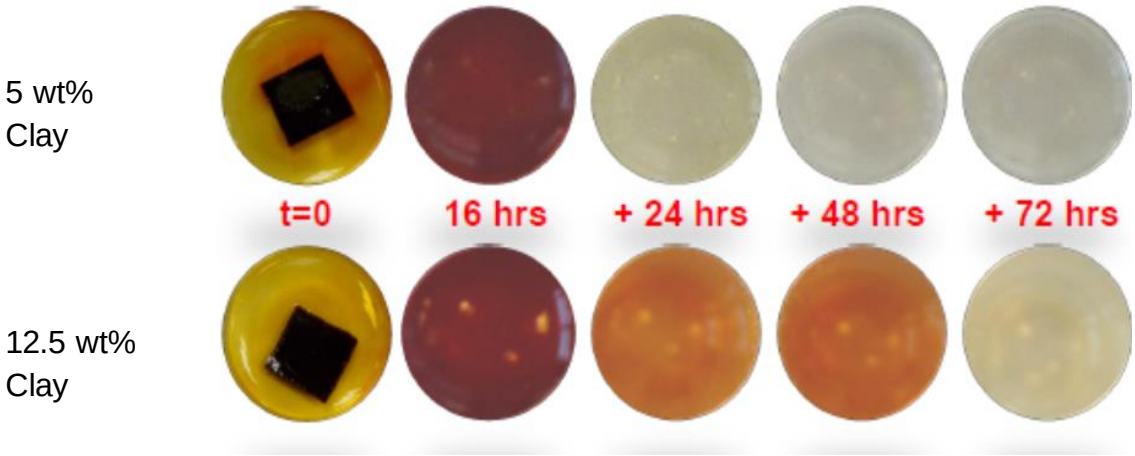


Figure 4.8: Visible image of iodine release into a fixed volume of water. Top row sample contains a 5wt% Cloisite® Na^+ sample and the bottom row contains a 12.5wt% Cloisite® Na^+ sample. Both samples contained 6wt% Iodine.

After a further 24 hours (total of 40 hours submerged in water) the water containing the 5wt% sample was much paler than that of the 12.5wt% and after a further 48 hours (total of 64 hours) the 5wt% sample water was clear suggesting that it was no longer releasing iodine. The 12.5wt% clay sample was still producing a slight discolouration of the water after 72 hours (a total of 88 hours submerged in water) indicating it was still releasing iodine. This would support the hypothesis that increasing the concentration of clay platelets in the composite material slows the release of iodine. However, this simple test used water as the experimental media which does not contain the organic elements such as proteins found in wound exudate which may impact on the release of iodine from the material.

4.5. Formulation of PVOH/Povidone iodine (PVP-I)/Clay Composites

4.5.1. Introduction

Having demonstrated that there appeared to be a difference in the release characteristics of the two different clay containing samples the next series of experiments were designed to investigate further the impact of altering the clay

concentration on the release of iodine. The theory was that the higher the concentration of clay platelets the slower the release of iodine so the smaller the area of the zone of inhibition (ZOI) of bacterial growth.

4.5.2. Methods Summary

In these experiments three different concentrations of clay were added to the PVOH/PVP-I composite; 2.5wt%, 5wt% and 12.5wt%. The concentration of iodine in the composites was fixed at 6wt% in all three formulations. The method of preparation of the composites is described in Section 2.3.2.4.

Another consideration that was made at this point in the material development was the choice of a suitable backing material. In all previous experiments the composites had been cast onto glass microscope cover slips.

When the cover slips, with no dressing applied, had been used as negative controls on the three test bacteria they appeared to be reduced microbial growth under the 2 x 2cm square of glass. In order to eliminate the effect that the glass may have on bacterial growth it was decided to cast the composite on either a cardboard backing (Figure 4.9) or to produce a composite with no backing material. The composites which had no backing were described as unsupported films (Figure 4.10).

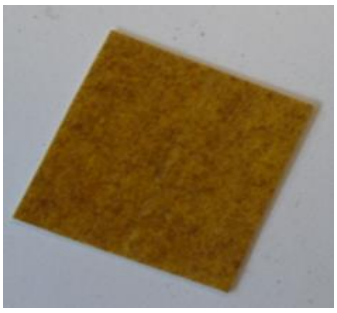


Figure 4.9: Cardboard backed composite

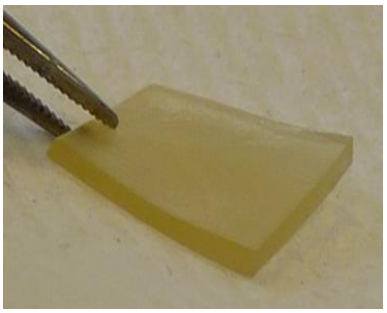


Figure 4.10: Unsupported film

A series of formulations with differing clay (Cloisite®Na+) concentrations shown in Table 4.9 were produced as either unsupported films (Samples 20 - 31) or cast onto cardboard (Samples 32 - 45).

Four controls were created: pure cardboard (Sample 32), PVOH on cardboard (Sample 33 and unsupported films of PVOH/PVP (Sample 20) and pure PVOH (Mowiol 98-99) (Sample 27).

The samples were cut into 2 x 2cm squares and UV sterilised as previously described and their antimicrobial activity tested against *S*. *aureus*, *E*. *coli* and *P. aeruginosa* for 24 hours.

Table 4.9: Formulations with differing clay and iodine concentrations, with and without cardboard backings

Sample	**Formulation**	**Backing**	**Clay wt%**	**Approx iodine wt%**
20	PVOH/PVP = 40/60 (Control)	None	None	**0**
21	PVOH/PVP/Clay = 35/52.5/12.5	None	**12.5**	**0**
22	PVOH/PVP/Clay = 38/57/5	None	**5**	**0**
23	PVOH/PVP/Clay = 39/58.5/2.5	None	**2.5**	**0**
24	PVOH/Clay = 87.5/12.5	None	**12.5**	**0**
25	PVOH/Clay = 95/5	None	**5**	**0**
26	PVOH/Clay = 97.5/2.5	None	**2.5**	**0**
27	PVOH (98-99) (Control)	None	None	**0**
28	PVOH/PVP-I = 40/60	None	None	**6**
29	PVOH/PVP-I/Clay = 35/52.5/12.5	None	**12.5**	**6**
30	PVOH/PVP-I/Clay = 38/57/5	None	**5**	**6**
31	PVOH/PVP-I/Clay = 39/58.5/2.5	None	**2.5**	**6**
32	Cardboard substrate (Control)	Cardboard	None	**0**
33	PVOH (98-99) / cardboard (Control)	Cardboard	None	**0**
34	PVP-I Pure cardboard	Cardboard	None	**6**
35	PVOH/PVP = 40/60	Cardboard	None	**0**
36	PVOH/Clay = 87.5/12.5	Cardboard	**12.5**	**0**
37	PVOH/Clay = 95/5	Cardboard	**5**	**0**
38	PVOH/Clay = 97.5/2.5	Cardboard	**2.5**	**0**
39	PVOH/PVP/Clay = 35/52.5/12.5	Cardboard	**12.5**	**0**
40	PVOH/PVP/Clay = 38/57/5	Cardboard	**5**	**0**
41	PVOH/PVP/Clay = 39/58.5/2.5	Cardboard	**2.5**	**0**
42	PVOH/PVP-I = 40/60	Cardboard	None	**6**
43	PVOH/PVP-I/Clay = 35/52.5/12.5	Cardboard	**12.5**	**6**
44	PVOH/PVP-I/Clay = 38/57/5	Cardboard	**5**	**6**
45	PVOH/PVP-I/Clay = 39/58.5/2.5	Cardboard	**2.5**	**6**

4.5.3. Results

During these experiments it was noted that the unsupported films containing PVP-I behaved differently depending on which side of the dressing was placed in contact with the agar. When the smooth/darker coloured side of the sample was placed in contact with the agar and incubated for 24 hours the sample curled up at the corners and a pool of dark brown liquid appeared on the top of the composite (Figure 4.11). When the rough / lighter side was placed in contact with the agar and incubated for 24 hours it remained flat and produced a larger ZOI than when the smooth/dark side was placed on the agar. Many of the flat samples swelled and increased in size up to 25 x 25 mm. They also went white around the edges with a central dark brown area after 24 hours incubation (Figure 4.12).

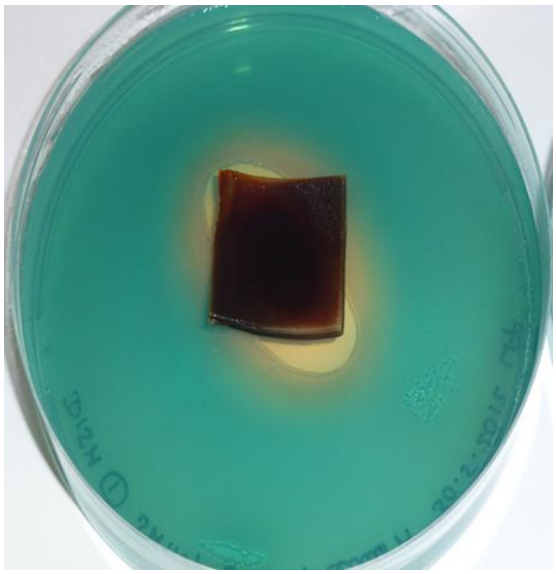


Figure 4.11: Dark/smooth side of 12.5wt% clay sample which curls.

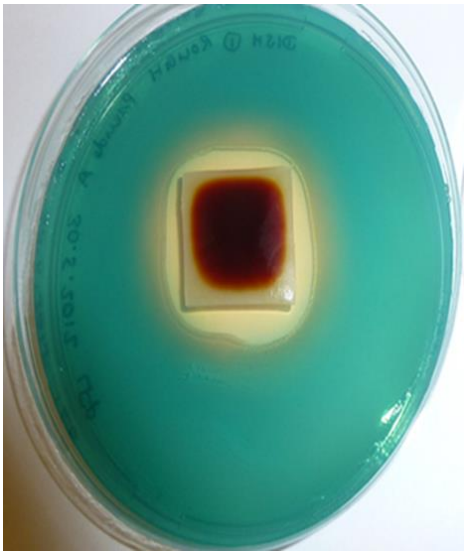


Figure 4.12: Light/rough side of 12.5wt% clay sample which remains flat.

The surface area of the ZOI was measured for each replicate. Having identified the different behaviours of the two sides of the material the results were split into two groups; the curled and the flat samples and the mean surface area calculated. The number of samples exhibiting the different states is given as (n) after the mean calculation.

4.5.3.1. *S. aureus* - Composites without cardboard backing (20-31)

The PVOH/PVP = 40/60 unsupported film control (Sample 20) curled up leaving a small central area in contact with the agar. There was evidence of a reduction in microbial growth where the material remained in contact with agar.

The samples which contained PVOH, with or without PVP and different concentrations of clay (Samples 21-26) but no iodine did not appear to inhibit microbial growth. This would suggest that the composite material without the antimicrobial iodine did not have any antimicrobial properties.

The pure PVOH (Mowiol 98-99) control (Sample 27) curled up at the edges and appeared to have an area of reduced microbial growth at the points where the samples remained in contact with the agar. The samples which contained 6wt% iodine in the form of PVP-I (Samples 28-31) all produced large clear ZOI (Table 4.10). After 24 hours incubation the samples had either curled up and had a pool of brown iodine on the top and some eluent, presumably containing iodine, free in the agar plate or they remained flat. The samples that remained flat appeared to produce larger ZOI than the samples which curled. The surface areas of the ZOI of all samples were measured and the results recorded as either “curled” or “flat”. The means and standard deviation were calculated for the curled and flat samples.

Table 4.10: ZOI produced by different PVOH/PVP-I composites, with and without Cloisite®Na$^+$clay, against *S. aureus*

Sample	Formulation PVOH/PVP-I(6wt%) With and Without Cloisite®Na+ Clay	Curled Samples Mean SA (mm^2) (Dark)	Flat Samples Mean SA (mm^2) (Light)
28	No Clay	$2233 \pm 186.30 mm^2$ (n=3)	$2744 \pm 365.57 mm^2$ (n=3)
29	12.5wt% Clay	$823 \pm 37.81 mm^2$ (n=4)	$1220 \pm 127.94 mm^2$ (n=5)
30	5wt% Clay	$743 \pm 99.74 mm^2$ (n=4)	$2413 \pm 152.67 mm^2$ (n=5)
31	2.5wt% Clay	$1173 \pm 259.47 mm^2$ (n=6)	$2553 \pm 625.84 mm^2$ (n=8)

4.5.3.2. *S. aureus* - Composites with cardboard backing (32-45)

The cardboard backed materials that did not contain iodine (Samples 32,33,35,36,37,38,39,40 and 41) all had complete microbial growth under the samples.

The cardboard sample with PVP- I (6wt%) (Sample 34) appeared to have no microbial growth under the sample and a clear ZOI of 1mm from each edge of the square sample.

The cardboard samples which all contained 6wt% PVP-I and differing concentrations of clay 0-12.5wt% (Samples 42 - 45) all appeared to have no microbial growth under the samples and produced a very small ZOI of between 0.5 and 1mm from each edge of the square sample.

4.5.3.3. *E. coli* - Composites without cardboard backing (20-31)

The PVOH/PVP = 40/60 unsupported film control (Sample 20) curled up leaving only a small central area still in contact with agar. There was evidence of reduced microbial growth in the areas where the material remained in contact with the agar.

All the PVOH/ PVP/Clay composites (Samples 21-26) had complete microbial growth under the samples.

The PVP-I (6wt%) unsupported films (Samples 28-31) all produced clear ZOI however some samples curled, and some remained flat. The surface areas of all the ZOI were measured and the mean and standard deviation calculated for the curled and flat samples (Table 4.11).

Table 4.11: ZOI produced by different PVOH/PVP-I composites, with and without Cloisite ®Na^+ clay, against *E. coli*

Sample	Formulation PVOH/PVP-I (6wt%) With and Without Cloisite®Na+ Clay	Curled Samples Mean SA (mm^2) (Dark)	Flat Samples Mean SA (mm^2) (Light)
28	No Clay	1086±SD 25.50mm^2 (n=2)	1455±SD 396.48mm^2 (n=8)
29	12.5wt% Cloisite	559±85.07mm^2 (n= 10)	1153±91.2mm^2 (n=6)
30	5wt% Clay	770±363.71mm^2 (n=10)	1330±102.58mm^2 (n=6)
31	2.5wt% Clay	491±97.00mm^2 (n=11)	1923±127.88mm^2 (n=6)

4.5.3.4. *E. coli* - Composites with cardboard backing (32-45)

All Samples 32 - 45 had complete microbial growth under all replicates tested.

4.5.3.5. *P. aeruginosa* - Composites without cardboard backing (20-31)

All the samples which did not contain PVP-I (Samples 20-27) curled and there was evidence of reduced microbial growth where the sample had remained in contact with the agar. The dressings had a green area on the side that had been in contact with the agar which corresponded to the shape of the area of reduced microbial growth. The samples which contained PVP-I (6wt%) (Samples 28-31) all produced clear ZOI as shown in Table 4.12.

Table 4.12: ZOI produced by different PVOH/PVP-I composites, with and without Cloisite®Na^+clay, against *P. aeruginosa*

Sample	Formulation PVOH/PVP-I (6 wt%) With and Without Cloisite®Na+ Clay	Curled Samples Mean SA (mm^2) (Dark)	Flat Samples Mean SA (mm^2) (Light)
28	No Clay	2036±180.96mm^2 (n=2)	1141 ±396.20mm^2 (n=4)
29	12.5 wt% Clay	312±119.42mm^2 (n=5)	759±49.15mm^2 (n=5)
30	5 wt% Clay	351±82.13mm^2 (n=5)	897±65.39mm^2 (n=5)
31	2.5 wt% Clay	450±163.24mm^2 (n=5)	854±SD 0mm^2 (n=2)

4.5.3.6. *P. aeruginosa* - Composites with cardboard backing (32-45)

Viewed from underneath the agar plates all showed a full green lawn of microbial growth but the 2 x 2cm area covered by the dressing was paler than the surrounding green colour. The sides of the material in contact with the agar were green but the topside of the material remained white.

4.5.4. Discussion

After 24 hours incubation there was complete microbial growth under all the control samples. The four cardboard backed samples which contained the active agent, 6wt% iodine, all inhibited the growth of *S. aureus* under the dressing and produced a small ZOI of between 0.5 - 1mm from each edge of the dressing. However, there were no ZOI produced against *E. coli* and *P. aeruginosa* and limited evidence of reduced microbial growth under the cardboard samples. The four unsupported samples containing 6wt% iodine all produced clear ZOI against each of the test bacteria. The samples containing no clay produced the largest ZOI against all three bacteria. The only exception was against E*. coli* where the flat side of the no clay sample produced a smaller ZOI than the 2.5wt% clay sample but a larger ZOI than the 5wt% clay sample.

Conversely the samples containing the highest concentration of clay, 12.5wt%, consistently produced the smallest ZOI. There were two exceptions to this trend, the curled sample of the 5wt% clay material produced the smallest ZOI against *S. aureus* and the curled sample of the 2.5wt% clay material produced the smallest ZOI against *E. coli*. These smaller ZOI could have been due to the reduced size of the area where the samples remained in contact with the agar.

An important finding from this experiment was that the flat samples produced larger ZOI than the curled samples against all the test bacteria. In the *S. aureus* experiments the flat samples perfectly illustrated the predicted phenomena demonstrating that increasing the clay concentration reduced the size of the ZOI. However, the activity of the composites with different clay concentrations was not as consistent against the other bacteria, particularly between the 2.5 and 5wt% clay samples. The 5wt% curled samples produced slightly larger ZOI than the 2.5wt% curled samples against *E. coli* and similarly the flat 5wt% samples produced slightly larger ZOI than the 2.5wt% flat samples against *P. aeruginosa*.

The effect of the different clay concentrations was less marked in the *P. aeruginosa* experiments. For both the flat and curled samples the ZOI was smallest with the higher concentration of clay (12.5wt %). Interestingly the curled sample containing no clay produced a much larger ZOI against *P. aeruginosa* than the flat sample.

These results suggested that there was a difference in behaviour of both sides of the material and between their effects on the different test bacteria. The samples that remained flat were identified as the paler /rougher surface of the material that had been in contact with the base of the Petri dish when the material was created. The samples that curled were darker in colour and smooth and had been the upper surface of the material exposed to the atmosphere as it dried in the Petri dish.

Due to the marked difference in colour between the two layers the different sides were described as Light and Dark and in all further experiments both sides were tested and the results reported separately. The Light side of the material which remained flat during testing consistently produced larger ZOI

than the dark side samples that curled. This suggests that the paler, light side of the material released the iodine faster than the darker smooth side of the material. It was concluded that the two different surfaces of the material have different release characteristics.

One possible explanation for this phenomenon is that the light side remains flat so has a larger surface area in contact with the surface of the agar than the dark side which curls away from the surface. (Figures 4.13 a,b,c and 14.4 a,b,c)

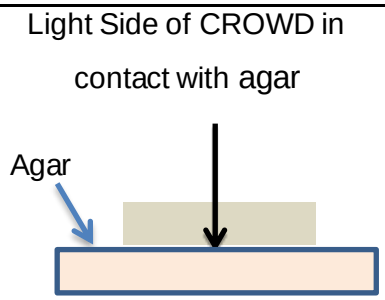


Figure 4.13a: Diagram of the cross section of a CROWD sample with the Light side in contact with the agar showing how the sample remains flat with the whole surface in contact with the agar.

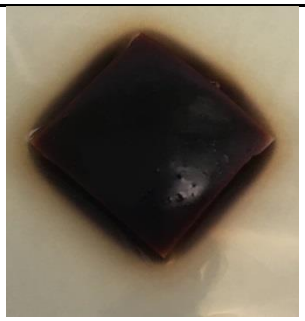


Figure 4.13b: Photograph of Light side 12.5wt% CROWD after 24 hours incubation at 37°C on an agar plate with no bacteria.

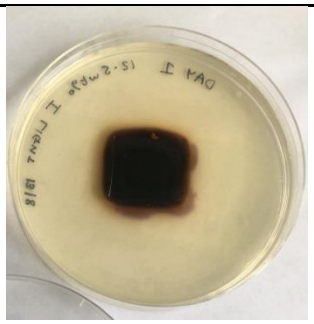


Figure 4.13c: Photograph of agar after a Light side 12.5wt% composite was removed following 24 hours incubation at 37°C showing the residual iodine containing liquid.

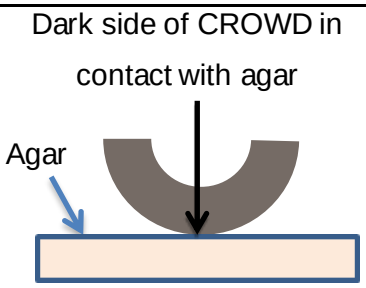


Figure 4.14a: Diagram of the cross section of a CROWD sample with the dark side in contact with the agar showing how the sample curls away from the surface of the agar leaving a small central circular portion in contact with the agar.

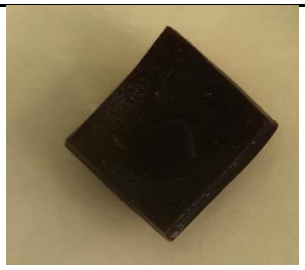


Figure 4.14b: Photograph of Dark side 12.5wt% CROWD after 24 hours incubation at 37°C on an agar plate with no bacteria.

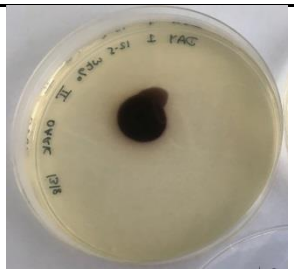


Figure 4.13c: Photograph of agar after a Dark side 12.5wt% composite was removed following 24 hours incubation at 37°C showing the residual iodine containing liquid.

The results from the previous experiment were relabelled from curled to dark side and flat to light side and then represented graphically to illustrate the effect of increasing the clay concentration and the effects of the different sides of the composite (Figure 4.15).

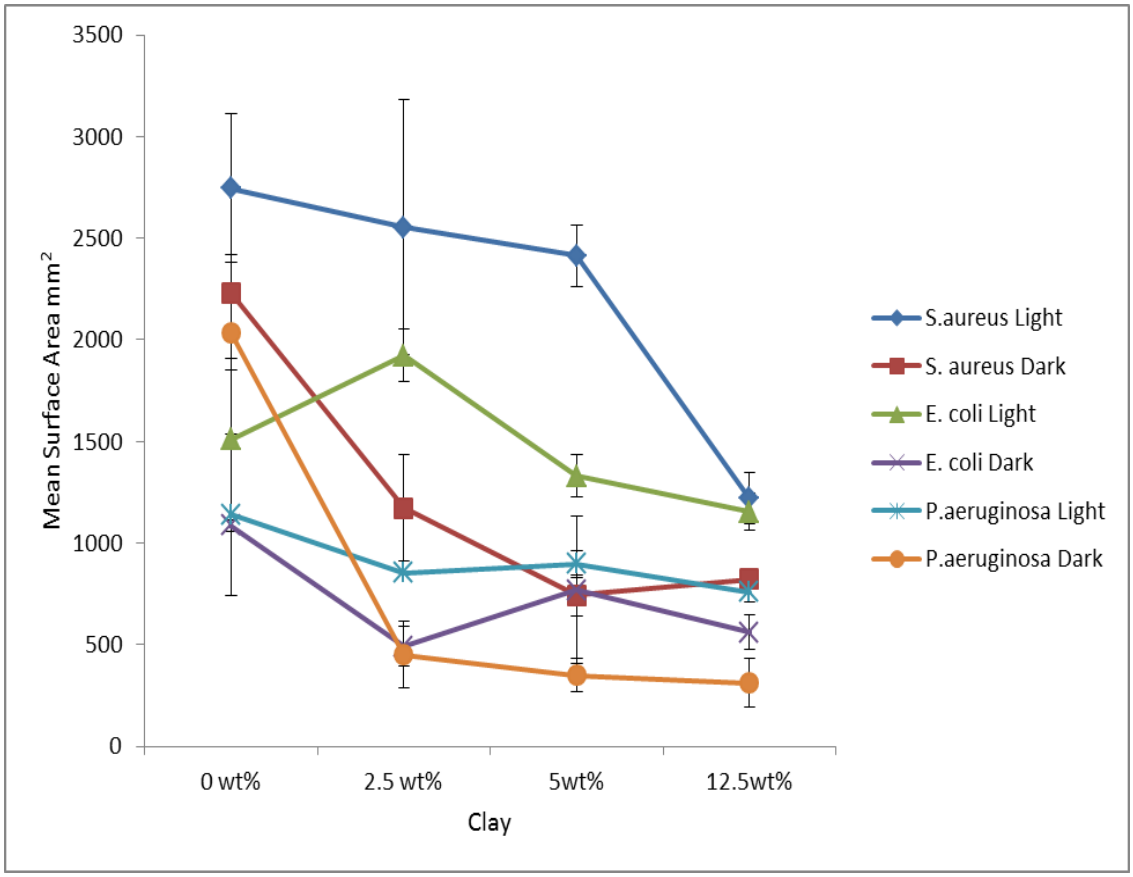


Figure 4.15: Influence of the light and dark sides of composites containing different clay concentrations on the ZOI produced against the three test bacteria (Error bar = Standard Deviation, n values shown in Tables 4.10, 4.11 and 4.12)

The figure clearly illustrates that for all the clay containing samples, the light side of the dressings produced much larger ZOI than the dark side of the dressing against all the test bacteria.

Due to the anomalies identified in these experiments, particularly with the 2.5wt% samples, another series of experiments were undertaken to further investigate the effect of the different sides of the material containing the two higher strength clays 5wt% and 12.5wt% on the release profile of the iodine. The difference in activity of the two sides of the material was a serendipitous observation so it was important to repeat the experiment in a more controlled manner in order to validate these results.

4.6. Comparison of Light and Dark sides of 5 and 12.5wt% Clay composites

4.6.1. Introduction

Further samples of the PVOH/PVP-I/Clay formulations containing fixed 6wt% iodine and either 5wt% Cloisite®Na^+ (Sample 30) and 12.5wt% Cloisite®Na^+ (Sample 29) were prepared for antimicrobial testing.

4.6.2. Methods Summary

Square 2 x 2cm samples of the two different concentrations of clay composite were UV sterilised as described previously. The dark side of the composite was placed in contact with agar in a Petri dish which had been inoculated with the test bacteria either *S. aureus*, *E. coli* or *P. aeruginosa*.

Six replicates were tested against each bacteria.

The experiment was repeated using 2 x 2cm samples of the light side of the composites.

4.6.3. Results

All samples placed on the agar light side down retained their shape and dark brown colour and remained flat. The dark samples however curled at all four corners and had a pool of what appeared to be an iodine containing solution on the top of the sample. When the samples were moved what appeared to be liquid iodine was visible in the agar plate.

Figure 4.16. illustrates that as seen in previous experiments the zones of inhibition produced by both clay concentrations were largest against *S. aureus*, then *E. coli* and smallest against *P. aeruginosa*. In almost all experiments the light side of the dressings produced larger ZOI than the dark side of the dressings against all three test bacteria. This would suggest that through contact with the light side of the material, iodine is released faster than through the dark side. The only exception to this was the 5wt% Cloisite®Na+ composite which produced very similar sized ZOI for both the dark side ($2010 \pm 117.79 mm^2$) and the light side ($1980 \pm 306.18 mm^2$) against *S. aureus*.

The standard deviation for the dark side samples was very high because one of the samples produced a very large ZOI measuring 3363mm^2.

Against *E. coli* the light side of the 5wt% clay samples produced a much larger ZOI (1829±301.81mm^2) than the light side of the 12.5wt% clay dressing (937±192.04mm^2). However unexpectedly, the dark side of the 5wt% clay samples produced a smaller ZOI (484±65.79mm^2) than the dark side of the 12.5wt% clay samples (731±163.09mm^2).

In the *P. aeruginosa* experiments, the light side of both samples produced ZOI more than three times the size of the ZOI created by the dark side of the material. The 12.5wt% clay light side samples produced slightly larger ZOI than the 5wt% clay light samples. Both the dark side ZOI were smaller than the original sample size (400mm^2) this was because the samples had curled away from the agar and were only able to reduce microbial growth where the material remained in contact with the agar.

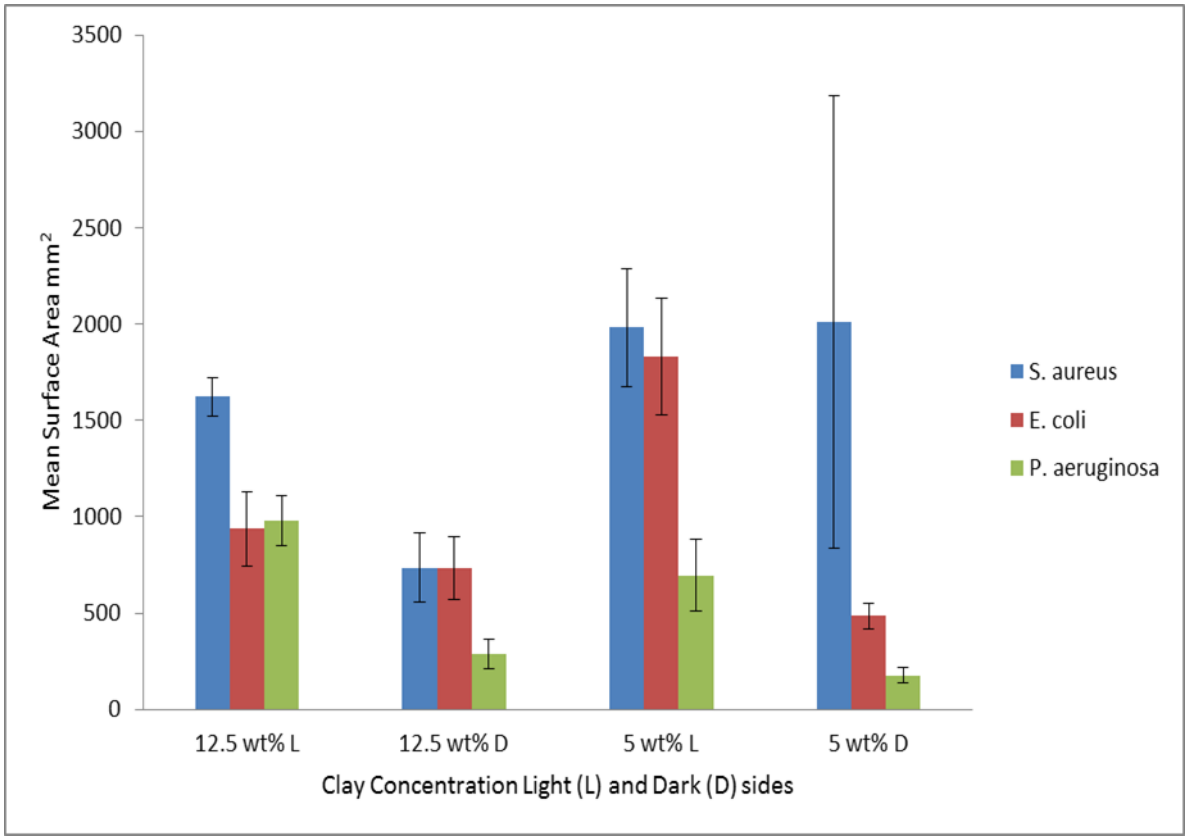


Figure 4.16: Comparison of the antimicrobial activity of the Light and Dark sides of the 5wt% and 12.5wt% clay composites against the three test bacteria. (Error bar = Standard Deviation, n = 6)

4.6.4. Discussion

These experiments demonstrate that increasing the concentration of clay in the material reduces the size of the ZOI which would suggest that increasing the clay concentration effectively slows the release of the iodine.

The clay is distributed within the material in the form of discrete platelets i.e. as an exfoliated composite but more predominantly as assemblies (stacks) of platelets intercalated with polymer; this arrangement is likely to control the release of iodine (Figure 4.17). The clay layers within a stack are about 1nm thick and between 200-500 nm in diameter.

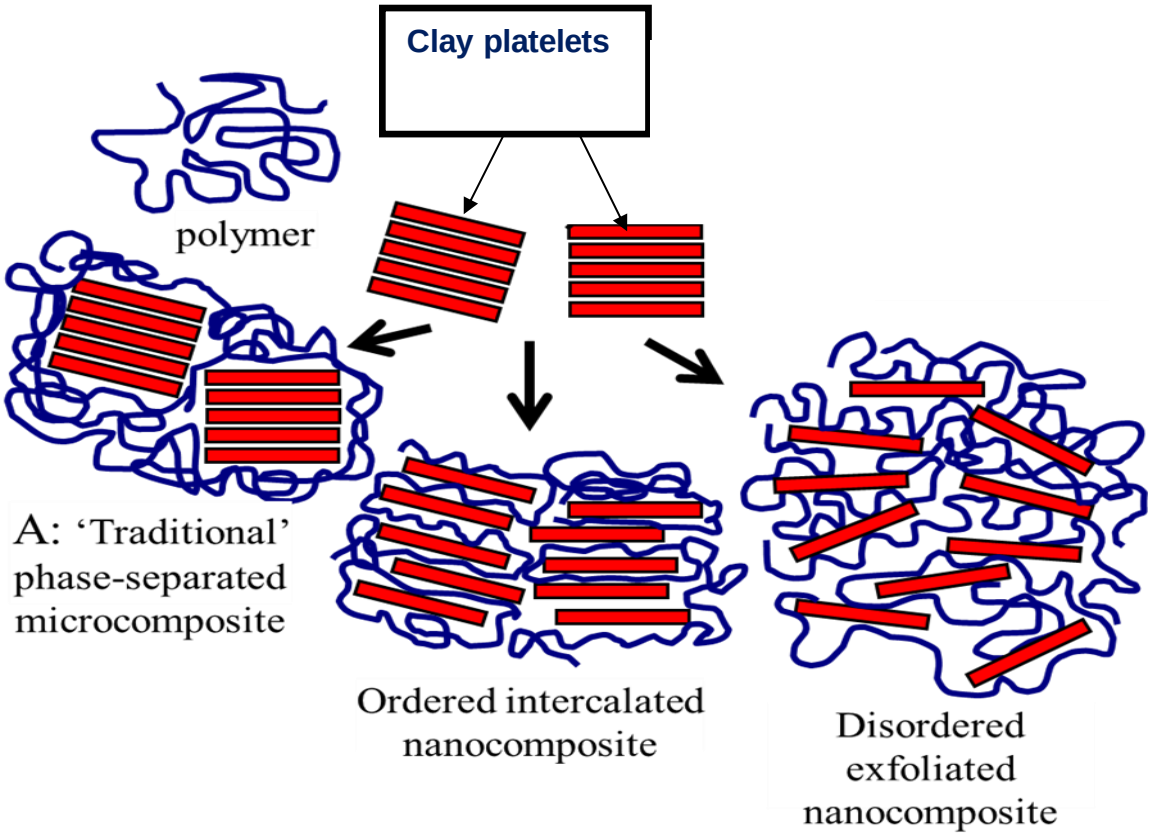
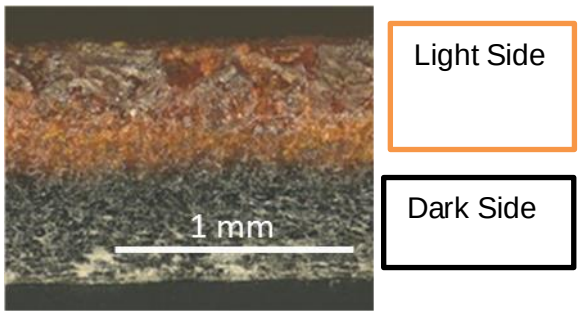


Figure 4.17: Behaviour of clays in composite materials (Breen 2015 Personal Communication)

In the 12.5wt% clay containing material there was a clear demarcation between the light and dark side of the material which was described as a self - stratifying film (Figure 4.18).

Figure 4.18: Photograph of the 12.5wt% clay composite showing the self – stratification into two layers: Light (brown outer layer) and Dark (grey inner layer)

The clay is heterogeneously distributed in the two self-stratifying layers. Figures 4.19 and 4.20 show optical microscope photographs of cross sections through the material and illustrate the clear demarcation in layers between the two layers in the both the 12.5wt% and 5wt% clay composites. (Magnification x 315)



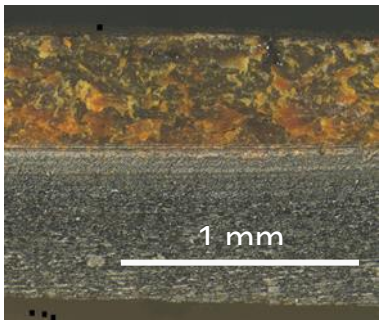


Figure 4.19:

5wt% Cloisite – Na (928 µm thick)

Figure 4.20:

12.5wt% Cloisite – Na (1098 µm thick)

The formulation containing the higher clay concentration 12.5wt% and 6wt% iodine was chosen for further investigation to determine the duration of antimicrobial action of the material. This preferred formulation was given the acronym CROWD, “Controlled Release in Open Wound Dressings”.

4.7. Duration of Antimicrobial Action of Clay/ PVP- I/Polymer Composites

4.7.1. Introduction

To determine the duration of the antimicrobial effect of the different sides of the preferred formulation (CROWD); PVOH/PVP-I/Clay 12.5wt% containing 6wt% PVP-I, samples were tested against the same strains of *S. aureus, E. coli* and *P. aeruginosa* as in the previous experiments.

4.7.2. Methods Summary

Passage Studies were undertaken as described in section 2.1.2.3.

Square 2 x 2cm samples of the dark side of the composite were placed in contact with the inoculated agar and a second series of 2 x 2cm samples from the same batch but with the light side placed in contact with the agar.

The experiment was repeated every day for seven days to test the efficacy of the antimicrobial agent over a period of a week. This time period was chosen because in clinical practice a dressing would be changed within a week or more often if there were signs of clinical infection (Brindle and Farmer 2019).

4.7.3. Results

Figure 4.21 shows the ZOI produced by the light and dark side of the material against the three test bacteria over a period of 8 days. For the first 48 hours, the PVOH/PVP-I/Clay 12.5wt% composite produced the largest ZOI against *S. aureus*, then *E. coli* and the smallest against *P. aeruginosa*. The light side of the material consistently produced a larger zone of inhibition after 24 hours than the dark side. Against *S. aureus* the light side produced the largest ZOI after 24 hours of all the ZOI created against all the test organisms. However, the size of the zone decreased after 48 hours and was less than 500mm^2 by day 4. The ZOI produced by the dark side against *S. aureus* was initially smaller than the light side but continued to increase in size and reached the maximum size at day 4 and was still above 500mm^2 after 8 days continuous use. Against *E. coli* both the light and the dark side produced their largest ZOI after 48 hours. The light side ZOI had reduced to less than 500mm^2 after 3 days, but the dark side

ZOI remained much larger until day 5 when they had reduced in size to less than 500mm^2. Against *P. aeruginosa* the light side ZOI had decreased to less than 500mm^2 by day 3 however the dark side ZOI increased in size between 24 and 48 hours and then dropped below 500mm^2 after 4 days.

By day 8 only the dark side was still producing a clear ZOI which was only against *S. aureus,* all the other samples were no longer producing a ZOI against any of the test bacteria.

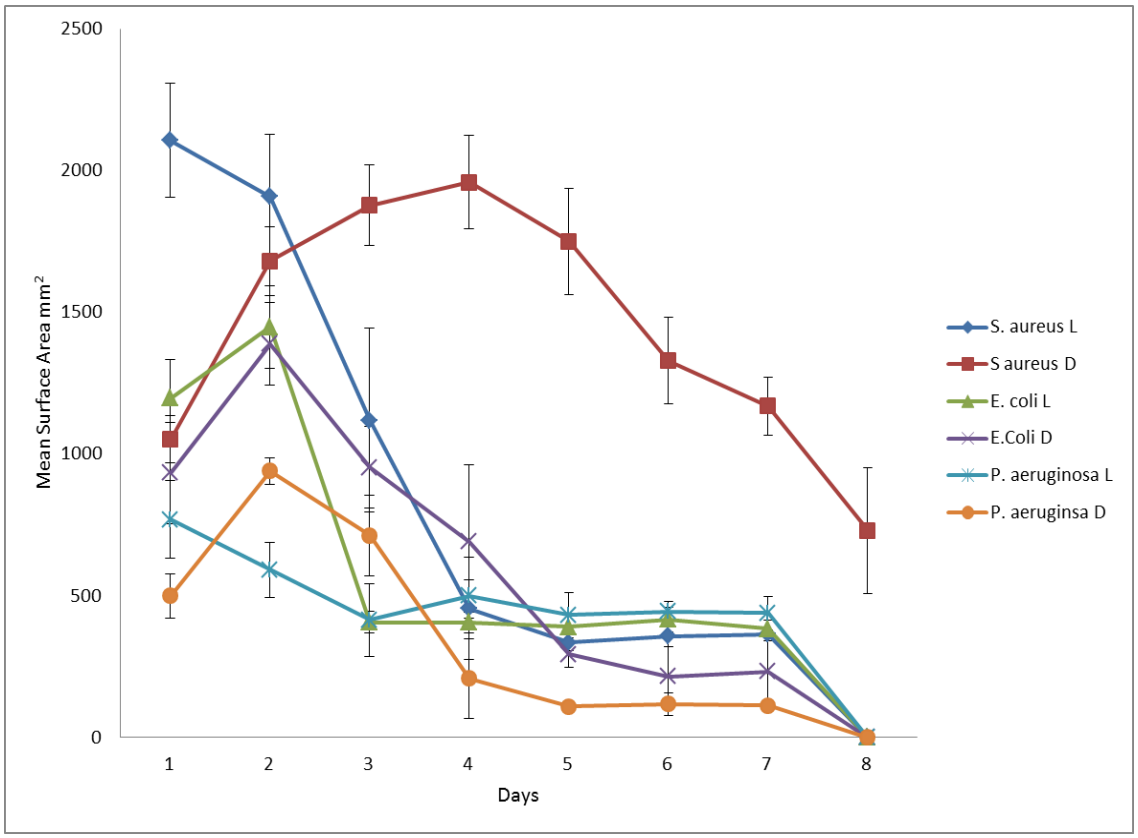


Figure 4.21: ZOI produced by the Light (L) and Dark (D) sides of the preferred CROWD formulation against three test bacteria (Error bar = Standard Deviation, n = 6)

4.7.4. Discussion

The CROWD material demonstrated a more prolonged duration of antimicrobial activity than any of the three commercial iodine dressings tested previously. The ability of the CROWD material to continue to produce large ZOI after 8 days of continuous use against *S. aureus* was far superior to the limited 2 day

antimicrobial effect demonstrated by the commercial iodine containing dressings: Iodoflex, Inadine and Iodozyme against this organism. Against *E. coli* and *P. aeruginosa* the light side of the CROWD material was still producing a ZOI greater than the sample size (400mm^2) on day 7 whereas none of the commercial iodine dressings were having an antimicrobial effect on either of these bacteria after 2 days.

This suggests that potentially the CROWD material could be left on a wound for up to 7 days continuous use. However, in a clinically infected wound dressing changes would have to be more frequent as regular visual inspection of the wound is important to ensure that the infection is not spreading, and the wound condition is not deteriorating. During the passage studies it was observed that the CROWD material changed colour from dark brown to white as the experiment progressed. The colour change appeared to be linked to the efficacy in that the darker the sample, the larger the ZOI, and when the samples were completely white they did not produce a ZOI suggesting that the active iodine had all been utilised.

4.8. Methicilin-resistant *Staphylococcus aureus* (MRSA) Passage Studies

4.8.1. Introduction

The widespread use of antibiotics since their introduction in the 1940s has led to the growing problem of antibiotic resistance. One of the resistant clinical pathogens often identified in wounds is methicillin-resistant *S. aureus* (MRSA). Early studies reported that Povidone iodine cream (5%) and solution (10%) were effective against MRSA. Povidone Iodine has also been shown to be 100% efficient against 33 clinical isolates of MRSA (McLure and Gordan 1992). Having demonstrated the antimicrobial efficacy of the CROWD material against a methicillin-sensitive strain of *S. aureus* (MSSA) a series of experiments were conducted to determine the efficacy and duration of antimicrobial activity against clinical strains of MRSA. Two strains of MRSA were included in the study: *S. aureus* USA300 and Epidemic methicillin - resistant *S. aureus* (EMRSA-16).

S. aureus USA300 is a community acquired strain of MRSA which can cause rapidly progressive fatal diseases such as necrotizing pneumonia, severe sepsis and nectrotising fasciitis (Boyle-Vavra S and Daum RS (2007). *S. aureus* USA300 often contain Panton-Valentine leucocidin (PVL). PVL is a pore-forming toxin encoded by the *lukSF-PV* genes which has been associated with necrotic infections in humans and forms lytic pores that destroy human neutrophils, monocytes, and macrophages *in vitro* (Brown et al. 2012). EMRSA-16 is a hospital associated pathogen responsible for a variety of dieases from uncomplicated skin infections to life threatening bacteraemia and pneumonia. It was first identified in the UK in the 1990s (Watkins, David and Salata 2012).

4.8.2. Methods Summary

Passage studies were undertaken to test the activity of the dark and light sides of the PVOH/PVP-I/Clay 12.5wt% (6wt% iodine) material against two different clinical strains of MRSA, *S. aureus* USA 300 (Community Acquired) and EMRSA -16 (Hospital Acquired).

4.8.3. Results

The PVOH/PVP-I/Clay 12.5wt% CROWD composite produced clear ZOI against both strains of MRSA for 96 hours (Figure 4.22). The CROWD material produced larger ZOI after 48 hours than it had after the first 24 hours. On day 1 the light side ZOI were larger than the dark side ZOI. However, on the second day the light side and dark side produced very similar sized ZOI. From the third day onwards, the dark side produced larger ZOI than the light side. This demonstrates how the dark side takes longer to release the iodine. The largest ZOI were produced against the *S. aureus* USA 300, the community acquired strain. After 5 days the dark side of the material was observed to be still inhibiting the growth of both bacteria whereas the light side was no longer producing a ZOI after 96 hours.

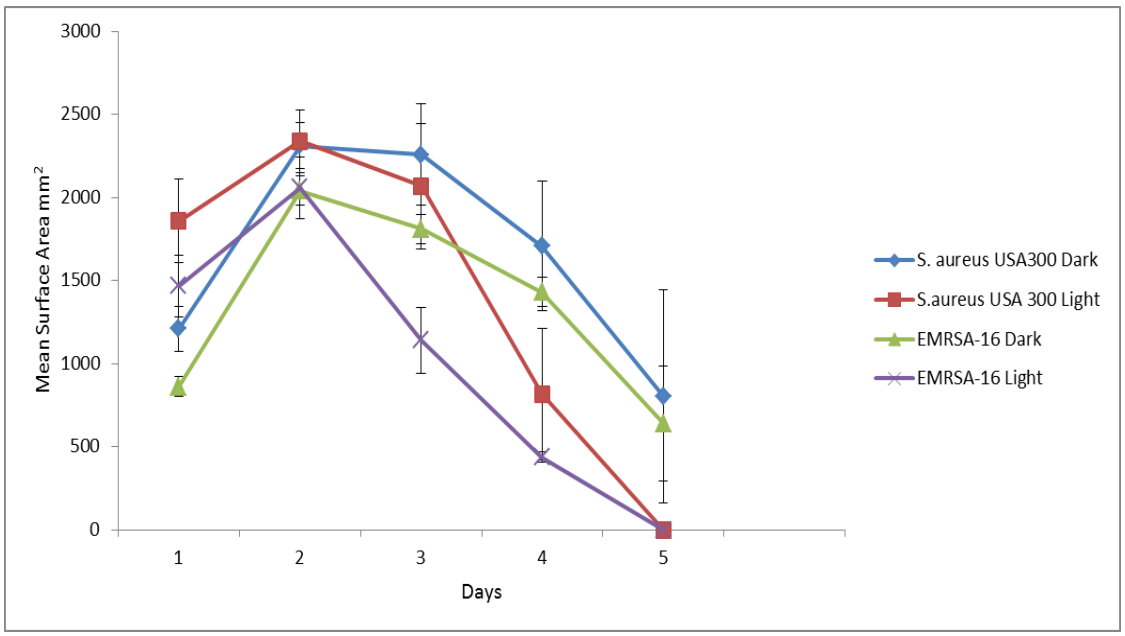


Figure 4.22: ZOI produced by the Light and Dark side of the CROWD material against two strains of MRSA (Error bar = Standard Deviation, n = 6)

4.8.4. Discussion

Both strains of MRSA were sensitive to the antimicrobial effects of the CROWD material for at least 4 days. However, the duration of anti-microbial action against the two strains of MRSA was shorter than seen previously against the methicillin-sensitive *S. aureus* (MSSA) where the dark side was still producing ZOI after 8 days of continuous use and the light side produced a ZOI for 7 days. (Section 4.7.3., Figure 4.21).

MRSA develop resistance by the acquisition of a non native gene encoding a penicillin-binding protein (PBP2a) which has a lower affinity for β-lactams anti-biotics. This resistance allows the bacteria to maintain cell-wall biosynthesis, the target of β-lactams, even in the presence of typically inhibitory concentrations of antibiotic (Peacock and Paterson 2015). As the resistance mechanism of MRSA has no relationship with the mechanism of action of iodine no cross resistance would be expected. This experiment demonstrated that in terms of the production of large ZOI against the three *S. aureus* species the antimicrobial efficacy of the iodine was not dimimished against the resistant strains.

This is important as the global increase in antibiotic resistance and the lack of development of new classes of antibiotics has led to calls for improved antimicrobial stewardship to try to reduce the incidence of antibiotic resistance (WHO 2017; NICE 2015; Baur et al. 2017). As antiseptics have multiple sites of action on the bacterial cell, unlike antibiotics which usually have one specific site of antimicrobial action, they are less likely to induce resistance. This suggests that the appropriate use of antimicrobial dressings for the management of wound infection has the potential to reduce the overall use of antibiotics and help preserve the use of antibiotics for specific infections (Roberts et al. 2017). Another advantage of using topical antimicrobial dressings in comparison with systemic antibiotics is that the low concentrations of an antimicrobial can produce high local levels at the wound surface and are not affected by arterial disease which can reduce the concentration of antibiotic reaching ischaemic tissue (Lipsky et al. 2016).

4.9. XRF Analysis of Iodine Release

4.9.1. Introduction

Having demonstrated the difference in the duration of the antimicrobial effect of the light and dark sides of the CROWD composite it was decided to further investigate the effect of the clay component of the composite on the release of the antimicrobial iodine.

X-Ray fluorescence is often used to determine the elemental composition of materials. X-Rays irradiate samples which excite the elements present in the material causing it to generate X-Ray fluorescence.

The nucleus of an atom contains positively charged protons and non-charged neutrons and is surrounded by layers of shells containing electrons. When an atom is irradiated with X-Rays the photons emitted can expel an electron from the atom producing a hole in the shell which puts the atom in an unstable excited state with a higher energy level. To repair the hole, an electron which has a higher energy level is transferred from an outer shell into the hole. This

surplus energy is emitted as X-Ray fluorescence radiation (Schlotz and Uhlig 2006).

The intensity of the radiation is proportional to the concentration of the element(s) in the sample. XRF is used to determine the elements quantitatively and qualitatively by measuring the characteristic radiation released (Schlotz and Uhlig 2006).

Measuring the energies produced by the sample make it possible to identify which elements are present in the sample. Semi quantitative analysis of the intensity of the energy produced by different elements can be used to determine how much of each element is present in the sample (PANalytical 2010).

4.9.2. Methods Summary

XRF analysis, described in section 2.3.2.7. was used to investigate the effect of the addition of Cloisite®Na+ clay platelets on the release of iodine from the PVOH/Povidone Iodine (PVP-I) composites.

This experiment involved a semi quantitative measurement of the release of iodine from composites containing different amounts of clay. All three test samples contained 6wt% PVP-I. A sample of the standard CROWD formulation containing 12.5wt% Cloisite®Na^+ was prepared by the method described in Section 2.3.2.4.

A second composite which contained PVOH and PVP-I (6wt% iodine) but no clay was prepared by the method described in Section 2.3.2.3.

The Light and Dark side of the 12.5 wt% Cloisite®Na^+ was compared with the composite containing no clay to determine the effect of the addition of the clay platelets on the release of iodine.

The samples were cut into 2x2 cm squares and UV irradiated for 15 minutes on both sides as described in section 2.1.2.2.

Individual samples were placed in the centre of a Mueller Hinton (MH) agar plate and the base of the Petri dish was labelled with the day from Day 1 to 7 and the % weight of clay that the sample contained. Three experiments were

conducted simultaneously to test the Light and Dark side of the 12.5wt% clay sample against the sample containing no clay. The Petri dishes with the samples were placed in a static incubator at 37^{o}C for 24 hours.

After 24 hours all the samples were removed from the incubator. The samples labelled “Day 1” were removed from the agar using sterile tweezers and placed in an individual sterile container with a screw cap and labelled with the Day and clay weight. These samples were then sent to the Materials Engineering Research Institute (MERI) for XRF analysis by Francis Clegg (Section 2.3.2.7.).

The Day 2 to Day 7 samples were aseptically transferred to individual fresh MH agar plates and returned to the incubator for a further 24 hours. After the second 24 hours the Day 2 samples were removed for XRF analysis and the other samples were transferred to fresh agar plates for a further 24 hours in the incubator. This process was repeated for 7 days when the final samples were sent for analysis. Due to manufacturing problems, there were only enough “no clay” samples for six days of analysis.

4.9.3. Results

The iodine content of each sample was measured using XRF. The reading on the first day before the samples had been placed on agar was considered to be 100%. Subsequent readings were measured and amount remaining was calculated as a percentage of the reading on day zero for that formulation.

Figure 4.23 shows the percentage reduction in iodine in the 3 samples over the seven days. After the first 24 hours the iodine level in the PVOH/PVP-I composite that contained no clay had decreased to 17%. By day 2 it was 10% of the original iodine concentration and by day 4 no iodine was detected.

The light side of the composite containing 12.5wt% Cloisite®Na^{+} had reduced to 71% of the original iodine concentration after 24 hours. On day 2 it was 46% and day 3 it was 41% of the original. By Day 4 the iodine had dropped to 20% of the original and day 5 no iodine was measured in the sample.

The dark side of the composite containing 12.5wt% Cloisite®Na^{+} after the first 24 hours had only dropped to 87% of the original iodine measured. After 48 hours it was still measuring 64% of the original amount but by day 3 had

decreased to 49%. There was little change at day 4 with 48% remaining but by day 5 the percentage remaining had dropped to 24%. After 7 days of passage there was still measurable iodine in the sample which equated to 9% of the iodine measured in the day zero samples.

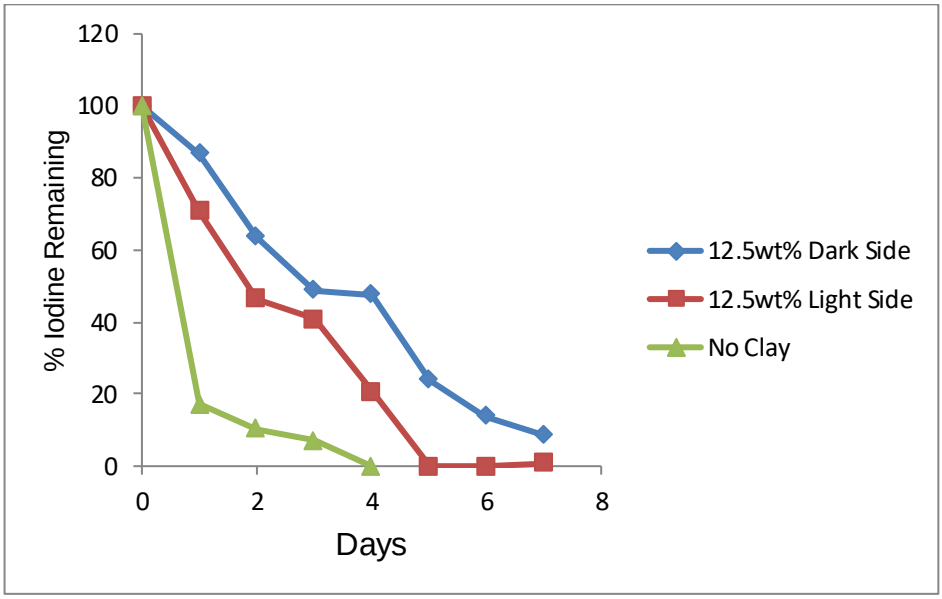


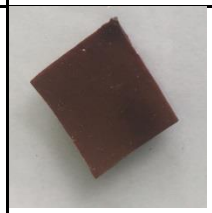
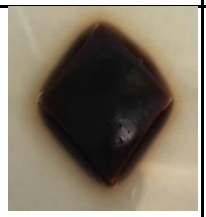
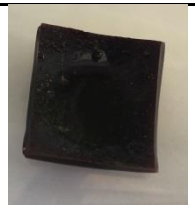
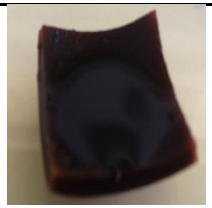
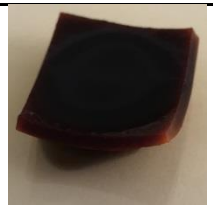
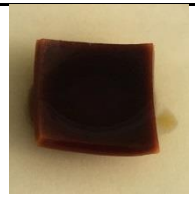
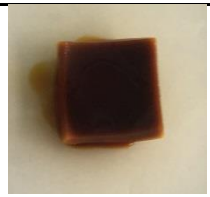
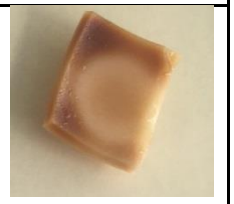
Figure 4.23: The reduction in the percentage iodine over time in the Light and Dark side of the 12.5wt% Cloisite®Na^+ composite compared to the PVOH/PVP-I composite containing no clay.

The photographs in Table 4.13 show the samples just before they were removed from the agar and sent for XRF analysis. The composites containing PVOH/PVP-I 6wt% but no clay, look very different to composites containing 12.5wt% clay and did not stratify into layers. After 24 hours incubation on agar the sample had curled and appeared to have a pool of iodine on top. By day three most of the colour was lost from the sample and this corresponds to the point when the XRF analysis did not register any iodine.

The difference in colour of the two sides of the PVOH/PVP-I 6wt%/Cloisite®Na^+ can be seen in the day 0 photographs where the Light side is a paler brown colour. As described previously the Light side remained flat and in contact with the surface of the agar. By day 3 the edges had started to lose colour and by

day 5 virtually the whole sample had discoloured. In comparison the dark side samples that were in contact with the agar curled away from the agar and retained their colour through to day 5 when the percentage iodine remaining was 24%. On day six the sample was much lighter in colour where it remained in contact with the agar but was still darker around the edges. By day 7 when 9% of the original iodine remained the sample still demonstrated a pale circular area in the centre of the sample but residual colour around the curled edges.

Table 4.13: Photographs of the PVOH/PVP-I 6wt% composites with and without Cloisite®Na^+ on agar just prior to removal for XRF Analysis

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
No Clay								
12.5wt % Clay Light Side on Agar								
12.5wt % Clay Dark Side on Agar								

4.9.4. Discussion

Whilst the XRF analysis provides only semi quantitative analysis of the iodine levels within the samples the results clearly illustrate the reduction in the percentage iodine in the samples compared to the day zero untreated samples. The composites containing no clay were shown to have no iodine remaining by Day 4. The light side of the composite containing 12.5wt% Cloisite®Na^+ appeared to be releasing iodine at a faster rate than the dark side and was shown to have no iodine remaining by day 5. This is interesting when considering the results of the passage studies described in Section 4.8 which demonstrated that the light side of the composite was still showing some antimicrobial activity after 6 days. This might suggest that the presence of bacteria on the agar may affect the release characteristics of the iodine.

The photographs also illustrate that the dark side of the clay containing composite retained its dark brown colour until day 6. At this point the percentage iodine had reduced to 14%. By day 7 the sample had a slightly larger paler central area but still retained 9% of the original iodine level and this corresponds to the results in the original antimicrobial passage studies shown in Figure 4.21 Page 154 where the dark side of the clay sample was still producing large ZOI against *S. aureus* and demonstrating antimicrobial activity against *E. coli* and *P. aeruginosa.*

These experiments suggest that whilst the addition of Cloisite®Na^+ platelets appears to slow the release of the antimicrobial iodine there are probably other factors that also influence the release of iodine from the composite, such as the different bacteria.

4.10. Determination of the composition of the preferred CROWD material

4.10.1. Introduction

To try to understand why the material formed a self- stratifying film and why each side of the composite appeared to have different release characteristics a series of experiments were undertaken by the material scientists in the

Materials Engineering Research Institute (MERI) to determine the different proportions of each component in the two different layers.

4.10.2. Methods Summary

To investigate the composition of the two layers of the preferred CROWD material, PVOH/PVP-I 6wt%/Cloisite®Na^+ 12.5wt%, Lukáš Petra, Francis Clegg and Chris Breen from MERI developed a methodology to extract the four different components: Clay, PVOH, PVP and iodine from the different layers which is described in Section 2.3.2.6.

4.10.3. Results

The first finding was that the mass of the layers in the composite were not equal. The bottom layer was heavier than the top layer. The percentage by mass was 36.7% for the top layer and 63.3% for the bottom layer (Figure 4.24).

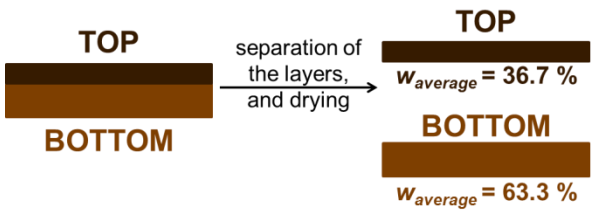


Figure 4.24: Weight of Top (Dark) and Bottom (Light) layers of the CROWD material expressed as a percentage of the total weight of the composite (Petra 2015).

The weights of the individual components in each layer were compared to the original amounts used to produce the composite and then used to calculate the percentage of the original amount of each component found in each layer (Table 4.14).

Table 4.14: Composition of the preferred CROWD composite layers (Petra 2015)

Component	% of amount added present in the Top (Dark) layer	% of the amount added present in the Bottom (Light) layer	Ratio Dark to Light
Cloisite®Na^+	13%	87%	0.2
PVOH	50%	50%	1
PVP	34%	66%	0.5
Iodine	42.0%	58.0%	0.7

4.10.4. Discussion

The separation of the composite into layers is probably caused by the sedimentation of clay-PVOH-PVP-Iodine particles from dispersion during the drying of the film.

The light layer contains nearly five times the amount of clay found in the dark layer. This is probably due to the clay settling at the bottom of the composite as it dries as the light layer is in contact with the base of the Petri dish.

Both layers contain the same amount of PVOH. However, the amount of PVP is double in the light layer, this is probably caused by a strong interaction between the clay and PVP.

A slightly higher concentration of iodine was found in the light layer which may account for increased size of the ZOI produced by the light side of the material in the passage studies in the first couple of days. What is still to be determined is the effect on the composition of the two layers over time, as the iodine is released, as this would help explain the mechanism of the slow-release action that has been observed.

4.11. Conclusions

This research has created a novel polymer clay composite which demonstrated prolonged *in vitro* antimicrobial activity against the five bacteria tested. In the experiments the size of the ZOI was used to compare the antimicrobial efficacy between the dressings. The clinical significance of the size of the ZOI is

unknown. In clinical practice the dressing would be required to kill bacteria which were in contact with the dressing and not necessarily release iodine into the surrounding area. It was clear from these experiments that increasing the concentration of iodine in the material produced a corresponding increase in the size of the ZOI. The optimum concentration of iodine required at the wound surface to inhibit microbial growth has yet to be determined. The material containing 6wt% iodine consistently produced ZOI greater than 500mm^2 against all the test bacteria, so this composite was chosen for the preferred CROWD material. Concentrations of iodine above this were also effective but in light of concerns about possible cytotoxicity it was decided to continue testing samples of the composite containing one of the lower proportions of PVP-I (6wt% iodine).

The onset of action of the bactericidal effect of iodine is very rapid, occurring within seconds of the topical administration. However, the effect is short lived as the iodine is rapidly inactivated by the destruction of the microorganisms. A review of iodine (Cooper 2007) concluded that a dressing that was able to provide sustained delivery of low doses of free iodine would have the advantage of delivering an antimicrobial action against a wide range of microorganisms, with a lower potential to develop resistance or induce cytotoxic effects.

The addition of clay to the composite was designed to increase the tortuosity of the path that small molecules have to travel through the material, to slow down the release of the antimicrobial. The experiments demonstrated that increasing the concentration of Cloisite®Na$^+$ clay produced smaller ZOI indicating that less iodine was being released into the surrounding agar. The highest concentration of clay tested, 12.5wt%, was chosen for the preferred formulation because it produced the smallest ZOI after 24 hours.

During the initial experiments it was noted that the samples appeared visibly different on each side. One side was rough and lighter brown in colour and the top side of the material was smoother and darker brown in colour. It also became apparent that the dressing behaved differently depending on which side was placed in contact with the agar. The light side when placed in contact with the agar produced a much larger ZOI than when the dark side was placed on the agar. This would suggest that the two sides offer different release

characteristics and that the light side released the iodine more rapidly than the dark side. The 12.5wt% clay composite formed a self-stratifying film with a clear demarcation between the two layers. This phenomenon could have interesting applications in clinical practice as placing the light side down in a wound would provide a more rapid release of iodine and the dark side should produce a more even, sustained release of the antimicrobial. Due to the marked difference in colour between the two sides it would be easy for practitioners to apply either side of the material to a wound depending on the desired effect. This could be particularly useful in situations where a prolonged duration of antimicrobial activity is required such as in a war zone where it may take days for a patient to be transported to a medical facility.

During the experiments it was noted that when the dark side of the material was placed in contact with the agar the sample curled and drew liquid to the top of the material. This absorptive capacity, referred to as wicking in wound care technology, is particularly useful for treating wounds with excess exudate. This wicking mechanism acts to draw exudate away from the wound and can also absorb bacteria and slough into the material and help promote healing. The CROWD material is very pliable and would conform to different sized wounds and could be cut into shape to treat awkward shaped wounds. After absorbing fluid, the material forms a coherent rigid gel which could easily be lifted from the wound surface. If the material became dry it can be rehydrated with sterile water enabling trauma free removal from the wound. In the hydrated form the material may be useful on dry wounds as there is the possibility that it could donate fluid. This potential property of the material has not yet been tested.

The passage studies clearly demonstrated that the CROWD material had a longer duration of antimicrobial activity against the bacteria tested than the commercial iodine dressings which only demonstrated *in vitro* antimicrobial activity for a maximum of 48 hours. This would be very useful in clinical practice as it should significantly reduce the number of dressing changes which could optimise the healing rate and produce cost savings in terms of the reduced number of dressings used and staff time required to change the dressings.

Another advantage of the CROWD material is that it provides a visual colour change from brown to colourless once the iodine has been consumed. This would act as an indicator to clinicians that the dressing requires changing.

Chapter 5: Comparison of CROWD with Commercial Dressings.

5.1. Introduction

Having created a novel antimicrobial composite it was important to compare various aspects of the material with the available commercial antimicrobial dressings to establish what advantages the CROWD material could offer in this highly competitive market.

To compare the antimicrobial efficacy of the CROWD material to the commercial iodine dressings, passage studies were undertaken against planktonic cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus aureus* USA300 and Epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA-16). Statistical analysis using these results and all of the passage study results for the commercial dressings tested in Chapter 3 was undertaken to establish if there was a significant difference between the activity of CROWD and the commercial dressings.

Bacteria found on chronic wounds are often living in biofilm colonies so the effect of iodine dressings on selected bacteria existing in biofilms was also investigated.

The minimum inhibitory concentration (MIC) of the eluted materials extracted from the iodine containing dressings was also determined. Having demonstrated the antimicrobial efficacy of CROWD the next important pre-clinical investigation required was to test the potential cytotoxicity of the material and compare this to the iodine dressings that are currently marketed for the treatment of infected wounds.

5.2. Passage Studies: CROWD compared to commercial iodine dressings

5.2.1. Introduction

The antimicrobial activity of the CROWD material was compared with the three commercial iodine containing dressings that were licensed for the treatment of infected wounds.

5.2.2. Methods Summary

Passage studies as described in Section 2.1.2.3. were undertaken to compare the duration of antimicrobial activity of the Light and Dark sides of the CROWD composite to the commercial iodine dressings**;** Iodoflex®, Inadine®, and Iodozyme®. Six 2 x 2cm samples of each dressing were tested against 5 microorganisms: *S. aureus* (SH100), *E. coli* (JM109), *P. aeruginosa* (H085180216), *S. aureus* USA300 and EMRSA -16.

5.2.3. Results

The mean surface area in mm^2 of the ZOI produced by each dressing after each 24 hour interval against the five different bacteria is shown in Figures 5.1.-5.5.

5.2.3.1. *E. coli* – Passage Study Results

All the iodine containing dressings produced clear ZOI against *E. coli* after 24 hours (Figure 5.1.). Both sides of the CROWD material were still producing a ZOI after 7 days. By day 5 the dark side of the CROWD material was producing very small ZOI where it remained in contact with the agar. The three commercial dressings were only effective for 48 hours. Their ZOI were smaller than the CROWD ZOI except for Iodoflex which produced the largest ZOI after 48 hours but then did not produce a ZOI on day 3.

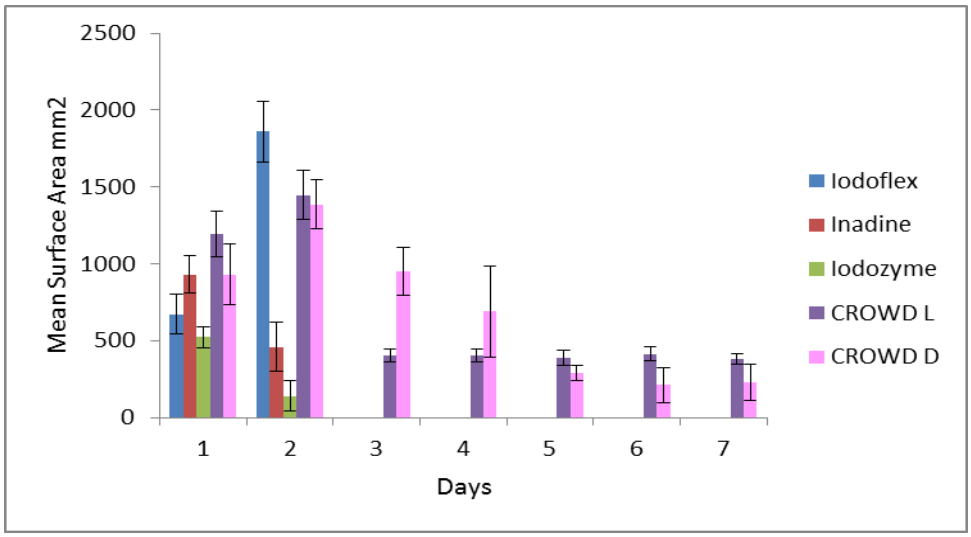


Figure 5.1: ZOI produced by Iodine dressings against *E. coli* (Error bar = Standard Deviation, n = 6)

5.2.3.2. *P. aeruginosa* – Passage Study Results

The ZOI produced by all the dressings were smaller against *P. aeruginosa* than those produced against the other four bacteria. Inadine produced a very small ZOI on the first day and had not produced a ZOI on the second day (Figure 5.2).

Iodoflex produced a very large ZOI in the first day and a much smaller ZOI the second day but was no longer producing a ZOI on day 3. Both sides of the CROWD material were still producing a ZOI on day 7 however the light side ZOIs were much larger than the dark side.

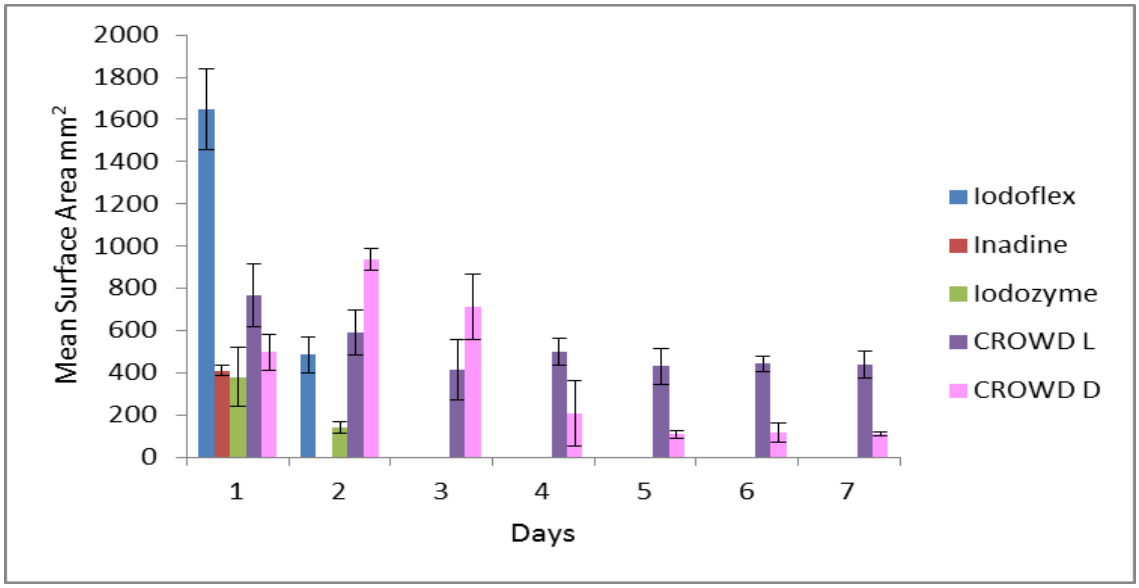


Figure 5.2: ZOI produced by Iodine dressings against *P. aeruginosa* (Error bar = Standard Deviation, n = 6)

5.2.3.3. *S. aureus* - Passage Study Results

As in all previous experiments the ZOI produced against *S. aureus* were larger than those produced against *E. coli* and *P. aeruginosa* (Figure 5.3). After the first 24 hours the light side of the CROWD material produced the largest ZOI of all the dressings tested. However, on the second day Iodoflex produced an even larger ZOI than the CROWD light side had produced on the first day. Interestingly the size of the ZOI produced by the CROWD dark side was also larger on the second day than it had been on the on the first day and continued

to increase in size reaching a peak on day 4. Both sides of the CROWD material were still producing a clear ZOI on day 7 and the dark side was still producing a large ZOI on day 8.

The commercial iodine dressings only produced ZOI for 48 hours. Inadine and Iodozyme produced very small ZOI on the second day compared with Iodoflex which produced a much larger ZOI on the second day.

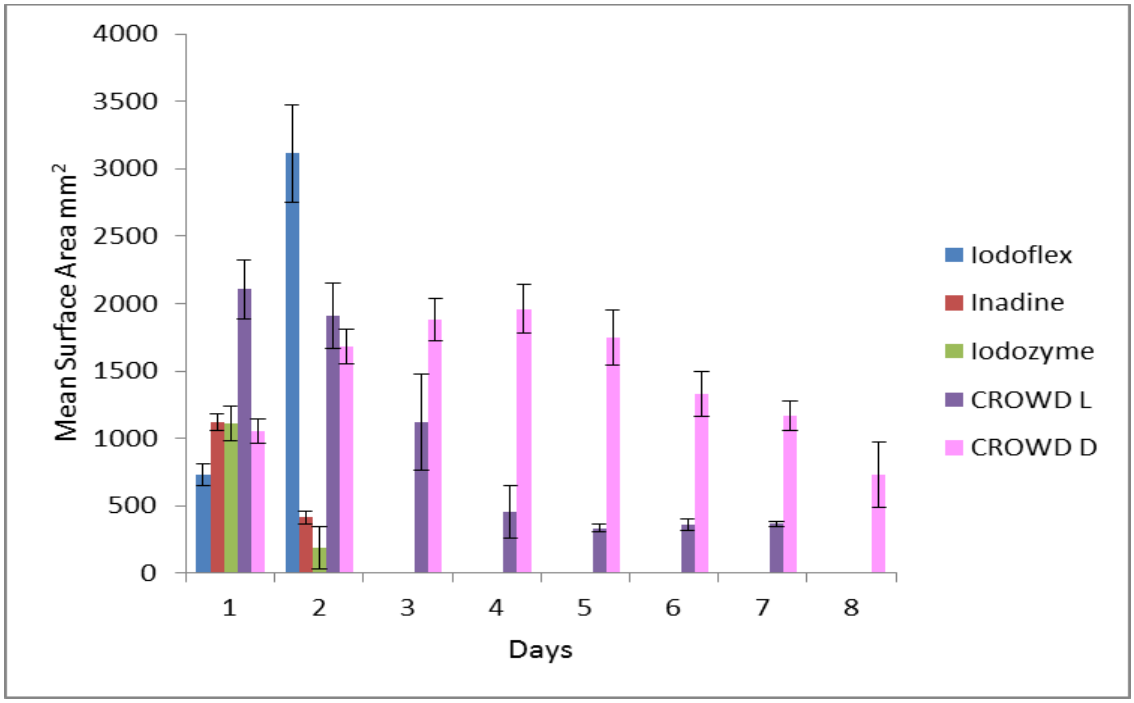


Figure 5.3: ZOI produced by Iodine Dressings against *S. aureus* (Error bar = Standard Deviation, n = 6)

5.2.3.4. *S. aureus* USA300 - Passage Study Results

After 24 hours the Iodoflex had cleared the whole Petri dish of microbial growth on all six replicates (Figure 5.4). It produced a smaller ZOI after 48 hours and had stopped producing a ZOI by day 3. Inadine was only effective for 24 hours. Iodozyme produced a similar sized ZOI to Inadine after 24 hours and a very small ZOI after 48 hours. As observed in the earlier MRSA passage studies in Section 4.8.3 the CROWD dark side continued to kill for 5 days but the light side only produced a ZOI for 4 days.

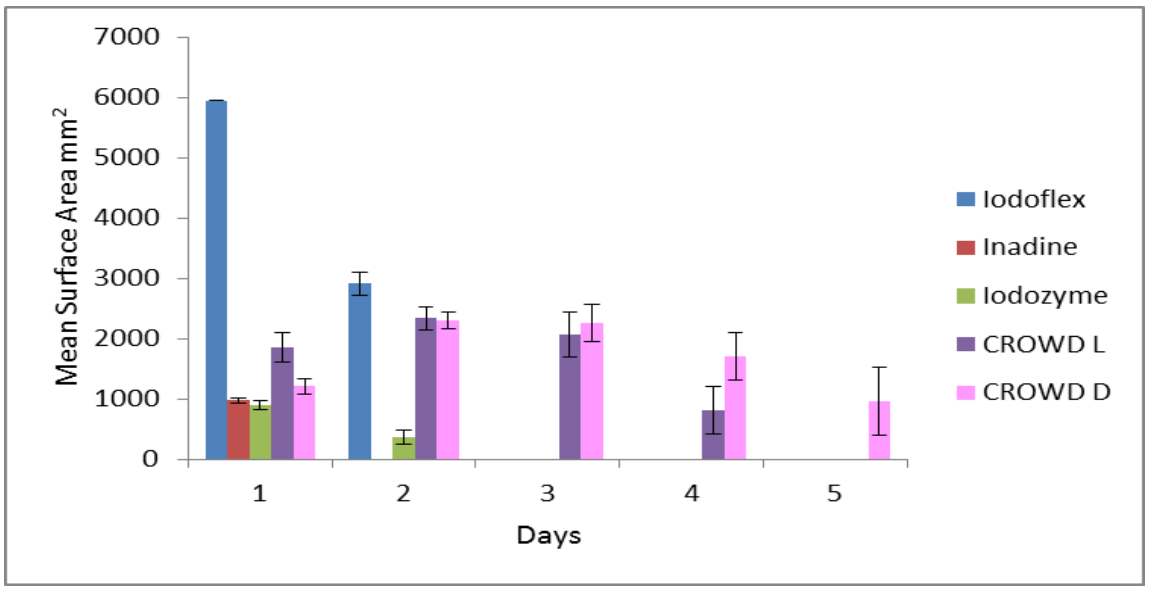


Figure 5.4: ZOI produced by Iodine dressings against *S.aureus* USA300 (Error bar = Standard Deviation, n = 6)

5.2.3.5. EMRSA -16 – Passage Study Results

After 24 hours Iodoflex had produced very large ZOI, almost clearing the whole surface of the Petri dish (Figure 5.5). On the second day Iodoflex produced a much smaller ZOI and there was no ZOI on the third day. Inadine and Iodozyme only produced ZOI against EMRSA-16 for 24 hours. As seen in the *S. aureus* USA300 experiment the CROWD dark side continued to produce a ZOI for 5 days, but the light side did not produce a ZOI after the fourth day.

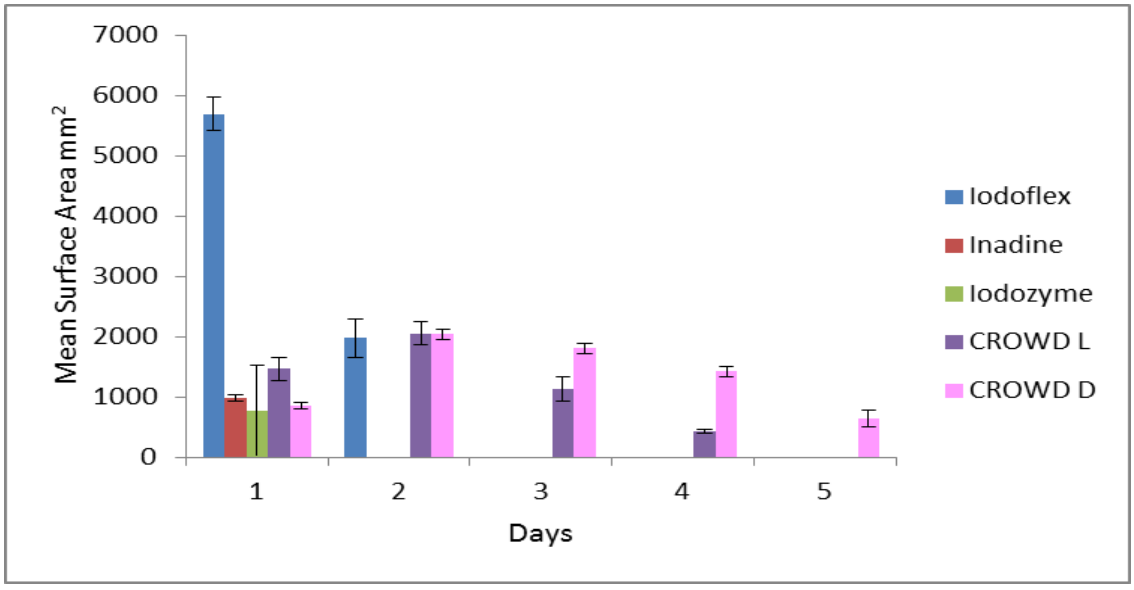


Figure 5.5: ZOI produced by Iodine Dressings against EMRSA-16 (Error bar = Standard Deviation, n = 6)

5.2.4. Discussion

The results of the passage studies would suggest that the duration of activity of the CROWD material was superior to the commercial iodine dressings both in terms of the size of the ZOI produced and more importantly the duration of action.

Inadine produced ZOI for 48 hours against *E. coli* and methicillin-susceptible *S. aureus* but only produced a ZOI against the two MRSA strains and *P. aeruginosa* for 24 hours. Of the three commercial iodine dressings, Iodozyme, consistently produced the smallest ZOI except against *S. aureus* were the ZOI it produced in the first 24 hours were similar to those produced by Inadine and larger than those produced by Iodoflex. By the second day the Iodozyme gel had curled and produced very small ZOI where it remained in contact with the agar. It produced ZOI on the second day against all the test bacteria except the EMRSA-16.

The Iodoflex produced very large ZOI against the five test bacteria for two days. However, the size of the ZOI varied on each day for the different bacteria. The ZOI produced against *P. aeruginosa* and the two MRSA strains was largest on the first day and smaller on the second. Conversely, the ZOI against *S. aureus* and *E. coli*. were smaller on the first day and much larger on the second day. The CROWD material had demonstrated a similar phenomenon that the material often produced a larger ZOI on the second day but with the CROWD material this was evident against all the test bacteria. It had been hypothesized that the increase in the size of the ZOI on the second day was possibly due to absorption of fluid from the agar reaching a critical saturation point which led to the increased release of iodine after the first 24 hours.

However, this would not fully explain why the Iodoflex produced a larger ZOI on the first day with certain bacteria and on the second day with others. What is particularly interesting was that the effect was different within the bacterial classes for example, with the Gram - positive bacteria, the MSSA had the largest ZOI on the second day, but the two MRSA strains had the largest ZOI on the first day. Similarly with the two Gram - negative bacteria; the *P.*

aeruginosa had the largest ZOI on day one but against *E. coli* the largest ZOI was on the second day. Whilst it would be interesting to investigate what was causing this phenomenon it probably would not be significant in clinical practice as the ZOI produced on both days against the five test bacteria were large suggesting effective antimicrobial activity for 48 hours.

As demonstrated in previous studies, the dark side of the CROWD material produced ZOI against *S. aureus* for 8 days (Section 4.7.4) and the two MRSA strains for 5 days (Section 4.8.3). The light side of the CROWD material was effective against *E. coli* and *P. aeruginosa* for 7 days (Section 4.8.3). Both sides of the CROWD material were effective against all five test bacteria for at least 72 hours clearly illustrating the prolonged duration of antimicrobial action compared to the other iodine containing dressings.

This supports the hypothesis that the addition of the clay has slowed the rate of iodine release from the composite and that this coupled with the higher original concentration of iodine has resulted in the elution of concentrations of iodine that have prolonged the duration of inhibitory activity compared to the commercial dressings.

5.3. Statistical Comparison of CROWD and Commercial Dressings.

5.3.1. Introduction

The graphical illustrations of the ZOI produced by the CROWD material against the five test bacteria indicate that in the *in vitro* tests CROWD produced larger ZOI and had a longer duration of antimicrobial action than most of the commercial antimicrobial dressings tested.

To test whether these results were significant, statistical analysis of the results of all the passage studies was undertaken.

5.3.2. Methods - Statistical Analysis

The raw data for the results for all the commercial wound dressings' passage experiments were imported into SPSS (version 24, IBM Corp., Armonk, New York, US) software. A full factorial three-way Analysis of Variance (ANOVA) was undertaken to compare each of the 18 different commercial dressings (Table 5.1) and the two sides of the CROWD material against each of the test

bacteria on the eight different days of the study. The dependent variable was the ZOI and the independent variables were the dressings, bacteria and day. Three Post Hoc tests; Student–Newman–Keuls (SNK), Tukey HSD and Scheffé were undertaken to construct homogenous subsets to try to identify means that were significantly different from each other.

Table 5.1: The 18 Commercial Dressings included in the Statistical Analysis

Dressing	Antimicrobial	Carrier Dressing
Bactigras	Chlorhexidine acetate 0.5% ointment	Tulle
Suprasorb X + PHMB	Polihexanide 0.3%	Biosynthetic cellulose fibre
Inadine	10% povidone iodine (1% w/w available iodine)	Tulle
Iodoflex	Cadexomer Iodine (0.9% w/w iodine)	Cadexomer iodine paste with gauze backing
Iodozyme	< 0.04% iodide ions and glucose oxidase	Hydrogel
Allevyn Ag	Silver sulfadiazine	Polyurethane foam film
Aquacel Ag	1.2 % w/w ionic Silver	Hydrocolloid Hydrofibre
Aquacel Ag Extra	1.2% w/w ionic Silver	2 layers of Hydrofibre
Biatian Ag	Patented Silver complex	Polyurethane foam film
Sorbsan Ag	1.5% Silver alginate fibres	Calcium Alginate
Urgosorb Ag	0.5 % ionic Silver	Calcium Alginate
Urgotul Ag	TLC – Ag Technology containing Silver particles	Soft Polymer
Urgotul SSD	Silver sulfadiazine	Tulle
Actilite Tulle	99% Medical grade Manuka honey and 1% Manuka oil	Tulle
Activon Tulle	100% Medical grade Manuka honey	Tulle
Algivon	100% Manuka honey	Calcium Alginate
Medihoney Antibacterial Honey Apinate	Medihoney	Calcium Alginate
Medihoney Gel	80% Medihoney and 20% Alginate	Sodium Alginate

5.3.3. Results

5.3.3.1. ANOVA Results

The difference between the three independent variables was shown to be highly statistically significant with a value of $p < 0.0005$, as were all 2 way and 3 way interactions.

As the total sum of squares represents the overall variance in a data set the separate contributions of the separate factors to the variance can be calculated by dividing the respective sum of squares by the corrected total. The results in the ANOVA table (Table 5.2) show that 97% of the variance in the whole data set is explained by the model. All three factors; type of dressing, species of bacteria and duration of exposure and their interactions were shown to be highly significant $p<0.0005$. This means that it can be concluded with a very high degree of confidence that the bacteria all behaved differently with respect to each other and when exposed to different antimicrobial dressings. Similarly, the significance level for the Dressing*Day interaction leads also to the conclusion that the dressings behave differently to each other and have different effects on different days. Also, the bacteria reacted differently on different days to different dressings (see Significance of 3 way interaction).

Table 5.2: ANOVA Test Table

Tests of Between-Subjects Effects

Dependent Variable: ZOI

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1493357649.000[a]	559	2671480.589	175.985	.000
Intercept	302342375.800	1	302342375.800	19916.911	.000
Dressing	170962483.400	19	8998025.440	592.748	.000
Bacteria	24264491.500	4	6066122.874	399.608	.000
Day	321003706.800	7	45857672.400	3020.890	.000
Bacteria * Day	71797891.060	28	2564210.395	168.918	.000
Dressing * Bacteria	202594653.600	46	4404231.599	290.130	.000
Dressing * Day	348056021.200	133	2616962.565	172.393	.000
Dressing * Bacteria * Day	275944150.000	322	856969.410	56.453	.000
Error	42383073.340	2792	15180.184		
Total	1939056458.000	3352			
Corrected Total	1535740723.000	3351			

Based on the actual passage study results the SPSS programme produced a predicted model comparing the estimated marginal means (based on the actual mean of the ZOI results from the experiments) of the ZOI for each dressing on each of the eight days against the five different bacteria (Table 5.3). Colour coding has been used to group the different classes of antimicrobial dressings based on the antimicrobial agent; blue (biguanide), lilac (iodine), green (silver), orange (honey) and pink (CROWD).

The differences in the antimicrobial activity of the individual commercial dressings against *S. aureus*, *E. coli* and *P. aeruginosa* were discussed in Chapter 3 Section 3.3.5. Comparison of the antimicrobial effects of the iodine containing dressings against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. aureus* USA 300 and EMRSA-16 have been described in Section 5.2.3.

Table 5.3. shows the estimated marginal means for the ZOI produced by all 18 commercial dressings and the two sides of the CROWD material against all five bacteria tested. It clearly illustrates that the CROWD material has a longer duration of antimicrobial action against all the test bacteria than any of the commercial dressings. Another observation from the tabulated data is the difference in duration of action between the light and dark side against different bacteria. If estimated means of < 400mm^2 are discounted, as these are smaller than the sample size, then there appears to be a difference between the behaviour in the two sides of the CROWD dressing against the Gram-positive and Gram- negative bacteria. Against the Gram - positive bacteria the dark side was active for 8 days compared with 4 days (Light side) against *S. aureus*, and 5 days against both strains of MRSA compared with the light side which was effective for 4 days. Conversely against *P. aeruginosa* the light side was active for 7 days whereas the dark side was only producing ZOI > 400mm^2 for 3 days. The difference in duration of action against *E. coli* was less marked with each side having an estimated mean ZOI > 400mm^2 on day 4. From day 5 the dark side was producing much smaller ZOI. However, the light side continued to produce ZOI around the 400mm^2 size for a further 3 days.

Table 5.3: Statistical model of estimated marginal mean ZOI for each AWD

E. coli	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Bactigras	460.16							
Suprasorb x = PHMB	1243.8	919.49						
Inadine	931.67	460.36						
Iodoflex	674.32	1860.84						
Iodozyme	523.68	141.93						
Allevyn Ag								
Aquacel Ag	448.88	540.86	548.19	544.26	414.74			
Aquacel Ag Extra	645.00	722.33	668.55	694.72	446.14			
Biatain Ag								
Sorbsan Ag	467.86	593.19	477.28	454.77				
Urgosorb Ag	542.82	672.35	524.24	470.98				
Urgotul Ag	624.5	447.83						
Urgotul SSD	587.18	390.66						
Actilite Tulle								
Activon Tulle	1061.3							
Algivon	1155.3	2686.92						
Apinate	964.76	2218.67						
Medihoney Gel	868.99							
CROWD D	932.33	1387.83	952.5	691	293.16	214.66	233	
CROWD L	1197	1448.66	406.5	406.33	390.16	416.33	383.33	

P. aeruginosa	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Bactigras	272							
Suprasorb X + PHMB	704.66	690.14	730.57					
Inadine	410							
Iodoflex	1648.9	486.43						
Iodozyme	380.99	70.65						
Allevyn Ag	482.83	571.66	1831.01	2135.5	1747.2	1910.8	1815.3	
Aquacel Ag	600.52	1759.05	1490.19	680.72				
Aquacel Ag Extra	794.99	1869.54	1660.66	1270.1				
Biatain Ag	503.44	1043.26	1819.63	2306.9	2388.8	2350.2	2401.9	
Sorbsan Ag	831.05	1602.83	1431.70	1118.36	82.1633			
Urgosorb Ag	648.62	1365.11	969.34	327.08				
Urgotul Ag	815.87	935.32	820.58	671.30				
Urgotul SSD	726.64	854.60	824.64	289.40				
Actilite Tulle								
Activon Tulle	616.5							
Algivon	916.74							
Apinate	558.00							
Medihoney Gel	692.37							
CROWD D	499.16	939	712.66	207.83	109.5	117.5	112.16	
CROWD L	767.98	592	414.83	499.83	431.5	443.83	438.83	

S.aureus	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Bactigras								
Suprasorb X + PHMB	1315.92	972.74						
Inadine	1117.44	414						
Iodoflex	730.79	3112.13						
Iodozyme	1110.77	186.77						
Allevyn Ag								
Aquacel Ag	679.15	652.33	588.88	609.02				
Aquacel Ag Extra	1283.9	877.10	825.68	684.12				
Biatain Ag								
Sorbsan Ag	896.60	746.01	564.93	555.12				
Urgosorb Ag	615.96	725.47	661.10	645.79				
Urgotul Ag	579.83	556.5						
Urgotul SSD	916.61	418.66						
Actilite Tulle	250.33							
Activon Tulle	1503.23	426.33						
Algivon	1244.74							
Apinate	1046.53							
Medihoney Gel	1043.26							
CROWD D	1052.16	1680.66	1877.16	1959.16	1750.16	1328.5	1169	729.83
CROWD L	2106.48	1907.80	1117.98	455.33	334.33	357.16	364.83	

S.aureus USA300	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Inadine	974.97							
Iodoflex	5941.66	2911.43						
Iodozyme	906.18	187.35						
CROWD D	1210.33	2309.99	2256.74	1706.59	802.53			
CROWD L	1858.11	2339.69	2066.77	814.96				
EMRSA-16	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Inadine	985.04							
Iodoflex	5691.25	1978.99						
Iodozyme	762.88							
CROWD D	861.33	2041.39	1808.77	1429.09	425.73			
CROWD L	1466.02	2057.48	1139.55	436.06				

5.3.3.2. Post Hoc Test Results

The post hoc tests compared the activity between different dressings and then grouped them based on their similarity. The results of the three individual tests can be found in Appendix 1. From the data a table was constructed (Table 5.4.) which lists the dressings in the order they were listed in all three sets of results. The Student – Newman - Kuels (SNK) test identified 13 sets whereas the Tukey HSD and Scheffé tests each produced 11 sets. The numbers in the table indicate that the tests had grouped the dressings together, for example all three tests grouped Actilite Tulle and Bactigras together. Conversely any dressing that had a single number that was different to all the other numbers in the list meant that it was unlike any of the other dressings tested. CROWD dark was found by all three tests to be in a set on its own indicating that it was very different to all the other dressings tested.

Where there were two or more numbers against a dressing then the action of that dressing was considered to overlap with the dressings listed above or below or both e.g. Urgotul Ag in the Tukey HSD test overlapped with Urgotul SSD above and Algivon below. There was a consistency in the ranking of the dressings, but some inter test variability. For example, CROWD L was considered to be a set on its own in the SNK test and Tukey HSD but was grouped with Iodoflex by the Scheffé test. Not surprisingly there was some overlap between many of the silver dressings. There would appear to be some trends in the similarity grouping in terms of the carrier dressings.

Table 5.4: Results of Post Hoc Tests

Dressing	Student- Newman-Kuels	Tukey HSD	Scheffé
Actilite Tulle	1	1	1
Bactigras	1	1	1
Iodozyme	2	2	2
Medihoney Gel	2	2	2
Inadine	2 3	2	2 3
Activon Tulle	3	2	2 3 4
Apinate	4	3	3 4 5
Urgotul SSD	4	3 4	4 5
Urgotul Ag	4 5	3 4 5	5
Algivon	5 6	4 5	5
Suprasorb X + PHMB	6	5	5 6
Urgosorb Ag	7	6	6 7
Aquacel Ag	8	7	7 8
Sorbsan Ag	8	7	7 8
Allevyn Ag	9	7	8
Aquacel Ag Extra	10	8	9
Biatain Ag	10	8	9
Iodoflex	11	9	10
CROWD L	12	10	10
CROWD D	13	11	11
Number of Sets	13	11	11

5.3.4. Discussion

The first five dressings listed in Table 5.4. from Actilite to Activon are all Tulles with the exception of the hydrogel Iodozyme and the alginate Medihoney, these dressings were not very absorbent and were effective for a short duration. The next four dressings from Apinate to Algivon are all alginates and soft polymers which are similar as they are more absorbent. There was some overlap with Suprasorb X + PHMB which also overlaps with the alginate, Urgosorb Ag which is in a group with other highly absorbent alginates and Hydrofibers. Aquacel Ag+Extra and Biatain Ag make a distinct pair which is interesting as Biatain Ag did not demonstrate any activity against two of the test bacteria but did have a long duration of action of 7 days against *P. aeruginosa* which could explain why it has been shown to be similar to the silver dressing which demonstrated

efficacy for up to 5 days against *E. coli* and 4 days against *S. aureus* and *P. aeruginosa.*

Both the SNK and the Tukey HSD use standardized range statistics however the SNK method uses different critical values for different pairs of mean comparisons (Sheskin 1989). Whilst the three test results were not identical they do however contain similar trends and provide a useful statistical tool to illustrate the differences between the test dressings.

In this study the size of the Zones of Inhibition created by the antimicrobial dressing were the continuous variables. Non parametric statistics do not offer a feasible alternative to a three way ANOVA. Applying the non parametric equivalent of various one or two way ANOVAS; Friedman or Kruscal Wallis tests would make it impossible to test for interactions and the absence of multiple tests e.g. Bonferroni correction would lead to inaccurate results. There would also be no way to test for the three-way interactions found in this study.

The nonparametric assumption that the data would be distribution free was highly improbable, neither is the data nominal or ordinal therefore even if the residuals were not normally distributed the data could be transformed and still an ANOVA would be applied.

It was decided to use an ANOVA because the less powerful parametric test alternatives would violate just as many, if not more, assumptions and would carry the danger of multiple comparisons and would not allow for the appropriate testing of the interactions which was central to the research question.

In hindsight this was considered the correct approach as it is highly improbable that such strong effects would be visible just due to the wrong choice of test.

Non normal data can lead to slightly optimistic significance estimation but considering all significance shown in the ANOVA Table are below 0.0005 (0.005%) it is extremely unlikely that these effects are only seen due to statistical error. Visual inspection of the data confirms very clearly these differences.

5.3.4.1. Normality Testing for Residuals in ANOVA using SPSS

Residuals were calculated by taking the sample value away from the mean. The residual for each observation is the difference between the predicted values of the dependent variable (ZOI) and the observed values.

Testing all the non-zero results produced a near normal histogram which was symmetrical, only very slightly skewed but with a slightly elevated kurtosis. Skewness measures the symmetry of the distribution while kurtosis determines the heaviness of the distribution tails. This can be seen in the histogram (Figure 5.6) where there is a peak shaped symmetrical distribution of the residuals. Compared to the bell-shaped curve of a nomal distribution the peak shape of the residual distribution is a bit sharper. This suggests a leptokurtic distribution. This is reflected in the higher kurtosis parameter calculated by the normality test meaning that the tails will contain a relatively higher proportion of data compared to a normal distribution. This effect is not very strong as is shown in the histogram (Figure 5.6), looking at the interval of the mean +/- 2 times the standard deviation the vast majority of the data is included within this interval. This means that whilst the tails might be slightly heavier, if we compare this small deviation to the distance of the p value in Table 5.2 to the cut off value of alpha = 0.05, we can conclude that the deviation is not of such a size that it could increase the p value enough to cast doubt on the significance. All p values in Table 5.2 are smaller than 0.0005 which is two orders of magnitude smaller than alpha. With only the slight deviation from normality seen in the histogram it is highly improbable that the deviation would make up for these two orders of magnitude.

The distribution depicted in the research histogram (Figure 5.6) looks very similar to an example of a leptokurtic distribution, the LapLace distribution, (Figure 5.7) and so it can be assumed that a Laplace distribution might be a suitable model. In 2018 Geraci and Borja applied a normal model for the confidence quantification on a Laplace distributed data set and quantified that the normal model would only be slightly optimistic i.e., a 99.9% confidence arising from a normal test would only have an actual confidence level of 99.3%. With that in mind, any p value near the alpha level would have to be met with scepticism as it would be unclear whether the binary conclusion of the test result would still be

true. In this case however, the results are not deviating from the generally accepted confidence level of 95% (α=0.05) as the study p-values are all below 0.0005, hence the error due to a normality assumption is not nearly large enough to invalidate the conclusions drawn on the basis of the ANOVA results.

The alternative would be to apply a non parametric test for the ZOI. The problem with that is two-fold. Non parametric tests are known for their lack of power but more importantly they are only defined for very specific cases. Non parametric equivalents do exist for the 1 and 2 way ANOVA's, for example the single factor Kruskal Wallis test and the Friedman's Two-Way Analysis of Variance by Ranks, but the latter does not allow for interaction testing. For an analysis with 4 factors and interactions as in this study, there is no non parametric test available. A sequence of single factor or two factor equivalents would be possible but their significance estimation would be much more optomistic due to the multiple testing problem. Also, it would not be able to quantify any interactions which was vital for this research. In conclusion, an ANOVA was the only possible choice for this data as a non parametric test could not provide the necessary models and the bias to be expected due to the Leptokurtosis is much smaller than the big difference between the study p values and the accepted confidence thresholds.

Residual for ZOI

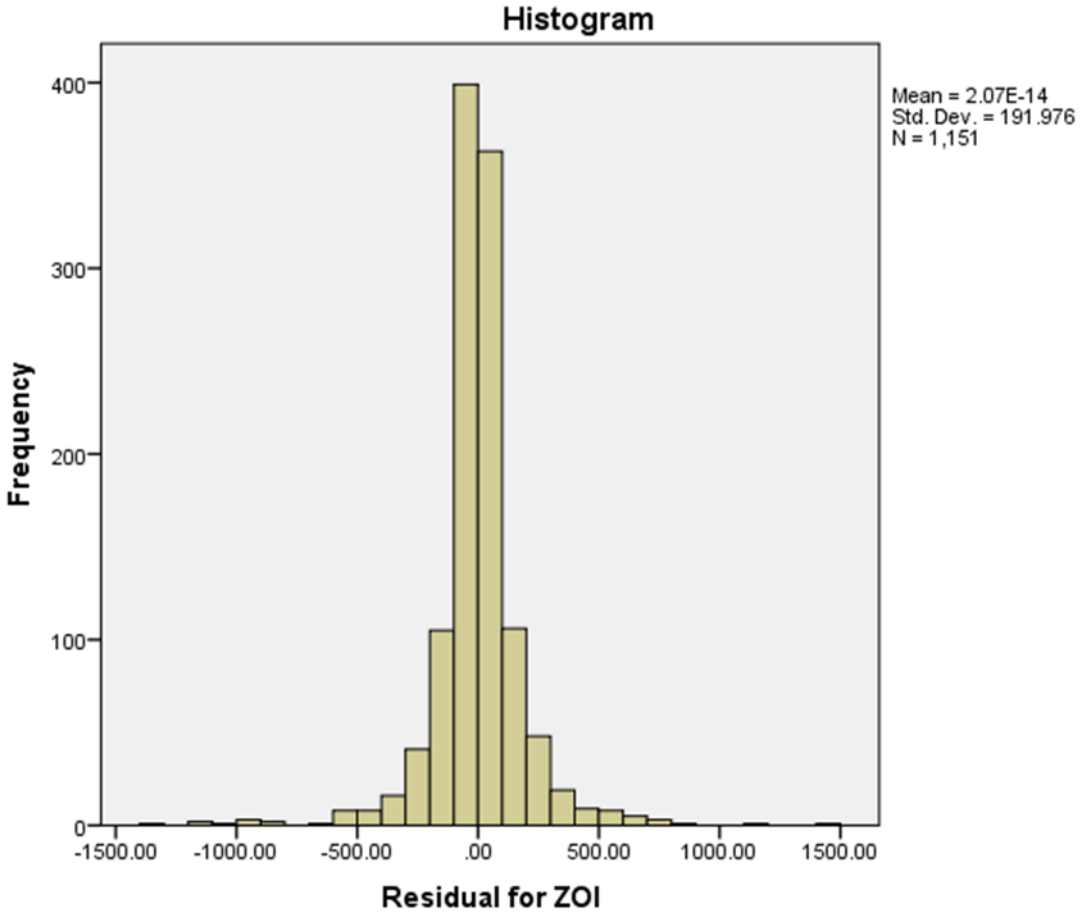


Figure 5.6 Histogram depicting the frequency of the Residual Zone of Inhibition

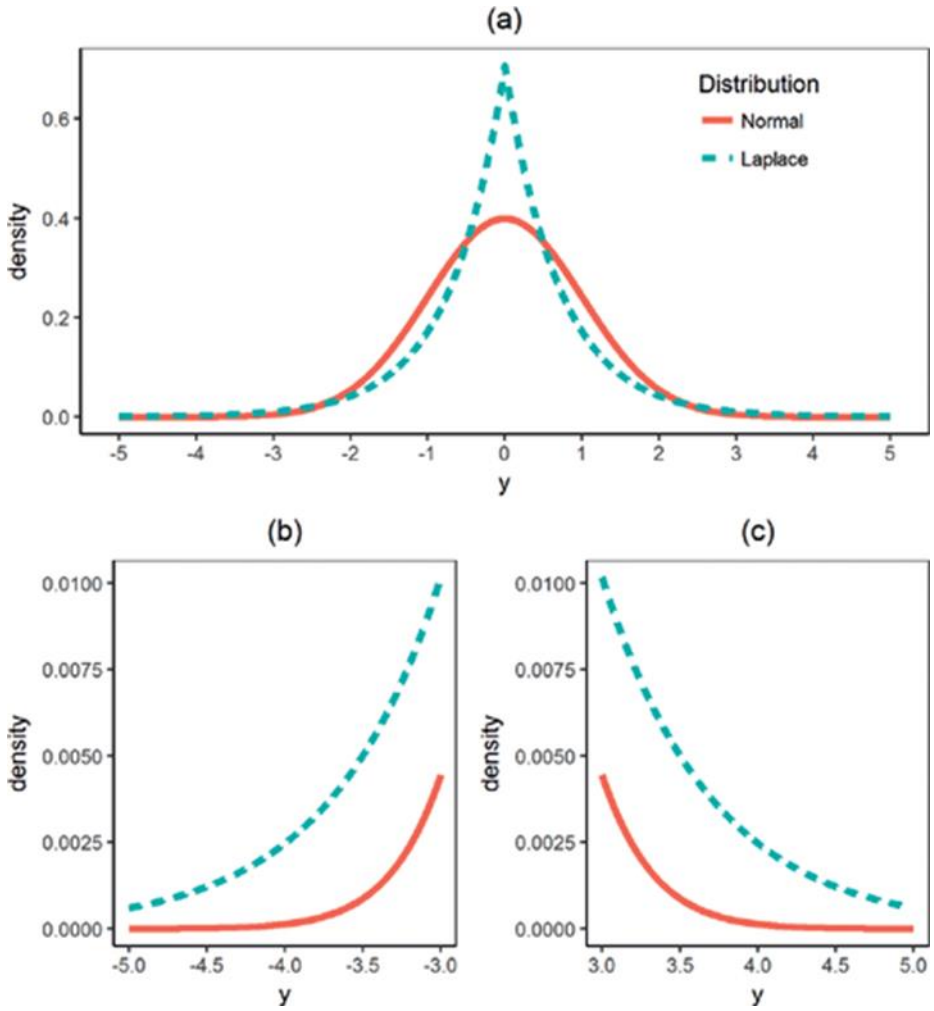


Figure 5.7 Laplace Distribution Curve showing (a) Laplace and normal densities; (b) and (c) show the considerably thicker lower and upper tails, respectively (Geraci and Borja 2018)(Reproduced with permission from John Wiley & Sons).

5.4. Anti - Biofilm activity - CROWD compared with the commercial iodine dressings.

5.4.1. Introduction

The previous *in vitro* experiments had been designed to test the antimicrobial efficacy of the new CROWD material against planktonic bacteria. Whilst the study produced encouraging results that indicate the dressings were effective against all five test organisms it is now known that bacteria in chronic wounds often exist in the biofilm phenotype (Malone et al. 2017).

Most chronic wound pathogens, including methicillin - resistant *S. aureus* (MRSA) and *Pseudomonas* spp., have been shown to produce biofilms (Leaper and Durani, 2008).

These pathogenic biofilms constitute a major concern in wound care because of their enhanced microbial activity and the diminished effectiveness of antimicrobial agents in their presence (Percival et al. 2008). It was therefore important to test whether the new antimicrobial material was able to kill bacteria existing in biofilms as well as inhibiting the growth of planktonic bacteria.

5.4.2. Methods Summary

The biofilm experiments were undertaken by Aneesah Mahmood in the Department of Biosciences and Chemistry, Sheffield Hallam University.

24, 48 and 72 hour Biofilms containing single species of *S. aureus* (SH1000)*, E. coli* (JM109) and *P. aeruginosa* (H085180216) were created as described in Section 2.1.2.4.

Square 2 x 2 cm samples of the test iodine dressings; Inadine®, CROWD Light side and CROWD Dark side were placed over the biofilms and incubated in a static incubator for 24 hours at 37^{o}C. A control was used which was a biofilm incubated in a static incubator at 37^{o}C without an antimicrobial dressing. After careful rinsing to remove planktonic bacteria the biofilm was suspended in 1% PBS and serially diluted for counting.

Each experiment was repeated twice.

The control biofilms that had not been treated with a wound dressing were removed from the MH broth after 24, 48 and 72 hours and transferred to 1ml PBS 1% to determine bacterial viability and to investigate the development of the untreated biofilms. BacLight reagents were added as described in Section 2.1.2.5. The biofilms were then placed on microscope slides, covered with a cover slip and the fluorescent images visualised using a confocal LSM 150 Laser microscope.

5.4.3. Results

5.4.3.1. Anti-Biofilm Activity of the Iodine Dressings

The results shown in Table 5.5 demonstrate that all the iodine dressings tested were able to completely eradicate the *S. aureus* and *E. coli* biofilms grown for 24, 48 and 72 hours.

However, the dressings all failed to kill any of the *P. aeruginosa* biofilms regardless of the maturity of the biofilm.

For each bacterium tested, the number of colony forming units (cfu) per filter increased as the biofilm aged.

Table 5.5: Effectiveness of iodine dressings against *S. aureus*, *E. coli* and *P. aeruginosa* Biofilms (No WD = No Wound Dressing applied) (Mahmood 2018)

Bacteria	Time (Hours)	Wound Dressing	cfu/filter (Std Dev)
S. aureus	24	CROWD Dark	0
		CROWD Light	0
		Inadine	0
		Control (No WD)	1.6×10^9 ($\pm$0.2)
	48	CROWD Dark	0
		CROWD Light	0
		Inadine	0
		Control (No WD)	1.56×10^9 ($\pm$0.1)
	72	CROWD Dark	0
		CROWD Light	0
		Inadine	0
		Control (No WD)	1.67×10^9 ($\pm$0.1)
E. coli	24	CROWD Dark	0
		CROWD Light	0
		Inadine	0
		Control (No WD)	1.62×10^9 ($\pm$0.004)
	48	CROWD Dark	0
		CROWD Light	0
		Inadine	0
		Control (No WD)	1.67×10^9 ($\pm$0.02)
	72	CROWD Dark	0
		CROWD Light	0
		Inadine	0
		Control (No WD)	1.71×10^9 ($\pm$0.1)
P. aeruginosa	24	CROWD Dark	$>3 \times 10^8$
		CROWD Light	$>3 \times 10^8$
		Inadine	$>3 \times 10^8$
		Control (No WD)	7.5×10^8 ($\pm$0.3)
	48	CROWD Dark	$>3 \times 10^8$
		CROWD Light	$>3 \times 10^8$
		Inadine	$>3 \times 10^8$
		Control (No WD)	9.6×10^8 ($\pm$3.9)
	72	CROWD Dark	$>3 \times 10^8$
		CROWD Light	$>3 \times 10^8$
		Inadine	$>3 \times 10^8$
		Control (No WD)	1.03×10^9 ($\pm$4.2)

5.4.3.2. Confocal Microscopy.

The confocal microscope images in Figures 5.8, 5.9 and 5.10 show the growth of bacterial cells that had not been exposed to an antimicrobial dressing at the three different time points 24, 48 and 72 hours. Confocal microscopy was used

to visualise the cells and determine the ratio of dead to live bacterial cells. The LIVE/DEAD™BacLight kit contains two stains; SYTO® 9 and Propidium Iodide (PI) which both stain nucleic acids. Green fluorescing SYTO® 9 is able to penetrate both live and dead cells. However red fluorescing PI has a greater affinity for nucleic acid but is only able to pass into cells with damaged membranes where it can displace SYTO 9 resulting in a red fluorescence as observed under the microscope. Green fluorescene produced by SYTO® 9 is only shown in live cells (Berney et al. 2007). The images in Figure 5.8. demonstrate the presence of adhered aggregated cells of *S. aureus* and *P. aeruginosa* but no *E. coli* cells after 24 hours.

Figure 5.8: Confocal Microscope BacLight images of untreated cells after 24 hours. (a) *S. aureus* (b) *E. coli* (c) *P. aeruginosa* (Mahmood 2018)

The number of cells in the 48 hour images (Figure 5.9) demonstrates a substantial increase in the number of live adhered bacterial cells, particularly of *E. coli* (b) and *P. aeruginosa* (c) compared with the 24 hour images (Figure 5.8)

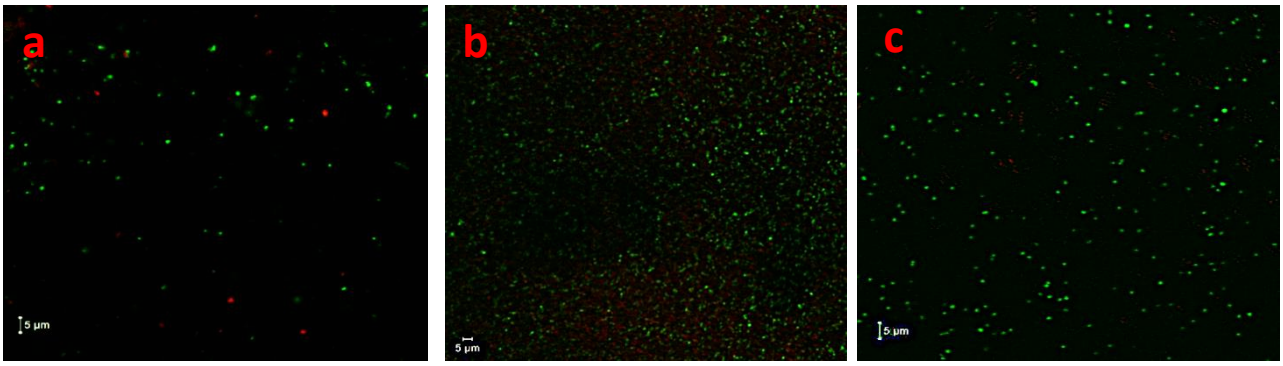


Figure 5.9: Confocal Microscope BacLight images of untreated cells after 48 hours. (a) *S. aureus* (b) *E. coli* (c) *P. aeruginosa* (Mahmood 2018)

Figure 5.10 illustrates the cell growth of the three bacteria after 72 hours. When compared with the 48 hour images (Figure 5.9) the amount of fluorescence had increased further indicating an even greater number of live bacterial cells of each species. The *P. aeruginosa* image (Figure 5.10 c) shows characteristics of a simple multicellular biofilm.

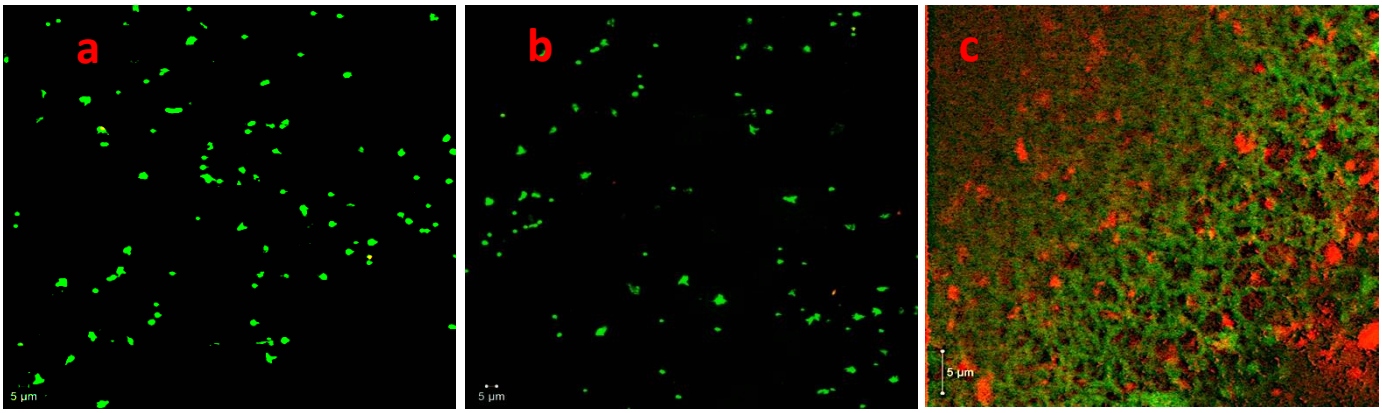


Figure 5.10: Confocal Microscope BacLight images of untreated cells after 72 hours. (a) *S. aureus* (b) *E. coli* (c) *P. aeruginosa* (Mahmood 2018)

Adhered cells are physiologically and metabolically different to their planktonic counterparts (Donlan 2002; Bischt and Wakeman 2019). The slow or non growing cells have adhered to the surface and are assumed to be acting as precusors for biofilm growth.

5.4.4. Discussion

The CROWD and Inadine dressings were able to effectively eradicate even the most mature biofilms containing *S. aureus* and *E. coli* but were ineffective against the *P. aeruginosa* biofilms. This would appear to follow the trends demonstrated in the planktonic studies which showed that the ZOI produced by both dressings against *P. aeruginosa* were usually smaller than those produced against the other two bacteria. These findings were similar to those reported by Halstead (2015) who found that Inadine slightly reduced biofilm formation of the clinical isolate *P. aeruginosa* (PS_1586) but this was not statistically significant, and it was ineffective against the control strain (ATCC_15692) originally isolated from an infected wound.

However, in a later study Povidone iodine ointment (10% PVP-I) was shown to be effective in four different concentrations: 3.3, 10, 33 and 100% against 48 hour mature biofilms of *P. aeruginosa* (NCIMB 10434). No viable cells were recovered after 4 and 24 hours treatment with PVP-I ointment at the different concentrations (Hoekstra, Westgate and Mueller 2017).

A number of *in vitro* studies have investigated the ability of different iodine containing dressings to disrupt and prevent the growth of bacterial biofilms. An early study by Akiyana et al. (2004) observed the activity of cadexomer iodine (Iodoflex) against *S. aureus* using confocal laser scanning microscopy, and found it was able to disrupt the biofilm structure enabling the bacteria to be killed by the iodine. In a later study Hill et al. (2010) created a mixed species biofilms containing six bacteria commonly found in wounds to test the activity of a range of iodine dressings. Analysis of the biofilm species composition revealed that *S. aureus* and *P. aeruginosa* predominated whilst other species were either not detected or on the borderline of detection. They found that PVP-I (1% w/v) solution demonstrated minimal efficacy against the 3 and 7 day biofilms. Prolonged PVP-I treatment caused a reduction in the *P. aeruginosa* and *S. aureus* counts of nearly 2-log fold but this effect was rapidly lost when PVP-I therapy stopped and both organisms returned to their pre-treatment levels. The authors also determined the MIC for both planktonically grown bacteria was 1% (w/v) so the biofilm results showed that this was not a high enough concentration to eradicate the bacteria in their biofilm form of growth. They also tested 1g Betadine cream (Povidone Iodine 1%) smeared on Topper Gauze on the biofilms and found it showed a slight reduction in *S. aureus* counts but no significant reduction in *P. aeruginosa*. However, when Inadine and Iodoflex were tested against the two bacteria no viable cells were found in either the 3 or 7 day biofilms after completion of the 7 day study. This study demonstrated that these dressings were effective against mature 7 day biofilms. Hill and colleagues (2010) proposed that the difference in anti-biofilm activity of the different iodine formulations could be due to the fact that the Inadine and Iodoflex form a gel excipient (polyethylene glycol and cadexomer) giving them a more prolonged efficacy.

The antimicrobial activity of Iodoflex and PVP-I (10% Solution – 1% available iodine) saturated gauze has also been tested on an *ex vivo* porcine skin explant biofilm model infected with *P. aeruginosa* (PAO1) (Phillips et al. 2015). A single application of PVP-I saturated gauze partially reduced biofilm CFUs whereas Iodoflex was more effective in reducing mature biofilms in 24 or 72 hours with a single exposure. The authors concluded that the ability to kill mature biofilms was influenced by the time of exposure, number of applications and the formulation of the antimicrobial. They deduced that the efficacy of Iodoflex was in part attributed to the "time" release of iodine from the cadexomer beads at a rate to maintain iodine availability at 1ppm.

The influence of the dressing formulations was also investigated in a study by Fitzgerald et al. (2017) which compared the efficacy of Inadine, Iodoflex and Iodosorb against *P. aeruginosa* (ATCC 9027) and *S. aureus* (ATCC 6538) 48 hour biofilms using colony and drip flow *in vitro* wound biofilm models. The Iodoflex and Iodosorb attained mean log reductions of > 9 following 24 hour treatment against both colony biofilms. In contrast Inadine had little/no effect against either biofilm and was not significantly different from the control. The authors considered that the efficacy of the cadexomer iodine dressings was probably due to the slow release of iodine. Iodine is known to kill planktonic cells in seconds (Koburger et al. 2010) however in this study, kill of the mature biofilms occurred over several hours when treated with the cadexomer iodine formulations. The authors concluded that the sustained/continuous release of iodine beyond the initial kill may be critical to continue to expose persister cells to the antimicrobial effects, potentially causing the death of these dormant but viable cells and so preventing biofilm reformation.

It would appear that there are a number of factors which may affect the efficacy of the antimicrobial dressings against biofilms such as the strain of the bacteria, the age of the biofilm and the duration of the application of the antimicrobial. The prolonged release of iodine demonstrated by the CROWD material may possibly enhance the efficacy in the same way that the cadexomer iodine had in the previous studies making it an interesting option for the treatment of biofilms. Further work is required to investigate the efficacy of the CROWD material against more complex biofilm models and against other iodine dressings.

5.5. Minimum Inhibitory Concentration (MIC) of the eluent from Iodine containing dressings

5.5.1. Introduction

Having compared the antimicrobial efficacy of the CROWD material to commercial iodine containing dressings it was important to investigate other differences between the materials. Minimum inhibitory concentrations (MICs) are considered the "gold standard" for determining the susceptibility of organisms to antimicrobials (Andrews 2001).

In this experiment the iodine dressings were tested in liquid cultures against *S. aureus* (SH1000), *E. coli* (JM109) and *P. aeruginosa* (H085180216) to compare their individual MICs.

5.5.2. Methods Summary

The dressings included in the experiment were Inadine®, Iodoflex®, CROWD and Povidone Iodine (PVP-I) Videne®10% aqueous solution. The iodine components of the dressings are listed below: -

Inadine® = 10% PVP-I Ointment (1% w/w available iodine)

Iodoflex ® = Iodine 0.9%w/w as cadexomer iodine

Povidone Iodine Aqueous Solution = 10% w/w (1% w/w available iodine)

CROWD 6wt% PVP-I made with the Povidone Iodine Aqueous Solution.

Iodozyme® was not suitable for testing by this method because the two layers of the gel need to remain in contact and so could not be submerged in MH broth.

The method used to determine the MIC of the eluent produced by the samples of the test dressings is described in Section 2.1.2.6.

5.5.3. Results

The minimum concentration of the eluent at which no bacterial growth was observed for each of the three test bacteria is shown in Table 5.6.

Table 5.6: Minimum Inhibitory Concentrations of eluent from iodine containing dressings.

Dressing	*S. aureus*	*E. coli*	*P. aeruginosa*
CROWD	25%	25%	25%
PVP-I	3%	6%	3%
Iodoflex	50%	100%	50%
Inadine	50%	100%	100%

5.5.4. Discussion

The CROWD material MIC for all three test bacteria was lower than the MIC for the two commercial dressings. Also, the MIC for the CROWD eluent was the same for all three bacteria tested.

However, the MIC for the CROWD eluent was higher than Povidone Iodine (PVP-I) aqueous solution which was the formulation used to create the CROWD composite. Interestingly the MIC for PVP-I aqueous solution was higher for *E. coli* (6%) than *S. aureus* and *P. aeruginosa* which were both 3%. In the *in vitro* growth inhibition studies the zones of inhibition of microbial growth produced by iodine dressings were consistently smaller against *P. aeruginosa* than those produced against *S. aureus* and *E. coli*. This would suggest that the antimicrobial effect is not just due to the concentration of the iodine but may also be due to factors associated with the bacteria.

Iodoflex followed a similar pattern to PVP-I aqueous solution in that the MIC of E. *coli* was higher than the other two bacteria. However, with Inadine the two Gram - negative bacteria; *E. coli* and *P. aeruginosa* both had an MIC of 100% but the MIC for the Gram - positive S. *aureus* was lower (50%).

Comparison of the three material antimicrobial dressings, Iodoflex, Inadine and CROWD is difficult as in clinical practice the release of iodine in wound exudate may be different to that demonstrated in MH Broth.

5.6. Toxicity of Iodine

5.6.1. Introduction

Reports of systemic effects following short term use of PVP-I are rare (Cooper 2007). In 1976 Pietsch and Meakins attributed two deaths in burns patients to topical use of PVP-I. There has also been one report of a patient who died ten hours after having had a continuous irrigation of Betadine (povidone – iodine solution) following surgical debridement of a hip wound (D'Auria et al. 1990).

In 1998 Burkes reported decreased renal function or renal failure in four patients aged between 50 and 83 years who all had pre-existing medical conditions. This occurred following either 10 hours continuous irrigation of their wounds with PVP-I or 17 days to 5 weeks of wound dressing with gauze soaked in PVP-I.

Concerns about systemic absorption of iodine through large open wounds which could lead to thyroid toxicity have also been reported. Nobukuni et al. (1997) suggested that long term use of PVP-I in 40 neurological patients had caused mild thyroid dysfunction.

There have also been concerns expressed about the potential adverse effects that iodine may have on wound healing. Numerous *in vitro* and *in vivo* studies have been undertaken which produced conflicting results regarding the bactericidal effects and cytotoxicity of povidone iodine. Kramer (1999) conducted an integrated review of clinical trials which compared PVP-I with other treatments for their impact on wound healing and concluded that PVP-I did not effectively promote wound healing and most studies showed either impaired wound healing, reduced wound strength or infection. However a later systematic review of 27 randomised clinical trials reported on chronic, acute, burn wounds, pressure ulcers and skin grafts (Vermeulen 2010) concluded that iodine did not lead to a reduction or prolongation of wound healing compared with other antiseptic wound dressings or agents. They also found that in individual trials iodine was significantly superior to other antiseptic agents.

Antibacterial activity and cytotoxicity are inherently in a trade-off relationship, therefore the behaviour of the topical antimicrobials on the wound surface need to be regulated to achieve sufficient antibacterial activity whilst simultaneously minimizing the cytotoxic effects (Yunoki et al. 2015). A hundred years ago Alexander Fleming (1919) (p127) stated that “antiseptics will only exercise a beneficial effect in a septic wound if they possess the property of stimulating or conserving the natural defence mechanism of the body against infection”. He advocated that when estimating the value of the antiseptic it is more important to study its effects on tissues than any effects on bacteria. A number of *in vitro* cytotoxicity studies have been conducted to investigate the potential detrimental effects of PVP-I on wound healing typically on fibroblasts and keratinocytes (Bigliardi et al. 2017).

Van Den Broek et al. (1982) demonstrated that PVP-I solutions at concentrations greater than 0.05% were toxic to granulocytes and only when the concentration was reduced to 0.005% were the viability and function of monocytes shown to be normal. Tatnall et al. (1987) found that concentrations greater than 0.004% were 100% toxic to keratinocytes and Balin and Pratt (2002) demonstrated that fibroblast growth was totally inhibited by 0.1% PVP-I solutions. However, an *in vitro* study by Akiyama et al. (2004) found that cadexomer iodine was not toxic to fibroblasts in culture. Zhou et al. (2002) investigated the effects of varying concentrations of cadexomer iodine on the viability of human fibroblasts in culture. At concentrations of up to 0.45% cadexomer iodine did not affect the cell viability of human cultured fibroblasts. They found that decreased fibroblast viability with concentrations greater than 0.45% of cadexomer iodine was dose dependent.

In 2003 Mertz et al. concluded that although many studies demonstrated deleterious effects of iodine on skin cells *in vitro* and *in vivo* this may be because the studies did not differentiate between the active agent iodine and the carrier vehicle. Many formulations contain detergents which are known to retard wound healing. They considered that another important factor influencing cytotoxicity was the rate the iodine was delivered to the wound. They concluded that preparations which release small amounts of iodine over long periods of

time, so reducing the iodine concentration at the wound bed, would be less toxic to cells.

This view was also the conclusion of a consensus meeting organized by the European Tissue Repair Society which deduced that slow-release formulations that generate low concentrations of iodine were effective and non-toxic (Gilchrist 1997). Usually the administration of low dose antimicrobials is not advisable as this may produce a powerful selection pressure for the emergence of resistance. However as there have been no reports of iodine resistance in over 150 years of clinical use then in this case the risk is extremely low (Lachapelle et al. 2103).

To investigate this phenomenon a series of *in vitro* experiments were developed to compare the cytotoxicity of the slow-release CROWD dressing with the commercially available iodine antimicrobial wound dressings Iodoflex, Inadine and Iodozyme.

5.7. Indirect cytotoxicity testing of CROWD and commercial iodine dressings.

5.7.1. Introduction

Funding for this work was obtained from an MRC Confidence in Concept Scheme Round 4 grant. Testing was undertaken by Dr Patrick Harrison, Post Doctoral researcher in the Biomolecular Sciences Research Centre, Sheffield Hallam University following protocols developed by Louise Freeman-Parry. The test methodology was adapted from an indirect cytotoxicity experiment described by Yunoki et al. (2015) which was based on ISO 10993-5:2009 (Biological evaluation of medical devices – Part 5: Tests for *in vitro* cytotoxicity).

5.7.2. Methods Summary

The cytotoxicity of 3.5g samples of Iodoflex®, Inadine® and the CROWD material were tested against HaCat keratinocytes and fibroblasts using the method described in Section 2.2.2.2

Povidone Iodine (PVP-I) 10% aqueous solution was used as a positive control.

5.7.3. Results

Iodozyme was not able to be tested by this method because it absorbed the Dulbeccos's Modified Eagles Medium (DMEM), swelled and turned solid in the test tube. The three other dressings produced eluents which were diluted. Three fluorescence readings were taken for each of the 8 eluent dilutions (100% eluent, 80%, 60%, 40%, 20%, 10%, 1% and 0% (100% DMEM). The mean and standard deviation were calculated for the three readings. This mean was then calculated as a percentage of the mean of the three readings for the 0% eluent (100% DMEM) control to produce an estimate of the % cell viability. The eight results were then entered into Prism Graph Pad and the EC_{50} of the eluent was calculated. The Prism Graph Pad fits the data to a sigmoidal dose response model and extrapolates the data to the maximum point and then calculates the 50% midpoint of the curve to determine the concentration at which 50% of the cells were dead (EC_{50}).

5.7.3.1. Results - Indirect cytotoxicity of the Standard CROWD 6wt% Iodine composite compared with commercial iodine Dressings.

The effect of the eluent produced by Inadine and Iodoflex compared to the standard CROWD formulation (PVOH/PVP-I 6wt%/Cloisite®Na^+ 12.5wt %) on the viability of HaCat keratinocytes is shown in Figure 5.11. 100% cell viability was achieved with the 20% eluent from the Inadine dressing and the 10% eluents from the Iodoflex and CROWD material. This suggests that the Inadine dressing is less cytotoxic to the keratinocytes than the Iodoflex and CROWD.

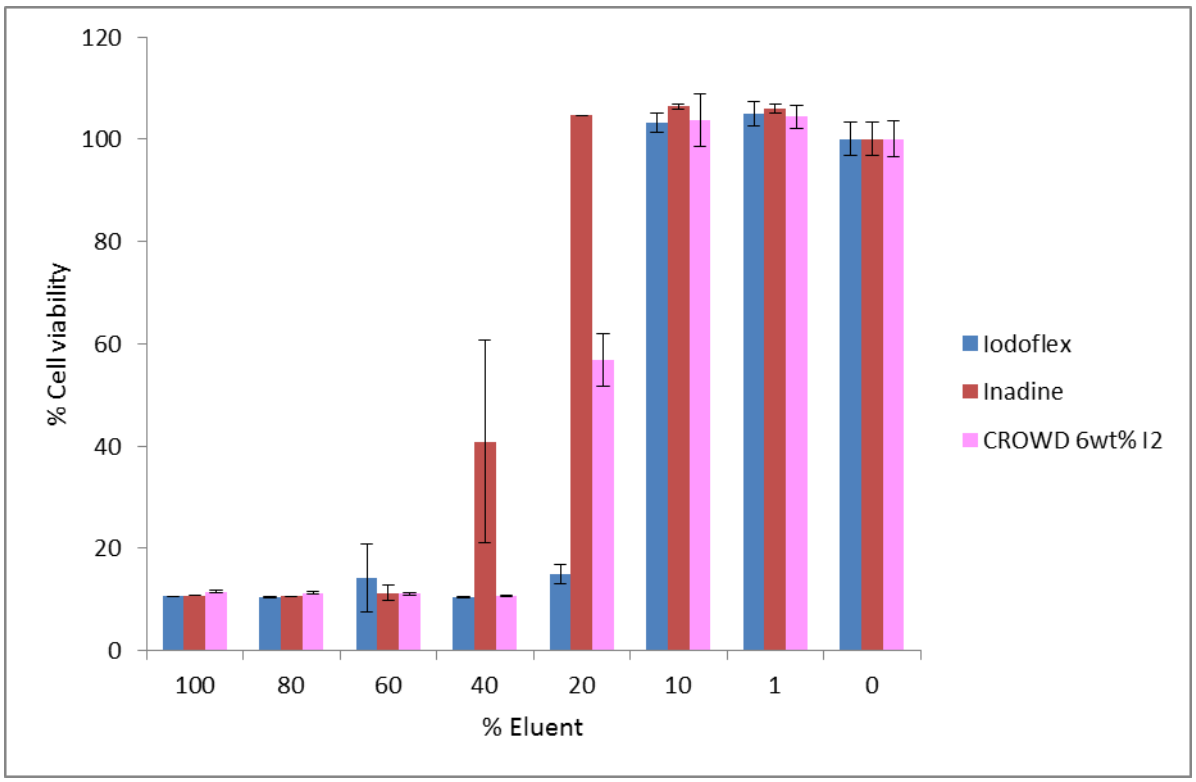


Figure 5.11: The effect of iodine dressings on the viability of HaCat keratinocytes (Error bar = Standard Deviation, n = 3)

The EC_{50} calculated for CROWD and the two commercial dressings, Iodoflex and Inadine are listed in Table 5.7.

The results show that the CROWD dressing eluent had a higher EC_{50} indicating a lower level of indirect toxicity than Iodoflex. However, CROWD had a lower EC_{50} than Inadine indicating it has a higher level of toxicity than Inadine.

Table 5.7: EC_{50} of Standard CROWD (PVOH/PVP-I 6wt%/Cloisite®Na^+) composite compared with commercial iodine dressings

Iodine Dressings	Cell Type	EC_{50} (% Eluent)	Standard Error
CROWD 6wt% (I_2) Cloisite®Na^+	Keratinocytes	20	0.3374
CROWD 6wt% (I_2) Cloisite®Na^+	Fibroblasts	18.57	0.02098
Inadine	Keratinocytes	37.53	0.04554
Inadine	Fibroblasts	24.21	0.03149
Iodoflex	Keratinocytes	16.86	3.305
Iodoflex	Fibroblasts	8.024	0.0509

In an attempt to reduce the cytotoxicity of the standard CROWD material which contained 6wt% iodine, three further formulations containing lower percentages of iodine; 1.5wt%, 3wt% and one composite without iodine were prepared and then tested using the indirect cytotoxicity assay. The method used to prepare the different strength iodine composites is described in Section 2.3.2.4.

5.7.3.2. Results - Indirect cytotoxicity of CROWD composites containing 6wt%, 3wt%, 1.5wt% iodine and no iodine.

When comparing the CROWD Cloiste®Na^+ formulations containing different iodine concentrations (Figure 5.12) 100% cell viability was reached by the 10% eluent obtained from the 6wt% and 1.5wt% iodine formulations and by the 1% eluent obtained from the 3wt% composite. This would suggest that reducing the iodine concentration in the Cloisite®Na^+ formulation did not markedly affect the cytotoxicity. Interestingly, the formulation containing no iodine did not produce 100% cell viability at all eluent dilutions suggesting that another element in the formulation was adding to the cytotoxic effect.

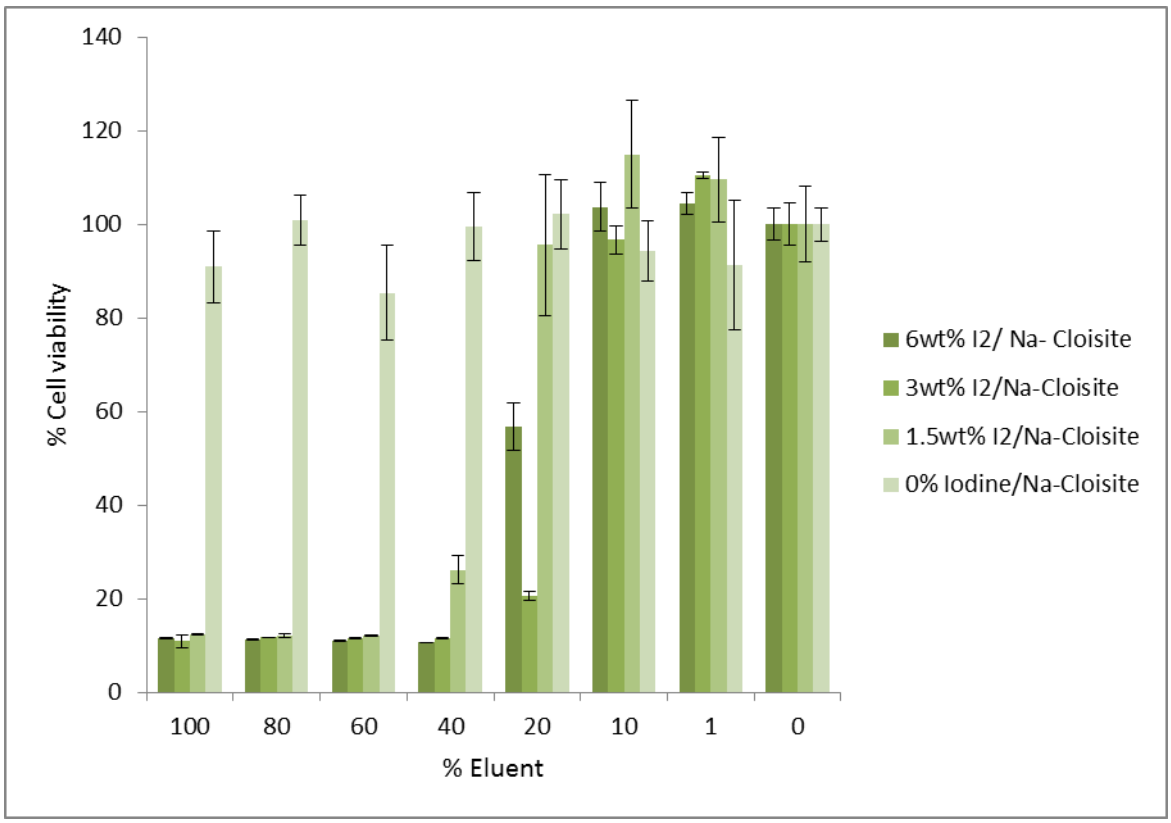


Figure 5.12: The effect of four different CROWD (PVOH/Cloisite®Na^+) composites containing different iodine concentrations 1.5wt%, 3wt% and 6wt% and no iodine on the viability of HaCat keratinocytes. (Error bar = Standard Deviation, n = 3)

The EC_{50} results from the cytotoxicity testing of the lower percentage iodine CROWD formulations produced similar cytotoxicity profiles to the standard 6wt% PVP-I CROWD dressing (Table 5.8). This would also suggest that there was another component of the dressing, not just the iodine, which was contributing to the cytotoxic effect on the test cells. However, the EC_{50} of the eluent from the material containing no iodine showed no inhibition of cell viability suggesting that the composite materials PVOH/PVP/Cloisite were not cytotoxic to the keratinocytes. This result does not concur with the graphical results illustrated in figure 5.12 that showed 100% cell viability was not achieved at all eluent concentrations for the composite containing no iodine. This discrepancy is likely to be representative of limitations in the line fitting model of Prism when determining EC_{50} values and that the overall level of cytotoxicity of Cloisite is low but measurable.

Table 5.8: EC_{50} of CROWD (Cloisite®Na^+) composites containing different Iodine concentrations

CROWD Formulation	Cell Type	EC_{50} (% Eluent)	Standard Error
CROWD 6wt% (I_2) Cloisite®Na^+	Keratinocytes	20	0.3374
CROWD 3wt% (I_2) Cloisite®Na^+	Keratinocytes	14.24	0.02558
CROWD 1.5wt% (I_2) Cloisite®Na^+	Keratinocytes	28.18	0.03344
CROWD 0% (I_2) Cloisite®Na^+	Keratinocytes	NC	-

(NC = Not Calculatable because of no Inhibition)

The next series of experiments were designed to investigate the indirect cytotoxicity of the individual components of the CROWD material.

5.7.3.3. Results - Indirect cytotoxicity of the individual components of the preferred CROWD Formulation.

The individual components of the dressing; PVOH, PVP and PVP-I were tested. Unfortunately it was not possible to test the Cloisite®Na^+ powder because when it was added to DMEM it swelled to form a solid gel.

The results in Table 5.9. show that the PVOH and PVP eluents produced no inhibition so were not considered to be cytotoxic against either the keratinocytes or fibroblasts. However, the povidone iodine eluent produced EC_{50} values against both cell types which were higher than the three iodine containing CROWD materials which would suggest it was less cytotoxic in its original liquid formulation than when it had been added to the polymer composite.

Table 5.9: EC_{50} of the eluent produced by the individual CROWD components

Material	Cell Type	EC_{50} (% Eluent)	Standard Error
PVOH	Keratinocytes	NC	-
PVOH	Fibroblasts	NC	-
PVP	Keratinocytes	NC	-
PVP	Fibroblasts	NC	-
PVP-I	Keratinocytes	35.21	19.8
PVP-I	Fibroblasts	36.77	5.14

(NC = Not Calculatable due to no Inhibition)

To try to reduce the cytotoxicity of the CROWD material it was decided to replace the Cloisite®Na^+ clay with the synthetic clay, Laponite®RD. Two CROWD materials were created containing Laponite with 0wt% and 6wt% Iodine using the method described in Section 2.3.2.4

5.7.3.4. Results – Indirect Cytotoxicity of Composites containing Laponite®RD instead of Cloisite®Na^+.

In the Laponite study (Figure 5.13) the eluent from the Laponite formulation containing no iodine produced a cell viability which fluctuated between 88% and 100%. The Laponite formulation containing 6 wt% iodine produced 61% cell viability with the 40% eluent this increased to 82% viability with the 20% eluent and reached 100% cell viability with the 10% eluent dilution.

Compared to this the 6wt% Cloisite®Na^+ eluent produced a much lower cell viability of 11% with the 40% eluent, 57% cell viability with the 20% eluent and only when the eluent had been diluted to 10% there was 100% cell viability.

This would suggest that the Laponite®RD clay composite was less cytotoxic than the Cloisite®Na^+. The EC_{50} of the Laponite eluent (Table 5.10) would appear to confirm this as values for Laponite were higher than those produced by the 6wt% Cloisite®Na^+ against both cell types (Table 5.7).

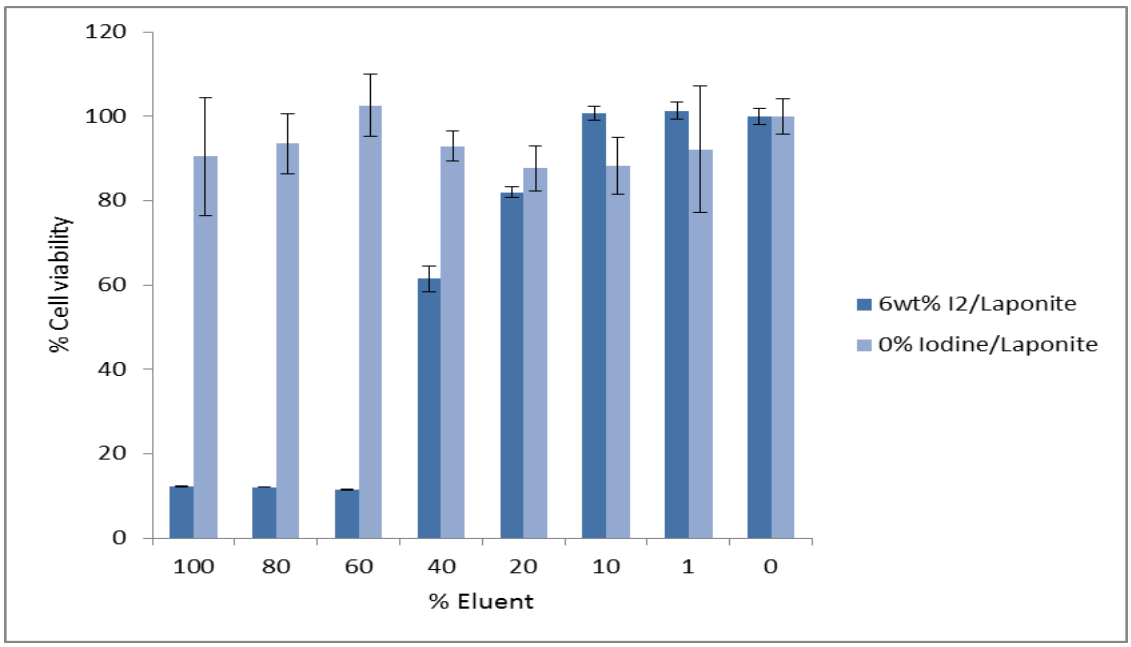


Figure 5.13: The effect of different Laponite®RD composites containing either 6wt% iodine or no iodine on the viability of HaCat keratinocytes. (Error bar = Standard Deviation, n = 3)

Table 5.10: EC_{50} of the eluent produced by the Laponite®RD composites

Laponite Formulations	Cell Type	EC_{50} (% Eluent)	Standard Error
CROWD 6 wt% (I_2) Laponite	Keratinocytes	40.55	1.827
CROWD 6 wt% (I_2) Laponite	Fibroblasts	41.17	0.2039
CROWD 0% (I_2)Laponite	Keratinocytes	NC	-
CROWD 0% (I_2)Laponite	Fibroblasts	NC	-

(NC = Not calculatable due to no Inhibition)

5.7.4. Discussion

These results indicate that whilst the PVP-I appeared to contribute to the cytotoxicity, removing the iodine from the material did not totally eliminate the problem. It also suggested that another component of the composite was contributing to the cytotoxic effects. The two polymers in the composite, PVOH and PVP did not demonstrate cytotoxicity activity against the test cells. This led to the suspicion that the clay, Cloisite®Na^+ may be contributing to the cytotoxicity. Unfortunately the cytotoxicity of the Cloisite®Na^+ powder could not be tested by this method. So to test the theory that the clay was contributing to the cytotoxicity of the material it was decided to replace the natural clay,

Cloisite®Na^+ with the synthetic clay, Laponite®RD to determine if a composite containing this clay would be less cytotoxic than the standard CROWD formulation. The results from the EC_{50} experiments showed that the EC_{50} value for the eluent from the 6wt% Laponite composite was double that of the 6wt% Cloisite composite suggesting it was less cytotoxic. This made the Laponite®RD clay composite a possible alternative formulation.

5. 8. Direct Cytotoxicity Testing using a Tissue Engineering (TE) model.

5.8.1. Introduction

The indirect method of measuring cell toxicity is limited by the two dimensional cell culture which does not reflect the complex environment at the wound/skin surface. The next series of experiments were designed to test the cytotoxicity of the dressings involved the use of a three dimensional tissue engineered (TE) model of human skin developed by Dr Joanna Shepherd at the University of Sheffield. (Shepherd et al. 2009)

Cytotoxicity testing was undertaken by Dr Patrick Harrison according to protocols developed by Louise Freeman-Parry listed in Section 2.2.2.4.

5.8.2. Methods Summary

The TE skin model was prepared using the methodology described in Section 2.2.2.3.

The Dressings tested were: -

Inadine®

Iodoflex®

CROWD 6wt % Iodine with Cloisite®Na^+ (Standard CROWD Formulation)

CROWD 0% Iodine with Cloisite®Na^+ (Control)

CROWD 6wt % Iodine with Laponite®RD

CROWD 0% Iodine with Laponite®RD

The PVP-I dressings were cut using a cork borer into 9mm diameter discs. The samples were then sterilised by UV irradiation for 15 minutes on both sides. The dressing discs were placed on top of the TE model in the holders and incubated at 37^{o}C for 24 hours.

After 24 hours the discs were removed, and the TE model was washed with sterile PBS 1% to remove excess iodine. The TE was then incubated with Alamar blue for 2 hours. After 2 hours incubation the fluorescence was read on a plate reader and the results compared with a positive control (DMEM + Alamar Blue + cells but no PVP-I disc) and a negative iodine control (DMEM + Alamar Blue + PVP-I disc but no cells).

5.8.3. Results

The % reduction in tissue viability produced by the commercial iodine dressings and different CROWD formulations is shown in Figure 5.14.

Inadine produced a 42% reduction in tissue viability in the TE model following 24 hours of exposure to the dressing material but still had 49% viable tissue after 72 hours. In contrast, 24 hours exposure of the TE model to Iodoflex®, 6wt% Cloisite®Na^{+} and the 6wt% Laponite®RD CROWD materials resulted in 1%, 0% and 2% tissue viability respectively. Both the Cloisite®Na^{+} and Laponite®RD CROWD composites which contained no iodine (0wt% PVP-I) were less cytotoxic than their corresponding 6wt% iodine composites.

After 24 hours the 0wt% PVP-I/Laponite®RD samples had reduced the tissue viability by only 2% compared with a reduction of 27% produced by the 0wt% PVP-I/Cloisite®Na^{+} formulation. After 48 hours the 0wt%PVP-I/Laponite®RD percentage tissue viability was 47% compared with 29% for the 0wt% PVP-I/Cloisite®Na^{+} composite. However, after 72 hours the % tissue viability with the Cloisite®Na^{+} had only reduced a further 2% to 27% whereas the Laponite®RD % tissue viability had decreased to 14%.

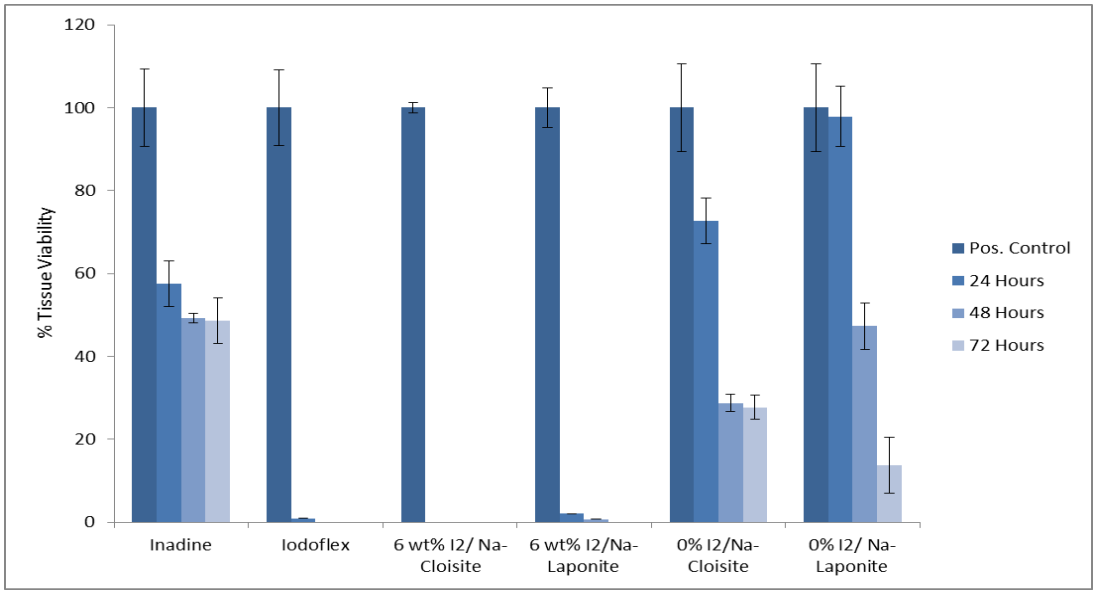


Figure 5.14: The effect of Commercial Iodine dressings compared with different CROWD formulations, with and without Iodine, on the tissue viability of a Tissue Engineered Skin (TE) Model (Error bar = Standard Deviation, n = 9)

Key: Positive Control = DMEM + Alamar Blue + Cells But no Iodine Dressing

5.8.4. Discussion

Inadine appeared to be the least cytotoxic of all the iodine containing antimicrobial materials tested. However, this may be associated with the lower concentration of iodine in the dressings which would explain the smaller ZOI it produced and the shorter duration of antimicrobial activity compared to Iodoflex and CROWD (Section 5.2.3).

Interestingly the cytotoxic effects demonstrated by Iodoflex in the TE model were similar to those shown by the two CROWD formulations containing 6wt% PVP-I.

From these experiments it can be concluded that removing the iodine from the Cloisite®Na^+ and Laponite®RD formulations did not eliminate the cytotoxic effects of the material. It would also appear that the Laponite®RD was marginally less cytotoxic than Cloisite®Na^+ clay composite.

5.9 Conclusions

The passage studies testing commercial dressings in Chapter 3 showed that most of the dressings were effective against *S. aureus*, *E. coli* and *P. aeruginosa* for either 24 or 48 hours. Only the silver dressings demonstrated a prolonged duration of action greater than 48 hours against all three test bacteria (Section 3.4). In this chapter the results of passage studies of both sides of the CROWD material and the commercial iodine dressings were compared. The experimental results show that both sides of the CROWD material were effective against the three test bacteria and two strains of MRSA for a minimum of 72 hours whereas none of the commercial iodine dressings produced a ZOI for longer than 48 hours (Section 5.2.4). The two different sides of the material demonstrated considerably longer durations of action against either the Gram - positive or Gram - negative bacteria. The dark side of the CROWD material produced ZOI against *S. aureus* for 8 days (Sections 4.7.4. and 5.2.3.3.) and MRSA for 5 days (Sections 4.8.3, 5.2.3.4 and 5.2.3.5). However, the Light side of the CROWD material produced ZOI > 400mm^2 against *E. coli* and *P. aeruginosa* for 7 days (Sections 4.7.4, 5.2.3.1 and 5.2.3.2).

The statistical analysis comparing CROWD to all the commercial dressings, used a three way ANOVA and showed that the difference between the three independent variables; Dressing, Day, Bacteria was statistically significant with a value of $p < 0.0005$ as were all the two way and three way interactions. The predicted model used to compare the estimated marginal means for the ZOI for each dressing on the 8 days against the 5 different bacteria clearly illustrated that the CROWD material had a longer duration of antimicrobial action against all the test bacteria than any of the other commercial dressings. The only exceptions were the two silver foam dressings; Allevyn Ag and Biatian Ag which were effective against *P. aeruginosa* for 7 days, the same duration as the CROWD light side. The three post hoc tests identified that the dark side of the CROWD material was in a subset on its own indicating that it was very different to all the other dressings tested.

These *in vitro* studies show, with statistical confirmation, that the CROWD material outperforms the commercial iodine dressings in terms of the duration of antimicrobial activity.

Having confirmed the extended duration of action of the CROWD material it was important to investigate the potential cytotoxicity of the CROWD material compared the commercial iodine dressings that have been used in clinical practice on open wounds.

The indirect cytotoxicity experiments showed that 100% keratinocyte cell viability was attained at 20% eluent for Inadine and 10% eluent for CROWD and Iodoflex indicating that Inadine was less cytotoxic than the other two dressings against this cell line.

Attempts to reduce the cytotoxicity of the CROWD material by decreasing the iodine content did not greatly reduce the cytotoxicity and even removing the iodine from the formulation did not eliminate the toxicity indicating that another component of the material was contributing to the cytotoxicity. Two of the other components of the composite, PVOH or PVP, were then tested separately and did not demonstrate any indirect toxicity which suggested that the clay component, Cloisite®Na^+, was responsible for the cytotoxicity.

The EC_{50} of PVP-I against both cell lines were higher than the EC_{50} of the iodine containing CROWD formulations which would suggest that the PVP-I was less toxic in its original liquid state than when it had been incorporated into the polymer composite. In the 10% povidone-iodine solution the iodine is complexed with polyvinyl pyrrolidone (povidone) and yields 1% available iodine (Burkes 1998). However, the relationship between povidone iodine and free iodine is not linear, as it forms a bell shaped curve, which peaks at 0.7%. Higher concentrations of povidone iodine can paradoxically bind more free iodine to the carrier molecule thereby lowering the available free iodine (Zamora 1986). What is not yet known is the effect on the PVP-I complex of mixing it with the other components in the CROWD formulation, this requires further investigation.

Cytotoxicity of PVP-I solutions have been investigated *in vitro* with differing results. Lineweaver et al. (1985) reported that 15 minute exposure to full strength 10% PVP-I killed 100% of exposed cultures of fibroblasts however they

considered 0.05% a safe concentration of PVP-I when tested against fibroblasts. In a later study, McKenna et al. (1991) reported that fibroblast and leucocyte function could only be maintained if povidone iodine was diluted to 0.001% at which concentration its antimicrobial activity was reduced.

Hirsch et al. (2009) compared the cytotoxicity of Betaisodona (10g povidone-iodine/100ml aqueous solution and Braunol (7.5g povidone iodine/100g in aqueous solution) against primary human keratinocytes, primary human fibroblasts and human keratinocyte cell line HaCat. Against the HaCat cell line the maximal toxicity of Betaisodona was at 0.7% and Braunol 4%. The pattern was similar with primary human keratinocytes with maximal toxicity of Betaisodona 4.9% and Braunol 9.3%. Fibroblast viability was also significantly reduced compared with controls for both solutions producing maximal cell cytotoxicity at 7.5% for Betaisodona and 12.5% for Braunol.

Having removed the potentially cytotoxic PVP-I from the CROWD material and demonstrated that PVOH and PVP were not cytotoxic the formulation still exhibited cytotoxicity. This led to the decision to try replacing Cloisite®Na^+ with the synthetic sodium clay Laponite®RD, to determine if this would reduce the cytotoxicity of the formulation. Indirect cytotoxicity testing demonstrated that the 6wt% PVP-I/Laponite®RD formulation produced an EC_{50} value twice the value of the EC_{50} from the eluent of the 6wt% PVP-I/ Cloisite®Na+ formulation so Laponite®RD appeared to be less cytotoxic. Laponite®RD is a highly purified synthetic, layered silicate clay (ByK Additives and Instruments) so is less likely to contain impurities than Cloisite®Na^+ which is a natural montmorillonite, organic intercalated nanoclay (ByK Atlanta Group).

Cells in monolayer culture are in isolation and as a result these studies do not encompass the intricate interactions that occur *in vivo* (Shepherd et al. 2009). The tissue engineered skin has similar properties and is histologically similar to human skin so provides a more realistic model for cytotoxicity testing. In the direct cytotoxicity test on the TE model after 24 hours exposure to both the 6wt% PVP-I Laponite®RD and Cloisite®Na^+ formulations there was less than 2 % tissue viability. As discussed Iodoflex produced similar results, after 24 hours it had reduced the tissue viability to 1%. The CROWD formulations containing no iodine produced different effects on the TE model with the Laponite®RD

sample resulting in 98% tissue viability compared to 73% for the Cloisite®Na^+ after 24 hours. This apparent reduction in cytotoxicity with the Laponite®RD led to the further testing of the antimicrobial activity of the Laponite formulations which is described in Chapter 6.

Fumal et al. (2002) reported that fibroblast function in chronic wounds was unaffected by treatment with PVP-I and interestingly Bennet et al. (2001) observed that fibroblast proliferation was increased with use of 10% PVP-I. More recent cytotoxicity tests have shown that povidone iodine had very low cytotoxicity compared with other antiseptics e.g., PHMB and Chlorhexidine when tested on skin (Van Meurs et al. 2014).

Cytotoxicity data from tests performed on isolated cells must be considered in perspective. *In vitro* cytotoxicity can be more pronounced than in a biological system with a three - dimensional matrix and vascular system and is not necessarily reflective of an *in vivo* or clinical setting (Leaper and Durani 2008). In spite of the general toxicity and the toxic effects of PVP-I on human cells even at low concentrations and brief exposure periods its use is advocated by many who claim that it is non-injurious to human tissue and actually promotes or is not detrimental to wound healing (Balin and Pratt 2002).

The CROWD materials produced similar *in vitro* cytotoxicity results to Iodoflex which has been widely used in clinical practice since it was introduced in 1981. It is hoped that the slow release of iodine from CROWD material would reduce the concentration of iodine at the wound surface minimising the cytotoxic effects. This would need to be tested on an animal model or in a clinical evaluation on human wounds.

Chapter 6: Commercialisation of the CROWD material

6.1. Introduction

Having compared the antimicrobial activity and cytotoxicity of the CROWD materials to a range of commercial antimicrobial wound dressings the next phase of the development process was to optimise the formulation and create a wound dressing.

The original CROWD samples were prepared in the laboratory in small circular 9cm^2 Petri dishes and cut into 2 x 2cm square samples. The next challenge was to transform the composite into a wound dressing which would be suitable to undergo clinical evaluation. In clinical practice much larger sheets would be required, most wound dressings are at least 10 x 10cm, so it was decided to try to create a uniform dressing measuring at least 20 x 20cm. All wound dressings applied to broken skin are required to be sterile so another challenge was to determine a suitable method of sterilisation. As the CROWD material is highly absorbent it would be unlikely to adhere to the wound surface so would require a secondary dressing to keep it in place. The choice of secondary dressing could impact on the release mechanism of the material so the impact of any cover material also needed to be assessed.

Before scale up of the manufacturing process, possible modifications to the formulation were considered. The results from the cytotoxicity experiments (Section 5.7.3.4) indicated that the composite containing the synthetic clay, Laponite®RD, were less cytotoxic than the composites containing Cloisite®Na$^+$. So an obvious consideration was to change the clay to Laponite®RD, however it was important to investigate whether this modification to the formulation would impact on the antimicrobial efficacy of the material. The first experiments were designed to determine the antimicrobial activity of a series of Laponite formulations against the five bacteria used in the previous passage studies.

6.2. Antimicrobial activity of different Laponite®RD Formulations

6.2.1. Introduction

Three Laponite composites were prepared containing a fixed amount of 12.5wt% Laponite®RD but with different concentrations of 3 and 6wt% Iodine and one containing no iodine. The method of preparation is described in Section 2.3.2.4. The lower strength iodine composite was prepared to determine if reducing the iodine concentration would impact on the antimicrobial efficacy. The 0wt% iodine sample had been prepared for the cytotoxicity testing so was used as a control.

6.2.2. Methods Summary

Six 2 x 2cm samples of the dark side of each composite were tested using passage studies described in Section 2.1.2.3. against *Staphylococcus aureus* (SH1000), *Escherichia coli* (JM109), *Pseudomonas aeruginosa* (H085180216), *Staphylococcus aureus* USA300 and Epidemic methicillin-resistant *Staphylococcus aureus* EMRSA-16. The passage experiments were undertaken for five days.

6.2.3. Results

The formulation containing Laponite®RD with no iodine did not demonstrate any antimicrobial activity against any of the test bacteria. This was a significant finding as it confirms that the Laponite does not contribute to the antimicrobial properties of the material.

Both the iodine containing Laponite®RD formulations produced clear ZOI against the five test bacteria (Figures 6.1. and 6.2).

The 3wt% iodine containing Laponite®RD samples produced ZOI against *S. aureus* for the 5 day duration of the study (Figure 6.1). However, they were only effective against the two MRSA strains for 4 days. The Laponite®RD samples produced much smaller ZOI against the *P. aeruginosa* and *E. coli* and only produced ZOI for three days.

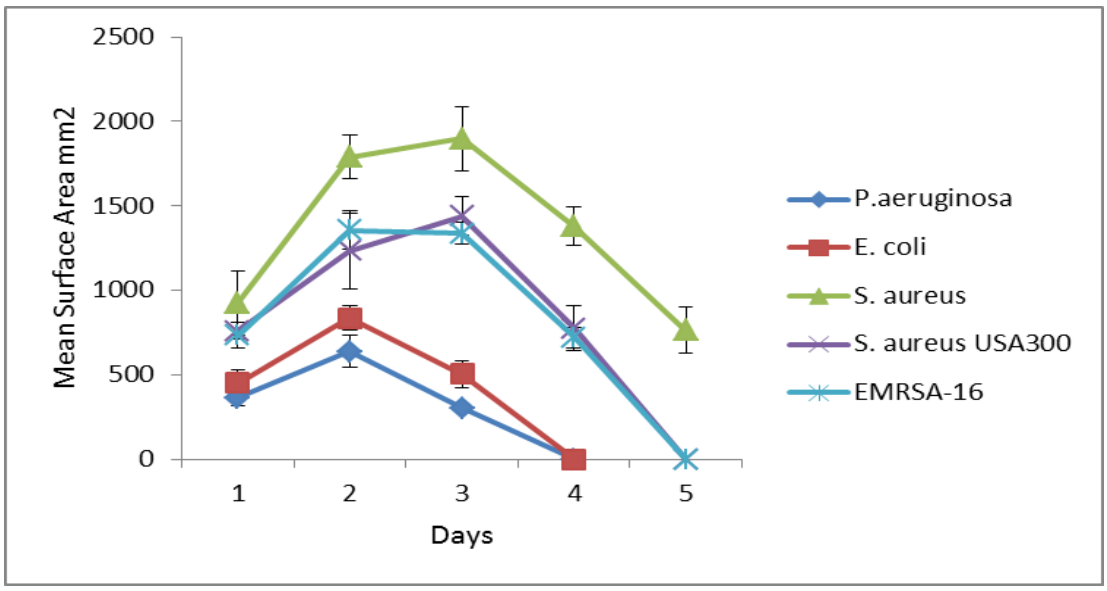


Figure 6.1: ZOI produced by the PVOH/PVP-I 3wt%/ Laponite®RD 12.5wt% composite against five different bacteria. (Error bar = Standard Deviation, n = 6)

The ZOI produced by the Laponite®RD samples containing 6wt% iodine were larger than those produced by the samples containing 3wt% iodine (Figure 6.2). As in the 3wt% experiment, the 6wt% iodine samples had stopped producing a ZOI against *P. aeruginosa* by day 4. However, the duration of action of the 6wt% samples against *E. coli* was 4 days compared with 3 days of activity observed with the 3wt% samples. Another difference between the two concentrations was that the 6wt% samples continued to produce clear ZOI against the three Gram- positive bacteria for the 5 day duration of the study.

Interestingly the community acquired, *S. aureus* USA300, appeared to be more susceptible to the 6wt% samples as the ZOI produced after 48 hours were the largest against this strain and twice the size produced by the 3wt% iodine samples.

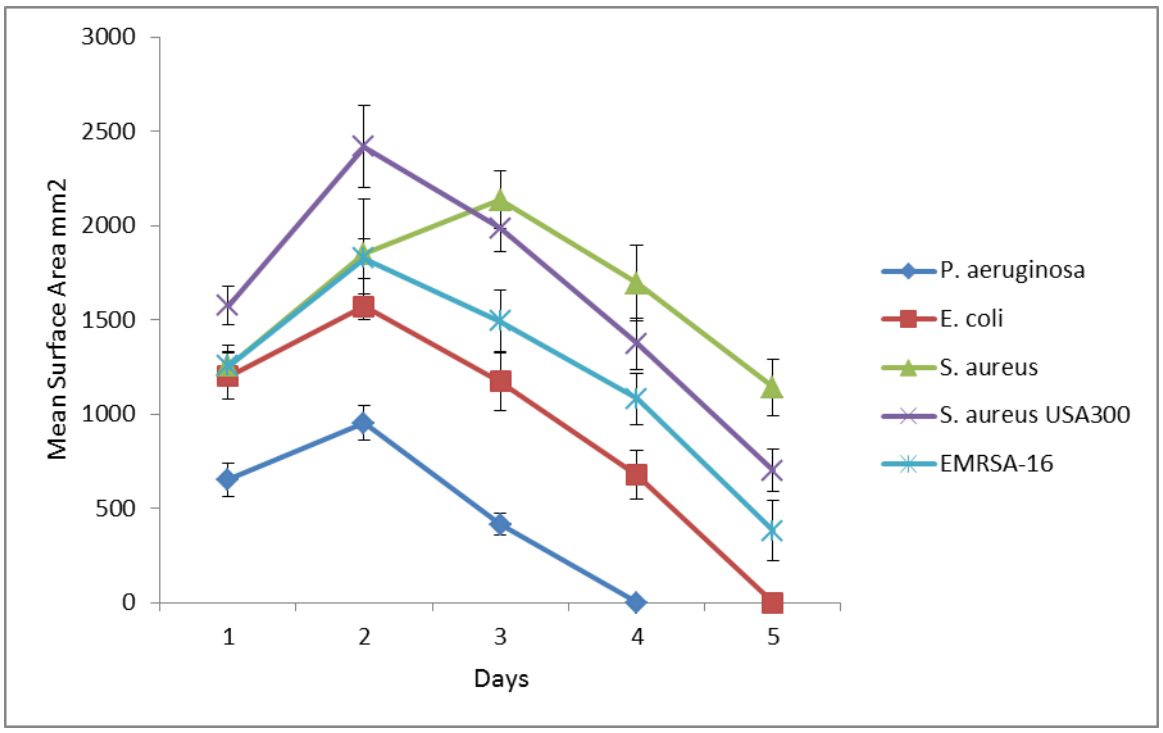


Figure 6.2: ZOI produced by the PVOH/PVP-I 6wt%/Laponite 12.5wt% Composite against five different bacteria. (Error bar = Standard Deviation, n = 6)

6.2.4. Discussion

When these results were compared with the results produced by the dark side of the standard 6wt% PVP-I/Cloisite®Na^+(12.5wt%) composite the 6wt% PVP-I/Laponite®RD (12.5wt%) composite had the same duration of action against the two Gram - negative bacteria; *E. coli* (4 days) and *P. aeruginosa (3 days).* After 5 days the 6wt% PVP-I/ Laponite®RD composite was still producing large ZOI against the three Gram - positive bacteria*. S. aureus, S. aureus* USA300 and EMRSA-16.

This experiment was terminated after 5 days so it is not known whether the Laponite®RD sample would match the 8 day duration of action against *S. aureus* demonstrated by the 6wt% PVP-I/Cloisite®Na^+ samples. However, the Laponite®RD samples were still producing a very large ZOI after 5 days which suggests they could have remained active if tested for longer.

The results from these experiments suggest that changing the form of the clay did not adversely affect the antimicrobial activity of the composite. The

antimicrobial activity of the 6wt% PVP-I/ Laponite®RD sample was not dissimilar to the 6wt% PVP-I/ Cloisite®Na^+ so in light of its reduced cytotoxicity could have been a suitable alternative antimicrobial material.

However, there was a problem with the formulation, after 24 hours on the agar the material split (delaminated) into two halves along the boundary between the two layers. This meant that during the passage studies care had to be taken when transferring the material to a fresh agar plate that the two layers were accurately positioned on top of each other. The layers could easily be displaced during the experiments and the lack of coherence between the layers may have affected the flow of iodine through the layers. This problem limited the duration of the study. By the 5^{th} day it had become impossible to keep the two layers correctly aligned.

It is unclear why the Laponite samples split. One of the differences between the two clays is the diameter of the platelets, the Cloisite®Na^+ platelets are between 200-500nm diameter whereas the Laponite®RD platelets are much smaller, approximately 20nm diameter. The self-stratifying layers are believed to occur because the clay settles as the dressing dries. The demarcation into two layers was more distinct in the Cloisite®Na^+ composites compared with the Laponite®RD composites. The coherent nature of the PVOH and PVP-I composite is thought to be reliant on the clay which helps to compatibilise them. There are a number of factors which may have contributed to the failure of the Laponite®RD composite to retain its form. It could be due the fact that the platelets are smaller and possibly settle out more than the Cloisite®Na+ platelets or that the polarity of the Laponite®RD or its different ionic charge means that it does not compatibilise as well (Clegg 2015 Personal communication)

However, despite attempts to alter the mixing techniques and try to produce a more consistent film it was not possible to produce a composite which remained intact under the experimental conditions. This meant that the focus of the research returned to the Cloisite®Na^+ composite.

6.3. Antimicrobial activity of different PVOH/PVP-I/Cloisite®Na^+ Formulations

6.3.1. Introduction

The patent application (PCT/GB2016/050658) stated that the antimicrobial material could contain a concentration of iodine between 0.1 to 15wt%. The initial experiments which investigated the antimicrobial effects of different iodine concentrations tested concentrations between 1 and 8.5wt% (Section 2.3.2.3.). However, these experiments were only undertaken over a 24 hour period so did not demonstrate the phenomena identified in the passage studies that the material consistently produced larger ZOI after 48 hours. The preferred CROWD formulation contained Cloisite®Na^+ (12.5wt%) and 6wt% PVP-I.

Whilst the cytotoxicity testing suggested that reducing the iodine concentration did not appear to greatly decrease the composite's cytotoxicity it was still considered important to try to reduce the amount of iodine in the composite to the minimum effective concentration. It was decided to investigate the impact of reducing the Iodine concentration on the antimicrobial efficacy by comparing the three iodine containing 6, 3, and 1.5wt% PVP-I/ CloisiteNa^+ formulations that were tested in the cytotoxicity experiments (Section 5.7.3.2.).

6.3.2. Methods Summary

Passage studies were undertaken using the dark side of the three formulations containing 1.5wt%, 3wt% and 6wt% PVP-I/Cloisite®Na^+ against the five test bacteria; *E. coli*, *P. aeruginosa, S. aureus*, *S. aureus* USA300 and EMRSA- 16. The passage experiments were undertaken for five days.

6.3.3. Results

Figures 6.3. to 6.7. illustrate the different ZOI produced by the Cloisite®Na^+ composites containing the three different iodine concentrations against the five test bacteria.

The 1.5wt% PVP-I samples produced very small ZOI (<200mm^2) against *P. aeruginosa* and had stopped producing a ZOI by day 4. The ZOI produced

against *E. coli* were similar to the size of the test sample (400mm^2) for the first 2 days and no ZOI was produced on the third day. However, the 1.5wt% samples did produce ZOI against the three Gram-positive bacteria for 4 days but had stopped producing a ZOI by day 5.

The ZOI produced by the 3wt% PVP-I samples were larger than those created by the 1.5wt% samples but smaller than the 6wt% samples. The 3wt% samples produced clear ZOI against *P. aeruginosa* for the first 2 days but by the third day the ZOI was smaller than the sample size and there was no ZOI on the fourth day. The results against the *S. aureus* strains differed in that the 3wt% samples produced clear ZOI against *S. aureus* (SH1000) and the community acquired USA300 for 5 days but was only effective against the EMRSA-16 for 4 days.

As in all previous studies the standard CROWD material, PVOH/PVP-I/Cloisite® Na^+ containing 6wt% PVP-I produced a large ZOI against all six test bacteria for the duration of the 5 day experiment.

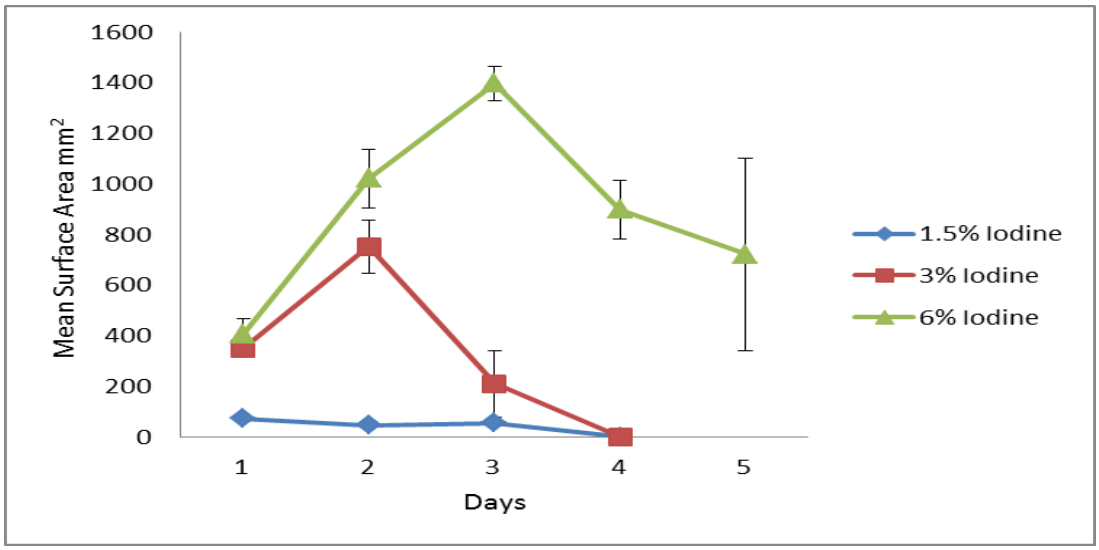


Figure 6.3: The effect of reducing the Iodine Concentration in the PVOH/PVP-I/Cloisite®Na^+ composite on the ZOI produced against P*. aeruginosa* (Error bar = Standard Deviation, n = 6)

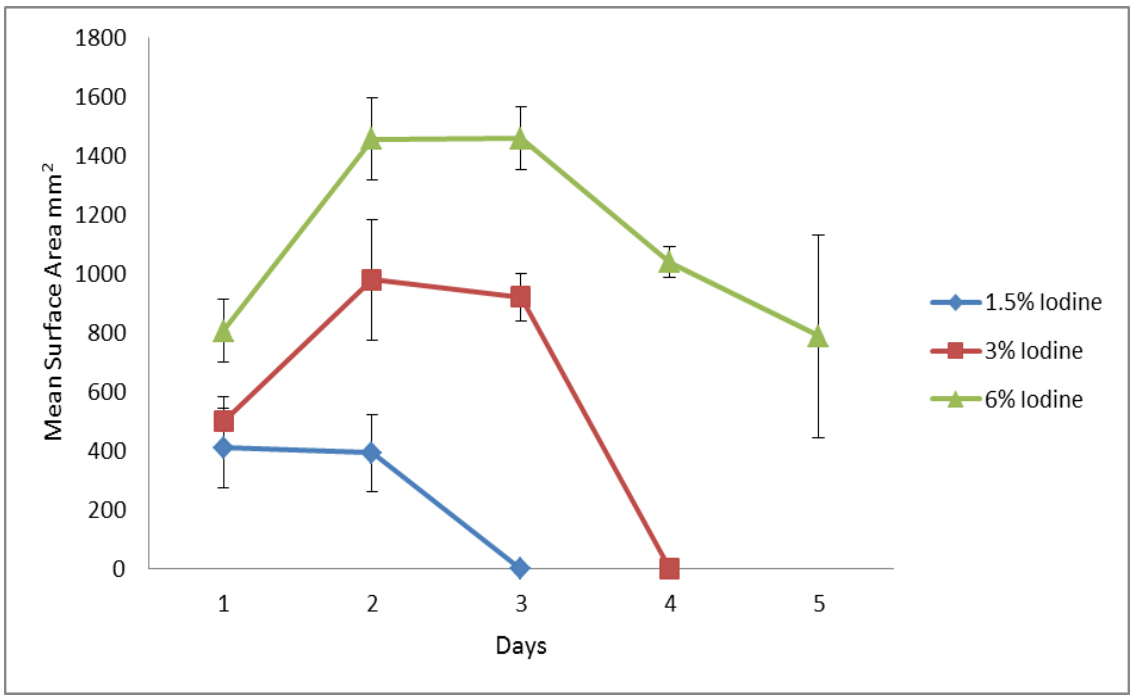


Figure 6.4: The effect of reducing the Iodine Concentration in the PVOH/PVP-I/Cloisite®Na⁺ composite on the ZOI produced against *E. coli* (Error bar = Standard Deviation, n = 6)

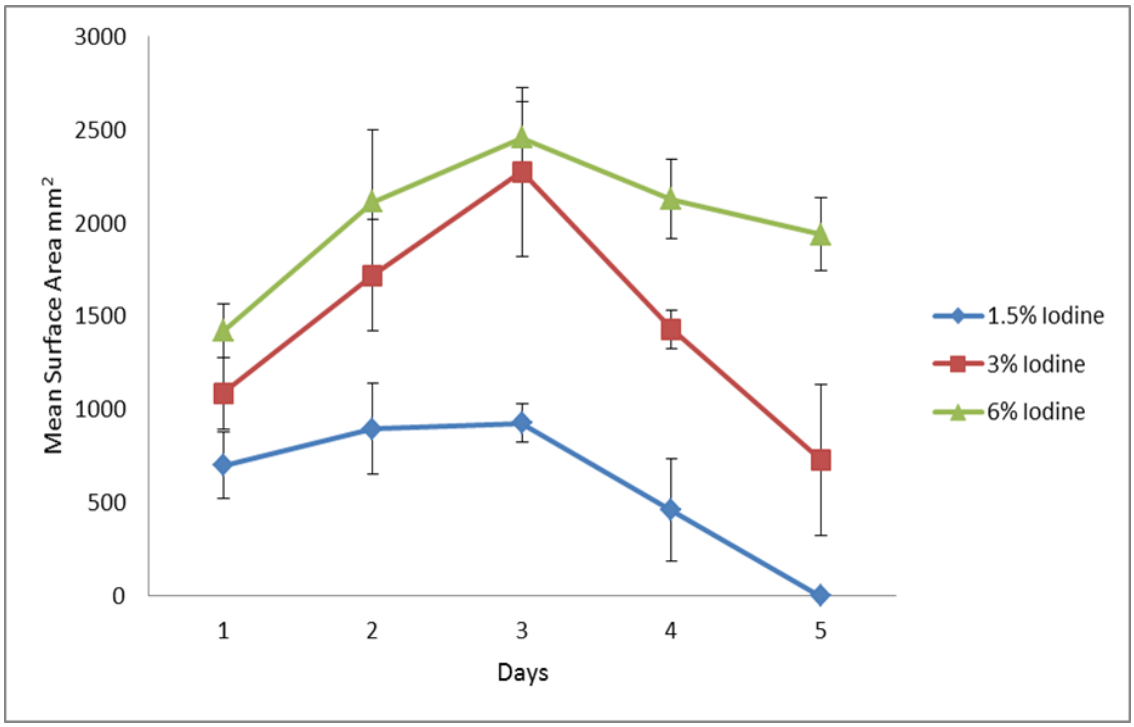


Figure 6.5: The effect of reducing the Iodine Concentration in the PVOH/PVP-I/Cloisite®Na⁺ composite on the ZOI produced against *S. aureus* (SH1000) (Error bar = Standard Deviation, n = 6)

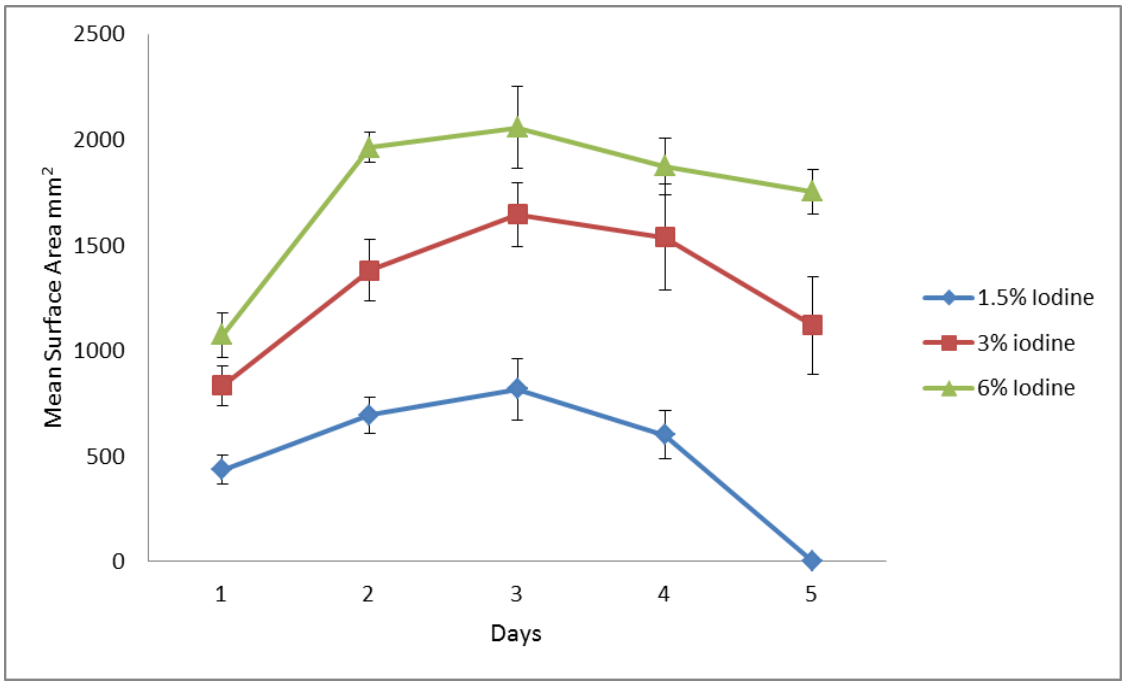


Figure 6.6: The effect of reducing the Iodine Concentration in the PVOH/PVP-I/Cloisite®Na⁺ composite on the ZOI produced against *S. aureus* USA300 (Error bar = Standard Deviation, n = 6)

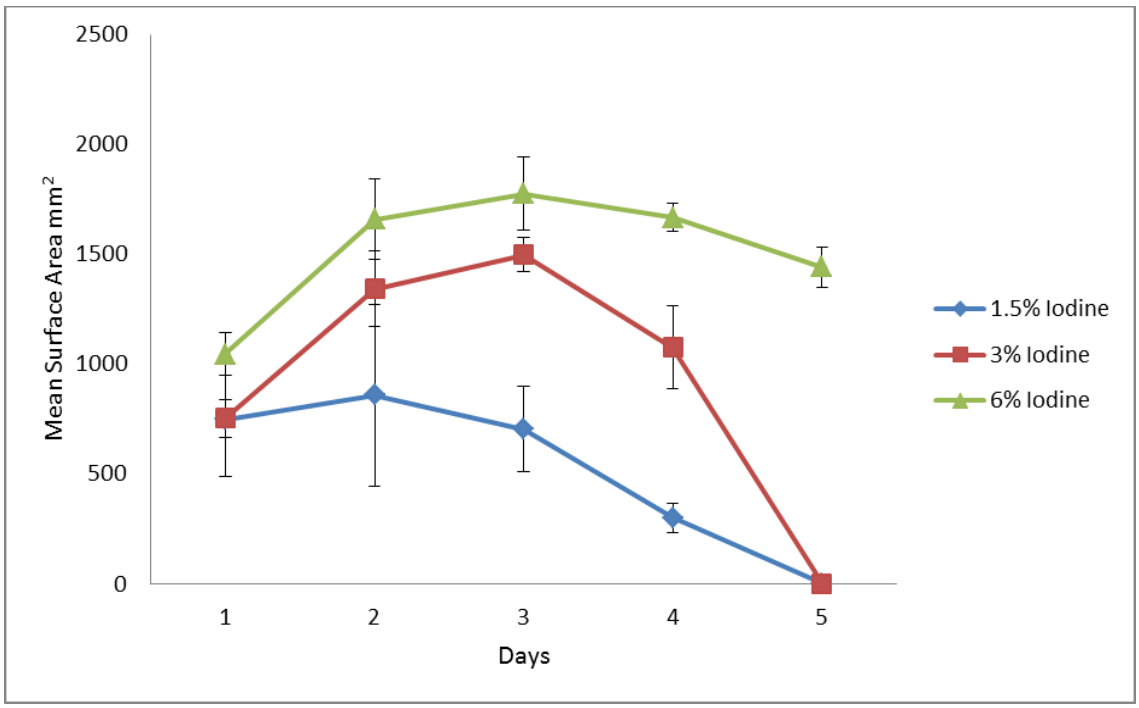


Figure 6.7: The effect of reducing the Iodine Concentration in the PVOH/PVP-I/Cloisite®Na⁺ composite on the ZOI produced against EMRSA-16 (Error bar = Standard Deviation, n = 6)

6.3.4. Discussion

These results show that reducing the iodine concentration would shorten the duration of antimicrobial action of the material, particularly against *P. aeruginosa* and *E. coli.* As the indirect cytotoxicity tests of these three formulations had demonstrated that reducing the iodine concentration did not appear to reduce the cytotoxicity it was decided to retain the more effective 6wt% PVP-I concentration as the preferred iodine concentration.

6.4. Scale up of manufacture of preferred CROWD formulation

6.4.1 Introduction

The initial CROWD test samples were created in the Materials Engineering Research Institute (MERI) on a small scale by mixing all the ingredients in a round bottom flask using magnetic stirrer bars. 60g of the suspension was then poured into 9cm circular petri dishes and dried in a vacuum oven over 7 days. The samples were then cut into 2 x 2cm squares for testing.

Scale-up of production and manufacture of a prototype wound dressing was undertaken by Dr David Hogg from Rejuvetech Ltd and Dr Francis Clegg (MERI, SHU). Specific challenges they had to address included the mixing of the ingredients on a larger scale, finding suitable moulds which could be used to cast the films and drying the films in different conditions/ environments to produce a polymer-composite gel.

The preferred CROWD formulation contained: -

PVOH/PVP-I 6wt%/ Cloisite® Na^+ 12.5wt%

6.4.2. Methods Summary

The manufacture of the large scale CROWD material is described in Section 2.3.2.5.

6.4.3. Results

The first large scale formulation which measured 22.5 cm x 22.5 cm was produced in MERI by Francis Clegg (Figure 6.8). In Figure 6.9. the material has been detached from the dish to demonstrate the stratification of the composite.

Figure 6.8: Large scale standard CROWD composite produced in MERI

Figure 6.9: Stratification in the CROWD Composite

The final manufactured material produced by Rejuvetech (Figure 6.10.) was very smooth, uniform and rubber like in consistency. In figure 6.11. the material has been conformed to demonstrate the flexibility of the material and clearly illustrates the self-stratifying layers which form the Light and Dark sides of the composite.

Figure 6.10: Large Scale CROWD 6wt% PVP-I/ Cloisite®Na+ composite produced by Rejuvetech Ltd

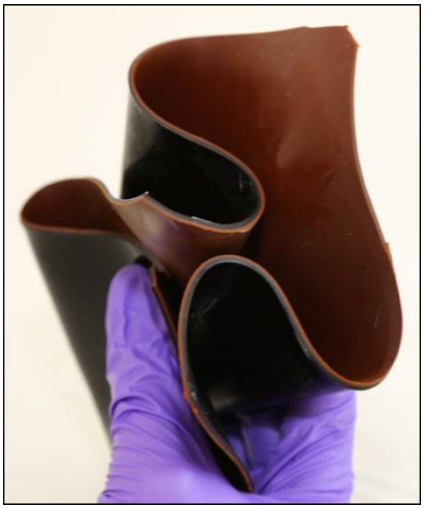


Figure 6.11: The flexible CROWD composite illustrating the light and dark sides.

6.4.4 Discussion

The large scale composite could be applied directly to a wound surface and should conform to the shape of the wound. It can easily be cut into any shape so could be applied to unusually shaped wounds.

6.5. Effect of hydration on the preferred CROWD composite.

6.5.1. Introduction

One of the interesting characteristics of the initial CROWD materials was the different states of hydration. Depending on the length of the drying time, the oven temperature or if they were dried at room temperature the samples could be wet and flexible or very dry and brittle. Dry samples could be rehydrated by the addition of sterile water. Having observed that the material could absorb fluid it was decided to investigate the effects of rehydrating the material and whether this would affect the antimicrobial properties of the material.

6.5.2. Methods Summary

The preferred CROWD formulation (PVOH/PVP-I 6wt%/ Cloisite®Na^+ 12.5wt%) was prepared using the standard mixing technique described in section 2.3.2.4.

Square 2 x 2cm samples were placed Top (Dark) side up in an open Petri dish and then placed in a Slimline-H humidity oven (Sharetree Ltd, Stonehouse, Gloucester, UK) set at 23^{o}C and 95% relative humidity. (Above 95% humidity partial dissolution of the film was observed). After 7 hours the films appeared more flexible. The samples were left in the humidity oven for a total of 50 hours and the increase in mass calculated.

The process was repeated using 2 x 2cm samples of the Bottom (Light) side of the material.

6.5.3. Results

After 50 hours at 95% humidity both the Top (Dark) and Bottom (Light) sides had increased in mass 40% (Figure 6.12).

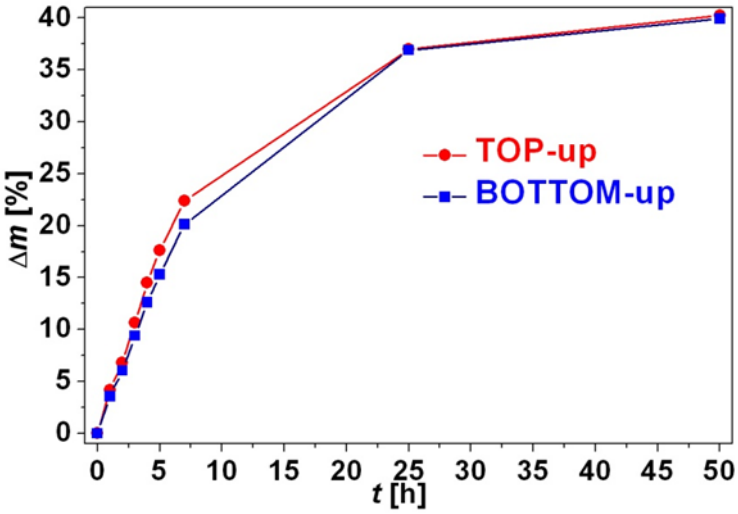


Figure 6.12: The increase in mass of the Top (Dark) and Bottom (Light) side of the standard CROWD formulation over 50 hours in a humidity oven at 23°C at 95% relative humidity (Petra 2015).

6.5.4. Discussion

This ability to absorb moisture whilst maintaining the integrity of the film could be very useful in clinical practice. This suggests that the material could be used on heavily exuding wounds to absorb large volumes of exudate. It could also be applied to dry wounds in the rehydrated form to provide a moist wound environment at the wound interface and this may help enhance auto debridement. It is possible that the material may also be able to donate fluid and behave similarly to a hydrogel. This potential advantage of the material warrants further investigation.

6.6. Effect of hydration in the Antimicrobial Activity of the CROWD Composite

6.6.1 Introduction

Having identified that the CROWD material could be rehydrated it was important to determine if the change in hydration would affect the antimicrobial release profile. Passage studies were undertaken to compare the antimicrobial activity of hydrated and non-hydrated samples of both the light and the dark sides of the standard CROWD material.

6.6.2. Methods Summary

The standard CROWD formulation containing PVOH/PVP-I 6wt%/Cloisite®Na+ was prepared using the standard mixing technique described in Section 2.3.2.4.

After the normal drying time half of the batch was placed in the humidity oven set at 23^{o}C and 95% relative humidity for 50 hours.

Square 2 x 2cm samples of the Standard CROWD material (S) were compared to the hydrated samples (HY). Both the Light (L) and Dark (D) sides of the standard and hydrated samples were tested.

The antimicrobial activity of the samples was tested against *S. aureus*, *E. coli* and *P. aeruginosa* using the passage method described in Section 2.1.2.3.

6.6.3. Results

The ZOI of produced against the three test bacteria by the standard formulation (S) compared to the samples that had been rehydrated at 95% relative humidity (HY) are shown in Figures 6.13 - 6.15.

All samples were still producing a large ZOI against *S. aureus* after 5 days (Figure 6.13). After the first 24 hours both sides of the hydrated samples were producing larger ZOI than the light and dark sides of the standard formulations. On day 2 all samples had produced larger ZOI than they had in the first 24 hours. Both the light side samples ZOI were much larger than the dark side samples. The light side of the standard formulation produced the largest ZOI.

After the third day the ZOI of all the samples had decreased but they all still produced a clear ZOI greater than 500mm^2 on day 5.

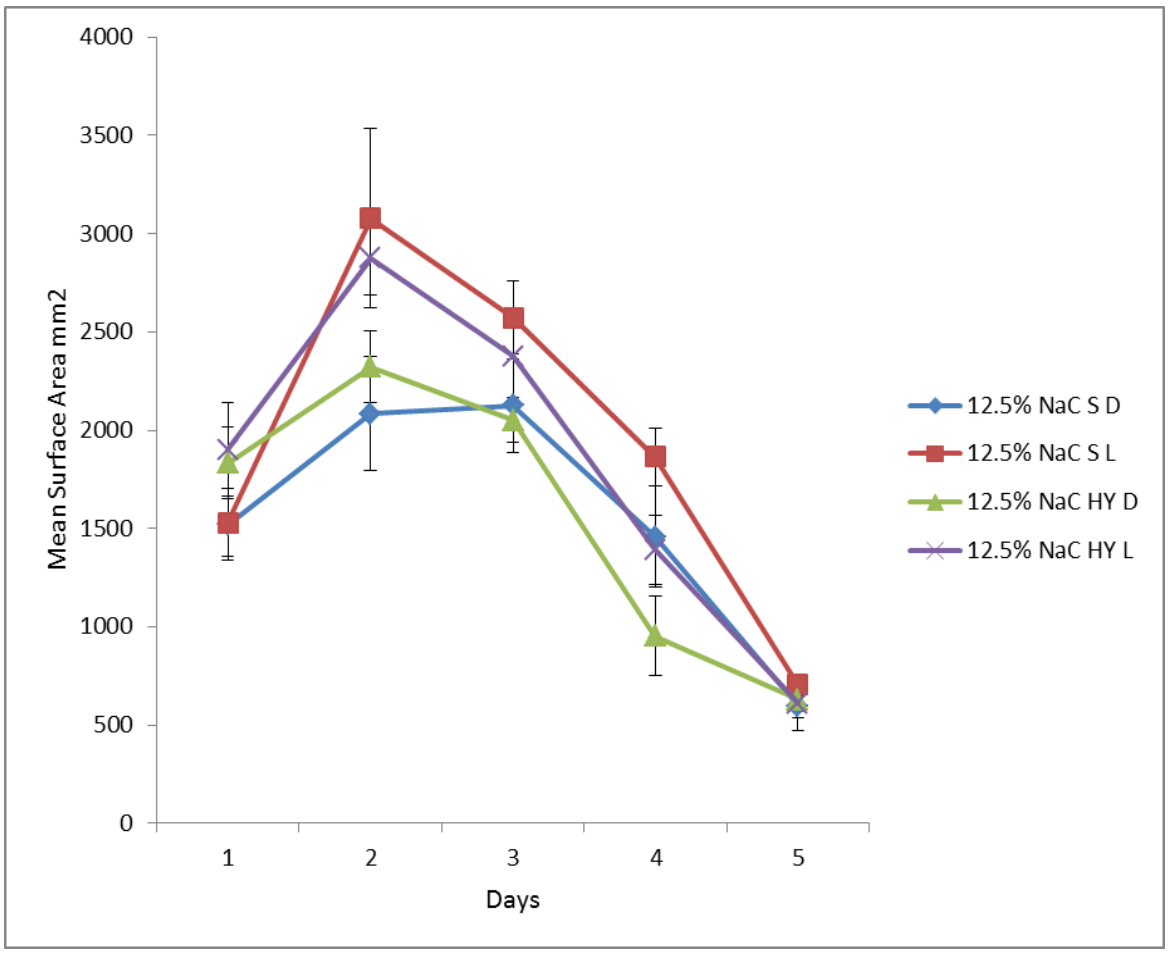


Figure 6.13: The ZOI produced by standard (S) and 95% hydrated (HY) samples of the preferred CROWD composite against *S. aureus*. (Error bar = Standard Deviation, n = 6) Key: L=Light side in contact with agar D=Dark side in contact with agar

Against *E. coli* the light sides of both the standard and hydrated samples produced very similar ZOI in the first 24 hours (Figure 6.14). Both the dark side samples produced smaller ZOI after 24 hours than the light sides. The ZOI of the hydrated dark side after 24 hours was larger than the standard dark side but by 48 hours it was smaller than the standard sample however it then increased in size by day 3 and was still producing a small ZOI on day 4. Both light side samples had stopped producing a ZOI after 4 days. Only the hydrated dark side was still producing a ZOI on day 4 and this was similar in size to the test sample size of 400mm^2.

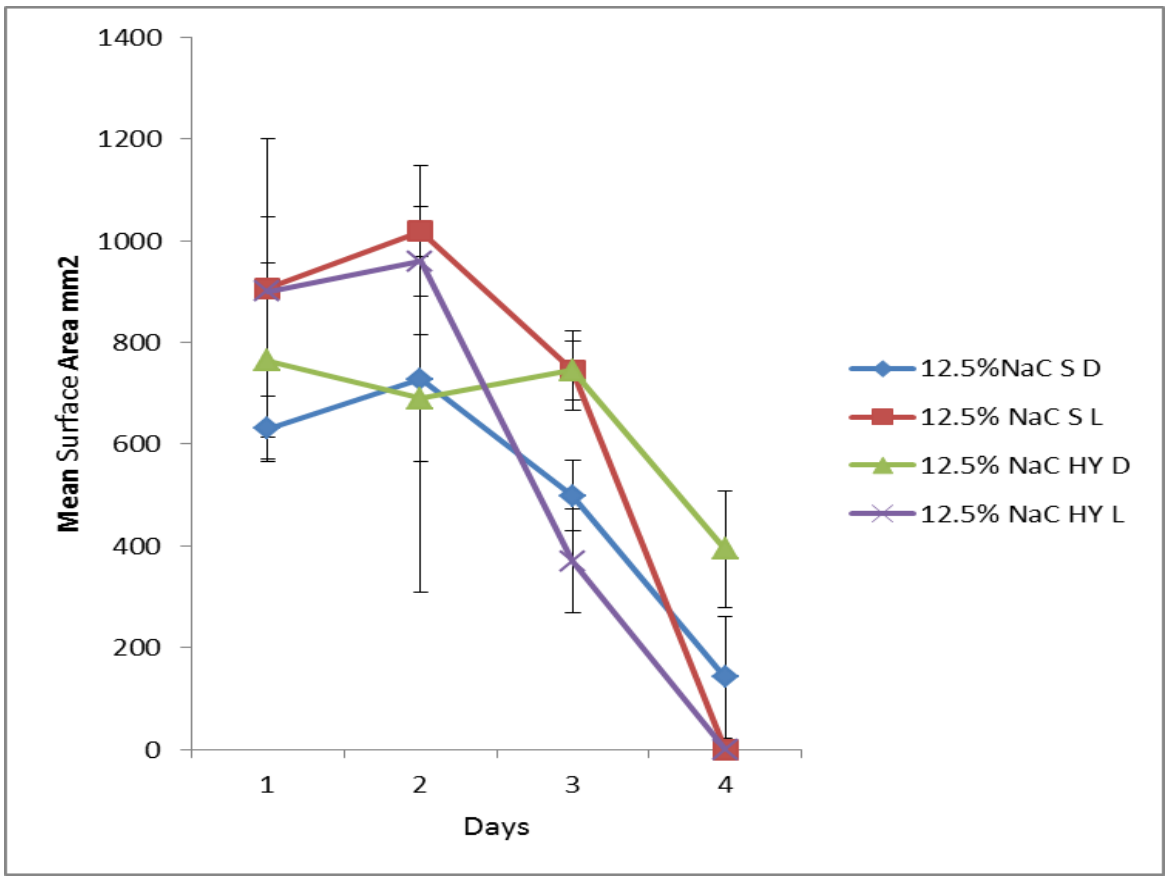


Figure 6.14: The ZOI produced by standard(S) and 95% hydrated (HY) samples of the preferred CROWD composite against *E. coli*. (Error bar = Standard Deviation, n = 6) Key: L=Light side in contact with agar D=Dark side in contact with agar

In the *P. aeruginosa* experiments shown in Figure 6.15, the light sides of both the standard and hydrated samples produced very similar ZOI after the first 24 hours and similarly the dark sides of the two different samples produced very similar ZOI. On day 1 both the light side samples produced ZOI which were more than double the size of the dark side ZOI. After 48 hours both the light side samples ZOI had reduced but the size of the dark side samples ZOI had increased slightly. By day 3 all samples were producing ZOI smaller than the 400mm^2 sample size. On day 4 the hydrated dark side had not produced a ZOI, but the other three samples were still producing small ZOI similar in size to the previous day.

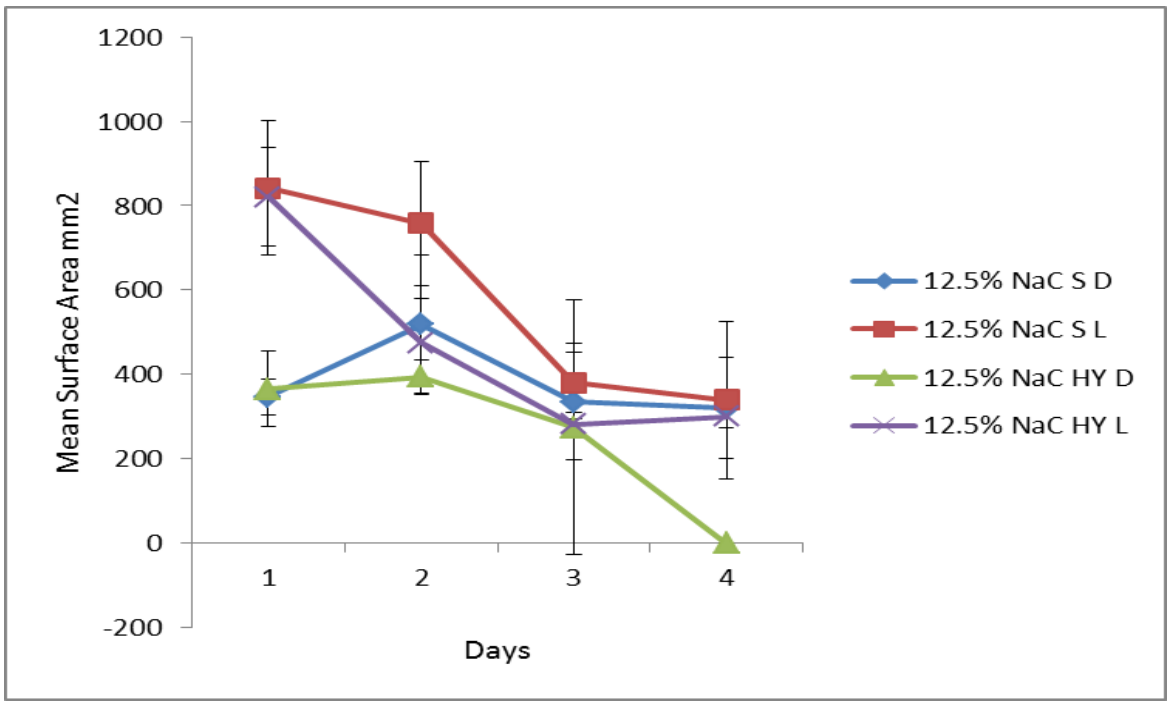


Figure 6.15: The ZOI produced by standard(S) and 95% hydrated (HY) samples of the preferred CROWD composite against *P. aeruginosa* (Error bar = Standard Deviation, n = 6) Key: L=Light side in contact with agar D=Dark side in contact with agar

6.6.4. Discussion

It would appear that in the first 24 hours both the standard and the hydrated samples dark sides and the light sides produced similar sized ZOI. Against all three bacteria the light side of the hydrated material followed a similar pattern to that of the light side standard samples but the ZOI were smaller. The most notable difference between the two formulations occurred in the *P. aeruginosa* experiments where the hydrated dark side stopped producing a ZOI after the third day whereas the other three samples were still producing a small ZOI on day 4.

As in previous experiments the ZOI produced by the different sides of the material and the duration of antimicrobial action varied between the different bacterial species. The duration of the antimicrobial effect against the Gram - negative bacteria; *E. coli* and *P. aeruginosa* was shorter than that seen against *S. aureus*.

The state of hydration of the sample did not appear to markedly affect the antimicrobial efficacy. This was important as it implies that the material could be used on wounds with differing levels of exudate and that high fluid levels should not affect the antimicrobial activity of the material. However further work is required to investigate fully the absorption capacity of the material.

6.7. Sterilisation of the preferred CROWD formulation.

6.7.1. Introduction

As mentioned previously, all dressings for clinical use are required to be sterile so it was important to find a method of sterilisation for the CROWD material which would conform to the industry standard for sterilisation of medicinal products and devices.

One of the most common forms of sterilisation used for commercial dressings is gamma (ɣ) ray sterilisation. The usual source of ɣ ray sterilisation used in the pharmaceutical industry is Cobalt 60 which emits two high energy rays: ɣ- rays and lower energy β particles. A cobalt source of 1-4x10^{16} Bq is used for industrial irradiation to deliver an absorbed radiation dose greater than 25kGy which is the standard pharmacopoeial dose in Europe (European Pharmacopoeia 2011).

The ɣ-rays target DNA and cause single or double strand breaks in the DNA which inhibits DNA synthesis and can cause errors in protein synthesis. They also cause damage to RNA, enzymes, bases and cell membranes (Aulton's Pharmaceutics, 2013).

Gamma irradiation is suitable for wound dressings because it can penetrate various materials effectively, it does not cause a significant rise in temperature and there is no radioactivity in the final product. However, there may be chemical changes that can affect polymers such as changes in tensile strength, colour, odour and gas formation. Materials known to be affected include acetal, Fluorinated Ethylene Propylene, Polytetrafluoroethylene, Polyvinyl Acetate, and Polyvinyl Alcohol (PVOH) (Aulton's Pharmaceutics 2013).

For this part of the research we were able to find a collaborator, Steve Cotton, the Managing Director of Brightwake, Mansfield, UK. Brightwake manufactures a wide range of commercial dressings including honey and other antimicrobial materials. Steve Cotton agreed to take samples of the CROWD material to be gamma irradiated by the contract sterilisation company Brightwake uses to sterilise their commercial wound dressings. In the UK two companies, Steris and Swann Morton, undertake contract sterilisation of pharmaceutical products and devices.

For these experiments samples of the large scale CROWD material (PVOH/PVP-I 6 wt%/Cloisite®Na^+ 12.5wt%) were sealed in plastic bags and covered in tin foil to protect from light to transport them to Brightwake. Having undergone the standard pharmaceutical gamma irradiation cycle the CROWD dressing was returned to SHU for antimicrobial testing in a silver foil package. (Figure 6.16)

Figure 6.16: Gamma irradiated sample of the CROWD material shown with foil outer package.

6.7.2. Methods Summary

Square 2 x 2cm samples were cut from an irradiated CROWD sample and from a non-irradiated sample of the same batch. The dark side of the 2 x 2 cm samples of the irradiated sample were then tested at the same time as the dark

side of the 2 x 2cm samples from the non-irradiated CROWD material to determine whether the ionizing radiation had affected the antimicrobial efficacy of the material. Passage studies as described in Section 2.1.2.3. were undertaken against *S. aureus* (SH1000), E. *coli* (JM109), *P*. *aeruginosa* (H085180216), *S. aureus* USA300 and EMRSA -16.

6.7.3. Results

The irradiated samples produced ZOI for 72 hours against all test bacteria except *P. aeruginosa* where they were only effective for 48 hours (Figure 6.17). The ZOI produced by the irradiated samples were larger for the first two days thereafter those produced by the non–irradiated samples were larger suggesting that the iodine was being released more rapidly by the irradiated material. By the fourth day of the experiment all of the irradiated samples were no longer producing a ZOI. The non irradiated samples were still producing clear ZOI against all five test bactetria after 5 days when the experiment was terminated.

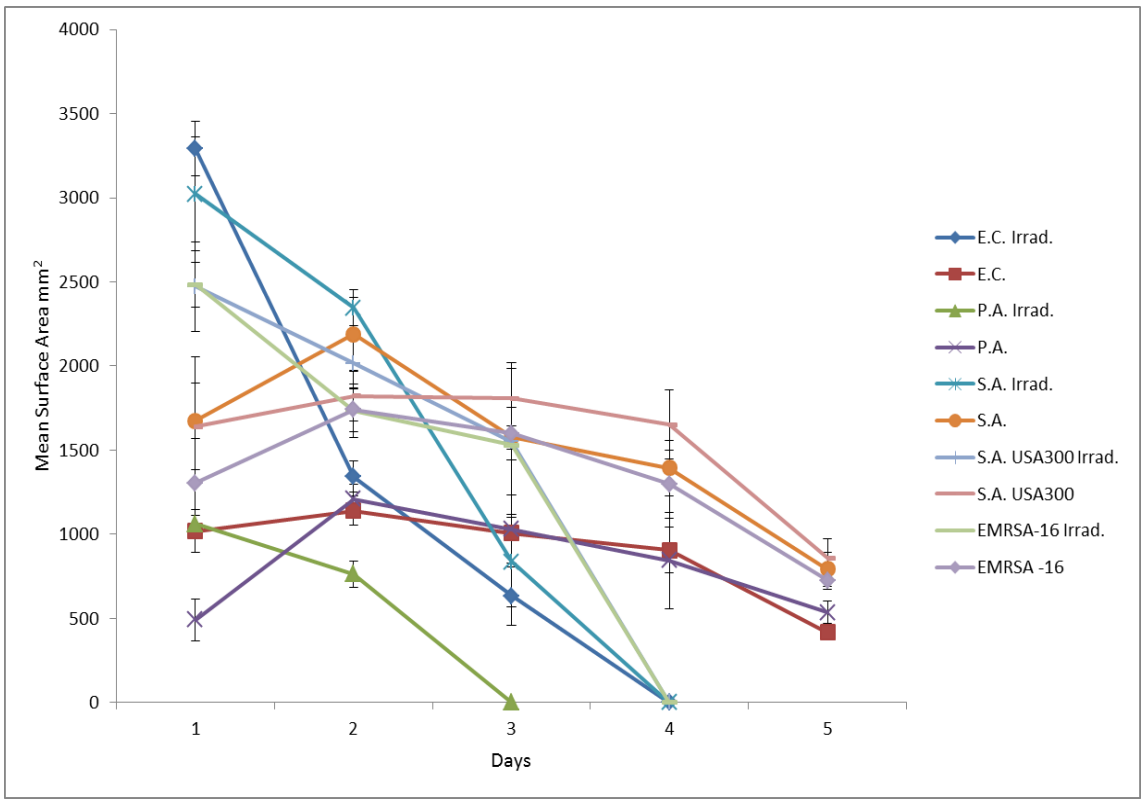


Figure 6.17: Comparison of the ZOI produced by the irradiated (Irrad.) and non-irradiated CROWD samples against the five test bacteria. (Error bar = Standard Deviation, n = 6) Key = "Irrad" indicates the samples which had undergone commercial ɣ irradiation.

6.7.4. Discussion

The irradiated samples were visibly wetter than the non - irradiated samples and iodine appeared to have leaked into the plastic packaging (Figure 6.16). This could possibly be due to a cross linking reaction occurring within the material, effectively shrinking the polymer network resulting in the elution of aqueous iodine solution. This experiment demonstrated that the ɣ irradiation had affected the release characteristics of the dressing effectively shortening the duration of antimicrobial action so an alternative form of sterilisation may be required to maintain the slow release characteristics.

6.8. Incorporation of the CROWD material into secondary dressings.

6.8.1. Introduction

In its original sheet form the CROWD material could be used as a primary dressing and be placed directly in contact with a wound surface. However, it would require a secondary dressing to keep it in place and to absorb any liquid that is wicked through the material from the wound to the top surface of the composite. The choice of secondary dressing is important as it must not interfere with the unique release characteristics of the CROWD composite. Most of the absorbent dressings such as alginates and hydrofibers would not be appropriate as they would absorb any moisture from the top of the CROWD dressing and form a gel. The most obvious candidate materials for use as a covering over the CROWD material would be foams, soft silicone polymers or vapour permeable films.

Our collaborators at Brightwake were interested in incorporating the CROWD material into existing carrier dressings to determine if the unique slow release properties of the composite would be retained.

6.8.2. Methods Summary

Samples of the pre-dried standard CROWD formulation containing PVOH/PVP-I 6 wt%/ Cloisite®Na^+ 12.5wt% were supplied to Brightwake in the form of a slurry. This was then applied by Brightwake, using different commercial coating techniques, to a foam dressing Advazorb and a gauze tulle dressing. Advazorb® (Advancis Medical) is a low - adherent, hydrophilic, polyurethane foam dressing with a breathable, bacteria proof film backing. The foam is claimed to have high fluid retention properties.

Three different dressing formulations were created; Foam with pattern print (Figure 6.18), Foam with flood coat iodine (Figure 6.19) and Iodine knitted dressings (Figure 6.20). The dressings were returned to Sheffield Hallam University (SHU), Biomolecular Sciences Research Centre (BMRC) for antimicrobial testing.

Figure 6.18: Foam with pattern print iodine

Figure 6.19: Foam with flood coat iodine

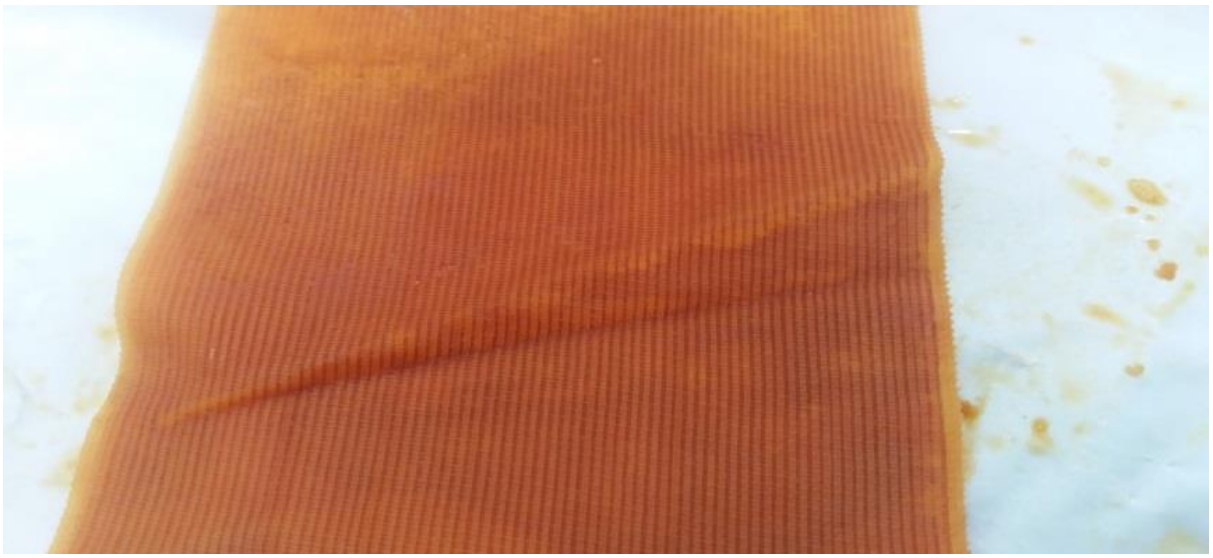


Figure 6.20: Iodine Knitted Dressing

The dressings arrived at SHU sealed in a foil wrapper but had not been sterilised. The samples were aseptically cut into 2 x 2cm squares and UV irradiated on both sides for 15 minutes prior to testing. Passage studies as described in Section 2.1.2.3. were undertaken against *S. aureus, P. aeruginosa and E. coli.*

6.8.3. Results

The two foam dressings curled and detached from the agar, so the experiment was repeated using sterilised 20 pence coins as weights.

After 24 hours incubation at 37^{o}C there was complete microbial growth of all three bacteria under both the different foam dressings.

The colour of the foam dressings was very pale which would suggest that there was very little iodine present and that the CROWD material may have been absorbed into the foam material and there was not sufficient remaining to produce an antimicrobial effect.

The iodine knitted dressing when opened had liquid iodine within the plastic packaging which was sealed within the outer foil wrapper (Figure 6.20). When tested this dressing produced a clear ZOI against each of the three test bacteria after 24 hours incubation (Figure 6.21) but when transferred after the second 24

hours there was complete microbial growth of each of the test bacteria indicating that the controlled release of iodine had been lost in this formulation. Interestingly this sample looked similar to the Inadine® commercial dressing. The results from the Inadine passage studies (Section 3.3.2) were included in Figure 6.21 as a comparison to the iodine knitted dressing results. The Inadine dressings produced similar sized ZOI as shown in Figure (6.21). However, unlike the iodine knitted dressing, Inadine was still effective against *S. aureus* and *E. coli* after 48 hours.

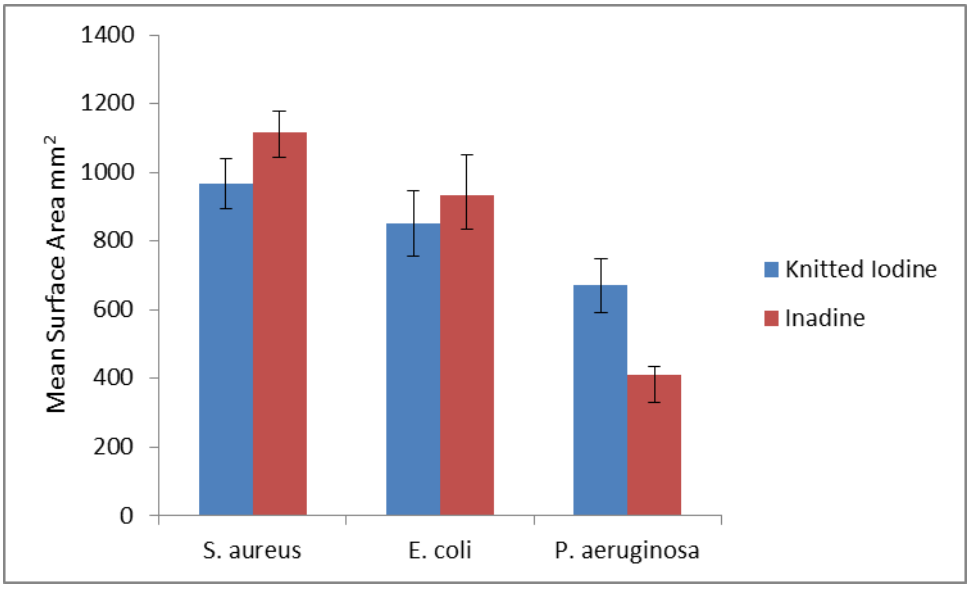


Figure 6.21: ZOI against the three test bacteria that were created by the Iodine Knitted Dressing compared to the ZOI produced by Inadine after 24 hours. (Error bar = Standard Deviation, n = 6)

6.8.4. Discussion

Unfortunately, none of the combined dressing formulations demonstrated the enhanced duration of antimicrobial activity exhibited by the original CROWD composite. It is likely that the active iodine may have been lost, possibly through evaporation, during the printing and coating processes. The knitted dressing appeared to have retained the characteristic iodine colour but had lost the original rubber like consistency of the CROWD material. Incorporation of the CROWD slurry into the gauze appears to have disrupted the clay/polymer composition leading to the loss of the slow release properties of the material.

6.9. Conclusions

The scale up of the manufacturing process produced a large flexible uniform material with two distinct different coloured layers. The different colours provide an indication of the release characteristics with the light brown side producing a faster release of iodine than the dark brown side.

As discussed in section 4.10.3. the light layer contains five times the amount of clay than the dark layer and it also contains a larger amount of iodine. The higher concentration of iodine in the light layer may account for increased size of the ZOI produced in the passage studies in the first couple of days. What is still to be determined is the effect on the composition of the two layers over time as the iodine is released, as this would help explain the mechanism of the slow release action that has been observed. The state of hydration of the material did not appear to markedly affect the release of iodine and this ability to rehydrate the material may be useful in clinical practice especially for the management of dry to low exuding wounds. The ability of the material to absorb fluid is also important particularly in the treatment of heavily exuding wounds.

The results of the gamma irradiation experiments were challenging. Firstly, there was obvious leakage of iodine from the material into the plastic wrapper which would make removal of the material difficult for clinicians. Secondly the duration of antimicrobial activity was reduced to less than 3 days against the *P. aeruginosa* and only 3 days for the other four test bacteria. This effect is most likely to be due the ionising radiation affecting the cross linking within the polymer or possibly an effect on the structure of the platelet stacks within the material which then made the material more porous to the iodine.

Finding an alternative sterilisation method may be difficult as the CROWD polymer is hydrophilic and probably heat sensitive or thermo labile, so steam or dry heat sterilisation methods would not be suitable. Ethylene oxide gaseous sterilisation is sometimes applied to elastic adhesive dressings but to be effective the dressing packaging has to be permeable to air, water and the gas. This would not be an option because the CROWD material has to be packed in a material such as foil which protects it from moisture and light. Early CROWD

samples left in a sealed Petri dish were observed to fade over time on exposure to light so since this was noted all samples have been stored in sealed Petri dishes covered with tin foil to protect from light.

The results of the antimicrobial testing of the CROWD incorporated into the Advazorb foam and the knitted dressing were disappointing. It is assumed that during the printing and coating of the foams the active iodine was lost. The knitted material was effective, but only for 24 hours, demonstrating that the novel sustained release effect of the CROWD material had been lost. One suggestion would be that the CROWD coating could be applied as a thicker layer or the gauze and foams could be incorporated into the solid composite rather than being mixed with the slurry.

Further work is required to identify a suitable secondary dressing for the CROWD composite. One experiment planned is to test the effect of the Advazorb foam as a secondary dressing to cover the CROWD material to determine if this combination would affect the release properties of the CROWD composite. Other suitable secondary dressings to be tested could include less absorbent foams such as polyurethane or silicone and vapour permeable films.

Chapter 7: General Discussion, Future Directions and Conclusion

7.1. Aims of Research and Contribution to Knowledge

The original aim of this research was to investigate the antimicrobial properties of commercial antimicrobial wound dressings (AWD) in order to determine if individual bacteria are more susceptible to a particular class of AWD. Having written wound care formularies for a number of different NHS Hospital Trusts, one of the challenges has been to find robust evidence to support any recommendations of the appropriate choice of antimicrobial dressing for a wound infected with a particular bacterium. The lack of evidence available stems in part from the fact that most wound dressings are classed as medical devices and as such are not required to undergo the rigorous clinical trials which are mandatory for medicinal products, which have to pass the three tests of quality, safety and efficacy in order to be granted a licence for sale. A review of manufacturers' websites for all the commercial dressings included in this reasearch illustrated the fact that most companies published very few references to evidence the efficacy of their products.

This study has shown that the dressings tested demonstrated antimicrobial activity but there were significant differences between individual dressings within the same class in terms of their effect on different bacteria and their duration of action. These differences are probably associated with the structural properties of the carrier dressings and the mechanism of antimicrobial release. This research highlights the need for the characteristics of individual antimicrobial dressing formulations to be defined so that clinicians can make evidence based choices when selecting a dressing to treat a particular wound infection.

Another key contribution to knowledge of this research was the creation of a novel antimicrobial material. The unique two side release profile, absorbent wicking properties and the colour change indicator make this material different to any of the dressings currently available in the UK and provide a novel material for the treatment of infected wounds.

Future work is however required to develop this technology to create a wound dressing prototype which could undergo clinical evaluation.

7.2. Future Microbiological Studies

7.2.1. Test Organisms

As discussed in Section 1.6. there are a number of pathogens which are commonly found in wounds. The bacteria used for this study were chosen because they are often associated with clinical infection in chronic wounds were; *Staphylococcus aureus,* a Gram - positive bacteria which is a common skin commensal, and two Gram - negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* both of which are frequent wound colonising organisms associated with wound infection. Later experiments included the methicillin – resistant *S. aureus* species, as these strains are particularly resistant to antibiotic treatment and are a problem in wound care, particularly in immunocompromised patients.

This study developed a fast, simple and easily reproducible *in vitro* model which could be used to test other wound pathogens. It would be interesting to extend the research to include other pathogens such as *Staphylococcus epidermidis,* a common skin commensal, which is an opportunistic pathogen capable of causing bacteraemia. As discussed previously, deep wounds are often colonised with anaerobic bacteria so it would also be useful to test activity against an anaerobe *such as Clostridium perfringens* which can cause gas gangrene by the production of lecithinase which destroys tissue, red blood cells and immune cells (Edwards - Jones 2016). Broadening the range of lab strains and clinical isolates examined within a species to determine if there are different susceptibilities to the antimicrobial agents would provide a more comprehensive analysis of the usefulness of specific dressings against pathogens based on speciation.

Other organisms known to colonise wounds are yeasts and a common contaminant is *Candida albicans* so the antimicrobial efficacy of dressings against yeasts could also be studied. For future biofilms experiments a series of

multispecies biofilms containing a selection of common wound pathogens will be developed as this would more effectively represent the polymicrobial nature of biofilms found in non-healing wounds.

7.2.2. Test Dressings

The commercial dressings included in this research (listed in Table 3.2. in Section 3.2.) were chosen to represent examples from the four main classes of antimicrobial dressings which contain biguanides, iodine, silver and honey. As it was not possible to test all the commercially available antimicrobial wound dressings a selection was made from each class to include different carrier dressings such as tulles, foams, alginates and hydrofibers. During the study some dressings have been withdrawn from the market, for example Iodozyme was withdrawn in the autumn of 2017. Similarly, a number of new dressings have been introduced and variations to formulations or carrier dressings have been made. The dressings chosen were all licensed for treatment of infected wounds at the time of selection. The dressings had to be available as sheets because the test methodologies could not accommodate antimicrobial gel formulations such as honey, Octenilin® (octenidine) and Prontosan (PHMB) wound gels.

The dressings included in the study were tested against lab strains of *S. aureus* and *E. coli* and an antibiotic susceptible clinical isolate of *P. aeruginosa* from a burns patient. Only the silver containing dressings were effective against all three bacteria for longer than 48 hours so it is planned that the next series of passage studies for these dressings will include the MRSA strains. Within the class of silver dressings the two foams; Allevyn Ag and Biatain Ag, produced the most interesting results. Their minimal efficacy against *S. aureus* and *E. coli* compared with their prolonged antimicrobial activity against *P. aeruginosa* warrants further investigation. These experiments will be repeated to compare the silver foams with plain Allevyn and Biatain non-adhesive foams to determine if the carrier dressing exert any antimicrobial activity which may be contributing to the efficacy of the silver foams. It is also the intention to investigate a wider range of silver foam dressings containing a variety of silver formulations either on the dressing surface or embedded within the carrier material. Three products have been identified for future study: Acticoat Moisture Control, PolyMem Silver

and Aquacel Ag Foam. The Acticoat dressing consists of a three layer polyurethane foam with a wound contact layer containing nanocrystalline silver whereas the PolyMem Silver consists of a polyurethane foam containing monocrystalline silver. The Aquacel Ag foam was chosen because it is a combination of the Aquacel Hydrofiber and polyurethane foam containing 1.2% ionic silver. It will be interesting to determine how the antimicrobial activity of the combined silver foam layer and hydrofiber layer compares with the unsupported silver hydrofibers previously tested, Aquacel Ag and Aquacel Ag+ Extra.

7.2.3. Experimental Methodologies

The Zone of Inhibition (ZOI) method used in this study provides a measure of the ability of the dressing to release an antimicrobial from within the carrier dressing. The test is designed to mimic the clinical use of the dressing and predict the capability of the dressing to kill or prevent bacterial growth within a wound. In order to exert a significant antimicrobial effect in this test the dressings must absorb moisture from the agar to activate or release the antimicrobial held within the material. The antimicrobial then has to diffuse into the agar to exert its antimicrobial effect (Thomas and McCubbin 2003). One of the limitations of this method is that dressings where the antimicrobial is immobilised within the material the antimicrobial may not diffuse far into the agar and so the material does not produce clear ZOI. This could be one of the reasons why the Tulle dressings Bactigras, Inadine and Actilite did not perform well in this study. Another issue identified with this methodology was with the dressings that were highly absorbent and swelled and obscured the developing clear zone, an example being the hydrogel Iodozyme. Similarly, the materials that curled away from the agar surface produced smaller ZOI where they remained in contact with the agar. In some circumstances it was possible to apply a weight to the test sample as was done in the experiments with the commercial foam dressings. However, with Tulle open weave dressings this was not possible because a weight would obstruct the pores in the fabric and could have impacted on the antimicrobial efficacy of the material.

Other factors can also influence the results of the diffusion assay. The diffusion rate of the antimicrobial and the rate of the lawn growth of the test bacteria and concentration are sensitive to the physicochemical effects of temperature, pH,

water content and the agar gel concentration and depth (Thorn, Greeman and Austin 2005). The test does not distinguish between bacteriostatic and bactericidal effects but did provide a reproducible visual and measurable distinction between the dressings.

The bactericidal activity of a dressing could be tested by taking a swab from the area under the dressing and then streak onto a fresh agar or a sample of the agar under the dressing could be taken and placed in broth for incubation. No growth would indicate that the dressings were bactericidal.

In future, challenge testing could be used to enable comparison of the speed of bacterial kill between different dressings and in the presence of different bacteria. It is designed to measure the ability of a dressing to kill bacteria applied to it in the form of a suspension. One method commonly used in antimicrobial dressing comparison studies is based on a method published by Wright et al. (1999). In this test, 0.2ml of a log phase culture of the test bacteria is added to a standardised portion of the dressing and incubated for two hours. The dressings are then transferred into 10ml of 0.1% peptone water (Oxoid) and vortexed to remove any viable organisms. Serial dilutions are performed in triplicate on each extract and the number of viable organisms present determined using a standard surface counting technique. If viable organisms are recovered, the test is repeated using a 4 hour contact time and then again for 24 hours. If no organisms are detected after 2 hours the dressing is placed in 10ml tryptone soya broth to detect very low levels of viable organisms. This test enables comparison of the speed of bacterial kill between different dressings and in the presence of different bacteria (Thomas and McCubbin 2003).

To provide a quantitative assessment of the performance of the dressings a logarithmic reduction assay could be undertaken. The antimicrobial dressings are incubated with the test bacterium of a known culture density for 0.5 – 24 hours. At various time points the bacteria are recovered and viable cells counted and the number expressed as a logarithm. The results are expressed as $\log_{10}$ reduction values ($\log_{10}$ initial count – $\log_{10}$ final count). A log reduction of >3 (>99.9% of bacteria are killed) may be used to define an antimicrobial that is bactericidal rather than bacteriostatic. Log reductions > 1 but < 3 indicate that

a proportion of the bacteria have been killed (Wounds International 2012). When reviewing published literature there are often variations in the $\log_{10}$ reduction methods used. Variation in the experimental design such as different inoculum concentration and exposure time to the antimicrobial and the use of a neutraliser to inhibit the action of the antimicrobial after the chosen exposure time may generate different results when evaluating the same dressings (Lindsay 2011).

The techniques used to test the antimicrobial efficacy of wound dressings are often not standardised so comparison between different studies may not be possible or may lead to incorrect assumptions.

On 9th June 2014 a draft British Standard "EN16756: Antimicrobial wound dressings - Requirements and test methods", was published for public consultation. Last accessed on 12th February 2020 it was still in draft form and was not an established British Standard. The draft standard describes a range of test methods for establishing whether a wound dressing exerts antimicrobial activity. The draft standard suggests the use of clinical strains of *S. aureus* (American Type Culture Collection (ATCC) 6538), *P. aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). *C. albicans* was recommended because it forms part of the normal microflora and acts as an opportunistic pathogen in immunocompromised patients or those on broad spectrum antibiotics, so is a key infection causing organism in many different types of wounds. The methods described involved the organisms being grown in Simulated Wound Fluid (SWF) containing 50% maximum recovery diluent and 50% Foetal Calf Serum (Parsons et al. 2005) which contain salts and proteins which are known to interfere with some antimicrobial substances used for dressings. The SWF is also thought to more closely simulate the role of wound exudate and create a more realistic challenge for the test organisms. Our pilot study (Bradshaw 2011), which tested 1 x 1cm samples of a variety of antimicrobial dressings, found that there was no significant difference in the size of the ZOI using Mueller Hinton broth compared with SWF for honey or silver ($p = 0.981, 0.567$) but a significant difference $p< 0.05$ was observed for iodine. However subsequent student research projects did not demonstrate such clear differences between the two growth media. Due to inconsistency in these

results the decision was made at the start of this study to undertake the initial passage experiments using Mueller Hinton (MH) broth. Restricting the experiments to a standard growth media also reduced the number of variables when making comparisons between the dressings.

Now that a preferred CROWD formulation has been identified future antimicrobial testing could be undertaken to compare the antimicrobial effects produced when bacteria are grown in MH broth compared with the growth of bacteria of the same species in growth media containing SWF. Another recommendation in the draft standard that differed from our research methodology was the suggested incubation temperatures, between 30-35^{o}C for bacteria and which are lower than the 37^{o}C used in this study. The first experiments in this study were undertaken in 2010 and the test bacteria were incubated at 37^{o}C. For consistency throughout this research all the experimental conditions, including the temperature for incubating the bacteria were kept the same. Future experiments could be undertaken using lower incubation temperatures to mimic wound conditions at the environmental facing surface but then the results would not be directly comparable to previous results. However, it would be possible in future studies to run parallel experiments at different temperatures to establish if lower incubation temperatures affect the size of the zones of inhibition of microbial growth.

The draft standard suggests three test methods; a direct contact method in which the inoculum is applied to the dressing (Gallant-Behn et al. 2005), a shaking method where the dressing is placed in liquid culture (Parsons et al. 2005) and a two compartment method where the dressing is suspended in a strainer in contact with the test inoculum (Ågren and Mirastschijski 2004). The draft standard does not recommend a particular test but advises that the first two methods are suitable for most dressings except super absorbent dressings, and the third method is suitable for dressings releasing antimicrobial agents. The standard does however advise that the contact time should be 24 hours +/- I hour which should ensure more consistency between tests. This research used a standard contact time of 24 hours for all antimicrobial studies.

When the British standard is formally published it would be advisable to repeat the testing on all of the commercial dressings and the CROWD material, using

one of the official methods. The shaking method would probably be most suitable because it is unlike the ZOI diffusion assay and would provide quantitative data on kill rates.

7.2.4. Biofilm Experiments

The initial *in vitro* tests measured the antimicrobial activity of the dressings against planktonic bacteria which are known to be more susceptible to antimicrobial agents than their counterparts living in a biofilm. A simple *in vitro* biofilm model was developed to test the ability of the CROWD and Inadine dressings to eradicate 24, 48 and 72 hour biofilms containing either *S. aureus, P. aeruginosa or E. coli*. Whilst both the CROWD and Inadine dressings were shown to completely eradicate biofilms containing *S*. *aureus* and *E*. *coli* they were ineffective against the *P*. *aeruginosa* biofilms. However, there were limitations with this simple biofilm model. The biofilms tested involved single species of bacteria which were grown in the presence of required nutrients for the set period of growth. Biofilms growing on wounds are likely to have very different compositions and may contain multiple bacterial species and altered physiology in terms of their metabolic rate and the presence of nutrients (Halstead et al. 2016).

Various biofilm models have been developed which vary in complexity from flat-bed perfusion models (Thorn and Greenman 2009) to complex bioreactors. In the flat–bed perfusion model the biofilms are grown within 1 cm^2 cellulose support matrices and are perfused with growth media. Dressings are applied to the biofilm and the biofilm can be removed at various time points to determine the total biofilm population. An alternative approach is the colony biofilm model where biofilms are grown on a semipermeable membrane that is placed on agar. The bacteria are provided with a new supply of nutrients by moving the membrane to a fresh agar plate.

Another commonly used model is the drip-flow reactor (DFR) where biofilms are grown on an inclined microscope slide inside a testing channel where it is continuously supplied with fresh medium (Buckingham-Meyers, Goeres and Hamilton 2007).

A combination of these methods, the colony-DFR model has been described by Lipp et al. (2010) which the authors claim is a practical method for growing *in vitro* biofilms in a manner that mimics a chronic wound environment.

The Biomolecular Sciences Research Centre at Sheffield Hallam University has recently acquired a commercial drip flow reactor so a further series of experiments are planned to compare the antibiofilm activity of the CROWD material with the remaining commercially available iodine dressings, Inadine and Iodoflex. The intention is to test against a greater variety of bacterial species and create more mature 7 day biofilms.

The commercial iodine dressings: Inadine, Iodoflex and Betadine cream (1g applied to Topper Gauze) have been tested against mixed species 3 and 7 day old biofilms produced in a constant depth film fermenter (CDFF) (Hill et al. 2010). The biofilms formed were derived from both aerobic and anaerobic chronic wound isolates. Quantitative analysis of the mature biofilms found that *S. aureus* and *P. aeruginosa* predominated in all the different wound consortia analysed. The authors concluded that this situation was directly analogous to that found in the quantitative analysis of the microflora in chronic wounds. Inadine and Iodoflex were shown to have killed all the bacteria in both the 3 and 7 day CDFF - generated biofilms. The Betadine® cream/ gauze produced a reduction in *S. aureus* counts to $1x10^4$ cfu/ml against the 3 day biofilm but a lower reduction to $1x10^7$ cfu/ml in the more mature 7 day biofilm. No significant reduction in the *P. aeruginosa* count was observed with the Betadine cream/ gauze. It would be interesting to try to repeat this experiment to assess the performance of the CROWD material against this more complex biofilm and to compare its anti-biofilm activity with the available commercial iodine dressings.

A recent study by Oates et al. (2018) described a novel wound biofilm model developed to evaluate the effectiveness of topical antimicrobials. The basal perfusion model was developed to reproduce the basolateral nutrient delivery of wounds and was able to sustain the growth of four functionally distinct wound pathogens in multi-species biofilms. The biofilms were exposed over 7 days to povidone-iodine (PVP-I) (10%w/v), polyhexamethyelene biguanide (PHMB) (0.5%v/v) and silver acetate (0.05%w/v). The authors found that the rank order of tolerance in the multi-species biofilm was *Pseudomonas aeruginosa*>

Methicillin resistant *Staphylococcus aureus* (MRSA) > *Bacteroides fragilis* (*Gram*-negative, anaerobic bacilli)> *Streptococcus pyogenes* (Gram-positive, facultative anaerobic coccus). None of the antimicrobials were able to eradicate the *P. aeruginosa* or MRSA from the biofilms. The rank order of effectiveness for the antimicrobials against the biofilm communities was shown to be PVP-I > PHMB > silver acetate. These experiments used liquid formulations of the antimicrobials however it may be possible to test the CROWD material using a similar methodology which could then help determine it's efficacy against multi-species biofilms containing mixed aerobic and anaerobic species commonly found in wounds.

7.2.5. Cell Biology Experiments

Whilst the Biofilm models are more representative of bacterial behaviour in a wound environment than the planktonic studies, they still do not reflect the complex biological interactions which occur within wounded skin. Traditionally wound dressings have been tested on animal skin, usually pig skin. Problems with the reproducibility of wounding animal skin and the inherent cost have led to the development of skin models. The cytotoxicity testing of CROWD and the iodine commercial dressings was undertaken using a 3D tissue engineered skin model developed by colleagues at the University of Sheffield (Shepherd et al. 2009). This Tissue Engineered (TE) skin has also been used to create a burn wound by the application of heated rods to the surface of a piece of skin at a set temperature and time duration. The wound can be infected with bacteria to produce a biofilm on the skin which could in future be used to test the anti-biofilm activity of CROWD and the commercial dressings. Another project currently being undertaken in the Biomolecular Sciences Research Centre (SHU) has involved the development of a wound model using a living skin equivalent (LSE) produced by Innovenn UK Ltd (Lewis et al. 2018). The skin was wounded using a scalpel blade and assessed at day 4 post wounding by histology and matrix-assisted laser desorption-mass spectrometry imaging (MALDI-MSI). Wounding the model was shown to disrupt the epidermal barrier function and stimulate keratinocytes to migrate and proliferate across the wound to close the damaged area. This model can be infected and used to test antimicrobial dressings.

7.3. Clinical Evaluation

In vitro models provide a very useful assessment of antimicrobial activity and can be used to screen and compare efficacy in a reproducible way between dressings. The skin models enable examination of the effect of antimicrobials on skin cells however they do not replicate all the factors involved in wound healing such as the impact of wound exudate, growth factors, proteases, antimicrobial peptides and the role of the vascular system. The ultimate test for the CROWD dressing would be an *in vivo* study on human wounds. The CROWD research has been presented at conferences and Universities within the UK and internationally and has generated a great deal of interest, particularly in developing countries such as India, Vietnam and Ghana where there are a limited range of effective and affordable antimicrobial dressings. A potential collaboration to investigate a possible clinical evaluation has been developed following a visit to the University of Ghana, Medical School in 2017. Medical colleagues are interested in testing the CROWD material on infected diabetic foot ulcers. A local pharmacist who runs a pharmaceutical manufacturing facility in Accra has also been involved in discussions about the feasibility of preparing the material in Ghana. The materials incorporated into the CROWD material are relatively cheap to buy or import. The most challenging part of the manufacturing process is the drying of the slurry which may require the purchase of a temperature and humidity controlled oven. At the time of writing various avenues for funding were being investigated to support this future work.

7.4. Antimicrobial Material Developments

7.4.1. Patent Application

An International patent application No. PCT/GB2016/050658 (Priority Date 13/3/2015) was filed on 19/3/2016 for An Antimicrobial Material by Sheffield Hallam University. There were four specific objectives of the invention listed in the Summary of the Invention:

The first was to provide an antimicrobial material for use in wound dressings that provides protection against a broad spectrum of bacteria and is configured specifically to provide a sustained and controlled release of the active antimicrobial agent.

The second objective was to provide an antimicrobial obtainable via a low cost manufacturing method that may be stored for prolonged periods of time without losing antimicrobial activity.

The third objective was that the antimicrobial material may be regarded as free – standing or self-supporting or a material that may be layered or backed by additional materials.

The fourth objective was that the antimicrobial material was capable of providing an easily identifiable visual indication of the level of antimicrobial depletion and hence an estimated time period for the continued antimicrobial properties of the material when applied as a wound dressing.

7.4.2. Novel Antimicrobial Composite Material.

The novel CROWD material has been shown in *in vitro* antimicrobial tests to be highly effective against planktonic and biofilm forms of growth of common wound pathogens. The material forms a self- stratifying film and is organised into a first and second layer. The first layer is lighter in colour and comprises of a higher concentration of clay and higher concentration of Iodine than the darker layer. The lighter layer has been shown to produce larger ZOI than the dark side suggesting that it releases the antimicrobial at a faster rate than the dark side. The dark side initially produced smaller ZOI but demonstrated a longer duration of antimicrobial action, still being effective against *S. aureus* after eight days continuous use.

Statistical analysis of the passage study results for all the antimicrobial wound dressings tested demonstrated a significant difference in the antimicrobial efficacy of the CROWD material compared with all the other commercial antimicrobial dressings tested.

7.4.3. Advantages of the CROWD material

Another advantage of the material identified during testing was the ability of the composite to draw water through the material when the dark side was placed in contact with agar. This wicking effect could be highly advantageous particularly in the management of heavily exuding wounds. The CROWD composite appears to be highly absorbent and further work is required to investigate the fluid handling properties of the material. British Pharmacopoeia standard tests will be used to determine the absorbent properties of the material. The most appropriate tests for the CROWD material would include the fluid-handling capacity (FHC) test which determines the ability of the composite to absorb and retain wound exudate whilst losing water vapour through the outer surface. Also the fluid affinity test would be useful to investigate the ability of the composite to donate moisture as well as absorb wound exudate. The ability to donate moisture would be very advantageous particularly in the management of dry necrotic wounds to facilitate rehydration and autolytic debridement (Aulton's Pharmaceutics 2013). When dehydrated the CROWD composite is brittle but it can be easily rehydrated so potentially in the dry form the material could be used on heavily exuding wounds and the more moist, flexible form, applied to wounds producing moderate to low levels of exudate. It is envisaged that either side of the dressing could be applied to a wound depending on the antimicrobial release characteristics required. The material should conform to the wound surface allowing no dead space and can be cut to fit awkward shaped wounds or to be wrapped around appendages such as finger or toes. One major advantage of the CROWD material over the only other iodine dressings suitable for the treatment of cavity wounds, Iodoflex, is that it can be removed intact from the wound bed. A problem associated with the cadexomer bead preparation is that the beads can dry out and adhere to the wound surface making removal difficult and sometimes traumatic. Iodoflex should not be used in cavity wounds with tracking sinuses due to the risk of beads being dislodged from the paste and becoming lost in a sinus where they could become a focus for infection.

7.4.4. Consideration of Secondary Dressing materials

Whilst the CROWD material is suitable for use as a primary dressing it will, like hydrogel dressings, require a secondary dressing to retain the material in position and to absorb any exudate that is wicked through the composite. The most obvious choice of secondary dressing would be absorbent foam, such as the Advazorb that was used in the commercialisation study. The impact of a secondary dressing on the unique release properties of the CROWD composite will require investigation before a clinical evaluation can be undertaken.

7.4.5. Storage Conditions

The one objective from the patent that has not yet been investigated is the determination of the storage requirements and shelf life of the material. This has proved difficult as the γ irradiation used to sterilise the composite appeared to affect the physical properties of the material and caused the release of iodine into the packaging. Wound dressings are required to be sterile so an alternative method of sterilisation has to be identified. Long term stability testing should be carried out in the exact packaging the material will be marketed in. Anecdotal evidence has shown the material fades on prolonged exposure to artificial light but the effect of this on the antimicrobial activity of the material has not been tested in a controlled manner. However, this observation did lead to the material being stored in sealed containers wrapped in tinfoil to protect from light. Due to the hygroscopic capacity of the CROWD material it will need to be sealed in a wrapper to protect it from moisture which is also suitable for the sterilisation process.

One of the key factors that could possibly affect the stability of the material is temperature. If the material is going to be used in very hot and humid countries such as Ghana then it will be required to be tested at higher temperatures and relative humidity than a product being used in a temperate climate. The World Health Organisation (WHO) (2009) classify Ghana as Climatic Zone IV, a hot and humid climate and advise long term test conditions should include testing at temperatures of 30^{o}C and 65% Relative humidity. CROWD samples will be required to be stored under various conditions for different time periods and

testing will be required to demonstrate that there has been no reduction in efficacy and any obvious degradation in the formulation.

7.4.6. Mechanical Properties

Various mechanical properties of wound dressings are also widely tested such as the tensile strength, performance under pressure and rheological properties. There are also some important clinical considerations when determining the appropriateness of material to be used as a wound dressing. The adhesive strength of the material is important as it is critical that the material does not adhere to the wound making removal traumatic and potentially damaging new tissue. As discussed, the CROWD material is highly absorbent so is unlikely to adhere the wound surface. If the material dehydrates it can easily be rehydrated with water to facilitate pain free removal from a wound. Another potential problem associated with application of a dressing to skin is possible irritation and sensitisation. This could be tested using a skin model and assessed using MALDI – MSI.

The current focus of the CROWD project is to obtain further funding to develop the material and identify a suitable secondary dressing to enable it to undergo a clinical evaluation.

7.5. Conclusion

The original aim of this research was to investigate the antimicrobial properties of commercial antimicrobial dressings available in the UK. The *in vitro* passage studies involving 20 different commercial antimicrobial dressings found that most of the dressings tested were effective for up to 48 hours against all three test bacteria. Only the silver dressings demonstrated a prolonged duration of action greater than 48 hours. The two silver foam dressings behaved differently to the other silver dressing in that they were only effective against *P. aeruginosa* and that they had an extended duration of antimicrobial action of 7 days.

In our original pilot study (Bradshaw 2011) the antimicrobial efficacy of samples of different antimicrobial dressings were tested over a 24 hour period. The iodine cadexomer dressing, Iodoflex®, was shown to completely eradicate the

test bacteria from the whole surface of the Petri dish. This observation combined with an interest in iodine chemistry led to the development of the CROWD project designed to try to create a slow release iodine containing dressing. At this point the focus of this research was diverted into the development and testing of the iodine containing clay composite material.

Having compared the antimicrobial activity of the CROWD dressing to all the commercial antimicrobial dressings tested CROWD has been shown to be more effective, particularly in terms of the prolonged duration of activity. It is not only active against planktonic bacteria but is also able to eradicate bacteria growing in simple biofilms. Differences in the release properties of the two sides of the material offer the clinician a choice depending on the required duration of action. The highly absorptive properties, combined with the colour indicator and the two side release profile make this a unique material suitable for use on infected open wounds. Since the withdrawal of Iodozyme from the market in 2017 the only remaining iodine containing dressing which claims to have slow release properties is Iodoflex. CROWD has been shown to have a similar cytotoxicity profile to Iodoflex but a superior antimicrobial duration of action and has the added advantage that it can be easily removed intact from the wound.

The ideal wound dressing would combine proven clinical effectiveness with high tolerability, low cost and have a simple, reliable method of manufacture and sterilisation. The antimicrobial dressings investigated in this study have a limited clinical evidence base, and in this *in vitro* testing have been shown to have performance limited to the short term.

The unique CROWD material offers the opportunity to develop a product with a sustained antimicrobial effect, supported by evidence of activity against biofilms and a level of cytotoxicity similar to the only other iodine dressing marketed for the treatment of infected wounds (Iodoflex). Further development work is required to optimise the manufacturing and sterilisation processes before this promising material can be made available to improve the care of patients with infected wounds.

References

Abdelrahman T, Newton H (2011) Wound dressings: principles and practice. *Surgery*, 29(10): 491-495.

Adams CJ, Boult CH, Deadman BJ, Farr JM, Grainger MNC, Manley-Harris M, Snow MJ (2008) Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydr. Res,* 343: 651–659.

Adams CJ, Manley-Harris M, Molan PC (2009) The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydr. Res,* 344: 1050–1053.

Agrawal K, Chauhan N (2012) Pressure ulcers: Back to the basics. *Indian journal of plastic surgery: official publication of the Association of Plastic Surgeons of India*, *45*(2): 244–254.

Agency for Healthcare Research and Quality (2014) Chronic venous ulcers: a comparative effectiveness review of treatment modalities. *Comparative Effectiveness Review*, 127.

Agostinho A, Hartman A, Lipp C, Parker K, Stewart PS, James GA (2011) An *in vitro* model for the growth and analysis of chronic wound MRSA biofilms. *J of Applied Micorbiology,* 111(5):1275-1282.

Ågren MS (1996) Four alginate dressings in the treatment of partial thickness wounds: A comparative study. *J. Plast Surg*, 49:129-134.

Ågren MS, Mirastschijski U (2004) The release of zinc ions from and cytocompatilibity of two zinc oxide dressings. *J Wound Care*, 13(9):367-9.

Akiyama H, Oono T, Saito M, Iwatsuki K (2004) Assessment of cadexomer iodine against *Staphylococcus aureus* biofilm *in vivo* and *in vitro* using confocal laser scanning microscopy. *J Dermatol*, 31:529-34.

Alhede M, Bjarnsholt T, Jensen PØ, Phipps KR, Moser C, Christophersen L, Christensen LD, van Gennip M, Parsek M, HØiby N, Rasmussen TB, Givskov M (2009) *Pseudomonas aeruginosa* recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. *Microbiology,* 155 (Pt 11):3500-8.

Alam F, Islam MA, Gan SH, Khalil MI (2014) Honey: A potential Therapeutic Agent for Managing Diabetic Wounds. *Evidence based Complement Alternat Med*: 169130.

Alavi A, Sibbald RG, Phillips TJ, Miller OF, Margolis DJ, Marston W, Woo K, Romanelli M, Kirsner RS (2016) What's new: Management of venous leg ulcers: Treating venous leg ulcer. *J Am Acad of Dermatol*, 74(4): 643-664.

Allen MJ, Morby AP, White GF (2004) Cooperativity in the binding of the cationic biocide polyhexamethylene biguanide to nucleic acids. *Biochem Biophys Res Commun,* 318(2):397-404.

Aramwit P, Muangman P, Namviriyachote N, Srichana T (2010) *In vitro* evaluation of the Antimicrobial effectiveness of Moisture Binding properties of Wound Dressings. *Int J Molecular Science*s, 11(8):2864-2874.

Ammons MC, Copié V (2013) Mini-Review: lactoferrin: a bioinspired, anti-biofilm therapeutic. *Biofouling*, 29(4):443-55.

Anderson I (2001) The properties of hyaluronan and its role in wound healing. *Prof Nurse,* 17:232-5.

Andrews JM (2001) Determination of minimum inhibitory concentrations. *J Antimicrobial Chemotherapy,* 48 (Suppl 1):5-16.

Andriessen A, Eberlein TH (2008) Assessment of a wound cleansing solution in the treatment of problem wounds. *Wounds,* 20(60):171-5.

Angel DE, Lloyd P, Carville K, Santamaria N (2011) The efficacy of two semi-quantitative wound – swabbing techniques in identifying causative organism(s) in infected cutaneous wounds. *Int Wound J,* 8(2):176-185.

Archimed (2010a). Smart science — wound healing. http://www.archimed.co.uk/images/stories/smartscience1.pdf

Archimed (2010b). OxyzymeTM and iodozymeTM.

http://www.biologiq.nl/UserFiles/OXYZYME%20internet.pdf.

Archimed (2010c). Anti-microbial effectiveness of iodozyme. http://www.biologiq.nl/UserFiles/Presentatie%20Antimictrobiele%20effectiviteit%20van%20Iodozyme.pdf.

Armstrong D, Boulton AJM, Bus SA (2017) Diabetic foot ulcers and their recurrence. *New Eng J Med*, 376:2367-75.

Arya AK, Tripathi R, Kumar S, Tripathi K (2014) Recent advances on the association of apoptosis in chronic non-healing diabetic wounds. *World J Diabetes*, 5(6):756-62.

Attinger CE, Jasnis JE, Steinburg J, Schwartz J, Al-Attar A, Couch K (2006) Clinical approach to wounds: debridement and wound bed preparation including the use of dressings and wound – healing adjuvants. *Plast Recontrs Surg,* 117(Suppl):72S-109S.

Aulton's Pharmaceutics. (2013) The Design and Manufacture of Medicines. Fourth Edition. Editors Aulton ME and Taylor KMG. Churchill Livingstone, Elsevier, London, UK.

Bæk KT, Frees D, Renzoni A, Barras C, Rodriguez N, Manzano C, and Kelley WL (2013) Genetic Variation in the *Staphylococcus aureus* 8325 Strain Lineage Revealed by Whole-Genome Sequencing PLoS One. 2013; 8(9): e77122.

Balin AK, Pratt L (2002) Dilute povidone iodine solutions inhibit human skin fibroblast growth. *Dermatologic Surgery,* 28(3):210-4.

Banin E, Brady KM, Greenberg EP (2006) Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells. *App Environ Microbiol*, 72:2064-9.

Bansal E, Garg A, Bhatia S, Attri AK, Chander J (2008) Spectrum of microbial flora in diabetic foot ulcers. *Indian J Pathol Microbiol,* 51:204-8.

Barillo DJ, Marx DE (2014) Silver in Medicine: a brief history BC 335 to present day. *Burns* 40 (Suppl 1): S3-8.

Baur D, Gladstone BP, Burkert Carrara E, Foschi F, Döbele S, Tacconelli E (2017) Effect of antibiotic stewardship on the incidence of infection and colonisation with antibiotic – resistant bacteria and clostridium difficile infection: a systematic review and meta-analysis. *Lancet,* 17:990-1001.

Beidler SK, Douillet CD, Berndt DF, Keagy DF, Rich PB, Marston WA (2008) Multiplexed analysis of matrix metalloproteinases in leg ulcer patients with chronic venous insufficiency before and after compression therapy. *Wound Repair Regen*, 16(5):642-8.

Bennett LL, Rosenblum RS, Perlove C, Davidson JM, Barton RM, Nanney LB (2001) An *in vitro* comparison of topical agents on wound repair. *Plast Reconstr Surg,* 108:675-87.

Bergaya F, Theng BKG, Lagaly G (2006) Handbook of Clay Science-Developments in Clay Science Volume1, 1st Edition. Elsevier Science, Oxford, UK.

Berney M, Hammes F, Bosshard F, Welienmann HU, Egli T (2007) Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BackLight Kit in combination with Flow Cytometry. *Appl Environ Microbiol*, 73(10):3283-3290.

Bessa LJ, Fazii P, Di Giulio M, Cellini L (2015) Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection. *Int Wound J*, 12(1):47-52.

Bianchi J (2001) Cadexomer-iodine in the treatment of venous leg ulcers: what is the evidence? *J Wound Care,* 10:225-9.

Bigliardi PL, Alsagoff S A L, El-Kafrawi HY, Pyon J-K, Wa CTC, Villa MA (2017) Povidone iodine in wound healing: A review of current concepts and practices. *Int J of Surgery,* 44:260-268.

Bischt K, Wakeman CA (2019) Discovery and Therapeutic Targeting of DifferentiatedBiofilmSubpopulations.FrontMicrobiol/https://doi.org/10.3389/Fmicb2019.01908

Bjarnsholt T, Eberlein T, Malone M, Schultz G (2017) Management of wound Biofilm Made Easy. London: *Wounds International*, 8(2):1-6.

Bjarnsholt T, Kirketerp-Moller K, Jensen P, Kit M, Krogfelt K, Phipps R, Holby N, Glyskov M (2008) Why chronic wounds won't heal: a novel hypothesis. *Wound Repair Regen,* 1:2-10.

Blake FA, Abromeit N, Bubenheim M, Li L, Schmelzle R (2007) The biosurgical wound debridement: experimental investigation of efficiency and practicality. Wound *Repair Regen*, 15:756-761.

Blueman D, Bousfield C (2012) The use of larval therapy to reduce the bacterial load in chronic wounds. *J Wound Care*, 21:244-253.

BNF: British National Formulary (September 2015 – March 2016) No. 70. BMJ Group and Royal Pharmaceutical Society of Great Britain, London, UK.

BNF: British National Formulary (September 2019 – March 2020) No 78. BMJ Group and Royal Pharmaceutical Society of Great Britain, London, UK.

Boles BR, Theondel M, Singh PK (2004) Self-generated diversity produces insurance effects in biofilm communities. *Proc Natl Acad Sci* USA **,**101:166630-5

Bonham PA (2003) Assessment and management of patients with venous, arterial, and diabetic/neuropathic lower extremity wounds. *AACN Clin,* Issues 2003, 14: 442–456.

Bourdillon KA, Delury CP, Cullen BM (2017) Biofilms and delayed healing – an in *vitro* evaluation of silver and iodine containing dressings and their effect on bacterial and human cells. *Int Wound J*, 14(6):1066-1075.

Bowler PG (2003) The 10^5 bacterial growth guideline: reassessing its clinical relevance in wound healing. *Ostomy Wound Manage,* 49:44-53.

Bowler PG, Duerden BI and Armstrong DG (2001) Wound Microbiology and associated Approaches to Wound Management. *Clinical Microbiology Reviews*, 14(2):244-269.

Boyko TV, Longaker MT, Yang G P (2018) Review of the Current Management of Pressure Ulcers. *Adv in Wound Care*, *7*(2): 57–67.

Boyle-Vavra S, Daum RS (2007) Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab. Invest*, 87 (1): 3–9.

Bradshaw CE (2011) An *in vitro* comparison of the antimicrobial activity of honey, iodine and silver wound dressings. *Bioscience Horizons,* 4(1):61-70.

Braga IA, Pirett CC, Ribas RM, Gontijo Filho PP, Diogo Filho A (2013) Bacterial colonisation of pressure ulcers: assessment of risk for blood stream infection and impact on patient outcomes. *J Hosp Infect,* 83(4):314-20.

Bragg PD, Rainnie DG (1974) The effect of silver ions on the respiratory chain of *E. coli*. *Can J Microbiol*, 2096: 883-9.

Braza ME, Fahrenkopf MP (2020) Split-Thickness Skin Grafts. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK551561/

Brenes RA, Sobotka L, Ajemian MS, Manak J, Vyroubal P, Slemrova M, Adamkova V, Zajic J, Dudrick SJ (2011) Hyaluronate-iodine complex. A new adjunct for the management of complex sternal wounds after cardiac operation. *Arch Surg*, 146(11):1323-1325.

Brin YS, Mumcuoglu KY, Massarwe S, Wigelman M, Gross E, Nyska M (2007) Chronic foot ulcer management using maggot debridement and topical negative pressure therapy. *J Wound Care,* 16:111-3.

Brindle T, Farmer P (2019) Undisturbed wound healing: a narrative review of literature and clinical considerations. *Wounds International* 10 (20):40-8.

BritishStandardEN16756
https://standards.globalspec.com/std/1688355/PREN%2016756

Broderick C, Pagnamenta F, Forster R (2020) Dressings and topical agents for arterial leg ulcers. Cochrane Database of Systematic Reviews 2020, Issue 1. Art. No.: CD001836. DOI: 10.1002/14651858.CD001836.pub4

Brown JA (2004) The role of hyaluronic acid in wound healing's proliferative phase. *J Wound Care,* 13:48-51.

Brown ML, Aldrich HC, Gauthier JJ (1995) Relationship between glycocalyx and povidone iodine resistance in *Pseudomonas aeruginosa* (ATCC 27853) Biofilms. *Appl Environ Microbiol*, 61(1):187-193.

Brown ML, O'Hara FP, Close NM, Mera RM, Miller LA, Suaya JA, Amrine-Madsen H (2012) Prevalence and Sequence Variation of Panton-Valentine Leukocidin in Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Strains in the United States. *J Clin Microbiol*, 50(1): 86–90.

Broxton P, Woodcock PM, Gilbert P (1984) Injury and recovery of Escherichia coli ATCC 8739 from treatment with some polyhexamethylene biguanides. *Microbios,* 40:161-162, 187-193.

Buckingham-Meyer K, Goeres DM, Hamilton MA (2007) Comparative evaluation of biofilm disinfectant efficacy tests. *J Microbiol Methos,* 70(2): 236-244.

Burnand KG, Whimster I, Naidoo A, Browse NL (1982) Pericapillary fibrin in the ulcer-bearing skin of the leg: the cause of lipodermatosclerosis and venous ulceration. *Br Med J* (Clin Res Ed), 285:1071–2.

Burrell RE (2005) Comparison of *in vitro* disc diffusion and time-kill kinetic assays for the evaluation of antimicrobial wound dressing efficacy. *Wound Rep Reg,* 13(4):412-421.

Burkes RI (1998) Povidone- Iodine Solution in wound treatment. *Phys Ther*, 78: 212-218.

Butcher M (2012) PHMB: an effective antimicrobial in wound bioburden management. *Br J of Nursing* (Tissue Viability Supplement), 21(12): S16-21.

ByK (Atlanta Group) Cloisite®Na+ Data Sheet: https://polymer-additives.specialchem.com/product/a-byk-altana-group-cloisite-na

ByK Additives and Instruments. Laponite Performance Additives. Technical information B-R1 21 https://www.byk.com/.../technical_brochures/BYK_B-RI21_**LAPONITE**_EN.pdf

Cascone S, Lamberti (2020) Hydrogel based commercial products for biomedical applications: A review. *Int J Pharmaceutics*, 2020: 1-19.

Calissendorff J, Falhammer H (2017) Lugol's solution and other iodide preparations: perspectives and research directions in Grave's disease. *Endocrine*, 58(3): 467-473.

Centre for Evidence-based Purchasing (2008) Buyer's guide: advanced wound dressings. Online. https://www.nice.org.uk/advice/esmpb2/resources/chronic-wounds-advanced-wound-dressings-and-antimicrobial-dressings-pdf-1502609570376901

Chhabra S, Chhabra N, Kaur A, Gupta N (2017) Wound Healing Concepts in Clinical Practice of OMFS. *J Maxillofacial and Oral Surgery*, *16*(4):403–423.

Chakraborty A, Saralaya V, Adhikari P, Shenoy S (2017) Virulence property, phylogenetic background, amd resistance pattern of *Escherichia coli* isolates from wound infections. CHRISMED *J. Health Res* 4(4):248-252.

Chaw KC, Manimaran M, Tay FEH (2005) Role of silver ions in destabilization of intermolecular adhesion forces measured by atomic force microscopy in Staphylococcus epidermidis biofilms. *Antimicrob Agents Chemother,* 49(12):4853-59.

Chen C, Campbell L, Blair SE, Carter DA (2012) The effect of heat treatment on the antimicrobial properties of honey. *Front Microbiol,* 3(265):1-8.

Cokcetin NN, Pappalardo M, Campbell LT, Brooks P, Carter DA, Blair SE, Harry EJ (2016) The Antimicrobial Activity of Australian Leptospermum Honey Correlates with Methylglyoxal Levels. PloS ONE 11(12): e0167780.

Coleridge Smith PD, Thomas P, Scurr JH, Dormandy JA (1988) Causes of venous ulceration: a new hypothesis. *Br Med J* (Clin Res Ed), 296(6638):1726–7.

Collier M (2004) Recognition and management of wound infections. *World Wide Wounds*. http://www.worldwidewounds.com/2004/january/Collier/Management-of-Wound-infections.html

Collins F, Hampton S, White R (2002) A-Z Dictionary of Wound Care. Quay Books. London, UK.

Comerota A, Lurie F (2015) Pathogenesis of venous ulcer. *Seminars in Vascular Surgery*, 28:6-14.

Cooper RA (2007) Iodine revisited. *International Wound Journal,* 4:124-137.

Cooper RA, Jenkins L (2009) A comparison between Medical Grade Honey and Table honeys in relation to antimicrobial efficacy. *Wounds*, 21(2):29-36.

Cooper P (2011) Managing Wound infection and Pain using Suprasorb X + PHMB. *Wounds UK*, 7(2) Supplement: 10-14.

Cooper R, Bjarnsholt T, Alhede M (2014) Biofilms in Wounds: A review of present knowledge*. J Wound Care,* 23(11):570-582.

Copeland-Halperin LR, Kaminsky AJ, Bluefeld N and Miraliakbari R (2016) Sample procurement for cultures of infected wounds; a systematic review. *J Wound Care,* 25(4): S4-S10.

Cray A (2010) Honey treatments for wounds. *J Community Nursing,* 24 (2):22-7.

Cutting K, McGuire J (2015) Safe bioburden management. A clinical review of DACC technology. *J Wound Care*, 24(5): S1-30.

Cutting KF, White R (2004) Defined and refined: criteria for identifying wound infection revisited. *Br J Community Nurs*, 9: S6-S15.

Cutting KF, White RJ (2005) Clinical Identification of Wound Infection: A Delphi Approach. Identifying Criteria for Wound Infection, EWMA Position Document. London: MEP

Cutting K, White R, Hoekstra H (2009) Topical silver-impregnated dressings and the importance of the dressing technology. *Int Wound J*, 6:396-402.

Dabiri G, Damstetter E, Phillips T (2016) Choosing a Wound Dressing Based on Common Wound Characteristics. *Adv Wound Care*, *5*(1): 32–41.

Darby I, Skalli O, Gabbiani G (1990) α-Smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest*, 63: 21–29.

Daubney L (2007) Smith & Nephew Wound Management Data on File Report-0706038.

D'Auria J, Lipson S, Garfield JM (1990) Fatal iodine toxicity following debridement of a hip wound: case report. *J Trauma*, 30:353-5.

Davies D (2003) Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discovery*, 2:114-122.

Davis SC, Martinez L, Kirsner R (2006) The diabetic foot: the importance of biofilms and wound bed preparation. *Curr Diab Rep,* 6:439-45.

Davis PJ (2007) How might we achieve oxygen balance in wounds? *International Wound Journal*, 4:18-24.

Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM (2008) Microscopic and physiologic evidence for biofilm - associated wound colonization *in vivo*. *Wound Repair Regen*, 16:23-29.

De Kievit TR (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol*, 11(2):279-288.

de Oliveira S, Rosowski EE, Huttenlocher A (2016). Neutrophil migration in infection and wound repair: going forward in reverse. *Nature reviews. Immunology*, *16*(6), 378–391.

Desmouliere A, Redard M, Darby I, Gabbiani G (1995) Apoptosis mediates the decrease in cellularity during the transition between granulation-tissue and scar. *Am J Pathol*, 146: 56–66.

Dhivya S, Padma VV, Santhini E (2015) Wound Dressings a Review. *Biomedicine*, 5(4):24-8.

Diabetes.org. 2019 - https://www.diabetes.org.uk/about_us/news/new-stats-people-living-with-diabetes.

Diegelmann RF, Evans MC (2004) Wound healing: An overview of acute, fibrotic and delayed healing. *Front Biosci,* 9: 283–289.

Dinman S (2007) Medical Maggots. *Plast Surg Nurs,* 27:212-4.

Donlan RM (2002) Biofilms: Microbial Life on Surfaces. *Emer Infect Dis*, 8(9):881-890.

Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*., 15:167-193.

Dow G, Browne A, Sibbald RG (1999) Infection in Chronic Wounds: Controversies in Diagnosis and Treatment. *Ostomy/Wound Management*, 45(8):23-40.

Dowsett C (2013) Biofilms: a practice based approach to identification and treatment. *Wounds UK*, 9(2):68-72.

Dowsett C, Bielby A, Searle R (2014) Reconciling increasing wound care demands with available resources. *J Wound Care*, 23(11):552-562.

Dryden MS, Johnson AP, Ashiru-Oredope D, Sharland M (2011) Using antibiotics responsibly: right drug, right time, right dose, right duration. *J Antimicrob Chemother*, 66: 2441–3.

Dumville JC, Hinchliffe RJ, Cullum N, Game F, Stubbs N, Sweeting M, Peinemann F (2013) Negative pressure wound therapy for treating foot wounds in people with diabetes mellitus. *Cochrane Database of Systematic Reviews* 2013, Issue 10. Art. No.: CD010318. DOI: 10.1002/14651858.CD010318.pub2

Edmonds M, Foster AVM, Vowden P (2004) Wound bed preparation for diabetic foot ulcers. In EWMA Position Document. Wound bed preparation in practice. MEP Ltd, London: UK.

Edwards R, Harding KG (2004) Bacteria and Wound Healing. *Curr Opin Infect Dis*, 17(2):91-6.

Edwards - Jones V (2009) The benefits of silver in hygiene, personal care and healthcare. *Lett Appl microbial*, 49:147-52.

Edward - Jones V (2013) Wound Infection. In Flanagan M, ed. (2013) Wound healing and Skin Integrity: Principles and Practice. Wiley-Blackwell, Sussex, UK.

Edward – Jones V (2016) Essential Microbiology for Wound Care. Oxford University Press, Oxford, UK.

Elbright JR (2005) Microbiology of chronic leg and pressure ulcers: clinical significance and implications for treatment. *Nurs Clin N Am,* 40(2):207-16.

Eming SA, Smola- H, Hartmann B, Malchau G, Wegner R, Krieg T, Smola-Hess S (2008) The inhibition of matrix metalloproteinase activity in chronic wounds by a polyacrylate superabsorber. *Biomaterials*, 29(19): 2832-40.

Eming SA, Smola-Hess S, Kurschat P, Hirche D, Kreig T, and Smola H (2006) A novel property of povidone-iodine: inhibition of excessive protease levels in chronic non-healing wounds. *J Invest Dermatol,* 126:2731-3.

European Pharmacopoeia (2011) Methods of Preparation of Sterile Products. 5.1.1, 5th Council of Europe, Strasbourg.

Evans R, Dudley E, Nigam Y (2015) Detection and partial characterisation of antifungal bioactivity from the secretions of medicinal maggot*, Lucilia seicata*. Wound *Repair Regen*, 23:361-368.

EWMA - European Wound Management Association (2006) Position Document: Identifying criteria for wound infection. MEP, London

EWMA (2013) Antimicrobials and Non-Healing wounds: Evidence, controversies and suggestions. *J Wound Care,* 22(5) EWMA Document

Fair RJ, Tor Y (2014) Antibiotics and bacterial resistance in the 21st century. *Perspectives Medicinal Chem*, *6*: 25–64.

Fazli M, Thomas Bjarnsholt T, Kirketerp-Møller K, Jørgensen B, Andersen AS, Krogfelt KA, Givskov M, Tolker-Nielsen T (2009) Non-random Distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in Chronic Wounds. *J Clinical Microbiology,* 47(12):4084-4089.

Fisher TK, Wolcott R, Wolk DM, Bharara M, Kimbriel HR, Armstrong DG (2010) Diabetic foot infections: a need for innovative assessments. *Int J Low Extrem Wounds*, 9:31-36.

Fitzgerald DJ, Renick PJ, Forrest EC, Tetens SP, Earnest DN, McMillan J, Kiedaisch BM, Shi Lei, Roche ED (2017) Cadexomer Iodine provides superior efficacy against bacterial wound biofilms *in vitro* and *in vivo*. *Wound Repair Regen*, 25 (1):13-24.

Fleck CA (2006) Fighting infection in chronic wounds. *Adv. Skin Wound Care*, 19:184-188.

Fleming A (1919) The action of chemical and physiological antiseptics in a septic wound. *Br J Surg,* 7:127.

Flemming HC, Wingender J (2010) The biofilm matrix. *Nat Rev Microbiol*, 8:623-33.

Fonder MA, Lazarus GS, Cowan DA, Aronson-Cook B, Kohli AR, Mamelak AJ (2008) Treating the chronic wound: A practical approach to the care of non-healing wounds and wound care dressings. *J Am Acad Dermatol*, 58:185–206.

Forster R, Pagnamenta F (2015) Dressings and topical agents for arterial ulcers. Cochrane Database of Systematic Reviews issue 6:CD001836

Foster TJ, Hook M (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol*, 6:484-488.

Fox CL (1983) Topical therapy and the development of silver sulphadiazine. *Surg Gynecol Obstet,* 157:82-8.

Frankova J, Kubaka L, Velebny V (2006) The effect of hyaluronan combined with K13 complex (Hyiodine wound dressing) on keratinocytes and immune cells. *J Mater Sci Mater Med*, 17:891-8.

Franks PJ, Barker J, Collier M, Gethin G, Haesler E, Jawien A, Laeuchli S, Mosti G, Probst S (2016) Management of Patients with Venous Leg Ulcers: Challenges and Current Best Practice. *J Wound Care,* 25(6), S1-S67.

Frykberg RG, Banks J (2015) Challenges in the Treatment of Chronic Wounds. *Adv Wound Care* (New Rochelle), 4(9):560-582.

Fumal I, Braham C, Paquet P, Franchimont C, Pierard GE. (2002) The beneficial toxicity paradox of antimicrobials in leg ulcer healing impaired by a polymicrobial flora: a proof-of-concept study. *Dermatology*, 204(Suppl 1):70-74.

Gallant – Behm CL, Yin HQ, Liu S, Heggers JP, Langford RE, Olson ME, Hart DA, Burrell RE (2005) Comparison of *in vitro* disc diffusion and time-kill kinetic assays for the evaluation of antimicrobial wound dressing efficacy. *Wound Rep Reg*, 13(4):412-421.

Gardner SE, Frantz RA (2008) Wound Bioburden and infection-related complications in diabetic foot ulcers. *Biol Res Nurs*, 10(1):44-53.

Gardner SE, Frantz RA, Doebbeling BN (2001) The validity of the clinical signs and symptoms used to identify localized chronic wound infection. *Wound Repair Regen*, 9(3):178-86.

Gardner SE, Frantz R, Hillis SL, Park H, Scherubel M (2007) Diagnostic validity of semi quantitative swab cultures. *Wounds*, 19 (2): 31-8.

Garraud O, Hamzeh-Cognasse H, Cognasse F (2012) Platelets and cytokines: How and why? *Transfus Clin Biol*, 19:104-8.

Garraud O, Hozzein WE, Badr G (2017) Wound healing: time to look for intelligent, “natural” immunological approaches? *BMC Immunology,* 18(Suppl 1)23:39-46.

Gauglitz GG, Korting HC, Pavicic T, Ruzicka MG, Jeschke MG (2011) Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. *Mol Med*, 17:113-125.

Geraci M, Borja MC (2018) Notebook: The Laplace distribution. *The Royal Statistical Society Significance,* 15(5):10-11.

Gilbert P, Moore LE (2005) Cationic antiseptics: diversity of action under a common epithet. *J Appl Microbiol*, 99:703-715.

Gilchrist B (1997) Should iodine be reconsidered in wound management? European Tissue Repair Society. *J Wound Care*, 6(3):148-50.

Gill SE, Parks WC (2008) Metalloproteinases and their inhibitors: regulators of wound healing. *Int J Biochem Cell Biol,* (40): 1334-47.

Gjødsbøl K, Christensen JJ, Karlsmark T, Jørgensen B, Klein BM, Krogfelt KA (2006) Multiple bacterial species reside in chronic wounds: a longitudinal study. *Int Wound J,* 3(3):225-31.

Goldman R (2004) Growth factors and chronic wound healing: Past, present, and future. *Adv Skin Wound Care*, 17:24–35.

Gottardi W (1985) The influence of the chemical behaviour of iodine on the germicidal action of disinfectant solutions containing iodine. *J Hosp Infect*, 6 Suppl. A: 1-11.

Gottardi W (1991) Iodine and iodine compounds, p152-166. In: Block SS (ed.) Disinfection, sterilization and preservation. 4th edn. Lea & Febiger Philadelphia, Pa.

Gottardi W (1999) Iodine and Disinfection: Theoretical Study on Mode of Action, Efficiency, Stability, and Analytical Aspects in Aqueous System. *Arch Pharm*, 332(5):151-157.

Gottrup F, Apelqvist J (2020) The challenge of using randomized trials in wound healing. *British J Surg,* 97:303-4.

Gottrup F, Bjarnsholt T, Cooper R, Moore Z, Peters EJG, Probst S (2013) EWMA document: antimicrobials and non-healing wounds. Evidence, controversies and suggestions. *J Wound Care*, 22(5): S1-89:1 –92.

Gottrup F, Melling A, Hollander DA (2005) An overview of surgical site infections: Aetiology, incidence and risk factors; World Wide Wounds http://www.worldwidewounds.com/2005/september/Gottrup/Surgical-Site-Infections-Overview.html

Gottrup F, Jorgensen B (2011) Maggot debridement: an alternative method for debridement. *Eplasty*, 11: e33.

Grant SS, Hung DT (2013) Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. *Virulence*, 4:83:274-283.

Gray D, Acton C, Chadwick P, Fumarola S, Leaper D, Morris C, Stang D, Vowden K, Vowden P, Young T (2011) Consensus guidance for the use of debridement techniques in the UK. *Wounds UK*, 7(1):77-84.

Gray D, White RJ, Cooper P, Kingsley AR (2010) Applied Wound Management and using the Wound Healing Continuum in Practice. *Wound Essentials*, 5:131-9.

Grey JE, Harding KG, Enoch S (2006) ABC of wound healing: venous and arterial leg ulcers. *BMJ*, 332(7537): 347-350.

Grey JE, Harding KG, Enoch S. (2006) Venous and arterial leg ulcers. *BMJ*, 332: 347–350.

Guest JF, Ayoub N, McIlwraith T, Uchegbu I, Gerrish A, Weidlich D, Vowden K, Vowden P (2015) Health Economic burden that wounds impose on the National Health Service in the UK. *BMJ* Open 2015 (5): e009283.doi:10.1136/bmjopen-2015-009283

Guest JF, Ayoub N, McIlwraith T, Uchegbu I, Gerrish A, Weidlich D, Vowden K, Vowden P (2017) Health economic burden that different wound types impose on the UK's National Health Service*. Int Wound J,* 14(2):322–330.

Guest JF, Fuller GW, Vowden P (2018) Venous leg ulcer management in clinical practice in the UK: costs and outcomes. *Int Wound J,* 15(1): 29-37.

Guo S, DiPietro LA (2010) Factors affecting wound healing. *J Dent Res*, 89:219-29.

Gupta A, Maynes M, Silver S (1998) Effects of halides on plasmid-mediated silver resistance in *Escherichia coli*. *Applied Environ Micro*, 64(12):5042-45.

Gurtner GC, Evans GRD (2000) Advances in head and neck reconstruction. *Plast Reconstr Surg*, 106:672–682.

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. *Nature*, 453(7193):314-21.

Halstead F, Rauf M, Bamford A, Wearn C, Bishop, Johnathan RB, Burt R, Fraise AP, Moiemen S, Oppenheim BA, Webber MA (2015) Antimicrobial dressings: Comparison of the ability of a panel of dressings to prevent biofilm formation by key burn wound pathogens. *Burns,* 41(8):1683-1694.

Halstead FD, Webber MA, Rauf M, Burt R, Dryden M, Oppenheim BA(2016) *In vitro* activity of an engineered honey, medical-grade honeys, and antimicrobial wound dressings against biofilm-producing clinical bacterial isolates. *J Wound Care,* 25: 93–102.

Hartman (2020) ttps://www.hartmann.info/en-gb/our-products/wound-management/advanced-wound-care/hydro-responsive-wound-dressing/hydroclean®-plus (Last accessed 3 May 2020)

Han G, Ceilley R (2017) Chronic Wound Healing: A Review of Current Management and Treatments. *Adv Ther,* 34:599-610.

Hannigan GD, Grice EA (2013) Microbial ecology of the skin in the era of meta genomics and molecular microbiology. *Cold Spring Harb Perspect Med*, 3(12): a015362

Harbison MA, Hammer SM (1989) Inactivation of human immunodeficiency virus by Betadine products and chlorhexidine. *J Acquir Immune Def Syndr*, 2:16-20.

Hardwicke J, Schmaljohann D, Boyce D, Thomas D (2008) Epidermal growth factor therapy and wound healing-past, present and future perspectives. *Surgeon*, 6(3):172-7.

Hatch RA, Schiller NL (1998) Alginate lyase promotes diffusion of aminoglycosides through the extracellular polysaccharide of mucoid *Pseudomonas aeruginosa*. *Antimcrob Agents Chemother*, 42:974-7.

Haukipuro K, Melkko J, Risteli L, Kairaluoma M, Risteli J (1991) Synthesis of type I collagen in healing wounds in humans. *Ann Surg*, 213 (1):75-80.

Heath K, Angel D, Campbell C, Jackson M, Kourtis S, Legg A (2019) Cleansing and debridement. Therapeutic Guidelines: ulcer and wound management Version 2 Melbourne: Therapeutic Guidelines Limited 109.

Heilborn JD, Nilsson MF, Kratz G, Weber G, Sørensen O, Borregaard N, Stahle-Bäckdahl M (2003) The cathelicidin anti-microbial peptide LL-37 is involved in the re-epithelialization of human skin and is lacking in chronic ulcer epithelium. *J Invest Dermatol,* 120:379-89.

Heinzelmann M, Scott M, Lam T (2002) Factors predisposing to bacterial invasion and infection. *Am J Surg,* 183: 179-90.

Helaine S, Kugelberg E (2014) Bacterial persisters: formation, eradication and experimental systems. *Trends Microbiol*, (22):417-424.

Henriques AF, Jenkins RE, Burton NF, Cooper RA (2010) The intracellular effects of manuka honey on *Staphylococcus aureus*. *Eur J Clin Microbiol Infect Dis,* 29:45-50.

Henriques AF, Jenkins RE, Burton NF, Cooper RA (2011) The effect of manuka honey on the structure of *Pseudomonas aeruginosa*. *Eur J Clin Microbiol Infect Dis,* 30(2):167–71.

Hess C. (1999) When to use hydrocolloid dressings. *Nursing*, 29:11- 20.

Herrick SE, Sloan P, McGurk M, Freak L, McCollum CN, Ferguson MW (1992) Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. *Am J Pathol*, 141(5):1085-95.

Hill KE, Malic S, McKee R, Rennison T, Harding KG, Williams DW, Thomas DW (2010) An *in vitro* model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities. *J Antimicrob Chemother,* 65(6): 1195-1206.

Hinman CD, Maibach H (1963) Effect of air exposure and occlusion on experimental human skin wounds. *Nature,* 200: 377-378.

Hirsch T, Koerber A, Jacobsen F, Dissemond J, Steinau, H-U, Gatermann S, Al-Benna, S, Kesting M, Seipp, H, Steinstraesser L (2009) Evaluation of Toxic Side Effects of Clinically Used Skin Antiseptics *In vitro*. *Journal of Surgical Research*: 1-7.

Hoekstra MJ, Westgate SJ, Mueller S (2017) Povidone–Iodine ointment demonstrates *in vitro* efficacy against biofilm formation. *Int Wound J*, 14(1):172-179.

Honnegowda TM, Kumar P, Udupa EGP, Kumar S, Kumar U, Rao P(2015) Role of angiogenesis and angiogenic factors in acute and chronic wound healing. *Plast Aesthet Res,* 2:243-249.

HSE (2018) *HSE National wound management guidelines. Health Service Executive*.https://healthservice.hse.ie/filelibrary/onmsd/hse-wound-management-guidelines-2018.pdf

Hunter S, Langemo D, Thompson P, Hanson D, Anderson J (2009) Maggot Therapy for Wound Management. *Adv in Skin & Wound Care*, 22(1):25-27.

Hutchinson JJ, Mcguckin M (1990) Occlusive dressings: a microbiologic and clinical review. *Am J Infect Control*, 18: 257.

Hutchinson JJ, Lawrence JC. (1991) Wound infection under occlusive dressings. *J Hosp Infect*, 17: 83-94.

Ikeda T, Ledwith A, Bamford CH, Hann RA (1984) Interaction of polymeric biguanide biocide with phospholipid membranes. *Biochem Biophys Acta,* 769(1):57-66.

Innes ME, Umraw N, Fish JS, Gomez M, Cartotto RC (2001) The use of silver coated dressings on donor site wounds: a prospective, controlled matched pair study. *Burns*, 6:621-7.

International Consensus. Appropriate use of silver dressings in wounds. An expert working group consensus. London: Wounds International, 2012. Available to download from: www.woundsinternational.com

International Wound Infection Institute (IWII) (2016) Wound Infection in Clinical Practice: Principles of best practice. Wounds International.

Ivins N, Simmonds W, Turner A, Harding K (2007) The use of an oxygenating hydrogel dressing in venous leg ulcers. *Wounds UK*, 3:1–5.

James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS (2008) Biofilms in Chronic Wounds. *Wound Repair Regen,* 1:37-44.

Jenkins RE, Cooper RA, Burton NF (2008) Poster presentation “The determination of antimicrobial activity of 3 honey impregnated wound dressings by challenge test with EMRSA- 15” at 3rd Congress of the World Union of Wound Healing Societies held in Toronto between 4-8 June 2008.

Jenkins R, Burton N, Cooper R (2011) Effect of manuka honey on the expression of universal stress protein A in methicillin-resistant *Staphylococcus aureus*. *Int. J. Antimicrob. Agents,* 37:373–376.

Jenne CN, Kubes P (2015) Platelets in inflammation and infection. *Platelets,* 26:286-92.

Jensen PO, Bjarnsholt T, Phipps R, Rasmussen TB, Calum H, Christoffersen L, Moser C, Williams P, Pressler T, Givskov M, Høiby N (2007) Rapid necrotic killing of Polymorphonuclear leukocytes is controlled by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology*, 153(Pt 5): 1329-1338.

Jiang D, Liang J, Noble PW (2007). Hyaluronan in tissue injury and repair. *Ann Rev Cell Dev Biol,* 23:435-61.

Jones V, Milton T (2000) When and how to use hydrogels. *Nursing Times,* 96(23) (Suppl): 3-4.

Kaqlan LR, Pepin DM, Ul-Haq I, Miller SB, Hay ME, Precht RJ (2017) Targeting biofilms of multidrug-restistant bacteria with silver oxynitrate. *Int J Antimicrob Agents*, 49:719-726.

Kato Y, Umeda N, Maeda A, Matsumoto D, Kitamoto N, Kikuzaki H (2012) Identification of a novel glycoside, leptosin, as a chemical marker of manuka honey. *J. Agric. Food Chem,* 60: 3418–3423.

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister Cells and tolerance to antimicrobials. *FEMS Microbiol Lett*, (230):13-18.

Kilty SJ, Duvel M, Chan FT, Ferris W, Slinger R (2011) Methylglyoxal (active agent of manuka honey) *in vitro* activity against bacterial biofilms. *International Forum of Allergy & Rhinology,* 1 (5):348-350.

Kingsley A, Tadej M, Colbourn, Kerr A, Bree-Aslan C (2009) Suprasorb X + PHMB: antimicrobial and hydrobalance action in a new wound dressing. *Wounds UK*, 5 (1):72-77.

Kingsley A (2005) Practical use of modern honey dressings in chronic wounds. In: White R, Cooper R, Molan P, eds. Honey: A Modern Wound Management product. *Wounds UK*, Aberdeen: 57-8.

Koburger T, Hubner NO, Braun M, Siebert J, Kramer A (2010) Standardised comparison of antiseptic efficacy of triclosan, PVP-iodine, octenidine dihydrochloride, polyhexanide and chlorhexidine gluconate. *J Antimicrob Chemother*, 65:1712-9.

Koo H, Allan RN, Howlin RP, Stoodley P and Hall-Stoodley L (2017) Targeting microbial biofilms: current and prospective therapeutic strategies. *Nature reviews. Microbiology*, *15*(12), 740–755.

Kostenko V, Lyczak J, Turner K, Martinuzzi RJ (2010) Impact of silver-containing dressings on bacterial biofilm viability and susceptibility to antibiotics during prolonged treatment. *Antimicrob Agents Chemoth,* 52 (12):5120-31.

Koziel J, Potempa J (2013) Protease-armed bacteria in the skin. *Cell Tissue Res,* 351:325-337.

Kramer SA (1999) Effect of povidone iodine on wound healing: A review. *J Vascular Nursing*, 17 (1): 17-23.

Kramer A, Dissemond J, Kim S, Willy C, Papke R, Tunchmann F, Assadian O (2017) Consensus on Wound Antisepsis: Update 2018. *Skin Pharmacol Physiol*, 31(1):28-58.

Krzyszczyk P, Schloss R, Palmer A, Berthiaume F (2018) The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. *Frontiers in Physiology*, *9*(419):1-22.

Kunisada T, Yamada K, Oda S, Hara O (1997) Investigation into the efficacy of povidone iodine antiseptic- resistant species. *Dermatology*, (Suppl):14-18.

Kwakman PHS, Sebastian AJZ (2011) Antibacterial components of honey. *IUBMB Life,* 64(1):48-55.

Kwakman PHS, Van den Akker JPC, Guclu A, Aslami H, Binnekade JM, de Boer L, Boszhard L, Paulus F, Middelhoek P, te Velde AA, Vandenbroucke-Grauls CMJE, Schultz MJ, Zaat SAJ (2008) Medical-Grade honey kills antibiotic –resistant bacteria in vitro and eradicates skin colonisation. *Clin Infect Dis,* 46(11): 1677-1682.

Kwakman PHS, te Velde AA, de Boer L, Vandenbroucke-Grauls CM, Zaat S A(2010) How honey kills bacteria. *FASEB J*, 24(7);2576-82.

Kwakman PHS, te Velde AA, de Boer L, Vandenbroucke-Grauls CM, Zaat S A (2011) Two major medicinal honeys have different mechanisms of bactericidal activity. *PLoS ONE* 6: e17709 10.1371/journal.pone.0017709

Labet M (2011) CROWD Controlled Release from Open Wound Dressings. Project Report, Sheffield Hallam University, UK.

Landis SJ (2008) Chronic wound infection and antimicrobial use. *Adv Skin Wound Care*, (21):531-540.

Lansdown ABG, Williams A (2004) How safe is silver in wound care? *J Wound Care*, 13:131-6.

Lachapelle JM (2012) Antiseptics and Disinfectants. In: Rustemeyer T, Elsner P, John SM, Maibach HI (eds) Kanerva's Occupational Dermatology. Springer, Berlin, Heidelberg.

Lachapelle J-M, Castel O, Casado AF, Leroy B, Micali G, Tennstedt D, Lambert J (2013), Antiseptics in the era of bacterial resistance. A focus on povidone iodine. *Future Med,* 10(5):579-592.

Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA (2006) Risk factors for individuals with diabetes. *Diabetes Care*, 29:1288-1293.

Leaper D, Assadian O, Edmiston CE (2015) Approach to chronic wound infections. *Br J Dermat*, 173(2)351-358.

Leaper DJ, Durani P (2008) Topical antimicrobial therapy in chronic wounds healing by secondary intention using iodine products. *Int Wound J,* 5:361-368.

Leaper DJ, Schultz G, Carville K, Fletcher J, Swanson T, Drake R (2012) Extending the Time concept: what have we learned in the past 10 years? *Int Wound J*, 9 (Suppl 2):1-19.

Lee YK, Park YK, Koo YT, Baek RM, Heo CY, Eun SC, Lee TS, Lee KM, Kim BK (2014) Analysis of multiple risk factors affecting the result of free flap transfer for necrotising soft tissue defects of the lower extremities in patients with type 2 diabetes mellitus. *J Plast Reconstr Aesthet Surg*, 67(5):624-8.

Leid JG, Wilson CJ, Shirtliff ME, Parsek MR, Jeffers AK (2005) The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-α-mediated macrophage killing. *J Immunol*, 175:7512-7518.

Lemire JA, Harrison JJ, Turner RJ (2013) Antimicrobial activity of metals, mechanisms, molecular targets and applications. *Nat Rev Microb*. 11(6):371-84.

Lewis EEL, Barrett MR, Freeman-Parry L, Bojar RA, Clench MR (2018) Examination of the skin barrier repair/wound healing process using a living skin equivalent (LSE) model and matrix-assisted laser desorption-ionization-mass spectrometry imaging (MALDI-MSI). *Int J Cosmetic Science,* 40 (2), 148-156.

Lewis K (2001) Riddle of biofilm resistance. *Antimicrob Agents Chemother*, 45:999-1007.

Lewis K (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol*, (5):48-56

Lewis K (2012) Persister cells: molecular mechanisms related to antibiotic tolerance. In Antibiotic Resistance 121-133. Springer, Berlin, Heidelberg.

Liao S, Zhang Y,Pan X, Zhu F, Jiang C, Liu C, Liu Q, Cheng Z, Dai G, Wu G, Wang L, Chen L (2019) Antimicrobial activity and mechanism of silver nanoparticles against multi-drug-resistant *Pseudomonas aeruginosa*. *Int J Nanomedicines*, 14:1469-1487.

Li J, Chen J, Kirsner R (2007) Pathophysiology of acute wound healing. *Clin Dermatol*, 25: 9–18.

Lim CS, Baruah M, Bahia SS (2018) Diagnosis and management of venous leg ulcers. *BMJ*, 2018 Aug 14;362:k3115. doi: 10.1136/bmj.k3115.

Lindley LE, Stojadinovic O, Pastar I, Tomic-Canic M (2016) Biology and biomarkers for wound healing. *Plast Reconstr Surg*, 138 (Suppl 3):18S-28S.

Lindsay S (2011) Silver White Paper; Everything you ever wanted to know about the use of silver in wound therapy. Systagenix, UK.

Lindsay S, Oates A, Bourdillon K (2017) The detrimiental impact of extracellular bacterial proteases on wound healing. *Int Wound J*, 14:1237-1247.

Lineweaver W, McMorris MD, Soucy D, Howard R (1985). Cellular and bacterial toxicities of topical antimicrobials. *Plast Reconstr Surg*, 75:394-6.

Linger RJ, Belikoff EJ, Yan Y, Li F, Wantuch HA, Fitzsimons HL, Scott MJ (2016) Towards next generation maggot debridement therapy: transgenic Lucilia sericata larvae that produce and secrete a human growth factor. *BMC Biotechnol*,16:30. https://doi.org/10.1186/s12896-016-0263-z

Lipp C, Kirker K, Agostinho A, James G, Stewart PS (2010) Testing wound dressings using an in vitro wound model. *J Wound Care,* 19(6):220-6.

Lipsky BA, Berendt AR, Cornia PB, Pile JC, Peters EJ, Armstrong DG, Deery HG, Embil JM, Joseph WS, Karchmer AW, Pinzur MS, Senneville E (2012) Infectious Diseases Society of America clinical practice guideline for the diagnosis and treatment of diabetic foot infections. *Clin Inf Dis*, 2012: e132-e173.

Lipsky BA, Dryden M, Gottrup F, Nathwani D, Seaton RA, Stryja J (2016) Antimicrobial stewardship in wound care: A Postion Paper from the British Society for Antimicrobial Chemotherapy and European Wound Management Association. *J Antimicrobi Chemother*, 71:3026-35.

Lipsky BA, Hoey C (2009) Topical Antimicrobial Therapy for Treating Chronic Wounds. *Clin Infect Dis,* 40(10):1541-9.

Liu Y, Min D, Bolton T, Nubé V, Twigg SM, Yue DK, McLennan SV (2009) Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers. *Diabetes Care*, 32(1):117-9.

Lohmmanrauscher:https://lohmannrauscher.co.uk/woundcare/suprasorb/suprasorb-x-phmb

Löfmark S, Edlund C, Nord CE (2010) Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infect Dis*, 50(Suppl):s16-23.

Lu J, Carter DA, Turnbull L, Rosendale D, Hedderley D, Stephens J, Gannabathula S, Steinhorn G, Schlothaurer RC, Whitchurch CB, Harry EJ (2013) The effect of New Zealand kanuka, manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species. *PLoS ONE* 8: e55898 10.1371/journal.pone.0055898

Maddocks SE, Lopez M S, Rowlands RS, Cooper RA (2012) Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins. *Microbiology,* 158: 781–790.

Mahmood A (2018) Antimicrobial activity of iodine containing wound dressings against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Project Report, Sheffield Hallam University, UK.

MacNeil S, Shepherd J, Smith L (2011) Production of tissue- engineered skin and oral mucosa for clinical and experimental use. *Methods Mol Biol*, 695:129-53.

Majno G (1975) The healing hand: man and wound management in the ancient world. Harvard University Press, Cambridge (Mass), USA.

Majtan J, Bohova J, Prochazka E, Klaudiny J (2014) Methylglyoxal may affect hydrogen peroxide accumulation in manuka honey through the inhibition of glucose oxidase. *J Med Food*, 17:290–293.

Majtan J (2014). Honey: an immunomodulator in wound healing. *Wound Repair Regenerat,* 22: 187–192.

Malone M, Bjarnsholt T, McBain AJ, James GA, Stoodley P, Leaper D, Tachi M, Schultz G, Swanson T, Wolcott RD (2017) The prevalence of biofilms in chronic wounds: a systematic review and meta-analysis of published data. *J Wound Care*, 26(1): 20—25.

Mandal MB, Mandal S (2011) Honey: its medicinal properties and antimicrobial activity. *Asian Pac J Trop Biomed*, 1(2):154-160.

Mani R (2011) The Basic Needs to Achieve Wound Healing. Jaypee Brothers Medical Publishing Ltd.

Marx DE, Barillo GJ (2014) Silver in medicine; the basic science. *Burns*, 40(Suppl1): S9-18.

Mast BA, Schultz GS (1996) Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen,* 4(4):411-20.

Mavric E, Wittmann S, Barth G, Henle T (2008) Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Mol Nutrit Food Res*, 52 (4):483–489.

McCarty SM, Cochrane CA, Clegg PD, Percival SL (2012) The role of endogenous and exogenous enzymes in chronic wounds: a focus on the implications of aberrant levels of both host and bacterial proteases in wound healing. *Wound Repair Regen*, 20:125-136.

McDonnell G, Russell AD (1999) Antiseptics and disinfectants: activity, action and resistance. *Clin Microbiol Rev,* 12:147-79.

McKenna PJ, Lehr GS, Leist P, Welling RE (1991) Antiseptic effectiveness with fibroblast preservation. *Ann Plast Surg*, 27:265-268.

McLure AR, Gordin J (1992) *In-vitro* evaluationof povidone-iodine and chlorhexidine against methicillin-resistant *Staphylococcus aureus*. *J Hosp infection,* 21(4):291-9.

Merckoll P, Jonassen TO, Vad ME, Jeansson SL, Melby KK (2009) Bacteria, biofilm and honey: A study of the effects of honey on ‘planktonic’ and biofilm embedded chronic wound bacteria. *Scandinavian J Infectious Diseases*, 41:341-7.

Mertz P (2003) Cutaneous biofilms: friend or foe? *Wounds,* 15(5): 129-32.

Mertz PM, Oliveira-Gandia MF and Davis SC (1999) The Evaluation of a Cadexomer Wound Dressing on Methicillin Resistant *Staphylococcus aureus* (MRSA) *Dermatol Surg,* 25:89-93.

Michaels JA, Campbell WB, King BM, Macintyre J, Palfreyman SJ, Shackley P, Stevenson MD (2009) A prospective randomised controlled trial and economic modelling of antimicrobial silver dressings versus non-adherent control dressings for venous leg ulcers: the VULCAN trial. Health Technology Assessment 13:1-114.

Mills JL, Conte MS, Armstrong DG, Pomposelli FB, Schanzer A, Sidawy AN, Andros G (2014) The Society for Vascular Surgery Lower Extremity Threatened Limb Classification System: Risk stratification based on Wound, Ischaemia, and foot infection (WIfI). *J Vasc Surg*, 59(1):220-34.

Milne J (2015) Wound bed preparation: the importance of rapid and effective desloughing to promote healing. *Br J Nurs*, 24: S52-S58.

Milstone AM, Passaretti CL, Perl TM (2008) Chlorhexidine: Expanding the Armamentarium for Infection Control and Prevention. *Clinical Infectious Disease,* 46:274-81.

Molan PC (1992) The antibacterial activity of honey: 2. Variation in the potency of the antibacterial activity. *Bee World,* 73:59-76.

Molan PC (2001) Honey as a Topical Antimicrobial Agent for the treatment of infected wounds. www.worldwidewounds.com/2001/november/Molan/honey-as-topicalagent.html

Molan PC (2008) An explanation of why the MGO level in manuka honey does not show the antibacterial activity. *New Zealand Beekeeper,* 16: 11–13.

Monroe D (2007) Looking for Chinks in the Armor of Bacterial Biofilms. PLoS Biol 5(11): e307. https://doi.org/10.1371/journal.pbio.0050307

Moore K, Gray D (2007) Using PHMB antimicrobial to prevent wound infection. *Wounds UK,* 3(2):96-102.

Moore K (2006) The interaction of a “Second Generation” Hydrogel with the Chronic Wound Environment. Poster Presentation, European Wound Management Association.

Morgan D (2004) Formulary of Wound Management Products (9th edn). Euromed Communications.

Morrison MJ (1989) Wound Cleansing – which solution? *Professional Nurse,* 4: 220-5.

Moshakis V, Fordyce MJ, Griffiths JD, McKinna JA (1984) Tegaderm versus gauze dressing in breast surgery. *Br J Clin Pract,* 38:149-152.

Moues CM, Heule F, Hovious SER (2011) A review of topical negative pressure therapy in wound healing: sufficient evidence? *Am J Surg,* 201:544-556.

Muller M (2018) Bacterial Silver resistance gained by Cooperative Interspecies Redox bahavior. *Antimicrob Agents and Chemother*, 62: e00672-18

Mumcuoglu KY, Miller J, Mumcuoglu M, Friger M, Tarshis M (2001) Destruction of bacteria in the digestive tract of the maggot of *Lucilis sericata* (Diptera: Calliphoridae). *J Med Entomol*, 38:161-6.

Murdoch R, Lagan KM (2013) The role of povidone and cadexomer iodine in the management of acute and chronic wounds. *Physical Therapy Reviews*, 18(3):2017-216.

Nagaraja S, Wallqvist A, Reifman J, Mitrophanov AY (2014) Computational approach to characterize causative factors and molecular indicators of chronic wound inflammation. *J Immunol*, 192: 1824–1834.

Nathwani D (2012) Antimicrobial stewardship. In: Mayhall CG, editor. Hospital Epidemiology and Infection Control. 4th edn. Philadelphia: Lippincott, Williams and Wilkins

Naik G, Harding KG (2017) Maggot debridement therapy: the current perspectives. *Chronic Wound Care Management and Research*, 4:121-128.

Nestjones D, Vandeputte J (2012) Clinical evaluation of Melladerm Plus: A honey-based wound dressing Gel. *Wounds UK,* 8 (2): 106.

Newman GR, Walker M, Hobot JA, Bowler PG. (2006) Visualisation of bacterial sequestration and bactericidal activity within hydrating hydrofiber wound dressings. *Biomaterials,* 27(7):1129-39.

NICE (2014) Oxyzyme and Iodozyme 2-layer hydrogel wound dressings with iodine for treating chronic wounds. Nice.org.uk/guidance/mib11

NICE (2015) Antimicrobial stewardship and processes for effective antimicrobial medicine use.NG15. www.nice.org.uk/guidance/ng15

NICE (2016) Chronic wounds: advanced wound dressings and antimicrobial dressings. Evidence Summary [ESMPB2] *National Institute for Health and Care Excellence*. www.nice.org.uk

NICE (2019a) Wound care products: Key therapeutic topic (KTT14). *National Institute for Health and Care Excellence*. www.nice.org.uk

NICE (2019b) Antimicrobial stewardship: prescribing antibiotics (Key therapeutic topics). *National Institute for Health and Care Excellence*. www.nice.org.uk

NICE (2019c) Draft guideline on leg ulcer infection: antimicrobial prescribing. *National Institute for Health and Care Excellence*. www.nice.org.uk

NICE 2019 Draft Guidance NG152: Leg Ulcer infection: antimicrobial prescribing https://www.nice.org.uk/guidance/ng152/documents/draft-guideline

Nigam Y, Bexfield A Thomas S, Ratcliffe NA (2006) Maggot therapy: the science and implication for CAM part ii – maggots combat infection. *Evid Based Complement Alternat Me,* 3:303-308.

Nikolaev I, Plakunov VK (2007) Biofilm- "City of Microbes" or an Analogue of Multicellular Organsisms? *Mikrobiolgila,* 76:149-63.

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature*, 414(6862):454-7.

Nobukuni K, Hayakawa N, Namba R, Ihara Y, Sato K, Takada H, Hayabara T, Kawahara S (1997) The influence of long – term treatment with povidone-iodine on thyroid function. *Dermatology*, 195(Suppl):60-72.

Noli C, Miolo A (2001) The mast cell in wound healing. *Vet Derm*, 12: 303–313.

Norman G, Dumville JC, Mohapatra DP, Owens GL, Crosbie EJ (2016) Antibiotics and antiseptics for surgical wounds healing by secondary intention. *The Cochrane database of systematic reviews*, *3*(3), CD011712. https://doi.org/10.1002/14651858.CD011712.pub2

Norman G, Westby MJ, Rithalia AD, Stubbs N, Soares MO, Dumville JC (2018) Dressings and topical agents for treating venous leg ulcer (Cochrane Review/Cochrane Intervention Protocol). John Wiley & Sons, Ltd. www.cochranelibrary.com/

Norton AM, McKenzie LN, Brooks PR, Pappalardo LJ (2015) Quantitation of dihydroxyacetone in Australian *Leptospermum* nectar via High-Performance Liquid Chromatography. *J Agric Food Chem,* 63: 6513–6517.

Nouraldin AAM, Baddour MM, Harfoush RAH, Essa SAM (2016) Bacteriophage-antibiotic synergism to control planktonic and biofilm producing clinical isolates of *Pseudomonas aeruginosa*. *Alexandria J Med*, 52(2):99-105.

Oates A, Lindsey S, Mistry H, Ortega F, McBain AJ (2018) Modelling antisepsis using defined populations of facultative and anaerobic wound pathogens grown in a basally perfused biofilm model. *Biofouling*, 34(5):507-518.

O'Brien M (2007) Knowledge: Understanding critical colonisation of wounds. *Nursing Time,* http://tinyurl.com/p8gkukq

Office of National Statistics (2019) https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/articles/overviewoftheukpopulation/august2019

O'Donnell TF Jr, Passman MA (2014) Clinical practice guidelines of the Society for Vascular Surgery (SVS) and the AmericanVenous Forum (AVF) — management of venous leg ulcers, Introduction. *J Vasc Surg*, 60(Suppl):1S–2S.

O'Meara SM, Cullum NA, Majid M, Sheldon TA (2001) Systematic review of antimicrobial agents used for chronic wounds. *British J Surg,* 88:4-21.

O'Meara SM, Cullum NA, Nelson EA, Dumville JC (2012) Compression for venous leg ulcers (Cochrane Review/Cochrane Intervention Protocol). John Wiley & Sons, Ltd. www.cochranelibrary.com/

O' Neill AJ (2010) *Staphylococcus aureus* SH1000 and 8325-4: comparative genome sequences of key laboratory strains in staphylococcal research. *Lett Appl Microbiol* 2010 Sep;51(3):358-61.

Ousey K (2018) The roles of topical metronidazole in the management of infected wounds. *Wounds* UK, 15(5):105-9.

Ousey K, Cook L, Young T, Fowler A (2012) Hydrocolloids in practice: Made Easy. *Wounds* UK 8(1):1-6.

Ousey K, Roberts C, Leaper D (2016) Silver-containing dressings. In M Agren (Ed.) *Wound Healing Biomaterials- Volume 2*, Woodhead Publishing, Elsevier Ltd., London, UK.

Ousey, K, Rogers AA, Rippon MG (2016) HydroClean® plus: a new perspective to wound cleansing and debridement. *Wounds UK*, 12(1):94-104.

Ousey K, Stephenson J, Barret S, King B, Morton N, Fenwick K, Carr C (2013) Wound Care in five English NHS Trusts: results of a survey. *Wounds UK,* 9(4):20-28.

Ovington LG (2004) The truth about silver. *OWM 50*, (9A Suppl):1S-10S.

Ovington LG (2007) Advances in Wound Dressings. *Clinics in Dermatology,* 25(1):33-38.

PANalytical (2010) Theory of XRF http://www.chem.bg.ac.rs/~grzetic/predavanja/Nedestruktivna%20hemijska%20analiza%20%20odabrana%20poglavlja/XRF/Literature/PANanalytical%20XRF%20theory.pdf

Parsons D, Bowler PG, Myles V, Jones S (2005) Silver antimicrobial dressings in wound management a comparison of antimicrobial, physical and chemical characteristics. *Wounds*, 17(8):222-32.

Peacock SJ, Paterson GK (2015) Mechanisms of Methicillin resistance in *Staphylococcus aureus*. *An Rev Biochem*, 84(1):577-601.

Percival SL, Bowler P (2004) Biofilms and their potential role in wound healing. *Wounds,* 16:234-40.

Percival SL, Bowler PG, Russell D (2005) Bacterial resistance to silver in wound care. *J Hosp Inf*, 60:1-7.

Percival SL, Bowler P, Woods EJ (2008) Assessing the effect of an antimicrobial wound dressing on biofilms. *Wound Repair Regen,* 16(1): 52-57.

Percival SL, Emanuel C, Cutting KF, Williams DW (2012) Microbiology of the skin and the roles of biofilms in infection. *Int Wound J*, 9:14-32.

Percival SL, McCarty SM (2015) Silver and alginates: role in wound healing and biofilm control. *Adv Wound Care*, 4(7):407-14.

Percival SL, McCarty SM, Lipsky B (2015) Biofilms and Wounds: An overview of the Evidence. *Advances in Wound Care*, Volume 4, (7):373-381.

Percival SL, Suliman L (2015) Slough and biofilm: removal of barriers to wound healing by desloughing. *J Wound Care*, 24(11):498-510.

Petra L (2015) IN-CROWD Results Report, Sheffield Hallam University, UK.

PHE (2014) UK standards for microbiology investigations: Investigation of skin, superficial and non-surgical wound swabs. Public Health England. www.gov.uk

Phillips PL, Wolcott RD, Fletcher J, Schultz GS (2010) Biofilms Made Easy. *Wounds International*, 1(3):1-6.

Phillips P, Sampson E, Yang Q, Antonelli P, Progulske-Fox A, Schultz G (2008) Bacterial biofilms in wounds. *Wound Healing Southern Africa*, 1(2):10-12.

Phillips PL, Yang Q, Davis S, Sampson EM, Azeke JI, Hamad A, Schultz GS (2015) Antimicrobial dressing efficacy against mature *Pseudomonas aeruginosa* biofilm on porcine skin explants. *In Wound J*, 12:469-483.

Pierce GF, Tarpley JE, Tseng J, Bready J, Chang D, Kenny WC, Rudolph R, Robson MC, Vande Berg J, Reid P (1995) Detection of platelet -derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic non-healing wounds. *Clin Invest*, 96(3):1336-50.

Polakovičova S, Polák Š, Kuniaková M, Čambal M, Čaplovičová M, Kozánek M-I, Danišovič L, Kopáni M (2015) The Effect of Salivary Gland Extract of *Lucilia sericata* Maggots on Human Dermal Fibroblast Proliferation within Collagen/Hyaluronan Membrane In Vitro: Transmission Electron Microscopy Study. *Adv Skin Wound Care,* 28 (5): 221–226.

Posnett J, Franks P (2008) The burden of chronic wounds in the UK. *Nurs Times,* 104:44–5.

Prestinaci F, Pezzotti, P, Pantosti A (2015) Antimicrobial resistance: a global multifaceted phenomenon. *Pathogens and global health*, *109*(7): 309–318.

Pritchard DI, Nigam Y (2013) Maximising the secondary beneficial effects of larval debridement therapy. *J Wound Care*, 22:610-611,614-616.

Prompers L, Schaper N, Apelqvist J, Edmonds M, Jude E, Mauricio D, Uccioli L, Urbancic V, Bakker K, Holstein P, JIrkocska A, Piaggesi A, Ragnarson –Tenvall G, Reike H, Spraul M, Van Acker K, Van Metode F, Ferreira I, Huijberts M (2008) Prediction of outcome in individuals with diabetic foot ulcers: focus on the differences between individuals with and without peripheral arterial disease: The EURODIALE Study. *Diabetologia*, 51:747-755.

Purner SK, Babu M (2000) Collagen based dressings- A Review. *Burns,* 26: 54-62.

Qin Y (2005) Silver- containing alginate fibers and dressings. *In Wound J*, 2:172-176.

Rafii F, Hart ME (2015) Antimicrobial Resistance in clinically important biofilms. *World J. Pharmacology*, 4(1):31-46

Rackur H (1985) New aspects of mechanism of action of povidone-iodine. *J Hosp Infect,* 6 Suppl A: 13-23.

Rahim K, Saleha S, Zhu X. Huo L, Basit A, Franco OL (2017) Bacterial Contribution in Chronicity of Wounds. *Microb Ecol*, 73:710-721.

Rayment EA, Upton Z, Shooter GK (2008) Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the severity of the ulcer. *Br J Dermatology*, 158(5):951-61.

Reimer K, Schreier H, Erdos G, Konig B, Konig W, Fleischer W (1998) Molecular effects of a microbiocidal substance on relevant microorganisms: electron microscope and biochemical studies on povidone-iodine. *Int J Hyg Environ Med,* 200:423-34.

Reinke JM, Sorg H (2012) Wound repair and regeneration. *Eur Surg Res*, 49: 35–43.

Rippon MG, Ousey K (2015) HydroClean® plus. Hydro-Responsive Wound Dressing (HRWD): Clinical and Scientific Monograph. Heywood: Paul Hartmann Ltd. ISBN 978-194478863-9.

Rippon MG, Ousey K, Cutting KF (2016) Wound healing and hyper-hydration: a counterintuitive model. *J Wound Care*, 25(2):68-75.

Roche ED, Woodmansey EJ, Yang Q, Gibson DJ, Zhang H, Schultz GS (2019) Cadexomer iodine effectively reduces bacterial biofilm in porcine wounds *ex vivo* and *in vivo*. *Int Wound J*, 16(3): 674-683.

Roberts CD, Leaper DJ, Assadian O (2017) The role of topical antiseptic agents within antimicrobial stewardship strategies for prevention and treatment of surgical site and chronic open wound infection. *Adv Wound Care*, 6(2):63-71.

Roberts AD, Simon GL (2012) Diabetic foot infections: the role of microbiology and antibiotic treatment. *Semin Vas Surg*, 25:75-81.

Rossaint J, Zarbock A (2015) Platelets in leukocyte recruitment and function. *Cardiovasc Res*, 107:386-95.

Russell AD, Hugo WB (1994) Antimicrobial activity and action of silver. *Prog Med Chem,* 31:351-71.

Sackett WG (1919) Honey as a Carrier of Intestinal diseases. *Experiment Station of the Colorado Agricultural College Bulletin*, 252:1-18.

Said J, Walker M, Parsons D, Stapleton P, Beezer AE, Gaisford S (2014) An *in vitro* test of the efficacy of an anti-biofilm wound dressings. *Int J Pharm,* 474:177-81.

Sandoz H (2015) Negative pressure wound therapy: clinical utility. *Chronic Wound Care Management and Research*, 2:71-79.

Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol*, 184(4):1140-1154.

Schintler MV (2012) Negative pressure therapy: theory and practice. *Diabetes Metab Res Rev*, 28 (Suppl 1):72-77.

Schlotz DR, Uhlig DS (2006) Introduction to X-ray Fluorescence Analysis (XRF). Retrieved from http://www.fem.unicamp.br/~liqcqits/facilities/xrf/%5BBruker_2006%5D%20Introduction%20to%20X-ray%20Fluorescence%20(XRF).pdf.

Schmidt RJ, Kirby AJ, Chung LY (1995) Cadexomer iodine formulations modulate the redox environment of wounds. In: Hunt TK and Middelkoope E (eds) *Iodine and wound physiology.* Cambridge, UK.

Schreier H, Erdos G, Konig B, Konig W, Fleischer W (1998) Molecular effects of povidone-iodine on relevant microorganisms: an electron microscope and biochemical study. *Dermatology,* 195 Suppl 2:111-6.

Schultz DR, Miller KD (1974) Elastase of *Pseudomonas aeruginosa*: inactivation of complement components and complement-derived chemotactic and phagocytic factors. *Infect Immun*, 10:128-135.

Schultz GS, Sibbald RG, Falanga V (2003) Wound bed preparation: a systematic approach to wound management. *Wound Rep Regen,* 11 (Suppl 1): S1-28.

Seaman S (2002) Dressings Selection in Chronic Wound management. *J Am Podiatr Med Assoc,* 92 (1):24-33.

Serra R, Grande R, Buffone G, Molinari V, Perri P, Perri A, Amato B, Colosimo M, deFranciscis S (2016) Extracellular matrix assessment of infected chronic venous ulcers: role of metalloproteinases and inflammatory cytokines. *Int Wound J*, 13(1):53-58.

Serra R, Grande R, Butrico L, Rossi A, Settimio UF, Caroleo B, Amato B, Gallelli L, de Franciscis S (2015) Chronic Wound infections: The role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Expert Review Anti Infect Ther*. Early online: 1-9

Seth AK, Geringer MR, Hong SJ, Leung KP, Mustoe TA, Galiano RD (2012) In vivo modelling of biofilm-infected wounds: a review. *J Surg Res*, 178:330-8.

Seth AK, Zhong A, Nguyen KT, Hong SJ, Leung KP, Galiano RD, Mustoe TA (2014) Impact of a novel, antimicrobial dressing on *in vivo*, *Pseudomonas aeruginosa* wound: quantitative comparative analysis using a rabbit ear model. *Wound Repair Regen*, 22: 712-9.

Shah CB (2007) Polyhexamethylene Biguanide (PHMB) Treated Wound Dressings and Vancomycin- resistant Enterococci (VRE) – Efficacy of PHMB treated dressings against VRE. *Managing Infection Control*, 26-34.

Sharma S, Parmar A, Mehta S (2018) Hydrogels: from simple networks to smart molecules- advances and applications. In Drug Targeting and Stimuli Sensitive Delivery systems. Elsevier pp 627-672.

Sharp A (2009) Beneficial effects of honey dressings in wound management. *Nursing Standard, 24*(7):66-74.

Shepherd J, Douglas I, Rimmer S, Swanson L, MacNeil S (2009) Development of Three-Diminsional Tissue-Engineered Models of Bacterial Infected Human Skin Wounds. *Tissue Engineering*, Part C, 15(3):475-484.

Sheskin DJ (1989) Handbook of Parametric and Nonparametric Statistical Procedures (3rd ed) Boca Raton, FL: CRC Press: 665 - 756.

Sibbald RG, Goodman L, Woo KY, Krasner DL, Smart H, Tariq G, Ayello EA, Burrell RE, Keast DH, Mayer D, Norton L, Salcido RS (2011) Special considerations in wound bed preparation: An Update. *Adv Skin Wound Care*, 24:415-436.

Sibbald RG, Leaper DJ, Queen D (2011) Iodine Made Easy. *Wounds International*, 2(2):1-6.

Siddiqui AR, Bernstian JM (2010) Chronic wound infections; facts and controversies. *Clin Dermatol*, 28:519-526.

SIGN (2010) Management of chronic venous leg ulcers. Scottish Intercollegiate Guidelines Network. www.sign.ac.uk

Simpson JA, Smith SE, Dean RT (1989) Scavenging by alginate of free radicals released by macrophages. *Free Radic Biol Med*, 6:347-53.

Singer AJ, Tassiopoulos A, Kirsner RS (2017) Evaluation and Management of Lower-Extremity Ulcers. *New Eng J Med,* 377(16):1559-1567.

Singh N, Armstrong DG, Lipsky BA (2005) Preventing foot ulcers in patients with diabetes. *JAMA*, 293:217-228.

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002) A component of innate immunity prevents biofilm development. *Nature*, 417:552-5.

Skrepnek GH, Mills JL, Lavery LA, Armstrong DJ (2017) Health care service and outcomes among an estimated 6.7 million ambulatory care diabetic foot cases in the US. *Diabetic Care*, 40(7):936-42.

Smith-nephew.com:https://www.smithnephew.com/canada/products/advanced-wound-management/bactigras/

Smola H, Maier G, Junginger M et al. (2016) Hydrated polyurethane polymers to increase growth factor bioavailability in wound healing. Hydrotherapy Symposium. A New Perspective on Wound Cleansing, Debridement and Healing, London, UK, 3 March 2016.

Sood A, Granick MS, Tomaselli NL (2014) Wound Dressings and Comparative Effectiveness Data. *Adv Wound Care*, 3(8):511-519.

Sorensen OE, Cowland JB, Theilgaard-Monch K, Liu L, Ganz T, Borregaard N (2003) Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol,* 170(11):5583-9.

Spruce P, Bullough L, Johnson S, O'Brien D (2016) Benefits of HydroClean plus in wound bed preparation: a case study series. *Wounds Int*, 7(1):26-32.

Stadelmann WK, Deginis AG, Tobin GR (1998) Physiology and healing dynamics of chronic cutaneous wounds. *Am J Surg*, 176(2a Suppl):26S-38S.

Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358(9276):135-138.

Stewart PS, Roe F, Rayner J, Elkins JG, Lewandowski Z, Ochsner UA, Hassett DJ (2000) Effect of catalase on hydrogen peroxide penetration into *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol*, 66:836-838.

Stickler D, Hewett P (1991) Activity of antiseptics against biofilms of mixed bacterial species grown on silicone surfaces. *Eur J Clin Microbiol Infect Dis*, 10(3):157-162.

Storm-Versloot MN, Vos CG, Ubbink DT, Vermeulen H (2010) Topical Silver for preventing wound infection. Cochrane Database Issue 3:CD001103.

Streubel PN, Stinner DJ, Obremskey WT (2012) Use of negative pressure wound therapy in orthopaedic trauma. *J Am Acad Ortho Surg,* 20(9):564-74.

Strohal R, Apelgvist J, Dissemond J, O'Brien JJ, Piaggesi A, Rimdeika R, Young T (2013) EWMA document: Debridement. *J Wound Care*, 22: S1-S52.

Su WH, Cheng MH, Lee WL, Tsou TS, Chang WH, Chen CS, Wang PH (2010) Nonsteroidal anti-inflammatory drugs for wounds: pain relief or excessive scar formation? *Mediat Inflamm*, Article ID 413238, 8 pages doi:10.1155/2010/413238

Sun X, Jiang K, Chen J, Wu L, Lu H, Wang A, Wang J (2014) A systematic review of maggot debridement therapy for chronically infected wounds and ulcers. *Int J Infect Dis*, 25:32-37.

Szweda P, Gorczyca G, Tylingo R (2018) Comparison of activity of selected, commercially available wound dressing materials. *J Wound Care,* 27(5):320-326.

Tam K, Torres VJ (2019) *Staphylococcus aureus* Secreted Toxins and Extracellular Enzymes.*Microbiol.Spec.*, 7(2):10.1128/microbiolspec.GPP3-0039-2018.

Tandara AA, Mustoe TA (2004) Oxygen in wound healing- more than a nutrient. *World J Surg*, (28): 294-300.

Tate S, Price A, Harding K (2018) Dressings for venous leg ulcers. *BMJ* ,362: K3115.

Tatnall FM, Leigh IM, Gibson JR (1987) Comparative toxicity of antimicrobial agents on transformed keratinocytes. *J Invest Dermatol*, 89:316-317.

Therattil PJ, Pastor C, Granick MS (2013) Sacral pressure ulcer. *Eplasty*, *13*, ic18.

Thomas PR, Nash GB, Dormandy JA (1988) White cell accumulation in dependent legs of patients with venous hypertension: a possible mechanism for trophic changes in the skin. *Br Med J* (Clin Res Ed), 296(6638):1693–5.

Thomas S (2000) Alginate dressings in surgery and wound management: Part 3. *J Wound Care,* 9(4):163-166.

Thomas S (2006) Cost of managing chronic wounds in the UK, with particular emphasis on maggot debridement therapy. *J Wound Care*, 15(10):465-469.

Thomas S, Loveless PA (1997) A comparative study of twelve hydrocolloid dressings. *World Wide Wounds*, 1:1-12

Thomas S, McCubbin P (2003) An *in vitro* analysis of the antimicrobial properties of 10 silver containing dressings. *J Wound Care*, 12(8): 305-8.

Thorn RMS, Austin AJ, Greeman J, Wilkins JP, Davis PJ (2009) *In vitro* comparison of antimicrobial activity of iodine and silver dressings against biofilms*. J Wound Care,* 18(8):343-46.

Thorn RMS, Greenman J, Austin AJ (2006) An *in vitro* study of antimicrobial activity and efficacy of iodine generating hydrogel dressings. *J Wound Care*, 15:305–310.

Thorn RMS, Greenman J (2009) A novel *in vitro* flat-bed perfusion biofilm model for determining the potential antimicrobial efficacy of topical wound treatments. *J Applied Microbiol*, 107 (6): 2070-2079.

Thorn RMS, Greenman, J, Austin AJ (2005). *In vitro* method to assess the antimicrobial activity and potential efficacy of novel types of wound dressing. *J Applied Microbiol*, 99:895–901.

Tonnesen MG, Feng X, Clark RA (2000) Angiogenesis in wound healing. *J Invest Dermatol Symp Proc*, 5:40-6.

Travis J, Potempa J, Maeda H (1995) Are bacterial proteinases pathogenic factors? *Trends Microbiol,* 3(10): 405-7.

Trengove NJ, Bielefeldt-Ohmann H, Stacey MC (2000) Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcer. Wound Repair Regen, 8(1):13-25.

Trengove NJ, Stacy MC, MacAuley S, Bennett N, Gibson J, Burslem F, Murphy G, Schultz G (1999) Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen*, (7)442-52.

Trengove NJ, Stacey MC, Mc Gechie DF, Mata S (1996) Qualitative Bacteriology and Leg Ulcer Healing. *J Wound Care*, 5:277-80.

Tuttle MS (2015) Association between microbial bioburden and healing outcomes in venous leg ulcers: a review of the evidence. *Adv Wound Care*, 4:1-11.

Uçkay I, Gariani K, Pataky Z, Lipsky BA (2013) Diabetic foot infections: state of the art. *Diabetes Obes Metab*, 16:305-316.

Uppuluri P, Lopez - Ribot JL (2016) Go forth and colonize: Dispersal from clinically important microbial biofilms. *PLoS Pathog*, 12(2): e1005397

Urgomedical.my/faqs/2020

Van Den Broek PJ, Buys LMF, Van Furth R (1982) Interaction of povidone-iodine compounds, phagocytic cells and microorganisms. *Antimicrob Agents Chemother,* 22:593-597.

Van Meurs SJ, Gawlitta D, Heemstra KA, Poolman RW, Vogely HC, Kruyt MC (2014) Selection of an optimal antiseptic solution for intraoperative irrigation: an *in vitro* study. *J Bone Jt Surg Am*, 96:285-291.

Vasudevan B (2014) Venous leg ulcers: Pathophysiology and Classification. *Indian Dermatol Online Journal,* 5(3): 366-370.

Vermeulen H, Van Hatten JM, Storm-Versloot MN, Ubbink DT, Westerbos SJ (2007) Topical Silver for treating wounds. Cochrane Database of Systematic Reviews Issue 3:CD006478.

Vermeulen H, Westerbos SJ, Ubbink DT (2010) Benefit and Harm of Iodine in wound care: a systematic review. *Journal of Hospital Infection,* 76(3):191-9.

Vowden K, Vowden P (2017) Wound Dressings: Principles and Practice. *Surgery*, 35(9): 489-493.

Vuong C, Kocianova, Voyich JM, Yao Y, Fischer ER, DeLeo FR, Otto M (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion and virulence. *J Biol Chem*, 279(52): 54881-54886.

Walker M, Cochrane CA, Bowler PG, Parson D, Bradshaw P (2006) Silver deposition and tissue staining associated with wound dressings containing silver. *Ostomy Wound Manage,* 52: 222-231.

Waters CM, Bassler BL (2005) Quorum sensing: cell-cell communication in bacteria. *Annu Rev Cell Dev Biol*, 21:319-346.

Watkins RR, David MZ, Salata RA (2012) Current concepts on the virulence mechanisms of methicillin-resistant *Staphylococcus aureus*. *J Med Micro*, 61:1179-1193.

Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. *Physiol. Rev*, 83: 835–870.

Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydern A Molin S, Pitts B, Stewart PS (2004) Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol*, (70):6188-6196.

Werthen M, Davoudi M, Sonesson A, Nitische DP, Morgelin M, Blom K, Schmidtchen A (2004) *Pseudomonas aeruginosa*-induced infection and degradation of human wound fluid and skin proteins ex vivo are eradicated by a synthetic cationic polymer. *J Antimicrob Chemother*, 54(4):772-9.

White JW, Subers MH, Schepartz AI (1963) The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochimica Biophysica Acta,* 73:57-70.

White RJ, Cooper RA (2005) Silver sulphadiazine: a review of the evidence. *Wounds UK*, 1(2):51-6.

White R, Cowan T, Glover D (2015) Supporting evidence – based practice: Clinical Review of TLC healing matrix. *J Wound Care*, Vol 24 (8) (Suppl 1):S1-47.

White R, Gardener S, Cutting K, Waring M (2012) What is the current status of foam dressings? *Wounds UK*, 8(3): 21-24.

Wiegend C, Abel M, Ruth P, Hipler UC (2009) HaCat keratinocytes in co-culture with *Staphylococcus aureus* can be protected from bacterial damage by polihexanide. *Wound Repair Regen*, 17(5):730-8.

Wilkinson JM, Cavanagh HMA (2005) Antibacterial activity of 13 honeys against *Escherichia coli* and *Pseudomonas aeruginosa*. *J Med Food*, 8:100–103.

Wilkinson LJ, White RJ, Chipman JK (2011) Silver and nanoparticles of silver in wound dressings: A review of efficacy and Safety. *J Wound Care*, 20(11):543-9.

Williams S, King J, Revell M, Manley-Harris M, Balks M, Janusch F, Kiefer M, Clearwater M, Brooks P, Dawson M (2014). Regional, annual, and individual variations in the dihydroxyacetone content of the nectar of manuka (*Leptospermum scoparium*) in New Zealand. *J Agric Food Chem*, 62: 10332–10340.

Winter GD (1962) Formation of the scab and the rate of epithelialization of superficial wounds in the skin of the young domestic pig. *Nature,* 193: 293-294.

Wolcott R (2015) Disrupting the biofilm matrix improves healing outcomes. *J Wound Care,* 24(8):366-71.

Wolcott RD, Costerton JW, Raoult D, Cutler SJ (2013) The polymicrobial nature of biofilm infection. *Clin Microbiol Infect*, 19(2):107-112.

Wolcott RD, Kennedy JP, Dowd SE (2009) Regular debridement is the main tool for maintaining a healthy wound bed in most chronic wounds. *J Wound Care*, 18(2):54-6.

Wolcott RD, Rhoads DD (2008) A study of biofilm-based wound management in subjects with critical limb ischaemia. *J Wound Care*, 4:145-148.

Woo KY, Ayello EA, Sibbald RG (2008) Silver versus other antimicrobial dressings: best practices. *Surg Technol Int*, 17:50-71.

Wood L, Wood Z, Davis P, Wilkins J (2010) Clinical experience with an antimicrobial hydrogel dressing on recalcitrant wound. *J Wound Care*, 19:287–293.

World Health Organisation (2009) Stability testing of active pharmaceutical ingredients and finished pharmaceutical products. Annex 2 of WHO Technical Report Series No. 953, 43rd Report of WHO Expert Committee on Specifications for Pharmaceutical Preparations. WHO, Geneva, Switzerland.

World Health Organisation (2016) Global Report on diabetes. Available at: http://apps.who.int/news-room/Fact-sheets/details/diabetes

World Health Organisation (2017) Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. Geneva: World Health Organisation (WHO/EMP/IAU/2017.11).

Wounds International (2012) International Consensus: Appropriate use of silver dressings in wounds. An expert working group consensus. Wounds International 2012, London. Available at: www.woundsinternational.com

Wounds International (2013) International Best Practice Guidelines: Wound Management in Diabetic Foot Ulcers, 2013. Available at: http://www.woundsinternational.com/media/bestpractices/ /673/files/dfubestpracticeforweb.pdf.

World Union of Wound Healing Societies (2016) Position document: Local Management of diabetic foot ulcers. Available at: http://www.woundsinternational.com/wuwhs/view/position-document-local-management- of diabetic- foot-ulcers

World Union of Wound Healing Societies (WUWHS) (2008) Principles of Best Practice: Wound Infection in clinical practice. An international Consensus (2008) MEP Ltd, London.

Wound Care Handbook 2019-2020. Cowan T (Ed) 12th edition. MA Healthcare 2019.

Wounds UK (2013) Best Practice Statement: The use of topical antimicrobial agents in wound management. Available online from www.wounds-uk.com

Wounds UK (2019) Best practice statement. Addressing complexities in the management of venous leg ulcers. Available online from www.wounds-uk.com

Wright JB, Lam K, Hansen D, Burrell RE (1999) Efficacy of topical silver against fungal burn wound pathogen. *Am J Infect Control*, 27(4):344-350.

Wu S, Driver VR, Wrobel JS, Armstrong DG (2007) Foot Ulcers in the diabetic patient, prevention and treatment. *Vasc Health Risk Manag*, 3(1):65-76.

Wu Y-K, Cheng N-C and Cheng C-M (2019) Biofilms in Chronic Wounds: Pathogenesis and Diagnosis. *Trends in Biotechnology*, 37(5):505 -517.

Wysocki AB, Grinnell F (1990) Fibronectin profiles in normal and chronic wound fluid. *Lab Invest*, 63(6):825-31.

Xue M, Jackson CJ (2015) Extracellular matrix reorganization during wound healing and its impact on abnormal scarring. *Adv. Wound Care* (New Rochelle), 4(3):119-136.

Yang J, Weinberg RA (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell,* (14): 818-829.

Young A, McNaught C-E (2011) The physiology of wound healing. *Surgery,* 29(10):475-9.

Yunoki S, Kohta M, Ohyabu Y, Iwasaki T (2015) In vitro parallel evaluation of antimicrobial activity and cytotoxicity of commercially available silver-containing wound dressings. *Plast Surg Nurs*, 35(4):203-211.

Zamora JL (1986) Chemical and microbiological characteristics and toxicity of povidone- iodine solutions. *Am J Surg,* 151:400-406.

Zamora JL, Price MF, Chuang P, Gentry LO (1985) Inhibition of povidone-iodine's bactericidal activity by common organic substances: an experimental study. *Surgery,* 98:25-29.

Zhang YM, Rock CO (2008) Membrane lipid homeostasis in bacteria. *Nature Rev Microbiol*, 6:223-233.

Zhao G, Usui ML, Lippman SI, James GA, Stewart PS, Fleckman P, Olerud JE (2012) Biofilms and Inflammation in Chronic Wounds. *Advances in Wound Care*, 2 (7):389 -399.

Zhao R, Liang H, Clarke E, Jackson C, Xue M (2016) Inflammation in Chronic Wounds. *Int J Mol Sci*, 17(12): 1-14.

Zhou LH, Nahm WK, Badiavas E, Yufit T, Falanga V (2002), Slow release iodine preparation and wound healing: in vitro effects consistent with lack of *in vivo* toxicity in human chronic wounds. *British J Derm,* 146:365-37.

Zumla A, Lulat A (1989) Honey: a remedy rediscovered. *J Royal Soc Med*, 82(7):384-5.

Appendix 1. Post Hoc Test Results

Table 1: Scheffé Test

Dressing		1	2	3	4	5	6	7	8	9	10	11
Scheffe[a,b,c]	Actilite Tulle	11										
	Bactigras	31										
	Iodozyme		107									
	Medihoney Gel		109									
	Inadine		132	132								
	Activon Tulle		150	150	150							
	Apinate			199	199	199						
	Urgotul SSD				209	209						
	Urgotul Ag					227						
	Algivon					250						
	Suprasorb					274	274					
	Urgosorb Ag						340	340				
	Aquacel Ag							398	398			
	Sorbsan Ag							409	409			
	Allevyn Ag								437			
	Aquacel Ag Extra									518		
	Biatain Ag									534		
	Iodoflex										626	
	CROWD L										676	
	CROWD D											845

Table 2: Tukey HSD Test

								Subset				
Dressing		1	2	3	4	5	6	7	8	9	10	11
Tukey HSD[a,b,c]	Actilite Tulle	11										
	Bactigras	31										
	Iodozyme		107									
	Medihoney Gel		109									
	Inadine		132									
	Activon Tulle		150									
	Apinate			199								
	Urgotul SSD			209	209							
	Urgotul Ag			227	227	227						
	Algivon				250	250						
	Suprasorb					274						
	Urgosorb Ag						340					
	Aquacel Ag							398				
	Sorbsan Ag							409				
	Allevyn Ag							437				
	Aquacel Ag Extra								518			
	Biatain Ag								534			
	Iodoflex									626		
	CROWD L										676	
	CROWD D											845

Table 3: Student –Newman- Keuls Test

Dressing		Subset 1	2	3	4	5	6	7	8	9	10	11	12	13
Student-Newman-Keuls[a,c]	Actilite Tulle	11												
	Bactigras	31												
	Iodozyme		107											
	Medihoney Gel		109											
	Inadine		132	132										
	Activon Tulle			150										
	Apinate				199									
	Urgotul SSD				209									
	Urgotul Ag				227	227								
	Algivon					250	250							
	Suprasorb						274							
	Urgosorb Ag							340						
	Aquacel Ag								398					
	Sorbsan Ag								409					
	Allevyn Ag									437				
	Aquacel Ag Extra										518			
	Biatain Ag										534			
	Iodoflex											626		
	CROWD L												676	
	CROWD D													845

Table 4: Normality Testing for Residuals in ANOVA using SPSS - Descriptives

Descriptives

			Statistic	Std. Error
Residual for ZOI	Mean		.0000	5.65861
	95% Confidence Interval for Mean	Lower Bound	-11.1024	
		Upper Bound	11.1024	
	5% Trimmed Mean		1.0344	
	Median		-.5233	
	Variance		36854.846	
	Std. Deviation		191.97616	
	Minimum		-1323.38	
	Maximum		1498.33	
	Range		2821.71	
	Interquartile Range		124.17	
	Skewness		-.314	.072
	Kurtosis		12.379	.144