

Epithelialization of hydrogels achieved by amine functionalization and co-culture with stromal cells

RIMMER, Stephen, JOHNSON, Claire, ZHAO, Bojun, COLLIER, Joyleen, GILMORE, Louisa, SABNIS, Subodh, WYMAN, Paul, SAMMON, Chris <<http://orcid.org/0000-0003-1714-1726>>, FULLWOOD, Nigel J. and MACNEIL, Sheila

Available from Sheffield Hallam University Research Archive (SHURA) at:

<http://shura.shu.ac.uk/12664/>

This document is the author deposited version. You are advised to consult the publisher's version if you wish to cite from it.

Published version

RIMMER, Stephen, JOHNSON, Claire, ZHAO, Bojun, COLLIER, Joyleen, GILMORE, Louisa, SABNIS, Subodh, WYMAN, Paul, SAMMON, Chris, FULLWOOD, Nigel J. and MACNEIL, Sheila (2007). Epithelialization of hydrogels achieved by amine functionalization and co-culture with stromal cells. *Biomaterials*, 28 (35), 5319 - 5331.

Copyright and re-use policy

See <http://shura.shu.ac.uk/information.html>

Editorial Manager(tm) for Biomaterials
Manuscript Draft

Manuscript Number: jbmt7409R1

Title: Epithelialization of hydrogels achieved by amine functionalization and co-culture with stromal cells

Article Type: FLA Original Research

Section/Category: Biomaterials & Tissue Engineering

Keywords: hydrogels; cornea; co-culture; Epithelialization

Corresponding Author: Dr. S. Rimmer,

Corresponding Author's Institution: University of Sheffield

First Author: S. Rimmer

Order of Authors: S. Rimmer; Claire Johnson, BSc; Joyleen Collier, MChem; Bojun Zhao, MD; Louisa Gilmore, MChem; Subodh Sabnis, MSc; Paul Wyman, MChem, PhD; Christopher Sammon, BSc, PhD; Nigel J Fullwood, BSc, PhD; Sheila MacNeil, BSc, PhD

Manuscript Region of Origin:

Abstract: Abstract

The aim of this study was to develop a hydrogel which would be suitable for corneal cell re-epithelialization when used as a corneal implant. To achieve this, a series of hydrogels were functionalized with primary amines by post-polymerization reactions between amine compounds and glycidyl ether groups attached to the hydrogels. We report a strong correlation between the structure of the amine and the viability of stromal cells and epithelial cells cultured on these hydrogels. Subsequent co-culture of epithelial and stromal cells on the amine modified hydrogels allowed successful expansion of epithelial cells on surfaces functionalised with alkyl α - ω diamines with carbon chain lengths of between 3 and 6. Analysis of variance showed that corneal epithelial cells had a strong preference for surfaces functionalized by the reaction of excess 1,3-diaminopropane with units of glycidyl methacrylate compared to the reaction products of other amines (ammonia; 1,2-diaminoethane; 1,4-diaminobutane or 1,6-diaminohexane). We suggest this approach of

amine functionalization combined with stromal/epithelial co-culture offers a promising new approach to achieving a secure corneal epithelium.

AUTHOR DECLARATION

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.


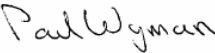
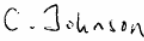



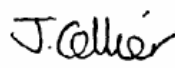
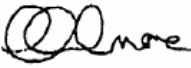
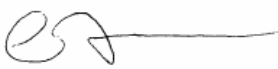
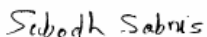
We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from biomaterials@online.be.

Signed by all authors as follows:

Dr Stephen Rimmer	
Dr Paul Wyman	
Ms Claire Johnson	
Dr Bojun Zhao	
Dr Nigel J. Fullwood	
Prof. Sheila MacNeil	
Ms Joyleen Collier	
Ms. Louisa Gilmore	
Dr. Chris Sammon	
Mr. Subodh Sabnis	

Rimmer et al Epithelialization of hydrogels

Epithelialization of hydrogels achieved by amine functionalization and co-culture with stromal cells

Stephen Rimmer,^{a*} Claire Johnson,^c Bojun Zhao,^b Joyleen Collier^a, Louisa Gilmore,^a Subodh Sabnis^d, Paul Wyman^a, Christopher Sammon^d, Nigel J. Fullwood^b, Sheila MacNeil^{c*}

^a*Polymer and Biomaterials Chemistry Laboratories, Chemistry Department, University of Sheffield, Sheffield, South Yorkshire, S3 7HF, UK*

^b*Biomedical Sciences, Department of Biological Sciences, Lancaster University, Lancaster, LA1, 4YQ, UK*

^c*Department of Engineering Materials, The Kroto Research Institute, University of Sheffield, Broad Lane, Sheffield, South Yorkshire, S3 7HQ, UK.*

^d*Materials and Engineering Research Institute, Sheffield Hallam University, City Campus, Sheffield, South Yorkshire, S1 1WB, UK*

**Corresponding authors*

Rimmer et al Epithelialization of hydrogels

Abstract

The aim of this study was to develop a hydrogel which would be suitable for corneal cell re-epithelialization when used as a corneal implant. To achieve this, a series of hydrogels were functionalized with primary amines by post-polymerization reactions between amine compounds and glycidyl ether groups attached to the hydrogels. We report a strong correlation between the structure of the amine and the viability of stromal cells and epithelial cells cultured on these hydrogels. Subsequent co-culture of epithelial and stromal cells on the amine modified hydrogels allowed successful expansion of epithelial cells on surfaces functionalised with alkyl α - ω diamines with carbon chain lengths of between 3 and 6. Analysis of variance showed that corneal epithelial cells had a strong preference for surfaces functionalized by the reaction of excess 1,3-diaminopropane with units of glycidyl methacrylate compared to the reaction products of other amines (ammonia; 1,2-diaminoethane; 1,4-diaminobutane or 1,6-diaminohexane). We suggest this approach of amine functionalization combined with stromal/epithelial co-culture offers a promising new approach to achieving a secure corneal epithelium.

Introduction

Synthetic hydrogels containing high densities of hydroxyl groups are generally known as non-fouling biomaterials that are not conducive to the adhesion of most mammalian cell types. On the other hand, they have distinct advantages over other potential materials when used as supports or scaffolds in tissue engineering. In particular, their non-adhesive nature allows for modification with molecules that can guide cell growth[1] and their swollen state allows diffusion and release of essential biomolecules throughout the construct[2]. In this connection we previously showed that mammalian cells can adhere and proliferate on amphiphilic conetworks[3-5]. In that work, the polymer conetworks, formed from copolymerization of preformed oligomers[6] and low molecular weight monomer, phase separated from the reaction medium during synthesis to give porous structures and cell adhesion and proliferation was dependant both on overall polymer composition and on morphology.

In this study we address the problem of developing hydrogel surfaces for corneal implants. The use of phase separated materials in ophthalmic applications is problematic because of light scattering from either water/polymer interface (in porous materials) or micro-phase separated polymer domains. Careful optimization of random copolymer amphiphilic networks, which do not phase separate and are often optically clear, can provide materials for cell culture scaffolds but considerable improvements are needed if they are to be of use in clinical situations[7]. One strategy for further improvement of the cell-adhesive properties of hydrogels is to incorporate specific cell-adhesive peptide sequences[8-18]. Other non-peptide chemical surface modifications particularly creating

charged surfaces have also been shown to improve cell adhesion and proliferation on hydrogels. For example, it has been shown that neurite outgrowth and viability was enhanced [19,20] and proliferation of keratinocytes improved[21] as the amine content of chitin hydrogels increased. Also, Pokharna et al observed increased growth of fibroblasts on poly(hydroxyethyl methacrylate) hydrogels containing amine functionality[22]. These authors also suggested that the length of the branch from the polymer chain to the amine group might have a critical effect on biocompatibility. Hydrogels containing quarternary ammonium functionality have also been reported to be good substrates for some cell types[23].

In bioengineering of hydrogel-based corneal implants it is necessary to have a surface that promotes attachment, migration and proliferation of corneal epithelial cells. This has not yet been achieved to the best of our knowledge. In this paper we describe how we have combined amine modification of the surface of a hydrogel with a knowledge of epithelial/stromal cell dependency (as detailed in 24-28) to control and optimise epithelialization on hydrogel surfaces. In brief our prior experience with stromal cell/epithelial cell co-culture has shown that it is possible to achieve epithelial cell growth (both primary human keratinocytes [25-28] and primary human corneal epithelial cells) [29] on a range of substrates providing one co-cultures them with stromal cells. Cell culture conditions can be achieved where stromal cells act as initial support/feeder cell for epithelial cells so that epithelial cells can be grown serum-free on substrates where they would not previously grow unaided [25-29]. .

In this study we first modified the hydrogel surfaces with amines of different chain lengths and showed that the carbon chain length of these amine modifications is a critical parameter in the attachment of stromal cells or epithelial cells. We then combined this with corneal epithelial cell/stromal cell co-culture to provide the crucial epithelial-fibroblast interactions which then enabled the epithelial cells to survive and proliferate on the hydrogel surface.

Methods

Materials

Unless stated otherwise all reagents and chemicals were obtained from Aldrich (UK) Ltd. Laurylmethacrylate (DM) and ethyleneglycol dimethacrylate (EGDMA) were passed through a column of basic alumina to remove the inhibitors. Glycerol monomethacrylate (GM) (Rohm Haas) was stored at -18°C and used as received. Glycidylmethacrylate (GME) was distilled *in vacuo* prior to use. All amines were obtained from Fluka (UK), stored under nitrogen and used without further purification. Photoinitiator, 2-hydroxy-2-methylpropiophenone (Irgacure 1173), was stored in the dark at -18°C and used as supplied. Dioxane (“sureseal”, Aldrich (UK)) and 2-propanol (IPA), HPLC grade, (Fischer) were used as received.

Photopolymerization

The monomers, DM, GM, EGDMA and GME in varying ratios (total 9g) were dissolved in IPA (4ml) along with 2-hydroxy-2-methylpropiophenone (90mg, 1wgt.% monomers). The mixtures were added to a polymerization mould and irradiated with 2” arc 400W mercury discharge lamp at a distance of 10cm in a Dimax model Bondbox on a rotating

table for 40seconds on each side. The polymerization mould consisted of two 4mm thick glass sheets covered with 100 μm PET film (hifi films pmx727, no slip) attached by the minimum amount of 3M spray-mount[®] adhesive. The plates were separated with a rectangular 500 μm PTFE spacer. The monomer feeds used are shown in Table 1. The sheets were 125mm x 70mm x 0.5mm when removed from the mould.

Reaction with diamines or ammonia

Amine functional polymers (10g) were produced by soaking freshly prepared polymers in ethanolic solutions containing the required amine (250 cm^3 , 5 vol%) in large excess for 24 hours at room temperature. The polymers were modified with ammonia (**1**), 1,2-diaminoethane (**2**), 1,3-diaminopropane (**3**), 1,4-diaminobutane (**4**) or 1,6-diaminohexane (**5**). In the case of ammonia, concentrated ammonia was used. In all other cases the pure amines were used as supplied. The use of a large excess of the amine ensures that only one amine of the diamine compounds reacted with the glycidyl ether group of the GMA residues and that all of the glycidyl ether groups were quantitatively converted. This operation was conducted in screw-top polypropylene bottles. Polymers were soaked twice overnight in absolute ethanol to remove excess amine.

Characterization of hydrogels

The equilibrium water contents (EWC) were determined gravimetrically in deionized water on cylindrical samples cut using a cork borer. 6 sample disks per material were cut with a number 2 cork borer (6 mm diameter) and were hydrated over 24 hours in ultrapure water prepared by filtration (Milli-Q Systems). The equilibrium water contents (EWC) were calculated according to Eq. 1.

$$\text{EWC} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100 \quad (1)$$

The presence of the bound primary amines groups was confirmed by reaction with trinitrobenzenesulphonic acid (TNBS). The hydrogels were cut into disks (8mm diameter) with a size 4 cork borer and soaked in aqueous 0.01M sodium tetraborate solution (3 cm³ per disk) then an aqueous solution of TNBS (0.2 cm³, 0.03M dm⁻³) was added. This mixture was left to react at room temperature with agitation for 2 hours. Disks were then gently blotted with tissue paper, attached to the front of a quartz cuvette and the adsorbance at 430 nm was determined. The concentration of primary amine was calculated using the Beer-Lambert law using a molar extinction coefficient obtained by reacting a known concentration of isopropyl amine with TNBS. The adsorbance of solutions of this product gave a molar extinction coefficient at 430 nm of $\epsilon = 9426.5 \text{ molL}^{-1}\text{cm}^{-1}$. Raman spectroscopy was used to confirm the presence and reaction of the glycidyl ether group. Raman spectra were collected on a Renishaw 1000 system, with a 785nm laser excitation and a typical exposure time of 200 seconds. The micro pKas of the amine units were estimated by producing model compounds in the Marvin Sketch 4.1.4 (ChemAxon Ltd) and then running the pKa plugin, which also calculates the predicted microspecies distributions as a function of pH.

Preparation of hydrogels for cell culture

Polymer sheets were washed five times in phosphate buffered saline (PBS) under aseptic conditions to remove the ethanol and then cut into 13mm x 500 μm discs with a No. 9 cork borer and stored in 0.1% NaN₃ solution. Before the addition of cells, the polymer discs were washed three times in PBS, including one overnight wash.

Cell culture

Bovine stromal cells (keratocytes) were isolated from bovine corneas obtained within a few hours of slaughter from a local abattoir. The corneal epithelium and endothelium were scraped off with a scalpel blade and the stroma was cut into small pieces (2x3mm). Three pieces of stromal explants were placed onto each pre-scratched well of 6-well culture plates (3.5 cm diameter wells) and foetal calf serum (FCS, 0.5 cm³) was added to each explant. The plates were left 20-30 minutes before 2 ml of Dulbecco's Modified Eagle's Medium (DMEM, 2 cm³) supplemented with FCS (20wt%), fungizone (2.5 µg cm⁻³), streptomycin (100 µg cm⁻³), kanamycin (100 µg cm⁻³), benzyl penicillin (100 U cm⁻³) and 2 mMol.dm⁻³ glutamine was added to the each well. The plate was returned to the incubator and left for 2-3 days until keratocyte outgrowth was observed. Explants were then removed from the plate. Culture medium was changed every 2-3 days and the cells were allowed to reach confluence before subculture. BK cells were cultured on tissue culture plastic (TCP) in DMEM (Sigma-aldrich, UK) supplemented with 20% fetal bovine serum (Biosera, UK), 2mMol.dm⁻³ L-glutamine, penicillin (100 U cm⁻³), streptomycin (100µg cm⁻³) and amphotericin B solution (0.625µg cm⁻³). The human corneal epithelial cell line (HCEC) was obtained from LGC Promochem UK (the European distributor for the American Tissue Culture Collection (ATCC)) and cultured in Epilife[®] medium with Human Cornea Growth Supplement (HCGS) (Cascade Biologics, UK) on TCP flasks pre-coated with collagen I (0.03mg/ml) and fibronectin (0.01mg cm⁻³) for 2h at 37°C, as these cells grow poorly on TCP. Both cell types were grown in a humidified atmosphere of 5% CO₂/95% air at 37°C until nearly confluent.

Culture of cells on hydrogel discs

Hydrogel discs were transferred to a 24-well tissue culture plate and incubated overnight in either serum-free DMEM for the culture of bovine keratocytes, or EpiLife[®] medium with antibiotics for the culture of HCEC. For single culture, 2×10^4 cells were seeded in their cell specific fresh medium (1.0ml) onto each of the polymers (in triplicate). For co-culture, 1×10^4 of each cell type were seeded using serum-free conditions in EpiLife media (1.0ml) appropriate for the culture of HCEC. The control substrate used for co-culture was TCP. All cultures, single culture and co-culture, were cultured for 8 days and photographs were taken at 1, 2, 3 and 8 days. Cell viability of the BK cultures was assessed using the MTT assay of cellular dehydrogenase activity, as described in reference 5, on days 3, 8 and 16 and the HCEC and BK/HCEC cultures were assessed on days 3, 6 and 8.

Labelling of cells with Red CellTracker[™]

A confluent monolayer of bovine keratocytes were detached from a flask, re-suspended in DMEM (serum-free) (5ml) containing CellTracker[™] Red CMTPX (50 μ g in 20 μ l DMSO) (C34552, Molecular Probes) and incubated for 40min at 37°C (final concentration 14.5 μ Mol. dm⁻³). Cells were centrifuged and resuspended in DMEM (with serum) for 30min at 37°C and then centrifuged again before seeding onto the hydrogels.

Specific identification of epithelial cells by immunolabelling for keratin 3

HCEC's and HCEC/bovine keratocyte co-cultures were washed once with PBS, fixed in 10% buffered formalin for 30minutes at room temperature and then washed 3 times with PBS. To block nonspecific binding, the tissues were incubated with 2% bovine serum albumin. The cells were then permeabilised by treatment with 0.1% TritonX for 5

minutes. Subsequently, the samples were incubated at room temperature for 2 hours with a mouse monoclonal antibody against K3 (anti-K3) (Progen Biotechnik, Heidelberg, Germany), then washed several times in PBS. Control incubations were with the appropriate normal mouse IgG at the same concentration as the primary antibody and omission of the primary antibody for the respective specimen. After staining with the primary antibody, the sections were incubated at room temperature for 2 hours with a fluorescein (FITC)-conjugated goat anti-mouse IgG (Sigma, UK). After several washings with PBS, the samples were imaged using a Leica confocal microscope (SB2-AOBS).

Statistics

All cell biology experiments were based on triplicate wells containing hydrogels and two separate batches of hydrogel were examined (referred to as Experiments 1 and 2). Student's non-paired t-test (EXCEL) was used to assess differences in viability between cells cultured on the differing polymers and the controls. One way ANOVA (MINTAB[®] 14.20) was used to assess any differences between sets of data obtained when cells were grown on the aminated polymers. The MTT data were grouped into sets in which the following were held constant: the composition of the monomer feed; cell type(s) and culture time. Post hoc analysis was carried out by examination of the 95% confidence intervals.

Results

Preparation of amine modified hydrogels.

The materials strategy takes advantage of the fact that glycidyl methacrylate repeat units react with primary amines in a coupling reaction that can be used to functionalize the hydrogels, as shown in Figure 1. The use of excess diamines produced polymers

containing primary amines, from non-reacted amine groups, and secondary amines, from amines that have reacted with glycidyl ether groups. We used this amination reaction to create a series of amine-functional polymers in which the length of an alkyl spacer between the amine functionality and the backbone of the polymer was altered. The reaction of the glycidyl ether group was confirmed by FT-IR Raman spectroscopy as shown in Figure 2. Prior to treatment with amines the spectra show the glycidyl ether band at $\sim 807\text{ cm}^{-1}$. This was absent after reaction with each of the amines as shown in Figure 2 b and c.

Two formulations (**A** and **B**) were prepared that contained the hydrophobic monomer, DM, whereas the latter was omitted from formulation **C** as summarised in Table 1. Figure 1 shows the series of amines that were attached to the glycidyl ether groups of the terpolymer and the resultant functionality. The equilibrium water contents were measured gravimetrically as described previously [29] and are shown in Figure 3. The data show that the structure of the amine does not have much effect on equilibrium swelling and, therefore, this aspect is not a major factor in the EWC attained by these hydrogels. The **A** and **C** materials vary in EWC between 39 – 49 wt%. Also, the EWC of polymers based on formulation **B** are, for all amines, between 45 and 54 wt% and higher than those based on formulations **A** and **C**. Thus series **A** and **C** have the same EWC but **A** contains the hydrophobic component DM, whereas **C** does not. On the other hand both series **A** and **B** contain DM but their compositions were designed to give different EWCs.

The reaction of primary amines with glycidyl ether groups in these reaction conditions is expected to go to completion and the use of a large excess of the amine compound should ensure that only a small fraction of the amine-functionality form crosslinks. TNBS reacts with primary amines to yield a coloured product which in this case is covalently linked to the polymer backbone. The amount of this coloured compound was determined and this was used to calculate the concentration of primary amine functionality as shown in Figure 4. The procedure showed that all of the materials contained between 1.4 and 2.4 $\mu\text{mol cm}^{-3}$ of primary amine functionality and ANOVA showed that there was no significant difference between the amount of primary amine present on each of these materials ($F=1.91$, $p = 0.073$).

Viability of cells cultured on amine functionalised hydrogels.

The MTT assay of cell viability was used to examine the effect of amine functionalization on the survival and expansion of stromal cells (BKs) and of epithelial cells (HCECs cells) cultured separately and in co-culture. Two separate experiments were performed using different batches of the substrate polymers (referred to as Experiments 1 and 2 throughout.) Figure 5 shows the data from these experiments with BK cells cultured on the modified polymers and viability assessed at days 3, 8 and 16. On tissue culture plastic (TCP) these cells showed an increase in MTT activity over 16 days in Experiment 1 but no such increase in Experiment 2. The two experiments clearly give different results. However, consistent conclusions can be arrived at by comparisons within the two experiments; ie by comparing results from individual time points to the relevant result with TCP. Therefore, using the MTT value on TCP as the reference point

throughout it was clear that changing the length of the hydrocarbon spacer affected cell viability. When hydrogels were functionalized with **1** cells showed the lowest viability. This was particularly marked in Experiment 1 where there was negligible cell survival on each class of material, **A**, **B** and **C**, when modified with **1** ($p < 0.01$ or greater for all time points) and less dramatic for Experiment 2 ($p < 0.05$ for days 3 and 16 compared to values on TCP). Cell viability on the substrates **A** or **B** modified with **2**, **3**, **4** or **5** showed no significant variation from the TCP. The results from material **C** however indicated that modification with **2** was less effective than modifications with **3**, **4** or **5**; MTT values for days 3, 8 ,and 16 in Experiment 1 and days 3 and 16 in Experiment 2 were significantly lower ($p < 0.05$) than for cells on TCP.

Figure 6 shows the same protocol for HCEC cells. In contrast to the stromal derived BK cells, HCECs do not usually adhere or grow well on TCP but require a substrate of collagen plus fibronectin, which was used as the positive control for this set of experiments. The overall MTT values obtained were low throughout the 8 days of culture even on the positive collagen/fibronectin substrate. The viability of these cells cultured on each of the hydrogels was much reduced compared to those on collagen and fibronectin ($p < 0.05$). Results using the **C** substrate were poorest overall and the MTT data showed that this was a very poor substrate for these cells regardless of the addition of any of the amine modifications. Although HCEC monoculture was much less successful than the BK monocultures, ANOVA within the **A** and **B** sets of materials showed a significant difference in cell viability between modifications **1** to **5** (see later).

In contrast Figure 7 shows the results of two experiments in which co-cultures of HCECs with BKs were examined on the full range of functionalized hydrogels. The co-cultures were deliberately carried out under conditions optimized for HCEC proliferation, using serum-free conditions in Epilife media, but the reference substrate used for co-culture was TCP, which supports BK but not HCEC culture. The supposition made was that this substrate would support BK cells which would in turn support HCEC cells if HCEC media were used.

The data show a clear increase in cell viability over 8 days for these co-cultures in both experiments on the control substrate (TCP) and also on hydrogels that were functionalized with **3**, **4** or **5**, whilst functionalization with **1** or **2** produced poor substrates. The relationship between amine chain length and cell response to it was consistent for both batches of hydrogel materials. A statistical analysis of cell preference for hydrogels based on all cell culture experiments was then undertaken.

A series of one way ANOVA procedures were used to compare cell performance on the hydrogels functionalized with the different amines; in each experiment $p < 0.01$. ANOVA was carried out on each of the groups of data shown in Figures 5-7. Post hoc analysis of the results of the ANOVA then allowed us to place each amine into sets that were not significantly different by using the 95 % confidence intervals. We then plotted the frequency of an amine appearing in the sets with the highest cell viability results as shown in figure 8a. The same procedure was used to identify those that gave the poorest support to cells (Figure 8b).

Figure 8a clearly indicates that in the case of single culture of HCECs, materials functionalized with **3** produced more viable cultures than any of the other amines. In co-culture, materials functionalized with **4** and **5** also performed well, but materials functionalized with **3** were more frequently in the best performing group. Differences between **3**, **4** or **5** appeared to be less pronounced for the culture of BKs. It was evident that materials functionalized with **2** only rarely appeared in the best performing group and there were no instances of materials functionalized with **1** in these groups. Figure 8 b shows that both **1** and **2** were most frequently observed in the worst performing groups. Materials functionalized with **1** were, in all instances, in this group regardless of cells cultured on them or polymer composition.

Identification of cell type in co-cultures on hydrogels.

The MTT data in the previous experiments did not allow one to distinguish between the cell types in these co-culture experiments. As the culture medium was optimized for HCEC proliferation we expected that the increase in overall cell viability detected with the MTT assay was derived from proliferation of the HCECs and this was supported by visual inspection of the cultures using optical microscopy. Figure 9 shows optical micrographs of examples of cells cultured for 6 days on the materials **1- 5**. The most striking aspect of these data is the very significant change in cell organization of the HCECs on materials functionalized with **1** and **2** when compared to those functionalized with **3**, **4** or **5** in both mono-culture and co-culture with BKs. On materials **1** and **2** the HCECs are present but they form rounded colonies with the cells apparently stacked into aggregates. The MTT data on these cultures shows that the overall viability of these cells is very low. In contrast, HCECs cultured on materials **3**, **4** or **5** are well separated and

have the appearance of HCECs cultured on their optimized substrate (collagen and fibronectin). The HCECs appear to be slightly less numerous than the control, but their appearance and cell number is superior to HCECs co-cultured with BKs on TCP; the reference surface used for the BKs.

Specific identification of cells was then undertaken by immunostaining HCEC for keratin 3 (K3), which is specific to cornea epithelial cells, and prestaining BK cells with red CellTracker®, so that it was possible to identify these cells in co-culture. Preliminary experiments were undertaken to confirm the identification of cells in monoculture. BKs were cultured on TCP and HCECs were cultured on collagen and fibronectin then both cultures were examined for the presence of K3. Figure 10 shows that the BKs could not be identified with anti-K3 and were only visualized with the red cell tracker procedure. On the other hand, anti-K3 bound to the HCECs giving a clear indication of their phenotype. We then used these two visualization techniques to examine co-cultures on the hydrogels modified with amines **3**, **4** or **5**. Figure 9 shows the results of cell culture on the **B** substrate modified with **3**, **4** and **5**. Examination of the co-cultures with anti-K3 clearly shows that by day 6 the dominant cell type was the HCECs despite seeding hydrogels with equal numbers of both cells on day 0. The HCEC achieved more than 70% confluence by day 6 as shown in Figure 11 and continued to increase in confluence as indicated by the MTT data in Figure 11 for day 8. Of considerable importance there was, in contrast, no comparable increase in BK cells, which were present in only low numbers by the end of this culture period.

Discussion

The aim of this work was to develop a hydrogel that would be suitable for corneal cell culture when used as a corneal implant. The addition of amine functionality has previously been used to enhance the adhesion of mammalian cells to synthetic substrates. Most authors ascribe the observed enhancements to a combination of increased surface charge and increased hydrophilicity. In this study a decrease in surface hydrophilicity might be expected on progressing from amine **1** to **5**. However, the culture data indicates a general preference for the more hydrophobic amines with alkyl spacers of 3 to 6 carbons. Hydrogels modified with **1** have a lower total (primary + secondary) amine density than those modified with **2** to **5**. Also, the proximity of the two amine groups in hydrogels modified with **2** would act to reduce the overall pK_a of the system and, therefore, we should expect hydrogels modified with **1** or **2** to possess lower charge densities than those modified with **3** to **5**. In order to further investigate these changes in charge density with structure, we calculated the distributions of charge states at pH 7.2 by calculating (using the plugin available in Marvin) the theoretical micro pK_a s of the units carrying the amine groups as shown in Figure 11. The material functionalized with **1** is predicted to be fully ionized. Figure 12 shows that all the materials are charged at pH 7.2 and that we should expect an increase in charge density on these materials in going from **1** to **2** to **3**. The material functionalized with **1** is expected to be fully ionized but it contains only one amine group per unit. In **2** the amine groups are separated by 2 carbons which means that one amine group reduces the basicity of the other so that 56 mol.% of these groups are predicted to be in the dication state and the rest are monoprotonated at one of the amine groups. Adding an extra carbon has a large effect so that materials

functionalized by **3** contain 96 mol% of the diamines in the dication state. Functionalization with **4** or **5** then produces materials that are essentially fully ionized. These changes in charge density may be a factor in the performance of these materials because the materials which have the highest charge density produce the best cell culture substrates. However, it is likely that other factors are also important and further work would be needed before the role of charge could be confirmed. Our previous work showed that human fibroblasts did not adhere to poly(GMA-co-EGDMA) hydrogels but they did adhere to the same networks modified with LMA, poly(GMA-co-EGDMA-co-LMA) [5]. In this work we studied one set of aminated materials based on poly(GMA-co-EGDMA) and two similar materials (with different EWCs) based on poly(GMA-co-EGDMA-co-LMA). Each of these materials performed well when modified with **3**, **4** or **5**, but performed poorly when modified with **1** or **2**. ANOVA was applied to examine the effect of polymer composition in this limited set of polymer formulations. Although there were differences between the response of cells to the three hydrogel formulations **A**, **B** and **C**, these were much less than the differences seen between aminated and non-aminated hydrogels. Therefore, it seems reasonable to conclude that the main factor controlling cell adhesion and proliferation on these materials is the presence and structure of the amine group.

The approach of co-culture which was introduced in these experiments was based on prior work from our laboratories on skin cell culture, where we have shown that fibroblasts will promote keratinocyte proliferation in the absence of serum on a variety of substrates [25-28]. The fibroblast feeder layer also reduces the degree of differentiation

of keratinocytes, which may be advantageous for initial wound healing, and the fibroblasts can be derived from several sources - murine, human, donor or autologous cells[25]. Similarly with respect to the cornea we recently confirmed that corneal fibroblasts will support freshly isolated human corneal epithelial cells in 2D co-culture on a range of surfaces produced by plasma polymerization under serum free conditions[29]. As discussed in [24] extensive prior work on the use of fibroblast feeder layers to support keratinocyte expansion from Rheinwald and Green [30] has shown that fibroblasts provide both adhesive extracellular matrix proteins such as fibronectin as well as a range of growth factors which stimulate epithelial cell proliferation. In our own work we have shown extensive fibronectin production by fibroblasts in monoculture which is attenuated when epithelial cells are co-cultured with them [31] . Fibronectin acts as an adhesive protein in binding cells to both natural and synthetic substrates. Thus, in the current study we extended the co-culture approach to these amine modified hydrogels using bovine keratocytes (BKs) to provide an environment conducive to human corneal epithelial cell adhesion and proliferation.

Conclusions

We have developed a system in which it is possible to achieve epithelial cell culture on previously low-adhesive hydrogels by surface modification of hydrogels using functionalization with primary amines and co-culture of epithelial cells with stromal cells. Both epithelial and stromal cells adhered well to surfaces functionalized with alkyl α - ω diamines with carbon chain lengths of between 3 and 6. Lower carbon chain lengths were less effective. Co-culture with stromal cells in media supporting epithelial cell

growth resulted in a securely attached epithelial layer with relatively few residual stromal cells. We suggest this approach could be translated to the clinic using autologous corneal epithelial and stromal cells.

Acknowledgements

We thank the BBSRC (UK) for funding post-doctoral fellowships for Paul Wyman and Bojun Zhao and for funding to support Ms. C. Johnson. We also thank EPSRC (UK) for studentships for Ms. L. Gilmore and Ms. J. Collier and Sheffield Hallam University for a studentship for S. Sabnis.

Rimmer et al Epithelialization of hydrogels

References.

- [1] Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat. Biotech.* 2005; 23: 47 - 55
- [2] Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications *Biomaterials* 2003;24: 4337–4351
- [3] Rimmer S, Tattersall P, Ebdon JR, Fullwood N New strategies for the synthesis of amphiphilic networks, *React. Funct. Polym.* 1999;41:177-184
- [4] Rimmer S, German MJ, Maughan J, Sun Y, Fullwood N, Ebdon J, MacNeil S. Synthesis and properties of amphiphilic networks 3: Preparation and characterization of block conetworks of poly(butyl methacrylate-block-(2,3 propandiol-1-methacrylate-stat-ethandiol dimethacrylate)) *Biomaterials* 2005;26:2219-2230
- [5] Sun Y, Collett J, Fullwood NJ, Mac Neil S, Rimmer, S. Culture of dermal fibroblasts and protein adsorption on block conetworks of poly(butyl methacrylate-block-(2,3 propandiol-1-methacrylate-stat-ethandiol dimethacrylate)) *Biomaterials* 2007;28:661-670
- [6] Ebdon JR, Rimmer S. A one pot method for the preparation of latices of telechelic oligomers, by ozonolysis of latices of polymers containing main-chain unsaturation *J. Polym. Sci. A Polym. Chem. Ed.* 1997; 35:3255-3262
- [7] Haigh R, Fullwood N, Rimmer S. Synthesis and properties of amphiphilic networks 2: A Differential scanning calorimetric study of poly(dodecyl methacrylate-*stat*-2,3 propandiol-1-methacrylate-*stat*-ethandiol dimethacrylate) networks and adhesion and spreading of dermal fibroblasts on these materials, *Biomaterials* 2002;23:3509-3516

- [8] Yang F, Williams CG, Wang D-A., Lee H., Manson PN, Elisseeff J. The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells *Biomaterials* 2005;26:5991–5998
- [9] Bokharia MA, Akaya G, Zhang S, Mark A, Birch MA. The enhancement of osteoblast growth and differentiation in vitro on a peptide hydrogel—polyHIPE polymer hybrid material *Biomaterials* 2005;26:5198–5208
- [10] Jacob JT, Rochefort JR, Bi J, Gebhardt BM. Corneal Epithelial Cell Growth Over Tethered-Protein/Peptid Surface-Modified Hydrogels *J Biomed Mater Res Part B: Appl Biomater* 2005;72B:198–205
- [11] Park K-H, Na K, Chun H-M. Enhancement of the adhesion of fibroblasts by peptide containing an Arg-Gly-Asp sequence with poly(ethylene glycol) into a thermo-reversible hydrogel as a synthetic extracellular matrix *Biotech. Lett.* 2005; 27: 227–231
- [12] Gümüşderelioğlu M., Müftüoğlu O, Karakeçili A.G. Biomodification of thermosensitive copolymer of ethylene glycol vinyl ether by RGD and insulin. *React. Funct. Polym.* 2004;58:149–156
- [13] Reinhart-King CA, Dembo M, Hammer DA. Endothelial Cell Traction Forces on RGD-Derivatized Polyacrylamide Substrata *Langmuir* 2003;19:1573-1579
- [14] Rowley JA, Sun Z, Golman D, Mooney DJ. Biomaterials to spatially regulate cell fate *Adv. Mater.* 2002;14:886-889
- [15] Kim, MR, Jeong JH, Park TG. Swelling Induced Detachment of Chondrocytes Using RGD-Modified Poly(*N*-isopropylacrylamide) Hydrogel Beads *Biotechnol. Prog.* 2002; 18:495-500

- [16] Woerly S, Pinet E, de Robertis L, Van Diep D, Bousmina M. Spinal cord repair with PHPMA hydrogel containing RGD peptides (NeuroGel) *Biomaterials* 2001;22:1095-1111
- [17] Stile RA, Healy KE. Thermo-Responsive Peptide-Modified Hydrogels for Tissue Regeneration *Biomacromolecules* 2001; 2:185-194
- [18] Hern DL, Hubbell JA. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing *J. Biomed. Mater. Res.* 1998;39:266 – 276
- [19] Freiera T, Montenegro R, Koha HS, Shoichet MS. Chitin-based tubes for tissue engineering in the nervous system *Biomaterials* 2005;26:4624–4632
- [20] Freiera T, Koha HS, Kazaziana K, Shoichet MS, Controlling cell adhesion and degradation of chitosan films by N-acetylation *Biomaterials* 2005;26:5872–5878
- [21] Chatelet C, Damour O, Domard A. Influence of the degree of acetylation on some biological properties of chitosan *Biomaterials* 2001;22: 261-268
- [22] Pokharna HK, Zhong Y, Smith DJ, Dunphy MJ. Copolymers of hydroxyethyl methacrylate with Quadrol methacrylate and with various aminoalkyl methacrylamides as fibroblast cell substrata *J. Bioact Comp Polym* 1990; 5: 42-52
- [23] Alejandro S., Sefton M.V. Methylation of Poloxamine for Enhanced Cell Adhesion *Biomacromolecules* 2006; 7: 331-338
- [24] Higham M, Dawson C, Szabo M, Short R, Haddow DB, MacNeil S. Development of a stable chemically defined surface for the culture of human keratinocytes under serum free conditions for clinical use. *Tiss Engin* 2003;9:919-930
- [25] Sun T, Mai S, Norton D, Haycock J.W., Ryan AJ, MacNeil S. Self-organisation of Skin Cells in 3D-Electrospun Polystyrene Scaffolds. *Tiss Engin* 2005;11:1023-1033

Rimmer et al Epithelialization of hydrogels

[26] Sun T, Higham M, Layton C, Haycock J, Short R, MacNeil S. Development in xenobiotic-free culture of human keratinocytes for clinical use. *Wound Repair and Regeneration*. 2004;12:626-634

[27] Bullock AJ, Higham MC, MacNeil S. Use of human fibroblasts in the development of a xenobiotic-free culture and delivery system for human keratinocytes. *Tiss Engin* 2006;12:245-255

[28] Notara M, Bullett N, Deshpande P, Haddow D, MacNeil S, Daniels J. Plasma polymer coated surfaces for serum-free culture of limbal epithelium for ocular surface disease *Journal of Materials Science: Materials in Medicine* 2007;18:329-338

[29] Haigh R, Fullwood N, Rimmer S. Synthesis and Properties of Amphiphilic Networks 1: The effect of hydration and polymer composition on the adhesion of Immunoglobulin-G to Poly(Laurylmethacrylate-*stat*-Glycerolmethacrylate-*stat*-Ethylene-glycol-dimethacrylate) networks *Biomaterials*. 2000;21:735-739

[30] Rheinwald J G, Green H Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;6:331-343.

[31] Ralston DR, Layton C, Dalley AJ, Boyce SG, Freedlander E, MacNeil S. Keratinocytes contract normal human dermal extracellular matrix and reduce soluble fibronectin production by fibroblasts in a skin composite model. *Brit. J. Plast. Surg.* 1997;50:408-415

Captions to figures

Table 1 The composition of the hydrogels **A**, **B** and **C**

Figure 1. Reaction of diamines or ammonia with GMA residues and table showing the structures of the resultant structural units

Figure 2. FT-IR Raman spectra of: (a) **A**; (b) **A** modified with **1** and (c) **A** modified with **4**. ← indicates the peak derived from the glycidyl ether group

Figure 3. Mean equilibrium water contents (EWC) of materials **A** (■), **B** (●) or **C** (▲) functionalized with amines **1** – **5**. (Error bars are 99% confidence limits).

Figure 4. Primary amine concentrations in each of the hydrogels **A**, **B** and **C** determined by colourimetry of TNBS adducts at 430 nm.

Figure 5. Viability of BKs cultured on amine functional hydrogels study by MTT assay ● control; ◇ **1** ; ■ **2** ; ▲ **3** ; ■ **4** ; ◆ **5** (MTT measured in units of optical density at 410 nm)

Figure 6. Viability of HCECs cultured on amine functional hydrogels study by MTT assay ● control; ◇ **1** ; ■ **2** ; ▲ **3** ; ■ **4** ; ◆ **5** (MTT measured in units of optical density at 410 nm)

Figure 7. Viability of HCEC/BKs cocultured on amine functional hydrogels study by MTT assay ● control; ◇ **1** ; ■ **2** ; ▲ **3** ; ■ **4** ; ◆ **5** (MTT measured in units of optical density at 410 nm)

Figure 8. Aggregated assessment of performance (MTT assay) of amine functionalized materials obtained following ANOVA. Frequency of observing an amine-functionalized material in (a) the best performing group and (b) the worst performing group

Figure 9. Optical micrographs of HCECs cultured for 6 days, on hydrogels functionalized with amines **1** – **5**, in mono-culture and coculture with BKs. Magnification x 100

Figure 10. Micrographs a and b are HCECs on tissue culture plastic (collagen + fibronectin) and c and d are BKs on tissue culture plastic. Micrographs a and c are phase contrast images, b shows HCEC's labelled for keratin 3 and d shows keratocytes loaded with cell tracker. Scale bars 40 µm

Figure 11. Micrographs showing HCECs – BK cocultures on different hydrogels. Micrographs a, b and c are of hydrogel **B** with modification by **3**; d, e and f are of hydrogel **B** with modification by **4** and g, h and i are of hydrogel **B** with modification by **5**. Micrographs a, d and g are phase contrast images; b, e and h show keratin 3 labelling and c, f and i show BKs loaded with cell tracker. Scale bars, 80µm

Figure 12. Distributions of charge states for modifications with diamines **2 – 5** calculated from predicted pK_a s of model compounds with the general structures:

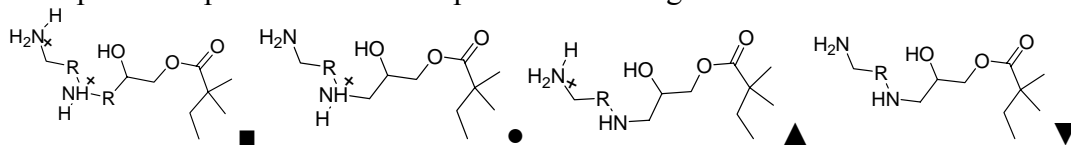
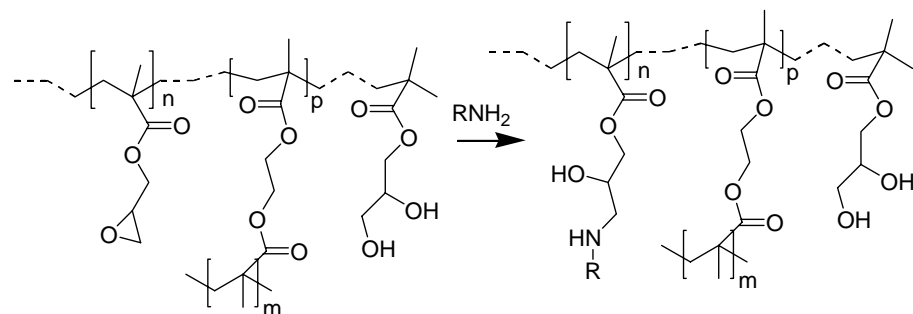


Figure 1



	Amine	Modified structure
1	NH ₃	
2		
3		
4		
5		

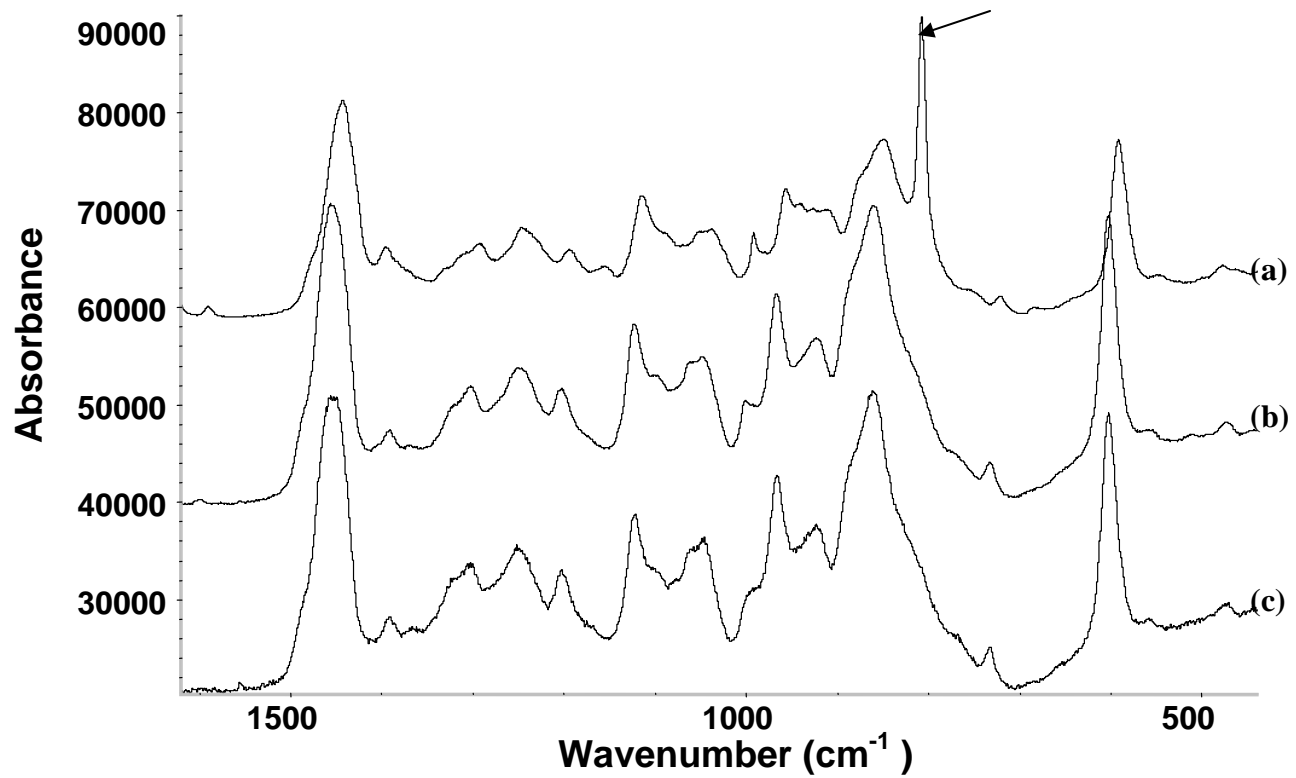


Figure 2

Figure 3

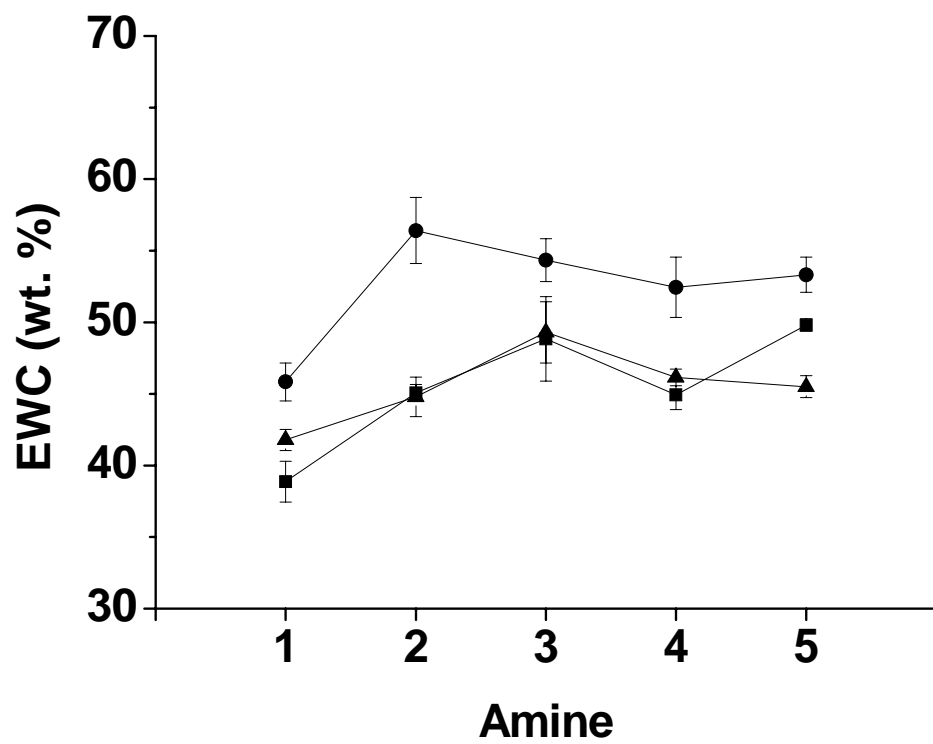


Figure 4

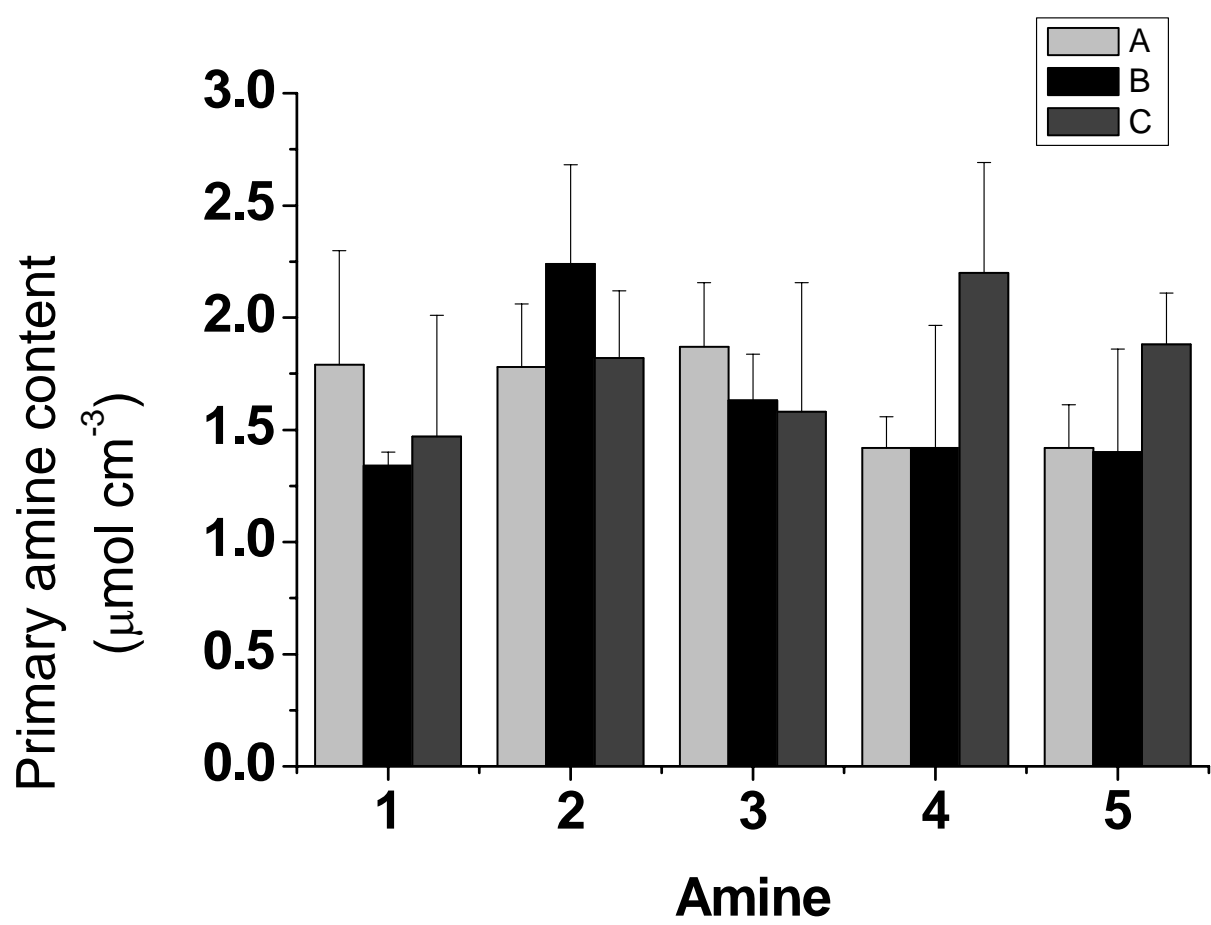


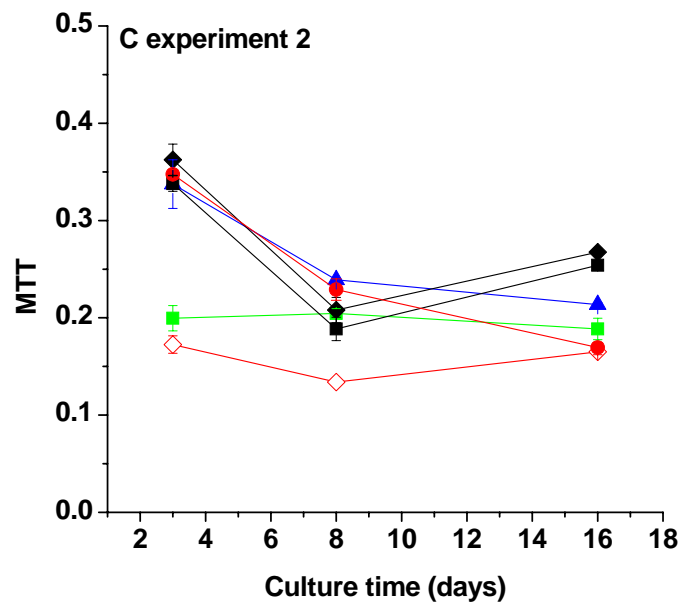
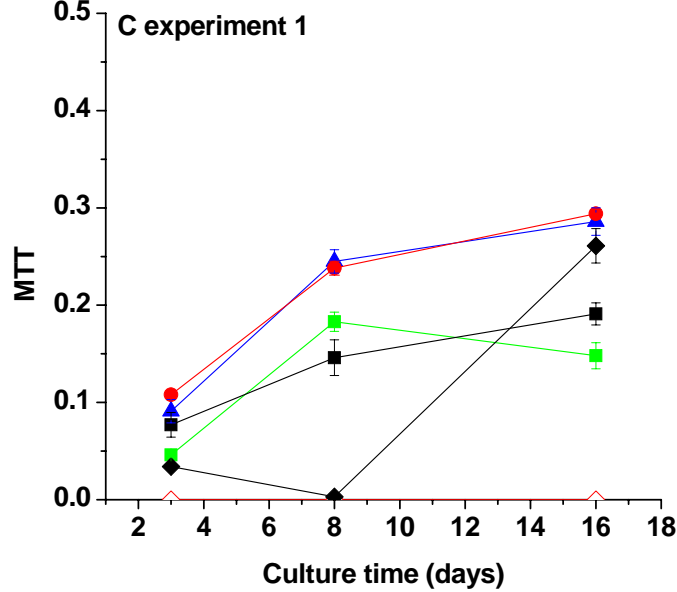
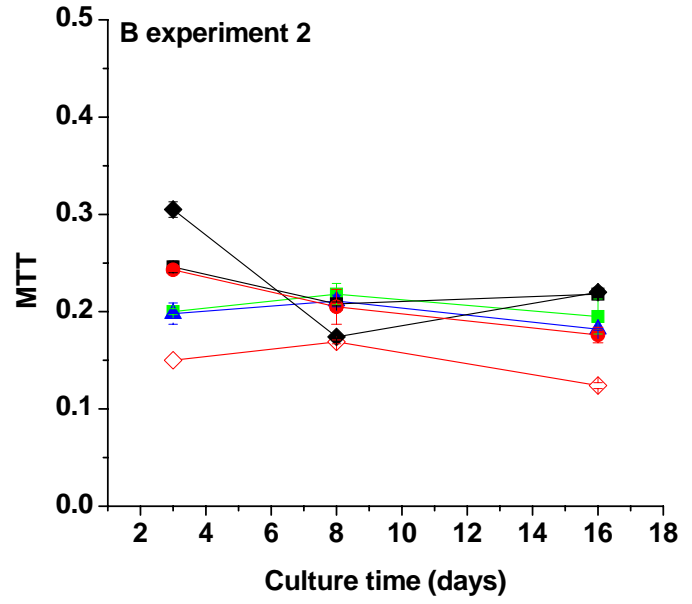
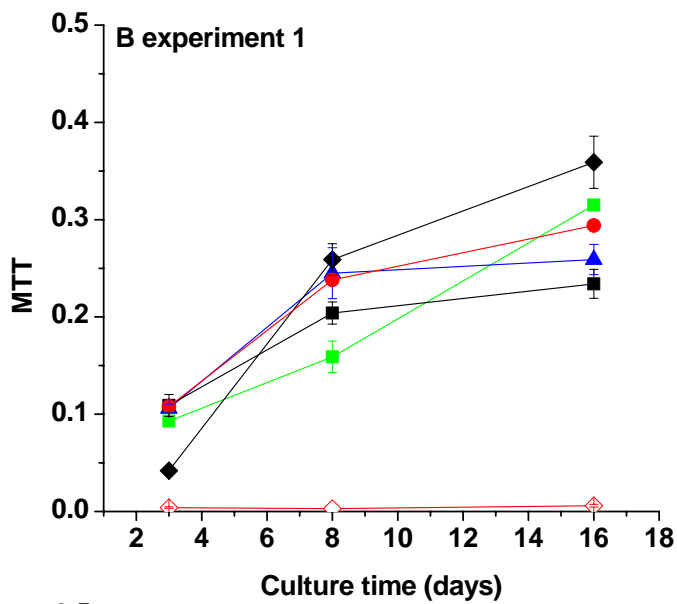
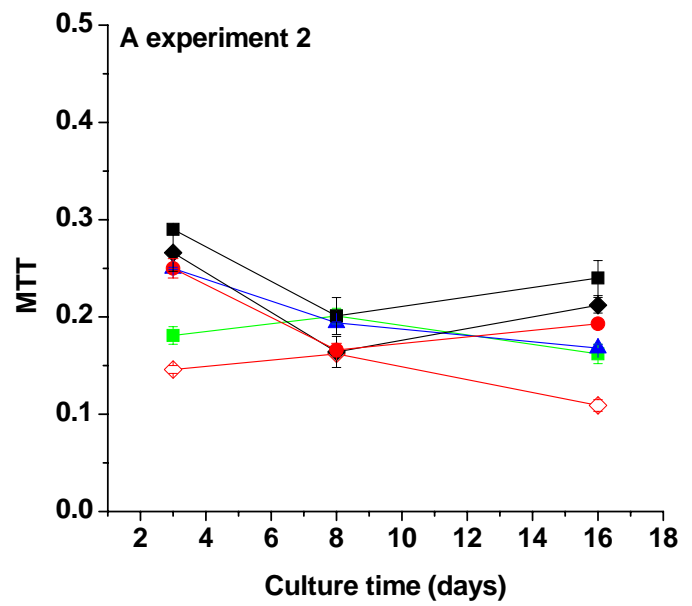
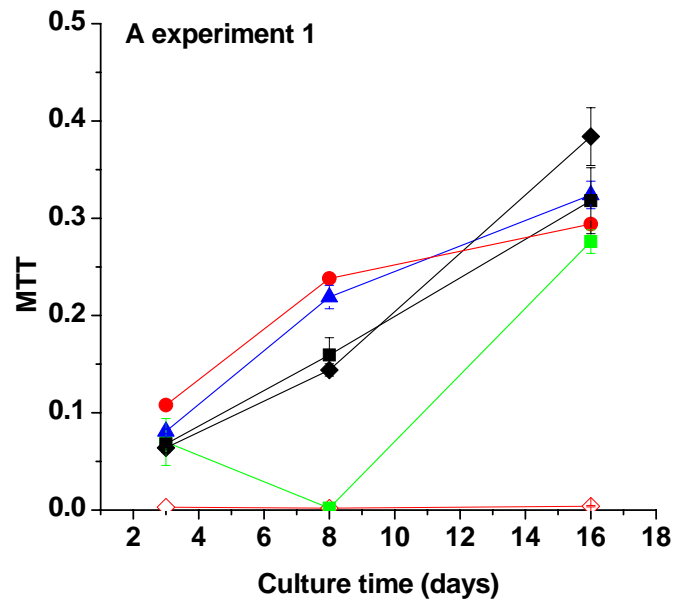
FIGURE 5

Figure 6

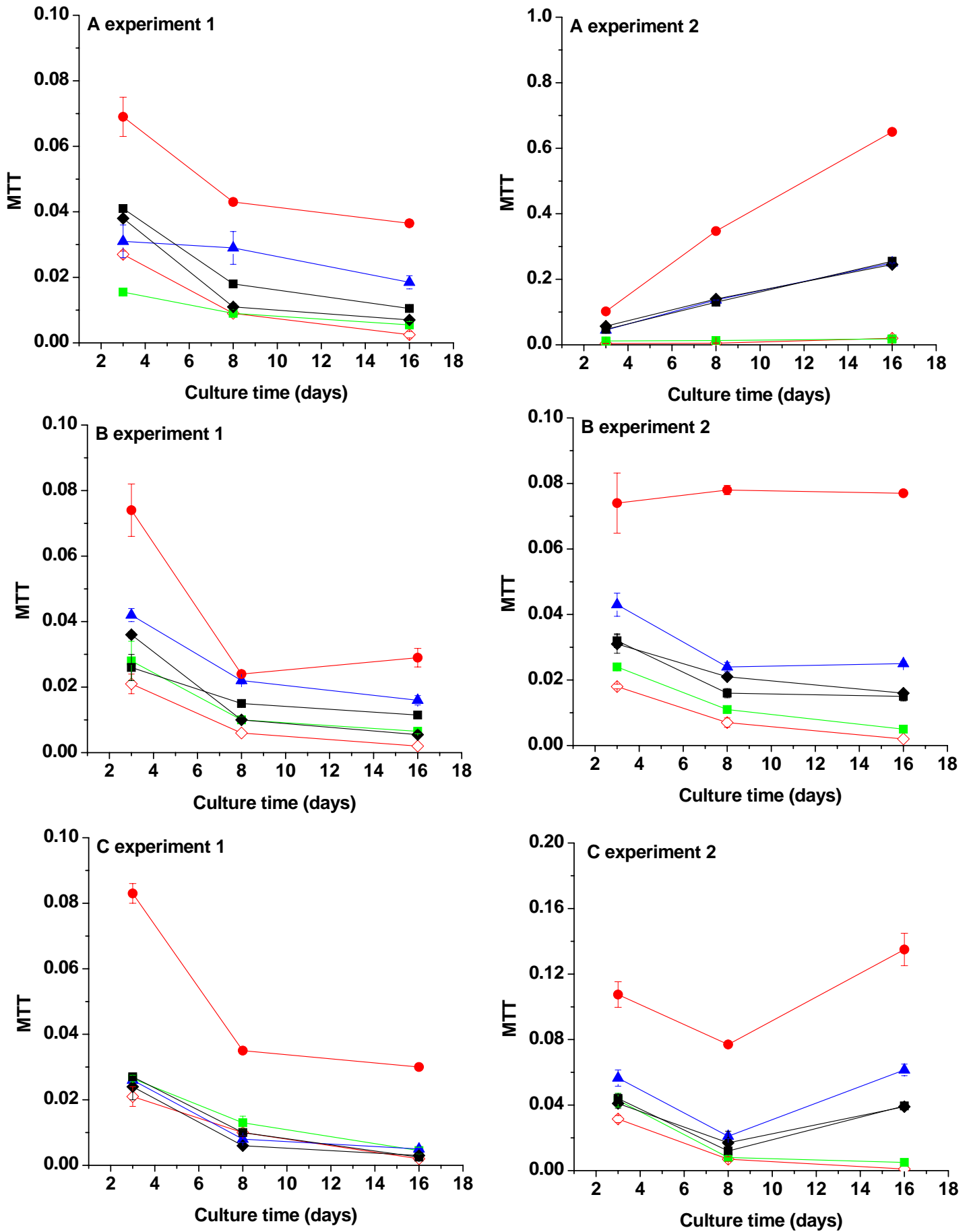


Figure 7

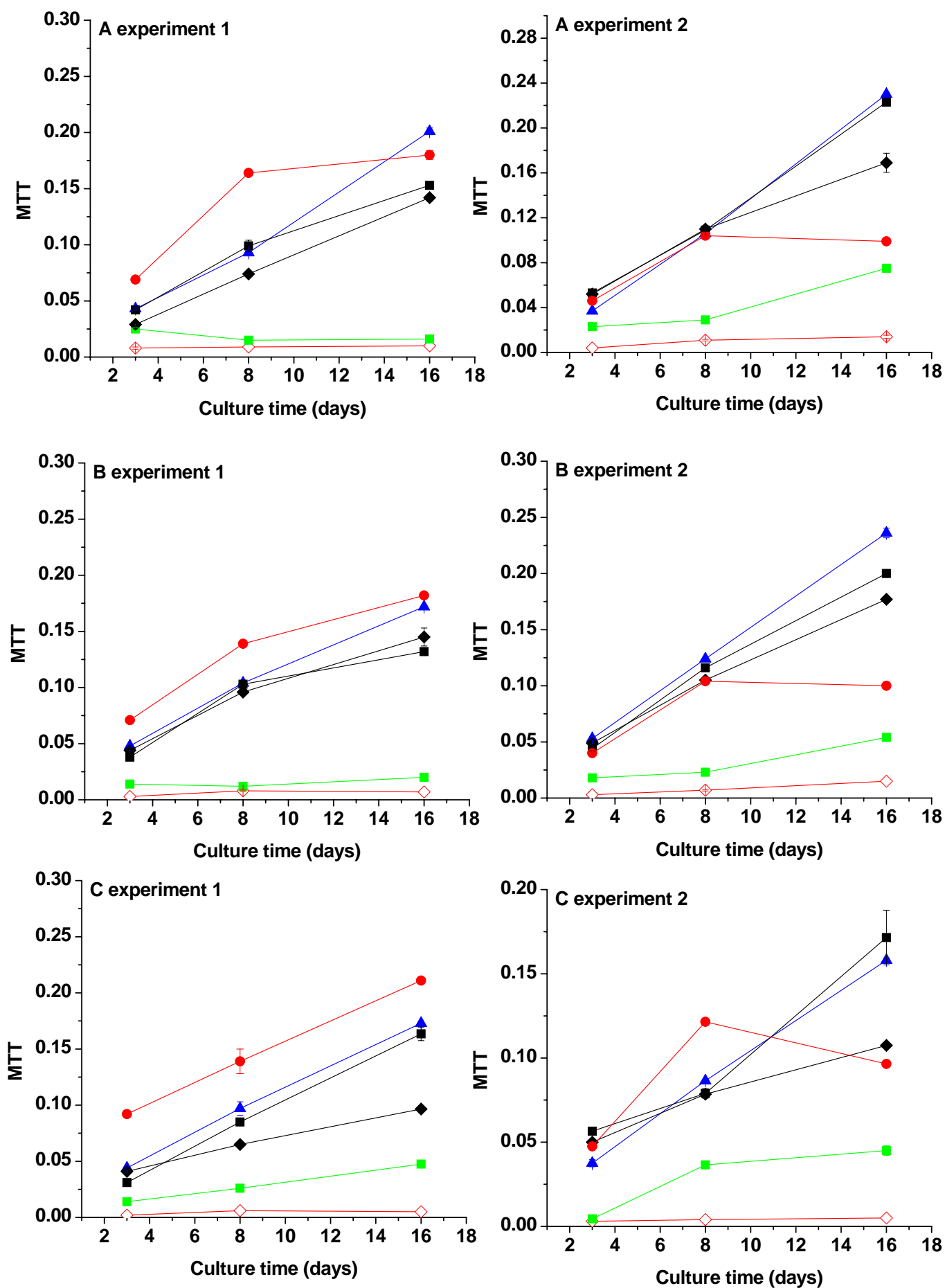


Figure 8

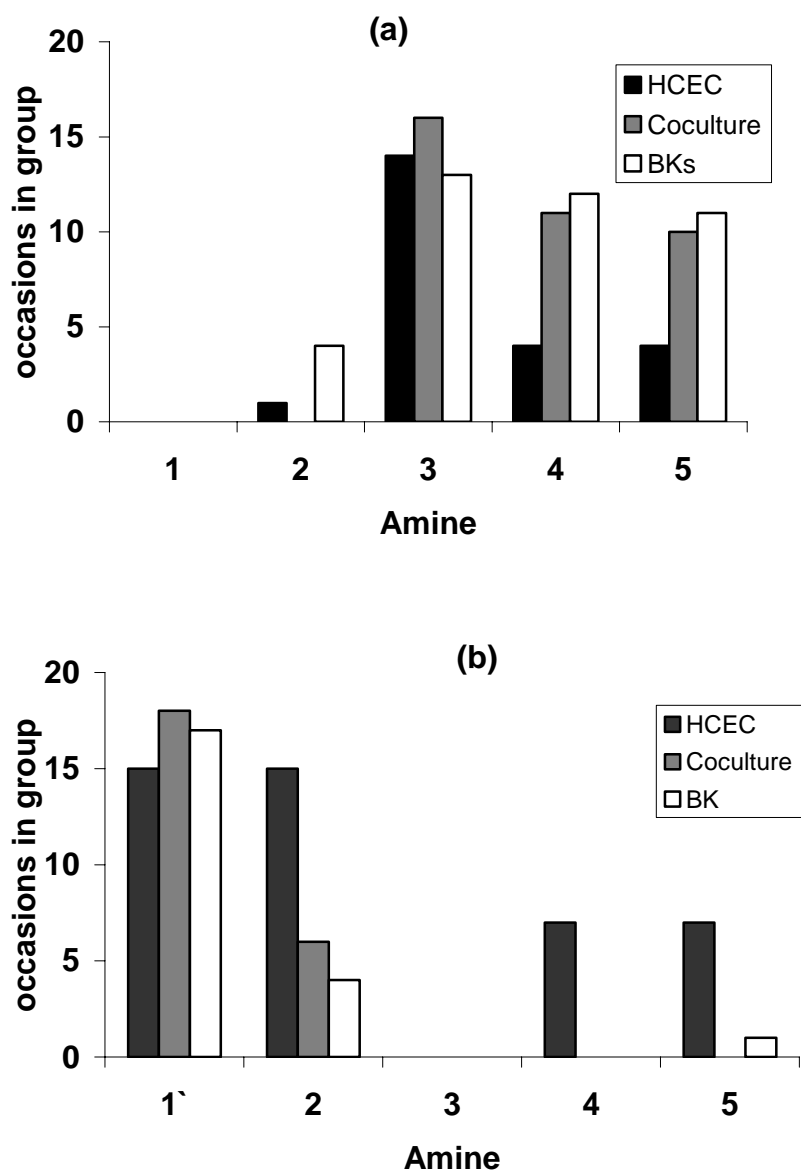


Figure 9

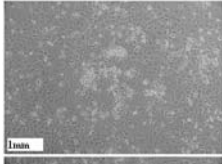
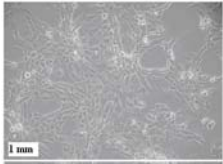


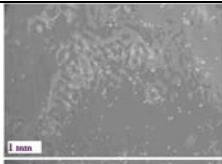
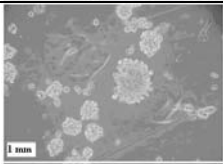
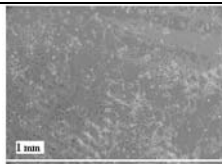
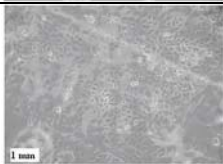
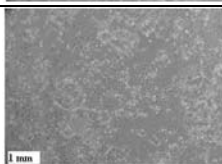


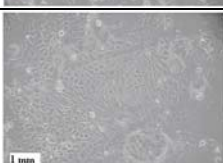
	HCEC	BK/HCEC
Coll 1 + Fn		
TCP		
1		
2		
3		
4		
5		

Figure 10

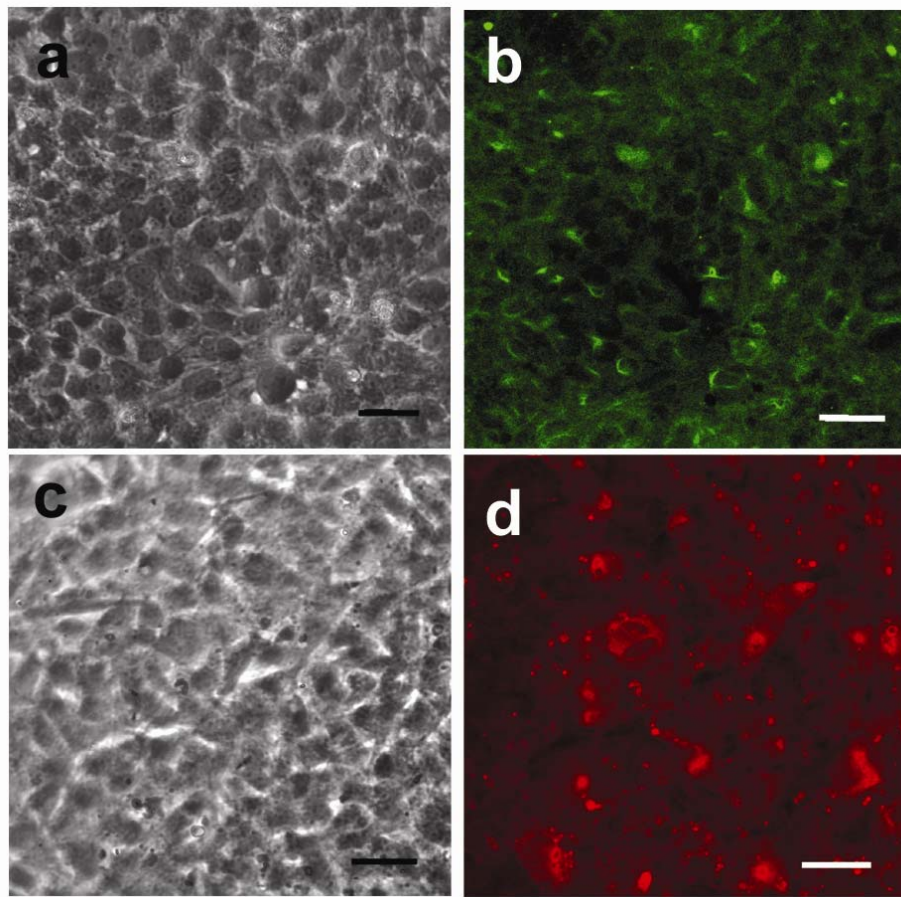


Figure 11

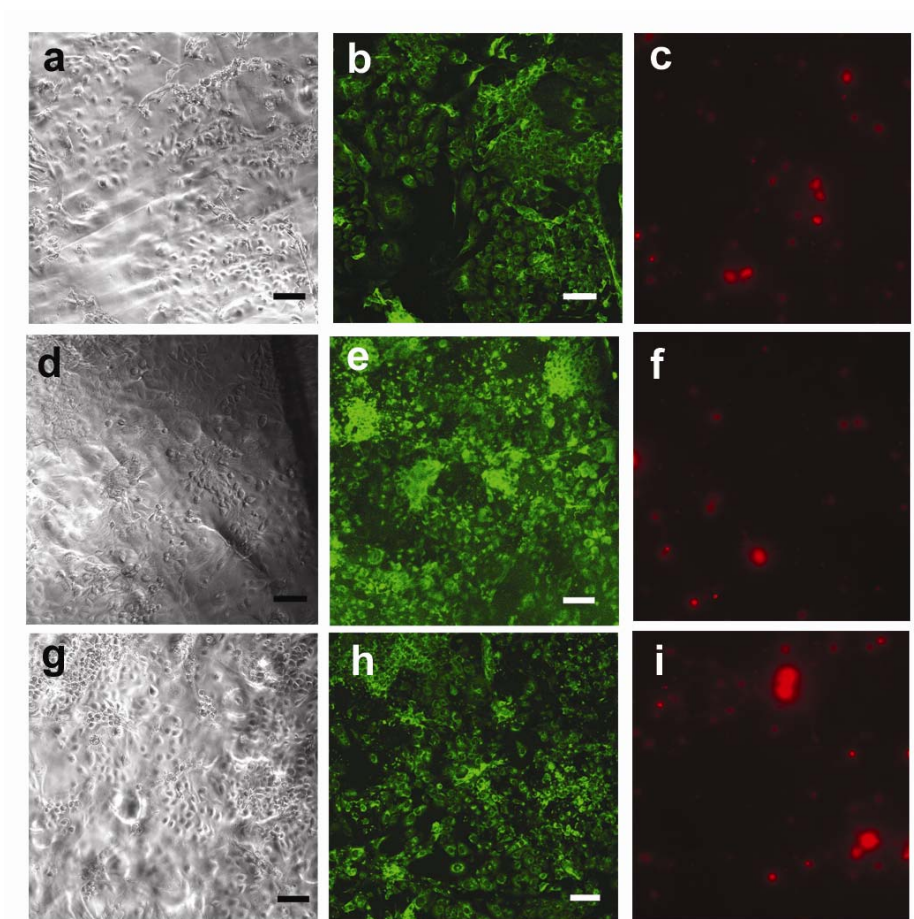


Figure 12

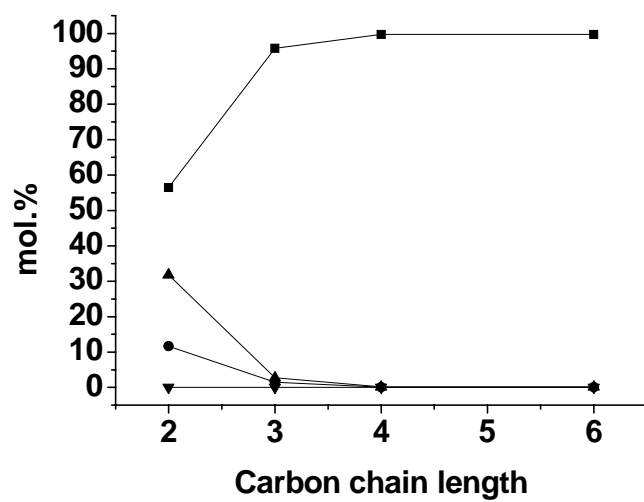


Table 1

Hydrogel	Glycerol monomethacrylate (pbw)	Lauryl methacrylate (pbw)	EGDMA (pbw)	Glycidyl methacrylate (pbw)
A	75	12	8	5
B	75	14	6	5
C	85	0	10	5