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Comparison of the Performances of Two RNA-Based Geno-Sensing Principles for the Detection of lncPCA3 Biomarker †

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Abstract: The most common prostate cancer (PCa) diagnostics which is based on detection of prostate-specific antigen (PSA) in blood has specificity limitations often resulting in both false-positive and false-negative results; therefore, improvement in PCa diagnostics using more specific PCa biomarkers is of high importance. Studies have shown that the long noncoding RNA Prostate Cancer Antigen 3 (lncPCA3) over-expressed in the urine of prostate cancer patients is an ideal biomarker for non-invasive early diagnostics of PCa. Geno-sensors based on aptamer bioreceptors (apta-sensors) offer cost- and time-effective, and precise diagnostic tools for detection PCa biomarker. In this study, we report on further development of RNA-based aptasensors exploiting two different detection strategies, i.e., electrochemical (CV and IS) and optical (spectroscopic ellipsometry) measurements. These sensors were made by immobilization of thiolated CG-3 RNA aptamers on the surface of gold. Aptamer labelled with redox group (ferrocene) was used in electrochemical measurements, while non-labelled aptamer was used in total internal reflection ellipsometry (TIRE) measurements. The results obtained by these two methods were compared; the sensitivity in sub-pM level of concentration was achieved, and the required selectivity is provided by high affinity of PCA3-to-aptamer binding with KD in 10^{-9} M range. The spectroscopic ellipsometry measurements provided additional information on the processes PCA3 to aptamer binding of proposed detection approaches allow the reliable detection of PCA3 at low concentrations, thus providing a background for future development of novel, highly sensitive and cost-effective diagnostic methodologies for prostate cancer detection.

Keywords: Aptamer; mRNA; PCA3; biosensor; TIRE

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1. Introduction

Prostate cancer (PCa) is considered as one of the most common types of cancer worldwide, and is the second leading cause of mortality among men after lung cancer [1,2]. There are clinical challenges for PCa early-stage diagnosis related to asymptomatic nature of the disease and the similarity of its symptoms to benign prostatitis [3]. Early diagnosis of PCa can reduce mortality rates and increase the opportunity for effective medical interventions, therefore, the development of reliable diagnostics of PCa is of high importance [4,5]. Current diagnostics of PCa is based on the detection of total serum prostate-specific antigen (PSA) in blood followed by (if PCa suspected) digital rectal examination and imaging studies [6,7]. However, the lack of specificity of PSA marker often leads to both false-positive and false-negative results of PSA test [8]. Hence, identifying alternative specific prostate cancer biomarkers and developing methods for their detection in the early stage of the disease is required [9,10]. A wide range of PCa biomarkers have been identified as being over-expressed in prostate tumours [11]. The differential display code 3 (DD3) gene, also known as prostate cancer antigen 3 (PCA3), the long non-coding RNA (lncRNA) discovered in 1999 [12] has been widely accepted as one of the specific biomarkers for malignant PCa [13–15]. PCA3 levels can predict prostatic biopsies' outcome, especially in combination with other PCa biomarkers such as PSA and can reduce the likelihood of false-positive results. Prognesa® test based on simultaneous detection of PCA3 and PSA using quantitative nucleic acid amplification with high sensitivity and specificity is approved in USA [16]. However, such a test is time-consuming and expensive. The development of PCA3 biosensors for express, accurate and cost-effective diagnostics of PCa is a subject of high importance. Recent developments in biosensing technologies related to the use of aptamers, synthetic bioreceptors having specifically designed sequences of RNA or DNA oligonucleotides to provide the antibody-like function towards a wide range of analytes, leads to substantial progress in cancer diagnostics (including prostate cancer) [17,18]. The RNA-based aptamer (CG3 aptamer) having high affinity towards 277-bases section of PCA3 transcript has been developed recently [19]. The successful application of the CG3 aptamer functionalized with ferrocene at C5′ terminal and immobilized on the surface of gold screen-printed electrodes via thiol group at C3′ terminal for electrochemical *in-vitro* detection of PCA3 was reported for the first time in [20].

One of the most attractive optical biosensing technology developed in the last decade was the method of total internal reflection ellipsometry (TIRE) which is a combination of spectroscopic ellipsometry (SE) and surface plasmon resonance (SPR) [21]. The method of TIRE having high sensitivity (10 times higher than conventional SRP) was particularly attractive for detection of small molecules, such as mycotoxins, in concentrations down to ppt level [22,23]; it is also suitable for the study of adsorption kinetics and subsequent evaluation of the affinity of bioreceptors (antibodies and aptamers). This work is mostly focused on TIRE detection of PCA3 in direct assay with unlabelled CG3 aptamers immobilized on the surface of gold. The results are compared to our data of electrochemical detection of PCA3 using redox-labelled CG3 aptamer. Our observations are a step towards the long-term aim of developing a novel, accurate, simple, and cost-effective diagnostic tool for early detection of prostate cancer.

2. Materials and Methods

2.1. Chemicals

HEPES binding buffer (HBB) pH 7.2–7.6, sodium phosphate di-basic (Na_2HPO_4), potassium phosphate mono-basic (KH_2PO_4), potassium chloride (KCl), magnesium chloride (MgC_{l2}), dithiothreitol (DTT), and sodium chloride (NaCl), were procured from Sigma-Aldrich (UK). All reagents were of analytical grade. The biological target: The 277 nt target analyte fragment of lncRNA PCA3, were purchased from Eurofins Genomics (Germany). The label-free CG-3 RNA-based aptamer (5′- AGUUUUUGCGUGUG-CCCUUUUUGUCCCC-3′SH) for optical and transducer was acquired from Sangon-Biotech, China. The same aptamer but labelled with ferrocene at 5′ was used previously [20] for electrochemical detection of PCA3. Before immobilization, the stock solution of aptamer (100μM) was diluted at desired concentration with PBB supplemented with 2 mM of DTT, then diluted aptamer solution was activated by thermocycler (Prime TC3600) PCR unit by heating to 90 °C for 5 min and cooling down to 4 °C for 5 min. Immobilization buffer consisted of 100 M HEPES buffer (pH 7.4) with the addition of 2.5 mM DTT and 3 mM MgC_{l2}. Detection buffer the 100 mM PBS (pH 7.4) was prepared by dissolving 10 mM Na_2HPO_4, 1.56 mM KH_2PO_4, 2.5 mM KCl, and 135 mM NaCl. Milli-Q water was used for all preparations.

2.2. TIRE-Optical Bio-Transducer.

The TIRE experimental set-up schematically shown on inset in Fig. 1 is based on J.A. Woollam M2000 spectroscopic ellipsometer with the addition of a 68° glass prism (providing the light coupling at total internal reflection conditions) optically connected via index matching fluid with the gold coated glass slide. The PTFE cell of 0.2 ml in volume was sealed against the gold coated glass slide; the inlet and outlet tubes allow the injection of the required liquid reagents in the cell. The principles of total internal reflection ellipsometry (TIRE) measurements and data acquisition were described in detail previously in [20], [24]

Standard microscopic glass slides were cleaned in hot piranha solution (3: 1 mixture of H_2SO_4 and H_2O_2) for 10 min. followed by rinsing with di-ionized Milli-Q water and drying under a stream of nitrogen gas. Gold layers of about 20 to 25 nm in thickness were evaporated on glass slides using Edwards E306A metal evaporator unit; an intermediate layer of Cr (3 to 5 nm) was used to improve the adhesion of a gold layer to glass. For TIRE measurements gold coated glass slides functionalized with thiolated label-free aptamer.

The PCA3 solutions were prepared by diluting the original stock solution (100μM) in PBS buffer to obtain the required concentrations of 0.09, 0.5, 1, 10, 100 nM . TIRE measurements were performed in a sequential adsorption manner (starting with the injection of lowest concentration of PCA3) and rinsing the cell after each adsorption step; the initial TIRE measurements of pure buffer were used as a reference. The TIRE setup allows two types of ellipsometric measurements: (i) single spectroscopic spectral scans performed in PBS after completing each stage of molecular adsorption, and (ii) dynamic measurements, e.g., recording of several spectroscopic scans during the binding of analytes (PCA3) to receptors (CG3 aptamer) which give the information on the reaction's kinetics.

3. Results and Discussion

3.1. Results of TIRE Single Spectroscopic Measurements

Typical TIRE spectra of Ψ and Δ of Au/Cr layer on glass slide functionalised with aptamers are shown in Fig. 1. The maximum and minimum in the spectrum of an amplitude related parameter Ψ correspond respectively to the conditions of total internal reflection (TIR) and surface plasmon resonance (SPR), while a sharp drop in a spectrum of phase related parameter Δ is a new quantity non-existent in traditional SPR. Position of such phase drop is highly sensitive to changes in the optical density of a molecular layer adsorbed the surface of gold; the increase in the molecular layer thickness causes its "red" spectral shift, while thickness decrease causes the "blue" spectral shift of Δ spectrum. As one ca see in Fig. 1a, binding of PCA3 from its 0.5 nM solution in PBS to its specific aptamer results in a blue shift of both Ψ and Δ spectra (dotted lines) as compared to the spectra of unperturbed aptamer layer (solid lines). As shown in Fig. 1b, the increase in concentration of PCA3 causes progressive increase in the blue (negative) spectral shift until the saturation of binding sites, e.g., aptamers) occurs at concentrations larger than 1nM. The saturation of the TIRE sensor response at the level of about -9.5 nm shown as inset in Fig. 1b corresponds to decrease in the film thickness of around 2 nm. The negative control tests were carried out by adsorbing molecules having scrambled sequence of PCA3 resulted in the "red" (positive) spectral shift of about 20 nm associated with the thickness increase of about 4 nm.

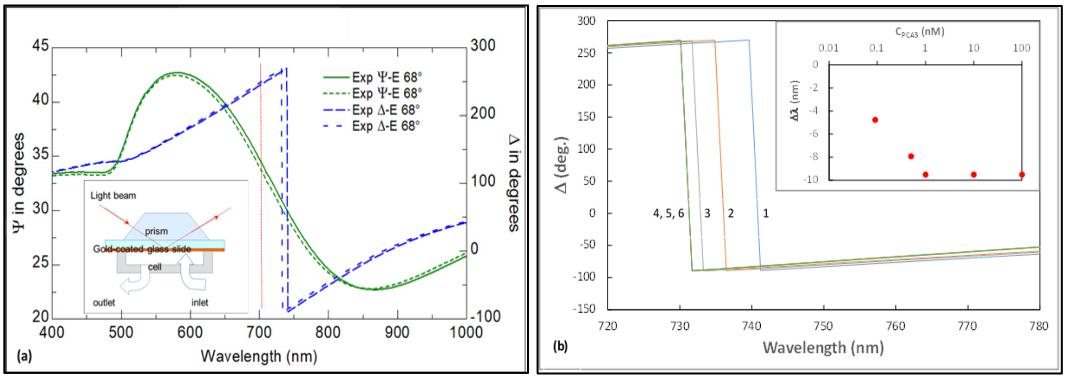

Figure 1. Results of TIRE spectra measurements: (**a**) TIRE spectra for Au layer with aptamers immobilized on the surface (solid line) and the same sample after binding 1 nM of PCA3 to aptamer (dotted line); (**b**) A series of TIRE D-spectra demonstrating the "blue" spectral shift caused by binding PCA3 of different concentrations: aptamer before exposure (1) and after exposure to PCA3 0.09 nM (2), 0.5 nM (3), 1 nM (4), 10nM (5), and 100 nM (6).

These results can be understood using the model shown in Fig. 2 which schematically illustrate the process of PCA3 to aptamer binding during which the aptamer engulf the target resulting in the thickness decrease. Contrarily, non-specific binding of scrambled PCA3 to the aptamer results in the thickness increase. Interestingly, that prolong exposure of aptamer to large concentrations of PCA3 (10 nM and 100 nM) also causes the red spectral shift which is due to the non-specific adsorption (or piling up) of PCA3 molecules. Another explanation of the saturation of the sensor response at relatively low concentrations of PCA3 is in high density of the immobilized aptamers which have no room for coiling around large target molecules of PCA3; optimization of the aptamer concentration is therefore required.

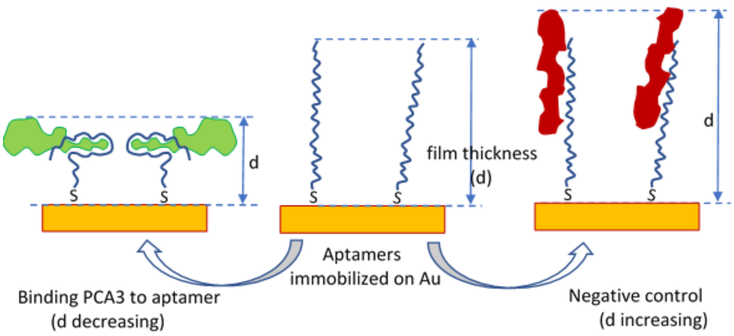

Figure 2. Schematic diagram of specific and non-specific binding of molecules to unlabelled aptamers immobilized on the surface of gold. .

It is worth mentioning that the binding of 90 pM (the lowest concentration used) of PCA3 causes a substantial spectral shift of about 5 nm. Considering high accuracy of ellipsometry measurements with the noise level of Ψ and Δ in the second decimal digits the limit of detection (LOD) could be at least two orders of magnitude lower, e. g. in sub-pM range.

Additional spectroscopic ellipsometry measurements were carried out on dry samples in order to evaluate the thickness of the aptamer layer covalently bound on the surface of gold. The thicknesses of Au (17 nm to 20 nm) and Cr (5 nm to 7 nm) layers were evaluated first from the measurements on bare metal layers. The aptamer layer thickness of 2.5 nm was found. The thermocycling of gold coated slides with immobilized aptamer in PCR unit (e. g. heating up to 95 °C and cooling down to 5 °C during 10 min.) has resulted in the aptamer layer thickness increase up to 4.5 nm. It shows that aptamer molecules tend to coil in dry state, while thermocycling in buffer solution containing Mg^{2+} ions stabilised the aptamer structure in the original stretched form suitable for sensing. Such procedure is recommended for "refreshing" samples of aptamers immobilized on gold prior to sensing tests.

3.2. TIRE Study of the Binding Kinetics.

TIRE spectral measurements were carried out (at certain time intervals) during binding PCA3 to aptamers immobilized on the surface of gold. The resulted massive data files can be processed by plotting the time dependencies of either Ψ or Δ at fixed wavelength typically selected on the left side of the resonance (see red dotted line in Fig. 1a). Typical example of TIRE binding kinetics of PCA3 (0.5 nM) to aptamers is given in Fig. 3a as the time dependence of Ψ at 700 nm.

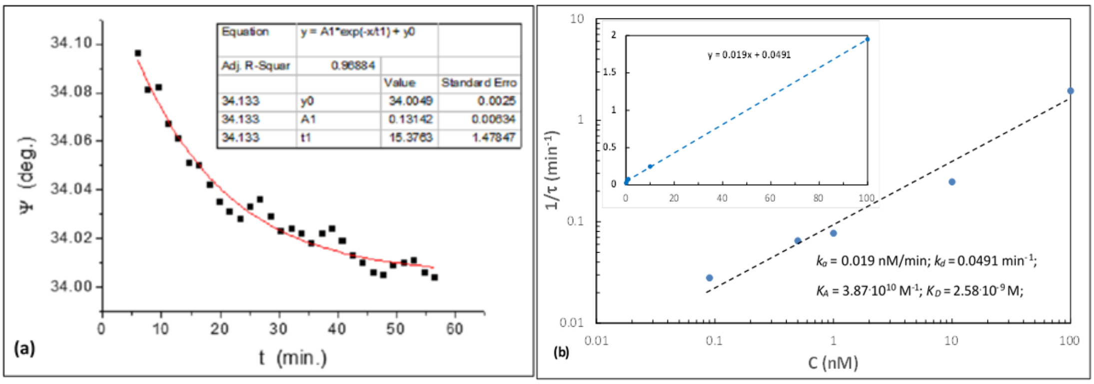

Figure 3. Evaluation of the PCA3 to aptamer binding affinity from dynamic TIRE measurements: (**a**) Example of Ψ time dependence upon binding PCA3 (0.5 nM) to aptamer immobilized on the surface of Au; The values of Ψ at 700 nm were presented; (**b**) Evaluation of K_A and K_D from the 1/τ(C) dependence given in logarithmic and linear scales.

These data were fitted to the rising exponential function with the parameters of equation given as inset. The parameter of interest was the time constant (τ). Such measurements were carried out at different concentrations of PCA3 (C) and the characteristic time constants (τ) were evaluated at each concentration. According to the theory of molecular adsorption [20], the rates of adsorption and desorption (k_a and k_d) can be found, respectively, as the gradient and intercept of the following linear equation : $\frac{1}{\tau} = k_a C + k_d$, then the association and affinity constants (K_A and K_D) can be found as $K_A = k_a/k_d$, $K_D = 1/K_D$. Linear dependence of 1/(τ) vs. C given in **Fig. 3b** in both logarithmic and linear coordinates yields the values of KA = 3.87.1010 M-1 and KD = 2.58.10-9 M which are very similar to those obtained earlier by electrochemical method of CV [20]. Also, the TIRE experiments revealed anomalous kinetics at high concentrations of PCA3 when soon after reaching the saturation the response started to rise again. This is most-likely associated with non-specific adsorption of PCA3.

4. Conclusions: Comparison of the Electrochemical and Optical Detection Strategies

The use of TIRE method for detection of PCA3 in direct assay with aptamer immobilized on the surface proved to be promising. One of the main outcomes of our preliminary optical experiments is high sensitivity of TIRE detection method, potentially in sub-picomolar range, which is comparable with the values of LOD reported earlier for electrochemical CV (0.35 pM – 0.78 pM) and EIS (0.26 pM) methods [20], More detailed optical study in a wider concentration range of PCA3 and optimization of aptamer concentration is currently underway. The high affinity of unlabelled aptamer towards PCA3 was confirmed by TIRE kinetics study which gave similar values KA and KD to those obtained previously from CV measurements for aptamer labelled with ferrocene [20].

From the point of view of sensing, electrochemical methods are more attractive because of the low cost and simplicity of use, however the optical method of TIRE provides important complementary information on the thickness of molecular layers which allow better understanding of processes of aptamer-target interaction.

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