SPR and AFM study of engineered biomolecule immobilisation techniques.

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***Abstract*— A comparative study into two novel and diverse schemes designed to improve immobilization of biomolecules for biosensing purposes is presented. In the first method a silicon rich matrix is created using PECVD. The second method involves creating nano-patterns on the sensor surface to create a large number of surface discontinuities to which the proteins will bind preferentially. The basic theory of SPR is provided to show the importance of the surface sensitive nature of this optical transduction technique. The present work suggests that both may prove both for SPR and other biosensing applications. Of the two schemes proposed, the results for nano-patterning seem to suggest that it is promoting better surface attachment of biomolecules. The results of SPR and AFM studies are presented that have shown that each of these schemes promotes improved binding of various proteins.**

I. INTRODUCTION

Advances in nanotechnology are presenting opportunities in a diverse range of scientific fields, including biosensing applications. A plethora of techniques exist for the immobilisation of biorecognition elements to a sensing surface [1]. As yet the development of an optimum technique in terms of packing density, orientation and the total retention of activity have proven to be elusive for researchers. The purpose of this research has been to investigate a number of novel methods that have emerged, that potentially could offer improvements for each of these unresolved issues. Regardless of the biosensing transduction method being used it is necessary to immobilise a thin layer of a material that is known to show specific binding to the biomolecule of interest [2] at the interface between analyte and transducer. The sensor surface may be coated with binding molecules which may be antibodies, DNA probes, enzymes or other reagents. Each binding molecule will be chosen because they react almost exclusively with a selected target, analyte or molecule. Immobilisation techniques have for the most part come about through trial and error experimentation [1]. Surface plasmon resonance (SPR) is an optical transduction mechanism that demonstrates extremely high sensitivity to surface binding events and is used in this research to monitor protein attachment. AFM has been shown to provide an effective means of imaging proteins bound to a surface and is used to corroborate the results obtained from SPR monitoring.

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II. SURFACE PLASMON RESONANCE

SPR is a highly sensitive label free biosensing method. In the Kretschmann configuration light is totally internally reflected beyond a critical angle dictated by the refractive index of the mediums on both sides of the reflecting interface, in accordance with Snell's law. At angles beyond the critical angle a portion of the electromagnetic wave will travel in the x direction (along the interface) as a spatially decaying field in the z direction in accordance with electromagnetic theory with a determinable propagation constant (k_x). This field known as an evanescent wave oscillates at a frequency equal to that of the incoming light. A surface plasmon wave (SPW) is a charge density oscillation that can exist at the interface between materials of opposite dielectric sign, typically a metal and a dielectric (n_p) [3]. In the visible and near infra-red region of the electromagnetic spectrum gold and silver are the most commonly used metals because the complex dielectric function of these metals is strongly supportive of surface plasmons [4]. The propagation constant of a surface plasmon is dependant on the refractive index of the materials on both sides of the interface (ε_m & ε_d) and the free space wave number of incoming light (k_o). If the momentum of the evanescent wave and the SPW are matched a significant portion of the electromagnetic energy can transfer through the interface and this is measurable as a significant dip in the amount of light being reflected at the resonance angle ($\sin\theta_{sp}$). A typical SPR curve showing a dip at the resonant angle is shown in Fig. 1. Small amounts of molecules binding at or close to the sensing region of an SPR biosensor will effect change in the bulk dielectric value, resulting in a slightly different SPW propagation constant (k_x'). The angle required to satisfy the resonant condition will therefore shift slightly. This shift is measurable and refractive index changes as little as 2×10^{-7} is frequently reported in literature [5]. The expression for the matching condition is given as:

$$k_x = k_x^{'}$$
$$k_x = k_o n_p \sin\theta_{sp} \quad (1)$$
$$k_x^{'} = k_o \sqrt{\left(\frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d}\right)}$$

One of the most important properties of SPR is its highly surface sensitive nature. This property can be shown to be a result of the SPW being an exponentially decaying electromagnetic field. The penetration depth (t_d) of the SPW into the dielectric material is governed by the formula [6]:

$$t_d = \frac{1}{k_o \varepsilon_d} \left| \mathrm{Im}\left\{ \sqrt{\varepsilon_m + \varepsilon_d} \right\} \right| \quad (2)$$

For the experimental setup used in our work the wavelength of light used is 833 nm this provides a maximum depth of 365 nm in liquid with a bulk refractive index of 1.33. Importantly biomolecules directly immobilised to the sensing region have the greatest influence on the resonance. Liedberg and co-workers investigated the benefits of immobilisation of biomolecules in a carboxylated dextran matrix on an SPR sensing system and were able to demonstrate enhanced sensitivity over traditional methods [7].

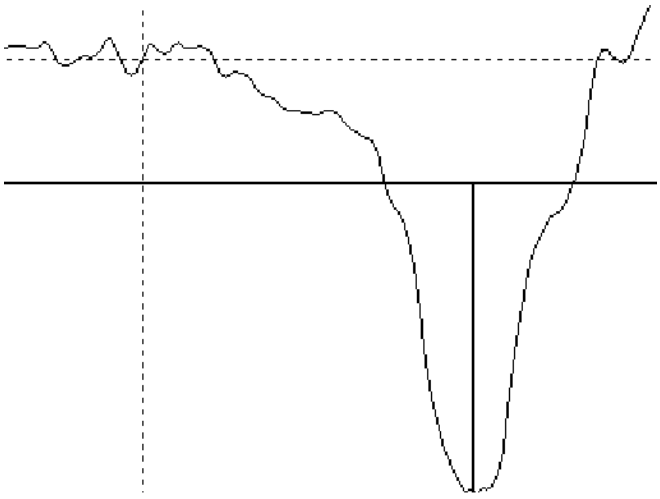

Fig. 1. A typical SPR resonance dip.

III. Protein Attachment Methods Used

Surface silanisation in conjunction with carbodiimide coupling or using glutaraldehyde is a method that has been employed in the past. The silanisation process has previously been implemented using a silane layer chemically attached to the surface in liquid form. In this research we created a silicon rich nano-dimensional matrix on the sensing area by plasma enhanced chemical vapour deposition (PECVD). In the second method nano-scale discontinuities were created on the sensing surface to promote protein adhesion using a focussed ion beam (FIB) system. Proteins have previously been shown to preferentially attach to discontinuities on surfaces [8]

The monitoring of blood glucose levels for diabetic management is one of the relatively few commercial successes in biosensor development. GOx was chosen as one of the protein structures for evaluation due to the large volume of research into its use previously making it relatively straightforward to compare and evaluate. Chaperonin 60 is from the family of heat shock proteins. It has previously been successfully used in protein templating studies. Alignment of these proteins has been promoted by the use of grids, monolayers and micropatterning. The size and structure of these proteins has previously been determined to be a double heptameric ring structure of 15-20 nm diameter and 3-4 nm height [8]. Chaperonin 60 is a promising protein not least because it has been previously genetically modified [9].

Fibronectin is a large extracellular glycoprotein which has been shown to be important as a precursor to cell adhesion. Fibronectin adhesion to patterned surfaces with grooves of 25 micrometer separation have been conducted previously [10].

IV. Experimental Work.

A. Materials

Proteins chosen for surface immobilisation were:- Fibronectin from Bovine plasma, Chaperonin 60 from E-coli and Glucose Oxidase which were all purchased from Sigma–Aldrich, as were all other chemicals used in this work. Phosphate Buffer Solution (PBS) was mixed from Na_2HPO_4 and NaH_2PO_4 in de-ionized water and stored at 4^0 C. The ratios were adjusted to provide the appropriate Ph levels. Chaperonin 60 was mixed in Ph 7.0 PBS to a ratio of 1 mg per 10 ml aliquoted and stored at -20^0 C. Fibronectin was aliquoted in PBS (Ph 7.0) to a ratio of 10 mg/100 ml of buffer and also stored at -20^0 C. Glucose Oxidase was freshly prepared prior to immobilisation in 10 mg/100 ml ratios of Ph 7.0 PBS buffer.

B. Method 1 PECVD treatment.

A Spreeta sensor was placed in a Diavac 320 PECVD system. The PECVD system is composed mainly of a radio frequency (13.56 MHz) power supply, with an auto-matching network and an aluminium reaction chamber fitted with a capacitively coupled parallel-electrode. In each experiment the chamber was evacuated to a working pressure of ~6.0 x 10^{-6} Torr. Bias voltage was set at 400 V

and power was ~ 130 W. The sensor underwent a plasma etch stage in Argon (Ar) gas with a flow of 60 sccm for 5 minutes prior to a 5 second deposition stage in Ar and tetramethysilane (TMS) with flow rates of 10:20 sccm respectively. On removal from the chamber the sensing surface was placed in 2.5% solution of glutaraldehde for 30 mins prior to protein immobilisation.

C. *Method 2- FIB nano-patterning.*

Spreeta sensing surfaces were nano-patterned in a Quanta 200 3D system to create grating-like structures to which proteins would show preferential attachment. The FIB system utilises a gallium ion source and patterning was performed with source currents ranging from 10 pA to 1 nA. Separation distances of 200 nm and line widths of ~50 nm were achievable with this system. In addition to creating trenches on the surfaces the system has the capability to deposit a range of materials. Both carbon and tungsten were each deposited on different Spreeta sensors for evaluation. On removal from the FIB system proteins were flowed past the sensors for 2 hours and sensograms were saved using the Nomadics software.

D. *SPR monitoring*

The SPR system used throughout this work was the Spreeta™ device from Texas Instruments and supplied by Nomadics Inc. Following initialisation and calibration steps, protein in PBS buffer solution were flowed past the activated sensing surfaces for a minimum of 2 hours. Flow rates in each instance were set to 0.2 ml/min. In the case of GOx immobilisation following a PBS rinse cycle the sensors were exposed to β- D glucose solutions within the range normally experienced within the body to assess the ability of the sensors to discriminate between the various concentrations and evaluate the stability of the sensors over time.

E. *Atomic force microscopy*

AFM studies of the surfaces of patterned and non-patterned surfaces were carried both before and after immobilization of chaperonin 60. The characterisation of the surfaces was carried out in a meniscus of buffer solution on the surface and was preceded by conducting force curves to ensure that a minimum force was being exerted on the proteins [11]. Previous AFM studies have shown that imaging of chaperonin 60 in tapping mode in liquid is possible and can be used to resolve the individual toroidal structure of the protein [12]. Images were collected using a Nanoscope IV, using cantilevers with a nominal spring constant of 0.32 N/m. Tips were cleaned immediately prior to use in pirhana solution for ~1 min and rinsed in deionised water. In all cases post-imaging analysis was conducted using nanoscope v6.11r1 software and was subjected to a first order plane fitting flattening procedure.

V. RESULTS AND DISCUSSION.

The time and flow rate selected for the establishment of a silicon rich matrix was known to provide a layer of approximately 10 nm and this has previously been confirmed by Raman and Profilometry [13]. Collected sensograms of each protein binding to the surface were compared with those for immobilisation using simple adsorption and compared favourably taking into account the dielectric layer and penetration depth required. Further work into this method is ongoing and the results of this shall be reported in due course.

In comparison with simple protein adsorption sensograms, patterned surfaces demonstrated a reduced amount of immobilisation within the first hour, however in the later stages of attachment the overall increase in refractive index showed a much greater volume of molecular binding to the sensing area (see Fig. 2). This increased binding activity was demonstrated for each of the three proteins. Additionally, in the case of bound GOx, the detection of glucose in the millimolar range showed good resolution, although it was seen to drift slightly ($<10^{-5}$) over a period of several hours (see Fig. 3). AFM scans revealed a dense layer of chaperonins bound to the surface. The dimensions of the toroidal chaperonin structures bound to the surface were in good general agreement with observations from similar studies. Fig. 4 is an AFM amplitude image for the patterned surface. The brighter region seen to run diagonally upwards from left to right in the image is the patterned line. Further work is ongoing in order to determine how well the proteins become tethered to the surface due to the patterning.

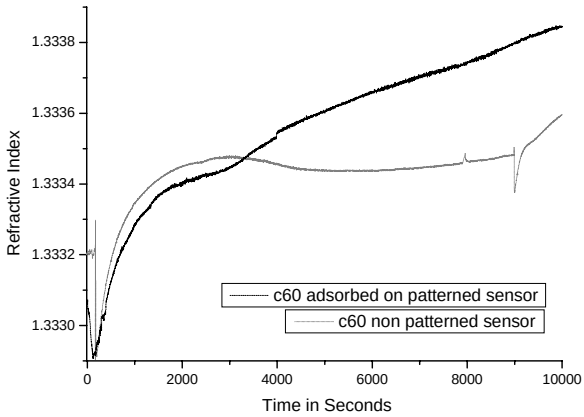

Fig. 2. Sensogram comparing binding of Chaperonin 60 unto patterned and non-patterned surfaces. Black line is for

a patterned surface and grey line is for a non-patterned substrate.

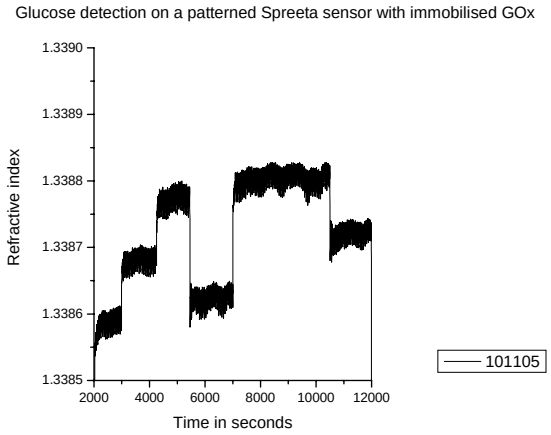

Fig. 3. Glucose detection on a patterned Spreeta sensor immobilised with GOx.

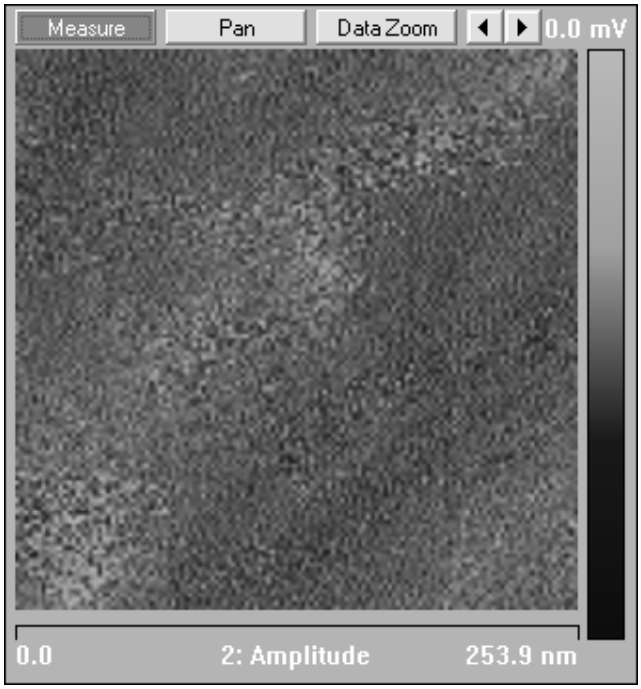

Fig. 4. AFM image showing chaperonin 60 densely packed unto a patterned gold substrate.

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