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Development of capacitance based immunosensors on mixed self-assembled monolayers

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Abstract

The construction and characterisation of a capacitance based immunosensor on mixed mercaptohexadecanoic acid and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-caproyl biotinyl self-assembled monolayers on gold are reported. Immobilisation of IgG antibodies was achieved using the strong non-covalent bond formed between Neutravidin and biotin to tether anti-human haemoglobin goat IgG to the mixed monolayers. Analysis of sensor construction and of the functional immunosensors was characterized via physical techniques, including quartz crystal microbalance, and atomic force microscopy for surface topographical measurement. Sensor assembly and the binding affinity of immobilised antibodies were independently determined using [^{125}I]-tagged components. Finally, the electrochemical properties of partially constructed and fully functional immunosensors were analysed by electrochemical impedance spectroscopy in a ferricyanide mediated system. The haemoglobin sensors show a linear response of the imaginary component of impedance (Z'') across a wide range of concentrations, from 10^{-10} (according to Fig. 8, linearity is restricted over the concentration range 10^{-8} to 10^{-6} M haemoglobin), at the lower range of frequencies examined.

Keywords: Impedance; Immunosensor; SAM; Affinity lipid; Biotin–avidin

1. Introduction

Immunosensors detect and quantify analytes based on binding, with detection being related to changes in mass, resistance or capacitance at the sensor surface.

Key to their development is the ability to immobilise antibodies, including monoclonal antibodies (Mab), Fab fragments and single chain synthetic antibodies (ScFv) via procedures that allow specific binding affinity to be retained. Increasingly, the use of self-assembly has been employed to create functionalised platforms for the immobilisation of these receptor molecules. Currently, many sensors designed for receptor immobilisation are based upon the self-assembly of homogenous monolayers, many of which are then subjected to in situ chemical modification [1–5]. Evaluations of heterogeneous self-assembled monolayers (SAMs) prepared from mixtures of thiolated compounds [6–8] or mixtures of phospholipids and proteins, generally conclude that it is possible to incorporate compounds into self-assembled monolayers at the time of deposition. Immobilisation supports comprising mixtures of alkanethiols and phospholipids have previously been reported. Nevertheless, these experiments focussed on the creation of solid supported bilayers, maintaining the two components segregated within the resulting layers [9–12]. The development of mixed heterogeneous alkanethiol/functionalised-phospholipid monolayers currently remains poorly investigated.

Physical detection of analyte binding using flow through QCM biosensors has previously been reported [13], although they remain a slow and often unpredictable method of analyte detection. This is due in part to the assumption that deposited matter is present in a rigid format, to which the Sauerbray equation may be fitted, although for many biopolymers this is incorrect, and it has been reported that the equation may not apply to viscoelastic media [14,15]. However, QCM does remain a useful tool for investigating the kinetics of the component assembly from bare electrode to functional sensor, including analyte detec-

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tion. Using data obtained via QCM measurements, optimum assembly conditions have been determined for the construction of immunosensors based on mixed SAMs on Au electrodes. The immunosensors prepared were directed against haemoglobin as the analyte. However, this construction format can be applicable to almost any analyte for which an antibody is available.

Additionally, the surface topography of functionalised gold surfaces at the nano-scale was investigated using atomic force microscopy (AFM) to gain an insight into the 2D distribution of the affinity phospholipids used within the SAM, as well as to investigate the nature of the coverage and topographical changes occurring following sequential addition of the various sensor components.

Finally, the affinity of the anti-haemoglobin sensors was analysed using [^{125}I] radio-labeled haemoglobin. Data obtained from this direct measurement of analyte binding was found to confirm analyte binding to the constructed sensor.

2. Materials

Impedance electrodes: Sensor electrodes were from the Tyndall institute, Cork and comprised sputter coated gold (200 nm) onto a 50 nm Cr adhesion layer on SiO_2. Except for the 0.7 mm diameter working electrode and contact pad, the surface was passivated using Si_3N_4.

Chemicals: The phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-caproyl biotin (biotinyl-cap-DPPE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) were purchased from Avanti Polar Lipids Inc. Neutravidin was purchased from Pierce.

All other chemicals were purchased from Sigma–Aldrich unless stated otherwise.

Unless stated, all procedures were performed in 100 mM K_2HPO_4, pH 7 buffer (PB).

3. Experimental details

3.1. SAM deposition

The electrode surface was cleaned by immersing the electrode for 1 min in a hot piranha solution containing a 3:7 (v/v) ratio of H_2O_2 to H_2SO_4. Electrodes were then rinsed thoroughly in de-ionised water, and dried under a N_2 stream. QCM chips were also cleaned this way immediately prior to use.

Mixed biotinyl-functionalized monolayers were created by immersing freshly cleaned gold electrodes or QCM chips for 30 min in an ethanol solution of 0.5 mM MHDA and 0.05 mM biotinyl-cap-DPPE at a 10:1 molar ratio unless otherwise specified. Samples were then rinsed in de-ionised water for 5 min, and dried under a N_2 stream.

3.2. Neutravidin attachment

Specific binding of the Neutravidin (Pierce), which is a re-engineered, non-glycosylated avidin, to the biotinyl-functionalised surface was achieved by incubating the biotinyl-functionalised SAMs in 10 ml of PB solution containing 10^{-6} M Neutravidin for 45 min at room temperature. Samples were then rinsed in PB solution prior to testing or further assembly.

3.3. Antibody attachment

Antibody immobilisation was studied using goat anti-human haemoglobin IgG (Biomeda). In order to remove NaN_3 preservative, the IgG were concentrated by centrifugal filtration in a Centricon 30 ultrafilter then diluted again in PB. This step was performed three times. The IgG was biotinylated using biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma), according to manufacturers instructions. Samples were then passed through a G-25M Sephadex column to remove free biotin and the biotinylated antibody recovered. Specific coupling of anti-human haemoglobin biotinyl-IgG to Neutravidin functionalized SAMs was performed in 10 ml, PB solution containing 10^{-6} M biotinyl-IgG for 30 min at room temperature. Sensors were then rinsed in PB solution prior to testing or further assembly.

3.4. Quartz crystal microbalance measurements

QCM analysis was carried out using a Maxtek RQCM instrument using 5 MHz, 1 in. diameter piezoelectric crystals. Real time deposition studies were obtained by setting up a flow through system using a 100 μl flow chamber and at a flow rate of 100 μl min^{-1}. Quartz crystals were cleaned and SAMs deposited as described in Section 3.1, and then placed in the flow cell and equilibrated with PB at 100 μl min^{-1} until a steady trace was obtained. Samples were introduced to the system using a two-way valve, set to deliver PB, or PB containing sensor components. Data was collected and processed using Maxtek software, and plotted as μg deposited cm^{-2}.

3.5. Atomic force microscopy

The surface topography of partially constructed and fully functionalised gold surfaces was studied by atomic force microscopy (AFM) using a Nanoscope III instrument. Atomically flat gold surfaces were obtained using template stripped gold [16] from silica wafers. Sensor construction was then performed using freshly obtained gold surfaces following the methods described above.

3.6. Electrochemical measurements

Impedance spectroscopy was performed using sensor electrodes at various stages of construction or after being exposed to haemoglobin solutions, between 10^{-6} and 10^{-10} M, in PB for 30 min at room temperature. The real and imaginary components of the complex capacitance plane were determined in PB solution containing 10 mM $K_3Fe(CN)_6$, using a Autolab PSGTA12/FRA2 (EcoChemie) instrument over a frequency range of 100 KHz–0.1 Hz at an applied voltage of 0.4 V and with 10 mV amplitude.