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A new application of scanning electrochemical microscopy for the label-free interrogation of antibody–antigen interactions

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ABSTRACT

Within this work we present a 'proof of principle' study for the use of scanning electrochemical microscopy (SECM) to detect and image biomolecular interactions in a label-free assay as a potential alternative to current fluorescence techniques. Screen-printed carbon electrodes were used as the substrate for the deposition of a dotted array, where the dots consist of biotinylated polyethyleneimine. These were then further derivatised, first with neutravidin and then with a biotinylated antibody to the protein neuron specific enolase (NSE). SECM using a ferrocene carboxylic acid mediator showed clear differences between the array and the surrounding unmodified carbon. Imaging of the arrays before and following exposure to various concentrations of the antigen showed clear evidence for specific binding of the NSE antigen to the antibody derivatised dots. Non-specific binding was quantified. Control experiments with other proteins showed only non-specific binding across the whole of the substrate, thereby confirming that specific binding does occur between the antibody and antigen at the surface of the dots. Binding of the antigen was accompanied by a measured increase in current response, which may be explained in terms of protein electrostatic interaction and hydrophobic interactions to the mediator, thereby increasing the localised mediator flux. A calibration curve was obtained between 500 fg mL^{-1} to 200 pg mL^{-1} NSE which demonstrated a logarithmic relationship between the current change upon binding and antigen concentration without the need for any labelling of the substrate.

1. Introduction

In recent years there has been an increasing demand for the development of parallel analytical testing platforms and this has led to increased research into miniaturised biosensors, biosensor arrays, and chip based testing systems. Within the commercial market it is already possible to obtain DNA-based testing systems, however there is still an unmet need when considering the use of proteins as the functional units. This is possibly due in part to proteins being less stable and also their characteristic to denature once in contact with the surface to which they are immobilized.

The incorporation of antibodies into conducting polymer films was first reported in 1991 [1]. Anti-human serum albumin (anti-HSA) was incorporated into a (poly)pyrrole film, which was galvonostatically polymerized onto a platinum wire substrate. When the pyrrole anti-HSA was exposed to 50 μg mL^{-1} HSA for 10 min, a new reduction peak was observed at a potential of +600 mV vs. Ag/AgCl. Since this preliminary work there has been an increase in the development of electrochemical immunosensors, as reviewed previously elsewhere [2–4].

Previous work within our group has shown that up to 2–3 μg of antibodies for bovine serum albumin (BSA) and digoxin may be successfully incorporated into conducting polymer films by entrapment in an electrochemically grown (poly)pyrrole film with no detrimental effect to antibody activity [5]. Further work utilized an ac impedance protocol as the method for interrogation for these films [6]. A protocol was then developed for immobilizing antibodies onto polyaniline-coated screen printed carbon electrodes which utilized the classical avidin–biotin interaction. This enabled the construction of immunosensors for the antibiotic ciprofloxacin [7,8], the heart drug digoxin [9] and for myelin basic protein [10] – which could detect their respective targets with detection limits of 1 ng mL^{-1} of the target species. Later work utilized polyaniline microarrays as a substrate for the assembly of immunosensors for prostate specific antigen (PSA) [11] and the stroke marker proteins neuron specific enolase (NSE) [12] and S100[β] [13], with greatly lowered limits of detection down to the level of 1 pg mL^{-1} of the target.

Biotin/avidin chemistry has been used extensively to modify electrode surfaces by protein immobilization. The attachment of a biotin molecule allows the immobilization of any biomolecule of the avidin family. Neutravidin protein is a deglycosylated version of avidin with four identical binding sites. It has a near neutral isoelectric point (IEP) which minimizes non-specific interaction and

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like avidin itself has a strong affinity with biotin ($kD = 10^{-15}\ M^{-1}$) [14,15]. Due to this strong interaction, the complex formation is nearly unaffected by extremes of pH or temperature, organic solvents, and denaturing agents. The tetravalent binding of neutravidin to biotin allows the construction of a molecular sandwich effect where the bound neutravidin is free to couple to a biotinylated antibody with the appropriate characteristics needed for the construction of a biosensor.

Enolase is a 78 kDa homo- or heterodimeric cytosolic protein produced from [α], [β], and [γ] subunits. The [γ][γ] enolase isoform is most specific for neurons, and is referred to as NSE. Elevations of NSE in serum can be attributed to cerebral injury due to physical damage or ischemia caused by infarction or cerebral haemorrhage, coupled with increased permeability of the blood brain barrier. The serum concentration of NSE has also been reported to correlate with the extent of damage (infarct volume) and neurological outcome [16]. Additionally, a secondary elevation of serum NSE concentration may be an indicator of delayed neuronal injury resulting from cerebral vasospasm [17]. NSE, which has a biological half-life of 48 h and is normally detected in serum at an upper limit of 12.5 ng mL^{-1} (160 pM), is typically elevated after stroke and cerebral injury. Serum NSE is elevated after 4 h from onset, with concentrations reaching a maximum between 1 and 3 days after onset [18]. After the serum concentration reaches its maximum (maybe >300 ng mL^{-1}, 3.9 nM), it gradually decreases to normal concentrations over approximately one week.

Scanning electrochemical microscopy (SECM) is a surface scanning probe technique that allows for the collection of high resolution electrochemical data on a variety of surfaces and has previously been used successfully to investigate various biological systems including cells [19–23], enzymes [24–27], and DNA [28–30].

We have, within this work, attempted to utilize the SECM to detect the binding of antibody layers to electrodes and to determine if these systems can be used for the label-free detection of NSE. This work is intended as a 'proof of principle' study to show the feasibility of antigen adsorption and imaging rather than the fabrication of either a sensitive antigen sensor or the detailed examination of the polymer and biomolecular films absorbed. Once a proof of principle has been demonstrated we will in future work move towards studying a range of antigens of various molecular sizes and investigate the possibility of fabricating one array containing a number of different antibodies of interest.

2. Experimental

2.1. Chemicals and reagents

Ferrocene carboxylic acid, biotinylation kit (part no. BK101), neutravidin, polyethyleneimine (PEI) (MW = 50000) and BSA were purchased from Sigma–Aldrich (Gillingham, Dorset, UK). Disodium hydrogen orthophosphate monohydrate, sodium dihydrogen orthophosphate 12-hydrate and sodium chloride (all 'AnalaR' grade) were purchased from BDH (Poole, Dorset, UK). All reagents were used without further purification. Commercial screen-printed carbon electrodes were obtained from Microarray Ltd. (Manchester, UK). NSE and monoclonal antibody against NSE – both with sodium azide preservative, and PSA were supplied by Canag Diagnostics, Ltd. (Gothenburg, Sweden).

For antibody and PEI biotinylation, the procedure outlined in the BK101 kit was followed (see manufacturer's instructions for details). Biotinylated antibodies were kept frozen in aliquots of 200 μL at a concentration of 0.2 mg mL^{-1} until required.

All water used was purified with an ELGA Purelab UHQ-II water system (Elga, High Wycombe, UK). Phosphate buffer (PBS), pH 7.0 contained NaH_2PO_4 (4 mmol mL^{-1}), Na_2HPO_4 (6 mmol mL^{-1}) and NaCl (132 mmol mL^{-1}).

SECM experiments were carried out using a Uniscan SECM270 (Uniscan Instruments Ltd, Buxton, UK). The SECM instrument (shown schematically in Fig. 1a) is composed of (1) an electrochemical cell, (2) a translational stage capable of high resolution movement in the *X*, *Y* and *Z* planes (sub-micron), (3) a bipotentiostat for the accurate control of the potential applied at the tip and/or substrate, (4) a hardware interface enabling the control of (1) and (2), and (5) a PC which provides an interface with the hardware – and allows the operator to accurately control the parameters of the SECM procedure. Pt counter electrodes and Ag/AgCl reference electrodes were also utilized as shown in Fig. 1b. Ferrocene carboxylic acid (5 mmol L^{-1} in pH 7.0 phosphate buffer) was used as the mediator (Fig. 1c).

2.2. Substrate patterning

In the immunosensors previously developed within our laboratory, cyclic voltammetry was utilized to deposit polyaniline films on the carbon electrodes [7–13]. This was deemed inappropriate for the preparation of electrodes to be interrogated by SECM as it is not possible to compare a modified region with an unmodified region. Any changes in the tip current obtained may be due to fluctuations in the background current and not due to changes in the charge transfer properties of the modified substrate. It was therefore decided that for SECM interrogation, the polyelectrolyte film should be patterned in an array dot format. By producing this pattern, background effects in the measured current could therefore be eliminated and any changes in the current over the modified surface would contrast with the current over regions of unmodified carbon. Since it would not be possible to use polyaniline in this format it was decided to use PEI which has previously been used within the group when interrogating DNA arrays with SECM [30].

A borosilicate glass capillary was pulled to an internal diameter of 80–100 μm using a Narishige PP-830 pipette puller (Narishige International Limited, London, UK) and the tip polished to a flat finish. This capillary was then filled with a 1% biotinylated PEI solution and, using the micropositioning stage on the Uniscan SECM270, used to fabricate a biotinylated PEI array (Fig. 2). Each of the dots was distanced 300 μm centre to centre and approximately 200 μm in diameter. After patterning, the substrate was then rinsed with UHQ water. Once the area was dry 20 μL of neutravidin (10 mg mL^{-1} in water) was placed on the dotted microarray for 1 h, followed by rinsing with water. 20 μL of biotinylated antibody (0.2 mg mL^{-1} in water, 1 h) was then added followed by further rinsing. Finally non-specific interactions were blocked by BSA (10^{-6} mol L^{-1} in PBS, 1 h).

2.3. SECM studies

A screen-printed carbon electrode substrate was placed in a plastic Petri dish and exposed to 5 mmol L^{-1} mediator solution. The SECM working electrode tip and counter and reference electrodes were then immersed into the mediator solution. Prior to undertaking an area scan over the antibody/PEI functionalised regions, an approach curve experiment was conducted over the polycarbonate, non-conductive region of the substrate to estimate the tip-to-substrate distance (tip potential (E) = +0.45 V vs. Ag/AgCl; step size = 10 μm). The tip was positioned at a distance at which the measured current was half that of the observed current with the tip a few mm distant from the surface of the screen printed electrode (effectively infinite on this scale) (approximately 70 μm