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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 labeling of the substrate.

Keywords: Scanning Electrochemical Microscopy, Neuron Specific Enolase, Label-Free, Antibody

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 versus 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 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 hours 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 hours from onset, with concentrations reaching a maximum between 1-3 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 Section

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 figure 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 figure 1b. Ferrocene carboxylic acid (5 mmol l^{-1} in pH 7.0 phosphate buffer) was used as the mediator (figure 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 (figure 2). Each of the dots were 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) were 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) were 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 functionalized 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 from substrate surface); this was to allow for the variation in topography and the height differential between this non-carbon region and the slightly raised carbon electrode surface - while also serving to reduce the risk of tip crash. After tip positioning, an area scan over the functionalised region was conducted with a step size of 10 µm. At no point in the experiment was the substrate touched; this allowed any change in the observed tip current

to be attributed solely to changes in the charge transfer properties of the surface. In all cases the tip diameter was 8.5 µm; the scan rate used was 10 µm per step throughout and there was no potential applied to the substrate.

After the first area scan experiment was conducted, the tip was retracted a known safe distance from the substrate. The mediator solution was then removed and the substrate gently rinsed with UHQ water before applying a solution containing complementary antigen over a range of concentrations or a non-complementary antigen. After exposure for 1 hour, the antigen solution was then removed and the substrate again rinsed before the re-introduction of fresh mediator solution. A second area scan experiment was then conducted over the same functionalised area as measured previously. The tip current data from the area scan before exposure was then subtracted from the tip current data following this exposure. Throughout all these experiments the sample substrate does not move at all, i.e. all exposures, rinsing steps etc are performed with the sample *in situ.* It is worth noting here that after each exposure and rinsing step the Petri dish is refilled with fresh mediator solution and the tip exactly repositioned, made possible using the XYZ micro-positioning stage of the SECM270. This allows precise and reproducible imaging of the same area of the electrode.

3. Results and Discussion

To ensure that changes occurring on the sensor surface were directly related to the hybridization of antibody and antigen, and not due to interference of mediator solution or

poor stability of the substrate, surface scans were undertaken of the antibody modified dotted arrays (figure 3a). The modified surface displays an array of peaks which correspond to decreases in the tip current. This can be accounted for by the polymer/antibody composite acting as a barrier to mediator diffusion to the surface, thereby diminishing the current since polyelectrolytes are known to act as barriers to ion migration [31].

After this initial scan, samples were incubated in purified water for 30 mins in parallel to the immunochemical exposures but in the absence of antigen. Following rinsing, fresh mediator solution was introduced. A scan of this surface is shown in figure 3b. Subtraction of figure 3a from 3b (figure 3c) clearly shows only minimal variations, indicating that minimal changes in background current are seen and that there is no loss of material from the surface. Other techniques employed to dot down the array were investigated. These included the direct dotting of antibody onto the carbon surface, however, these proved to be unstable and deterioration of the array could be visualised as the scan progressed.

Binding experiments were performed by soaking the modified antibody dotted arrays in solutions of varying concentrations of the complementary antigen for 30 mins, followed by scanning in fresh mediator solution. A control was also run using 5 pg mL^{-1} PSA to ensure changes could be related to specific antigen/antibody binding rather than simple non-specific absorption of proteins to surfaces. Binding experiments were carried out using NSE solution with concentrations ranging from 500 fg mL^{-1} - 200 pg mL^{-1} in water.

Detailed results (figures 4a – c) are presented for the exposure to 200 pg mL^{-1}. Figure 4a depicts the scan over the dotted array before incubation, with figure 4b showing the scan taken after exposure to 200 pg mL^{-1} NSE. Figure 4c shows the difference between the two scans indicating changes to the tip current over both the carbon surface and the dotted substrate. Figure 4c shows that across the whole of the array there is a general increase in measured tip current. The change in tip current is, however, seen to be much greater in the areas of the PEI/biotinylated antibody dots than for the untreated carbon surface.

NSE is known to have both positively and negatively charged areas on the surface of the protein [32]. The cationic areas of the protein are capable of electrostatically attracting the ferrocene carboxylic acid (which is anionic at pH 7 due to ionisation of the acid group), thereby increasing the flux of the mediator to the microelectrode tip. There is also the possibility of hydrophobic interactions between the ferrocene unit and any hydrophobic regions of the NSE protein. These could effectively increase the concentration of ferrocene carboxylic acid at the surface and thus enhance the current flow.

Evidence for this type of behaviour comes from earlier work on DNA where the charge on the mediator is found to determine the electrochemical response at the surface. DNA is an anionic polymer due to the presence of phosphate groups and when an anionic mediator is utilised (ferricyanide), increasing the amount of DNA at the surface by hybridisation led to a decrease in current transfer due to repulsion and inhibition of

mediator diffusion [33]. However when a cationic hexaamine ruthenium mediator was used, increasing the amount of DNA led to an increase in current transfer [30]. This is thought to be due to the cationic mediator being bound by the anionic DNA, thereby increasing the local concentration of mediator at the surface and enhancing electron transfer between the surface and the probe tip. In a similar fashion we believe the binding of antigen causes a localised increase of mediator (in this case ferrocene carboxylic acid) by the same principle and leads to increased current transfer.

The results demonstrate that NSE is bound at the surface in greater concentration in the modified areas, indicating that specific binding is indeed occurring. There appears to be some non-specific binding as shown by the increase in current over the unmodified carbon surface however, this non-specific binding was only observed a the highest concentration of 200 pg mL^{-1} and not when investigating the lower antigen concentrations. This confirms our earlier results obtained with microelectrode arrays.[9] Further confirmation was obtained by control experiments with PSA which showed a low level of non-specific binding over the entire surface but no specific binding to the anti-NSE; this is demonstrated in figure 5a.

In each investigation, an array of twelve dots was imaged with a surface profile being obtained for each separate dot. The twelve profiles could be combined to give a mean peak magnitude response for these dots. Figure 5a shows the mean traces for 12 dotted samples, exposed to a variety of concentrations (0.5-200 pg ml-1) of NSE and it is clear that an increase in tip current as the concentration of antigen increases occurs over the

modified regions. For 200 pg mL^{-1} the dot profile appears larger and this is believed to be due to saturation of the area resulting in a much greater level of non-specific binding to the carbon being observed. There is also sign of the dot swelling in size at 20 and 50 pg mL^{-1} antigen concentration however, at the lower concentrations (0.5-5 pg mL^{-1}) there is clearly very little change in current to the carbon region. Figure 5b shows a calibration profile obtained by plotting the peak mean dot responses with respect to antigen concentration. A linear relationship can be seen between the response and the log of the concentration in the range 0.5 pg mL^{-1} to 200 pg mL^{-1}. A logarithmic relationship has also been found between ac impedance and antigen concentration in previous immunosensor work [8,10]. This demonstrates the potential for this technique to detect and quantify the presence of a target in solution.

The limits of detection for NSE in this experiment (0.5 pg mL^{-1} to 200 pg mL^{-1}) are comparable to those obtained with sonochemically microfabricated arrays (0.5 pg mL^{-1}) [12] - and substantially lower than we obtained for similar systems based on macroelectrodes [7,8,10]. Previous work has succeeded in imaging layers of immobilised antibodies [34], however their technique required the use of enzyme modified antigens and also only reported the use of excess labelled antigen whereas our technique is label-free and is capable of producing a calibration curve. The sensitivity of our method also compares well with fluorescence based assays, for example when microarrays on silicon were utilised, antibody-peptide specific interactions could be observed with a detection limit of 0.5-1 ng mL^{-1} [35, 36].

The key significance of this work is that for the first time we have demonstrated the feasibility of SECM antibody based arrays for the development of high throughput labeless affinity based analysis. This highly simplified approach could in some contexts directly challenge the fluorescence based approach for molecular diagnostic and/or other applications.

4. Conclusions

We have within this paper demonstrated the potential for utilising the SECM to image arrays of immobilised antibodies and furthermore detect binding of the antigen using a label-free protocol. A clear relationship between the antigen concentration and the changes in the signals obtained for binding to the arrays is observed. A calibration profile showed a clear correlation of change in current with respect to antigen concentration with detection limits of 0.5 pg mL^{-1} to 200 pg mL^{-1} . The low (pg mL^{-1}) limits of detection will aid in the analysis of physiological samples since they can be diluted before use therefore diminishing the effects of interferents.

The results obtained within this work demonstrate the feasibility of using the SECM to analyse surface binding of antigens. This technique although slower than fluorescent methods, has a significantly lower cost base and does not require labelling of any kind, including fluorescence of samples. Further work to be conducted will include the improvement of the preparation of arrays to increase the number of samples being tested at one time and also to allow for ‘control dots’ being incorporated within an array.

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426 **Figure Captions**
427
428 Figure 1. (a) Schematic representation of SECM instrumentation- the microelectrode
429 probe is clamped into a high resolution XYZ micropositioning device and scanned across
430 the sample surface. Scan parameters and tip electrochemistry are controlled via a PC
431 interface. (b) The arrangement of electrodes in the electrochemical cell – i.e. WE, RE and
432 the CE. (c) Redox coupling of ferrocene carboxylic acid between the microelectrode tip
433 and the substrate.

Figure 2. Photograph of biotinylated PEI deposition on carbon electrode by pulled microcapillary using the XYZ micro-positioning stage of the SECM270 and of the final array pattern on the screen printed carbon electrode surface (dot size ~ 200 µm).

Figure 3. Area scan of PEI/avidin/biotinylated antibody arrays on screen printed carbon electrode (a) before and (b) following rinsing and 30 mins exposure to water; (c) absolute change in measured current. (Scan rate was 10 µm per step). (Note Z axis scales are in reverse for visual aid).

Figure 4. SECM scan of a PEI/avidin/biotinylated antibody array on a screen-printed carbon electrode; (a) following exposure to biotinylated antibody NSE, (b) following further exposure to complementary NSE antigen at 200 pg mL^{-1}, (c) absolute change in measured current. (Scan rate was 10 µm per step). (Note Z axis scales are in reverse for visual aid).

Figure 5. (a) Mean changes of a twelve dot array scan (n = 12) taken over PEI/avidin/biotinylated antibody surface area to various antigen concentrations (no error bars are included for clarity, data points on individual curves represent the tip current values taken in different sites of the dots); (b) Calibration plot showing changes in current measured (n = 12) vs. NSE concentration. Equation is $y = 0.54\ln(x) + 0.51$, $R^2=0.97$ and the error bars show the standard deviation between the 12 individual dots.

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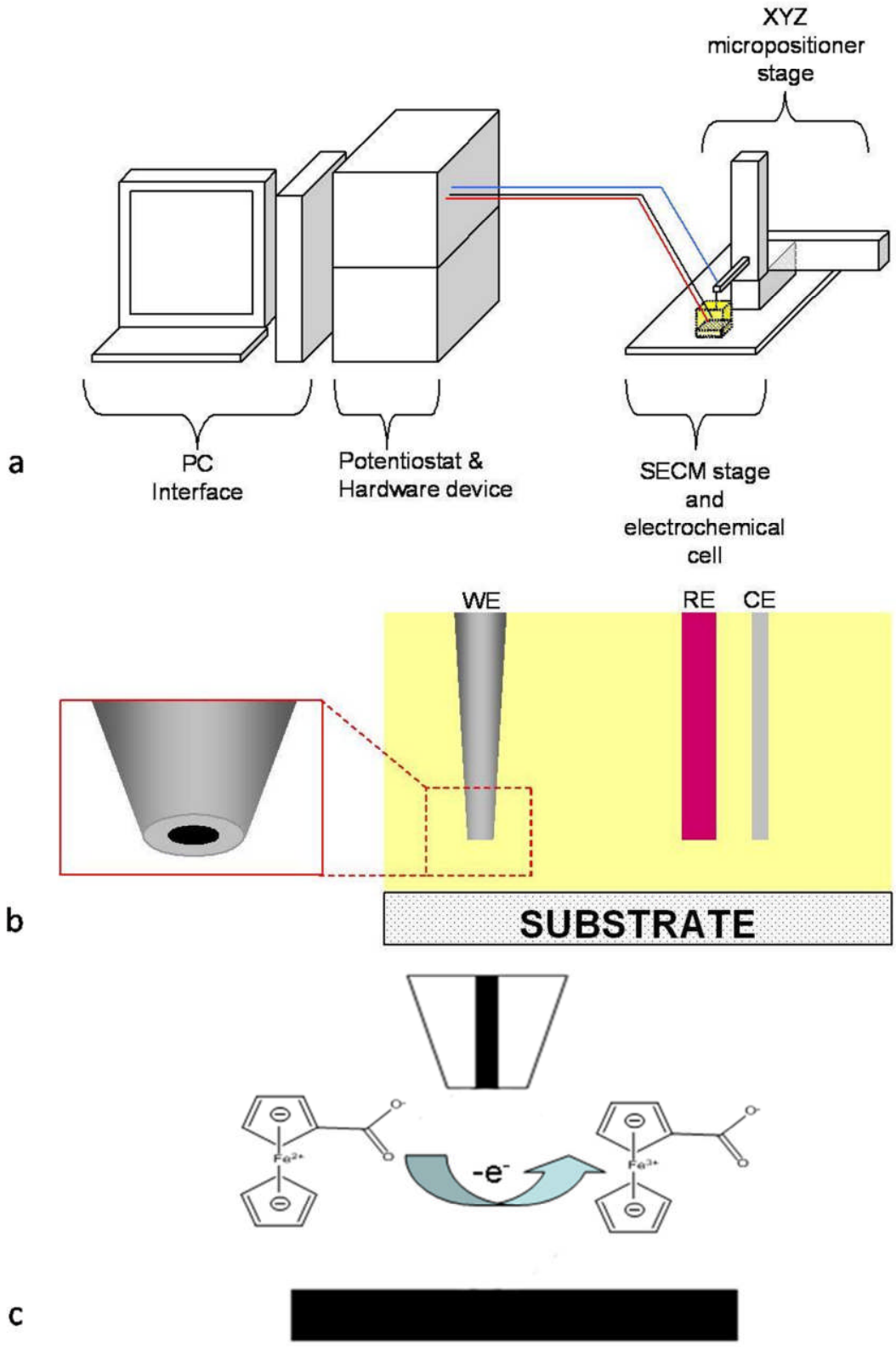

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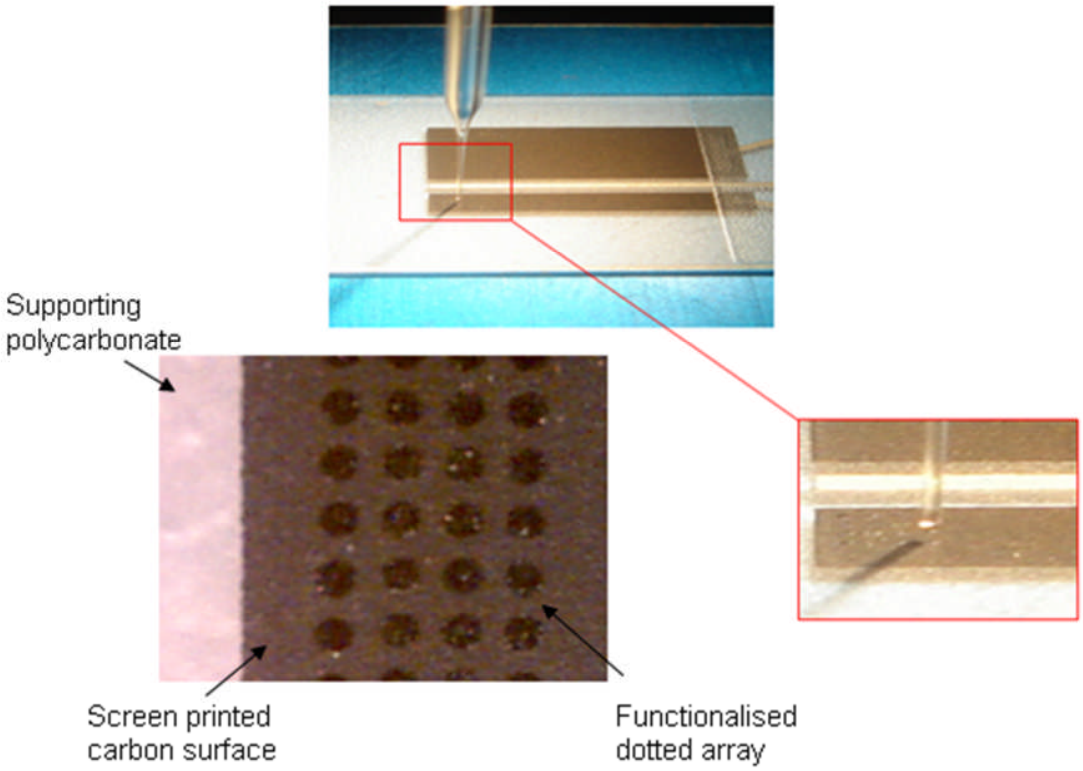

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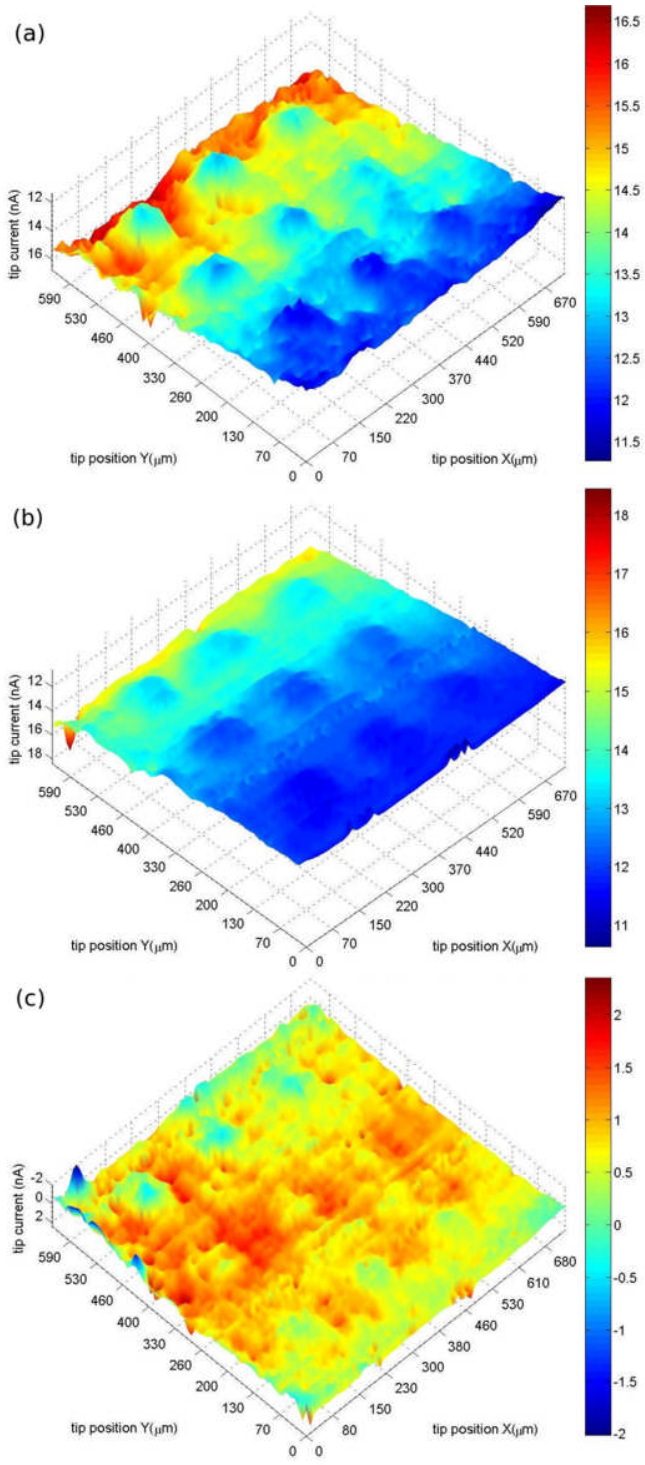

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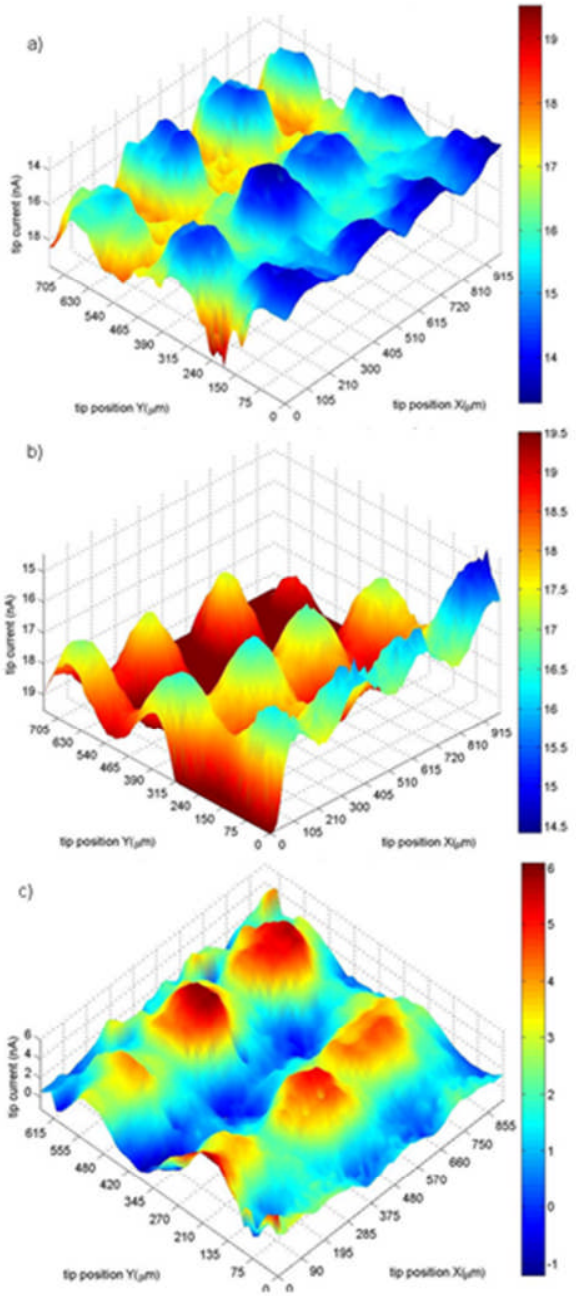
Figure 4.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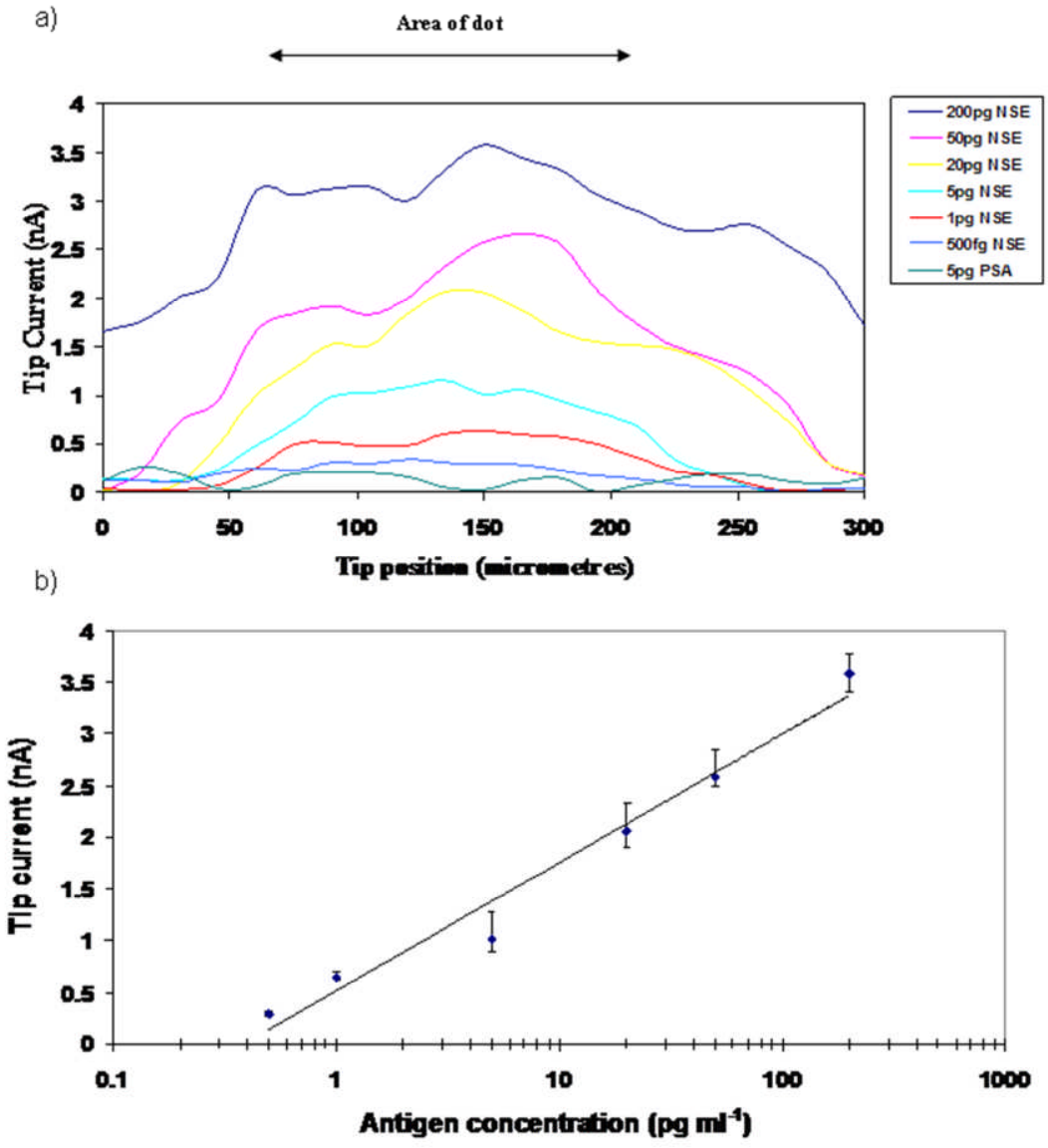
tip current (nA)
tip position Y(μm)
tip position X(μm)
b)
tip current (nA)
tip position Y(μm)
tip position X(μm)
c)
tip current (nA)
tip position Y(μm)
tip position X(μm)

468 Figure 5.



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