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Construction and interrogation of enzyme microarrays using scanning electrochemical microscopy - optimisation of adsorption and determination of enzymatic activity.

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Abstract

Scanning electrochemical microscopy (SECM) has been used to image and study the catalytic activity of horseradish peroxidase (HRP) immobilised in a patterned fashion onto glass slides. Microarrays of HRP islands could be deposited on amino-modified glass slides using glutaraldehyde crosslinking combined with the SECM being used as a micro-deposition device. The enzymatic activity of the immobilised enzyme on the surface was in the presence of its substrate observed to give rise to substantial positive feedback between the slide and the SECM microelectrode tip. Conversely when either blank slides - or slides coated with HRP which had been subsequently thermally denatured were utilised, these showed negative feedback effects. Various conditions such as enzyme concentration, incubation time and substrate concentration were systematically varied to optimise sensitivity. Regular arrays of HRP could be assembled and when imaged, displayed lower limits of detection of 1.2×10^{-12} mol ml^{-1} of benzoquinone.

Introduction

Scanning electrochemical microscopy (SECM) is a surface scanning probe technique that allows for the collection of high resolution electrochemical data at a variety of surfaces. The technique involves interrogation of a discrete area by scanning a probe electrode across its surface in a similar manner as utilised in atomic force microscopy except that in SECM the electrochemical nature of the surface is investigated. A wide range of distinctly different bioanalytical problems have been examined utilizing SECM, as reviewed elsewhere [1]. One common theme is to immobilise biological species such as enzymes onto a surface. The enzymes can then be used to catalyse the consumption or formation of an electrochemically active product, such as hydrogen peroxide, when exposed to a suitable substrate. This process can either be detected directly or by the use of a suitable mediator such as a ferrocene compound or other electroactive systems such as methylene blue.

Oxidase enzymes have been studied using SECM; one early paper for example discussed the immobilisation of carbodiimide activated glucose oxidase onto an aminothiol coated gold surface with the resultant surface being analysed with SECM in the presence of glucose and a ferricyanide mediator [2]. Using this method both the topography and electrochemical activity of the composite surface could be imaged. Much of the early work on patterned enzyme surfaces and their imaging by SECM has been reviewed [3].

Horseradish peroxidase could be grafted onto glass slides using biotin-avidin interactions, immobilisation within crosslinked hydrogels on glass slides - or within the

pores of a polycarbonate membrane [4]. When the filters were imaged using SECM, the enzyme-filled pores could be clearly resolved. Patterns of oxidase enzymes could be deposited by soft lithography and imaged using SECM [5]. In other work glucose dehydrogenase could be bound to strepavidin modified magnetic microbeads, deposited on a surface and the resultant microarray imaged by SECM [6, 7]. Multiple enzyme arrays have also been studied; galactoxidase and glucose dehydrogenase have, for example, previously been combined in microspots and imaged by SECM [8]. SECM and AFM techniques have also been combined using a single probe to enable both topographic and electrochemical imaging of a patterned enzyme surface [9]. Carbon microdisc electrodes have been used to image HRP patterns [10] and further work successfully developed carbon disc electrodes with diameters of a few hundred nm [11]. Enzymatic arrays were produced using inkjet printing techniques in which individual square spots in a grid pattern could be deposited. These contained different proportions of glucose oxidase and a polymer matrix - and therefore yielded different levels of activity which could be followed using SECM [12]. SECM has also been used to investigate polyelectrolyte multilayers containing cationic polymer and anionic glucose oxidase layers and showed that the catalytic activity within these systems was essentially restricted to the uppermost enzyme layer [13].

The activity of enzymes has led them to be utilised as labels for the determination of other biochemical binding events in SECM. For example workers immobilised single stranded DNA on surfaces, and exposed this to the complementary strand which had been labeled with glucose oxidase [14]. Monitoring the enzyme activity at the surface with SECM allowed the determination of hybridization events. Similarly target DNA strands

could be labeled with biotin and then biotin-avidin interactions utilised to conjugate with a biotinylated HRP label [15]. These types of systems can also be used within immunoassays; antibodies to the cancer-related marker CA15-3 could be immobilised on surfaces and then exposed to the antigen. Further exposure to a HRP-labeled second antibody gave rise to a sandwich type assay which could be visualised and quantified by SECM [16]. Besides these examples a number of other proteins have been imaged by SECM as reviewed previously [1].

In earlier work we have within our laboratory studied the label-free monitoring of biochemical interactions at electrode surfaces. Besides utilizing the SECM as an imaging procedure, we also simplified the generation of the biochemical arrays by utilizing the SECM as a tool for their fabrication. We have previously demonstrated both hybridization of genomic DNA [17] and also immunochemical detection of a stroke marker protein, Neuron Specific Enolase [18]. To improve detection of these types of events such as protein expression in cell membranes, we are developing labeled techniques to increase sensitivity. As an early stage within the development of these techniques we have studied the use of the SECM in fabricating enzyme microarrays, developed a relatively simple chemistry to ensure enzyme immobilisation and also optimised the imaging process to maximise sensitivity.

Experimental

Chemicals and array fabrication.

Nitric acid (70%), sulphuric acid (95-98%), 3-aminopropyltriethoxysilane (APTES), hydrogen peroxide, horseradish peroxidase and hydroquinone were obtained 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 chemicals were used without further purification. All water used was purified with an ELGA Purelab UHQ purifier. Phosphate buffer (PBS), pH 7.1 contained NaH_2PO_4 (4 mM), Na_2HPO_4 (6 mM) and NaCl (132 mM).

Glass microscope slides were cleaned by immersion in a 1:1 solution of 95% H_2SO_4 and 70% HNO_3 for ten minutes. These were then removed from the acid, washed, rinsed with sterile UHQ water and dried. Following drying, slides were immersed in acetone and dried before immersion in a 1% solution of APTES in acetone for 2 minutes - after which they were allowed to dry. Screen printed electrodes consisting of carbon working and counter electrodes together with an Ag/AgCl reference were supplied by Microarray Ltd. (Manchester, UK).

5 mg of horseradish peroxidase was dissolved in 1 ml of 1% glutaraldehyde and consecutive dilutions prepared down to 0.0625 mg ml^{-1}. For experiments involving approach curves only, a small volume (50 μl) was deposited onto the surface; this was then allowed to crosslink with the excess being rinsed off after 10 minutes. In all cases, the location of the deposited enzyme was marked by the use of a glass-cutter pen on the underside of the glass slide.

A borosilicate glass capillary was pulled to an internal diameter of 20-30 μm using a Narashige PP-830 pipette puller and the tip polished to a square finish. Deposition of arrays of HRP was carried out as described previously for DNA and antibodies [17, 18] using the pulled microcapillary to deposit the enzyme/glutaraldehyde solution onto the silanised glass slides by micromanipulation of the tip position using the SECM270 XYZ stage. After patterning, the slide was dried for 10 minutes, rinsed with UHQ water and stored in pH 7.1 phosphate buffer until required.

SECM interrogation

SECM experiments were carried out using an Uniscan SECM270 (Uniscan Instruments Ltd, Buxton, UK) as described previously [1, 17, 18]. The SECM instrument is composed of an electrochemical cell, a translational stage capable of high resolution (sub-micron) movement in the X, Y and Z planes and a bipotentiostat all interfaced with a PC using bespoke software for computer control[18]. Pt working and counter electrodes and Ag/AgCl reference electrodes were used throughout.

The patterned slide was positioned on the SECM stage and the entire assembly leveled. 1 ml of 2 mM hydroquinone in pH 7 buffer was then placed on the slide and the three electrodes positioned within the droplet. The microelectrode tip was then set at +0.8 V which was sufficient for the oxidation of hydroquinone to benzoquinone. An approach curve was conducted over the functionalised region; when the observed tip current begin to fall, the approach curve was halted and the tip retracted 1000 μm. The tip was stopped at this stage so as to prevent tip crash and any damage to the immobilised enzyme layer. 1 ml of 2 mM H_2O_2 was then injected into the system, resulting in a mediator solution

composed of 1 mM hydroquinone and 1 mM H_2O_2. After a specific period (0, 2, 4, 6, 8 or 10 min), the tip was biased at -0.4 V to detect the benzoquinone produced by the immobilised enzyme and an approach curve was conducted over the enzyme modified region at a rate of 10 μm per step (10 μm s^{-1}); the tip was stopped when the approach curve profile began to fall off – i.e. when negative feedback was observed. Each scan therefore took just over a minute to run. It was not possible to monitor the production of benzoquinone immediately after the introduction of the hydrogen peroxide using the approach curve because of the time delay between the commencement of the scan and the point at which the tip comes into close proximity to the immobilised enzyme For patterned slides, area scans were conducted over the region of interest at a rate of 20 μm per step over areas up to 1200x1200 μm in size. Figure 1 shows a schematic for the interrogation process. One challenge with SECM is accurate determination of the absolute nature of surfaces since many factors such as tip-to-surface distance or surface homogeneity affect the response. The usage of a patterned array allows visualization of the contrast between enzyme modified and unmodified regions of the surface which allows the cancellation of non-specific effects.

Results and Discussion

Measurement of enzyme activity and diffusional characteristics.

The first step of our investigation was to determine approach curves over inactive control surfaces. Two slides were used as controls in this study – a silanised glass microscope slide along with a slide which had been functionalised with glutaraldehyde-crosslinked

horseradish peroxidase and then exposed to a heat treatment (placed in an oven at 80^{o}C for 15 mins). Approach curves were conducted over these slides in a mediator solution containing 1 mM hydroquinone and 1 mM hydrogen peroxide in a pH 7.1 buffered solution. The resulting approach curve for the heat-treated HRP-modified glass slide is presented in figure 2. There is a small background current observed at large distances from the slide even in the absence of electrochemical activity, this is because the tip is charged at the potential applied and ions from the electrolyte diffuse towards the tip to form a double layer, this is known as the capacitive current (I_c). As the tip is moved into close proximity to the slide, diffusion of ions around the microelectrode is hindered and potentially limits the thickness of the double layer. This leads to a drop in tip current (known as negative feedback) due to the hindered diffusion around the microelectrode. These observations suggest that the heat treatment denatures the HRP immobilised at the surface, diminishing its ability to catalyse the reduction of hydrogen peroxide to water via the oxidation of hydroquinone to benzoquinone. Almost identical approach curves to those obtained for denatured HRP-slides were also obtained using silanised, insulating glass slides.

By contrast studies utilizing glass slides with immobilised, active HRP showed very different behavior. On approaching a HRP functionalised surface, the tip current is observed to increase to a peak value before exhibiting a sharp fall (figure 2). This is in contrast to the negative feedback curves obtained when approaching an insulating, non-functionalised slide or denatured slide. The increase in current can be explained in terms of the generation of benzoquinone by the immobilised enzyme at the slide surface. Since the rate at which the benzoquinone molecules can diffuse away from the surface is

slower than the rate at which they are being produced by the enzyme, a concentration gradient arises and extends into the solution; in this way, the concentration of benzoquinone at the functionalised surface is greater than that in bulk solution. As a result of this increase in concentration, there is a proportional increase in the faradaic current as the tip approaches the surface. However, as the tip comes into close proximity to the HRP-functionalised surface, the diffusion of the enzymatic product from the surface to the tip is hindered by the insulating glass sheath around the conductive region of the probe, resulting in a lowering in current akin to that observed for a probe approaching an insulating slide.

Control experiments were also made using active-HRP slides in buffer, in buffer containing 1 mM hydroquinone (but no H_2O_2) and in buffer containing 1 mM H_2O_2 but no hydroquinone. In all three cases no current flow due to enzymatically produced benzoquinone could be detected.

To allow comparison with the control curves, the approach curves were normalised to the current observed in bulk solution (figure 2) i.e. the plot shows the tip currents obtained at each distance divided by that observed in bulk solution. In this particular example, at a distance of 600 μm from the slide surface, the tip current is equal to the tip current obtained in bulk solution. However, as the tip approaches the slide, the tip current increases to a peak, indicating a local benzoquinone concentration higher that observed 600 μm away in bulk solution. Approach curves were also carried out with H_2O_2 concentrations of 0.2 mM, 0.4 mM and 0.6 mM, these displayed no significant differences to those at 1 mM indicating that there is a large enough excess of H_2O_2 for it not to be a determining factor in the reaction kinetics.

To determine the effect of enzyme loading on the observed current, the silanised glass slide was functionalised using solutions containing different concentrations of enzyme – specifically, 5, 4, 3, 2, 1, 0.75, 0.5, 0.05 and 0.025 mg of HRP per ml of 1% glutaraldehyde solution. A 50 µl droplet of each loading solution was pipetted onto the silanised glass surface and allowed to crosslink for five minutes before the slide was rinsed and stored in pH 7.1 buffer until used. Approach curves were measured as before. The average background subtracted peak currents (i.e. the maximum currents observed for each concentration) were then plotted (figure 3). As can be seen, there is a steady increase of peak tip current with enzyme concentration between 0-1 mg ml^{-1}. Above this limit, further increases in enzyme concentration have minimal effect. The lowest measured current peak detected was for the slide functionalised with 0.025 mg ml^{-1} enzyme solution and was calculated to be due to a benzoquinone concentration of 1.2 x 10^{-12} mol ml^{-1}. The observed relationship between the average peak tip current and the enzyme concentration in the loading solution may be due to two possible reasons, as described below.

The first possible explanation relates to the density of enzyme on the surface; on treating the silanised glass with a 1 mg ml^{-1} solution, if the surface is saturated with the enzyme and all cross-linking sites are occupied, the use of enzyme loading solutions with 2, 3, 4 and 5 mg ml^{-1} may result in no further increase in the concentration of enzyme immobilised at the slide surface. The effect of such surface saturation would be that the peak current observed over each of these saturated slides was similar in magnitude.

A second hypothesis for this observation is based on the diffusional kinetics of the immobilised enzyme. When the rate of diffusion of the reactants to the enzyme is greater

than the rate of the enzymatic reaction, the overall rate of the enzymatic reaction would be under the kinetic control of the enzyme. Under these conditions, if one were to increase the concentration of the enzyme at the surface, the enzymatic activity per unit area would also increase, resulting in an enhancement in the production of benzoquinone and consequently a higher peak current when interrogated by SECM. At the lower enzyme concentrations, on increasing the concentration of enzyme at the surface, an incremental increase in the peak current would be observed. However, on increasing the concentration of the loading solution to 1 mg ml^{-1} and above, the peak current plateaus. This is thought to be due to the fact that at this level of enzymatic activity per unit area, the rate of the enzymatic reaction per unit area is faster than the rate at which the reactants, hydroquinone and hydrogen peroxide, can reach the immobilised enzyme by diffusion, i.e. the rate of the enzymatic reaction is limited by the rate of diffusion of the substrate to the enzyme.

To ascertain the diffusional kinetics of this system, a series of approach curves were conducted at specific time periods. This strategy allows monitoring of the diffusion of the enzymatic product, benzoquinone, from the slide with time. In this way, a measurement of the rates of benzoquinone diffusion may be obtained which would aid the acquirement and interpretation of area scan data. Approach curves were conducted at t = 0, 2, 4, 6, 8 and 10 minutes from the introduction of hydrogen peroxide to the mediator solution. An example of the approach curves observed is shown in figure 4.

Approach curves were conducted at a scan rate of 5 μm per point so as to minimise disturbance of the diffusion layer emanating from the HRP functionalised slide. If approach curves were conducted at any greater speed, whilst they would allow

measurements to be taken faster, the movement of the tip towards the surface could disturb the diffusion layer by mechanical convection.

In the example given (figure 4), at t = 0, the bulk concentration of benzoquinone is very low, resulting in a low initial tip current up to 600 μm from the surface. As the tip approaches the enzyme functionalised slide, the tip current was observed to increase to approximately 3.2 nA above the background current, due to the reduction of benzoquinone produced by the enzyme catalysed reaction. A second approach curve begun two minutes after of the addition of hydrogen peroxide showed similar behaviour except that the current began to rise much sooner, indicating that benzoquinone has diffused away from the surface and is being detected at the microelectrode at a greater distance from the slide. When t=4 minutes this behaviour is even more pronounced, with benzoquinone being detected as soon as the approach curve begins and with longer time intervals the benzoquinone concentration is becoming much more uniform throughout the system; at t=10 min there is only a relatively small difference between tip current close to the surface and in bulk solution.

The SECM was also used to determine the distribution of the benzoquinone product in solution with increasing time. The first step was to determine the diffusion coefficient for benzoquinone under the conditions of the protocol described within this paper. A cyclic voltammagram for benzoquinone (2 mM) in buffer was measured utilising the screen printed electrodes described in earlier work [17]. The Randles-Sevcik equation (a simplified version is shown in equation 1) can be used to determine the diffusional coefficient of benzoquinone from the peak current of the CV.

Equation 1: $$i_p = (2.687 \times 10^5) n^{3/2} v^{1/2} D^{1/2} AC$$

In this relationship, the peak current (i_p) in a cyclic voltammogram for the species under interrogation is expressed as a function of *n,* the number of electrons involved in the charge transfer reaction (two in this case), *A* the electrode area (cm^2), *v* the sweep rate, *D* the analyte diffusion coefficient ($cm^2\ s^{-1}$) and *C* the concentration in mol ml^{-1}. For an electrode area of 0.24 cm^2, a sweep rate of 0.05 V s^{-1} and a concentration of 2 mM, a peak current of 2.01 μA was obtained. This led to a diffusion coefficient of 2.46 x $10^{-5} cm^2 s^{-1}$ for benzoquinone in buffer being calculated under these specific conditions. This figure is in good agreement with previous results [19, 20].

The faradaic current observed at a microelectrode in bulk solution is described below where a is the radius of the microelectrode and F the Faraday constant [21].

Equation 2: $$i_{T\infty} = 4nFDCa$$

During the approach curves over HRP modified glass, at any point in bulk solution, the current may be described by equation 2. Given the tip current, the area of the working electrode and the diffusion coefficient for the species under detection, it is possible from this equation to estimate the concentration of benzoquinone at each point along each approach curve. The change in the benzoquinone concentration profile over time for a 5 mg ml^{-1} HRP functionalised slide is presented in figure 4, which demonstrates the slow diffusion of the benzoquinone out into solution. Initially, the concentration gradient is high as the rate at which benzoquinone can diffuse away from the site of the enzymatic

reaction is slower than the rate at which it is produced by the enzyme. As time elapses, the rate of diffusion of the enzymatic substrate to the enzyme becomes slower than the rates at which benzoquinone is created by the enzyme and later diffused to the bulk solution, thus, kinetically controlling the whole process. The result is that this decrease in the rate of benzoquinone production allows benzoquinone to become more evenly distributed in the volume of solution in the vicinity of the enzyme – i.e. the difference in benzoquinone concentration between adjacent points along the approach curve axis becomes smaller. After 10 minutes there is only a small difference between the peak current and that measured in bulk solution - hundreds of nm from the surface. This is important in the context that if we wish to image arrays there must not be a long interval between substrate addition and the imaging procedure, otherwise the benzoquinone product will diffuse away from the active sites and become more evenly distributed throughout the solution, rendering the acquirement of high resolution scans problematic.

Imaging horseradish peroxidase activity

The next stage of our work was to develop an appropriate method for imaging HRP activity. Using the same patterning techniques as those employed earlier [17, 18], patterned HRP arrays were fabricated using the XYZ micro-positioner. The spacing of the arrays was an essential consideration to avoid the merging of diffusion layers – which would affect the resolution of the array. If the array dots were placed too close together, there would be a risk of their diffusion layers overlapping, resulting in two dots appearing as one. It was found that by positioning them up to 500μm apart in a grid format, they were sufficiently distant from one another to appear as two distinct dots.

As described previously within this paper, a potential issue is the fact that benzoquinone diffuses away from its site of production. In the case of a patterned array, this could lead to imaging becoming problematic, since if the benzoquinone is evenly distributed throughout the solution, no resolution of the pattern can occur. At the scanning rate used (20 μm per step), imaging of a 1200x1200 μm area requires approximately one hour. Increasing the scan rate beyond this could well lead to interference due to rapid tip movement. Figure 4 indicates that after 10 minutes the benzoquinone distribution with respect to tip distance is quite even, however these results were for a completely coated slide. In the case of small patterned areas, much less benzoquinone is produced overall (less than 10% of the area imaged is coated with HRP) and such small areas experience hemispherical diffusion profiles (in a similar manner to microelectrodes) which means benzoquinone concentrations will fall more rapidly with distance from the enzyme coated areas. Therefore there will still be a measurable contrast between the coated islands and the uncoated slide.

Before conducting area scans over the HRP arrays, approach curves were carried out over a non-functionalised region of the microscope slide patterned with the array. The tip was positioned 50 μm above the point at which the tip current was 50% of that in bulk. The choice to position the probe at this height was based on observing the behaviour of the tip current when approaching the HRP functionalised slide in the previous series of approach curves. It was at this height where the peak current was obtained and where negligible feedback effects were observed. After positioning the tip in this Z axis, the tip was then moved through solution and positioned proximal to the horseradish peroxidase array,

prior to hydrogen peroxide being introduced into the system. The area scan was then conducted over the functionalised slide.

This initial set of experiments were carried out to determine the effect of substrate concentration on the enzymatic reaction. Area scans were conducted in a buffered mediator solution comprising 1 mM hydroquinone and hydrogen peroxide at concentrations of 1 mM, 0.6 mM, 0.4 mM and 0.2 mM. We observed that on using smaller concentrations of the hydrogen peroxide substrate, the resolution of the images depreciated. Using hydrogen peroxide concentrations of 0.6 mM or above, the area scans recorded distinct and well defined regions of enzymatic activity (figure 5). Those scans which have been obtained using hydrogen peroxide in lower concentrations however have poorly defined functionalised regions, indicative of lowered enzymatic activity (images not shown). From these experiments, optimal imaging seems to occur at hydrogen peroxide concentrations of 0.6 mM, results somewhat different from the simple approach curve data.

Background subtraction

Background subtractions were performed to improve the resolution of the area scan images obtained. In doing so, the current range of the images was lowered. The advantages of utilizing background subtraction include removing the tip current variation due to slide slope as well minimizing variation due to the concentration of benzoquinone in bulk solution. The subtraction was achieved post-scan by constructing a background array from a line of data extracted from the experimental area scans.

A line of data from an unsubstituted portion of the array was analyzed and gave a measurement of the slope of the slide. This could be utilised to generate a model sloped background area. This background area was then subtracted from the original experimental area scan to obtain the background subtracted, optimally resolved area scan images, of the array.

An alternative strategy to the background subtraction method employed here would be to have conducted an area scan before and following the addition of the hydrogen peroxide and then to have subtracted the ‘before’ scan from the ‘after’ scan. The reasons for not doing so are threefold: firstly, although the un-catalysed reaction between hydroquinone and hydrogen peroxide is very slow, the addition of hydrogen peroxide to the system may have an effect on the concentration of benzoquinone in bulk, resulting in an increase in the bulk concentration of benzoquinone that would not have been catered for by the simple subtraction of the ‘before’ data. Secondly, on the addition of the hydrogen peroxide to the system, the bulk concentration of the mediator is altered, which would have implications for the validity of comparing the two scans. Thirdly, on conducting the first scan, a tip crash might occur and also the tip may become fouled on the first scan, which again would introduce error to the data, affecting the validity of the subtraction.

Using the methodology described, well defined, high resolution images were obtained which illustrate the applicability of the technique to imaging enzymatic activity. Figure 6 shows a well resolved scan of a HRP array. When a single cross-sectional line of data is extracted and plotted to show a profile of three of the dots, it is apparent that there is good correlation of the dot footprints. A significant limitation of the substrate generation, tip collection mode is that during scanning, the electrode is moved through the diffusion

layers extending into solution around the area of interest, which may lead to the redistribution of electroactive species over the slide. Such movement of these species may then lead to a poorly resolved image whereby it is difficult to determine where on the slide the reaction is occurring. Despite this potential problem, it is apparent from this work that well resolved images may be obtained.

Conclusions

The principle aim of this work was to explore the possibility of using SECM to both construct and characterise regular arrays of enzymes on surfaces. We have developed an optimised procedure for the deposition and imaging of HRP arrays.

In the series of experiments described here, the use of SECM in generation collection (GC) mode allowed for the sensitive imaging of HRP activity. On conducting approach curves over HRP modified glass, the low detection limits of the SECM technique were demonstrated. Silanised glass was loaded with glutaraldehyde-crosslinked horseradish peroxidase present in concentrations down to 0.025 mg ml^{-1}.

By observing the difference between the peak currents obtained by approach curve over slides loaded with differing concentrations of HRP, it was found that this technique is capable of differentiating between surfaces functionalised using different concentrations of enzyme loading solution in the range 0-1 mg ml^{-1}. Above this limit, further increases led to only minimal changes in tip current. Using approach curves, benzoquinone concentrations down to as low as 1.2×10^{-12} mol ml^{-1} were detected, demonstrating excellent sensitivity.

The imaging capabilities of the SECM to this system have been demonstrated. Imaging an array fabricated by the deposition of a HRP/glutaraldehyde solution on glass allowed a well resolved array to be defined and dots spaced 500 μm apart were clearly resolved from one another. Obtaining such a well resolved pattern was, however, unexpected. Since the generation/collection mode involves imaging the enzymatic product in the diffusion layer, it was thought that there would be a considerable lack of resolution in the images obtained as the tip 'dragged' enzymatic product from the vicinity in which it was produced as it was moved through the diffusion layer. This was not the case however and very good resolution was observed.

Varying the concentration of hydrogen peroxide had a significant effect on the quality of image obtained. By limiting the concentration of enzyme substrate, the rate of enzymatic reaction was lowered, which in turn had the effect of lowering the quantity of enzymatic product generated by the enzyme and so, the size of the diffusion layer. The result of this effect was to lower the resolution of the image with significant inter-dot variation.

Both investigations into the diffusional kinetics affecting surfaces modified with HRP have been presented, along with demonstrating a methodology by which horseradish peroxidase activity could be imaged. One challenge with the study of this reaction is that the enzymatic reaction is exceptionally rapid, even though the rate of diffusion is relatively slow. Benzoquinone is produced rapidly by the enzyme, but the rate at which it diffuses from the slide and into solution is subsequently slow, resulting in a difficulty in differentiating diffusion profiles due to slides functionalised with similar surface enzyme concentrations.

However, since the intention was to use the enzyme as an electroactive label for following surface binding events, the results presented here demonstrate the suitability of the detection method as a viable route for the further development of this assay. In addition, the micropatterning capabilities of the Uniscan micro-positioning device have been further demonstrated, enabling rapid array construction without the necessity to purchase another piece of equipment.

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Figure Captions

Figure 1: Schematic diagram of proposed detection mechanism of horseradish peroxidase using the hydroquinone / benzoquinone redox couple and the SECM in GC mode.

Figure 2: Normalised approach curves over a control slide (heat treated HRP functionalised glass) and an active HRP functionalised glass slide. Each approach curve was conducted in a pH 7.1 buffered mediator solution containing 1 mM hydroquinone and 1 mM H_2O_2.

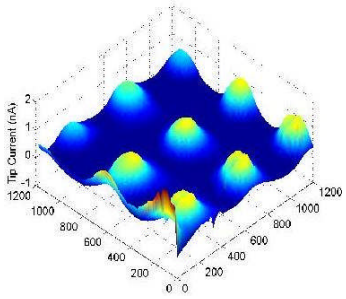
Figure 3: Variation in peak tip current with enzyme concentration in loading solution. Error bars are 95% confidence intervals for calculated means.

Figure 4: A series of approach curves obtained at various time intervals after the introduction of 1 mM H_2O_2 and to the system. Both the measured current and calculated benzoquinone concentration from bulk solution towards the enzyme functionalised surface are shown.

Figure 5: Background subtracted area scan over HRP modified array using pH 7.1 buffered solution containing 1mM hydroquinone and A) 1 mM H_2O_2 and B) 0.6 mM H_2O_2.

Figure 6: An area scan across a HRP modified array. A cross section was extracted from the area scan array along the dotted line and the resulting data plotted as a line scan.

Graphical Abstract



Patterned arrays of horseradish peroxidase have been deposited and imaged by scanning electrochemical microscopy and shown to be capable detecting a substrate at levels as low as 1.2×10^{-12} mol ml^{-1}.

Figure 1.

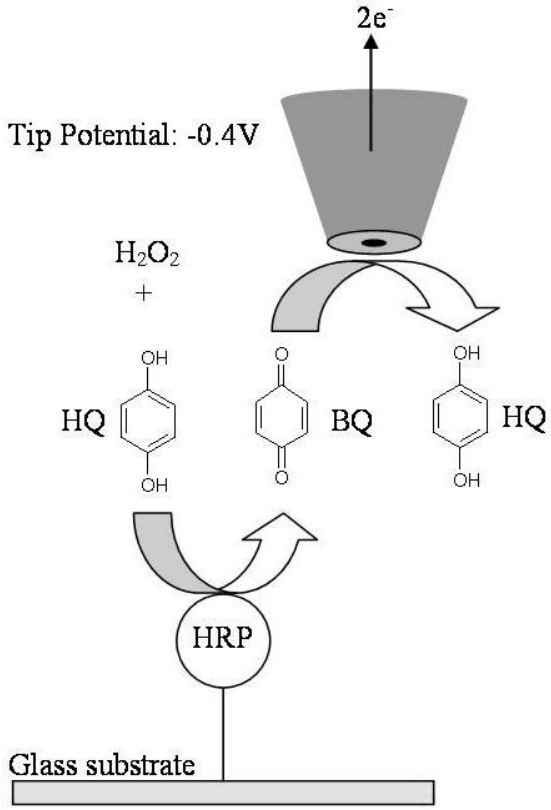


Figure 2.

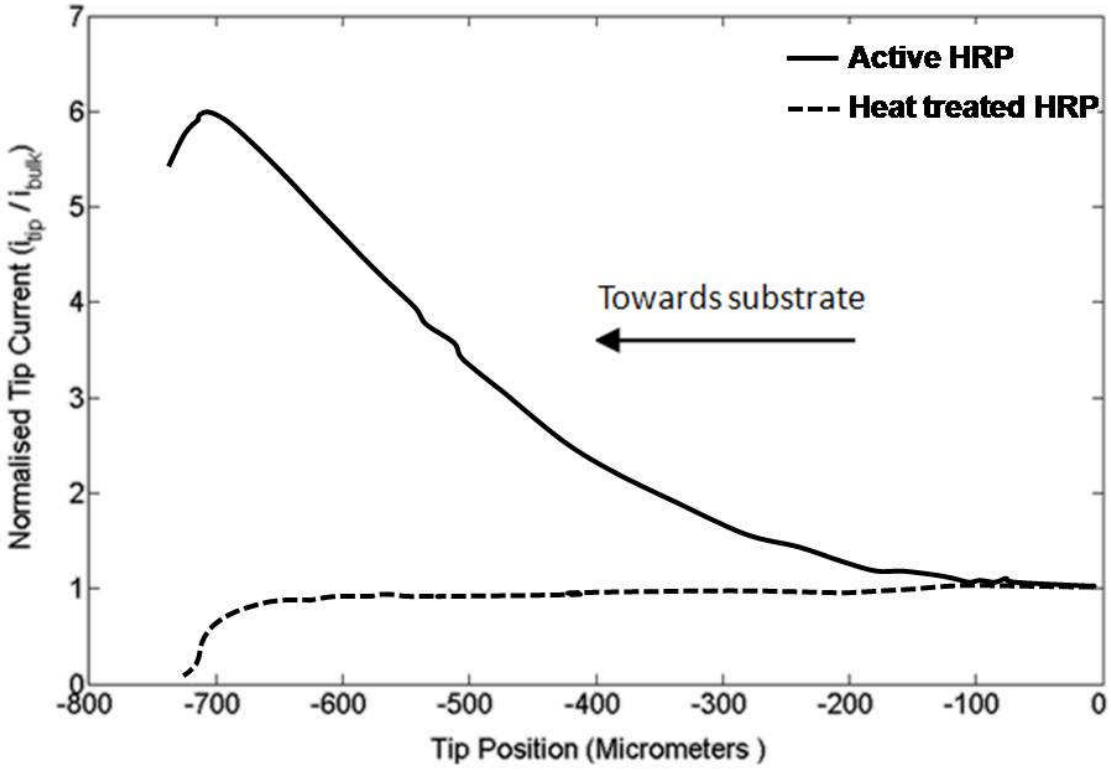

Active HRP
Heat treated HRP
Towards substrate
Normalised Tip Current (i_{tip} / i_{bulk})
Tip Position (Micrometers)
7
6
5
4
3
2
1
0
-800
-700
-600
-500
-400
-300
-200
-100
0

Figure 3.

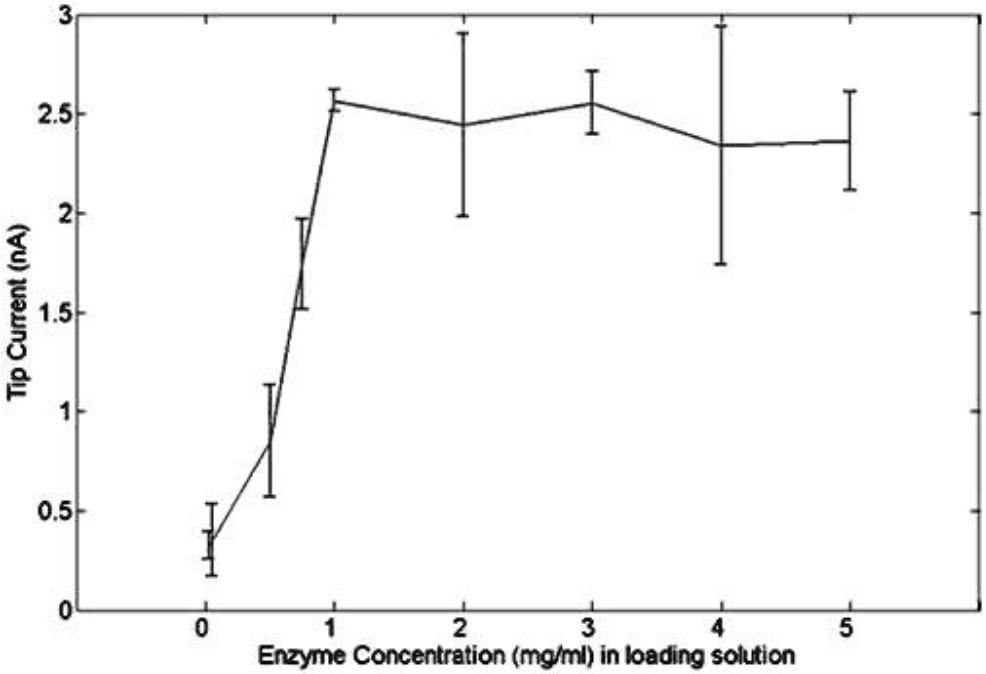

Tip Current (nA)
Enzyme Concentration (mg/ml) in loading solution
0
0.5
1
1.5
2
2.5
3
0
1
2
3
4
5

Figure 4.

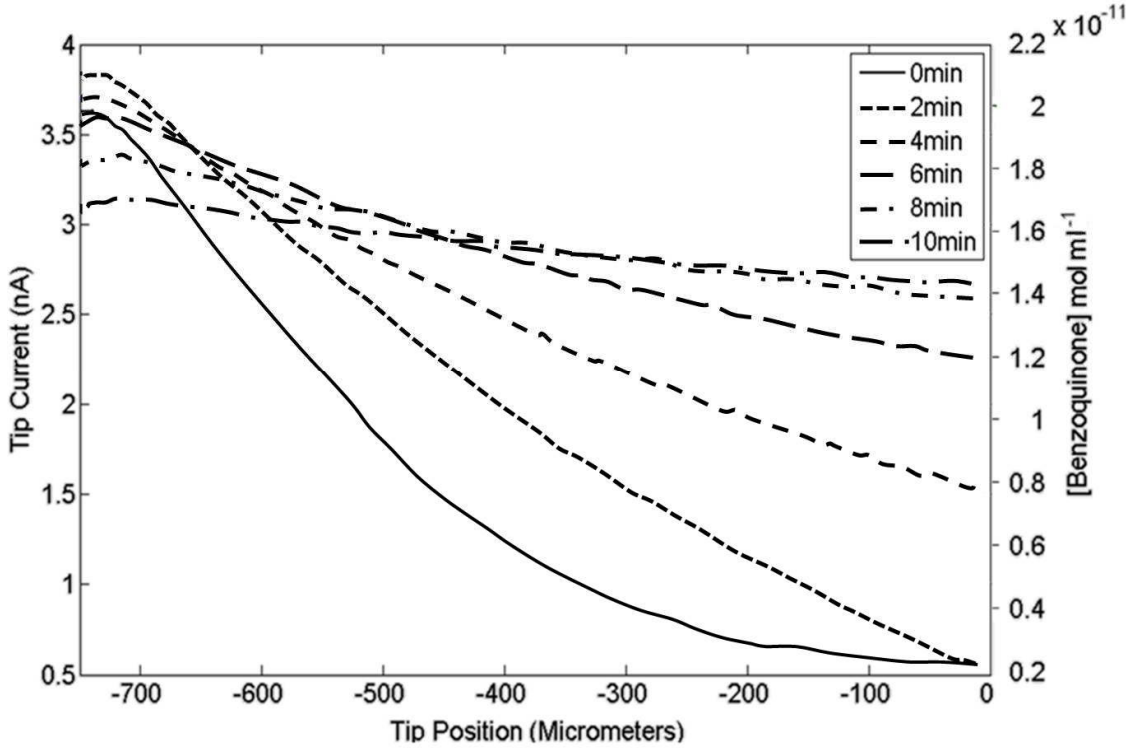

0min
2min
4min
6min
8min
10min
Tip Current (nA)
[Benzoquinone] mol ml-1
x 10-11
Tip Position (Micrometers)
4
3.5
3
2.5
2
1.5
1
0.5
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
-700
-600
-500
-400
-300
-200
-100
0

Figure 5.

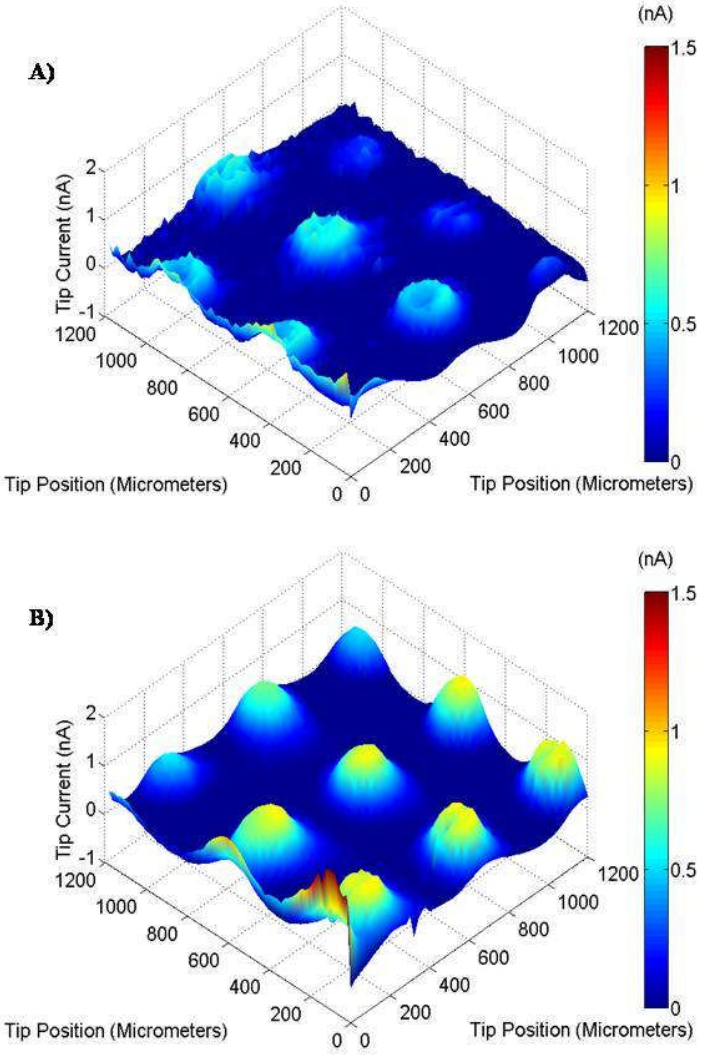
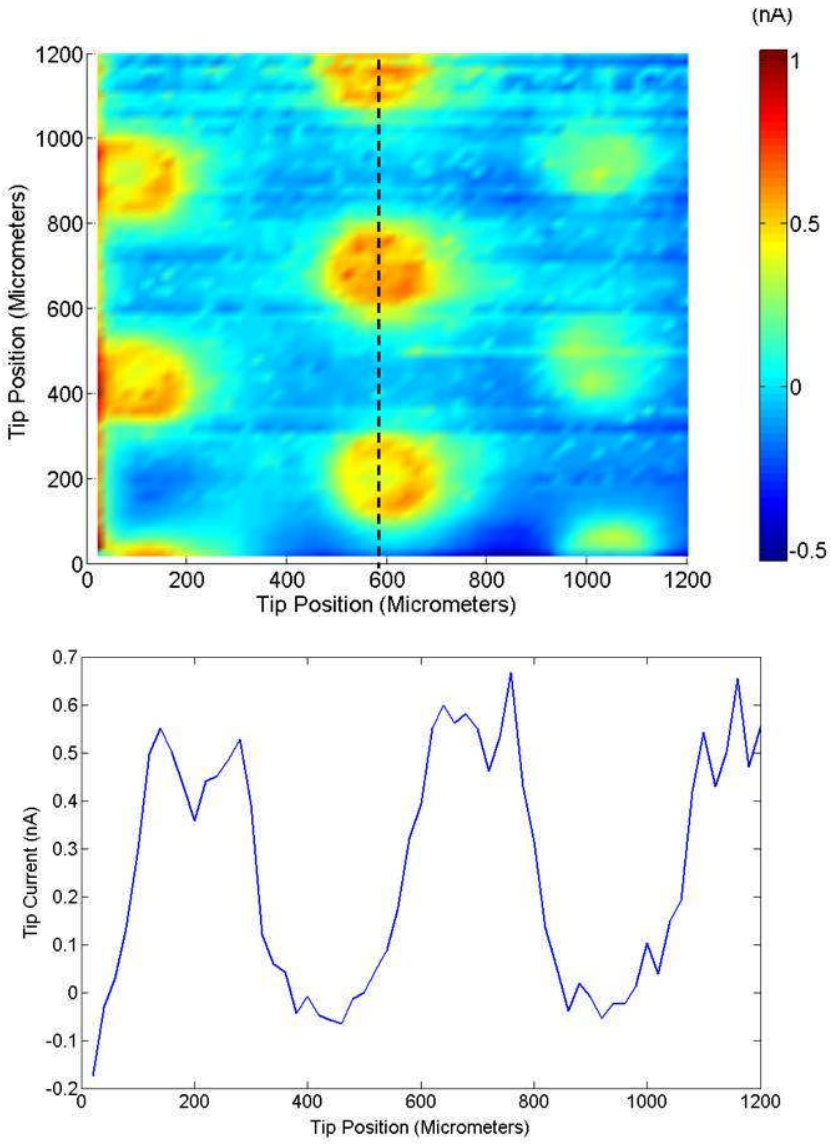

A)
(nA)
1.5
1
0.5
0
Tip Current (nA)
2
1
0
-1
1200
1000
800
600
400
200
0
Tip Position (Micrometers)
Tip Position (Micrometers)
B)
(nA)
1.5
1
0.5
0
Tip Current (nA)
2
1
0
-1
1200
1000
800
600
400
200
0
Tip Position (Micrometers)
Tip Position (Micrometers)

Figure 6.


(nA)
1
0.5
0
-0.5
Tip Position (Micrometers)
Tip Position (Micrometers)
Tip Current (nA)
Tip Position (Micrometers)

Construction and interrogation of enzyme microarrays using scanning electrochemical microscopy - optimisation of adsorption and determination of enzymatic activity.

Roberts, William St John

2011-12-21T00:00:00Z

Roberts WS, Davis F, Collyer SD, Higson SP. (2011) Construction and interrogation of enzyme microarrays using scanning electrochemical microscopy - optimisation of adsorption and determination of enzymatic activity. Analyst. 2011 Volume 136, Issue 24, pp. 5287-5293. http://dx.doi.org/10.1039/c1an15589j