Scanning Electrochemical Microscopy. 44. Imaging of Horseradish Peroxidase Immobilized on Insulating Substrates

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Scanning electrochemical microscopy (SECM) was used to study horseradish peroxidase (HRP) immobilized with copolymer on insulating substrates (glass slide or polycarbonate membrane filter). Two methods were used to immobilize HRP: In the first, HRP was coimmobilized by cross-linking on a glass slide with a copolymer swelled in water to form a hydrogel; in the second, the same copolymer and avidin were coimmobilized on the glass slide and biotin-labeled HRP was conjugated to the avidin of the film. SECM was then used to detect the presence of the bound enzyme by observing the feedback current in a solution of benzoquinone and hydrogen peroxide, when hydroquinone was generated at the tip. A detection limit less than 7×10^5 HRP molecules within a $\sim$7-μm-diameter area was demonstrated.

Biosensors are widely used and methods of characterizing these are of interest. In many biosensors, an enzyme is used to produce a signal that indicates the presence of an analyte and correlates with its concentration. The method of attachment of the enzyme to the substrate in the sensor, the stability of the attachment, and communication between the immobilized enzyme and the substrate, e.g., in an electrochemical system, are important issues. Attachment techniques used include covalent linkage of the enzyme to a functionalized electrode surface,[1] noncovalent coupling of enzymes to solid electrodes via affinity binding,[2] and coupling by electrostatic, hydrophobic, and hydrophilic interactions.[3] Covalent linkage via cross-linking with polymers has the advantage of strongly binding the enzyme to the electrode surface, but the enzyme may denature whether cross-linking is excessive.[4] Electrostatic, hydrophobic, and hydrophilic attachment of enzymes to an electrode surface is simple, but the attachment may not be sufficiently strong to securely hold the enzyme, thus resulting in short sensor lifetimes. The affinity interaction between avidin and biotin shows a very high binding constant of 10^{15} M^{-1} having the advantage of strong interaction under mild binding conditions.[5] Additionally, since each avidin can bind up to four biotins, the density of binding sites can be high. Oxidized horseradish peroxidase (HRP) is one of the few enzymes that can be directly electroreduced on electrodes. Electroreduction requires, however, proper orientation of the enzyme. Because immobilization prevents the tumbling of the enzyme, only a small fraction of the directly adsorbed enzyme is electroreduced. When the enzyme is coimmobilized with a redox polymer, this fraction is further reduced and can be negligibly small. Diffusing mediators dramatically improve the communication between HRP and electrodes.[6] Communication is also improved when tethered electron relays are attached to the enzyme[7] and when the enzyme is coimmobilized with a redox polymer and electrons transfer via polymer-bound redox relays.[8−10]

Scanning electrochemical microscopy (SECM) is a powerful electrochemical technique and has been applied extensively during the past decade.[11−15] It is a versatile technique for extracting electron-transfer kinetics information,[16−23] micropatterning,[24−29] and studying biological systems.[30−42] The two unique features of

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the SECM study of this paper are that a bare tip is used rather than an enzyme-modified tip and that the enzyme probed is immobilized on an insulating substrate. These features enable the investigation of the electrochemical response when the enzyme-catalyzed reaction is mediated by a diffusing redox couple and can indicate when the distance between enzyme and conductive substrate exceeds the distance across which electrons can be transferred. In this paper, we demonstrate the use of SECM to chemically image immobilized zones of enzyme sites and to estimate the number of enzymes within a site.

EXPERIMENTAL SECTION

Materials. Hydrogen peroxide, 30% solution, was from EM Science (Catalog No. HX0635-1; Gibbstown, NJ). 1,4-Benzoquinone (BQ) (Catalog No. B1,035-8) and potassium ferricyanide (Catalog No. 20,402-3) were from Aldrich (Milwaukee, WI). Horseradish peroxidase (Catalog No. P-6782), biotinamidocaproyl-labeled horseradish peroxidase (B:HRP, Catalog No. P-9568), and avidin (Catalog No. A-9390) were purchased from Sigma (St. Louis, MO). The copolymer of acrylamide and 1-vinylimide (PAA−PVI) was prepared as previously described.[9] Poly(ethylene glycol) (400) diglycidyl ether (PEG; Catalog No. 08210) was purchased from Polysciences (Warrington, PA). The nonlabeled HRP was dissolved in 30 mM phosphate buffer (pH 7). The labeled HRP was dissolved in 0.1 M NaCl, 30 mM Na_2HPO_4 (pH 7.4). All solutions were prepared with deionized water (Milli-Q, Millipore Corp.). The polycarbonate (PC) membrane filter (14-μm-diameter pore size) was from Osmonics (Livermore, CA).

Tip and Substrates. The SECM tips were carbon fiber (CF; 7-μm diameter) and Pt (10-μm diameter) microelectrodes. The CF microelectrode was prepared as described earlier.[13,43] The Pt microelectrode was from CH Instruments (Austin, TX).

HRP Immobilization. (A) Equal volumes of HRP (5 mg/mL), PAA−PVI (5 mg/mL), and PEG (0.21 mg/mL) were mixed. A 20-μL drop of this mixture was then placed on a 2.5 by 2.5 cm^2 glass microscope slide to form the HRP/hydrogel film. The total HRP added was 3.3×10^{-5} g or 7.6×10^{-10} mol. Before the SECM experiment, the sample was soaked in eight drops of pH 7 phosphate buffer solution (PBS) for 10 min.

(B) Thiol-avidin was prepared by treating avidin with *N*-succinimidyl *S*-acetylthioacetate and deblocking with hydroxylamine.[2] It was coimmobilized by mixing *x* μg of the thiol-avidin (where *x* is 9, 4.5, 2.25, 0.9), PAA−PVI (73.5 μg), and PEG (0.3 μg). The mixture (20 μL) was dropped on a glass microscope slide and dried for 2 days to form an ~8-mm-diameter circle. Before the SECM experiment the film was soaked in pH 7.4 PBS for 10 min and then in eight drops of biotin-labeled HRP in pH 7.4 PBS (1 mg/mL) for 30 min. The slide was washed with 1 mL of pH 7.4 PBS before the SECM experiment.

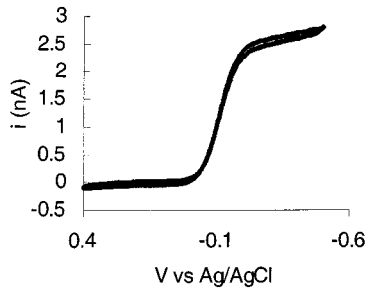

Figure 1. Cyclic voltammogram of 1 mM BQ and 1 mM H_2O_2 in 30 mM pH 7 phosphate buffer. Working electrode, 7-μm-diameter carbon fiber ultramicroelectrode. Reference electrode, saturated Ag/AgCl. Auxiliary electrode, Au.

Electrochemistry. The SECM images, approach curves, and cyclic voltammograms were measured with a model CHI 900 SECM (CH Instruments, Austin, TX). The SECM instrument basically consists of two parts: piezoinchworms, a stage, a controller that can move the tip in three dimensions, and a bipotentiostat. In recording approach curves (tip current vs tip−substrate distance), the approach speed was 3 μm/s. A saturated Ag/AgCl reference electrode and an Au auxiliary electrode were used in all experiments. Images shown are *x*,*y*-scans with *x* and *y* in units of micrometers, with the indicated currents in units of amperes. In these images, yellow represents higher, and green, lower current. The substrate was the HRP/hydrogel film on a glass slide; the polycarbonate filter membrane; the polycarbonate filter membrane whose pores were filled with the HRP/hydrogel, or the avidin-containing hydrogel on a glass microscope slide, conjugated with biotin-labeled HRP.

RESULTS AND DISCUSSION

SECM allows one to locate inactive or insulating zones and differentiate them from active, enzyme-containing zones. SECM can also provide information about the substrate and attachment-mode dependence of the electrochemical reaction kinetics.

Catalytically Regenerated Positive Feedback on HRP/Hydrogel. Before imaging the HRP/hydrogel on the membranes, CV and approach curves for the BQ/hydroquinone (HQ) couple were obtained. Figure 1 shows the voltammogram of 1 mM BQ and 1 mM H_2O_2 in phosphate buffer with a 7-μm CF microelectrode. The voltammogram shows the reduction of BQ to HQ; BQ/HQ $E_{1/2} = -0.12$ V versus Ag/AgCl. The voltammograms with and without H_2O_2 were essentially the same, establishing that the rate of reaction of H_2O_2 with HQ, although thermodynamically favorable, is negligibly slow in the absence of an appropriate catalyst, such as HRP. A steady-state reduction current, $i_{T,\infty}$, was observed at potentials negative of about −0.3 V, where BQ is reduced to HQ.

SECM approach curves, where the tip approaches a substrate, were then taken. In SECM nomenclature, the term "negative feedback" implies that the current drops, and is always smaller than $i_{T,\infty}$, as the tip approaches the substrate because the substrate blocks diffusion to the tip. "Positive feedback" is shown when the

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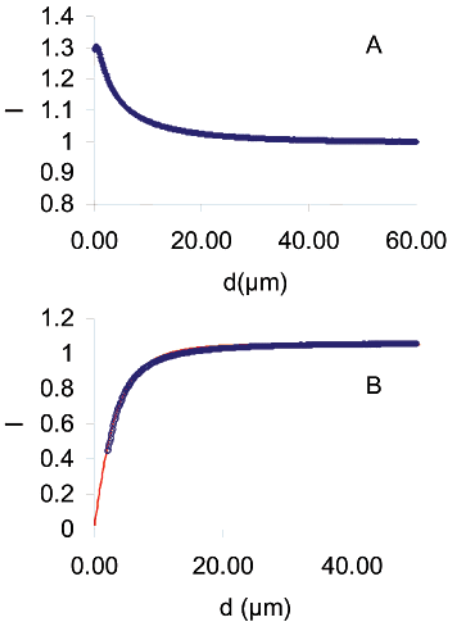

Figure 2. Approach curves. Solution, 1 mM BQ and 1 mM H_2O_2 in 30 mM pH 7 phosphate buffer. Working electrode, 7-μm-diameter carbon fiber ultramicroelectrode. Reference electrode, saturated Ag/AgCl. Auxiliary electrode, Au. Substrates: (a) HRP/hydrogel on glass microscope slide; (b) glass microscope slide. Thin solid line is the theoretical curve for negative feedback over an insulator.

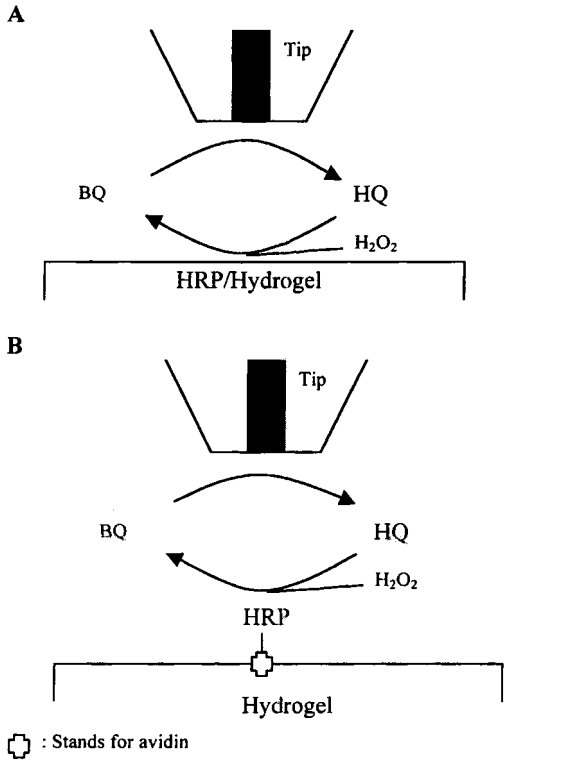

Figure 3. Scheme of the BQ/HQ reactions at (A) the tip and the HRP/hydrogel and (B) the tip and HRP-biotin/avidin/hydrogel.

current increases, and is always larger than $i_{T,\infty}$, because the reactant is regenerated at the substrate. Figure 2A shows the approach curve to the HRP/hydrogel on a glass slide in the same solution, for a CF microelectrode tip held at −0.4 V versus Ag/AgCl. Positive feedback was observed for the HRP-modified glass slide, because HRP catalyzed the reaction of HQ with H_2O_2 (Figure 3A). BQ was reduced to HQ at the tip, and HQ was oxidized to BQ by H_2O_2 in a reaction catalyzed by HRP, on the substrate. To confirm that the catalytically regenerated positive feedback was produced by the HRP-catalyzed H_2O_2 oxidation of HQ, approach curves to a position on the glass substrate away from the HRP/hydrogel spot and to a hydrogel spot without HRP, where the catalyzed oxidation of HQ could not occur, were recorded. Both showed, as expected, negative feedback behavior typical of insulators. (Figure 2B). These experiments demonstrated that the catalytically regenerated positive feedback was produced only by the HRP-catalyzed HQ oxidation.

SECM Imaging of the Polycarbonate Filter Membrane. To demonstrate the imaging capability of the SECM, a 6-μm-thick polycarbonate filter membrane substrate was imaged. The nominally 14-μm pores were filled with solution. When a 10-μm Pt tip (−0.4 V vs Ag/AgCl) electrode approached the membrane, the tip current decreased. The tip approach was stopped when the current had decreased to 15% of $i_{T,\infty}$, indicating that the tip was ~0.25 tip radii, or ~1 μm, away from the surface. An x,y-image was then obtained, Figure 4. The tip current was higher above the pores than above the surrounding polycarbonate because the tip is a larger distance at these locations from the insulator (polycarbonate) surface.

SECM Images of the Polycarbonate Membrane/HRP/Hydrogel. To image zones loaded with the HRP catalyst, the pores of the polycarbonate membrane filter were filled with the HRP/hydrogel. Figure 5 shows the SECM images of a polycarbonate filter membrane with HRP/hydrogel-filled pores obtained with a CF microelectrode tip held at −0.4 V versus Ag/AgCl to reduce BQ to HQ. Before this $x-y$ scan, the tip was moved in a vertical (z) direction over an insulating portion of the membrane to a position where the tip current was 75% of the steady-state tip current. The separation between the tip and the membrane was calculated to be 6.2 μm, based on the equation for SECM at insulating substrate.[13] Figure 5 reveals the following: first, from the positive feedback observed above the surface of the HRP/hydrogel filled into the pores of the membrane, the separation

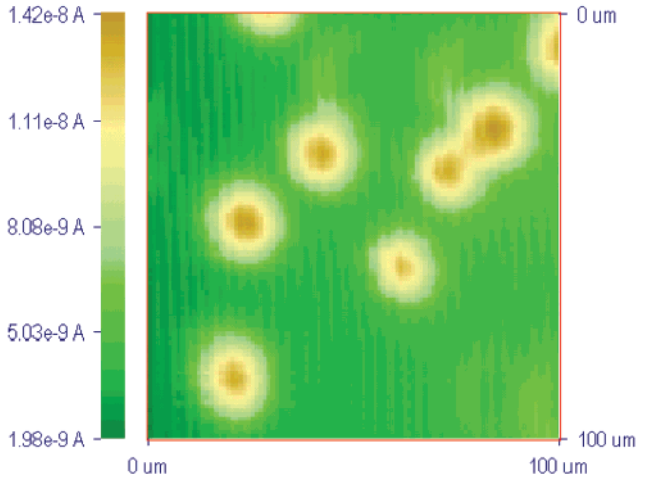

Figure 4. SECM image of the polycarbonate filter membrane supported by an electrode. Solution: 10 mM ferricyanide, 0.1 M KCl. Counter electrode: Pt wire. Tip potential: −0.4 V vs Ag/AgCl. Tip: 10-μm-diameter Pt ultramicroelectrode. $i_{T,\infty}$ −14.2 nA. Separation was close to 1 μm.

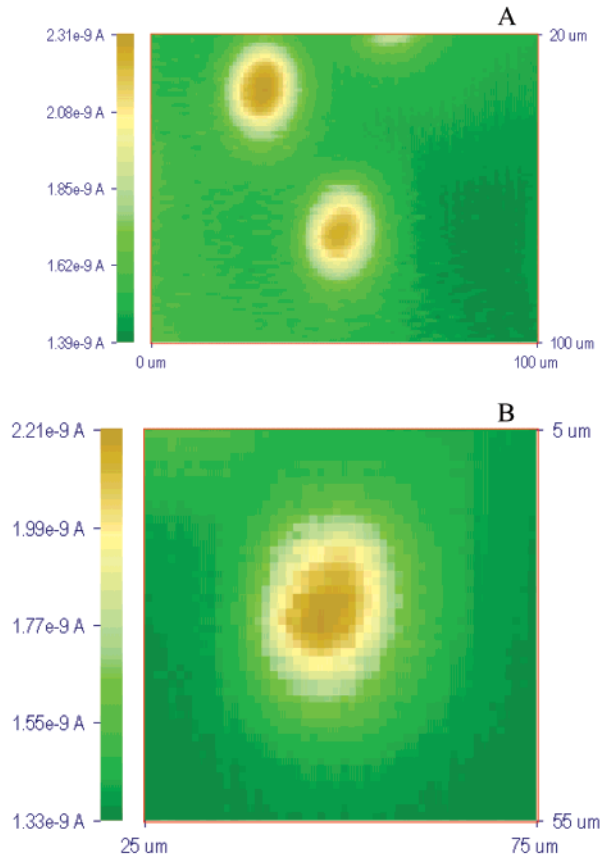

Figure 5. SECM images of a polycarbonate filter membrane with HRP/hydrogel-filled pores. Solution: 1 mM BQ, 1 mM H_2O_2 in 30 mM pH 7 phosphate buffer. Working electrode: 7-μm-diameter carbon fiber ultramicroelectrode. Reference electrode: saturated Ag/AgCl. Auxiliary electrode: Au. $i_{T,\infty} = 2.5$ nA.

between the tip and the HRP/hydrogel was fitted as 5 μm. The thickness of the PC membrane was 6 μm, and the tip was ~6.2 μm from the insulating polycarbonate portion of the membrane. This implies that the HRP/hydrogel in the pores protruded above the PC membrane by ~1 μm. The images of the pores had centers with a higher current than the surrounding rings. The high current centers had 12−15-μm diameters, close to the nominal diameter of the pores. This high center current was produced by the positive feedback over HRP/hydrogel as shown in Figure 3A. The rings were 2−5 μm wide, slightly less than the diameter of the CF tip. The current of the rings corresponded to the transition from the pore center where catalytically regenerated positive feedback, due to HRP-catalyzed hydrogen peroxide oxidation of HQ to the PC, occurs to the catalytically inactive region and represents the resolution limits of the image as governed by the tip size. The characteristics of the HRP/hydrogel-filled pores of Figure 5, where catalytically regenerated positive feedback is observed ($i_T > i_{T,\infty}$) differed from those of the empty pores of Figure 4, where the feedback was not significant due to combined larger separation and diffusion blocking by pore walls ($i_T \Leftarrow i_{T,\infty}$).

SECM Detection of Biotin-Labeled HRP Bound to Immobilized Avidin. The biotin/avidin affinity reaction also allowed

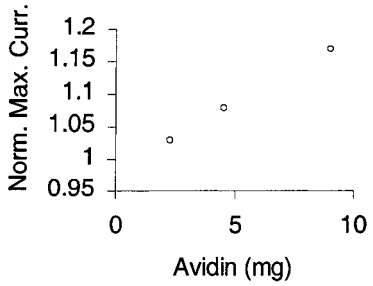

Figure 6. Dependence of the normalized maximum approach currents and the avidin loadings in an 8-mm-diameter hydrogel spot on a glass substrate.

immobilization of HRP on a glass slide. The immobilization and the reactions are shown schematically in Figure 3B. The approach curve for the avidin−biotinylated-HRP was similar to that for the cross-linked-immobilized HRP of Figure 2A. Figure 6 shows the linear dependence of the maximum normalized feedback current on the amount of avidin in the 8-mm-diameter hydrogel circle on the slide. The *y*-axis of the figure is the ratio of the maximum current and the $i_{T,\infty}$ of the approach curves. Measuring this ratio, rather than the current itself, minimizes the effect of differences in limiting currents. The HRP concentration can, however, be calculated from the maximum current. This figure also shows that the tip current at these HRP concentrations was smaller than the diffusion-controlled value and was governed by the amount of HRP. If this were not the case, a tip current independent of the avidin concentration would result. When the separation between the tip and the substrate was very small, then, at maximum tip current the tip only addressed the ~7-μm-diameter area of the spot beneath the tip. From the maximum tip current, the surface HRP concentration was estimated as follows. The reported maximum turnover number of an HRP molecule is 756/s.[44] Because two electrons are transferred, this corresponds to a current of 2.4×10^{-16} A/HRP molecule. At the lowest avidin concentration in the film, the maximum feedback current was 0.17 nA (1.7×10^{-10} A) (Figure 6). Thus, ~7×10^5 HRP molecules were detected within the ~7-μm-diameter area probed, in agreement with the value measured by electrically contacting HRP labels of DNA with a redox hydrogel on the tip of a 7-μm carbon fiber electrode by hybridization.[45]

CONCLUSIONS

Positive feedback from the HRP-catalyzed oxidation of HQ, produced by reduction of BQ on a CF tip ultramicroelectrode was observed by SECM. SECM images of pores of a polycarbonate membrane filter filled with HRP/hydrogel showed positive feedback only above the pores. Positive feedback was also observed for biotinylated-HRP conjugated to avidin immobilized in a hydrogel on a glass slide. The current produced by fewer than 7×10^5 HRP molecules in a 7-μm-diameter circular area was detected.

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