# Study of Specific Binding of Maltose Binding Protein to Pyrrole-Derived Bipyridinium Film by Quartz Crystal Microbalance

Kazutake Takada,[†] Zeki Naal,[†] Joo-Heon Park,[‡] James P. Shapleigh,[§] Stefan Bernhard,[†] Carl A. Batt,[‡] and Héctor D. Abruña*,[†]

*Department of Chemistry and Chemical Biology, Baker Laboratory, Department of Food Science, Stocking Hall, and Department of Microbiology, Wing Hall, Cornell University, Ithaca, New York 14853*

*Received November 19, 2001. In Final Form: March 22, 2002*

The affinity of the maltose binding protein−nitro reductase fusion (MBP−NR) to electropolymerized films of *N*-(3-pyrrol-1-ylpropyl)-4,4′-bipyridinium (PPB) has been studied in aqueous medium by the quartz crystal microbalance (QCM) technique. It was found that the MBP domain of MBP−NR exhibits specific binding toward PPB films through the maltose binding site and that the immobilized MBP−NR retains its enzymatic activity toward trinitrotoluene (TNT) reduction. Although some MBP−NR was nonspecifically adsorbed onto a maltose-covered PPB film as well as to a bare Au electrode, in both cases the adsorbed enzyme exhibited no catalytic activity. Nitro reductase (NR) also appeared to adsorb onto the PPB films but did not exhibit any enzymatic activity. The electropolymerization of *N*-(3-pyrrol-1-ylpropyl)-4,4′-bipyridinium (PPB) onto a Au electrode in acetonitrile solution was studied by electrochemical quartz crystal microbalance (EQCM). It was found that the extent of polymerization upon pyrrole-centered oxidation decreased with continuous potential scanning. The bipyridinium-localized (quaternized nitrogen) redox reaction appeared to be of the anion-exchange type.

## Introduction

The study of the electrochemical behavior of redox enzymes has received a great deal of attention because of the interest in electron transport in biological systems and the desire to construct practical enzyme electrodes for biosensor applications.[1,2] One of the problems attendant to the use of enzymes for biosensor applications is that direct electron transfer between the redox sites and an electrode surface is often quite slow. This is due to the fact that the redox centers of enzymes are generally located well within the protein body, which insulates them from the electrode surface. Thus, redox mediators have been used to establish electrical communication between enzymes and an electrode.[3−5] It is also well-known that redox enzymes often lose their enzymatic activity when they are immobilized on an electrode surface. This is due to the fact that an enzyme's activity is strongly dependent on its structure and immobilization almost always results in changes in it. Therefore, there has been a great deal of interest to new enzyme immobilization methodologies that allow for maximal retention of activity.[6−10] One of the more recent strategies has been to introduce functional groups into biological molecules that allow the oriented immobilization of a protein onto a chemically modified electrode surface.[11]

We have been interested in use of redox polymers to construct enzyme-modified electrodes, since redox polymers could serve both as a support anchor for the enzyme and as an electron mediator to shuttle electrons between the immobilized enzyme and the electrode. Among many enzymatic systems, we are particularly interested in denitrification processes and in the development of analytical methodologies using redox enzymes, since they are some of the most important processes in ecosystems. In this context, we have previously demonstrated that electropolymerized films of $[Cr(v\text{-}tpy)_2]^{3+}$ show high electrocatalytic activity toward the reduction of NO.[12] We also developed an amperometric biosensor for the determination of nitrite based on a maltose binding protein−nitrite reductase (MBP−Nir) fusion immobilized on a *N*-(3-pyrrol-1-ylpropyl)-4,4′-bipyridinium (PPB) modified electrode.[13] The advantage of utilizing PPB is that it can serve both as a support for the enzyme and as an electron transfer mediator by shuttling electrons between the enzyme and the electrode, ostensibly through the 4,4′-bipyridinium group.

More recently, we reported on an amperometric biosensor for the determination of nitro derivatives (with emphasis on TNT, trinitrotoluene) based on the immobilization of a maltose binding protein−nitro reductase (MBP−NR) fusion on a electrode previously modified with a film of PPB.[14] In that paper, we reported that the MBP domain of MBP−NR exhibits a high affinity toward PPB

[†] Department of Chemistry and Chemical Biology.
[‡] Department of Food Science.
[§] Department of Microbiology.

(1) Göpel, W.; Heiduschka, P. *Biosens. Bioelect.* **1995**, *10*, 853.
(2) Armstrong, F. A.; Wilson, G. S. *Electrochim. Acta* **2000**, *45*, 2623.
(3) Allen, P. M.; Hill, H. A. O.; Walton, N. J. *J. Electroanal. Chem. Interfacial Electrochem.* **1984**, *178*, 69.
(4) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285.
(5) Degani, Y.; Heller, A. *J. Am. Chem. Soc.* **1989**, *111*, 2357.
(6) Turner, A. P. F. *Methods Enzymol.* **1988**, *137*, 90.
(7) Riklin, A.; Katz, E.; Willner, I.; Stocker, A.; Buckmann, A. F. *Nature* **1995**, *376*, 672.
(8) Cosnier, S.; Innocent, C.; Jouanneau, Y. *Anal. Chem.* **1994**, *66*, 3198.
(9) Cosnier, S.; Galland, B.; Innocent, C. *J. Electroanal. Chem.* **1997**, *433*, 113.
(10) Ramsay, G.; Wolpert, S. M. *Anal. Chem.* **1999**, *71*, 504.
(11) Piervincenzi, R. T.; Reichert, W. M.; Hellinga, H. W. *Biosens. Bioelect.* **1998**, *13*, 305.
(12) Maskus, M.; Pariente, F.; Wu, Q.; Toffanin, A.; Shapleigh, J. P.; Abruña, H. D. *Anal. Chem.* **1996**, *68*, 3128.
(13) Wu, Q.; Storrier, G. D.; Pariente, F.; Wang, Y.; Shapleigh, J. P.; Abruña, H. D. *Anal. Chem.* **1997**, *69*, 4856.
(14) Naal, Z.; Park, J.-H.; Bernhard, S.; Shapleigh, J. P.; Batt, C. A.; Abruña, H. D. *Anal. Chem.* **2002**, *74*, 140.

10.1021/la0116950 CCC: $22.00 
Published on Web 05/15/2002

films and that the immobilized MBP−NR retains virtually all of its enzymatic activity. We also suggested that the specific affinity of MBP−NR to PPB films was through the nonquaternized pyridine nitrogen of PPB. In the present context, it is worth mentioning that Marvin et al.[15] have reported that *Escherichia coli* maltose binding protein binds through its maltose binding site.

In the present paper, we present electrochemical and quartz crystal microbalance (QCM) studies aimed at confirming the specific binding between MBP−NR and PPB film. We also describe an electrochemical quartz crystal microbalance (EQCM) study of the electropolymerization of PPB onto a Au electrode.

## Experimental Section

**Materials.** MBP−NR and nitro reductase (NR) were overexpressed by the method reported previously.[14] *N*-(3-Pyrrol-1-ylpropyl)-4,4′-bipyridinium hexafluorophosphate (PPB) was prepared by modification of the syntheses of Carpio et al.[16] Water was purified with a Hydro purification system connected in series to a Millipore Milli-Q system. Acetonitrile (AN) was purchased from Burdick and Jackson (distilled in glass) and dried over 4 Å molecular sieves for 72 h. Tetra-*n*-butylammonium hexafluorophosphate (TBAH; G. F. Smith) was recrystallized three times from ethyl acetate and dried under vacuum for 72 h. All other reagents were of at least reagent-grade quality and were used without further purification.

**Instrumentation.** AT-cut quartz crystals (5 MHz) of 24.5 mm diameter with Au electrodes deposited over a Ti adhesion layer (Maxtek Co.) were used for QCM measurements. An asymmetric keyhole electrode arrangement was used, in which the circular electrodes' geometrical areas were 1.370 $cm^2$ (front side) and 0.317 $cm^2$ (back side). The quartz crystal resonator was set in a probe (TPS-550, Maxtek) made of Teflon, in which the oscillator circuit was included. One of the electrodes of the quartz crystal resonator, in contact with the solution, was also used as the working electrode in the electrochemical experiments. The potential of the working electrode was controlled with a potentiostat (CV-27, BAS). A sodium chloride-saturated Ag/AgCl and a coiled Pt wire were used as reference electrode and counterelectrode, respectively, useless otherwise noted. The frequency response, measured with a plating monitor (PM-740, Maxtek), and the current, measured with the potentiostat, were simultaneously recorded by a personal computer, which was interfaced to the above instruments through LabVIEW (National Instruments). In the experiments in which we monitored the binding process of the enzymes, a water-jacketed beaker (which served as the cell) connected to a thermostated bath (digital temperature controller 9101, Fisher Scientific) was used. The changes in admittance of the quartz crystal resonator were measured by an impedance analyzer (HP4194A, Hewlett-Packard) equipped with a test lead (HP16048A). The utility of admittance measurements for characterizing the viscoelastic properties of films has been previously documented.[17]

**Preparation of PPB Films on Electrodes.** PPB films were electropolymerized onto quartz crystal resonators by scanning the potential between −0.40 and +1.60 V vs Ag/AgCl (five scans) in a thoroughly degassed 0.10 M TBAH/AN solution containing 2.0 mM PPB. The coated electrodes were rinsed thoroughly with pure AN and dried with nitrogen gas. The electrodes were transferred to a 0.10 M phosphate buffer solution (pH 7.5). The surface coverage of the polymer film was determined, after the solution was degassed with nitrogen gas for 30 min, from the charge passed during the reduction reaction of the cyclic voltammogram obtained at 10 mV $s^{-1}$, where the peak current was directly proportional to the potential scan rate.

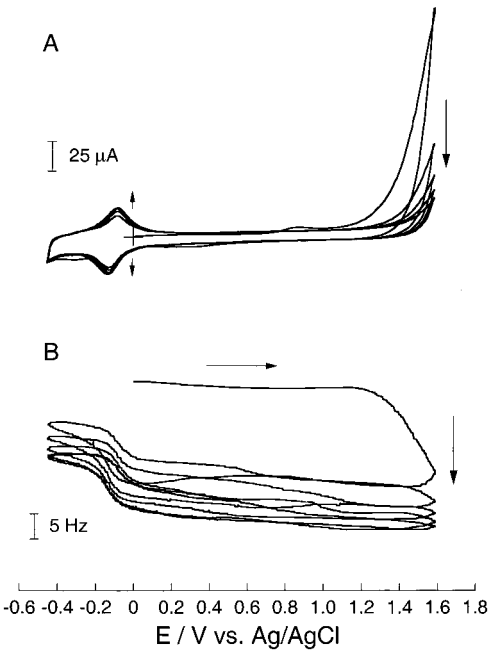


**Figure 1.** (A) Current (CV) and (B) frequency responses as a function of applied potential at 50 mV $s^{-1}$ for a Au EQCM electrode in contact with a 0.10 M TBAH/AN solution containing 2.0 mM PPB.

**Monitoring Enzyme Binding with the QCM.** After the surface coverage of the PPB film was determined, the frequency of the modified quartz crystal resonator was monitored in a 0.10 M phosphate buffer solution (pH 7.5), which was stirred with a Teflon-coated magnetic stirrer bar and thermostated at 25.0 ± 0.1 °C. We have previously reported that the stirrer does not affect the frequency stability, which was maintained to within ±1.7 Hz in water for 10 h after the establishment of a constant temperature (25.0 ± 0.1 °C).[18] Once the frequency had reached a steady state, an enzyme or maltose dissolved in water was injected into the electrolyte solution with a gastight syringe. The volume injected varied according to the desired final concentration of the enzyme or maltose. Although the injection of the solution sometimes caused a sudden frequency change, the frequency rapidly returned to its original value within a few minutes. Enzymatic activity was measured electrochemically following the method mentioned previously.[14]

## Results and Discussion

**Modification of a Au QCM Electrode with PPB.** Figure 1 shows (A) the typical current (cyclic voltammogram, CV) and (B) frequency response as a function of applied potential for a Au QCM electrode in contact with a 0.10 M TBAH/AN solution containing 2.0 mM PPB. The first anodic scan showed an irreversible oxidation at ca. +1.50 V vs Ag/AgCl, which we ascribe to a pyrrole-localized process. The amplitude decreased upon continuous scanning, and this might be due to the formation of a polypyrrole film, which blocks mass and electron transfer to the electrode surface. On the subsequent cathodic scan there was the appearance of a reversible peak with a formal potential $E^{\circ\prime}$ of −0.11 V, which we ascribe to a bipyridinium-localized process (quaternized nitrogen) on the electrode surface, analogous to a viologen. This peak increased in amplitude upon consecutive scanning, indicating the accumulation of an electroactive film on the electrode surface.

(15) Marvin, J. S.; Corcoran, E. E.; Hattangadi, N. A.; Zhang, J. V.; Gere, S. A.; Hellinga, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4366.

(16) Carpio, H.; Galeazzi, E.; Greenhouse, R.; Guzman, A.; Velarde, E.; Antonio, Y.; Franco, F.; Leon, A.; Perez, V.; Salas, R.; Valdes, D.; Ackrell, J.; Cho, D.; Gallegra, P.; Halpern, O.; Koehler, R.; Maddox, M. L.; Muchowski, J. M.; Prince, A.; Tegg, D.; Thurber, T. C.; Vanhorn, A. R.; Wren, D. *Can. J. Chem.* **1982**, *60*, 2295.

(17) Takada, K.; Storrier, G. D.; Pariente, F.; Abruña, H. D. *J. Phys. Chem. B* **1998**, *102*, 1387.

(18) Takada, K.; Abruña, H. D. *J. Phys. Chem.* **1996**, *100*, 17909.

An overall decrease in frequency upon continuous potential scanning also demonstrated that polymerization of the PPB was taking place on the electrode surface (Figure 1B). It is also apparent from the figure that the frequency decreased most significantly in the first oxidative sweep. The magnitude of the decrease in the frequency upon pyrrole-centered oxidation gradually became smaller with continuous potential scanning. These results are also in good agreement with the changes in the anodic peak current seen in Figure 1A. The changes in frequency upon bipyridinium-localized (quaternized nitrogen) redox reaction appeared to be of the anion-exchange type. That is, the frequency increased during the reductive process, indicating the expulsion of anion from the film, whereas the frequency decreased during the oxidative process, indicating the incorporation of anion, as well as possible changes in solvation, to compensate the changes in charge generated by the redox reactions. The fact that the bipyridinium-localized redox process appeared to be the anion-exchange type is reasonable since the polymer is cationically charged, making the anions the mobile species.

Admittance measurements of the quartz crystal resonator were also carried out to ascertain if the decrease in frequency was solely related to changes in mass. Theoretical aspects of admittance measurements (resistance parameter) have been described previously.[19] When the potential was scanned between −0.40 and +1.60 V vs Ag/AgCl with the all other experimental conditions being the same as in Figure 1, the resistance parameter remained essentially constant within experimental error, even upon pyrrole-based oxidation where polymerization takes place. This indicates that the PPB film on the electrode is relatively thin and/or rigid, so that the film properties such as viscoelasticity and roughness remained virtually constant. The electrochemically determined surface coverage of the film was $1.2 \times 10^{-9}$ mol cm$^{-2}$, which represents approximately four equivalent monolayers (vide infra).[13] On the basis of this result and by using the Sauerbrey equation, with 17.7 ng cm$^{-2}$ Hz$^{-1}$ as a proportionality constant, the increase in mass during polymerization was calculated, from changes in the frequency ($\Delta F = 27.8$ Hz), to be 492 ng cm$^{-2}$.[20] This value, in turn, corresponds to a surface coverage of $1.2 \times 10^{-9}$ mol cm$^{-2}$, which is in excellent accordance with that obtained by integration of the charge as mentioned above. It should be noted that this result also suggests that the polymer film on the electrode does not incorporate solvent.

When the electrode was transferred to a 0.10 M phosphate buffer aqueous solution (pH 7.5), the CV response exhibited a redox process at −0.34 V vs Ag/AgCl, corresponding to a bipyridinium-localized process (quaternized nitrogen) (Figure 2A). This value is negatively shifted by ca. 200 mV from that obtained in acetonitrile. This potential shift might reflect differences in solvation between aqueous and nonaqueous solvents. The surface coverage was determined to be $1.2 \times 10^{-9}$ mol cm$^{-2}$ from integration of the cyclic voltammetric peaks at 50 mV s$^{-1}$, where the peak current was directly proportional to the scan rate. It should be noted that the peak potential separation was not zero as would be anticipated for a surface-confined redox process. This might be due, at least in part, to IR drop effects as the electrode used was relatively large (1.370 cm$^2$), rather than to slow electron-transfer kinetics. When a smaller Au (or glassy carbon) electrode was used, sharp and symmetric peaks were observed, indicating fast electron-transfer kinetics.

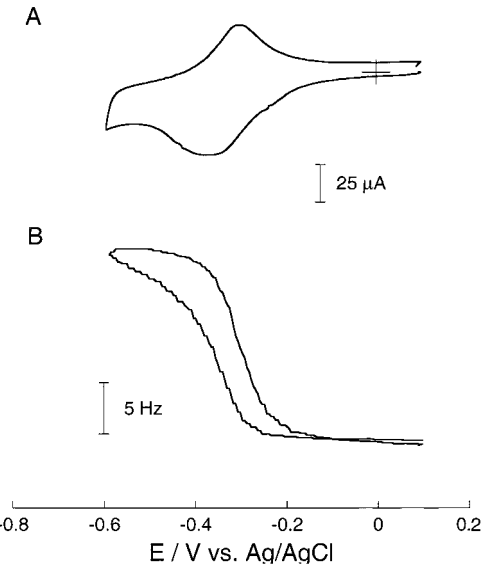


**Figure 2.** (A) Current (CV) and (B) frequency responses as a function of applied potential at 50 mV s$^{-1}$ for a PPB-coated Au EQCM electrode in contact with a 0.10 M phosphate buffer solution (pH 7.5).

Figure 2B shows the frequency response obtained concurrently with the CV. The frequency profile again appeared to be of the anion-exchange type. Qualitatively, similar results have been previously reported in aqueous solution for gold electrodes modified with self-assembling viologen derivatives with a thiol group.[21] At pH 7.5 the predominant anionic species in phosphate buffer solution is $HPO_4^{2-}$. Therefore, the changes in frequency would be primarily due to the movement of $HPO_4^{2-}$ anions across the film/solution interface. The mass transfer equivalent, $M_{eq}$ (grams per mole), which provides an indication of the change in mass per redox (bipyridinium) site of the film during the redox reaction, was calculated to be 267 g mol$^{-1}$ by dividing the change in mass (0.33 $\mu$g cm$^{-2}$) by the bipyridinium surface coverage ($1.2 \times 10^{-9}$ mol cm$^{-2}$). The changes in mass were determined from the changes in frequency (18.8 Hz) of the quartz crystal resonator by use of the Sauerbrey equation.[20] Since the formula weight of the $HPO_4^{2-}$ ion is 96, the $M_{eq}$ value of 267 g mol$^{-1}$ indicates that each $HPO_4^{2-}$ ion is accompanied by about 10 water molecules. This value is estimated, again, on the basis of the assumption that the species compensating the charge in the film is the $HPO_4^{2-}$ anion.

It is also clear that whereas in the TBAH/AN solution there was little if any solvent incorporation by the film, in the aqueous phosphate buffer there was significant solvent incorporation (10 water molecules/$HPO_4^{2-}$ anion). These differences might be also be responsible, at least in part, for the shift in the formal potential and the kinetics of charge transfer/propagation.

**Monitoring Enzyme Binding with QCM.** To study the interaction between PPB and enzymes (MBP−NR and NR), changes in the frequency of a quartz crystal resonator modified with a PPB film were monitored upon exposure to the enzyme in solution. Measurements were carried out immediately after modification of the resonator with PPB. Curve A in Figure 3 shows the typical frequency changes after injection of an MBP−NR aliquot diluted to 50 $\mu$g mL$^{-1}$, which is equal to 0.8 $\mu$M, into a 0.10 M

(19) (a) Beck, R.; Pittermann, U.; Weil, K. G. *Ber. Bunsen-Ges. Phys. Chem.* **1988**, *92*, 1363. (b) Martin, S.; Granstaff, V. E.; Frye, G. *Anal. Chem.* **1991**, *63*, 2272. (c) Yang, M.; Thompson, M. *Anal. Chem.* **1993**, *65*, 1158. (d) Buttry, D. A.; Ward, M. D. *Chem. Rev.* **1992**, *92*, 1355.

(20) Sauerbrey, G. *Z. Phys.* **1959**, *155*, 206.

(21) Long, H. C. D.; Buttry, D. A. *Langmuir* **1990**, *6*, 1319.

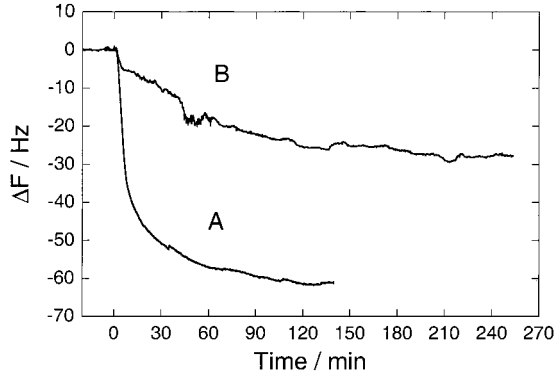


**Figure 3.** Frequency vs time plot for (A) PPB-coated and (B) bare Au QCM electrodes in contact with 50 $\mu$g mL$^{-1}$ (0.8 $\mu$M) MBP−NR in 0.10 M phosphate buffer solution (pH 7.5) at 25.0 ± 0.1 °C.

phosphate buffer solution (pH 7.5). It is apparent that the frequency decreased rapidly right after the injection, followed by a much more gradual decrease, after which a steady state was obtained.

To check for changes in film properties, such as viscoelasticity and roughness, following enzyme binding, admittance measurements of the quartz crystal resonator were carried out under the same experimental conditions as in Figure 3. The resistance parameter was found to remain essentially constant (ca. 323 Ω) for 150 min, during which time the binding of the enzyme to the surface was complete, as is clear from the QCM data (Figure 3A). Thus, this result indicates that the layer of bound MBP−NR, in a 0.10 M phosphate buffer solution, can be regarded as rigid and one whose film properties are essentially constant. Thus, frequency changes, as in Figure 3, can be directly related to changes in mass. We assume that this also holds for the other enzymes as well as maltose, used in these experiments, since their molecular sizes/masses are smaller than that of MBP−NR, which should not make the films thicker, more viscous, nor rougher. From these results, we conclude that the observed decreases in frequency correspond to increases in mass due to the binding of the enzyme onto the PPB-modified QCM resonator. The decrease in frequency (curve A in Figure 3) after the injection of MBP−NR was 61.2 Hz, which corresponds to surface coverage of $1.6 \times 10^{-11}$ mol cm$^{-2}$, assuming that the enzyme does not carry any solvent and/or ions. Since the surface coverage of the PPB film used in this experiment was $1.2 \times 10^{-9}$ mol cm$^{-2}$, the ratio of PPB to MBP−NR is 75.

As a control experiment, a bare Au resonator was also used. In this case, an MBP−NR-modified electrode was prepared by immersing, for 4 h, the Au electrode in 0.10 M phosphate buffer (pH 7.5) containing 50 $\mu$g mL$^{-1}$ MBP−NR. Curve B in Figure 3 presents the frequency changes of the resonator with the other experimental conditions the same as in Figure 3A. The magnitude of the changes in frequency appeared to be less than half of the one in which the resonator was modified with a PPB film. This points to the affinity of MBP−NR toward films of PPB. In addition, it should be mentioned that electrodes modified with MBP−NR only (ostensibly via nonspecific binding) exhibited no enzymatic activity. This is in contrast to electrodes where the binding of MBP−NR was to an electropolymerized film of PPB. In this case, a very high degree of catalytic activity (toward TNT, trinitrotoluene) was observed. These results are in accord with those of a previous study with electrodes modified with PPB/MBP−Nir (Nir = nitrite reductase).[13]

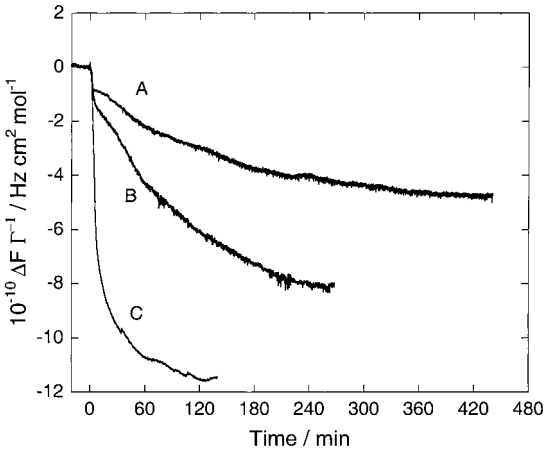


**Figure 4.** Frequency, normalized to the surface coverage of the PPB film, vs time plot for PPB-coated Au QCM electrodes in contact with (A) 13 $\mu$g mL$^{-1}$ (0.2 $\mu$M), (B) 25 $\mu$g mL$^{-1}$ (0.4 $\mu$M), and (C) 50 $\mu$g mL$^{-1}$ (0.8 $\mu$M) MBP−NR in 0.10 M phosphate buffer solution (pH 7.5) at 25.0 ± 0.1 °C.

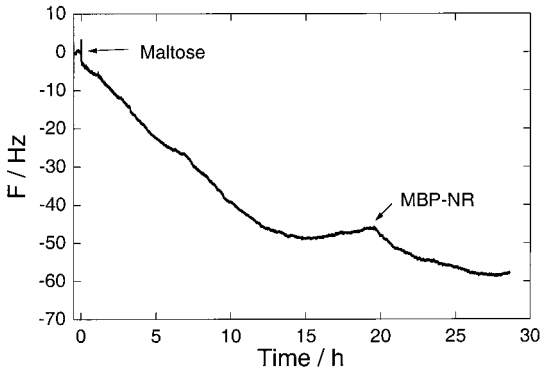


**Figure 5.** Changes in frequency of a PPB film-coated Au QCM electrode after injection of a maltose aliquot diluted to 0.5 mM into a 0.10 M phosphate buffer solution (pH 7.5) at 25.0 ± 0.1 °C, followed by another injection of a MBP−NR aliquot diluted to 0.4 $\mu$M.

The dependence of the changes in frequency on the concentration of MBP−NR were also studied. Figure 4 shows the changes in frequency, normalized to the surface coverage of the PPB film, upon immobilization of MBP−NR onto a PPB-modified Au electrode at various MBP−NR concentrations. It is apparent that the highest concentration gave rise to the largest decrease in the frequency, indicating that not all sites in the PPB film are bound to MBP−NR at the lower concentrations (13−50 $\mu$g L$^{-1}$, which correspond to 0.2−0.8 $\mu$M). Alternatively, it could indicate that MBP−NR forms multilayers on the PPB film.

We were particularly interested in determining whether there was a specific interaction between the PPB film and the MBP domain in the MBP−NR. We have previously suggested that the nonquaternized nitrogen of PPB might bind to a specific site in the MBP domain.[14] To ascertain this, changes in the frequency of a PPB-modified Au resonator were monitored following the injection of maltose and MBP−NR. As can be seen in Figure 5, upon the injection of maltose (to a final concentration of 0.5 mM), the frequency decreased about 49 Hz. This value corresponds to 0.87 $\mu$g cm$^{-2}$ maltose (assuming no solvent and/or ions are incorporated), which, in turn, is equal to a surface coverage of $2.4 \times 10^{-9}$ mol cm$^{-2}$. Since the surface coverage of the PPB film used in this experiment was $4.6 \times 10^{-10}$ mol cm$^{-2}$ and sizes of PPB and maltose are almost the same, this result would indicate that maltose forms

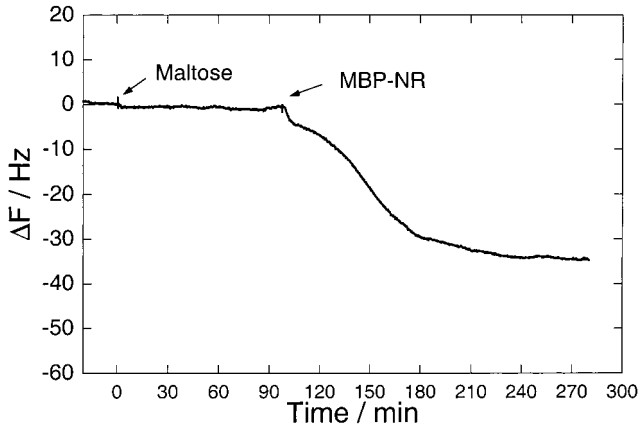


**Figure 6.** Changes in frequency of a bare Au QCM electrode after injection of a maltose aliquot diluted to 0.5 mM into a 0.10 M phosphate buffer solution (pH 7.5) at 25.0 ± 0.1 °C, followed by another injection of a MBP−NR aliquot diluted to 0.4 $\mu$M.

a multilayer equivalent layer on the PPB film, rather than specifically binding to the PPB. If maltose were to specifically bind to PPB, the coverage ratio of the maltose and the PPB should be equal to or less than 1.

After the frequency had reached a steady state, MBP−NR was injected to the solution. As can be seen in Figure 5, the frequency decreased (ca. 12 Hz), indicating the adsorption of MBP−NR onto the maltose that is already adsorbed onto the PPB film. Since the concentration of maltose is much higher (0.5 mM) than that of MBP−NR (0.4 $\mu$M), most if not all the maltose binding sites of MBP−NR should bind to maltose. Thus, the decrease in frequency may reflect nonspecific adsorption of MBP−NR onto the maltose-covered PPB film. In addition, the surface-coverage-normalized decrease in the frequency appeared to be smaller than that without maltose in solution. This might indicate that nonspecific adsorption of MBP−NR onto the PPB film is weaker than the specific adsorption of MBP−NR by its maltose binding site. In essence, the MBP−NR has a lower affinity for a PPB surface that is saturated with maltose. Although, in principle, a better control experiment to check the affinity of maltose-bound MBP−NR to the PPB film would be to inject maltose-bound MBP−NR, in practice, such an experiment is complicated by the fact that the binding events are reversible. Thus, this would raise some ambiguities. Moreover, when MBP−NR was adsorbed onto a PPB film in phosphate buffer solution (pH 7.5) containing maltose, no catalytic activity was evident for TNT reduction, indicating that in order to retain its enzymatic activity, the MBP−NR needs to bind directly to the PPB film.

As mentioned above, the direct immobilization of enzymes onto a bare electrode surface often gives rise to a large if not complete loss of activity. From these results, we conclude that the maltose binding sites of MBP−NR specifically bind to the PPB film. This specific binding might be due to electrostatic and/or steric interactions between the nonquaternized pyridine nitrogen of PPB films and the negatively charged MBP in the pH 7.5 solution (p*I* value of MBP is around 6.5).

When a bare Au electrode was used for a similar experiment (i.e., without a PPB film on the electrode but with other experimental conditions being the same as in Figure 5), the frequency decreased 1.4 Hz upon injection of maltose to a final concentration of 0.5 mM (Figure 6). This value corresponds to 25 ng cm$^{-2}$ maltose, which, in turn, is equal to a surface coverage of $6.9 \times 10^{-11}$ mol cm$^{-2}$. If maltose were to bind in a parallel fashion to a Au surface, then on the basis of the diameter of maltose (ca.

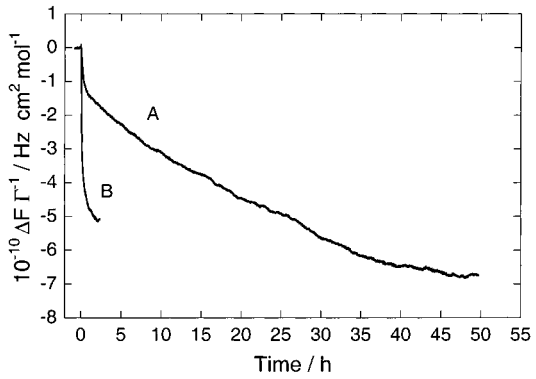


**Figure 7.** Frequency, normalized to the surface coverage of the PPB film, vs time plot for PPB-coated Au QCM electrodes in contact with (A) 13 $\mu$g mL$^{-1}$ (0.5 $\mu$M) NR and (B) 13 $\mu$g mL$^{-1}$ (0.2 $\mu$M) MBP−NR in 0.10 M phosphate buffer solution (pH 7.5) at 25.0 ± 0.1 °C.

10 Å) and with the assumption of hexagonal packing, the value of a monolayer equivalent coverage can be calculated to be $1.8 \times 10^{-10}$ mol cm$^{-2}$. The experimentally determined coverage would then correspond to about a third of a monolayer. On the other hand, if maltose were to bind in a perpendicular fashion to the Au surface, with an effective diameter of ca. 5 Å (taking the width), the value of a monolayer equivalent coverage would be $2.3 \times 10^{-9}$ mol cm$^{-2}$. Thus, under these conditions, the actual surface coverage ($6.9 \times 10^{-11}$ mol cm$^{-2}$) would correspond to about 3% of a monolayer. Note that these values were, again, estimated on the basis of the assumption that maltose does not carry any solvent upon adsorption. A comparison of the decrease in frequency due to maltose adsorption obtained with and without the PPB film would indicate that maltose has a much higher affinity toward the PPB film. This is likely due, at least in part, to hydrogen bonding between the hydroxyl groups of maltose and the non-quaternized nitrogens present in the PPB film.

In Figure 6, once the frequency became stable, MBP−NR was injected to the solution (to a final concentration of 25 $\mu$g mL$^{-1}$). This gave rise to a decrease in frequency of 34 Hz, which corresponds to about 600 ng cm$^{-2}$ MBP−NR. As mentioned above, on a bare Au electrode, maltose forms a layer with a surface coverage of at most a third of a monolayer (compared to a multilayer equivalent film in the presence of PPB, vide supra), leaving some bare Au electrode surface exposed to the solution. Thus, upon comparison with the results shown in Figure 5, where the decrease in frequency was about 12 Hz, it could be concluded that the maltose-bound MBP−NR has a higher affinity to a bare Au surface than to a PPB film covered with maltose.

Binding of NR (nitro reductase) to a Au electrode modified with a PPB film was also studied to ascertain if there were noticeable differences relative to MBP−NR. Since NR is an enzyme that lacks the MBP domain, it was anticipated that it would not strongly bind to PPB films. Curve A in Figure 7 shows the typical frequency changes (normalized to the surface coverage of the PPB film) after injection of NR, diluted to 13 $\mu$g mL$^{-1}$ (0.5 $\mu$M), into a 0.10 M phosphate buffer solution (pH 7.5). As can be seen in the figure, the frequency decreased about 65 Hz ($6.8 \times 10^{10}$ Hz mol$^{-1}$ cm$^2$) after the injection, indicating the adsorption of NR to the PPB-coated electrode. This decrease in the frequency corresponds to a surface coverage of $4.3 \times 10^{-11}$ mol cm$^{-2}$, assuming that NR does not carry solvent molecules. This normalized value of the change in frequency is slightly larger than that for MBP−NR ($5.0 \times 10^{10}$ Hz mol$^{-1}$ cm$^2$ ) also shown in Figure 7, curve B.

Since the molecular weight of NR ($2.7 \times 10^4$) is about 70% that of MBP−NR ($4.0 \times 10^4$), the amount of NR adsorbed on the PPB film would be anticipated to be larger than that of MBP−NR. However, the NR adsorbed on the PPB film did not show any catalytic effect toward TNT reduction. In addition, it should be also mentioned that it took about 50 h for the frequency to reach a steady state. This time scale is too long for enzymatic experiments since the enzymatic activity could drastically decrease over such a time period.

When a bare Au electrode was used, with other experimental conditions being the same as with the PPB film, the frequency decreased 13 Hz upon injection of NR and reached a steady state within 2 h. The changes in the frequency correspond to a surface coverage of $9.0 \times 10^{-12}$ mol cm$^{-2}$. The smaller decrease in the frequency, relative to the case where a PPB film was present, indicates that NR does not adsorb onto a bare Au surface as much as onto a PPB film, probably due to the lack of electrostatic interactions in the former vs the latter. This result is qualitatively similar to the MBP−NR mentioned above.

## Conclusions

The electropolymerization of *N*-(3-pyrrol-1-ylpropyl)-4,4′-bipyridinium (PPB) onto a Au electrode in acetonitrile solution was studied by the electrochemical quartz crystal microbalance (EQCM). It was shown that the extent of polymerization upon pyrrole-centered oxidation became smaller with continuous potential scanning. The bipyridinium-localized (quaternized nitrogen) redox reaction appeared to be of the anion-exchange type. Impedance measurement of a quartz crystal resonator revealed that the PPB film on the electrode is relatively thin or rigid, so that the film properties such as viscoelasticity and roughness remained virtually constant.

The affinity in aqueous medium of a maltose binding protein−nitro reductase (MBP−NR) fusion to electropolymerized films of PPB was also studied by the QCM technique. It was found that the MBP domain of MBP−NR exhibits a specific binding toward PPB films, ostensibly through the maltose binding site, and that the immobilized MBP−NR retains virtually all of its enzymatic activity toward TNT reduction. Impedance measurement indicated that the PPB-bound MBP−NR film was rigid. Although some MBP−NR nonspecifically adsorbed onto a maltose-covered PPB film as well as to a bare Au electrode, it exhibited no catalytic activity. Similarly, nitro reductase (NR) also appeared to adsorb to a PPB film but did not exhibit catalytic activity.

These studies indicate that the interaction(s) between electropolymerized films of PPB and the MBP domain of fusion proteins is specific and could thus be used as a general platform for the oriented immobilization of enzymes with retention of virtually all enzymatic activity.

**Acknowledgment.** This work was supported by the Department of Energy (95ER20206) and Kraft. Z.N. acknowledges support by Universidade de São Paulo (USP) and FAPESP (99/06745-9). S.B. acknowledges a fellowship for advanced researchers from the Swiss National Science Foundation (Grant 8220-053387).

LA0116950