Amperometric TNT Biosensor Based on the Oriented Immobilization of a Nitroreductase Maltose Binding Protein Fusion

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The preparation and characterization of an amperometric 2,4,6-trinitrotoluene (TNT) biosensor based on the surface immobilization of a maltose binding protein (MBP) nitroreductase (NR) fusion (MBP–NR) onto an electrode modified with an electropolymerized film of N-(3-pyrrol-1-ylpropyl)-4,4′-bipyridine (PPB) are described. The MBP domain of MBP–NR exhibits a high and specific affinity toward electropolymerized films of PPB with the immobilized enzyme retaining virtually all of its enzymatic activity. Under similar conditions, the wild-type NR exhibits a high and specific affinity toward electropolymerized films of PPB with the immobilized enzyme retaining virtually all of its enzymatic activity. The kinetics of the catalytic reaction between the biosensor and TNT and 2,4-dinitrotoluene (DNT) were characterized using cyclic voltammetry techniques, and values of 1.4 × 10^4 and 7.1 × 10^4 M⁻¹ s⁻¹ were obtained for TNT and DNT, respectively. The apparent Michaelis–Menten constants (K_m) for MBP–NR in solution and on the surface, using TNT as substrate, were determined to be 27 and 95 μM, respectively. The corresponding value for “wild-type” NR in solution containing TNT was 78 μM, which is very close to the value obtained for MBP–NR on the surface. The limits of detection for both TNT and DNT were estimated to be 2 μM, and the sensitivities were determined to be 205 and 222 nA/μM, respectively.

Since the introduction of “biosensors” that employ biologically derived materials as sensing components, much progress has been made in this area. Among the numerous types of biosensors, amperometric biosensors have been some of the most successful and appear most promising for a wide range of applications. Coupled to this area, the study of the electrochemical behavior of redox enzymes has received a great deal of attention, driven in many cases by the desire to construct practical, self-contained enzyme electrodes for biosensor applications. Closely related to such studies has been the use of various types of electroactive polymers employed in conjunction with redox enzymes for immobilization of the enzyme itself and for the acceleration of electron-transfer kinetics.

For example, a conducting polymer containing glucose oxidase immobilized in poly(ferrocenylpyrrole)7–9 and a redox polymer containing glucose oxidase immobilized in poly(ferricyanopyrrole) have been previously reported.7

Sung and Bae8 developed an enzyme immobilization method by electropolymerization of a polypropylene conducting polymer onto which glucose oxidase (GOx) was conjugated with a polyanion, poly(2-acylamido-2-methylpropanesulfonic acid), via a poly-ethylene oxide spacer to improve the efficiency of enzyme immobilization into the conducting polymer. In an earlier study, Bartlett et al.9 reported on a reticulated vitreous carbon electrode coated with poly(aniline), which was used as a substrate for the adsorption of horseradish peroxidase (HRP), which was subsequently immobilized with a layer of poly-1,2-diaminobenzene. In this study, more stable modified electrodes were obtained if the HRP was also cross-linked with glutaraldehyde. Hendry et al. reported on a redox polymer containing glucose oxidase immobilized in poly(ferrocenylpyrrole).10 There have also been a number of reports of well-behaved electrochemical responses of redox proteins11 and redox enzymes12,13 at electrodes modified with conducting polymers.

The active sites of enzymes, where redox reactions occur, are often located inside the enzyme, so that the protein sheath presents a steric or insulating barrier between the redox center of the enzyme and the electrode. Because an enzyme’s activity is strongly dependent on its tertiary structure, and since virtually all immobilization approaches tend to alter it, it is often found

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that enzyme immobilization leads to partial, and sometimes complete, loss of activity. It is also the case that, for enzymes that contain redox-active prosthetic groups, their direct reaction at bare electrode surfaces often exhibits very slow kinetics for the reasons alluded to above. Redox mediators, and so-called enzyme wiring14,15 are often employed in order to accelerate interfacial kinetics and enhance enzymatic activity.16,17 M. E. Miller et al. demonstrated that, after immobilization, the activity of nitrate reductase, nitrite reductase, and nitrous oxide reductase is enhanced when methyl viologen, in solution, is used as an electron donor in place of the more conventional NADH.18

There is a great deal of interest in new methodologies for the immobilization of redox enzymes that will allow for maximal retention of activity as well as the acceleration of interfacial kinetics. Thus, many investigations have been carried out to overcome the steric hindrance problem as well as to achieve more efficient and direct electron transfer between the enzyme’s redox centers and the electrode.18,20 Simply put, there is a great need for enzyme immobilization strategies where enzymatic activity is largely retained. For example, Willner and co-workers19 improved the interaction between an enzyme and an electrode by modifying flavin adenine dinucleotide (FAD) with redox-active ferrocene-containing groups. Cosnier and co-workers22,23 developed a nitrate biosensor, which was prepared by coating a polypyrrole—viologen film on an electrode surface and subsequently immobilizing nitrate reductase. On the basis of that work, Ramsay and Wolpert24 proposed the use of “wiring” nitrate reductase by alkylpyrrole—viologen-based redox polymers for bioreactor applications. However, in that configuration, the immobilized enzyme gave a residual activity of only 0.18% of the amount originally applied.

Genetic engineering methodologies have also been employed in the development of biosensors.25,26 Such technologies can be used to introduce functional groups into biological molecules that allow for the oriented immobilization of a protein onto an appropriately chemically modified surface.27 M. Arvin et al.28 identified regions in Escherichia coli maltose binding protein that are allosterically linked to its maltose binding site. They demonstrated that the binding site could be manipulated without destroying the allosteric linkage with the corresponding group. This implicitly suggests that the sites can be independently manipulated as we have done in the present case.

Recently, we have been involved in the development of analytical strategies for the determination of products of the bacterial denitrification processes.29,30 In an earlier study, we demonstrated that the maltose binding protein (MBP) nitrite reductase (Nir) fusion (MBP—Nir), immobilized on an electropolymerized film of N-(3-pyrrol-1-ylpropyl)-4,4′-bipyridine (PPB), retained virtually all of its activity upon immobilization on the surface.29 That behavior was attributed to the fact that the MBP domain interacts strongly with the PPB polymer layer with ostensibly little, if any, effect on the tertiary structure of nitrite reductase. The choice of PPB was not arbitrary but rather stimulated by the fact that this material is similar to methyl viologen, which has been used as a redox mediator for Nir in solution.

In recent years, the reduction of nitroaromatic compounds, which are widely used in agrochemicals and explosives, has been studied in great detail, in part because of their environmental pollution effects. Various methods of analysis for the determination of nitro aromatics have been proposed including a membrane-based continuous-flow displacement immunoassay for the detection of nanomolar quantities of explosives,31 a miniaturized field-portable immunosensor (FAST 2000), which was field tested at former military munitions sites for the detection and quantitation of 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in groundwater,32 and a fiber-optic immunosensor for the simultaneous detection of the most common explosives, TNT and RDX.33

The nitroreductases of enteric bacteria are flavoproteins that catalyze the reduction of a variety of nitroaromatic compounds to metabolites that are highly toxic, mutagenic, or carcinogenic. Bryant et al. studied cloning, nucleotide sequence, and expression of the nitroreductase gene from Enterobacter cloacae.34,35 Koder and Miller36 studied the mechanism of E. cloacae nitroreductase and found that 2,4-dinitrotoluene (DNT) was the most efficient oxidizing substrate examined, with a $k_{cat}/K_M$ value that was 1 order of magnitude larger than those of p-nitrobenzoate, FMN, FAD, or riboflavin.

As part of our continued interest in the development of amperometric biosensors and analytical methodologies using fusion proteins, we have developed an amperometric biosensor for the determination of nitro derivatives based on the immobilization of a maltose binding protein nitroreductase (MBP—NR) fusion onto glassy carbon electrodes (GCE) previously modified with electropolymerized films of the N-(3-pyrrol-1-ylpropyl)-4,4′-bipyridinium cation.

In this paper, we describe the electropolymerization of PPB and the electrochemical characteristics of the resulting electro-
active films as well as the specific interaction between the MBP domain and the PPB film via the nonquaternized nitrogen of PPB. In addition, we have studied the kinetics of the reaction between the biosensor and TNT and DNT (used as substrates) in solution by cyclic voltammetry and rotated disk electrode techniques.

**EXPERIMENTAL SECTION**

**Reagents.** 2,5-Dimethoxytetrahydrofuran (98%), 3-bromopropylamine hydrobromide (98%), 4,4′-dipyridyl (98%), and ammonium hexafluorophosphate (99.99%) were obtained from Aldrich Chemical Co., Ltd. and were used as received. N-(3-Bromopropyl)pyrrole was prepared by modification of the syntheses of Dehaen and Hassner and N-(3-pyrrol-1-ylpropyl)-4,4′-bipyridinium hexafluorophosphate was prepared by modification of the syntheses of Carpio et al. Standard TNT and DNT ethanolic solutions were prepared daily.

**Tetra-butylammonium hexafluorophosphate (TBAH; G. F. Smith)** was recrystallized three times from ethyl acetate and dried under vacuum at 90 °C for 72 h. In aqueous media, potassium phosphate buffers (Fisher) were employed as supporting electrolytes. All other reagents were of at least reagent grade quality and were used without further purification. Solutions for electrochemical studies were purged with prepurified nitrogen gas for at least 15 min prior to use and a nitrogen stream was maintained over the solution.

**Bacterial Strain and Growth.** E. cloacae PB2 was grown in an LB medium at 30 °C with shaking, and E. coli TG1 was cultured at 37 °C with agitation. To induce the overexpression of proteins, 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the E. coli culture when the OD600 of the culture was 0.4. The incubation temperature was then shifted down to 30 °C, and the incubation was continued for 3 h. The E. coli competent cells were prepared as described by Sambrook et al.

**Cloning and Sequencing of nr Encoding NR.** The nr gene was amplified by PCR. A 100-μL aliquot of PCR mixture contained 4 μL of E. cloacae PB2 cell lysate, 10 μL of 5 pmol/μL each of primer, 10 μL of 10× Gibco PCR buffer (Rockville, MD), 8 μL of 2.5 mM dNTP, 2.5 units of Gibco Taq polymerase, and 54.5 μL of sterile H2O. The PCR was conducted under the following conditions: initial denaturing at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and an extension at 72 °C for 10 min, using the GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). The PCR product was cloned into pMAL-c2E (New England Biolabs, Beverly, MA). The primers used to clone the nr were designed to contain BamHI (5′) and HindIII (3′) restriction sites, which are compatible with the multiple cloning site of pMAL-c2E. The insert in the final construction was sequenced to confirm its identity. The synthesis of the primers and the sequencing were performed at the BioResource Center of Cornell University (Ithaca, NY).

**Purification of the MBP—NR Fusion Protein.** The MBP—NR fusion protein was purified using an amylose resin following manufacturer’s instructions (New England Biolabs).

**Enzyme Assay.** The specific activity of the MBP—NR fusion was determined by monitoring the NADH oxidation rate at 340 nm using a DU Series 600 spectrophotometer (Beckman Instruments, Fullerton, CA). The reaction mixture was 50 mM pH 7 phosphate buffer containing 150 μM NADH, MBP—NR, and TNT. For measurement of the Kₘ and kₘₐₜ of the fusion protein, the NADH oxidation rate was monitored at 340 nm with varying concentrations of TNT. The values of K_max and k_cat were calculated from the double-reciprocal (Lineweaver–Burke) plots of the reaction velocities and corresponding concentrations.

**Instrumentation.** Electrochemical experiments were carried out with either a PINE RDE-3 bipotentiotstat or an EG&G PARC 283 potentiostat. Three-compartment electrochemical cells (separated by medium-porosity sintered glass disks) were employed. All joints were standard tapers so that all compartments could be hermetically sealed with Teflon adapters. This is important to ensure that there is no gas leakage to or from the electrochemical cell. A GCE with a geometric area (ga) of 0.0314 cm² was used as a working electrode. It was polished prior to use with 1-μm diamond paste (Buehler) and rinsed thoroughly with water and acetone. A large-area platinum wire coil was used as a counter electrode. For rotated disk electrode experiments, a Pine Instruments rotator and RDE-3 bipotentiostat were employed. A glassy carbon rotated disk electrode from Pine (ga 0.283 cm²) was used as the working electrode. All potentials are referred to a saturated silver/silver chloride electrode (Ag/AgCl/NaCl_saturated) without regard for the liquid junction potential.

**Synthesis of N-(3-Bromopropyl)pyrrole.** 2,5-Dimethoxytetrahydrofuran (6.6 g, 0.05 mol), 3-bromopropylamine hydrobromide (11.2 g, 0.05 mol), sodium acetate (22 g, 0.26 mol), and glacial acetic acid (25 mL, 0.44 mol) in dioxane (50 mL) were heated to 140 °C and distilled for 1 h. After cooling, the remaining oil was poured into saturated aqueous NaCl solution (150 mL) and aqueous Na₂CO₃ was added until the solution was basic. The solution was extracted with CH₂Cl₂ and then the organic extract was washed with saturated aqueous NaCl, followed by saturated aqueous Na₂CO₃, and then again with a saturated aqueous NaCl solution. The organic layer was dried with MgSO₄ and concentrated in vacuo. The resulting oil was purified by column chromatography (silica gel with CH₂Cl₂ as eluent), and the first fraction was collected and concentrated to give N-(3-bromopropyl)pyrrole as a clear liquid (15.7g, 17%). 1H NMR (CDCl₃): J = 6.82 (d, 2H, J = 6.8 Hz, pyr(2,5)), 6.32 (d, 2H, J = 2.0 Hz, pyr(3,4), 4.18 (t, 2H, J = 6.3 Hz, CH₂Br), 3.42 (t, 2H, J = 6.2 Hz, NCH₃), 2.36 (q, 2H, J = 6.2 Hz, CH₂).

**Synthesis of N-(3-Pyrrol-1-ylpropyl)-4,4′-bipyridinium Hexafluorophosphate.** To a solution of 4,4′-bipyridine (0.41 g, 2.8 mmol) in acetonitrile (4 mL), N-(3-bromopropyl)pyrrole (0.36 g, 1.9 mmol) was added at room temperature. The solution was heated to 80 °C, stirred for 2 h, and then allowed to cool to room temperature. N-(3-Pyrrol-1-ylpropyl)-4,4′-bipyridinium hexafluorophosphate was obtained by dropwise addition of the reaction...
solution containing the bromide salt into a stirring aqueous solution containing excess ammonium hexafluorophosphate. The resulting precipitate was filtered, washed with water, and then recrystallized from acetonitrile/water to afford a yellow/tan crystalline solid (0.62 g, 80%). 1H NMR ((CD$_3$)$_2$SO) recrystallized from acetonitrile/water to afford a yellow/tan resulting precipitate was filtered, washed with water, and then solution containing excess ammonium hexafluorophosphate. The solution containing the bromide salt into a stirring aqueous solution containing excess ammonium hexafluorophosphate.

Preparation of Biosensor and Modified Electrodes. (1) Electrodes Modified with PPB. Electrodes were modified with electropolymerized films of PPB by scanning the potential (at 100 mV/s) between +1.5 and −0.50 V for a prescribed length of time (depending on the desired coverage) in a thoroughly degassed 2 mM solution of PPB in acetonitrile (0.1 M TBAH). Following the electropolymerization step, the modified electrode was rinsed thoroughly with acetone and water. The surface coverage of the polymer film, (Γ in mol/cm$^2$) was determined, in aqueous 0.1 M, pH 7.5 phosphate buffer solution, from the integrated charge under the cyclic voltammetric peak centered at −0.30 V at slow sweep rates (10 mV/s). Average coverage values of (2.0−3.0) × 10$^{-9}$ mol/cm$^2$ were obtained, which represent approximately four to six equivalent monolayers, respectively.

(2) Electrodes Modified with DPPB. Electrodes were modified with electropolymerized films of DPPB in a fashion similar to that employed for PPB except the potential was scanned between +1.4 and −0.90 V. The surface coverage of the polymer film, (Γ in mol/cm$^2$), was determined as for PPB, from the integrated charge under the cyclic voltammetric peak centered at −0.46 V at slow sweep rates (10 mV/s). Average coverage values of (3.0−4.0) × 10$^{-9}$ mol/cm$^2$ were obtained which represent approximately 8−10 equivalent monolayers, respectively.

(3) Biosensor. A glassy carbon (GC) electrode, previously modified with a PPB or DPPB film, was placed into a 200 µg/mL enzyme solution, prepared in pH 7.5 0.1 M phosphate buffer, for 60 min under slight stirring, followed by rinsing with buffer solution. When not in use, the biosensor was stored in a buffer solution at 4 °C.

Electrochemical Measurements and Biosensor Response. Cyclic voltammetric studies of the electrocatalytic reduction of nitro species at the biosensor were carried out at various sweep rates. Aqueous electrochemical experiments were conducted at pH 7.5, where the enzyme exhibits maximal activity. The rate constants for the reaction of the biosensor with TNT and DNT were determined by both cyclic voltammetry and rotated disk electrode (RDE) experiments. In the RDE experiments, the potential was swept at 5 mV/s from 0 to −0.5 V. The biosensor was placed in 5.0 mL of buffer solution at an applied potential of −0.35 V. The potential was scanned at 10 mV/s for two or three cycles from 0 to −0.50 V, until the background current decayed to a steady value. Aliquots of a solution of substrate were subsequently added. The catalytic current due to reduction of TNT or DNT was measured after stirring for 30 s and allowing the solution to stand for the same time period for equilibration.

RESULTS AND DISCUSSION

Electrode Modification and Electrochemical Characterization of the Electropolymerized Film. PPB readily undergoes electrooxidatively initiated polymerization to give rise to electrodes modified with an electroactive film of PPB. Figure 1A shows a series of consecutive cyclic voltammograms for an electrode in contact with a 2 mM solution of PPB AN/0.1 M TBAH. The first scan to negative potentials showed a featureless background up to −0.70 V. In the first anodic scan, there was an irreversible oxidation at ∼+1.50 V, whose amplitude decreased upon consecutive scanning and that we ascribe to a pyrrole-localized process. In the second and subsequent sweeps to negative potentials, there was a reversible peak with a formal potential, $E^\circ$ of −0.16 V, that we ascribe to a bipyridinium-localized process (quaternized nitrogen), analogous to a viologen. There was also an additional peak, with a formal potential of −0.60 V, that was attributed to the reduction of the nonquaternized nitrogen in the bipyridinium derivative. We recently carried out extensive and detailed studies of the electropolymerization of PPB which indicate that the reaction also involves partial quaternization of the free pyridine sites to give rise to what would ostensibly be a viologen-like species. The amplitude of these peaks, centered at −0.16 and −0.60 V, increased upon consecutive scanning between the aforementioned limits.

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With PPB, 7.5) at a sweep rate 20 mV/s for a glassy carbon electrode modified

Figure 2. Cyclic voltammograms in 0.1 M phosphate buffer (pH 7.5) at a sweep rate 20 mV/s for a glassy carbon electrode modified with PPB, \( I = 3.2 \times 10^{-9} \text{ mol/cm}^2 \) (solid line) and PPB/NR (dashed line), after (A) being in contact for 30 min with a 50 \( \mu \text{g/mL} \) solution of MBP/NR in the same buffer and (B) after being in contact for an additional 30 min with a 200 \( \mu \text{g/mL} \) solution of MBP/NR in the same buffer.

When such an electrode was removed from the polymerization solution, rinsed with acetone, and placed in an AN/0.10 M TBAH solution containing no dissolved PPB, the cyclic voltammetric response exhibited a reversible redox process centered at \(-0.16 \) V. In addition, the voltammetric peak was sharp and symmetric as would be anticipated for a surface-confined redox process.

The films were also electroactive in aqueous solution, and again, the redox response was that anticipated for a surface-immobilized redox couple, as can be seen in Figure 1B, which shows a cyclic voltammogram for a GCE modified with an electropolymerized film of PPB in a pH 7.5 phosphate buffer solution. In this case, however, the formal potential was \(-0.29 \) V, a value that is negatively shifted by \(-130 \) mV from that obtained in acetonitrile. This potential shift likely reflects differences in solvation between aqueous and nonaqueous solvents. As in the case of acetonitrile, a second peak was also observed with a formal potential at \(-0.59 \) V. It is worth noting that whereas the first voltammetric wave was shifted by \(-130 \) mV, the second remained essentially fixed. The \( \Delta E_p \) values for the peak centered at \(-0.29 \) V were typically small, 10 mV at a sweep rate range of 10–100 mV/s. The peak currents were directly proportional to the scan rate for values of up to 200 mV/s. The \( \Delta E_p \) values for the peaks centered at \(-0.59 \) V were 70 mV at a sweep rate range up to 100 mV/s and had a more “diffusional” shape, suggesting the involvement of a chemical reaction, such as protonation, associated with the redox process. However, the peak currents at \(-0.59 \) V were also directly proportional to the sweep rate for values of up to 200 mV/s, which indicates that the redox process is for a surface-immobilized couple. The charge-transfer rate constant of PPB-modified GC electrodes was determined in previous work. 29

Biosensor Description. Figure 2 shows cyclic voltammograms for a GCE modified with an electropolymerized layer of

PPB before (solid line) and after (dashed line) coming in contact with a solution of (A) 50 \( \mu \text{g/mL} \) MBP–NR during 30 min and (B) 200 \( \mu \text{g/mL} \) MBP–NR after an additional 30 min. It can be seen that the peak at \(-0.29 \) V became broader but without a shift in the formal potential. This indicates that the interaction between the PPB layer and MBP–NR does not affect the potential of the first redox process of PPB, centered at \(-0.29 \) V. It is also evident that the peak at \(-0.59 \) V is greatly altered after the immobilization of MBP–NR onto the PPB layer. This suggests that the nonquaternized pyridine nitrogen (in the PPB film) has an important role in the interaction between the MBP domain and PPB, which was speculated earlier for the nitrite biosensor. 29

To confirm the importance of the nonquaternized pyridine nitrogen in the PPB film, as well as potential electrostatic interactions between the film and the MBP domain, MBP–NR was immobilized onto an electrode modified with an electropolymerized layer of DPPB. The intent was to assess how the interactions between MBP–NR and PPB differed when DPPB was employed as modifier. This is possible since both nitrogen are quaternized in DPPB.

The \( \text{pH} \) value of the MBP is \(-6.5; \) thus, in \( \text{pH} 7.5 \) phosphate buffer, the protein will be negatively charged. In DPPB, both pyridine nitrogen are quaternized (with N-(3-pyrrol-1-ylpropyl) groups), so that the second nitrogen’s interaction with MBP would be anticipated to be largely electrostatic in origin. Figure 3 shows cyclic voltammograms of a DPPB-modified glassy carbon electrode (at a coverage of \( 3.6 \times 10^{-5} \) mol/cm²) before (solid line) and after (dashed line) exposure for 60 min to a of 200 \( \mu \text{g/mL} \) solution of MBP–NR.

![Cyclic voltammograms in 0.1 M phosphate buffer (pH 7.5) at a sweep rate 20 mV/s for a glassy carbon electrode modified with DPPB (I = 3.6 \times 10^{-9} \text{ mol/cm}^2) before (solid line) and after (dashed line) exposure for 60 min to 200 \( \mu \text{g/mL} \) solution of MBP–NR.](Image 371x493 to 504x752)
probably binds to a specific site in the MBP domain through the second nitrogen, which is not quaternized.

Moreover, in QCM experiments, it was observed that, in the presence of maltose in solution, the binding of MBP-NR to an electropolymerized film of PPB was inhibited, suggesting that they both (maltose and PPB) interact with the same region or domain of MBP-NR.40

The biosensor, schematically depicted in Figure 4, consists of two layers, the first being an electropolymerized film of PPB and the second the MBP-NR layer. The functioning of the biosensor can be described as follows. The PPB cationic layer is reduced at the electrode surface, to neutral PPB. This species transfers electrons to the enzyme layer reducing \( \text{NRox} \) to \( \text{NRred} \), which in turn, reduces nitro aromatic groups to the corresponding hydroxylamine derivative. This gives rise to an electrocatalytic current enhancement, which is used as the analytical signal.

Electrocatalytic Activities of Surface-Immobilized MBP-NR and NR. We were interested in ascertaining whether different NRs would retain their catalytic activity upon immobilization since it is often found that immobilization gives rise to loss of activity. In this case, we compared the behavior of MBP-NR with NR in which the former has the maltose binding domain whereas the latter does not. The catalytic activities of the enzymes, NR and MBP-NR, toward the reduction of TNT or DNT in aqueous media were evaluated by comparing the amperometric responses of the enzymes immobilized on a PPB-modified electrode. The response of GC electrodes modified with PPB and one of the two nitroreductases, NR or MBP-NR, in the presence and absence of the above-mentioned substrates was examined.

For electrodes modified with PPB and NR, there was no evidence of any catalytic current associated with the reduction of either of the two substrates (TNT or DNT) by the immobilized NR enzyme. It should be mentioned that, from QCM studies,40 we could unambiguously establish that there was some enzyme immobilized, albeit at low coverage, on the electrode surface, but as shown above, it exhibited no catalytic activity. On the other hand, a clear electrocatalytic response was observed for electrodes modified with PPB and MBP-NR. Figure 5 shows cyclic voltammograms in 0.1 M phosphate buffer for a GC electrode modified with PPB/MBP-NR (surface coverage of PPB was \( 2.9 \times 10^{-9} \text{ mol/cm}^2 \)) in the presence of 283 \( \mu \text{M} \) TNT.

![Figure 5. Cyclic voltammograms at 10 mV/s in 0.1 M phosphate buffer (pH 7.5) for a PPB/MBP-NR (\( \Gamma_{\text{PPB}} = 2.9 \times 10^{-9} \text{ mol/cm}^2 \)) glassy carbon electrode in the absence (dashed line) and in the presence of 283 \( \mu \text{M} \) TNT (solid line).](image)

For electrodes modified with PPB and MBP-NR, a well-defined voltammetric wave centered at \(-0.32 \text{ V}\). Upon the addition of TNT, the cyclic voltammogram (Figure 5, solid line) exhibited a dramatic enhancement of the cathodic peak current with virtually no current on the reverse sweep, typical of an EC\(_{\text{cat}}\) process. When the potential was scanned up to +0.2 V, a peak at +0.10 V was observed. This peak was ascribed to the formation of the hydroxylamine derivative, as has been previously observed for other nitro aromatic derivatives.41

The direct reduction of TNT and DNT at a bare glassy carbon electrode occurs at potentials that are significantly more negative than those observed for the immobilized enzyme. This selectivity is attributed to the presence of the maltose binding domain on the enzyme, which facilitates the specific recognition of TNT and DNT.


(by about 100 and 170 mV, respectively) than at the PPB/MBP-NR biosensor. In addition, the current was virtually the same as that obtained using the biosensor. Since the concentration of TNT in these two solutions was identical (in fact the same solution),

Figure 6. Electrocatalytic current in 0.1 M phosphate buffer (pH 7.5) at a potential of −0.35 V vs \( \omega^{1/2} \) (Levich plot) for the reduction of 80 \( \mu \)M TNT at a PPB/MBP-NR glassy carbon electrode (\( I_{PPB} = 8.2 \times 10^{-10} \) mol/cm\(^2\)) (A) and 1/i vs 1/\( \omega^{1/2} \) (Koutecky-Levich plot) (B).

Figure 7. Catalytic current of GC-PPB/MBP-NR biosensors vs [TNT] (\( I_{PPB} = 1.8 \times 10^{-9} \) mol/cm\(^2\)) (A) and DNT (\( I_{PPB} = 1.4 \times 10^{-9} \) mol/cm\(^2\)) (C) concentration. Inset: low concentration range. Sweep rate 10 mV/s; 0.1 M pH 7.5 phosphate buffer. Lineweaver-Burke plots for TNT (B) and DNT (D).
this suggests that, at the PPB/MBP–NR-modified electrode, the catalytic redox process is primarily controlled by diffusional mass transport.

**Kinetics of Nitro Reduction.** To assess the catalytic activity of PPB/MBP–NR biosensors toward the reduction of TNT and DNT, we employed both RDE and cyclic voltammetric methods. In the first case, the catalytic current for the reduction of TNT and DNT in solution (80 μM TNT and 180 μM DNT) was obtained as a function of the rate of rotation. A Levich plot of 1/ \( i_\text{lim} \) versus \( \omega^{1/2} \) deviated from linearity (Figure 6A) at high rates of rotation, indicating kinetic rather than transport control. On the other hand, a Koutecky–Levich plot of \( 1/ \omega \) versus \( 1/ i_\text{lim} \) (Figure 6B) was linear (as would be anticipated for a system that is kinetically controlled and assuming first-order kinetics) over the entire range of rates of rotation employed (up to 900 rpm). From the values of the intercepts, \( k_i \) values were determined to be 1.4 × 10^4 and 7.1 × 10^4 M⁻¹ s⁻¹ for TNT and DNT, respectively.

In the second case, using cyclic voltammetry, the catalytic currents for TNT and DNT reduction were obtained as a function of sweep rate using the same concentrations of TNT and DNT as above. Using the approach developed by Andrieux and Saveant, the rate constants were determined to be 1.3 × 10^1 M⁻¹ s⁻¹ for TNT and 7.3 × 10^4 M⁻¹ s⁻¹ for DNT. These values are virtually identical to those determined from rotated disk electrode experiments.

**Biosensor Response.** To compare results from different experiments and to assess the effect of different variables on the electrocatalytic response, the catalytic current was normalized to the surface coverage of the PPB film (\( i_\text{cat} / (i/\Gamma) \)) since it would be difficult to obtain identical surface coverage for multiple electrodes. In terms of pH dependence, the best response was obtained in pH 7.5 phosphate buffer. This value is in very good agreement with that reported for nitroreductase from E. cloacae. From the various supporting electrolytes (buffers) used (HEPS, BES, MOPS, Tris), phosphate buffer gave the best results. For immobilization of the enzyme onto the PPB film, the best results were obtained when the electrode was immersed in a 200 mg/mL enzyme solution for 1 h under slight stirring. These values were employed in all subsequent studies.

One of the objectives of the present work was the development of a biosensor for the determination of TNT. As was mentioned earlier, in the presence of TNT, the biosensor exhibits an enhancement in the current response for the peak centered at −0.35 V. As shown in Figure 7A, the amplitude of the catalytic current (in a cyclic voltammogram at 10 mV/s), defined as the difference of the peak currents in the presence and absence of TNT, is proportional to the solution concentration of TNT for values of up to 40 μM (Figure 7A, inset). However, at higher concentrations, the response deviates from linearity and appears to approach a concentration-independent value. Such behavior is typical of enzymatic processes where the reaction is eventually limited because of enzyme saturation. From an analysis of the data in Figure 7A for TNT and Figure 7C for DNT, using a Lineweaver–Burke plot of 1/i versus 1/concentration (Figure 7B,D), we estimated the values of the apparent Michaelis–Menten constants to be 95 μM for TNT and 105 μM for DNT. The value for TNT is very close to that for NR in solution (78 μM) and points to the fact that the immobilization of the enzyme results in retention of virtually all enzymatic activity.

The limit of detection was estimated by measuring the increase in current upon the addition of known amounts of TNT and DNT standard solutions. Even at a 2 μM substrate concentration, a clear increase in current was noted and we believe that the limit of detection is likely around this value. From the linear part of the calibration plot (Figure 7A and C, inset), the sensitivities were determined to be 205 nA/μM for TNT and 222 nA/μM for DNT. Thus, the results obtained indicate that glassy carbon electrodes modified with an immobilized film of PPB/MBP–NR can be used as amperometric biosensors for the determination of TNT and DNT.

**Stability and Reproducibility of Biosensors.** The reproducibility and stability of biosensors are particularly important, and experiments were conducted in order to investigate these aspects. In terms of reproducibility, it was found that the relative standard deviation of the catalytic current of TNT for six replicate determinations was 2.5%. After this, the biosensor was periodically used for 2 h and subsequently stored in a refrigerator for 2 days. After this storage period, the catalytic current for TNT reduction was 91.5% of the initial value.

It is especially important to investigate the reproducibility of several biosensors since it would be difficult to obtain identical surface coverages for multiple electrodes, a situation that would require a calibration curve for each biosensor. We compared the results of three different biosensors prepared under similar conditions but with somewhat different surface coverages. By constructing calibration curves normalized to the surface coverage of PPB (\( q_{cat} = 1 \text{s}^{-1} \text{mol cm}^{-2} \)), all three biosensors gave virtually the same coverage-normalized response, with an excellent linear correlation (Figure 8). This indicates that a universal calibration curve (Figure 8) may be constructed and employed in analytical determinations.

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CONCLUSIONS

Electropolymerized films of PPB on glassy carbon electrodes retain their redox activity in aqueous solutions. Further modification with MBP–NR gave rise to electrodes that exhibited very high electroactivity for the reduction of TNT and DNT. However, PPB electrodes modified with NR did not exhibit such activity. This suggests that the presence of specific interactions between the PPB layer and MBP fusions might represent a general way to immobilize enzymes onto surfaces with a high retention of activity as we had speculated in earlier work. A PPB/MBP–NR biosensor was shown to exhibit high activity toward the electrocatalytic reduction of TNT and DNT up to 50 μM in solution at a potential of −0.35 V versus Ag/AgCl. The catalytic response exhibits a linear dependency on the concentration of TNT and DNT in solution, with a limit of detection of ~2 μM. Rate constant values of $1.4 \times 10^4$ M$^{-1}$ s$^{-1}$ for TNT and $7.1 \times 10^4$ M$^{-1}$ s$^{-1}$ for DNT were determined via rotated disk and cyclic voltammetric techniques.

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