p-Ferrocenylaniline and *p*-Ferrocenylphenol: Promising Materials for Analytical Biochemistry and Bioelectrochemistry

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New substrates of horseradish peroxidase and laccase incorporating advantages of ferrocenes, on one hand, and typical aromatic substrates of peroxidase, on the other, viz. *p*-ferrocenylaniline ($FcC_6H_4NH_2$-*p*) and *p*-ferrocenylphenol (FcC_6H_4OH-*p*), have been introduced. The new substrates are superior in terms of (i) high reactivity, i.e., the second-order rate constants for their oxidation by the peroxidase compound II equal ca. 1×10^7 M^{-1} s^{-1} at 25 °C, pH 5.0, which are 10^2 times higher than the corresponding rate constants for oxidation of ferrocene, alkylferrocenes, and aniline; (ii) easy spectral control of redox transformations at 439 or 860−1000 nm (products are colored and the extinction coefficients are high); (iii) the products are water-soluble and do not inactivate enzymes. *p*-Ferrocenyl-aniline is readily electropolymerized on a rod carbon electrode in 0.1 M HCl by potential cycling in the range from −0.4 to +1.1 V (versus Ag/AgCl). The peak potentials are weakly pH-dependent in the range 0.5−7 and the slopes of potential versus pH plots are less than 30 mV. *p*-Ferrocenylaniline is also electrochemically active when adsorbed on a carbon electrode from an acetone solution. It has been demonstrated that the electrodeposited and adsorbed *p*-ferrocenylaniline films mediate the electro-oxidation of NADH with the sensitivity of 1.4 and 0.25 $\mu A/mM$ cm^2 at pH 5 and 7.2, respectively.

Introduction

A search for new materials used in analytical biochemistry and bioelectrochemistry emerges from the need for efficient mediated electron-transfer involving oxidoreductases[1−4] and electrochemical generation of conducting films on electrode surfaces.[5−8] We have recently learned that ferrocene derivatives are rather reactive toward oxidoreductases such as horseradish peroxidase (HRP)[9,10] and glucose oxidase from *Aspergillus niger*.[11,12] Products of the enzymatic transformations result from one-electron removal from ferrocene or one-electron addition to ferricenium ion in the case of HRP and glucose oxidase, respectively. In addition, these organometallic substrates are enzyme-friendly, especially in the HRP case, and the enzymatic products do not inactivate the biocatalysts. These features suggested an approach to novel artificial substrates of oxidoreductases based on the coupling of the ferrocenyl fragment and traditional organic substrate of the enzyme. To test the validity of the approach, first we looked at the peroxidase catalysis.[13−15] The essence of our strategy is illustrated in Scheme 1. It was decided to conjugate anilines and phenols, i.e., traditional substrates of HRP,[16] with ferrocene, a reactive organometallic substrate of HRP, to obtain a novel, artificial, enzyme-friendly organometallic substrate. An advantage of ferrocenes as HRP substrates[9] is that the enzymatic reaction ends in the formation of a water-soluble, relatively hydrophilic ferricenium cation. In a "bifunc-

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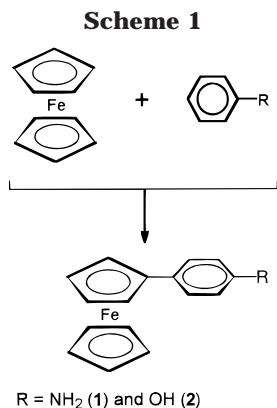(12) Ryabov, A. D.; Firsova, Y. N.; Nelen', M. I. *Appl. Biochem. Biotechnol.* **1996**, *61*, 25−37.
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Scheme 1

R = NH$_2$ (**1**) and OH (**2**)

tional" substrate such as **1** or **2**, the initial electron transfer at oxidized HRP could occur from the organic part of the molecule, rather than from the organometallic one, since aniline and phenol, as natural substrates, appeared to be better candidates as electron donors. The following intramolecular electron transfer from the ferrocenyl fragment could, in principle, give rise to a ferricenium cation of the type Fc$^+$-C$_6$H$_4$R-*p*. Compound **1** is an aniline derivative, and therefore it was also decided to test its ability to undergo electropolymerization, since some aniline−ferrocene conjugates have proved to be versatile precursors for the electrochemical generation of films at electrode surfaces.[17] In this work, we demonstrate that *p*-ferrocenylaniline and *p*-ferrocenylphenol are readily oxidized in the peroxidase- and laccase-catalyzed reactions, that the reactivity of *p*-ferrocenylaniline toward HRP is higher by a factor of 100 than that of aniline or ferrocene, that *p*-ferrocenylaniline is readily electropolymerized on electrode in acidic medium, and that the polymer formed does mediate the electrooxidation of NADH.

Results and Discussion

HRP- and Laccase-Catalyzed Oxidation of 1 and 2 by Hydrogen Peroxide. Compounds **1** and **2** are poorly soluble in water, even at pH 3. The solubility increases noticeably in the presence of MeOH or EtOH, but it becomes sufficient for kinetic measurements in the presence of the surfactant Triton X-100, even without alcohols. At the surfactant concentration of 0.0046 M and pH 5 the concentration of **1** was 0.008 M. Dissolved in the presence of the surfactant, compounds **1** and **2** readily undergo HRP-catalyzed oxidation by H$_2$O$_2$. The spectra of **1** and **2** remain unchanged in the presence of either H$_2$O$_2$ or HRP, while in the presence of both there is a rapid growth in the absorbance at 439 and 1013 nm or 436 and 851 nm in the case of **1** or **2**, respectively (Figure 1). After a rapid increase in absorbance due to the enzymatic conversion of **1** and **2**, a slower fading of the solutions is observed. The latter shows that the primary products of the enzymatic oxidation are unstable in the aqueous medium and undergo decomposition. In the case of **1**, a transient band at 369 nm is developed on degradation. The spectral patterns observed suggest that, in contrast to the HRP-catalyzed oxidation of alkylferrocenes,[9] the
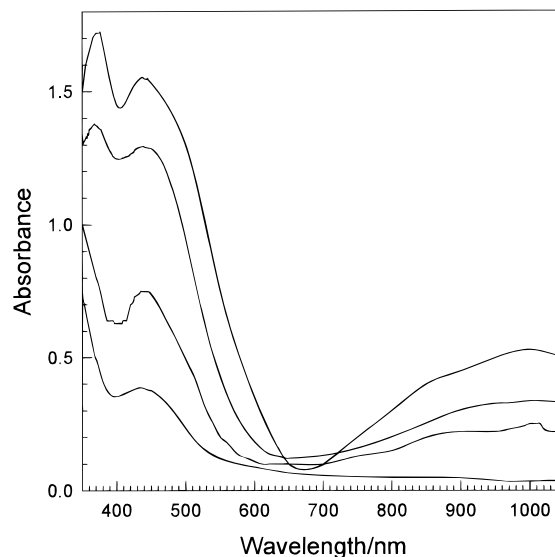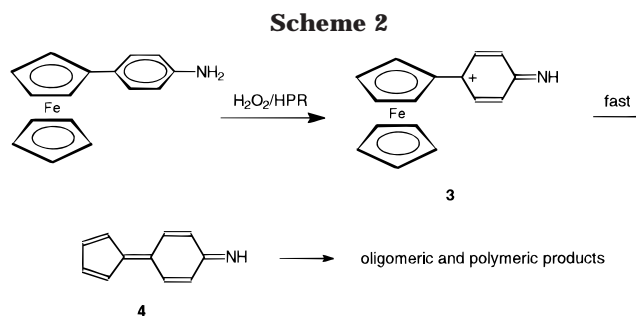
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Figure 1. Changes of the spectra of **1** in the presence of HRP (3.25 × 10^{-8} M) and H$_2$O$_2$ (2.0 × 10^{-4} M) after ca. 2 min; [Triton X-100] = 4.6 × 10^{-3} M, pH 5 (0.07 M citrate−phosphate buffer), 25 °C.

Scheme 2

oligomeric and polymeric products

primary products formed in the case of **1** and **2** are not the expected ferricenium cations. The spectra of products have no bands at ca. 610−630 nm, typical of the ferricenium dyes, and the solutions attain brownish-red rather than bright blue color at the end of the reaction. Our attempts to isolate and characterize the reaction product in the case of **1**, which, of course, should be the secondary one, ended in isolation of a small amount of iron-free, electrochemically inactive, according to the cyclic voltammetry data, brownish polymeric material. The true mechanism of the degradation has not been investigated, but it seems probable that the enzymatic electron transfer is followed by steps which afford the intermediate fulvenoid structures. It is known that fulvenes are readily produced from ferrocene derivatives bearing the α-carbenium ion.[18−20] The formation of such in the case of **1** can be envisaged as shown in Scheme 2. Here, the enzymatic electron transfer affords an unstable α-carbenium ion **3**, which transforms rapidly into intermediate fulvene **4**. It might be the fulvene **4** that accounts for the development of a strongly absorbing species.

On assumption that the *primary* oxidation product of **1** and **2** (such as **4**) is the only species formed in the

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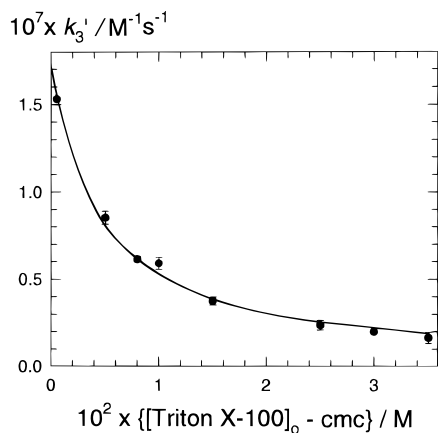
Figure 2. Observed second-order rate constants for HRP-catalyzed oxidation of **2** against the surfactant concentration at pH 5.0, 25 °C, [**2**] = 1.4×10^{-4} M, [H_2O_2] = 2.4×10^{-4} M, [HRP] = 10^{-8} M.

enzymatic reaction, the stoichiometry of the reaction was estimated using Job's method of continuos variations.[21] By the example of **2**, measuring the product absorbance at 1013 nm verified that H_2O_2 and **2** react in a 1:2 molar ratio. Using the procedure developed by us previously,[22] the extinction coefficients for **1** and **2** at 851 and 1013 nm were estimated to be 444 ± 4 and 152 ± 1 M^{-1} cm^{-1}, respectively.

Preliminary kinetic measurements showed that the HRP-catalyzed oxidation of **1** and **2** by H_2O_2 occurs much faster compared to that of alkylferrocenes. Since it is known that in an excess of H_2O_2 the activity of HRP diminishes due to inactivation of the enzyme,[9] the optimal concentration range of hydrogen peroxide was chosen. The rate of oxidation of **1** is independent of H_2O_2 concentration at [H_2O_2] = $(1.6-2.4) \times 10^{-4}$ M, but decreases slightly at higher concentrations. Therefore, the kinetics of oxidation of **1** and **2** was studied at [H_2O_2] = 2.4×10^{-4} M. The kinetic data was obtained at concentrations of **1** or **2** and HRP of $(1.5-14) \times 10^{-4}$ and $(0.6-3.2) \times 10^{-8}$ M, respectively. As in the case of alkylferrocenes,[9] the steady-state rates were linear functions of the substrate and enzyme concentrations in the indicated concentration ranges. Therefore, the rate law is given by eq 1.

$$v_0 = k_3'[\mathbf{1} \text{ or } \mathbf{2}][\text{HRP}] \qquad (1)$$

The observed second-order rate constants k_3' equal $(5.4 \pm 0.2) \times 10^6$ and $(9.2 \pm 0.5) \times 10^6$ M^{-1} s^{-1} for **1** and **2**, respectively, at 25 °C, pH 5, [Triton X-100] = 0.0046 M. These values are approximately by 2 orders of magnitude higher than those for ferrocene.[9] It should, however, be remembered that k_3' were measured in the presence of the surfactant, which strongly affects the rate of enzymatic oxidation.[9,23] To obtain the intrinsic, surfactant-independent rate constants, the effect of Triton X-100 on k_3' was studied. The representative data are shown in Figure 2.

Table 1. Rate Constants k_3 for the HRP-Catalyzed Oxidation of 1 and 2 by H_2O_2 in Aqueous Pseudophase and Distribution Coefficients P_{RFc} at pH 5, 25 °C, [H_2O_2] = 2.4×10^{-4} M

substrate	k_3, M^{-1} s^{-1}	P_{RFc}	reference
1	$(2.4 \pm 0.1) \times 10^7$	270 ± 25	this work
2	$(1.7 \pm 0.1) \times 10^7$	770 ± 50	this work
aniline	2.4×10^5		16
phenol	2.8×10^6		16
ferrocene	1.9×10^5	460 ± 60	9
ABTS[a]	$3.4 \times 10^{6\,b}$		29

[a] ABTS = 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).
[b] The k_{cat}/K_m value.

As seen, the steady-state rate of oxidation of **2** is soundly retarded by Triton X-100, as could be anticipated for such a hydrophobic molecule in the light of our recent work.[9,23] The approach to quantify the dependencies such as in Figure 2 in terms of the Berezin pseudophase model [24,25] applied to enzymatic reactions was described in detail previously.[9] If the reaction follows the rate law of eq 2, as in the present case, the target rate constant k_3 can be evaluated.

$$k_3' = \frac{k_3}{1 + P_{RFc}CV} \qquad (2)$$

Here P_{RFc} is the distribution coefficient for **1** or **2** between the micellar and aqueous phase (P_{RFc} = [RFc]$_m$/[RFc]$_w$), C is the total surfactant concentration without critical micelle concentration (cmc; C = [Triton X-100]$_t$ − cmc), k_3 is the intrinsic rate constant for the interaction between **1** or **2** and HRP in the aqueous pseudophase, and V is the molar volume of micelles (ca. 0.3 cm^3 mol^{-1}). Summarized in Table 1, the parameters of eq 2 (k_3 and P_{RFc}) were calculated by fitting the experimental data such as in Figure 2 to eq 2.

The rate constants in Table 1 show convincingly that the concept of conjugation of two peroxidase substrates of different nature appeared to be fruitful for creation of new ones with increased reactivity. As seen, the "bifunctional" substrates are 10−100 times as reactive as their precursor building blocks. Their reactivity is even higher than that of ABTS! It should also be mentioned that the increase in reactivity is not due to a trivial decrease in the formal redox potential of **1** and **2**. In fact, the $E^{o'}$ for **1** is 0.245 V (versus SCE) to be compared with that of 0.210 V for ferrocene. It seems likely that the conjugated substrates are capable of choosing the most favorable spatial mode of interaction with the HRP compound II, the electron transfer at which is the rate-limiting step under the steady state in the case of ferrocene derivatives.[10]

In addition to HRP, *p*-ferrocenylaniline was shown to be a reactive substrate of laccase. Semiquantitative experiments revealed that laccase catalyzes the oxidation of **1** at pH 5.0 (citrate−phosphate buffer). The enzymatic reaction was accompanied by a substantial absorbance increase, and the spectrum of the product formed was very similar to that in the case of HRP-catalyzed oxidation of **1** by H_2O_2. The product formed

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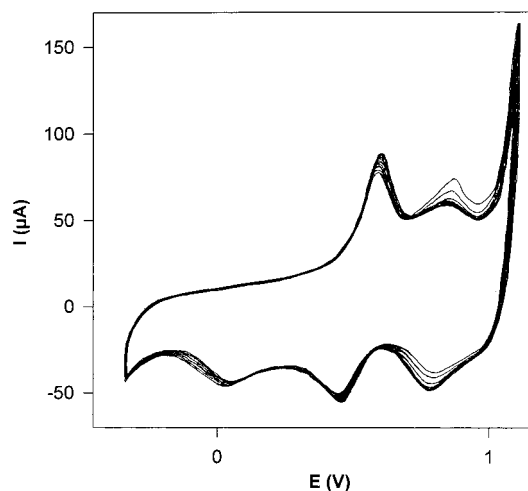
Figure 3. Cyclic voltammograms of electrodeposition of **1** on a carbon electrode at pH 2.4 (HCl) at potential sweep = 10 mV s^{-1} (versus Ag/AgCl).

Figure 4. Cyclic voltammograms of electrodeposited **1** on a carbon electrode in the absence (dashed line) and in the presence (solid line) of NADH (1 mM) in 0.1 M citrate−phosphate buffer, pH 5.0, [Triton X-100] = 0.45 mM, versus Ag/AgCl, scan rate = 10 mV s^{-1}.

in the laccase-catalyzed reaction, likewise the HRP case, is unstable in aqueous solution and undergoes slow decomposition.

Electropolymerization of *p*-Ferrocenylaniline. Films were generated on a carbon rod electrode by cycling the potential in the range from −0.4 to 1.1 V (versus Ag/AgCl) for 1 h at 50 mV s^{-1} from the acidic solution of **1** (0.1 M HCl) in the presence of Triton X-100. Such conditions are frequently used for the electropolymerization of aniline.[6] The cyclic voltammogram of the film thus formed is shown in Figure 3. Its profile is very similar to that formed in the case of aniline.[6] This observation is worth paying attention to, since it is generally assumed that aniline should contain a hydrogen atom in the position para to the amino group, and this is a key prerequisite for the film formation. In contrast to aniline, the para position of **1** is occupied by the ferrocenyl group. Nevertheless, the conducting film is readily formed in the case of **1**.

The film derived from **1** is electrochemically active in acidic and neutral solutions. It was investigated in a citrate−phosphate buffer in the pH range 0.5−7.0. As found, peak voltages were weakly dependent on pH and the slopes of the peak potential vs pH plots were always lower than 30 mV pH^{-1}, typical of the Nerstian behavior for a two-electron−one-proton process. Although the responses were pretty stable in acidic solutions, the peak current decreased gradually with cycling at pH 3−7. Treatment of such electrodes with 0.1 M HCl did not restore the initial peak currents. Thus, the poly-*p*-ferrocenylaniline film is stable in acidic medium only.

Poly-*p*-ferrocenylaniline Film in Mediation of Oxidation of NADH. Being interested in probing the electropolymerized film for biosensor design, we tested its ability to electrooxidase NADH. The data obtained at pH 5 is shown in Figure 4. As seen, there is an increase in anodic current on addition of 1 mM NADH to the poly-*p*-ferrocenylaniline film under such conditions. The sensitivity equals 1.4 μA/mM cm^2.

Mediation of NADH Oxidation by Adsorbed *p*-Ferrocenylaniline Film. Adsorption of the mediating structures onto carbon surfaces offers a simple and rapid technique to prepare chemically modified electrodes for electrocatalytic oxidation of NADH. A cyclic
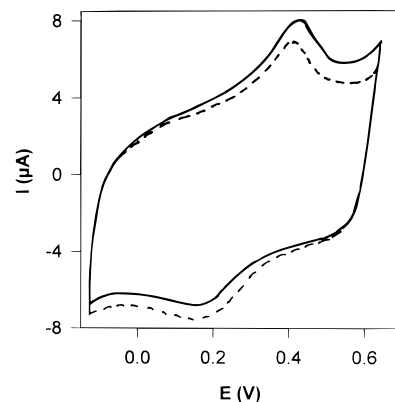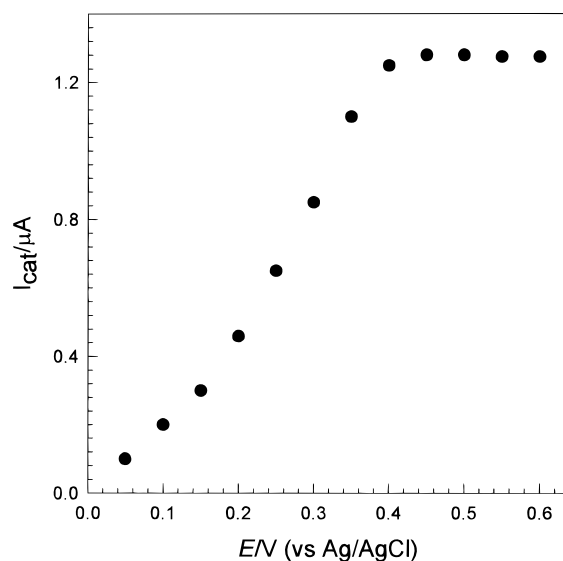

Figure 5. Current for NADH (8 mM) oxidation at a carbon rod electrode with adsorbed **1** as a function of potential (versus Ag/AgCl), pH 7.2 (0.1 M phosphate).

voltammogram of a carbon electrode with **1** adsorbed from the acetone solution recorded at pH 7.2 is characterized by ill-defined oxidation and reduction peaks at 0.4 and 0.1 V, respectively. The formal redox potentials of **1** and ferrocene, as it was mentioned above, are similar. In the presence of 8 mM NADH there is an increase in the anodic current, which is well-defined at potentials >0.05 V. As seen from Figure 5, the current increase is potential dependent, the highest current response being observed at 0.4 V. An example of the calibration curve for the quantifying of NADH is shown in Figure 6. Thus, the film derived from **1**, likewise the film of electropolymerized **1**, can be used for electrooxidation of NADH. The sensitivity of electrooxidation equals 0.25 μA/mM cm^2 at pH 7.2. The electrode thus made has good stability for quite a long time and this makes it more advantageous compared to the electrode obtained via the electropolymerization of **1**.

In conclusion, several examples presented in this work show that *p*-ferrocenylaniline and *p*-ferrocenylphenol are in fact promising materials for analytical biochemistry and bioelectrochemistry. They proved to be very reactive substrates of HRP, and the correspond-
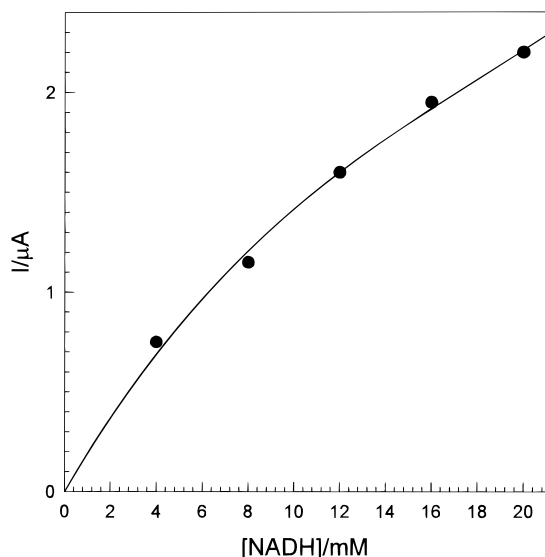
Figure 6. Calibration curve for the electrooxidation of NADH at a carbon rod electrode with adsorbed **1** at 0.4 V (versus Ag/AgCl) and pH 7.2 (0.1 M phosphate).

ing rate constants are comparable with the rate constants for the interaction between the resting state of the enzyme and hydrogen peroxide to form the HRP compound I.[15] Despite being a para-substituted compound, *p*-ferrocenylaniline undergoes electropolymerization to give electrochemically active films. When absorbed on a surface of graphite electrode, *p*-ferrocenylaniline is capable of electrooxidation of NADH, and such an assembly was shown to be a prototype of the corresponding biosensor. It seems convincing that the described above principle of the conjugation of typical substrates of oxidoreductases with organometallic, redox-active fragments could be very prolific in the comprehensive materials science.

Experimental Section

General. NADH (Reanal) was used as received. Triton-100 was a Sigma reagent. The buffer components and other inorganic and organic substrates used in this work were of the highest purity available. *p*-Ferrocenylaniline was prepared by reduction of *p*-ferrocenylnitrobenzene by zinc powder in the presence of HCl.[26,27] *p*-Ferrocenylnitrobenzene was synthesized by the reaction of ferrocene (Aldrich) with $[p\text{-O}_2\text{NC}_4\text{H}_4\text{N}{\equiv}\text{N}]^+$-

Cl⁻ obtained from *p*-nitroaniline and NaNO₂.[26,27] *p*-Ferrocenylphenol was prepared as described elsewhere.[28] HRP isoenzyme C was a Dia-M product (Russia) with RZ = 3.2 (ammonium sulfate precipitate). Laccase was obtained from Sigma.

Spectrophotometric Kinetic Studies. Spectrophotometric measurements were carried out on a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A cell positioner/temperature controller. The kinetic studies of HRP- and laccase-catalyzed oxidation of **1** and **2** were carried out as described in our previous work[9] in terms of preparation of solutions, order of mixing, and following the progress of reactions. In particular, solutions of **1** and **2** were prepared by adding 0.051 g of **1** or **2** to 60 mL of 0.046 M solution of Triton X-100. The mixture was stirred with a magnetic bar for 8 and 1 h in the case of **1** and **2**, respectively, to achieve homogeneity. The solutions were purged with nitrogen gas and kept at 5 °C.

Electrochemical Measurements. Cyclic voltammograms were recorded with a PA-2 polarograph equipped with a 4103 XY recorder. Amperometric measurements were carried out with a LP-7a polarograph. A working electrode was a carbon rod (surface 7 mm²) sealed in a Teflon tube and polished with a corundum abrasive. A saturated Ag/AgCl electrode was used as a reference. A platinum wire was used as an auxiliary electrode.

Two different methods were used for the electrode modification with **1**. In the first case, the working electrode was modified by cycling the potential between 1.1 and −0.4 V (at a sweep rate of 0.05 V s⁻¹) in a HCl solution diluted in 1:10 proportion with a solution of **1** (1 mg/0.5 mL) in citrate−phosphate buffer (pH 5, 0.45 mM Triton X-100). In the second case, the electrode was modified as follows. Compound **1** (1 mg) was dissolved in 6 mL of acetone. The working surface of the electrode was entrapped into a 2 μL of the acetone solution, dried, and rinsed with distilled water. Cyclic voltammograms of the electrode were run in the air-saturated solutions at pH 7.2 in a 0.1 M phosphate buffer. The potential sweep was 10 mV s⁻¹.

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