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Enzyme Containing Redox Polymer Networks for Biosensors or Biofuel Cells: A Photochemical Approach

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A photochemical approach to the generation of (microstructured) redox hydrogels with incorporated enzymes is presented and evaluated with respect to its potential in biosensor and biofuel cell applications. For this, poly(dimethylacrylamide) polymers containing both electroactive ferrocene moieties and photoreactive benzophenone groups are synthesized and deposited as thin films on electrode surfaces. Upon short irradiation with UV light, the polymer layer cross links and becomes firmly adhered to the glassy carbon electrodes. If glucose oxidase is mixed into the polymer solution prior to coating, then glucose-oxidizing electrodes with very high catalytic current responses are obtained. The influence of multivalent ions and proteins on the performance of the electrocatalytic films is studied. It is found that the interaction between bivalent HPO_4^{2-} and the oxidized redox moieties can shorten the lifetime of the redox electrodes significantly whereas the same electrodes are quite stable in the presence of monovalent ions and the reduced form of the mediator. Coating a thin, covalently attached poly(dimethylacrylamide) protective layer onto the redox polymer networks can greatly reduce the adsorption of proteins onto the surfaces and improve the long-term stability of the electrodes in physiological environments. Because the adsorption of proteins onto unprotected surfaces is one of the major causes of bioelectrode failure, this aspect is expected to contribute to the design of more biostable sensors and fuel cells.

Introduction

The immobilization and electrical "wiring" of functional enzymes at electrode surfaces has been the focus of intensive research efforts for several decades. Numerous methods for such electrode modification have been proposed in the literature, and potential fields of application include sensors,[1] fuel cells,[2] and biocomputing logic systems.[3] It is likely that the most successful approach is based on the incorporation of biomolecules into an electroactive redox polymer matrix.[4] Such modified electrodes are the core part of miniature, subcutaneously implantable glucose sensors for continuous diabetes control[5] and are likewise used in biofuel cells operating in a physiological solution.[6] A number of studies have been published that investigate the suitability of various polymers, enzymes, and mediators for the above-mentioned applications.[7−12] However, there are surprisingly few variations in how the actual immobilization reaction is achieved. The two most commonly used approaches are epoxide cross-linked networks[13,14] and electrodes based on the electropolymerization of suitable monomers from an enzyme-containing solution.[15] For both procedures, polymers with specific functional groups are required, which narrows the choice of possible monomers. For example, classical epoxide chemistry depends on nitrogen functionalities in the polymer because they are present in poly(vinylpyridine),[16] poly(vinylimidazole),[17] and poly(allylamine).[7] Electropolymerization, however, is possible only for selected polymerizable monomers, with pyrrole as the most prominent one.[18] Despite the widespread and highly successful use of both techniques, the amendment for restricted classes of base monomers remains a drawback because certain functional moieties will interfere with the intended use of the material. Investigations of the stability of glucose oxidation and oxygen reduction electrodes in the presence of physiologically relevant metabolites and ions revealed that the functionalities of the redox polymer contributed greatly to the rapid loss of catalytic current observed under testing conditions, for example, by physical cross linking in the presence of multivalent ions.[6,19]

Therefore, electrode modification approaches, which are not based on functional sites within the polymer, can contribute to the purposeful design and optimization of electrodes that retain their functionality in a complex chemical environment.

One possible approach is the photoinitiated polymerization of a deposited monomer film on an electrode surface.[20−22] Sirkar

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and Pishko published a respective approach based on the photopolymerization of a mixture of comonomers poly(ethylene glycol) diacrylate PEG-DA, vinylferrocene, and functional glucose oxidase.[23] The precursor solution was manually deposited onto gold electrodes and photo-cross-linked in the presence of an acetophenone photoinitiator, yielding functional, structured electrode coatings. However, because monomer solutions could not be deposited by typical thin film deposition techniques, the resulting films had a thickness of ~100 μm, which resulted in slow photopolymerization due to UV absorption by ferrocene units, thickness-dependent film properties, and a slow response time of the prepared sensors due to mass transport limitations. In contrast to this, the deposition of cross-linkable polymers would allow us to generate polymer coatings with varying thickness.

The entrapment of enzymes in photo-cross-linkable PEG-DA and polyphosphazene films for use in microstructured sensing platforms was also successfully demonstrated, but in both cases, the photostructured polymers did not contain redox units and were thus not intrinsically capable of contacting the immobilized biocatalysts.[24,25]

It was therefore interesting to develop a novel approach to the immobilization of enzymes in redox-active polymer networks, which requires no specific reactive units within the polymer and where the amount of deposited polymer as well as the degree of cross linking can be precisely controlled. For this purpose, we synthesized a redox polymer containing a small number of photoreactive benzophenone units.[26] This polymer can be deposited on the surface of glassy carbon electrodes and cross linked by UV irradiation on the basis of the photochemical reaction between benzophenone and adjacent C−H groups. If cross linking is done in the presence of a biocatalyst, then the latter is incorporated into the polymer network.

This article deals with the preparation and characterization of electrodes modified with thin films of redox polymer networks themselves as well as such films containing a biocatalyst using glucose oxidase as the model enzyme. The feasibility of the approach to the immobilization of the enzyme is evaluated, and the electrodes are characterized with respect to their glucose response, their stability against leaching, the influence of oxygen, and the effect of protein adsorption onto the polymer films.

Experimental Section

Materials. *N*,*N*-Dimethylacrylamide, *N*,*N*-dimethylformamide (DMF), and triethylamine were dried with CaH_2 and distilled under reduced pressure. Methanol was dried using magnesium and iodine and stored over activated molecular sieve (3 Å). All drying procedures were performed under a nitrogen atmosphere, and dried chemicals were stored and handled under nitrogen at all times. DMF was stored over activated molecular sieve (4 Å). All other chemicals were used as received. Glucose oxidase (GOx) from *Aspergillus niger* with a specific activity of 148.4 ku g^{-1} and fibrinogen from pig plasma were obtained from Sigma. 4-Methacryloyloxybenzophenone (**2**) was synthesized from 4-hydroxybenzophenone and methacryloylchloride in the presence of triethylamine in a standard esterification reaction.[27] *N*-Methacryloyl-β-alanine succinimide ester (**3**) was synthesized as reported by Murata et al.[28] The synthesis of [(6-aminohexyl)amino]methyl-ferrocene (**6**) has been reported previously.[26]

Polymerization and Aminolysis. The monomers were copolymerized in a free radical copolymerization procedure in DMF at a total monomer concentration of 1 mol/L and 0.1 mol % 2,2′-azobis(2-methylpropionitrile) (AIBN) initiator. Oxygen was removed by at least five freeze-and-thaw cycles, and polymerization was carried out at 60 °C for 16 h. The polymers were purified by repeated precipitation in diethyl ether and dried under vacuum. Ferrocene-containing polymers were obtained by the aminolysis of the active ester groups in DMF with a ferrocene-modified amino linker as described previously.[26] Again, the polymers were purified by repeated precipitation in diethyl ether and dried under vacuum before use.

XPS Measurements. XPS measurements were carried out using a Physical Electronics 5600 ci spectrometer equipped with a concentric hemispherical analyzer and using an Al Kα X-ray source (15 keV, filament current 20 mA). The samples were investigated under ultrahigh vacuum conditions at 10^{-9}−10^{-8} mbar. Spectra were taken at a 45° angle with respect to the electrode surface. The samples were sputtered for 10 s with argon before measurement. Surface sensitivity factors were used to determine the atomic concentrations. Spectra were calibrated with the C 1s peak of C−H/C−C carbon atoms at 284.8 eV.

Instrumentation. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 250 MHz instrument. Electrochemical experiments were conducted using an Ivium Compactstat electrochemical analyzer controlled by IviumSoft software. The UV irradiation process was carried out with an Oriel 69910 arc lamp from Newport, which was operated with an I-filter cutting off light with a wavelength below 365 nm with an illumination output of 50 mW cm^{-2}. GPC measurements were made in DMF containing 3 g/L LiCl against a PMMA standard. AFM measurements in the dry state were performed in tapping mode on a Digital Instruments IIIa AFM (Veeco) and on a Molecular Imaging Pico Plus (Agilent) instrument in the swollen state.

Electrochemical Experiments. Electrochemical experiments were conducted in a water-jacketed electrochemical cell in acetate buffer (150 mM sodium acetate, adjusted to pH 5.2) at 37 °C unless otherwise noted. The electrolyte volume was 30 mL for all experiments. Glucose concentrations were obtained by adding aliquots of 1 M glucose solution that had been allowed to mutarotate for at least 24 h. A saturated nitrogen atmosphere was obtained by purging the solution with nitrogen for 20 min before the start of an experiment unless stated otherwise. The gas was presaturated with water by passage through a water-filled washing flask. A platinum plate counter electrode and a Ag/AgCl/3 M KCl reference electrode were obtained from Metrohm. The working electrode was an EDI 101 glassy carbon rotating disk electrode obtained from Radiometer with 3 mm glassy carbon tips. The working electrodes were polished on alumina particles (0.3 μm), rinsed with ultrapure water, and dried before use. These steps were repeated several times, and no voltammetric features were observed on the cleaned electrodes in the measurement range in buffer at a 50 mV s^{-1} scan rate. When consecutive electrochemical cycles were recorded, a stabilization scan was conducted beforehand (scan 0).

Electrode Preparation. To obtain electrodes with electroactive polymer or polymer-GOx coatings, the glassy carbon electrode tips were spin coated with a solution containing a total mass concentration of 75 mg/mL. The active electrode area was covered with a 2.5 μL droplet of this solution and spin coated at 2500 rpm for 60 s. Afterwards, the electrodes were UV irradiated for 20 min through a foil mask with a transparent area identical to that of the central glassy carbon rod. The electrodes were washed extensively with ultrapure water and acetate buffer and stored in buffer before use.

Determination of Apparent Electron Diffusion Coefficients. Electron transport properties were studied using both cyclic voltammetry and potential step analysis. Cyclic voltammogramms were recorded at high scan rates between 250 and

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1000 mV s^{-1} to ensure diffusional electron transport. The peak current i of the oxidative scan was then plotted against the square root of the scan rate $v^{1/2}$ and evaluated according to the well-known Randles−Sevcik equation[29]

$$I_p = 0.4463\left(\frac{F^3}{RT}\right)^{1/2} n^{3/2} A D_e^{1/2} c v^{1/2} \quad (1)$$

with F as the Faraday constant, R as the gas constant, n as the number of electrons involved in the process, D_e as the apparent electron diffusion coefficient, c as the concentration of electroactive species, and v as the scan rate.

For potential step analysis, the electrodes were first held at a reducing potential of −0.2 V before the potential was raised to an oxidizing value of 0.7 V for 200 ms. The plot of current i versus the inverse square root of the time $t^{-1/2}$ was evaluated in the linear region according to the Cottrell equation[29]

$$i = \frac{nFAD_e^{1/2}c}{\pi^{1/2}} t^{-1/2} \quad (2)$$

where i is the current density and t is the time.

Calculations and Statistics. Averaged values are reported as the mean ± standard deviation of the mean (SEM). Errors in polymer composition also include an estimation of integral inaccuracy in polymer NMR data. Error propagation was used to estimate the errors in the apparent electron diffusion coefficient D_e.

Results and Discussion

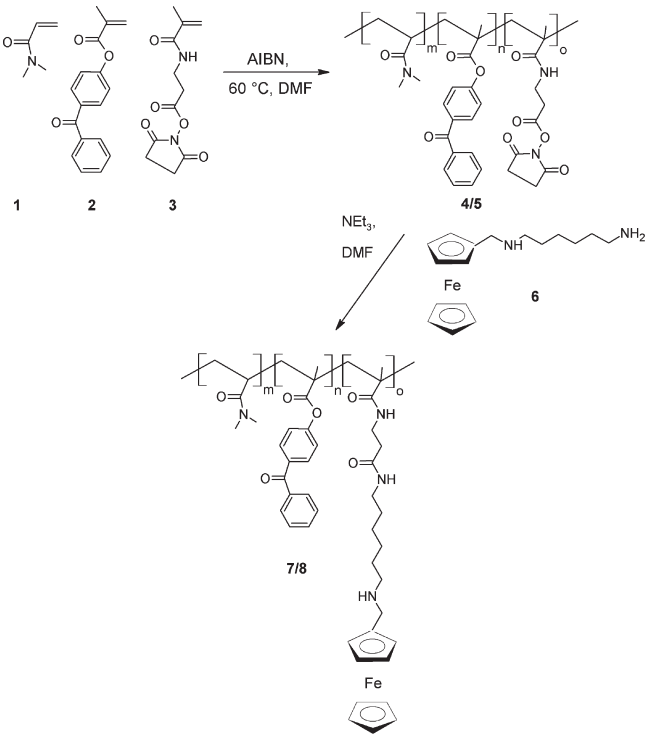
Polymer Synthesis. The structure of the ferrocene-containing redox polymer is shown in Scheme 1. It consists of three specifically chosen monomers. The base constituent dimethylacrylamide (**1**) was chosen because of its good water solubility and the stability of the corresponding polymer under a wide range of different conditions. Because the polymers are neutral, they are hardly affected by the presence of most ions or metabolites, and poly(dimethylacrylamide) polymers have been shown to possess protein-repelling properties.[30,31] Therefore, we consider them to be excellent candidates for the generation of bioelectrodes, which are to be used in a physiological environment.

To allow photochemical cross linking, monomer **2** containing the benzophenone functionality was incorporated. When irradiated with UV light, benzophenone moieties form covalent bonds to any hydrocarbon groups in close proximity. If these belong to adjacent polymer chains, then cross-linking points are formed.[26,27,32] This reaction has been widely used in the literature to immobilize functional biomolecules, and the mechanistic details of the photochemical process have been described previously.[33] In short, the reaction is initiated by a $\pi-\pi^*$ or $n-\pi^*$ transition (depending on the wavelength of light), which leads to hydrogen abstraction from a hydrocarbon group and subsequent radical−radical coupling. When this cross linking takes place in the presence of a codeposited biocatalyst, the latter is entrapped in and covalently attached to the formed 3D network.

The electrical "wiring" of the embedded active catalyst was achieved in this study by the incorporation of ferrocene moieties into the polymers. These were attached to the polymer backbone in a two-step process: First, NHS-active ester monomer **3** was copolymerized with the other two monomers in a free radical copolymerization step. The NHS functional groups can be used to introduce a variety of redox mediators functionalized with free amino groups. In the present case, aminolysis of the active ester was done with ferrocene-modified amino linker [(6-aminohexyl)-amino]methylferrocene (**6**). Test experiments had shown that the reaction of active ester monomer MAC_2AE with the amino linker takes place mainly at the primary and not at the secondary amino group, which is easily understood because the steric hindrance at the secondary amino group is much more pronounced than at the primary amine. The redox activity of the photo-cross-linkable polymers was thus based on ferrocenylmethylamino structures, which are known to be efficient mediators for the electron transfer from the glucose oxidase active centers.[34] Because the secondary amino group will be protonated under physiological conditions, the polymers carry under these conditions an overall positive charge, which is expected to enhance complexation with the negatively charged glucose oxidase. These attractive ionic forces between the catalyst and the polymeric "wire" should reduce the possibility of microphase separation during electrode coating.

Scheme 1 shows the described synthesis route toward ferrocene-containing photo-cross-linkable redox polymers. The final compositions of all polymers used in this study were determined from UV−vis and NMR measurements, and the molecular weights were determined by GPC (Table 1). Because of its good water solubility of up to 75 mg/mL, which allowed the easy codeposition with functional enzymes from an aqueous solution, polymer **7** was chosen for the electrochemical experiments shown in this study. It contained 7% benzophenone and 10% ferrocene, and GPC analysis indicated an M_w of 114 000 g mol^{-1} and a

Scheme 1. Synthesis of Photo-Cross-Linkable Ferrocene Redox Polymers[a]



[a] See the text for details.

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Table 1. Overview of Polymers Used in This Study

polymer	monomer content	content in polymer	M_w (g mol^{-1})	M_n (g mol^{-1})	D
4	5% MABP, 10% MAC_2AE	(6.8 ± 1.0) % MABP, (10.2 ± 0.5) % MAC_2AE[a]	105 000	32 600	3.22
5	5% MABP, 50% MAC_2AE	(5.3 ± 0.5) % MABP, (38.8 ± 2.0) % MAC_2AE[a]	189 000	58 300	3.24
7	not applicable	(6.8 ± 1.0) % MABP, (10.2 ± 0.5) % Fc	114 000	34 700	3.29
8	not applicable	(5.3 ± 0.5) % MABP, (38.8 ± 2.0) % Fc	129 900	59 700	2.17
9	2.5% MABP	(2.2 ± 0.2) % MABP	150 000	53 500	2.81

[a] Composition estimated assuming a 1:1 conversion of active ester units during aminolysis.

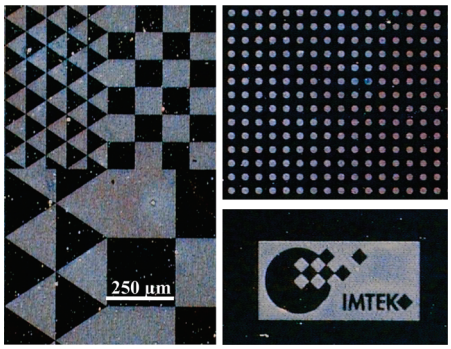

Figure 1. Microscope images of a silanized silicon wafer modified with thin films of the redox polymer in varying shapes. The modification procedure consisted of a spin-coating procedure from aqueous solution, followed by irradiation at 365 nm through a chrome mask and washing with water to remove any unbound material. The polymer-coated areas appear bright.

polydispersity of 3.3. Thus, on average, there are 16 potential cross-linking units per polymer chain. For XPS measurements, a high ferrocene content was desired, which was the motivation to use polymer **8**, whereas protein-repellent electrode coatings were obtained from polymer **9**.

Photostructured Polymer Networks. Stable coatings on glassy carbon substrates were obtained by applying a thin polymer coating in a spin-casting process and subsequently irradiating the layer with UV light. If desired, this step can be combined with standard mask technology to yield photolithographically structured redox polymer network arrays. These easily obtained structures may, for example, be used in the development of bioelectrode arrays or structured biofuel cell chips. To demonstrate the feasibility of the process, we spin coated a thin layer of the redox polymer onto a silicon wafer modified with photoreactive benzophenone silane.[35] The subsequent irradiation step was done through a chrome mask to generate different geometric structures. After cross linking, the polymer could be removed from the unexposed areas by extensive washing with water, and well-defined geometrical structures of the redox polymer network were obtained (Figure 1).

As can be seen, the surface-deposited redox polymer film could be structured in various shapes and sizes using the described fast, simple photolithographical procedure. Structures with sizes in the range from 250 to 25 μm with good spatial resolution were demonstrated. Still smaller structures are feasible but are beyond the scope of this work. These results demonstrate that photochemical cross linking can be used to generate arrays of electroactive polymer networks, which are of potential interest in applications based on structured bioelectrodes. This is a feature that clearly distinguishes the described photolithographical approach from thermal cross-linking strategies,[4−6] which do not allow the fabrication of structured polymer arrays.

The thickness of the films was analyzed by AFM measurements for a typical film on a silicon wafer in dry and swollen states. Accordingly, the dry film had a thickness of d_d = (103 ± 13) nm that increased upon hydration to a value of d_{sw} = (260 ± 19) nm. These values correspond to a swelling factor of ~2.5, which should be high enough to obtain redox hydrogels that contain sufficiently mobile redox centers but also possess sufficient mechanical stability.

Electrochemistry of Redox Polymer. For all electrochemical experiments, glassy carbon rod electrode tips were modified by spin coating the redox polymer or a mixed solution of redox polymer and GOx onto them and irradiating through a foil mask, which was transparent in the area of the central glassy carbon rod. Electrodes modified only with the redox polymer (i.e., without the presence of enzyme) gave a well-defined current response upon electrochemical cycling with a half-wave potential of about 410 mV versus Ag/AgCl in 150 mM acetate buffer. Figure 2a shows typical cyclic voltammograms at low scan rates. The peaks of the oxidation and the reduction process show a peak separation between 12 and 33 mV at scan rates increasing from 5 to 20 mV s^{-1}, the peak current increases nearly linearly with the scan rate, and the total charge over the baseline is constant. These findings suggest that the electron transport is mainly surface-controlled under these conditions, with the timescale of the experiment being long enough to allow complete charge depletion of the film. With increasing scan rate and thus decreasing experimental timescale, the peak current is proportional to the square root of the scan rate (Figure 2b,c), which is the expected characteristic of a redox polymer film controlled by semi-infinite diffusion.[36] Under these conditions, the experimental timescale is too short to allow the electrochemical conversion of all redox-active units and the electron exchange between the redox moieties determines the current response.

The electron-transport characteristics were evaluated using the Randles−Sevcik equation (eq 1), and a value for $cD_e^{1/2}$ of (7.2 ± 0.2) × 10^{-9} mol cm^{-2} s$^{-1/2}$ was obtained from three independent electrodes. Chronoamperometric experiments with the same electrodes (Supporting Information) gave a value of (1.2 ± 0.1) × 10^{-8} mol cm^{-2} s$^{-1/2}$.

Taking into account the film thicknesses in the dry and swollen states that were obtained from AFM measurements, the corresponding values for the apparent electron diffusion coefficients were calculated to be (6.7 ± 0.7) × 10^{-10} cm^2 s^{-1} and (1.9 ± 0.4) × 10^{-9} cm^2 s^{-1}, respectively. These values suggest relatively fast charge propagation through the redox hydrogels, which is a key requirement for effective electrode coatings. The absolute values are typical of ferrocene-containing redox hydrogels in which D_e varies in the range from 10^{-12} to 10^{-6} cm^2 s^{-1}.[23,37] It has also been shown that the absolute values for D_e can depend significantly on the applied method, and the values obtained by cyclic voltammetry

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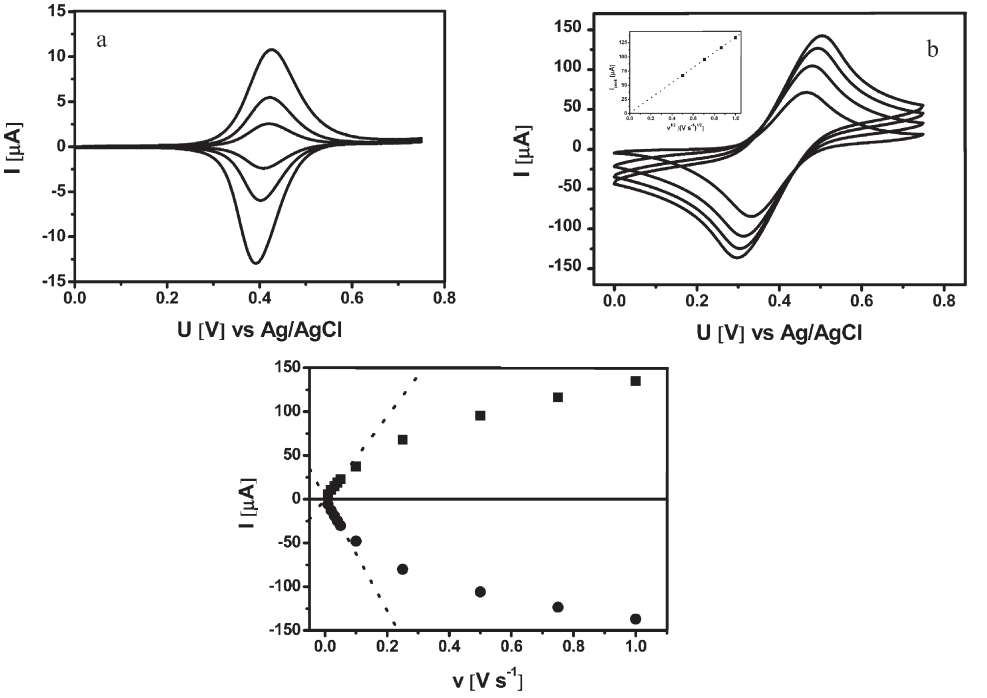

Figure 2. Cyclic voltammograms of polymer **7** on glassy carbon electrodes in acetate buffer (pH 5.2) (a) at a low scan rate (5, 10, and 20 mV s^{-1}) and (b) at a high scan rate (250, 500, 750, and 1000 mV s^{-1}) with an inset plot showing the peak current vs the square root of the scan rate. (c) Plot of peak currents against the scan rate with squares for the values of the oxidation step and circles indicating the reduction is shown with dashed lines showing the linear current increase with scan rate in the low-scan-rate region.

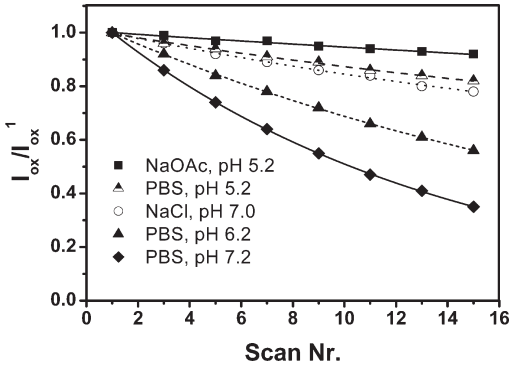

Figure 3. Typical oxidation peak currents I_{ox} for consecutive scans on glassy carbon electrodes modified with polymer **7**. The scans were recorded at a scan rate of 50 mV s^{-1} in various electrolytes given in the inset, and values are normalized to the oxidation current I_{ox}^{1} in scan 1.

are typically lower than those from potential step analysis by a factor of 2.5 to 10.[10]

Effect of Electrolyte on Redox Polymer Stability. In an electrochemical experiment, the electrolyte used is always of central importance. Therefore, the stability of the modified electrodes against repeated electrochemical cycling was tested under different conditions and was found to depend significantly on the type and pH of the electrolyte. Figure 3 shows the normalized oxidation peak currents for consecutive scans in different electrolytes. In acetate- and phosphate-buffered solution at pH 5, only small changes in the peak current with the scan number were observed. The same was true for physiological sodium chloride solution at pH 7, whereas in physiological PBS buffer at pH 7 the observed peak currents decreased by about 4% with each subsequent scan. No plateau was reached. In PBS buffer at pH 6, the electrodes were more stable than at pH 7 yet significantly less stable than at pH 5. This strong correlation between the repeatability of the redox process and the composition and pH of the electrolyte indicates that the observed peak current decrease is a function of the ions present in solution. The electrolyte plays a key role in electrochemical processes of redox polymers because any electronic charge-transport step is accompanied by an opposing ionic charge-transport step to maintain overall charge neutrality.[38,39] In ferrocene-containing redox polymers, the oxidation of the redox moieties leads to an uptake of anions into the hydrogels. If this process is irreversible, then the polymers will lose their electroactivity with time. PBS buffer at physiological pH contains three different anions that have to be considered: Cl^- as the main constituent; $H_2PO_4^-$, which is the dominant form of the phosphate anion at around pH 5; and HPO_4^{2-}, which becomes more abundant at pH >6. Thus, the redox polymer seems to be very stable in the presence of monovalent Cl^- and $H_2PO_4^-$ but less stable as the fraction of divalent HPO_4^{2-} in the buffer increases. To confirm the assumed irreversible uptake of phosphate anions, XP spectra were measured for electrodes modified with polymer **8**, which had been subjected to multiple electrochemical cycles in PBS buffer at pH 7 and washed with distilled water afterward. For comparison, a second electrode was measured, which had been soaked in PBS buffer, but not electrochemically cycled. Figure 4 shows the respective XPS survey and detail spectra for polymer **8**.

As can be clearly seen, only traces of phosphorus were found in the film after prolonged soaking of the electrodes in PBS solution. However, if an electrode was subjected to electrochemical cycling, then the P 2s and P 2p signals increased significantly. The P 2p signal was located at 133.3 eV, which is very close to the value

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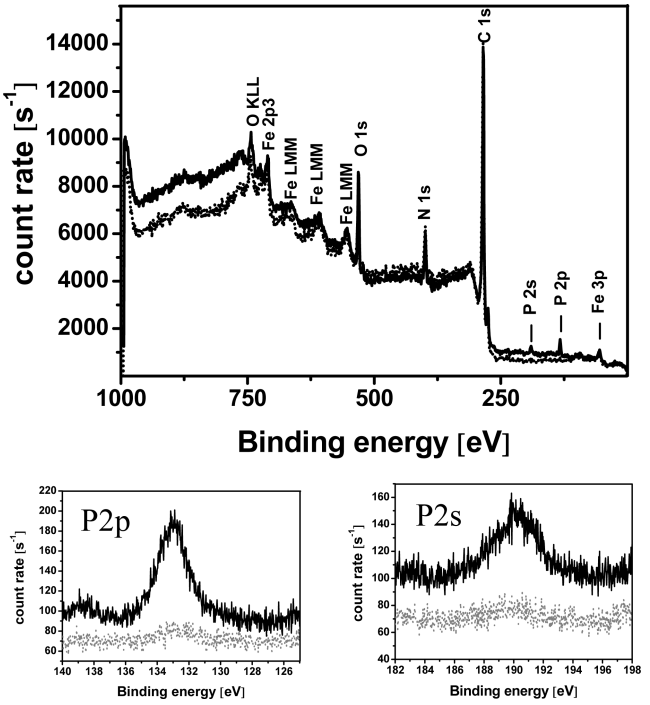

Figure 4. XP survey spectrum of glassy carbon electrodes modified with polymer **8** after soaking in PBS buffer for 3 h and subsequent washing with water (black solid line) and after subjection to 30 electrochemical cycles (50−750 mV vs Ag/AgCl, 50 mV s^{-1}) and subsequent washing with water (gray dashed line). The smaller graphs show detailed spectra for the P 2p and the P 2s peaks.

observed for Na_2HPO_4 (133.1 eV), while monobasic phosphate with sodium as a counterion (NaH_2PO_4) has a signal shifted to slightly higher energy (134.2 eV).[40] These values indicate that the incorporated phosphate is most likely in the dibasic form. The estimated atomic concentrations of phosphor and iron were 2.3 and 2.5%, respectively, implying a roughly 1:1 complexation of the ferrocinium ions with phosphate. The theoretical value calculated on the basis of the polymer composition and 1:1 complexation would be 2.1% for both elements, in close agreement with the observed values.

It is worth noting that electrodes containing active GOx that were measured at a low scan rate in the presence of glucose (i.e., under conditions in which the fraction of redox units in the oxidized form was kept to a minimum) were significantly more stable. A typical electrode made from polymer **7** with 25% GOx lost about 90% of the initial peak current in 15 consecutive scans at 5 mV s^{-1} in PBS buffer at pH 7, compared to 7 to 8% if 15 mM glucose was added.

In conclusion, the XPS analysis gives strong evidence that the loss of electroactivity is due to an irreversible uptake of phosphate into the polymer film upon oxidation of the redox sites. Electrochemical experiments carried out at different pH values suggest that the bivalent form of the phosphate anion, HPO_4^{2-}, is most likely incorporated. A possible explanation is that an ionic interaction between this anion and the oxidized ferrocinium structure might render the linker to the polymer backbone less mobile, thus reducing the ability of the redox moieties to undergo electron transfer. Such complexation may be enhanced by the presence of the nearby charged amino functionality, as is often the case for low-molecular-weight ferrocene structures.[41−43] These findings are in agreement with recent studies by Merchant et al. on ferrocene-modified PEI polymers, which were found to undergo an irreversible redox process in the presence of dibasic phosphate.[44,45] Besides the ferrocenylmethylamino structure, PEI polymers contain pH-sensitive backbone amino groups that are partially deprotonated by dibasic phosphate, resulting in a deswelling of the networks. The authors suggest two possible deactivation mechanisms based on an interaction between HPO_4^{2-} and either the PEI backbone or the ferrocinium ion itself. Because the polymers reported in this study do not contain any pH-sensitive backbone functionalities, it seems reasonable that the ferrociniummethylammonium structure that is formed at oxidizing potentials directly interacts with the multivalent dibasic phosphate.

On the basis of these considerations, we assumed that the electrochemical stability of the ferrocene polymer networks may be improved by substituting the amino group against an uncharged functionality. Respective studies in our laboratory recently confirmed this assumption.[26]

Enzyme Immobilization and Electrical Contacting. To demonstrate that the generated redox polymer networks are suitable for the immobilization and "wiring" of functional biomolecules, we prepared electrodes with varying contents of GOx. To obtain these, the polymer was mixed with enzyme before film deposition and photochemical cross linking. During photoprocessing, the benzophenone moieties do not differentiate between other polymer segments and enzyme. Even if the enzyme molecules should not participate in the photoprocess they will become entrapped in the film. Such a process has very strong advantages compared to film generation followed by diffusion of the enzyme into the film. This is especially important to the background that surface-attached (i.e., 2D) networks do not swell as strongly as the corresponding 3D networks and that the high segment concentration in the film caused by this leads to a more or less rapid diffusion of macromolecules out of the film for entropic reasons.[27]

Cyclic voltammograms were recorded at a low scan rate in acetate buffer, in which the redox hydrogels were sufficiently stable. As substrate for the enzymatic reaction, 15 mM glucose was present. This allows us to test whether the enzyme has been incorporated into the polymer network and has communicates effectively with the electrode. Figure 5a shows the voltammetric responses that were obtained for GOx contents of 0, 5, and 25%.

In the absence of GOx, only the redox response of the ferrocene moieties is detected, whereas the anodic current increases strongly in the presence of the enzyme, indicating the effective communication of the redox-active polymer with the active center of the reduced glucose oxidase. The oxidation peak currents are plotted in Figure 5b for enzyme contents between 5 and 55% in the as-prepared solution. High currents due to glucose oxidation were found for all electrodes, indicating that the photochemical immobilization of biocatalysts in the given redox-active polymer is an effective way to prepare bioelectrodes. The maximum catalytic current is observed for a glucose oxidase concentration

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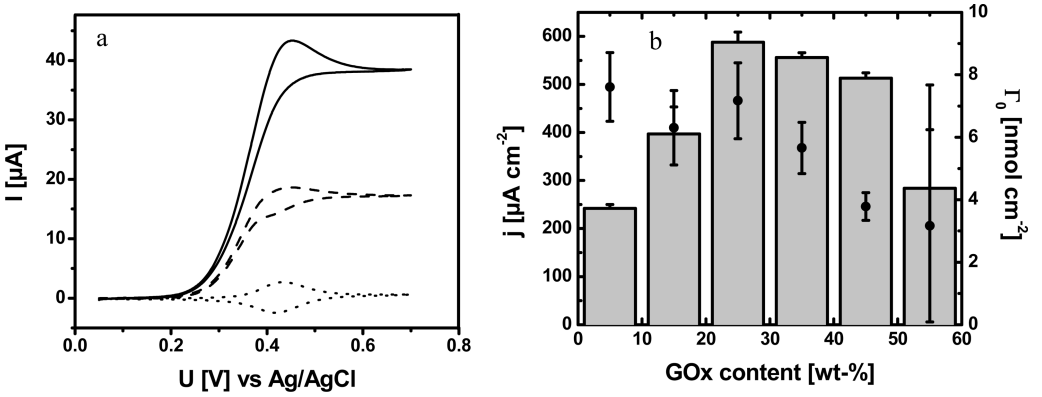

Figure 5. (a) Cyclic voltammograms at a 5 mV s^{-1} scan rate in the presence of 15 mM glucose in acetate buffer at pH 5.2, with electrodes prepared from solution containing 0 (···), 5 (- -), and 25 wt % GOx (—). (b) Peak current densities (bar chart) and ferrocene surface coverage (scatter diagram) depending on the content of enzyme in the spin-coating solution.

of 25−30 wt %, indicating an optimum ratio of enzyme active centers to electron mediator units in the respective concentration region. Therefore, electrodes were prepared in all further experiments with an initial GOx content of 25%.

The surface coverage with electroactive material as obtained from charge over baseline measurement decreases with increasing GOx content. This is the expected behavior if an increasing content of electroinactive material, such as an enzyme, is added to a redox-active thin film. At very high enzyme contents above 50 wt %, strong precipitation was observed due to ionic interactions between the enzyme and the redox polymer. All solutions prepared at such high concentration showed the precipitation of an enzyme−polymer complex, which led to problems with the reproducibility of the film-preparation process. At even higher GOx contents, no coherent electrode coatings could be obtained.

One of the central questions of any enzyme-immobilization strategy is how the generated surfaces are affected by the leaching of active material, which can significantly reduce the operating time of the device.[46] Therefore, we studied how the current response of the generated enzyme electrodes was affected by a combination of electrochemical load and mechanical stress. For this purpose, we measured the steady-state current response of shear-stressed electrodes rotating at 500 rpm in a 10 mM glucose solution. The experiments were conducted at an oxidizing potential of 0.45 V in a nitrogen atmosphere, and the results are presented in Figure 6. As expected from the findings described before, the stability of the electrodes was found to depend on the electrolyte used. In PBS buffer at pH 7, rapid current loss was observed, with only 16% of the initial current response remaining after the 3 h testing period. These findings are in agreement with our initial studies on the electrolyte-dependent stability of the redox hydrogel electrodes. In acetate and PBS buffer at pH 5.2, around 10% of the initial current density was lost within the test period of 3 h. The current loss was more pronounced in the presence of oxygen. For example, the current density decreased by about 30% when measured under ambient conditions in acetate buffer. These values are comparable to those obtained with established osmium-based polymers generated by conventional cross linking.[19]

After 48 h hours of storage in buffer at 4 °C, the current response of the electrodes to 5 mM glucose had dropped to about 60% of the initial current response. Thus, on a longer time scale,

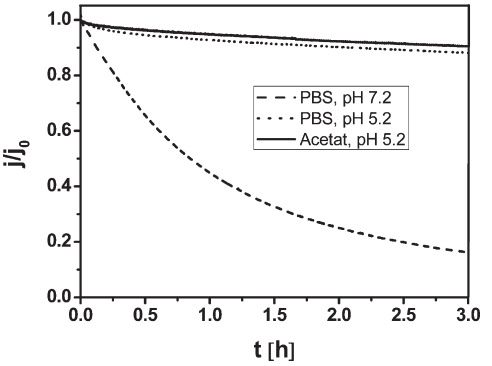

Figure 6. Stability of electrodes prepared from a solution of polymer **7** with 25% GOx in nitrogen-saturated 10 mM glucose solution, electrodes were poised at 0.45 V vs Ag/AgCl and rotated at 500 rpm, the current response with time is shown normalized to the initial value in acetate buffer (—), PBS at pH 5 (···), and PBS at pH 7 (---).

deactivation of the electrodes seems to become relevant, which is a typical observation in enzyme-based bioelectrodes.[46] A number of different factors could contribute to this, including bleeding out of polymer or enzyme, a loss in activity of either, or the failure of films to adhere to the glassy carbon electrode surface.

In conclusion, the cross-linking method demonstrated in this study is very efficient in terms of enzyme immobilization and has a number of additional strong points compared to other methods discussed before.

For further evaluation of the suitability of the electrodes, the glucose-dependent steady-state currents were measured. Figure 8 shows the plots of current density versus glucose concentration in a saturated nitrogen atmosphere and under ambient conditions for an electrode obtained from a 25% GOx solution. The current density follows Michaelis−Menten-like behavior in both cases. It is worth noting that the presence of ambient oxygen results only in a slight losses of current density. At 5 mM glucose concentration, the current density in the presence of ambient oxygen is about 12% lower than in a nitrogen-saturated solution, and this value decreases to 4% for glucose-saturated conditions. This indicates a very efficient electron transfer from the enzyme cofactor to the mediator even in the presence of the natural electron acceptor oxygen. Under ambient conditions, the apparent Michaelis−Menten constant K_M and the saturating current density j_{max} were measured for five independent electrodes, giving values of K_M = (11.5 ± 1.2) mM and j_{max} = (1193 ± 75) μA cm^{-2}, respectively. The sensitivity calculated from the current response at 5 mM

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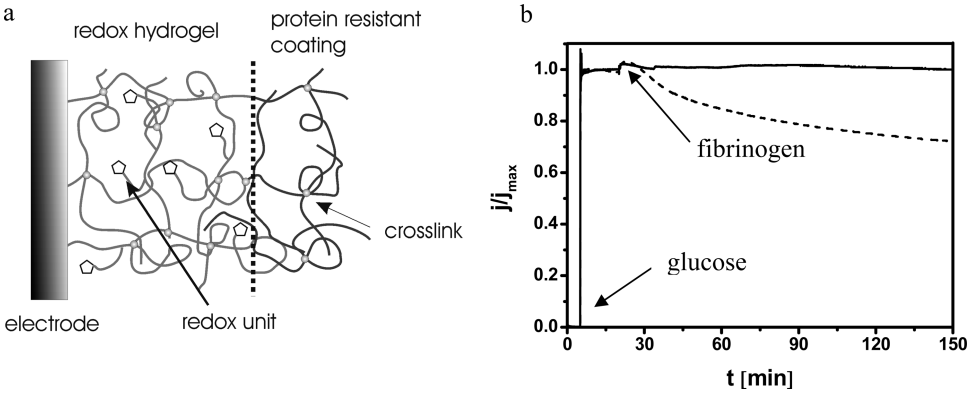

Figure 7. Increased stability of redox electrodes by entropic shielding: (a) scheme of the redox-active matrix (ferrocene units indicated by polygons) that is coated with a layer of a covalently attached, protein-repellent hydrogel and (b) current response in acetate buffer to the subsequent addition of glucose and fibrinogen with final solution concentrations of 10 mM glucose and 1 mg/mL fibrinogen. To the pure electrolyte, a glucose solution was added after 5 min, and a solution of fibrinogen in water was added after 25 min of total experiment time; the dashed line shows the response of the control electrode, and the solid line, that of an electrode modified with p(DMAA-2.5% MABP).

glucose concentration was 75.5 ± 5.2 μA mM^{-1} cm^{-2}. These values are promisingly high; other studies on ferrocene-based glucose oxidase electrodes often report significantly lower catalytic currents. For example, the saturating current in poly(acrylamide) systems was found to be 13.5 μA cm^{-2},[47] whereas 60 μA cm^{-2} was measured for poly(allylamine)-based electrodes.[7] To the best of our knowledge, the highest current density obtained so far for ferrocene-based sensors incorporating glucose oxidase is 1.2 mA cm^{-2}, a value that is identical to the one reported in this study.[45] These results show that the photochemical approach reported here can be used to prepare highly active glucose-oxidizing electrodes that are promising candidates in sensing and fuel cell applications. It is worth noting in the context of our previous report that the height of the electrooxidation current depends not only on the polymer itself but also on the morphology of the films and thus the method of electrode modification.

Fouling Caused by Protein Adsorption. When the application of any biosensor or biofuel cell in a physiological environment is considered, it is vitally important that the device retains its activity in the presence of various interfering substances. One of the main pathways for electrode deactivation that effectively prevents the use of continuous glucose sensors in the bloodstream to the present day is the unspecific adsorption of proteins to the surface of implants.[1,48,49] This surface fouling initiates thrombus formation and suppresses the response of the device to blood glucose. A promising technique to reduce this biofouling process is the coating of the electrodes with a protein-repelling polymeric membrane.[50,51]

In the present study, we therefore investigated whether protein adsorption can affect the electrochemical response of the glucose electrodes. We were especially interested to demonstrate that because of the versatility of the photochemical cross-linking procedure it could also be used to apply protein-repellent hydrogel layers on top of the electroactive films as shown in Figure 7a. To distinguish between deactivation caused by protein adsorption and by other deactivation mechanisms such as irreversible phosphate uptake, we performed these preliminary experiments in acetate buffer. The electrode response to the addition of fibrinogen was tested for a control electrode without a protein-repellent layer and a test electrode that had been modified by a p(DMAA-2.2% MABP) film using spin coating from *i*-PrOH and subsequent cross linking. The oxidative current responses with time are shown in Figure 7b. The baseline current was first measured in glucose-free electrolyte before 10 mM glucose was added after 5 min. Aqueous fibrinogen solution giving a final fibrinogen concentration of 1 mg/mL was added after 20 min of measurement time.

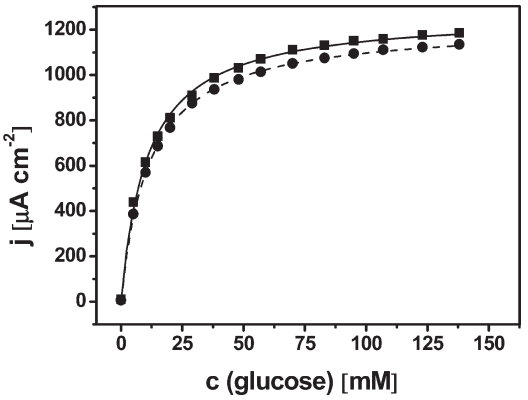

Figure 8. Steady-state current densities obtained for glucose-oxidizing electrodes in a nitrogen-saturated atmosphere (■) and in the presence of ambient oxygen (●); the electrodes were poised at 0.45 V vs Ag/AgCl and rotated at 1000 rpm, and the lines are fits to the Hill function.

As can be clearly seen in Figure 7b, the current of the control electrode drops to about three-fourths of its initial value within two hours after fibrinogen addition because the enzyme adsorbs to the surface of the electrode. In contrast, the current response of the pDMAA-coated test electrode is significantly more stable; it shows only some small changes during the experiment and remains essentially at the same level. Thus, it can be concluded that the approach to the immobilization of biocatalysts in electroactive films demonstrated in this work does not have only the promising features of fast enzyme immobilization and photostructuring but also is particularly interesting for applying additional polymer membranes to protect the electrodes from deactivation by protein fouling. Further work on this aspect, especially in long-term experiments, is currently being conducted in our laboratory.

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Conclusions

The incorporation of photoreactive benzophenone groups into ferrocene-group-containing polymers allows the photo-cross-linking of thin polymer coatings to form surface-attached, water-swellable redox hydrogels. Network formation is fast (minutes to seconds, depending on the wavelength and intensity of the light) and independent of the presence of specific functional groups in the polymer, which allows the combination of the redox-active groups with a wide range of polymer chemistries. Micropatterned redox hydrogel arrays can easily be obtained if the irradiation inducing the photoreaction is carried out through suitable masks. The obtained structures can be used for the generation of highly parallel bioelectrode arrays, a prerequisite for multianalyte/self-calibrating sensors. Enzyme electrodes photogenerated from mixed solutions of enzyme and redox polymer gave high catalytic current responses upon addition of glucose, even in the presence of ambient oxygen. Covalent binding and entrapping of the enzyme into the network lead to systems that are reasonably stable against leaching of active material even when exposed to solvent for prolonged periods of time.

A potential source of electrode failure in a physiological environment is the interaction of the mediator hydrogel with multivalent ions, especially phosphate, calcium, and certain transition-metal ions, which are all ubiquously present in physiological systems. The electroactive polymer containing ferrocenylmethylamino groups used in this study showed an irreversible uptake of bivalent phosphate (HPO_4^{2-}) ions if many ferrocinium units were in the oxidized state, with a concurrent loss of electroactivity. The interaction of the mediator with divalent ions in the contacting medium presumably causes additional (physical) cross linking and thus deswelling of the redox gels, which apparently reduces the electroactivity of the mediator film. However, electroactivity is retained for prolonged periods of time when the ferrocene mediator is kept mostly in the reduced form or if only monovalent ions are present. The latter renders systems containing phosphate ions susceptible to small changes in pH in the range where these changes influence the $HPO_4^{2-}/H_2PO_4^-$ equilibrium.

An important feature of all bioelectrode applications is the stability of the system against protein adsorption. The adsorption of proteins onto surfaces in a physiological environment will prevent the diffusion of substrate to the electroactive layer. On implanted devices, it will cause side effects such as thrombus formation or inflammatory reactions. We have addressed this important problem using the concept of "entropic shielding" by neutral, water-swollen polymer networks. For this, we deposited a second hydrogel coating obtained from uncharged poly(dimethylacrylamide) that did not carry any ferrocene units by the same photochemical process onto the glucose-oxidizing electrodes. Because the photoreaction leads to covalent binding of the newly forming network onto the redox hydrogel, delamination is prevented even when the networks are strongly swollen. Using this approach, we have demonstrated via the example of fibrinogen that protein adsorption can be suppressed and that glucose-oxidation electrodes can be stabilized significantly. Such nonfouling or at least reduced fouling systems are expected to help in the design of more biostable enzyme-based electrodes.

The results of this study show that the proposed strategy is suitable for the simultaneous immobilization and electrical wiring of a biocatalyst in an electroactive hydrogel matrix. The combination of the different features of the production process such as fast and reproducible film deposition, flexibility with respect to deposition protocol and polymer chemistry, and high functionality and stability of the obtained films makes the photochemical procedure described here very attractive for the generation of enzyme-containing redox hydrogel films on electrode surfaces.

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Supporting Information Available: AFM characterization, information on the calculation of D_e, and exemplary potential step current response. This material is available free of charge via the Internet at http://pubs.acs.org.