Fast and Easy Enzyme Immobilization by Photoinitiated Polymerization for Efficient Bioelectrochemical Devices

ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 2011

Impact Factor: 5.64 · DOI: 10.1021/ac200297r · Source: PubMed

4 AUTHORS, INCLUDING:

Vincent Studer
French National Centre for Scientific Research
43 PUBLICATIONS **1,475** CITATIONS
SEE PROFILE

Neso Sojic
Ecole Nationale Supérieure de Chimie, de Bi…
86 PUBLICATIONS **1,052** CITATIONS
SEE PROFILE

Nicolas Mano
Centre de Recherche Paul Pascal
119 PUBLICATIONS **3,684** CITATIONS
SEE PROFILE

analytical chemistry

TECHNICAL NOTE

pubs.acs.org/ac

Fast and Easy Enzyme Immobilization by Photoinitiated Polymerization for Efficient Bioelectrochemical Devices

Emmanuel Suraniti,[†] Vincent Studer,[‡,∥] Neso Sojic,[§] and Nicolas Mano*[,†]

[†]Université de Bordeaux, Centre de Recherche Paul Pascal, CNRS UPR 8641, 115 avenue Schweitzer, 33600 Pessac, France

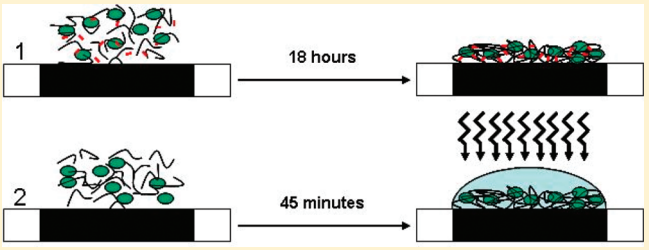
[‡]Université de Bordeaux, Interdisciplinary Institute for Neuroscience, UMR 5297, F-33000 Bordeaux, France

[∥]CNRS, Interdisciplinary Institute for Neuroscience, UMR 5297, F-33000 Bordeaux, France

[§]Groupe Nanosystèmes Analytiques, Institut des Sciences Moléculaires, CNRS UMR 5255, Université Bordeaux 1, ENSCPB, 16 avenue Pey-Berland, 33607 Pessac, France

Supporting Information

ABSTRACT: Immobilization and electrical wiring of enzymes is of particular importance for the elaboration of efficient biosensors and can be cumbersome. Here, we report a fast and easy protocol for enzyme immobilization, and as a proof of concept, we applied it to the immobilization of bilirubin oxidase, a labile enzyme. In the first step, bilirubin oxidase is mixed with a redox hydrogel "wiring" the enzyme reaction centers to electrodes. Then, this adduct is covered by an outer layer of PEGDA made by photoinitiated polymerization of poly(ethylene-glycol) diacrylate (PEGDA) and a photoclivable precursor, DAROCUR. This two-step protocol is 18 times faster than the current state-of-the-art protocol and leads to currents 25% higher. In addition, the outer layer of PEGDA acts as a protective layer increasing the lifetime of the electrode by 100% when operating continuously for 2000 s and by 60% when kept in dry state for 24 h. This new protocol is particularly appropriate for labile enzymes that quickly denaturate. In addition, by tuning the ratio PEGDA/DAROCUR, it is possible to make the enzyme electrodes even more active or more stable.



Immobilization and electrical wiring of enzymes have been the focus of intensive research for the past decade because they are two key steps for the elaboration of efficient enzymatic devices. Numerous strategies have been developed to immobilize enzymes on the electrode surfaces.[1,2] However most of these protocols are often cumbersome and time-consuming, some of them requiring up to 8 days.[3] One strategy pioneered by Heller and co-workers is based on the electrical "wiring" of enzymes that connects redox centers of enzymes to electrode surfaces through a redox hydrogel.[4] Redox hydrogels constitute the only electron-conducting phase in which the permeation of water-soluble biological reactants, products, ions, and electrons are rapid. In addition, the three-dimensional hydrogels obviate the need for orienting the enzyme molecules on the electrode surfaces. Phase separation between the redox hydrogel and the enzyme is avoided by forming an electrostatic adduct between the polyanionic enzyme and the polycationic redox polymer. To further increase the stability of the bioelectrocatalyst and prevent any leaching from the electrode surface, a step of cross-linking between the enzyme and the redox hydrogel using a poly(ethylene glycol) diglycidyl ether cross-linker is also needed. In these systems, an 18 h curing time is required to achieve efficient cross-linking.

Leaching is one of the main reasons advanced to explain the low operational stability of enzymatic electrodes,[5,6] a drawback that strongly contributes to slow down their direct application. This is the reason why different approaches have been proposed, such as covalent binding of the enzyme to the electrode surface,[7] or the addition of a protective membrane[8−10] such as carboxylated PVC, polyurethane,[11] lipids,[12] or Nafion[13] on top of the enzyme layer. However, even if these strategies tend to increase the lifetime of the enzymatic electrode, it is often to the detriment of the current. Recently, Pishko and co-workers developed a new approach based on photopolymerization and applied it to the immobilization of glucose oxidase (GOx). After the modification of gold electrodes for 20 min by a negatively charged thiol layer, the authors deposited on top of it an osmium based redox polymer and let it cure overnight. Then a mixture of poly(ethylene glycol) diacrylate (PEGDA), a photoclivable precursor (DAROCUR), and GOx was deposited on top of the redox polymer layer. It was then exposed to UV light which led to the formation of a PEGDA hydrogel immobilizing the enzyme on the gold surface by photoinduced radical polymerization.[14,15] However, in this system also, an overnight curing is needed for a complete electrostatic binding between the positively charged osmium-based redox polymer and the gold functionalized with a self-assembled monolayer (SAM) of carboxylated thiols.

Received: December 17, 2010
Accepted: February 23, 2011
Published: March 15, 2011

Here, we report a new approach for immobilizing efficiently enzymes on electrode surfaces. This new protocol is of particular interest for labile enzymes that quickly denaturate. As a proof of concept, we chose bilirubin oxidase, a labile enzyme widely used for the electroreduction of O_2 to water.[16−20] The electroreduction of oxygen to water is chosen as a key test because it is one of the most important reactions in energy and sensing electrochemical applications.[21−23] It is involved in the operation of air electrodes, oxygen sensors, biofuel cells, and most fuel cell cathodes in general.[24] With this new method, a mixture of the enzyme and the redox hydrogel is first deposited on a glassy carbon electrode, with no need of a long time curing step or prior modification of the electrode surface. Then, instead of typical chemical cross-linking, the bioelectrocatalyst is covered by an outer layer of a PEGDA hydrogel. We could dramatically shorten the preparation time of the electrodes down to only 1 h. In addition, the PEGDA hydrogel provides a protective environment that inhibits degradation of the electrode. Moreover, by tuning the photopolymerization parameters, we can modulate the final performance of the electrode and obtain at will a higher current or a better stability than the classical system based on chemical cross-linking.

EXPERIMENTAL SECTION

Purification of Bilirubin Oxidase. Bilirubin oxidase (BOD) from *Trachyderma tsunodae* was a gift from Amano, purified as already published,[25] and concentrated to a final concentration of 99 mg/mL.

Photopolymer Solutions Preparation. Poly(ethylene glycol) diacrylate (PEGDA) and 2-hydroxy-2-methyl propiophenone (DAROCUR) were purchased from Sigma. In an eppendorf tube covered with aluminum, pure DAROCUR was mixed to pure PEGDA in the desired volumetric ratio. After a brief vortexing, the solution was diluted with milli-Q water to the desired concentration and the solution was vortexed again during 10 s.

Preparation of Electrodes. Here, 5 or 3 mm diameter glassy carbon electrodes embedded in Teflon were purchased from Pine. They were polished on a felt using alumina powder (50 nm), rinsed, polished once more on a felt with water, and rinsed again. After 2 min in a bath sonicator, they were rinsed and their surface state was checked using electrochemical characterization. Cyclic voltammetry was performed between 0 and 1 V vs Ag/AgCl, 100 mV/s in 20 mM phosphate buffer, to check the cleanness and smoothness of the electrodes. They were then thoroughly rinsed and let dry at ambient temperature. Prior to their coating, electrodes were made hydrophilic by exposure to a 150 mTorr O_2 plasma for 5 min.

"Classical" PEGDGE Cross-Linked Electrodes. BOD-wiring redox polymer (PAA-PVI-$[Os(4,4'\text{-dichloro-}2,2'\text{-bipyridine})_2\text{-}Cl^-]^{+/2+}$, 10 mg/mL in water, 3.12 μL), additional water (3 μL), purified BOD (5 mg/mL in water, 1.23 μL), and poly(ethylene glycol) diglycidyl ether (PEGDGE, 3 mg/mL in water, 1 μL) were mixed in an eppendorf tube. A 1.47 μL portion of this solution was dropped on the glassy carbon electrode freshly cleaned with O_2 plasma. The electrodes were thus modified with 77.32 wt % polymer, 15.24 wt % BOD, and 7.43 wt % PEGDGE for a total loading of 100 $\mu g/cm^2$. Afterward, the electrodes were kept protected from dust and let dry for 18 h at ambient temperature.

Scheme 1. Elaboration of an Enzymatic Cathode by (A) Usual Chemical Cross-Linking[a] and (B) the New Protocol[b]

[a] BOD (green ovals), redox polymer (black lines), and PEGDGE (red segments) are mixed and deposited on glassy carbon electrodes and allowed to cure for 18 h. [b] BOD and the redox polymer are mixed and deposited on the GC electrode and let dry for 45 min. Then, a mixture of monomer (PEGDA) and photoiniator (DAROCUR) is spread on it and illuminated to achieve photoiniated polymerization.

Polymer/Enzyme Electrodes. Redox polymer (10 mg/mL in water) was diluted to 5 mg/mL with milli-Q water, and purified BOD (5 mg/mL in water) was added so as to get the desired polymer/enzyme weight ratio, with a total concentration of active species of 5.075 mg/mL. The solution was then homogenized on a vortex. A 1.4 μL portion was spread on a 3 mm diameter electrode for a total loading of 100 $\mu g/cm^2$. These electrodes were cured protected from dust at room temperature for 45 min before further covering with photopolymer.

Encapsulation with Photopolymer. A 2 μL portion of the desired PEGDA/DAROCUR solutions were spread on electrodes with a polymer/enzyme layer already on it. The electrodes were rapidly placed in front of the optical fiber output of the X-Cite mercury lamp (30 mW/nm) at a distance of 6.6 cm, and removed after the desired time of illumination. To control whether photopolymerization had taken place or not, the surface of the electrode was softly touched with a pipet tip to check the viscosity of the overlayer.

Electrochemical Measurements. All experiments were made using a CHInstruments potentiostat, in a three-electrode cell configuration. An Ag/AgCl and a platinum wire were used as reference and counter-electrode, while the working electrode was mounted on a rotating Pine motor. All experiments were performed in a 37 °C thermostatized cell in 20 mM phosphate buffer at pH 7.2, at 500 rpm. Except for electrodes made in the absence of BOD, the experimental protocol was fixed as follows: 5 scans of cyclic voltammetry (CV) (+0.6−+0.1 V vs Ag/AgCl) at 100 mV/s were performed followed by one CV at 5 mV/s in Ar-saturated buffer, and then 1 CV at 5 mV/s in O_2-saturated buffer. Amperometric measurements were done at +0.1 V vs Ag/AgCl to study the stability of the electrodes. For the storage experiments, electrodes were kept at 4 °C for 24 h in dry state after the CV under O_2. Then, the experiment protocol described above was repeated. When waiting for use, the electrodes were kept in 20 mM phosphate buffer at ambient temperature.

RESULTS AND DISCUSSION

Scheme 1A illustrates the general method developed by Heller and co-workers to "wire" enzymes to electrode surfaces. Enzymes (green), osmium based redox polymer (black), and cross-linker (PEGDGE, in red) are mixed together, deposited on a plasma treated glassy carbon electrode surface and allowed to cure for 18 h at room temperature. Scheme 1B represents the new two-step protocol that we developed. First, a mixture of enzymes and

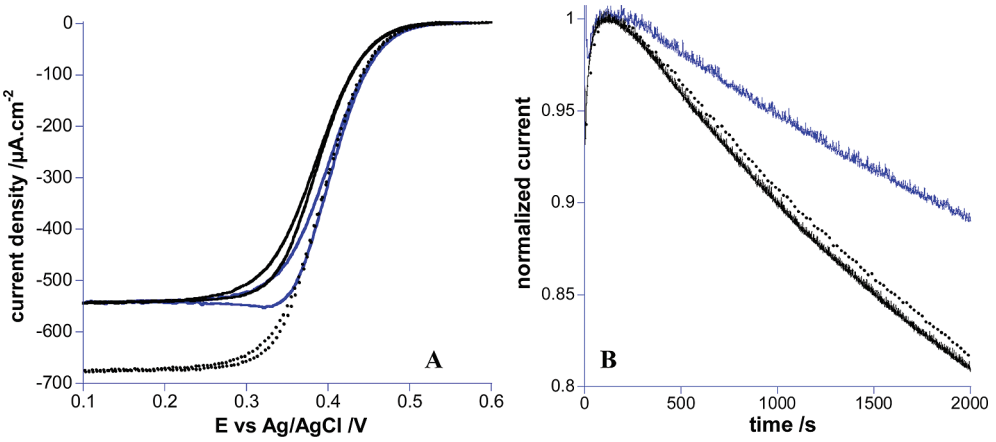


Figure 1. (A) Polarization curves under 1 atm O_2 of the modified cathode made by chemical cross-linking (black line) or by photopolymerization with a completely polymerized outer layer (PEGDA/DAROCUR 20:1 in volume diluted 8 times in water and illuminated 1.25s, blue line) or a partially polymerized outerlayer (PEGDA/DAROCUR 20:1 diluted 6 times in water and illuminated 0.5 s, dotted black line). (B) Corresponding profiles of normalized current measured at +0.1 V for 2000 s. See the Experimental Section for conditions.

osmium based redox polymer are deposited on a plasma treated glassy carbon electrode and allowed to cure for 45 min at room temperature. Then, a mixture of PEGDA/DAROCUR is spread on top of the enzyme/polymer layer and illuminated with UV visible light. Upon illumination, photopolymerization of the acrylate end groups of PEGDA by the photoinitiator DAROCUR occurs and entraps the enzyme and the redox polymer. This is a simple, straightforward method of modification that only takes 1 h instead of 18 h for the usual protocol and for the method developed by Pishko and co-workers (overnight curing). In addition, in our system, there is no need to functionalize the electrode surface via thiols to increase the interaction between the electrode and the bioelectrocatalyst. The porous structure of the hydrated overlayer allows for the diffusion of O_2 and the retention of BOD, permitting the catalytic activity of the enzyme.

In the first group of experiments, in order to achieve the highest electroreduction current of oxygen, we determined the optimal composition, i.e. the BOD/redox polymer ratio to deposit on the plasma-treated glassy carbon electrode. For a total loading fixed at 100 $\mu g \cdot cm^{-2}$, the optimal ratio was found to be 70 wt % of redox polymer and 30 wt % BOD giving a current of 900 $\mu A \cdot cm^{-2}$. The second group of experiments was dedicated to optimize the layer of PEGDA/DAROCUR to be spread on top of the BOD/redox polymer layer. Parameters such as dilution factor, PEGDA/DAROCUR ratio, and illumination time were studied (Supporting Information). Depending on the parameters used for photopolymerization, it was possible to tune the O_2 electroduction current. The black line in Figure 1A is the current obtained for a 15.24 wt % enzyme, 77.32 wt % polymer, and 7.43 wt % PEGDGE modified electrode and was found to be the best composition for cross-linked BOD hydrogels and is used as a control experiment. As seen in Figure 1A, for a complete polymerization, we could reach a current density of 536 $\mu A \cdot cm^{-2}$ (dark blue line) similar to the current obtained for the PEGDGE modified electrode (black). However, as shown in Figure 1B, the stability of the modified PEGDGE was 74% lower after 2000 s than that of the PEGDA electrode because of the redox polymer/enzyme adduct leaching (vide infra).

By concentrating the PEGDA/DAROCUR solution by a factor 1.33 (PEGDA/DAROCUR 20:1 in weight diluted 6 times in water instead of 8 times) and decreasing the illumination time to 0.5 s, preventing full photopolymerization, it was possible to increase the current by 25% (Figure 1A, dotted line) as compared to the current obtained for the full photopolymerization. However, the operational stability was decreased by 68% but remained similar with the one obtained for the PEGDGE system (Figure 1B, black line). These results can be explained by the following reasons. In these systems, electrons diffuse because of the collision between oxidized and reduced osmium complex and the highest electron diffusivities are being reached when the film is not cross-linked. However, in the absence of cross-linking, the film dissolves and the polymer leaks from the electrodes. Cross-linking increases the mechanical stability but reduces the segmental mobility on which the electron conduction in the redox polymer films depends. Thus mechanical strength and high apparent electron diffusion coefficients are not simultaneously achieved unless the redox functions are tethered to the cross-linked polymer networks by long and flexible spacers.

To elucidate the cause of the difference in loss of current observed in Figure 1B, we also evaluated the stability of electrodes only modified with redox polymer under continuous operation. Electrodes with total redox polymer loading of 75 $\mu g \cdot cm^{-2}$ were composed of either 92.5 wt % polymer and 7.5 wt % PEGDGE or made of a layer of pure redox polymer covered by an outer layer of PEGDA. After a series of 50 CVs at 100 $mV \cdot s^{-1}$, the electrodes cross-linked with PEGDGE lost 72% of their peak current, to finally stabilize at ~22.5 $\mu A \cdot cm^{-2}$. This decay is due to the leaching of the redox polymer which was evidenced by the direct observation of the brownish polymer in the buffer solution. For the electrodes modified with an overlayer of PEGDA, we could not observe any leaking and the loss was only 38%, the peak current stabilizing at a value 6 times higher than with PEGDGE (139 $\mu A \cdot cm^{-2}$). In addition after drying the electrode at room temperature for 1 h, PEGDA electrodes showed series of CVs almost superimposable to the first one, while a further loss was observed for PEGDGE electrodes.

These results suggest that the better stability observed in Figure 1B could be due to a better stability of the adduct made with the redox polymer and the enzyme on the electrode surface. In addition, the higher current densities can be explained because of the quickness of the process. By shortening the curing time, we

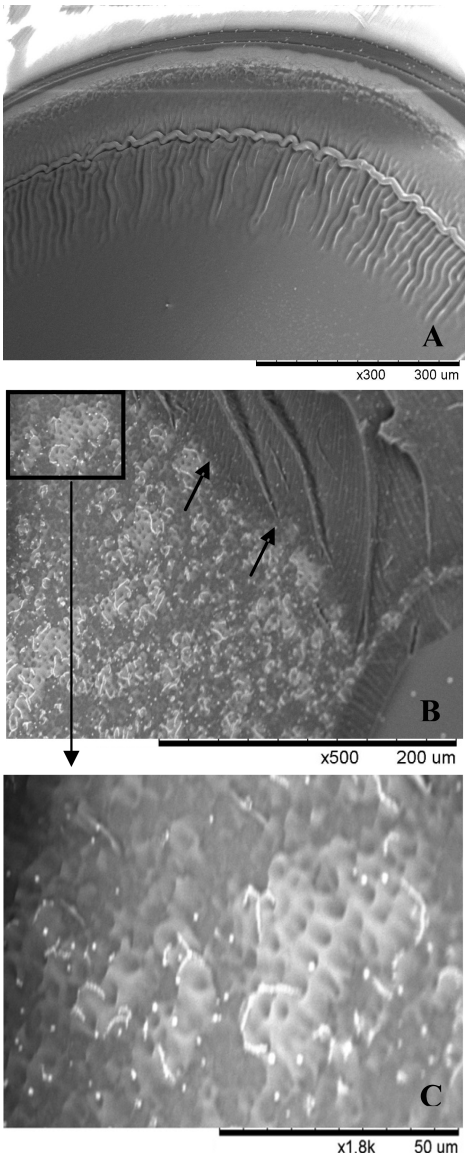


Figure 2. Scanning electron microscopy of the redox polymer/BOD electrode covered with a PEGDA outerlayer. (A) Edge of the electrode, on which the coating was remained intact. (B) Center of the electrode, on which the coating was removed. (C) Zoom in alveoli patterns on the uncovered part of the electrode.

limit the partial enzyme inactivation that can occur for a labile enzyme such as BOD and that becomes important after 18 h at room temperature. So, even though the photopolymerization step may be prejudicial to the enzyme because of the production of radicals,[26] reducing the illumination time permits limiting this loss and obtaining currents still higher than with PEGDGE, however with partial polymerization of the over layer. As seen earlier, the presence of the coating layer permits retaining the redox polymer on the surface of the electrode, and we can hypothesize that a layer for which the polymerization is not complete may be less efficient for retaining the redox polymer/enzyme layer. On the other side, a more polymerized layer will better retain the redox species but will need longer illumination time. Then, higher deactivation of the enzyme may occur and lead to lower catalytic currents, finally equal to those obtained for chemically cross-linked cathodes. Oxygen diffusion-limited like behavior could also be observed for a thick, fully polymerized layer (PEGDA/DAROCUR 20:1 × 6, 1.25 s illumination) but was not observed with the thinner and less dense covering layers we present here. A judicious choice of the photopolymerization parameters permits the rapid fabrication of cathodes exhibiting either better stability or higher catalytic currents than those made by the classical cross-linking method.

After modification with the PEGDA overlayer, we observed that this layer acted as a protective membrane. After CV under O_2, electrodes were stored at 4 °C in a dry state overnight, and new measurements were performed with the same electrodes. In the absence of an overlayer, the electroreduction current of O_2 for chemically cross-linked cathodes decreased by 47% while electrodes covered with a PEGDA layer only lost 29% of their current. Recently, it has been shown that BOD could be irreversibly deactivated during storage if not kept in its reductive state.[27] We believe that, when the overlayer of PEGDA is not hydrated, it forms a compact and airtight layer preventing O_2 to diffuse to the enzyme and preventing its reoxidation (vide infra).

Finally, we performed scanning electron microscopy (Hitachi, TM-1000) experiments to investigate the porous structure of the electrodes covered with the PEGDA layer. With a surgical blade and tweezers, the hydrogel layer was torn off in the center of the electrode to allow the observation on the same electrode of three distinct regions: the intact PEGDA coating, its section, and its junction with the redox polymer/enzyme underlayer (Figure 2). As can be seen in Figure 2A, on the edge of the electrode no neat separation can be observed between the redox polymer and the PEGDA layer that covers the whole electrode surface homogeneously. However, we could observe some wrinkles resulting from the dehydration of the PEGDA layer during the drying leading to the contraction of the layer. The arrows in Figure 2B show that the PEGDA layer is tightly bound to the underlying redox polymer/enzyme layer and that no separation between the redox polymer/BOD and PEGDA hydrogels is visible. The section of the PEGDA layer reveals a very compact structure that could be airtight enough not to let oxygen diffuse. This result is in agreement with our previous observation and could also explain why the lifetime of the electrode during storage is increased by photopolymer covering. Figure 2C represents a zoom of the uncovered part of the electrode of Figure 2B. We can observe alveoli; those may represent the imprint of water trapped during the photopolymerization process.

CONCLUSION

In this study, we developed a new method of enzyme immobilization and applied it as a proof of concept to a labile enzyme, bilirubin oxidase. First, a mixture of BOD and redox polymer was deposited on glassy carbon electrodes and further covered with an outer layer of PEGDA obtained by photoinitiated polymerization. Our new two-step protocol enables the fabrication of enzymatic electrodes in barely 1 h, when at least 18 h are needed for both the widespread method based on chemical cross-linking and other methods based on photodeposition of PEGDA/DAROCUR solutions. The two-state environment provided by the coating allows the diffusion of dissolved oxygen when the electrode is in solution and protects both the enzyme and the redox polymer from the atmosphere when dried, increasing the lifetime of the cathodes. Depending on the photopolymerization parameters, it is possible to build enzymatic

cathodes with enhanced performance when compared to the reference system based on chemical cross-linking. The catalytic current and/or the stability can be improved depending on the desired application. Finally, because of the simplicity of this new protocol and the performance obtained, we expect this novel way of enzyme immobilization to be a starting point for the elaboration of various high performance biosensors and electrochemical devices.

■ ASSOCIATED CONTENT

Supporting Information. Details on the influence of the illumination and of the PEGDA/DAROCUR on the BOD activity and optimization of the PEGDA/DARCOCUR ratio, dilution factor, and illumination time. This material is available free of charge via the Internet at http://pubs.acs.org.

■ ACKNOWLEDGMENT

We gratefully acknowledge the Agence Nationale de la Recherche for its financial support through the ANR BIOPUMP project (ANR-07-BLAN-0336-CSD 4).

■ REFERENCES

(1) Schummann, W. *J. Biotechnol.* **2002**, *82*, 425.
(2) Willner, I.; Katz, E. *Angew. Chem., Int. Ed.* **2000**, *39*, 1180.
(3) Merle, G.; Habrioux, A.; Servat, K.; Rolland, M.; Innocent, C.; Kokoh, K. B.; Tingry, S. *Electrochim. Acta* **2009**, *54*, 2998.
(4) Heller, A. *Curr. Opin. Chem. Biol.* **2006**, *10*, 664.
(5) Cooney, M.; Svoboda, V.; Lau, C.; Martin, G. L.; Minteer, S. *Energy Environ. Sci.* **2008**, *1*, 320.
(6) Moehlenbrock, M. J.; Minteer, S. D. *Chem. Soc. Rev.* **2008**, *37*, 1188.
(7) Pellissier, M.; Barrière, F.; Downard, A. J.; Leech, D. *Electrochem. Commun.* **2008**, *10*, 835.
(8) Scheller, F.; Pfeiffer, D.; Kuhn, M.; Hundertmark, J.; Quade, A.; Janchen, M.; Lange, G.; Holesch, H.; Dittmer, H. *Acta Biol. Med. German* **1980**, *39*, 671.
(9) Jimenez, C.; Bartrol, J.; de Rooij, N. F.; Koudelka-Hep, M. *Anal. Chim. Acta* **1997**, *351*, 169.
(10) Jimenez, C.; Bartroli, J.; de Rooij, N. F.; Koudelka-Hep, M. *Sens. Actuat. B* **1995**, *B27*, 421.
(11) Pauliukaite, R.; Schoenleber, M.; Vadgama, P.; Brett, C. A. *Anal. Bioanal. Chem.* **2008**, *390*, 1121.
(12) Rowinski, P.; Kang, C.; Shin, W.; Heller, A. *Anal. Chem.* **2007**, *79*, 1173.
(13) Kang, C.; Choo, J.; Shin, H.; Heller, A. *Electroanalysis* **2009**, *21*, 2709.
(14) Mugweru, A.; Clark, B. L.; Pishko, M. V. *Electroanalysis* **2007**, *19*, 453.
(15) Sirkar, K.; Pishko, M. V. *Anal. Chem.* **1998**, *70*, 2888.
(16) Shleev, S.; El Kasmi, A.; Ruzgas, T.; Gorton, L. *Electrochem. Commun.* **2004**, *6*, 934.
(17) Tsujimura, S.; Kano, K.; Ikeda, T. *J. Anal. Chem.* **2005**, *576*, 113.
(18) Zheng, W.; Zhao, H. Y.; Zhou, H. M.; Xu, X. X.; Ding, M. H.; Zheng, Y. F. *J. Solid State Electrochem* **2009**, *14*, 249.
(19) Schubert, k.; Goebel, g.; Lisdat, F. *Electrochim. Acta* **2009**, *54*, 3033.
(20) Fernandez, J. L.; Mano, N.; Heller, A.; Bard, A. J. *Angew. Chem., Int. Ed.* **2004**, *43*, 6355.
(21) Gao, F.; Viry, L.; Maugey, M.; Poulin, P.; Mano, N. *Nat Commun.* **2010**, 110.1038/ncomms1000.
(22) Flexer, V.; Mano, N. *Anal. Chem.* **2010**, *82*, 1444.
(23) Cinquin, P.; Gondran, C.; Giroud, F.; Mazabrard, S.; Pellissier, A.; Boucher, F.; Alcaraz, J.-P.; Gorgy, K.; Lenouvel, F.; Mathé, S.; Porcu, P.; Cosnier, S. *PLoS One.* **2010**, *5*, e10476.
(24) Barton, S. C.; Gallaway, J.; Atanassov, P. *Chem. Rev.* **2004**, *104*, 4867.
(25) Flexer, V.; Brun, N.; Backov, R.; Mano, N. *Energy Environ. Sci.* **2010**, *3*, 1302.
(26) Lin, C.-C.; Sawicki, S. M.; Metters, A. T. *Biomacromolecules* **2008**, *9*, 75.
(27) Kang, C.; Shin, H.; Heller, A. *Bioelectrochemistry* **2006**, *68*, 22.