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Glucose and Lactate Biosensors Based on Redox Polymer/Oxidoreductase Nanocomposite Thin Films

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Glucose and lactate enzyme electrodes have been fabricated through the deposition of an anionic self-assembled monolayer and subsequent redox polymer/enzyme electrostatic complexation on gold substrates. These surfaces were functionalized with a negative charge using 11-mercaptoundecanoic acid (MUA), followed by alternating immersions in cationic redox polymer solutions and anionic glucose oxidase (GOX) or lactate oxidase (LAX) solutions to build the nanocomposite structure. The presence of the multilayer structure was verified by ellipsometry and sensor function characterized electrochemically. Reproducible analyte response curves from 2 to 20 mM (GOX) and 2−10 mM (LAX) were generated with the standard deviation between multiple sensors between 12 and 17%, a direct result of the reproducibility of the fabrication technique. In the case of glucose enzyme electrodes, the multilayer structure was further stabilized through the introduction of covalent bonds within and between the layers. Chemical cross-linking was accomplished by exposing the thin film to glutaraldehyde vapors, inducing linkage formation between lysine and arginine residues present on the enzyme periphery with amine groups present on a novel redox polymer, poly[vinylpyridine Os(bisbipyridine)$_2$Cl]-*co*-allylamine. Finally, an initial demonstration of thin-film patterning was performed as a precursor to the development of redundant sensor arrays. Microcontact printing was used to functionalize portions of a gold surface with a blocking agent, typically 1-hexadecanethiol. This was followed by immersion in MUA to functionalize the remaining portions of gold with negative charges. The multilayer deposition process was then followed, resulting in growth only on the regions containing MUA, resulting in a "positive"-type pattern. This technique may be used for fabrication of thin-film redundant sensor arrays, with thickness under 100 Å and lateral dimensions on a micrometer scale.

The need for effective enzyme-based amperometric biosensors is present for many clinically relevant applications, with glucose and lactate sensing two of the most commercially important. Numerous types of amperometric devices for both substrates have been reported in the literature that take advantage of the enzymatic properties of glucose oxidase (GOX) and lactate oxidase (LAX). These electrodes include those based upon the electrochemical oxidation of H_2O_2 generated during the reaction between glucose or lactate and oxygen catalyzed by the corresponding oxidoreductase[1,2] and those that use charge mediators to transfer electrons between the redox center of the enzyme and the electrode surface.[3−7] The actual commercial application of biosensor technology, however, has been somewhat limited, typically to home glucose test meters and blood-gas instruments designed to detect glucose and lactate. Technically demanding applications such as the detection of multiple analytes in vivo require a high density of individual analyte-sensing electrodes, required for miniaturization, which themselves contain a large concentration of enzyme molecules necessary to provide high signal levels. In addition, these sensors must be fabricated easily, reproducibly, and at spatially distinct, readily addressable regions on a discrete portion of a surface. Biosensor development with a priority on spatial orientation and assembly on the nanoscale has thus become an important issue. Examples of such research include thin-film biosensors that use biotin/avidin linkages to immobilize enzymes in an ordered fashion[8,9] and assembled GOX/pyrroloquinoline (PQQ) multilayers using techniques including carbodiimide coupling of PQQ to surface polyamines.[10−12] Array or pattern development has also begun to receive attention for thin-film biosensing applications. Various macromolecules, including antibodies and proteins, have been deposited into discrete patterns using photolithography[13,14] and microcontact printing.[15,16]

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(1) Tatsuma, T.; Saito, K.-I.; Oyama, N. *Anal. Chem.* **1994**, *66*, 1002−1006.
(2) Shram, N. F.; Netchiporouk, L. I.; Martelet, C.; Jaffrezic-Renault, N.; Bonnet, C.; Cespuglio, R. *Anal. Chem.* **1998**, *70*, 2618−2622.
(3) Hale, P. D.; Boguslavsky, L. I.; Inagaki, T.; Karan, H. I.; Lee, H. S.; Skotheim, T. A.; Okamoto, Y. *Anal. Chem.* **1991**, *63*, 677−682.
(4) Pishko, M. V.; Michael, A. C.; Heller, A. *Anal. Chem.* **1991**, *63*, 2268−2272.
(5) Kenausis, G.; Chen, Q.; Heller, A. *Anal. Chem.* **1997**, *69*, 1054−1060.
(6) Sirkar, K.; Pishko, M. *Anal. Chem.* **1998**, *70*, 2888−2894.
(7) Osborne, P. G.; Niwa, O.; Yamamoto, K. *Anal. Chem.* **1998**, *70*, 1701−1706.
(8) Dontha, N.; Nowall, W.; Kuhr, W. *Anal. Chem.* **1997**, *69*, 2619−2625.
(9) Anzai, J.; Kobayashi, Y.; Nakamura, N.; Nishimura, M.; Hoshi, T. *Langmuir* **1999**, *15*, 221−226.
(10) Willner, I.; Heleg-Shabtai, V.; Blonder, R.; Katz, E.; Tao, G. *J. Am. Chem. Soc.* **1996**, *118*, 10321−10322.
(11) Willner, I.; Katz, E.; Willner, B. *Electroanalysis* **1997**, *9*, 965−977.
(12) Moore, A. N. J.; Katz, E.; Willner, I. *Electroanalysis* **1996**, *8*, 1092.
(13) Mooney, J.; Hunt, A.; McIntosh, J.; Liberko, C.; Walba, D.; Rogers, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12287−12291.
(14) Sundberg, S.; Barrett, R.; Pirrung, M.; Lu, A.; Kiangsoontra, B.; Holmes, C. *J. Am. Chem. Soc.* **1995**, *117*, 12050−12057.

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One technique that has received a great deal of attention recently involves the building of individual monolayers on surfaces based upon the attraction between oppositely charged species. Hammond et al. have addressed many issues in the development of patterned polyion multilayers.[17−19] Typically, these systems utilized sulfonated polystyrene as the anionic component and compounds such as poly(allylamine hydrochloride) as the cationic component. These polyion multilayers were grown in distinct patterns through the use of microcontact printing (μCP) and blocking agents anchored to a gold substrate. The effects of solution ionic strength[20,21] as well as the attachment of redox-active osmium complexes have been addressed in similar studies.[22]

Electrostatic complexation of the redox polymer and enzyme may be utilized to generate amperometric biosensors with high current densities, despite physical limitations on the total amount of sensor materials. This type of electrostatic complexation was demonstrated earlier with the complexation of polycationic poly(vinylpyridine) complexes of $[Os(bpy)_2Cl]^{2+}$ and polyanionic glucose oxidase at graphite electrodes.[23] Complexations of this type have been utilized in recent layer-by-layer deposition approaches for the development of glucose sensors. Chen et al. utilized alternating GOX and charge mediator layers for the fabrication of glucose enzyme electrodes.[24,25] Other groups also performed similar work utilizing ferrocene derivatives as their polycationic charge mediator.[26] Although these studies have demonstrated the fabrication of amperometric glucose sensors using the electrostatic layer-by-layer approach, no mention was made of the thickness of the sensing layers, attempts to improve sensor stability, patterning, or oxidoreductases other than GOX.

The present work expands on the concepts of electrostatic complexation between redox polymers and oxidoreductases. We describe glucose and lactate sensors that use a multilayer deposition approach based upon attraction between oppositely charged species, namely, a novel cationic redox polymer and anionic GOX or LAX. The thickness of the individual monolayers was determined via ellipsometry with the number of redox sites within a given monolayer established through cyclic voltammetry. The sensor function of the polyion multilayer was compared to that of multilayers stabilized via chemical cross-linking. Finally, a demonstration of patterning was performed through the utilization of microcontact printing of MUA and blocking agents such as 1-hexadecanethiol (C16-SH). This is a necessary precursor to the development of a single-analyte redundant sensor array or multianalyte sensor arrays.

EXPERIMENTAL SECTION

Reagents. Glucose oxidase (EC 1.1.3.4, type X-S, 128 units/mg of solid from *Aspergillus niger*), lactate oxidase (35 units/mg of solid, from *Pediococcus* species), and lactic acid (60% w/w in H_2O) were obtained from Sigma Chemical Co. (St. Louis, MO). Ammonium hexachloroosmate(IV), 1-hexadecanethiol, 11-mercaptoundecanoic acid (MUA), 2,2′-azobisisobutyronitrile (AIBN), poly(4-vinylpyridine), and 2,2′-dipyridyl (bpy) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Dextrose, methanol, ethyl alcohol, glutaraldehyde, ethylene glycol, hydrochloric acid (11 N), and acetonitrile were obtained from Fisher Scientific Co. (Pittsburgh, PA). An α-acryloyl, *ω-N*-hydroxysuccinimidyl ester of poly(ethylene glycol)propionic acid (PEG-NHS, MW 3400) was purchased from Shearwater Polymers (Huntsville, AL). All reagents, unless otherwise stated, were used as received. Phosphate-buffered saline (PBS) solution was composed of 1.1 mM potassium phosphate monobasic, 3 mM sodium phosphate dibasic heptahydrate, and 0.15 M NaCl in 18 MΩ·cm deionized water (E-pure, Barnstead).

Equipment. The equipment for electrochemical analysis included a CV-50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, IN), a C2 cell stand, a Ag/AgCl reference electrode, and a platinum counter electrode. The electrochemical apparatus was controlled and data were acquired using a Toshiba Pentium PC. Ellipsometry was performed using a Gaertner L2W26D ellipsometer (Gaertner Scientific Corp., Chicago, IL). Film thickness and refractive indexes were calculated using Gaertner software, utilizing a film model that assumed a refractive index (n_f) of 1.46. All measurements were made using a He/Ne (633.8 nm) laser and a 70.00 ± 0.02° angle of incidence. Micrographs of multilayer patterns were acquired using a Meiji optical microscope (Meiji Techno America, San Jose, CA) and a Kodak DC240 digital camera (Eastman Kodak Co., Rochester, NY).

Electrodes. Gold electrodes with diameters of 1.6 mm each were purchased from Bioanalytical Systems. Prior to deposition of the multilayers, the electrodes were polished with 1-μm diamond polishing slurry on nylon polishing pads and then with 0.05-μm alumina on microcloth pads followed by sonication along with water and methanol rinses. Gold-coated silicon wafers (200 Å Cr, 1000 Å Au on polished Si(111), Lance Goddard Associates, Foster City, CA) were fabricated into electrodes (6.45 cm^2) by soldering a conducting wire to the substrate surface. Gold-coated wafers were rinsed with ethanol, cleaned in an ozone cleaner (Boekel Industries, Inc., model 135500) for 10 min, and rinsed with ethanol again before use.

Polymer Synthesis. Osmium-based polycationic redox polymers were synthesized using modifications of established protocols. $Os(bpy)_2Cl_2$ was synthesized according to a standard procedure.[27] In brief, 2 equiv of bipyridine (720 mg) was mixed with 1 equiv of ammonium hexachloroosmate(IV) (1000 mg) in 50 mL of ethylene glycol. This mixture was heated to reflux for 45 min, precipitated with supersaturated sodium dithionite, and

(15) Kratzmuller, T.; Appelhans, D.; Braun, H.-G. *Adv. Mater.* **1999**, *11*, 555−558.
(16) James, C. D.; Davis, R. C.; Kam, L.; Craighead, H. G.; Isaacson, M.; Turner, J. N.; Shain, W. *Langmuir* **1998**, *14*, 741−744.
(17) Hammond, P. T.; Whitesides, G. M. *Macromolecules* **1995**, *28*, 7569−7571.
(18) Clark, S. L.; Hammond, P. T. *Adv. Mater.* **1998**, *10*, 1515−1519.
(19) Gregoriou, V. G.; Hapanowicz, R.; Clark, S. L.; Hammond, P. T. *Appl. Spectrosc.* **1997**, *51*, 470−476.
(20) Clark, S. L.; Montague, M. F.; Hammond, P. T. *Macromolecules* **1997**, *30*, 7237−7244.
(21) Sukhorukov, G. B.; Schmitt, J.; Decher, G. *Ber. Bunsen-Ges. Phys. Chem.* **1996**, *6*, 948−953.
(22) Bretz, R. L.; Abruna, H. D. *J. Electroanal. Chem.* **1996**, *408*, 199−211.
(23) Pishko, M. V.; Katakis, I.; Lindquist, S.-E.; Ye, L.; Gregg, B. A.; Heller, A. *Angew. Chem., Intl. Ed. Engl.* **1990**, *29*, 82.
(24) Hou, S.-F.; Fang, H.-Q.; Chen, H.-Y. *Anal. Lett.* **1997**, *30*, 1631−1641.
(25) Hou, S.-F.; Yang, K.-S.; Fang, H.-Q.; Chen, H.-Y. *Talanta* **1998**, *47*, 561−567.
(26) Hodak, J.; Etchenique, R.; Calvo, E. J.; Singhal, K.; Bartlett, P. N. *Langmuir* **1997**, *13*, 2708−2716.
(27) Kober, E. M.; Caspar, J. V.; Sullivan, B. P.; Meyer, T. J. *Inorg. Chem.* **1988**, *27*, 4587−4598.

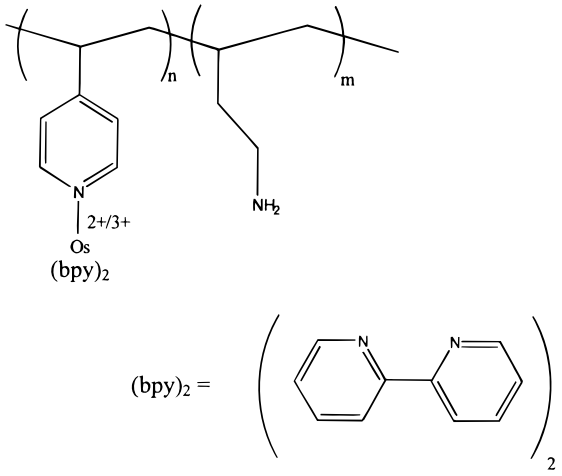

Figure 1. Structure of cross-linkable, organometallic osmium redox polymer used in development of electrostatic multilayers: poly[4-vinylpyridine Os(bisbipyridine)$_2$Cl]-*co*-allylamine (PVP−Os−AA).

repeatedly washed with water and ether. The Os(bpy)$_2$(Cl)$_2$ precursor was used in the synthesis of cross-linkable redox polymers used in these studies.

Poly[vinylpyridine Os(bis-bipyridine)$_2$Cl]-*co*-allylamine (PVP−Os−AA), was synthesized as follows: 1.0 g of 4-vinylpyridine, 1.0 g of allylamine, 0.2 g of AIBN, and 200 μL of HCl were mixed in 50 mL of acetonitrile. This solution was refluxed for 2 h and then concentrated using rotary evaporation for the removal of acetonitrile. The resulting polymer was dissolved in 1 mL of methanol and precipitated in 50 mL of ethyl ether. A 100-mg sample of Os(bpy)$_2$Cl$_2$ was then added to 100 mg of PVP-*co*-allylamine in 50 mL of ethylene glycol and refluxed under N_2 for 1.5 h. Ethylene glycol was removed and the product dissolved in a minimal amount of methanol followed by precipitation in 70 mL of diethyl ether. The ether was then decanted off and PVP−Os−AA was dried and collected. Figure 1 depicts the structure of PVP−Os−AA.

Multilayer Fabrication. Gold substrates were initially functionalized by immersing in 1 mM MUA in ethanol for ∼20 min. The substrates were removed, washed, sonicated, and dried under N_2. The substrates were then alternately placed first in a polycationic 10 mg/mL solution of PVP−Os−AA for ∼20 min and a polyanionic solution of GOX (10 mg/mL in PBS) or LAX (0.7 mg/mL in PBS) for ∼40 min. Between immersions, the substrates were rinsed with 0.1 M PBS, sonicated for ∼20 s, and dried under flowing N_2. A depiction of the final multilayer structure is presented in Figure 2.

Multilayer Cross-Linking. To chemically stabilize the multilayer structures, the cationic and anionic layers were chemically cross-linked using glutaraldehyde.[28] This was accomplished by exposing the coated substrates to glutaraldehyde vapors for ∼15 min and then removing to room air prior to electrochemical testing.

Multilayer Patterning. Stamps for microcontact printing structures were made via replica molding, using a slightly modified version of a published protocol.[29] In brief, Shipley STR1045

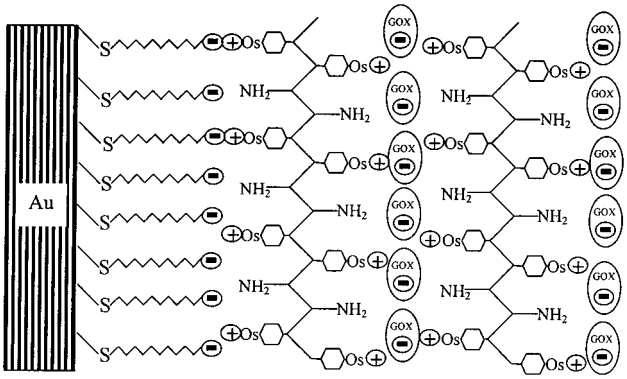

Figure 2. Depiction of the multilayer structure. Positive charges on redox polymer are electrostatically attracted to negative charges on enzyme and substrate surface.

photoresist (MicroChem Corp., MA) was deposited in a thin layer via spin coating at ∼5000 rpm on a clean Si wafer. This photoresist was exposed to 365-nm, 20 W/cm^2 UV light through an optical test mask (USAF resolution chrome test target, Melles Griot). After photoresist development, poly(dimethylsiloxane) (PDMS) (Sylgard 184 silicone elastomer and curing agent in a 10:1 ratio, Dow Corning Corp.) was deposited over the patterned photoresist. The uncured PDMS was confined with a small cylinder pressed down on the substrate with a slight weight. This prestamp was then placed in a vacuum oven to be degassed and cured for ∼24 h. Upon curing, the PDMS was lifted off the substrate resulting in the patterned stamp.

Application of the stamp to form a patterned substrate was performed as follows. Initially, the stamp was dipped in 1 mM C16-SH solution (ethanol) for ∼20 s. It was then dried, and pressed on a cleaned gold surface for ∼20 s. The stamp was then removed, the substrate exposed to 1 mM MUA for ∼20 min, and the multilayer deposition procedure followed as described earlier.

Electrochemical Characterization. Electrodes were tested in a cell containing Pt counter and Ag/AgCl reference electrodes as well as 0.1 M PBS degassed with N_2 (15 min prior to start of experiment and for the duration of experiment). Bubbling with N_2 was used to agitate the solution. Either of two experiments was conducted to characterize sensor response: cyclic voltammetry or constant-potential amperometry. For cyclic voltammetry, the applied potential was cycled linearly from 0 to 500 mV at a scan rate of 20 mV/s. The anodic peaks of individual scans were integrated to determine the surface coverage or the total amount of charge mediator present in any one layer. Constant-potential amperometry required the preconditioning (∼50 s) and operation of the electrode at a constant applied potential of 300 mV. When the current reached a baseline in the absence of substrate, aliquots of substrate were added and step changes in current monitored with time. Unless otherwise stated, $n = 3$ for all substrate response curves.

RESULTS AND DISCUSSION

The development of reliable and robust amperometric biosensor arrays is dependent upon the miniaturization of sensor features to increase both the density of sensing elements in a spatially distinct region and the density of biorecognition molecules within

(28) Wiseman, A. In *Ellis Horwood Series in Biochemistry and Biotechnology incorporating Molecular Biology*; Wiseman, A., Ed.; Ellis Horwood: London, 1995; p 738.

(29) Xia, Y.; Whitesides, G. M. *Angew. Chem., Intl. Ed.* **1998**, *37*, 550−575.

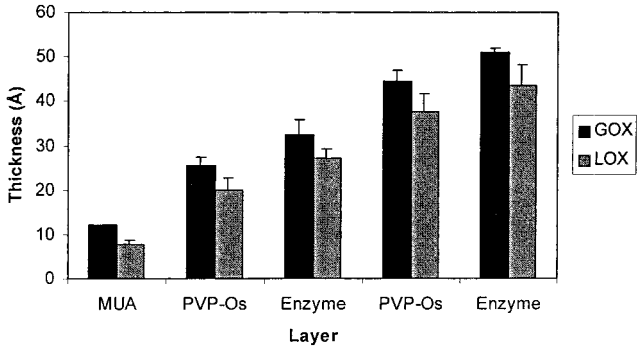

Figure 3. Increase in thickness as measured by ellipsometry upon attachment of each subsequent layer.

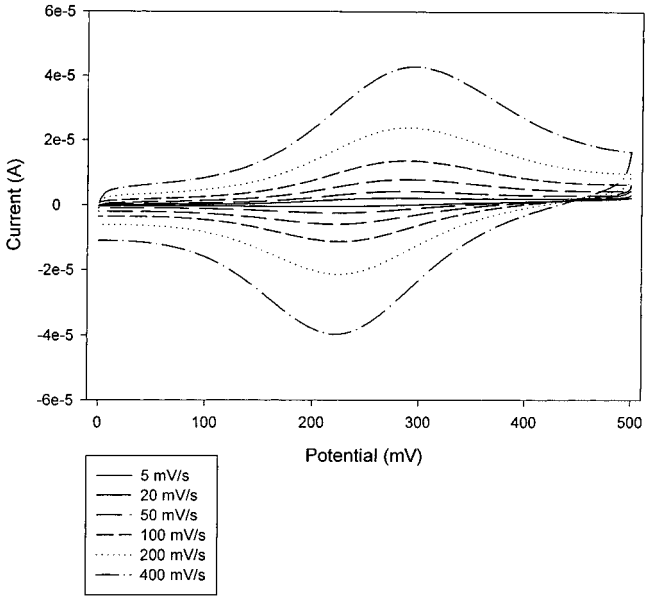

Figure 4. Cyclic voltammograms as a function of increasing scan rate (5−400 mV/s) for a GOX/PVP−Os−AA nanocomposite thin film. A formal potential of 250 mV was found with a peak separation of ~60 mV.

an individual sensor. Miniaturization, however, may result in low current densities and inadequate signal-to-noise ratios. To overcome this problem, we have formed redox polymer and enzyme complexes through an electrostatic layer by layer deposition scheme, a technique that has been shown to increase current densities in the resulting biosensors.[23] This scheme for sensor development minimizes the use of such components as retaining membranes or thick polymer matrixes, resulting in an increased density of the primary sensing components, namely, the enzyme and redox polymer. This permits rapid and efficient sensor performance, which may be further improved by chemically cross-linking the multilayer structure by exposure to glutaraldehyde. The goal of minimizing sensor dimensions was achieved by keeping the thickness of the sensing layers well under 100 Å through repeated attachment of single redox polymer/enzyme monolayers. The feasibility of depositing these layers in micrometer-scale patterns was then demonstrated using μCP techniques. This may eventually permit rapid generation of multilayer sensor arrays.

Multilayer Formation. The first step in the fabrication of these nanocomposite structures was the functionalization of a gold substrate for subsequent layer-by-layer deposition. As an adhesion layer, a monolayer of MUA[30] with a pK_a of ~6.5 was adsorbed to the gold substrate via its thiol end group. At the buffer pH of ~7.4, MUA presents the electrode−solution interface with an initial negative surface charge, via its partially deprotonated carboxyl acid groups, with which to begin the layer-by-layer deposition process. Poly(4-vinylpyridine) complexes of $Os(bpy)_2Cl_2$ are highly cationic[23] and will electrostatically adsorb to the negatively functionalized surface. GOX has an isoelectric point[31] of ~3 and LAX has a pI of ~4.6; thus, at pH 7.4, both enzymes possess a net negative charge, permitting their adsorption to the cationic redox polymer. Alternating deposition of positively charged redox polymers and negatively charged enzymes permitted the buildup, layer by layer, of a nanocomposite structure.

One of the requisite features for the formation of well-ordered and defined biosensors is the ability to tailor the organization of the sensor components as close to the molecular level as possible. With this goal in mind, ellipsometric data were obtained after adsorption of subsequent layers to verify that very thin layers were being deposited after each adsorption step. Figure 3 shows the increase in film thickness upon addition of each layer during the alternate immersions in polycation/polyanion solutions. For glucose enzyme electrodes, the increase in thickness was linear with each addition, with an $R^2 > 0.98$. The average thickness of the cationic multilayer, PVP−Os−AA, was larger, ~12.5 Å, than the average anionic multilayer (GOX), ~6.7 Å. GOX from *A. niger* in its native state is a globular glycoprotein with an elliptical shape and a maximum diameter as determined by X-ray crystallography of ~45 Å along its primary axis.[32] However, perpendicular to this axis, the diameter is smaller, in the range from 15 to 20 Å. On the basis of the ~6.7 Å increase in thickness after GOX adsorption, the enzyme is likely buried and intercalated within the redox polymer thin film. This is expected due to the nature of the electrostatic interactions between the polyions where very strong multipoint contacts are prevalent. The thickness determined for LAX multilayers is almost identical to those determined for GOX. The average thicknesses of LAX-containing films were ~11 Å for the redox polymer layers and 6.5 Å for the enzyme layers, with a linear increase in film thickness upon the addition of each layer ($R^2 > 0.98$). The thicknesses of these multilayered nanocomposites are comparable with the few reported values for nonbiosensor applications.[17,20] Controls were performed on gold substrates that were not functionalized with MUA, and it was found that there was a negligible change in thickness as measured via ellipsometry when an attempt was made to deposit both enzyme and/or redox polymer layers.

Electrochemical Characterization. Cyclic voltammetry, shown in Figure 4 for scan rates ranging from 5 to 400 mV/s, indicated a formal potential of ~250 mV with a difference in peak potential of ~60 mV. This indicated that the system is reversible, as the peak potential is independent of the scan rate and the peak current changes linearly with scan rate raised to the one-half power (not

(30) Jordan, C. E.; Corn, R. M. *Anal. Chem.* **1997**, *69*, 1449−1456.
(31) Schumann, W.; Ohara, T. J.; Schmidt, H.-L.; Heller, A. *J. Am. Chem. Soc.* **1991**, *113*, 1394−1397.
(32) Franchina, J. G.; Lackowski, W. M.; Dermody, D. L.; Crooks, R. M.; Bergbreiter, D. E.; Sirkar, K.; Russell, R. J.; Pishko, M. V. *Anal. Chem.* **1999**, *71*, 3133−3139.

shown). Repeated cyclic voltammetry without the presence of glucose produced scans whose peaks did not degrade appreciably over time, demonstrating that the electroactive components were not free to diffuse away from the electrode surface. Subsequently, cyclic voltammograms at a scan rate of 20 mV/s were used to characterize the number of electroactive osmium redox sites present in each layer, an important criterion for the assessment of the sensor's electrochemical function. This number was determined based upon the cyclic voltammogram of a wafer with only a single redox polymer layer adsorbed (MUA/PVP−Os−AA). The area under the anodic peak was integrated from the voltammogram and used to determine the surface coverage of osmium redox sites present per adsorbed layer. This value, ∼4.1 × 10^{-11} mol cm^{-2}, compares favorably with the values of (4−5) × 10^{-11} mol cm^{-2} reported for ferrocene monolayers[26] and 3 × 10^{-11} mol cm^{-2} reported for $Os(bpy)_3^{2+}$ layers.[33] On the basis of the redox polymer film thickness reported earlier and the surface coverage, the concentration of redox sites per monolayer is ∼3.2 × 10^{-4} mol cm^{-3}.

Substrate Response. The multilayer scheme has distinct advantages over other types of sensor fabrication methods reported in the literature. Consider specifically the sensing mechanism for enzymatic catalysis and electron transfer between polycationic osmium redox polymers and polyanionic glucose oxidase.

$$\beta\text{-D-glucose} + \text{GOX(FAD)} \rightarrow \text{gluconolactone} + \text{GOX(FADH}_2\text{)}$$

$$\text{GOX(FADH}_2\text{)} + 2\text{Os(bpy)}_2\text{(vp)}^{2+} \rightarrow \text{GOX(FAD)} + 2\text{Os(bpy)}_2\text{(vp)}^{1+}$$

$$\text{Os(bpy)}_2\text{(vp)}^{2+} + \text{Os(bpy)}_2\text{(vp)}^{1+} \rightarrow \text{Os(bpy)}_2\text{(vp)}^{1+} + \text{Os(bpy)}_2\text{(vp)}^{2+}$$

$$2\text{Os(bpy)}_2\text{(vp)}^{1+} \rightarrow 2e^- + 2H^+ + 2\text{Os(bpy)}_2\text{(vp)}^{2+}$$

Qualitatively, sensor response times should be minimized due to increased proximity between the enzyme and charge mediator in an ordered structure where enzymes are in direct contact with the redox polymer. Distances much larger than 10 Å result in very low rates of electron transfer;[34] the multilayer structure places redox sites well within 10 Å of each other and of enzymes.

The response of the enzyme electrodes to the addition of substrate was characterized using both constant-potential amperometry and cyclic voltammetry. Current response values for nanocomposite films consisting of native LAX and PVP−Os−AA are depicted in Figure 5. We see a linear response for a multilayer structure consisting MUA/PVP−Os−AA/LAX with a sensitivity of 0.027 μA cm^{-2} mM^{-1}, an R^2 value of ∼0.98, and a standard deviation of ∼12% between electrodes.

For a GOX multilayer, we see a relatively linear calibration curve from 2 to 20 mM substrate with a sensitivity of 0.015 μA cm^{-2} mM^{-1} and an R^2 value of ∼0.98 (Figure 6). The error bars indicate a standard deviation of ∼17% between electrodes. This

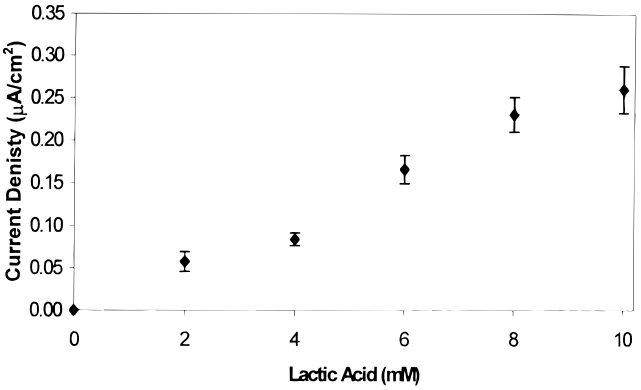

Figure 5. Increase in current density with substrate concentration for lactate enzyme electrode using multilayer scheme consisting of LAX and PVP−Os−AA (MUA/Os/LAX).

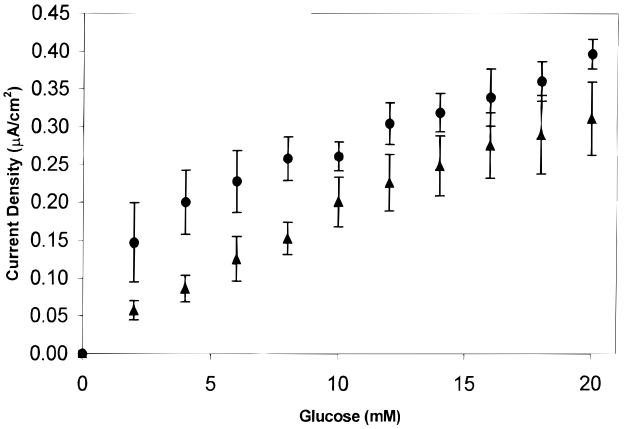

Figure 6. Response of glucose enzyme electrodes to substrate addition: (▲) electrostatically assembled multilayer structure; (●) chemically cross-linked multilayer structure.

compares favorably with the American Diabetes Association acceptable standard deviation of 15%.[35] The response in the absence of oxygen indicates electron transfer between GOX and osmium sites along with charge propagation through the redox polymer to the electrode surface. In the presence of oxygen, the response is reduced by ∼50%. This indicates, as expected, that oxygen does compete with osmium sites in the multilayer structure. The signal-to-noise ratio (SNR) was determined from the electrode response, under N_2, at 6 mM glucose and was calculated as the steady-state current at that substrate concentration divided by the standard deviation of the current (i.e., the noise). At 6 mM, the SNR was ∼100, indicating a relatively sensitive electrode at physiological glucose levels. As expected by the thickness of the nanocomposite thin film, the response times are quite rapid for both glucose and lactate enzyme electrodes, with times to reach 95% of the final response under 4 s for step changes in substrate concentration in the physiological range.

The lactate enzyme electrodes demonstrate a higher response as compared to a glucose enzyme electrode. The opposite might be expected since the glucose oxidase solution used for enzyme deposition was ∼2 orders of magnitude greater in activity than that of lactate oxidase. However, one must consider the size of

(33) Kuhn, A.; Anson, F. C. *Langmuir* **1996**, *12*, 5481−5488.
(34) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.
(35) American Diabetes Association. *Diabetes Care* **1987**, *10*, 95−99.

each of these macromolecules. GOX is a dimer of large molecular weight, ~180 000, more than twice the size of LOX, ~80 000. Considering enzyme intercalation in a highly charged redox polymer layer, it is possible that a larger amount of LOX was electrostatically incorporated into each layer. This may be true from a physical standpoint, as a larger number of smaller spheres (both GOX and LOX are globular) will pack into a given space than will larger spheres. This may also be true from an electrostatic standpoint, as the negative or anionic portions of LOX are potentially more accessible for electrostatic complexation, as the enzyme is smaller and these residues will more likely reside near the surface of the protein. This type of phenomenon has been observed in the past where Heller and colleagues[36] demonstrated higher current densities (~25−50%) for lactate enzyme electrodes as compared to glucose enzyme electrodes under analogous conditions.

The current densities determined in these experiments for both lactate and glucose enzyme electrodes, as expected, are relatively low due to the size of the electrodes used. As ellipsometry necessitated the use of larger Au substrates (6.45-cm^2 electrodes), the obtained current densities do not compare favorably to those present in the literature, including studies reporting 10−20 μA cm^{-2} mM^{-1} for polymer film-based sensors.[37,38] However, when these nanocomposite films were fabricated on gold microelectrodes ($d \sim 250$ μm), current densities approached 10 μA cm^{-2} mM^{-1}.

Multilayer Cross-Linking. Further covalent stabilization of the resultant nanocomposite thin films was desired not only to physically strengthen the film structure but also to eliminate potential desorption due to changes in pH, ionic strength, or high oxidation potentials. We have demonstrated that extreme changes in pH are capable of releasing biomolecules that are entrapped through electrostatic interactions.[32] Other studies have demonstrated the destruction or desorption of SAMs at negative applied potentials.[39,40] In addition, covalent bonds between the layers are likely to improve charge-transfer rates and, hence, sensor response. In redox polymers, electron transfer is believed to occur by two primary mechanisms: electron hopping between redox sites and routing along polymer chains. Although imparting covalent cross-links may suggest a decrease in redox polymer flexibility and subsequently reduced conductivity, cross-linking of the multilayer structure likely decreases the distance between enzyme active sites and redox sites while providing additional direct routes of charge transfer, both from the enzyme to redox sites and between redox sites via self-exchange.[41]

To further stabilize the structure, previously tested GOX enzyme electrodes were chemically cross-linked by exposure to glutaraldehyde vapors where it is anticipated that arginine and lysine residues of the enzyme will react with amines present on the redox polymer. Figure 6 also shows that there is an increase in current density upon cross-linking with glutaraldehyde as opposed to non-cross-linked films, with an average increase of ~60%. The standard deviation for electrodes based on cross-linked multilayer structures was ~13% between electrodes. With an R^2 ~ 88%, the response is not as linear as that for the non-cross-linked films. This appears to indicate that the Michaelis−Menten enzyme kinetics of the enzyme become rate limiting in the electrode reactions when the nanocomposite film is cross-linked, possibly due to improved electron transfer between the redox polymer and enzyme. These cross-linked films were able to retain nearly 100% of their activity for a minimum of three weeks when refrigerated (tested up to four weeks of retained activity), as opposed to a maximum of two weeks for non-cross-linked nanocomposites.

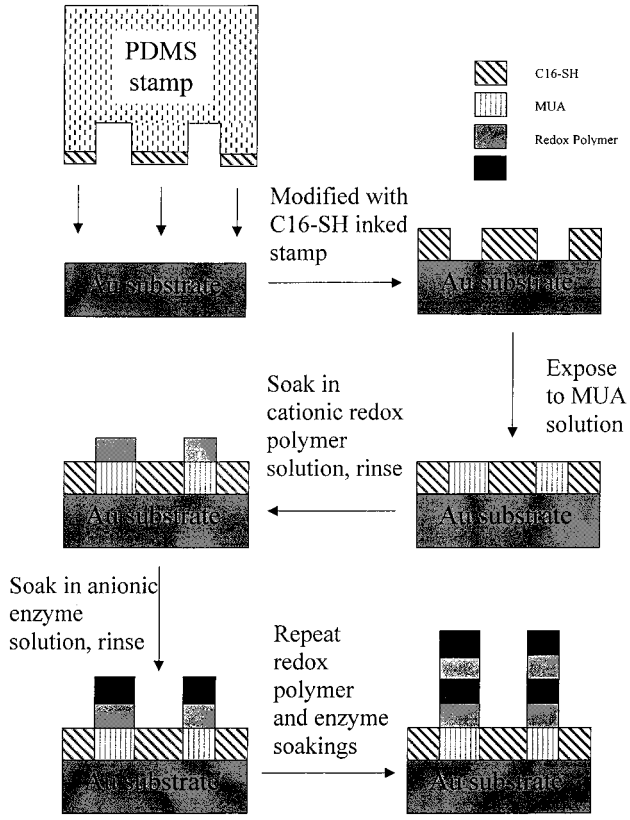

Figure 7. Schematic for buildup of multilayer films; repeated to generate the desired number of layers and sonicated between each step.

Patterning. With the goal of achieving spatially distinct and individually addressable biosensor array members, patterns of the multilayers were generated using the microcontact printing technology developed by Xia and Whitesides.[29] Stamps for μCP were fabricated by depositing and curing PDMS on a photolithographically generated master—a USAF test grid, consisting of rectangles and numbers of varying size. The scheme for patterning is depicted in Figure 7, with a resultant pattern shown in Figure 8. The bars shown here have the critical lateral dimensions of ~280 and 150 μm. This is true for both of the distinctly visible regions—those containing the multilayer structure and the bars blocked with C16-SH. Features as small as 10 μm have been identified, and 1−2-μm features are feasible using this technique.[29] Analogous to typical optical lithography techniques, the resulting film would be a "positive" pattern, as the regions stamped do not

(36) Delumleywoodyear, T.; Rocca, P.; Lindsay, J.; Dror, Y.; Freeman, A.; Heller, A. *Anal. Chem.* **1995**, *67*, 1332−1338.
(37) Malitesta, C.; Palmisano, F.; Torsi, L.; Zambonin, P. G. *Anal. Chem.* **1990**, *62*, 2735−2740.
(38) Ryan, M. R.; Lowry, J. P.; O'Neill, R. D. *Analyst* **1997**, *122*, 1419−1424.
(39) Garrell, R. L.; Chadwick, J. E. *Colloids Surf. A: Physicochem. Eng. Aspects* **1994**, *93*, 59−72.
(40) Widrig, C. A.; Chung, C.; Porter, M. D. *J. Electroanal. Chem.* **1991**, *310*, 335−359.
(41) Aoki, A.; Heller, A. *J. Phys. Chem.* **1993**, *97*, 11014−11018.

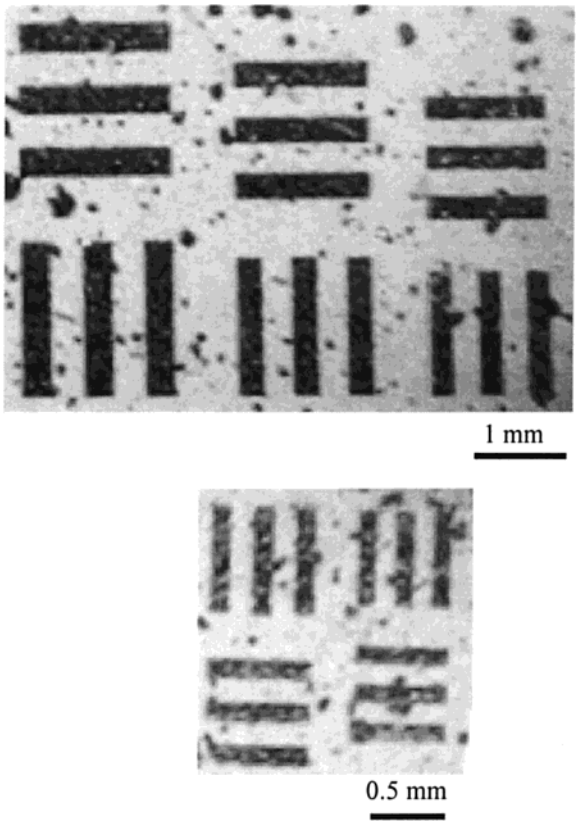

Figure 8. Patterns generated by μCP and subsequent multilayer buildup (dark regions on micrograph). Bars from pattern (top) have the critical lateral dimension of ~280 μm. Smaller features below have the critical lateral dimension of ~150 μm in width.

contain the multilayer sensing nanocomposite. If MUA or some other surface functionalizing agent were stamped instead of the blocking agent, or if an inverted stamp were fabricated, a "negative" pattern could be generated. This type of patterning may be utilized for the generation of redundant sensor arrays based upon nanocomposite electrostatic multilayers in the future.

CONCLUSIONS

We have developed robust glucose and lactate sensors using techniques of self-assembly and polyion adsorption. A gold surface was functionalized with a negative charge through reaction with MUA. Subsequently, cationic redox polymers were adsorbed followed by attachment of anionic GOX or LAX. This process was repeated as desired for the development of highly reproducible enzyme electrodes with standard deviations of ~12−17% between multiple electrodes. Sensor function was further improved through interlayer chemical cross-linking that stabilized the structure. Finally, a preliminary demonstration of patterning using the techniques of μCP was performed as a precursor to the development of a redundant glucose sensor array.

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