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Layer-by-Layer Assembly of Ferrocene-Modified Linear Polyethylenimine Redox Polymer Films

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Herein, both electrostatic and covalent layer-by-layer assembly were used for the construction of multicomposite thin films using a ferrocene-modified linear poly(ethylenimine) redox polymer (Fc-C_6-LPEI) as the cationic polyelectrolye, and poly(acrylic acid) (PAA), poly(glutamic acid) (PGA), or glucose oxidase (GOX) as the negative polyelectrolyte. The assembly of the multilayer films was characterized by cyclic voltammetry (CV), UV/Vis spectroscopy, and ellipsometry with the enzymatic response of the films containing GOX being characterized via constant potential amperometry. CV measurements suggested that the successful buildup of multilayer films was dependent upon the nature of the anionic polyelectrolyte used. Electrostatic assembly of films composed of Fc-C_6-LPEI and either PAA or PGA produced large oxidation peak current densities of 630 and 670 $\mu A\,cm^{-2}$, respectively, during cyclic voltammetry. Increased measured absorbance by UV/Vis spectroscopy and increased measured film thicknesses (400–600 nm) by ellipsometry provided additional evidence of successful film formation. In contrast, the films incorporating GOX that were electrostatically assembled surprisingly produced significantly lower electrochemical responses (12 $\mu A\,cm^{-2}$), low absorbance values, and reduced film thicknesses (~15 nm), and glucose electro-oxidation current densities less than 1 $\mu A\,cm^{-2}$, which all suggested unstable or minimal film formation. Subsequently, we developed a covalent layer-by-layer approach to fabricate films of Fc-C_6-LPEI/GOX by covalently linking the amine groups of Fc-C_6-LPEI to the aldehyde groups of periodate-oxidized glucose oxidase. Covalent assembly of the Fc-C_6-LPEI/GOX films produced oxidation peak current densities during cyclic voltammetry of 40 $\mu A\,cm^{-2}$ and glucose electro-oxidation current densities of 220 $\mu A\,cm^{-2}$. These films also showed an increase in their thicknesses (~140 nm) relative to the electrostatic GOX films. For the films containing either PAA or PGA, the pH of the polymer solutions used for construction was found to have a significant effect on the response of the multilayer films, and the electrochemical response of the Fc-C_6-LPEI/PAA, Fc-C_6-LPEI/PGA, or covalently assembled Fc-C_6-LPEI/GOX films could be tuned by varying the number of bilayers ($n = 1$–16) in the film. These results are important because this is the first report of the use of the novel Fc-C_6-LPEI redox polymer in the successful development of multicomposite layer-by-layer films. The electrochemical response achieved with the covalently assembled Fc-C_6-LPEI/GOX films demonstrates that this redox polymer and layer-by-layer assembly technique can be used for possible biosensor and biofuel applications, and the success of multiple anionic polyelectrolytes could lead to additional applications with other enzyme systems.

1. Introduction

Layer-by-layer self-assembly of polyelectrolytes is a popular method for constructing thin polymer films and can be used for a variety of applications, including biosensors,[1–4] biofuel cells,[5–7] and drug delivery.[8–10] Major advantages of this technique are its: 1) simplicity—alternating exposure of a surface to polycationic and polyanionic solutions;[11,12] 2) exquisite control of film composition, structure, and growth rate;[13] 3) low-cost fabrication of nanoscale film thicknesses; and 4) versatility—can be employed with various types of polymers, particles, and biological molecules (e.g. DNA,[14–16] enzymes,[6,17] antibodies[18,19]) and on both planar and non-planar surfaces.[20,21] In addition, the sequential buildup of these multilayer films is not restricted to only electrostatic interactions, but assembly can also be achieved through other non-covalent interactions such as hydrogen bonding[22] or biological affinity interactions(i.e. antigen-antibody binding,[23] lectin-sugar binding[24]), or by covalent bonding as well.[25,26]

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Several studies have demonstrated that a variety of factors are important in successful film formation by the LBL technique. In the case of electrostatic assembly, external factors such as temperature, pH, salt concentration, surface chemistry, deposition method (e.g. spin coating, dip-coating, spray coating),[27–29] and deposition time[30] are important parameters. Likewise the molecular nature of the polyelectrolytes (i.e. MW, charge density, flexibility, pH sensitivity) is also an important parameter in successful film formation.

Although there are a variety of cationic polymers [poly(allylamine) (PAH),[3,17,31] poly(dimethyldiallylammonium chloride) (PDDA),[16,17] and poly-L-lysine (PLL)[17,32]] that are routinely employed in electrostatic LBL assembly, both branched (BPEI) and linear (LPEI) polyethylenimine are attractive ones due to the fact that their molecular structure (two carbon groups for every amine nitrogen) provide the highest density of cationic charges (protonable amines) per molecule and thus ideal to promote electrostatic interactions. Applications of LBL films involving BPEI include: 1) characterizing the build-up of multilayer films using different water soluble proteins such as myoglobin, hemoglobin, and glucose oxidase through quartz crystal microbalance and UV/Vis spectroscopy;[11] 2) using magnetic field alignment to construct films with single-walled carbon nanotubes functionalized in an anionic surfactant (NaDDBS);[33] 3) developing sensors for the detection of DNA or for identifying DNA-binding drugs;[14] and 4) using Fc-BPEI with gold nanoparticles on an indium tin oxide (ITO) array electrode as a technique for detecting cocaine.[34] Similarly, applications involving LPEI include: 1) evaluating the effect of pH on the surface mobility of viruses;[13] 2) developing multilayer films to capture polymeric nanocarriers for drug delivery;[35] 3) assembly of polymer nanocomposites with Prussian Blue nanoparticles for electrochemical control over swelling and mechanical properties of film.[36]

Recently, the layer-by-layer approach has been recognized as a powerful technique to fabricate biosensors through the electrostatic assembly of redox polymers and enzymes.[3] As first demonstrated by Adam Heller and colleagues, redox polymers allow for the collection of electrons from the redox center of an oxidoreductase enzyme, such as glucose oxidase, when an electrostatic complex is formed between the enzyme and redox polymer.[37] Although a variety of redox polymers (e.g. osmium- and ferrocene-modified polyallylamine, osmium-modified polyvinylpyridine) have been incorporated into LBL films to produce nanoscale biosensors,[3,4,38–43] there have only been a handful of studies that have utilized poly(ethylenimine) based redox polymers in LBL assembly with a majority of these employing BPEI. Du et al. developed a sensor to cocaine by employing LBL to electrostatically assembled films of ferrocene modified BPEI (Fc-BPEI) and gold nanoparticles (Au-NPs) with the outmost layer of Au-NPs covalently containing a cocaine aptamer fragment.[34] In a similar strategy, sensors for thrombin and lysozyme were developed by LBL assembly of Fc-BPEI and multiwalled carbon nanotubes and aptamers.[44] Likewise Liu et al. reported LBL assembly of Fc-BPEI and Au-NPs for ascorbic acid determination.[45] Finally, Anzai's group has assembled LBL films of Fc-BPEI with either poly(vinyl sulfate) (PVS)[46] or DNA[14] to study their redox properties.

We have recently synthesized a series of novel ferrocene-modified LPEI polymers (Fc-C_n-LPEI) that are able to produce high current responses with both glucose oxidase and peroxidase.[47–49] Based on their high current densities, they appear to be excellent candidates for LBL assembly. In this study, we fabricate LBL films with the cationic redox polymer Fc-C_6-LPEI and the anionic polymers poly(acrylic acid) polymer (PAA), poly(glutamic acid) (PGA), or the anionic enzyme glucose oxidase(GOX). We report the effects of pH and method of assembly (i.e. electrostatic vs. covalent) on LBL film formation.

Experimental Section

Chemicals and Solutions

Glucose oxidase (GOX) from *Aspergillus niger* (EC 1.1.3.4, type X-S, 181 units mg^{-1} solid, 75% protein), poly(glutamic acid) sodium salt (PGA, $M_w = 50\,000$–$100\,000$), 11-mercaptoundecanoic acid (MUA), HEPES, and D-glucose were purchased from Sigma Chemical Co. (St. Louis, MO), and all chemicals were used as received. Poly(acrylic acid) (PAA, $M_w = 50\,000$, 25% aqueous solution) was purchased from Polysciences and used as received. The redox polymer designated as Fc-C_6-LPEI was synthesized according to a previously published procedure,[48] with 14.7% of the nitrogen in the polymer backbone substituted with ferrocene. A 50 mM sodium phosphate buffer was prepared by dissolving NaH_2PO_4 in Nanopure deionized water and adding sodium hydroxide pellets to adjust the pH to 7.0. Spectra/Por molecular porous membrane tubing (M_w cutoff 12 000–14 000) from Spectrum Labs was used for dialysis. The sodium phosphate buffer used for dialysis was prepared with 4.26 g of Na_2HPO_4 in 1500 mL of Nanopure deionized water. The pH of this buffer was adjusted to 7.4 using a 50% (v/v) HCl aqueous solution. Stock solutions of 2 M glucose were allowed to mutarotate for 24 h before use and were kept refrigerated at 4 °C when not in use.

Preparation of Electrostatic LBL Films

Gold electrodes (2.0 mm diameter, CH instruments, Austin, TX) were first polished with 1 and 0.25 μm diamond polishing slurry on nylon polishing pads before being polished on a microcloth pad with 0.05 μm alumina. For the films constructed with PAA and PGA, a 20 mM redox polymer solution based on the repeat-unit molecular weight of the redox polymer (Fc-C_6-LPEI $M_w = 44.077\ g\,mol^{-1}$) in Nanopure deionized water was used. Alternatively, a 0.23 M solution of Fc-C_6-LPEI in Nanopure deionized water was used for the films constructed with GOX. Similar to unmodified LPEI, which has a pK_a of 5.5,[13] Fc-C_6-LPEI is insoluble in water above neutral pH, and thus, solutions of Fc-C_6-LPEI were prepared by initially dissolving the polymer in water and adding small aliquots of 0.1 M HCl solution or a concentrated NaOH solution until the final concentration of the polymer solution was reached and the desired pH was achieved (3.0–5.0).[48,50] LBL films constructed with either PAA (repeat unit $M_w = 288.5\ g\,mol^{-1}$) or PGA (repeat unit $M_w = 129.12\ g\,mol^{-1}$) were built using 20 mM solutions of each polyanionic polymer in Nanopure deionized water. These concentrations were calculated based on the repeat-unit molecular weight of the polymers. Alternatively, a 0.125 mM solution of GOX based on the molecular weight of one dimer subunit of the enzyme ($M_w = 80{,}000$) in an aqueous 20 mM HEPES buffer (pH 7.4) was used for the LBL films containing GOX.

The first step in the LBL process was to functionalize the polished gold electrodes with a negatively charged layer of MUA by immersing the electrode in a solution of 5 mM MUA in ethanol for 20 min.[51] The electrodes were then removed and washed with ethanol followed by Nanopure deionized water. The electrode washing was performed by briefly squirting ethanol and then Nanopure water on the body of the electrode and then wiped clean with a chem-wipe to remove any excess fluid. The electrodes were next immersed in a polycationic solution of redox polymer (Fc-C_6-LPEI) for 5 min, washed with Nanopure deionized water to remove any excess material using the same washing procedure, and then they were immersed in a polyanionic solution of either the PAA polymer, PGA polymer, or GOX enzyme for 5 min. Multilayer films were constructed by repeating this procedure until the desired number of bilayers was reached. For all LBL films, the final layer was the Fc-C_6-LPEI polymer. In the nomenclature of this paper, *n* bilayers (1, 2, 4, etc.) refer to number of complete bilayers (Fc-C_6-LPEI/PAA; Fc-C_6-LPEI/PGA; or Fc-C_6-LPEI/GOX) plus the extra layer of Fc-C_6-LPEI polymer used as the final layer. The pH of both charged solutions was varied to determine the optimal pH conditions. These optimal pH conditions were then used in the experiments in which the number of bilayers was varied.

Preparation of Covalently Modified LBL Films

5 mL of a 20 μM solution of GOX in 10 mM sodium phosphate buffer, pH 6.8, was prepared. This solution was then reacted with 30 mg of sodium periodate. This reaction was allowed to take place for 1 hour in the dark at 4 °C. After 1 hour, 25 μL of a 25 mM ethylene glycol solution in Nanopure water was added to stop the reaction. This was allowed to occur in the dark at room temperature for 30 min. The reaction between the aldehyde groups of the periodate-treated GOX and the amine groups of the redox polymer occurs fairly easily and has been reported to not cause any deactivation of the enzyme.[31]

After this reaction occurred, dialysis was used to remove the periodate compound from the GOX enzyme solution. This step was critical to the construction of these sensors because if the periodate was still present in the solution, it oxidized the redox polymer. The ferrocene redox polymers are easily oxidized, which causes a denaturation of the protein. This prevented the sensors from producing any significant electrochemical response. Membrane tubing with a molecular weight cutoff of 12000–14000 was used for the removal of the sodium periodate. The dialysis procedure was performed for 12 h using 20 mM sodium phosphate buffer at pH 7.4. The buffer solution was changed every 4 h, and final GOX enzyme solution was stored at 4 °C.

The GOX sensors were constructed by first immersing the electrodes in a positively charged thiol. The thiol used for these sensors was a 20 mM solution of cystamine dihydrochloride in Nanopure water. The electrodes were incubated in this solution for 20 min. The electrodes were removed and washed with Nanopure water to remove excess material. The same washing procedure used for the electrostatic electrodes was also used in this case. Electrodes were then incubated in the 20 μM periodate-treated GOX solution for 10 min, washed with Nanopure deionized water, and then incubated in the polycationic Fc-C_6-LPEI solution for 10 min. The Fc-C_6-LPEI polymer solution was identical to the polymer solution prepared for the PAA and PGA electrostatic sensors (20 mM concentration at pH 5.0). Multilayer films were constructed by repeating this procedure until the desired number of bilayers was reached. In contrast to the previous sensors, an additional layer was not added after the final bilayer because the Fc-C_6-LPEI was the final solution deposited on the electrode surface.

UV/Vis Spectrophotometry and Ellipsometry

The assembly of the LBL films was also evaluated by constructing multilayer films on quartz substrates with Fc-C_6-LPEI combined with PAA, PGA, or GOX, and performing UV/Vis spectrophotometry. These films were constructed with the same polymer concentrations and incubation times as the films built on the gold electrodes, except the MUA thiol was not needed because the initial polymer solution layer absorbed to the quartz slide without having to functionalize the surface. The quartz slides were immersed in Nanopure water for 2 seconds between each layer to remove any excess material. Absorption spectra were taken with a UV-Vis spectrophotometer (DU 800, Beckman Coulter) to characterize the buildup of the multilayer films constructed with the different polyanionic species by a previously developed protocol.[51,52]

The thickness of the multilayer films was determined through ellipsometry by a method similar to that previously described.[52] These measurements were performed on gold-coated silicon wafers (100 nm Au layer on 5 nm of Cr on Si(100)) using a Gaertner LSE Stokes ellipsometer (Gaertner Scientific Corp., Chicago, IL) at a fixed angle of incidence of 70°. Films were constructed out to 8 bilayers (17 layers) with the same procedure used to assemble these films on Au electrodes, and measurements were taken after each layer. For each film type we conducted 3 separate trials in which the ellipsometric parameters were collected from 6 different points on each sample after each adsorption step. The films were dried under a stream of N_2 for 1.5 min after each layer was deposited. All measurements were made using a He-Ne laser at a wavelength of 632.8 nm, and the film thicknesses were calculated using the Gaertner software using a weighted refractive index for these films. The refractive index values for MUA and GOX was chosen as1.46 based on previous studies involving similar measurements of LBL films.[4,52] Additionally, the refractive index of PAA provided by Polysciences, Inc. is 1.52, and a refractive index of 1.52 for PGA has been used previously.[53] Because there are no reports of this Fc-C_6-LPEI being used in other studies, the refractive index of LPEI ($n = 1.58$) was used in this study.[54]

Electrochemical Characterization

Cyclic voltammetry measurements were performed with a CH Instruments biopotentiostat (CHI832, Austin, TX) in a three-electrodes configuration with a platinum-wire counter electrode and a saturated calomel reference electrode (SCE). All experimental measurements were conducted at room temperature (25 °C) and repeated a minimum of three times. The values presented are the mean ± standard error of the mean (SEM), unless otherwise specified.

2. Results and Discussion

2.1. Electrostatic LBL Assembly of Multilayer Films of Fc-C_6-LPEI and Polyacrylic Acid and Polyglutamic Acid

Previous studies have reported the successful construction of multilayer films of unmodified LPEI and polyacrylic acid by electrostatic LBL.[54,55] Likewise, nanoparticles for gene delivery have been fabricated by electrostatic LBL assembly of polyglutamic acid with branched PEI.[56,57] Thus, we hypothesized that multilayer films of Fc-C_6-LPEI with PAA and PGA could be constructed by electrostatic LBL assembly.

Since the redox polymer Fc-C_6-LPEI has well-defined redox properties,[48,50] we used cyclic voltammetry (CV) to characterize the build-up of the multilayer films of Fc-C_6-LPEI with PAA and PGA and to investigate the role of pH on film formation. Figure 1 A shows representative CV curves for LBL films constructed on gold electrodes using the positively charged Fc-C_6-LPEI redox polymer and the negatively charged PAA polymer in which the number of bilayers was held constant at 4 bilayers. The pH of both polymer solutions were the adjusted to the same value and varied between pH 3.0 and 6.0. We selected this pH range based on: 1) the pK_a of unmodified LPEI being 5.5;[13] 2) the pK_a of PAA being 4.2;[58] and 3) the previous studies on layer-by-layer assembly of LPEI/PAA layer-by-layer films by Yoo et al.[13] who demonstrated exponential film growth above pH 4. As shown in Figure 1 A, a pair of well-defined redox waves, corresponding to the oxidation and reduction of the redox polymer's ferrocene centers, were observed at ~260 and ~220 mV (vs. SCE), respectively. Although both the oxidation and reduction peak currents increased as the pH of the polymer solutions was increased from 3.0 to 6.0, the peak oxidation and reduction potentials remained fairly constant. Since the responses at pH 5.0 and 6.0 were similar, and because it has been suggested in the literature that assembly of LPEI/PAA films is optimal in the pH 4–5 range,[55] we utilized a pH 5.0 to investigate the effect of increasing the number of bilayers. Figure 1 B shows representative CVs of multilayer Fc-C_6-LPEI/PAA films as the number of bilayers was varied between1–16. As expected both the oxidation and reduction peak currents increased with the number of bilayers, with the oxidation peak current reaching a maximum of approximately 20 μA or 630 μA cm^{-2}. The results show that electrochemical properties of the films can be tuned by pH and the number of bilayers.

Figure 2 shows the effect of pH and the number of bilayers on the electrochemical response of films constructed by electrostatic LBL

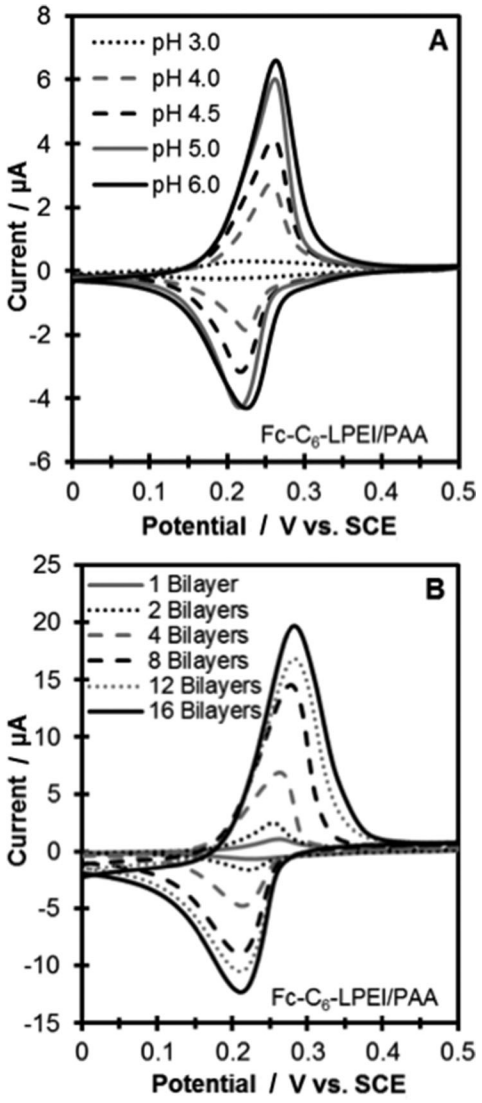

Figure 1. Effect of pH of assembly (A) and number of bilayers present (B) for Fc-C_6LPEI/PAA films on electrochemical response: A) Representative CVs of gold electrodes with the pH of both polymer solutions varied between 3.0 and 6.0 for films composed of four bilayers. B) Representative CVs of gold electrodes with number of bilayers varied between one and 16 and the pH of both polymers fixed at pH 5.0. All experiments performed in 50 mM sodium phosphate buffer at pH 7.0. Scan rate = 50 mV s^{-1}.

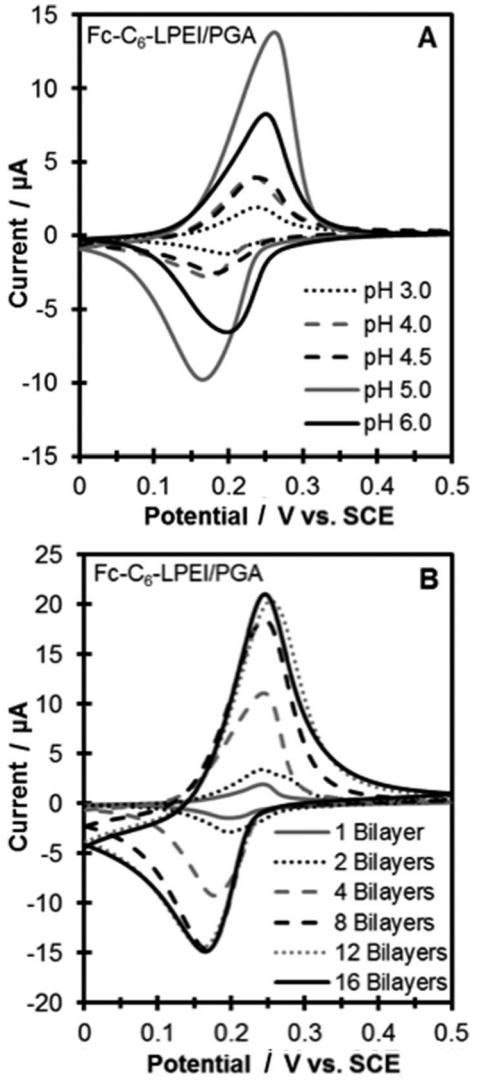

Figure 2. Effect of pH of assembly (A) and number of bilayers present (B) for Fc-C6-LPEI/PGA films on electrochemical response: A) Representative CVs of gold electrodes with PGA pH varied between 3.0 and 6.0 for films composed of 4 bilayers. B) Representative CVs of gold electrodes with number of bilayers varied between one and 16 with the pH of both polymers fixed at pH 5.0. All experiments performed in 50 mM sodium phosphate buffer at pH 7.0. Scan rate = 50 mV s^{-1}.

assembly with Fc-C_6-LPEI and PGA. Similar to the studies with PAA, the pH range for LBL formation with PGA was varied between pH 3.0 and 6.0. This pH range was selected because poly(glutamic acid) has an isoelectric point of 2.19,[59] and thus, its carboxyl groups will have a negative charge at these pH values. Once again a pair of well-defined redox waves corresponding to the oxidation and reduction of the redox polymer's ferrocene centers were observed with films fabricated with PGA. However, in contrast to the results with PAA, changing the solution pH did affect the oxidation and reduction peak potentials. For pH 3, 4.0, and 4.5, the oxidation peak potential was relatively constant at ~235 mV (vs. SCE), but the potential was shifted to more positive values as the pH was increased to 5.0 (260 mV) and 6.0 (250 mV). Likewise, the reduction peak potential varied between 165 mV (pH 5.0) and 200 mV (pH 3.0 and 6.0). The exact cause for this shift in redox potential is not known and under investigation. In addition to affecting the oxidation and reduction peak potentials, changing the pH also affected the magnitude of the oxidation and peak currents. Increasing the pH of the polymer solutions from 3.0 to 4.0 resulted in an initial increase in the peak currents; however, a further increase to 4.5 results in virtually no change in the response. Although increasing the pH to 5.0 resulted in a dramatic increase in both the oxidation and reduction peak currents, a further increase to pH 6.0 resulted in a drop in the oxidation and reduction peak currents. Since pH 5.0 gave the maximum current response in films fabricated with Fc-C_6-LPEI and PGA, this pH was used to study the influence of bilayer number. As shown in Figure 2B, the peak oxidation and reduction peak potentials remained fairly constant at 240 and 175 mV (vs. SCE) for the range of bilayers (n = 1–16) tested. As expected, the oxidation and reduction peak currents increased with bilayer number from 1–8 bilayers. However further increasing the bilayer number to 12 or 16 layers had only a minimal effect on the peak currents (suggesting that a plateau had been reached). The maximum oxidation peak current of 21 μA or 670 μA cm^{-2} produced with PGA was similar to maximum oxidation peak current obtained for films composed of PAA. Taking together the CV results for multilayer films composed with either PAA or PGA indicates that a) Fc-C_6-LPEI can be used as a cationic polymer in the electrostatic assembly of LBL films, and b) the assembly of films utilizing Fc-C_6-LPEI is pH dependent, with the optimum pH being ~5.0. In addition to cyclic voltammetry, we also performed UV/Vis spectroscopy studies to characterize the initial buildup of the multilayer films of Fc-C_6-LPEI with PAA and PGA at the optimum pH of 5.0 on quartz slides (Supporting Information, Figure S1). For films composed of Fc-C_6-LPEI and PAA (Figure S1 A), a significant increase in absorbance was observed as the number of bilayers was increased from 1 to 5 bilayers. The inset graph of the absorbance spectra for PAA shows that the absorbance at 280 nm increased linearly with the number of bilayers suggesting that the thickness of the films remains uniform as more bilayers are added. Similarly, the same trend of increasing absorbance with number of bilayers was observed for multilayers films constructed with Fc-C_6-LPEI and PGA (Figure S1B). These results provide additional evidence for the buildup of the films with PAA and PGA

To characterize the changes in film thickness with each layer at the optimum pH of 5.0, ellipsometry measurements were performed after each deposited layer. Films composed of Fc-C_6-LPEI/PAA and Fc-C_6-LPEI/PGA (Supporting Figure S2 A) both displayed an exponential increase in film thickness with additional layers. Films com-

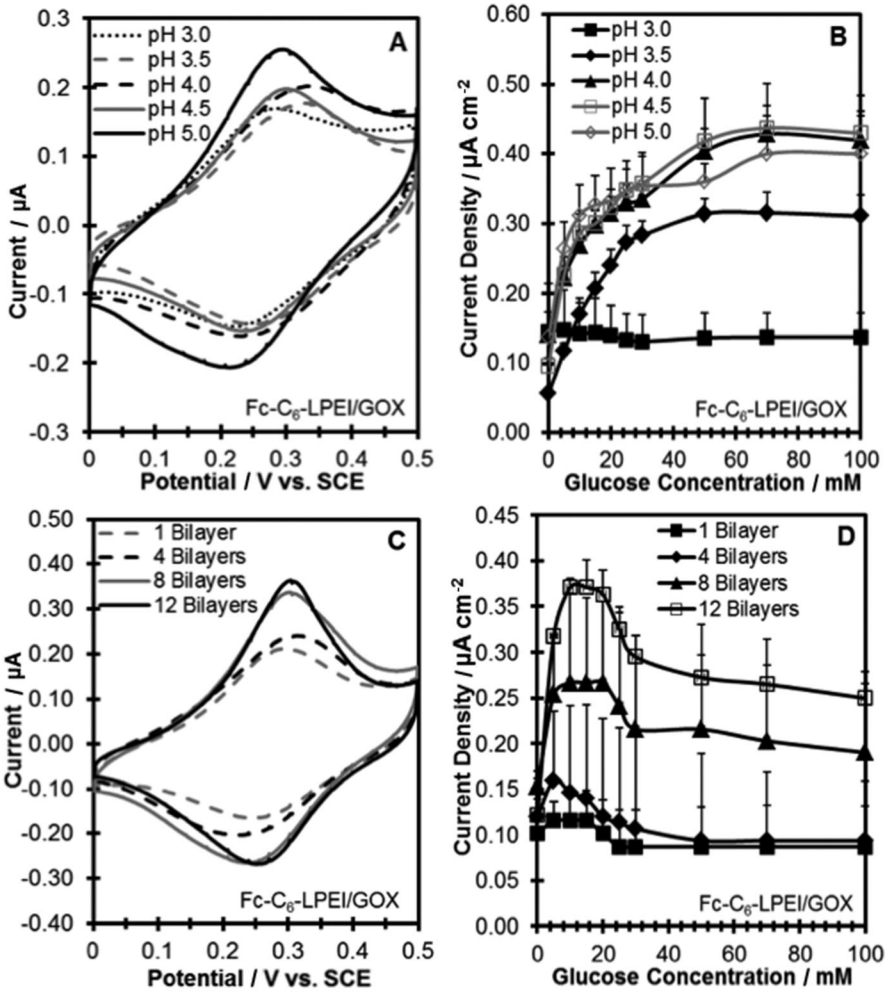

Figure 3. Effect of polymer solution pH and bilayer number on the LBL assembly of Fc-C_6-LPEI/GOX films. A) Representative CVs of gold electrodes coated with films containing four bilayers in which the Fc-C_6-LPEI polymer solution pH was varied between 3.0 and 5.0 and the GOX solution was kept constant at pH 7.4 during the film assembly process. B) Effect of polymer solution pH on glucose response (assembly conditions the same as those described for Figure A); C) Representative CVs of gold electrodes with coated with 1–12 bilayers, and the pH of both the Fc-C_6-LPEI polymer and GOX solutions were fixed at pH 5.0 during the film assembly process. D) Effect of number of bilayers on glucose response with pH 5.0 for both the redox polymer and enzyme. All electrochemical and enzymatic testing were performed in 50 mM sodium phosphate buffer at pH 7.0 (0.5 V vs. SCE, T = 25 °C). Scan rates = 50 mV s^{-1}.

posed of 8 bilayers reached thicknesses of approximately 410 nm for Fc-C_6-LPEI/PAA, and 680 nm for Fc-C_6-LPEI/PGA. Although the exponential growth behavior that we observe is similar to that reported by others for LPEI/PAA LBL films,[13] the film thicknesses with Fc-C_6-LPEI/PAA were considerably greater than with unmodified LPEI. At this time the exact cause of this is unknown and may be related to differences in assembly conditions (e.g. salt concentrations, relative humidity, etc.). These increased film thicknesses may also be related to the presence of the ferrocene and six-carbon spacer, which most likely impacts the structure of the cationic layer and its interaction with anionic polyelectrolytes.

2.2. Electrostatic LBL Assembly of Fc-C_6-LPEI and Glucose Oxidase

Given the results of the successful formation of multilayer films of Fc-C_6-LPEI/PAA and Fc-C_6-LPEI/PGA by electrostatic LBL assembly described above, we hypothesized that multilayer films of Fc-C_6-LPEI and GOX could be fabricated by electrostatic LBL assembly. Cyclic voltammetry was first used to determine the effect of solution pH on the formation of multilayer films of Fc-C_6-LPEI and GOX by LBL electrostatic assembly. In contrast to our studies with PAA and PGA, in which the pH of both solutions were held constant, we systematically varied the pH of the Fc-C_6-LPEI and GOX solutions separately. The rationale for this procedure is that we had determined that the optimal solution pH for films of Fc-C_6-LPEI was found to be pH 5.0 (Figure 1 and 2), while our previous experience of incorporating GOX into LBL films[51] produced improved multilayer assembly and enzymatic responses at pHs (6.0–8.5) much higher than the isoelectric point of 4.05 for GOX.[60] Figure 3 A shows representative CVs for films made with Fc-C_6-LPEI and GOX in which the pH of the Fc-C_6-LPEI polymer solution was varied between 3.0 and 5.0 in 0.5 level increments, while the GOX solution pH was held constant at 7.4. Similar to the results with PAA or PGA, changing the pH of the Fc-C_6-LPEI solution had an effect on the electrochemical response. Although there was an unexpected drop in the peak currents at pH 4.5, in general, the oxidation and reduction peak currents increased with pH. The maximum current response occurred at pH 5 similar to the optimum pH found with PAA and PGA films. Conversely, the oxidation and reduction peak potentials remained fairly constant at peaks at 370 and 315 mV (vs. SCE), respectively. A surprise to us was the low magnitude of the currents (~0.1–0.3 μA) which were approximately 50–100 fold lower than the films made with either PAA or PGA. These low current responses suggested limited or reduced film formation was occurring.

To determine whether the films formed contained any active GOX, the response of the multilayer films to glucose was measured by poising the electrodes at 500 mV (vs. SCE) and measuring the output current as aliquots of a stock 2 M glucose solution were added to a well-stirred phosphate buffer solution. Figure 3 B shows the glucose response of the LBL films that were assembled from Fc-C_6-LPEI polymer solutions of varying pH. Although all of the films assembled at the different Fc-C_6-LPEI polymer solution pHs showed a response to glucose that increased with glucose concentration, there was no consistent trend in the glucose current density (j) with pH ($j_{pH\,4.5} > j_{pH\,5.0} > j_{pH\,3.5} > j_{pH\,4.0} > j_{pH\,3.0}$) at saturating glucose levels. In addition, these values were considerably lower than the glucose current densities (12–220 μA cm^{-2}) that we have previous obtained with LBL assembly of GOX and PVP-Os.[51,52]

To determine whether the reduced electrochemical and enzymatic responses of the Fc-C_6-LPEI/GOX films were related to having only four bilayers present, we fabricated another set of films in which we varied the number of bilayers, but kept the at the Fc-C_6-LPEI polymer solution pH at 5.0 and the GOX at 7.4. Although there was a general increase in the enzymatic response with the number of bilayers, the absolute values of the glucose current densities were still low. Likewise, there was only an incremental response in the CVs, and essentially no difference between 8–12 bilayers.

One possible explanation to the unsuccessful results of electrostatic LBL formation in Figure 3 is that by exposing the deposited Fc-C_6-LPEI layer to a basic pH (i.e. 7.4) during the glucose oxidase adsorption step, the degree of protonation of the amines on the Fc-C_6-LPEI may have been changed to an extent that hindered its electrostatic interactions with GOX. To determine whether this was a cause, we repeated the LBL film assembly process a second time in which we varied the pH of the GOX enzyme solution between 3.0–5.0, while the pH of the Fc-C_6-LPEI polymer solution was held

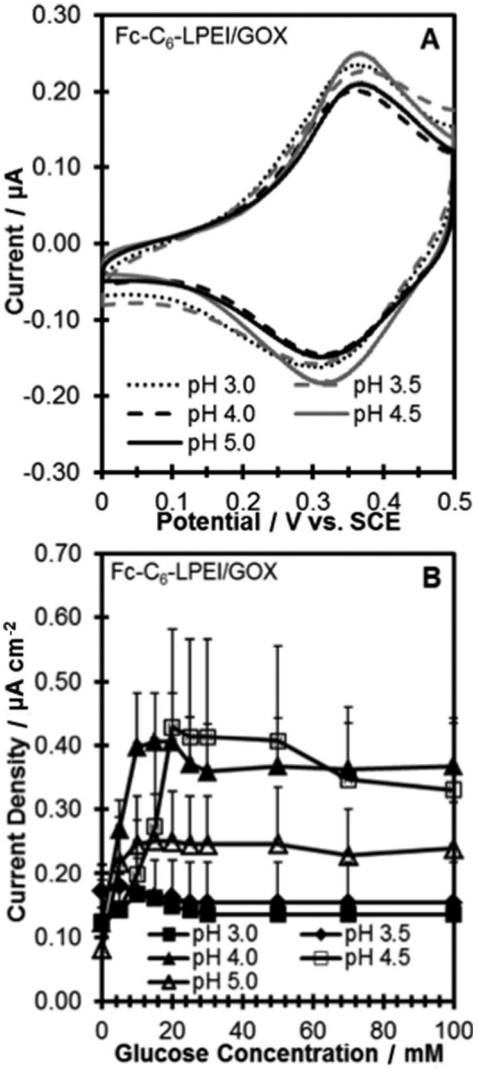

Figure 4. A) Effect of GOX solution pH on LBL Fc-C_6-LPEI/GOX films on electrochemical response. Representative CVs of gold electrodes coated with films containing four bilayers in which the GOX solution pH was varied between 3.0 and 5.0 and the Fc-C_6-LPEI solution was kept constant at pH 5.0 during the film assembly process. B) Effect of GOX enzyme solution pH on glucose response (assembly conditions were the same as those described for Figure A). All electrochemical and enzymatic testing were performed in 50 mM sodium phosphate buffer at pH 7.0. (0.5 V vs. SCE, $T = 25\,^{\circ}C$). Scan rates = 50 mV s^{-1}.

constant at 5.0. As shown in Figure 4A, changing the pH of the GOX solution had a minimal effect on the electrochemical response. Although there were well-defined redox peaks at 370 and 315 mV (vs. SCE), corresponding to the oxidation and reduction of the ferrocene redox centers, the peak currents of ~0.15–0.25 μA were approximately 50–100 fold lower then the films made with either PAA or PGA. Similar to the electrochemical results there was no consistent trend in the glucose response with pH, and the maximum current density values of $<0.5\ \mu A\,cm^{-2}$ were very low (Figure 4B). In addition, the glucose response curves exhibited a peculiar response in which the maximum response peaked at about 5–15 mM and then generally declined.

The limited film buildup for the electrostatic LBL assembly of Fc-C_6-LPEI and GOX were surprising to us given 1) our past success in LBL assembly of PVP-Os and GOX;[51,52] 2) significant glucose current densities (~400 $\mu A\,cm^{-2}$) produced by crosslinked films of Fc-C_6-LPEI and GOX;[48,50] 3) the successful LBL film formation between PAA and PGA with Fc-C_6-LPEI; 4) previous studies reporting the fabrication of LBL films of BPEI and GOX;[11,61–63] and 5) the observation that when solutions of Fc-C_6-LPEI and GOX are mixed together a gel and precipitates form almost immediately (data not shown). Taking the results of Figures 3 and 4 together, it appears that there was limited multilayer film formation between Fc-C_6-LPEI and GOX via the electrostatic LBL assembly process. This observation was further confirmed by the ellipsometry results shown in Supporting Figure S2B, which show minimal film thicknesses and film growth for electrostatically assembled Fc-C_6-LPEI/GOX films. The exact cause of this limited film formation is not known at this time. One possibility is that either the Fc-C_6-LPEI or the GOX molecules are removed from the film upon contact with the opposite solution through the formation of a water-soluble polyelectrolyte complex (WPEC).[64] This type of multilayer film erosion has been demonstrated in the literature for other systems,[64] and is currently under investigation in our lab.

2.3. Covalent LBL Assembly of Fc-C_6-LPEI and Glucose Oxidase

Recently, it has been reported that glucose oxidase can be assembled by covalent LBL into mulilayer films with poly(amidoamine) dendrimers,[65] poly(allylamine),[31] multi-wall carbon nanotubes functionalized with amino groups.[66] With this in mind we investigated whether a similar approach could be applied to our system of Fc-C_6-LPEI and GOX (Figure 5). The basic mechanism of this covalent approach is to first periodate-treat the glucose oxidase to convert the numerous hydroxyl groups on its surface into carbaldehyde groups. The aldehyde groups on the surface of GOX can initially be reacted with the amine groups of cystamine that has been immobilized on the gold electrode surface via a Schiff-base reaction and then can also react with the amino groups on the Fc-C_6-LPEI backbone. Similar to the electrostatic approach, this covalent bond formation process between polymer and enzyme can be repeated until the number of desired bilayers is achieved.

Figure 6A shows the CV responses as the number of covalently assembled Fc-C_6-LPEI/GOX bilayers was varied from one to 16 bilayers. Similar to the results with PAA or PGA, there was a clear trend of increased oxidation and reduction peak currents with the number of bilayers. In addition, the oxidation and reduction peak currents for the covalently assembled Fc-C_6-LPEI/GOX films were three to four times higher than the electrostatically assembled Fc-C_6-LPEI/GOX films. Conversely, there was no clear trend between oxidation/reduction peak potentials with bilayer number. Although each film displayed a pair of well-defined redox waves, the values varied randomly between 260–320 mV for the reduction peak and 300–370 mV (vs. SCE) for the oxidation peak. These electrochemical results provided initial evidence that bilayers of Fc-C_6-LPEI/GOX were being formed by the covalent assembly approach.

Figure 6B shows the enzymatic response of the covalently assembled Fc-C_6-LPEI/GOX films with increasing numbers of bilayers. Similar to the CV responses, the enzymatic responses showed a significant increase in the current density as the number of bilayers was increased. For the films fabricated with 16 bilayers, the sensitivity at 10 mM glucose was 15.9 $\mu A\,cm^{-2}\,mM^{-1}$, while a maximum enzy-

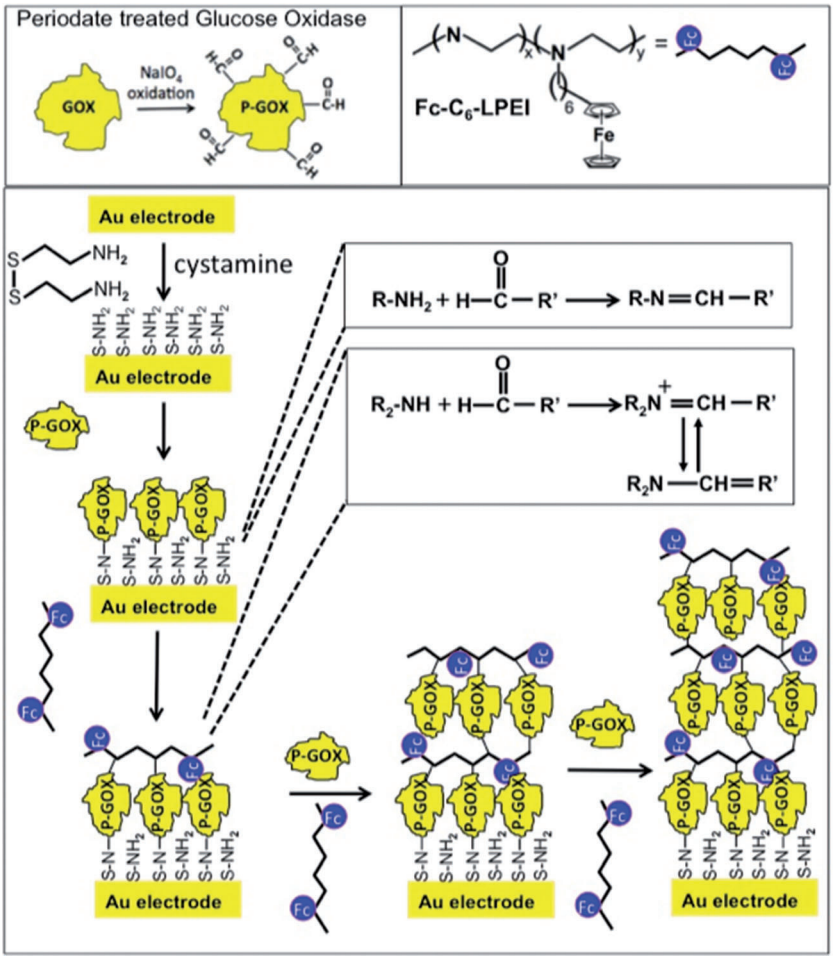

Figure 5. Schematic of the covalent layer-by-layer assembly of periodate-treated glucose oxidase with Fc-C_6-LPEI.

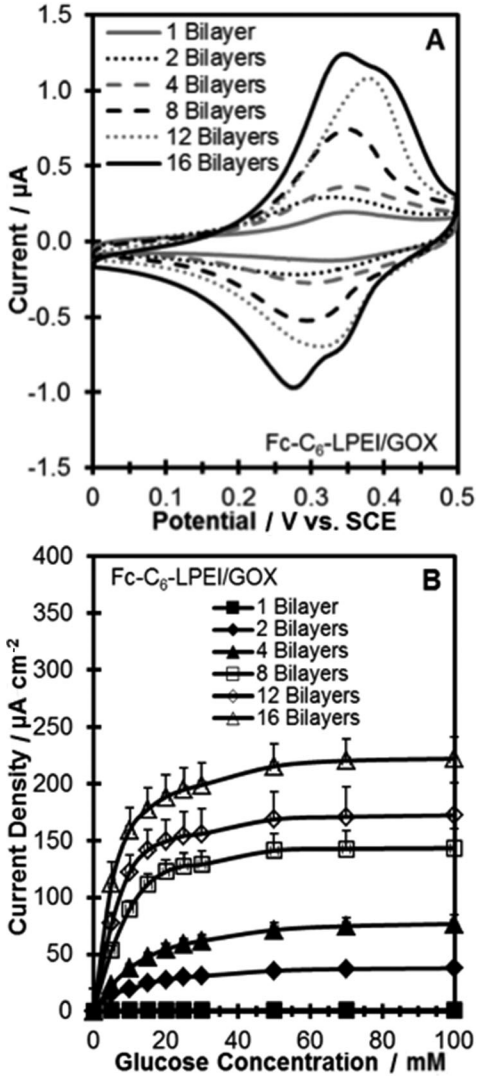

Figure 6. A) Effect of number of bilayers present on the electrochemical and enzymatic response for Fc-C_6-LPEI/GOX films using the covalent attachment technique. A) Representative CVs of gold electrodes with films constructed at Fc-C_6-LPEI pH 5.0 and GOX pH 6.8. Scan rates = 50 mV s^{-1}. B) Effect of number of bilayers present on glucose response for LPEI-GOX films. All experiments performed in 50 mM sodium phosphate buffer at pH 7.0 (0.5 V vs. SCE, $T = 25\,^{\circ}C$).

matic response of approximately 222 μA cm^{-2} was achieved under saturating glucose concentrations. These enzymatic results were particularly encouraging and significant for several reasons. The glucose sensitivities at 10 mM glucose compare favorably with LBL films assembled with osmium-based redox polymers, and are significantly higher than those of ferrocene-based redox polymers (Table 1). Similarly the maximum current densities generated are important because they 1) are similar to our previous results with electrostatically assembled PVP-Os/GOX films, which are among some of the highest current densities reported for LBL films of GOX and Osmium-based or Ferrocene-based redox polymers (Table 1); and 2) they approach previous results with solution cast biosensor films of Fc-C_6-LPEI and GOX that produced maximum glucose current densities around 400 μA cm^{-2}.[48,50]

We characterized changes in film thickness for covalently assembled films Fc-C_6-LPEI/GOX by ellipsometry (Supporting Figure S2B). The results show an increase in film thickness as additional layers were deposited. Compared to the electrostatic PAA and PGA films, covalently assembled films Fc-C_6-LPEI/GOX films exhibited smaller film thicknesses, which is in agreement with the lower electrochemical responses observed in the cyclic voltammograms. The covalently modified GOX films reached a thickness of approximately 140 nm at eight bilayers. These films showed similar results to those seen in previous studies involving the electrostatic LBL assembly of GOX films with PVP-Os[52] and PAH-Os.[67]

Table 1. Comparison of redox-polymer-based glucose sensors assembled by layer-by-layer assembly.

Redox polymer	Enzyme	Bilayer number	LBL assembly method	Current density[a] [μA cm^{-2}]	Sensitivity[b] [μA cm^{-2} mM]	Ref.
PAA-Os	GOX	4	electrostatic	35	3.0	[42]
PAA-Os	GOX	6	electrostatic	50	5.0	[38]
PAA-Os	GOX	4	electrostatic	210	16.0	[43]
PVP-Os	GOX	2	electrostatic	0.3	0.02	[4]
PVP-Os	GOX	6	electrostatic	12	0.6	[52]
PVP-Os	GOX	6	electrostatic	220	17.5	[51]
PAA-Fc	GOX	4	electrostatic	3.0	0.25	[3]
PMVF	GOX	10	covalent	0.5	0.05	[41]
Fc-C_6-	GOX	2	covalent	38	1.9	This
LPEI		4		77	3.8	work
		8		143	9.0	
		12		172	12.2	
		16		222	15.9	

PVP-Os = osmium-modified poly(vinylpyridine); PAA-Os = osmium-modified poly(allylamine); PAA-Fc = ferrocene- modified poly(allylamine); PMVF = Poly(2-methacryloyloxyethyl phosphorylcholine-co-p-vinylphenylboronic acidco vinylferrocene); [a] Current density values evaluated at saturating glucose concentrations. [b] Sensitivity values evaluated at 10 mM glucose.

3. Conclusions

In this study, electrostatic and covalent LBL assembly was used to construct multilayer films on gold electrodes composed of the polycationic redox polymer, Fc-C_6-LPEI, with the polyanionic polymers PAA and PGA, or the negatively charged enzyme GOX. We observed that the formation of LBL films with Fc-C_6-LPEI by electrostatic assembly was dependent upon the nature of the anionic polyelectrolye. Utilizing UV/Vis spectroscopy, ellipsometry, and cyclic voltammetry, we demonstrated that multilayer films composed of Fc-C_6-LPEI with poly(acrylic acid) or poly(glutamic acid) could be assembled by electrostatic LBL. The electrochemical response of the films was dependent upon both the number of bilayers deposited as well as the pH of the polymer solutions during the LBL deposition process. In contrast, we observed that stable multilayer films of Fc-C_6-LPEI and glucose oxidase could not be assembled by electrostatic LBL. The exact cause for this is not known, but may be due to the formation of water-soluble polyelectrolyte complexes (WPECs) in solution that favored more than complexes in the film under the conditions tested and subsequently led to film erosion. To overcome the limitation of electrostatic LBL assembly of Fc-C_6-LPEI and GOX films, we developed a covalent LBL assembly approach based on a reaction between the aldehyde groups on the surface of periodate-treated GOX and the amine groups on the Fc-C_6-LPEI backbone. Utilizing this covalent LBL assembly technique, we were able to assemble multilayer films of Fc-C_6-LPEI and GOX that produced current densities of ~225 μA cm^{-2} in response to glucose.

These results of this study are significant for a couple of reasons: 1) They are the first report of LBL assembly of the novel redox polymer Fc-C_6-LPEI; 2) these studies highlight the fact that a better understanding of the molecular interactions between LPEI and GOX is needed; 3) the buildup of LBL films of Fc-C_6-LPEI/poly(glutamic acid) may be useful in drug delivery applications where the biodegradability and non-toxic features of PGA are attractive while the redox properties of Fc-C_6-LPEI may allow for controlled release;[68] 4) the buildup of LBL films of Fc-C_6-LPEI/poly(acrylic acid) may be useful in the electrochemical detection of the neurotransmitter dopamine; and 5) the high glucose current densities produced by the covalent assembled LBL films suggest that these films may be useful in the development of nanoscaled biosensors for glucose monitoring[1] or as bioanodes in miniature enzymatic biofuel cells.[49]

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Keywords: biosensors · glucose · layer-by-layer · redox polymers · self-assembly

[1] T. Chen, K. A. Friedman, I. Lei, A. Heller, *Anal. Chem.* **2000**, *72*, 3757 – 3763.
[2] S. Mantha, V. A. Pedrosa, E. V. Olsen, V. A. Davis, A. L. Simonian, *Langmuir* **2010**, *26*, 19114 – 19119.
[3] J. Hodak, R. Etchenique, E. J. Calvo, K. Singhal, P. N. Bartlett, *Langmuir* **1997**, *13*, 2708 – 2716.
[4] K. Sirkar, A. Revzin, M. V. Pishko, *Anal. Chem.* **2000**, *72*, 2930 – 2936.
[5] P. Scodeller, R. Carballo, R. Szamocki, L. Levin, F. Forchiassin, E. J. Calvo, *J. Am. Chem. Soc.* **2010**, *132*, 11132 – 11140.
[6] S. Rengaraj, V. Mani, P. Kavanagh, J. Rusling, D. Leech, *Chem. Commun.* **2011**, *47*, 11861 – 11863.
[7] B. Kowalewska, P. J. Kulesza, *Anal. Chem.* **2012**, *84*, 9564 – 9571.
[8] X. Su, B. S. Kim, S. R. Kim, P. T. Hammond, D. J. Irvine, *ACS Nano* **2009**, *3*, 3719 – 3729.
[9] A. S. Zahr, M. de Villiers, M. V. Pishko, *Langmuir* **2005**, *21*, 403 – 410.
[10] K. Ariga, Y. M. Lvov, K. Kawakami, Q. Ji, J. P. Hill, *Adv. Drug Delivery Rev.* **2011**, *63*, 762 – 771.
[11] Y. Lvov, K. Ariga, I. Ichinose, T. Kunitake, *J. Am. Chem. Soc.* **1995**, *117*, 6117 – 6123.
[12] G. Decher, *Science* **1997**, *277*, 1232 – 1237.
[13] P. J. Yoo, N. S. Zacharia, J. Doh, K. T. Nam, A. M. Belcher, P. T. Hammond, *ACS Nano* **2008**, *2*, 561 – 571.
[14] H. Sato, J. Anzai, *Biomacromolecules* **2006**, *7*, 2072 – 2076.
[15] L. Dennany, R. J. Forster, B. White, M. Smyth, J. F. Rusling, *J Am Chem. Soc.* **2004**, *126*, 8835 – 8841.
[16] R. Pei, X. Cui, X. Yang, E. Wang, *Biomacromolecules* **2001**, *2*, 463 – 468.
[17] H. Ai, S. A. Jones, Y. M. Lvov, *Cell Biochem. Biophys.* **2003**, *39*, 23 – 43.
[18] E. J. Calvo, C. Danilowicz, C. M. Lagier, J. Manrique, M. Otero, *Biosens. Bioelectron.* **2004**, *19*, 1219 – 1228.
[19] T. Hoshi, H. Saiki, J. Anzai, *Biosens. Bioelectron.* **2000**, *15*, 623 – 628.
[20] G. Schneider, G. Decher, *Langmuir* **2008**, *24*, 1778 – 1789.
[21] D. V. Volodkin, N. I. Larionova, G. B. Sukhorukov, *Biomacromolecules* **2004**, *5*, 1962 – 1972.
[22] E. Kharlampieva, V. Kozlovskaya, S. A. Sukhishvili, *Adv. Mater.* **2009**, *21*, 3053 – 3065.
[23] C. Bourdillon, C. Demaille, J. Moiroux, J. M. Saveant, *J. Am. Chem. Soc.* **1994**, *116*, 10328 – 10329.
[24] J. Anzai, Y. Kobayashi, *Langmuir* **2000**, *16*, 2851 – 2856.
[25] Y. Y. Sun, H. Y. Wang, C. Q. Sun, *Biosens. Bioelectron.* **2008**, *24*, 22 – 28.
[26] L. Duan, Q. He, X. Yan, Y. Cui, K. Wang, J. Li, *Biochem. Biophys. Res. Commun.* **2007**, *354*, 357 – 362.
[27] J. Seo, J. L. Lutkenhaus, J. Kim, P. T. Hammond, K. Char, *Langmuir* **2008**, *24*, 7995 – 8000.
[28] P. A. Chiarelli, M. S. Johal, D. J. Holmes, J. L. Casson, J. M. Robinson, H. L. Wang, *Langmuir* **2002**, *18*, 168 – 173.
[29] J. B. Schlenoff, S. T. Dubas, T. Farhat, *Langmuir* **2000**, *16*, 9968 – 9969.
[30] C. H. Porcel, A. Izquierdo, V. Ball, G. Decher, J. C. Voegel, P. Schaaf, *Langmuir* **2005**, *21*, 800 – 802.
[31] S. X. Zhang, W. W. Yang, Y. M. Niu, C. Q. Sun, *Anal. Chim. Acta* **2004**, *523*, 209 – 217.
[32] A. Khademhosseini, K. Y. Suh, J. M. Yang, G. Eng, J. Yeh, S. Levenberg, R. Langer, *Biomaterials* **2004**, *25*, 3583-3592.
[33] Y. Tian, J. G. Park, Q. F. Cheng, Z. Y. Liang, C. Zhang, B. Wang, *Nanotechnology* **2009**, *20*.
[34] Y. Du, C. Chen, J. Yin, B. Li, M. Zhou, S. Dong, E. Wang, *Anal. Chem.* **2010**, *82*, 1556 – 1563.
[35] S. Park, S. H. Bhang, W. G. La, J. Seo, B. S. Kim, K. Char, *Biomaterials* **2012**, *33*, 5468 – 5477.
[36] D. J. Schmidt, F. C. Cebeci, Z. I. Kalcioglu, S. G. Wyman, C. Ortiz, K. J. Van Vliet, P. T. Hammond, *ACS Nano* **2009**, *3*, 2207 – 2216.
[37] A. Heller, *Acc. Chem. Res.* **1990**, *23*, 128 – 134.
[38] E. J. Calvo, R. Etchenique, L. Pietrasanta, A. Wolosiuk, C. Danilowicz, *Anal. Chem.* **2001**, *73*, 1161 – 1168.
[39] Q. Gao, M. R. Yang, *Chem. Commun.* **2004**, 30 – 31.
[40] E. J. Calvo, A. Wolosiuk, *ChemPhysChem* **2005**, *6*, 43 – 47.
[41] W. J. Li, Z. Wang, C. Q. Sun, M. Xian, M. Y. Zhao, *Anal. Chim. Acta* **2000**, *418*, 225 – 232.
[42] V. Flexer, E. S. Forzani, E. J. Calvo, *Anal. Chem.* **2006**, *78*, 399 – 407.
[43] V. Flexer, K. F. E. Pratt, F. Garay, P. N. Bartlett, E. J. Calvo, *J. Electroanal. Chem.* **2008**, *616*, 87 – 98.
[44] Y. Du, C. Chen, B. Li, M. Zhou, E. Wang, S. Dong, *Biosens. Bioelectron.* **2010**, *25*, 1902 – 1907.
[45] X. Q. Liu, F. Wang, S. Han, L. H. Shi, G. B. Xu, *Electroanalysis* **2010**, *22*, 963 – 968.
[46] A. H. Liu, Y. Kashiwagi, J. Anzai, *Electroanalysis* **2003**, *15*, 1139 – 1142.
[47] S. A. Merchant, T. O. Tran, M. T. Meredith, T. C. Cline, D. T. Glatzhofer, D. W. Schmidtke, *Langmuir* **2009**, *25*, 7736 – 7742.
[48] S. A. Merchant, M. T. Meredith, T. O. Tran, D. B. Brunski, M. B. Johnson, D. T. Glatzhofer, D. W. Schmidtke, *J. Phys. Chem. C* **2010**, *114*, 11627 – 11634.
[49] M. T. Meredith, D. Y. Kao, D. Hickey, D. W. Schmidtke, D. T. Glatzhofer, *J. Electrochem. Soc.* **2011**, *158*, B166 – B174.
[50] T. O. Tran, E. G. Lammert, J. Chen, S. A. Merchant, D. B. Brunski, J. C. Keay, M. B. Johnson, D. T. Glatzhofer, D. W. Schmidtke, *Langmuir* **2011**, *27*, 6201 – 6210.
[51] T. W. Tsai, G. Heckert, L. F. Neves, Y. Tan, D. Y. Kao, R. G. Harrison, D. E. Resasco, D. W. Schmidtke, *Anal. Chem.* **2009**, *81*, 7917 – 7925.
[52] Y. Wang, P. P. Joshi, K. L. Hobbs, M. B. Johnson, D. W. Schmidtke, *Langmuir* **2006**, *22*, 9776 – 9783.
[53] A. G. Frutos, S. C. Weibel, R. M. Corn, *Anal. Chem.* **1999**, *71*, 3935 – 3940.
[54] J. L. Lutkenhaus, K. McEnnis, P. T. Hammond, *Macromolecules* **2008**, *41*, 6047 – 6054.
[55] D. M. DeLongchamp, P. T. Hammond, *Chem. Mater.* **2003**, *15*, 1165 – 1173.
[56] T. Kurosaki, T. Kitahara, S. Fumoto, K. Nishida, J. Nakamura, T. Niidome, Y. Kodama, H. Nakagawa, H. To, H. Sasaki, *Biomaterials* **2009**, *30*, 2846 – 2853.
[57] Y. X. Yang, Y. F. Jiang, Z. Wang, J. H. Liu, L. Yan, J. X. Ye, Y. Z. Huang, *J. Mater. Chem.* **2012**, *22*, 10029 – 10034.
[58] M. D. Kurkuri, J. R. Lee, J. H. Han, I. Lee, *Smart Mater. Struct.* **2006**, *15*, 417 – 423.
[59] J. C. Antunes, C. L. Pereira, M. Molinos, F. Ferreira-da-Silva, M. Dessi, A. Gloria, L. Ambrosio, R. M. Goncalves, M. A. Barbosa, *Biomacromolecules* **2011**, *12*, 4183 – 4195.

[60] J. G. Voet, J. Coe, J. Epstein, V. Matossian, T. Shipley, *Biochemistry* **1981**, *20*, 7182 – 7185.
[61] M. Onda, K. Ariga, T. Kunitake, *J. Biosci. Bioeng.* **1999**, *87*, 69 – 75.
[62] M. C. Rodriguez, G. A. Rivas, *Electroanalysis* **2004**, *16*, 1717 – 1722.
[63] W. J. Zhang, Y. X. Huang, H. Dai, X. Y. Wang, C. H. Fan, G. X. Li, *Anal. Biochem.* **2004**, *329*, 85 – 90.
[64] S. A. Sukhishvili, E. Kharlampieva, V. Izumrudov, *Macromolecules* **2006**, *39*, 8873 – 8881.
[65] H. C. Yoon, H. S. Kim, *Anal. Chem.* **2000**, *72*, 922 – 926.
[66] W. W. Yang, J. X. Wang, S. Zhao, Y. Y. Sun, C. Q. Sun, *Electrochem. Commun.* **2006**, *8*, 665 – 672.
[67] E. S. Forzani, M. Otero, M. A. Perez, M. L. Teijelo, E. J. Calvo, *Langmuir* **2002**, *18*, 4020 – 4029.
[68] A. Fery, B. Scholer, T. Cassagneau, F. Caruso, *Langmuir* **2001**, *17*, 3779 – 3783.

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