

Adsorption of Glucose Oxidase onto Single-Walled Carbon Nanotubes and Its Application in Layer-By-Layer Biosensors

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In this study, we describe the use of a sodium cholate suspension-dialysis method to adsorb the redox enzyme glucose oxidase (GOX) onto single-walled carbon nanotubes (SWNT). By this method, solutions of dispersed and debundled SWNTs were prepared that remained stable for 30 days and which retained 75% of the native enzymatic activity. We also demonstrate that GOX-SWNT conjugates can be assembled into amperometric biosensors with a poly[(vinylpyridine)Os(bipyridyl)₂Cl^{2+/3+}] redox polymer (PVP-Os) through a layer-by-layer (LBL) self-assembly process. Incorporation of SWNT-enzyme conjugates into the LBL films resulted in current densities as high as 440 $\mu\text{A}/\text{cm}^2$, which were a 2-fold increase over the response of films without SWNTs. We also demonstrate that the adsorption pH of the redox polymer solution and the dispersion quality of SWNTs were important parameters in controlling the electrochemical and enzymatic properties of the LBL films.

The attachment of biological molecules (e.g., enzymes, antibodies, DNA) to single-walled carbon nanotubes (SWNTs) has gained considerable attention due to the high surface areas of SWNTs and their exceptional mechanical, electrical, and fluorescent properties. Protein-SWNT conjugates are being developed for a wide range of biomedical devices and therapies, including drug delivery,¹ cancer therapy,^{2,3} and biosensing applications.^{4–7} To achieve these applications it is desired that the protein-SWNT conjugates have the following characteristics: (i) they form stable

and homogeneous dispersions of individual SWNTs; (ii) the unique structure and properties of SWNTs are retained; and (iii) the native structure and activity of the protein is retained.

Unfortunately most SWNT syntheses produce large, aggregated bundles of SWNTs. These aggregated bundles often make processing difficult and mask the unique properties of individual tubes. Thus a number of covalent^{8,9} and noncovalent^{10–12} methods of protein attachment have been developed to debundle these aggregates into individual tubes. Benefits of covalent immobilization are that it provides a strong linkage of the protein to the SWNT and may increase the stability of the protein.¹³ However, covalent coupling normally damages the sidewalls of SWNTs and disrupts their unique electrical and optical properties.^{14–16} In contrast, noncovalent methods can allow for the electronic structure of the SWNTs^{5,11,12} to be retained but may in some cases lead to a loss of protein function.¹⁷

Recently, a noncovalent method of protein attachment to SWNTs utilizing a sodium cholate suspension-dialysis process has been developed.¹⁸ This method has been successful in coupling both concanavalin-A¹⁸ and the enzyme horseradish peroxidase to SWNTs.¹¹ In the case of HRP, it was demonstrated that both the enzymatic activity and the UV–vis-NIR spectra of the SWNTs were retained. In this study we investigate whether this method of protein attachment is applicable to other enzymes as well. To test this we used the enzyme glucose oxidase (GOX), which is

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commonly used in the development of biosensors for glucose detection^{19–21} as well as in miniaturized biofuel cells.^{22–24} Enzymatic activity measurements and UV–vis spectroscopy demonstrated that dispersions of the GOX-SWNTs conjugates were highly stable and biocatalytic. Based on these results we investigated the potential benefits of the GOX-SWNT conjugates in fabricating glucose biosensors.

A key issue in developing glucose biosensors based on GOX-SWNT conjugates is the detection method. The most widely used detection scheme for glucose based on GOX and SWNTs is amperometry,^{25–28} however conductivity,^{29,30} and fluorescent^{5,31} sensors have been developed as well. Important parameters in choosing one method over another often include: simplicity, sensitivity, selectivity, response time, necessity of specialized equipment, and cost. Reviews of the advantages and disadvantages of SWNTs in these detection methods are available.^{32–35} We chose amperometry as the basis for our detection method due to its simplicity, low cost, and fast response.

A second important issue in GOX-SWNT based biosensors is the method of immobilization. Ideally one desires a method that allows for high loading, retention of both biological activity and SWNT properties, simplicity, and control of the film architecture. In response to these challenges, a diverse set of strategies, such as solvent casting,^{28,36} carbon paste binding,³⁷ covalent immobilization,^{26,38,39} electropolymerization,⁴⁰ and layer-by-layer (LBL) assembly^{29,41,42} have been developed. Since the LBL assembly process can be performed in aqueous solutions under conditions that minimize protein denaturation, allows for precise control of the composition and film thickness, and is compatible with a variety of substrates and geometries, we opted for this technique. Furthermore recent studies have reported that incorporation of

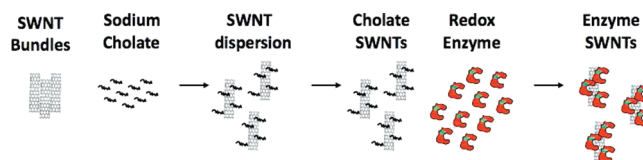


Figure 1. Schematic of the sodium cholate suspension-dialysis method to produce high quality dispersions of redox enzyme-single walled carbon nanotube conjugates.

SWNTs⁴³ or MWNTs⁴¹ into LBL films containing GOX have the added benefit of increasing sensor response by enhancing the electron diffusion through the films and/or the electrochemical surface area.

MATERIALS AND METHODS

Chemicals and Solutions. Glucose oxidase (GOX) from *Aspergillus niger* (EC 1.1.3.4, type X-S, 179 units/mg solid, 75% protein), 11-mercaptopundecanoic acid (MUA), D-glucose, horseradish peroxidase (HRP) (EC 1.11.1.7, type II, 181 units/mg), and sodium cholate were purchased from Sigma (St. Louis, MO) and used as received. Purified single-wall carbon nanotubes (SWNTs) produced by the CoMoCAT synthesis method⁴⁴ were kindly provided by SouthWest Nanotechnologies. To remove the silica support from the SWNTs, it was dissolved with a stirred 20% HF solution at 25 °C for 3 h. The resulting nanotubes (average diameter of 0.81 nm) were then filtered through a PTFE 0.2 μ m membrane and washed with deionized water to neutral pH. The redox polymer, designated as PVP-Os, was synthesized by partially complexing the pyridine nitrogens of poly(4-vinylpyridine) with Os(bpy)₂Cl^{+/2+} and then partially quaternizing the resulting polymer with 2-bromoethylamine according to a previously published protocol.⁴⁵ Phosphate-buffered saline solution (PBS), pH 7.4, was prepared from 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH₂PO₄, and 1.15 g/L Na₂HPO₄ in nanopure deionized water. Stock solutions of 2 M glucose in water were allowed to mutarotate for 24 h before use.

Adsorption of Enzyme to SWNTs. Highly dispersed and debundled SWNTs were prepared by the sodium cholate suspension-dialysis method.^{11,18} In this method (Figure 1) SWNTs were first debundled with the bile salt sodium cholate, the adsorbed cholate was subsequently replaced by glucose oxidase, and the sodium cholate was removed by dialysis. Initially a saturated suspension of SWNTs was made by adding 3 mg of pristine nanotubes to 7 mL of 2 wt % of sodium cholate solution. Next the mixture was sonicated for 40 min with a homogenizer (22% amplitude, Cole-Parmer model CPX) to disperse the SWNTs. The SWNTs solution was then centrifuged at 30100 g for 1 h at temperature 4 °C, and the supernatant containing a high fraction of individual SWNTs was retained. Sodium phosphate was added to 5 mL of supernatant SWNTs solution to give a concentration of 20 mM. Next 15 mg of GOX was added to the SWNTs solution and mixed well. The sodium cholate that was displaced as the GOX adsorbed onto the SWNTs was removed by dialysis with a 10 kDa dialysis membrane (Spectrum Laboratories) and 1.5 L of

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a 20 mM phosphate buffer (pH 7.4) for 12 h at 4 °C. Next the resulting GOX-SWNT solution was transferred to a 300 KDa dialysis membrane (Spectrum Laboratories) and dialyzed at 4 °C to remove any unadsorbed GOX. The dialysis buffer (20 mM phosphate, pH 7.4) was changed frequently (3, 6, 9, and 24 h). The resulting suspension was centrifuged at 30100 g for 1 h at 4 °C and the supernatant was retained.

Protein Loading on SWNTs. To determine the enzyme loading on the SWNTs, the following procedure was used: 1. *The concentration of GOX in the GOX-SWNT solution was determined.* A series of solutions of increasing concentrations of GOX were made, and the Bradford assay was used to make a standardized absorbance vs enzyme concentration curve. From the standard curve and the absorbance of the GOX-SWNT solution, the amount of protein in solution was determined. To verify that the measured GOX solution concentration was solely due to adsorbed GOX on the SWNTs and not GOX free in solution, any unadsorbed GOX (MW = 160 kDa) was removed by dialysis with a 300 KDa dialysis membrane. Testing of the final dialysis solution for GOX activity showed no activity, which provided evidence that all of the unadsorbed GOX was removed during the dialysis process. 2. *The concentration of SWNTs in the GOX-SWNT solution was determined.* A series of solutions of increasing concentrations of SWNTs dispersed by sodium cholate were made, and their UV-vis spectra were obtained (Supporting Information (SI) Figure S-1A). A standard curve of the absorbance vs SWNT concentration was generated (SI Figure S-1B) and demonstrated that the absorbance at 800 nm followed Beer's law and was proportional to the SWNT concentration. We chose to measure the absorbance at 800 nm since there is minimal influence from either the S11 transition or the adsorbed protein at this wavelength. From the UV-vis spectra of the GOX-SWNT conjugate suspension and the standard curve, the SWNT concentration was determined. The loading of GOX on the SWNTs was determined by combining the results of the individual SWNT and GOX measurements.

Enzyme Activity Assay. The activity of GOX was measured spectrophotometrically by the o-dianisidine-peroxidase method. Briefly, glucose oxidase catalyzed the oxidation of beta-D glucose to gluconolactone and H₂O₂. The generated H₂O₂ then oxidized o-dianisidine (Sigma-Aldrich F5803) in the presence of horseradish peroxidase. The oxidation of o-dianisidine was then measured spectrophotometrically. The initial reaction rates were measured with the increasing of fluorescence at an emission wavelength 500 nm and a temperature of 25 °C.

UV-vis Spectrophotometry. To monitor the LBL assembly process we fabricated PVP-Os/GOX-SWNT and PVP/GOX multilayer films on quartz substrates. Absorption spectra were taken with a UV-vis spectrophotometer (UV-2101PC, Shimadzu) of multilayer films fabricated with and without SWNTs.

Sensor Construction. Gold electrodes with diameters of 2.0 mm (CH instruments, Austin, TX) were used as working electrodes and polished with 1 and 0.25 μ m diamond polishing slurry on nylon polish pads before being polished with 0.05 μ m alumina solution on microcloth pads. Solutions of PVP-Os polymer (10 mg/mL in water), GOX (2 mg/mL in 20 mM sodium phosphate buffer, pH 7.4), and GOX-SWNTs were used for sensor construction. To facilitate the adsorption of the positively charged redox polymer, a negatively charged layer of MUA was first deposited on the

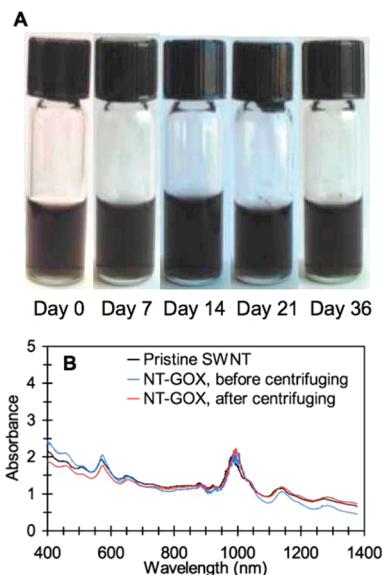


Figure 2. Stability and characterization of suspensions of GOX-SWNT conjugates. (A) Optical images of a GOX-SWNT suspension at different time points when stored at 4 °C. (B) UV-vis-NIR absorption spectra of pristine SWNTs, and SWNT-GOX conjugates before and after the final centrifugation.

surface of polished gold electrodes by immersing the electrode in a solution of 1 mM MUA in ethanol for 20 min. The electrodes were then removed and washed with nanopure deionized water. The electrodes were subsequently immersed in a polycationic solution of PVP-Os for 20 min, washed with nanopure deionized water to remove any excess material, and then immersed in a solution of GOX-SWNTs for 40 min. Multilayer films of PVP-Os/GOX-SWNTs were then created by repeated alternating exposure to the redox polymer (PVP-Os) and enzyme (GOX-SWNTs) solutions. As a control, we also fabricated multilayer films of PVP-Os/GOX by using GOX solutions (2 mg/mL) that did not contain any SWNTs. To monitor the LBL assembly process, multilayer films were fabricated with (PVP-Os/GOX) and without SWNTs (PVP-Os/GOX-SWNTs) on quartz substrates, and UV-vis spectra were taken after each layer.

Electrochemical Measurements. Cyclic voltammetry (CV) and constant potential measurements were performed with a CH Instruments bipotentiostat (model no. CHI832) in a three-electrode cell configuration with a saturated calomel reference electrode (SCE), and a platinum wire counter electrode. Glucose calibration curves were obtained by adding aliquots of a stock 2 M glucose solution to a well-stirred cell with the working electrode poised at 500 mV vs SCE.

Calculations and Statistics. Values are presented as mean \pm standard error of the mean (SEM) unless otherwise specified.

RESULTS AND DISCUSSION

SWNT Debundling and Enzyme Adsorption. Stable suspensions of debundled SWNTs that were functionalized with the enzyme glucose oxidase (GOX) were prepared by a sodium cholate suspension-dialysis method (Figure 1). Suspensions of the GOX-SWNT conjugates displayed a black colored solution and visually showed no signs of aggregation for over 30 days (Figure 2A). To assess whether the GOX-SWNTs were well dispersed,

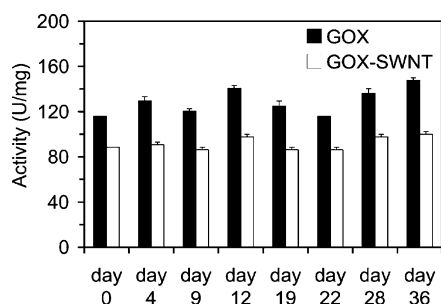


Figure 3. Retention and stability of glucose oxidase (GOX) enzymatic activity. The activity of native GOX in solution and GOX adsorbed onto single-walled carbon nanotubes (GOX-SWNT) as a function of time. GOX activity was measured spectrophotometrically at 25 °C by the o-dianisidine-peroxidase method. In between measurements the samples were stored at 4 °C. Values represent the average and SEM of three measurements.

we performed UV-vis spectroscopy. Figure 2B shows normalized absorption spectra for a pristine SWNT suspension and that for the GOX-SWNT suspension before and after the final centrifugation. The strong absorption band at 980 nm is characteristic of CoMoCAT SWNT samples and is ascribed to the S11 transition of (6,5) nanotubes.⁴⁶ The presence of the sharp peaks in the visible and infrared regions are a signature of well-dispersed SWNTs.⁴⁷ In order to quantify the dispersibility of the suspension we used the method of Tan et al.⁴⁸ to measure the resonance ratio. The resonance ratio is defined as the quotient of the resonant band area and its nonresonant background and is related to the fraction of individual nanotubes in the suspension. For the suspensions of the GOX-SWNTs conjugates described above we measured a resonance ratio of 0.129. This value compares quite well with resonance ratios values (0.11 – 0.15) measured for SWNTs dispersed by a number of anionic surfactants⁴⁸ and suggests a high fraction of individual GOX-SWNTs.

The GOX concentration on the SWNTs was determined by the Bradford assay to be 1.97 ± 0.73 mg/mL ($n = 13$) while the SWNT concentration was measured to be 0.095 ± 0.035 mg/mL ($n = 13$). Combining the results of both the GOX and SWNT concentration measurements, the loading of GOX on the SWNTs was calculated to be 20.7 ± 6.7 mg GOX/mg SWNTs. This high loading of GOX on the SWNTs is similar to the previous report in which high loadings of HRP on the SWNTs were obtained by the sodium cholate dialysis method.¹¹ These results suggest that this method is well suited to depositing high loadings of enzymes on SWNTs.

Retention of Enzymatic Activity. In addition to high enzyme loading, enzyme-SWNTs conjugates must also possess sufficient catalytic activity to be useful in biosensor or biofuel cell applications. To determine the activity of the GOX adsorbed onto the SWNTs, we used the o-dianisidine-peroxidase spectrophotometric method. Figure 3 shows the activity measurement of native GOX in solution and GOX adsorbed onto SWNTs. Approximately 75% of the native GOX activity was retained after GOX was adsorbed on the SWNTs. In addition, the activity of the native GOX and

GOX-SWNT conjugates remained relatively constant for over 36 days when stored at a temperature of 4 °C. Combined with the dispersion stability data in Figure 2, these results indicate that GOX-SWNTs conjugates not only remain highly dispersed but they also retain their enzymatic activity.

Layer-by-Layer Assembly of the PVP-Os/GOX-SWNT Films. Thin films composed of GOX-SWNTs conjugates and redox polymer PVP-Os were fabricated on gold electrodes by the layer-by-layer (LBL) technique (Figure 4). UV-vis spectroscopy was performed to characterize the deposition and buildup of the multilayer films (Figure 5A). Previous studies^{49,50} have reported that the absorption peak at 300 nm can be assigned to the $\pi-\pi^*$ transition of the bipyridine groups of PVP-Os and utilized to measure the relative amount of PVP-Os deposited. Figure 5A shows that the strong adsorption band at 300 nm increases linearly with the number of PVP-Os/GOX-SWNT bilayers deposited and suggests that the amount of material deposited can be easily controlled. As a control, films without SWNTs were also fabricated to determine the effect of SWNTs on the self-assembly process (Figure 5B). Similar to the films with SWNTs, a strong absorbance band at 300 nm was observed, and there was a linear increase in the absorbance intensity with the number of bilayers. It should be noted that for films made with the same number of bilayers, films with SWNTs had higher absorbance readings than those without SWNTs. Furthermore, visual inspection of the quartz slides showed a dark background with increasing bilayers of GOX-SWNT films while the slides without SWNTs remained transparent. These results suggest that the presence of SWNTs caused more material to be deposited.

Electrochemical Characterization of Multilayer Films. The buildup of multilayer films was also assessed by cyclic voltammetry. Figure 6A shows representative cyclic voltammograms (CVs) for electrodes made with one, two, four, and six bilayers in which GOX-SWNT conjugates were incorporated. A pair of well-defined redox waves corresponding to the oxidation and reduction of the redox polymer's Os(bpy) complexes were observed at 300 mV vs SCE. As the number of bilayers was increased both the oxidation and reduction peak current also increased. This observation corroborates the UV-vis absorption results that additional redox polymer was deposited with each bilayer. To determine the effect of SWNTs on the response, we also performed control CVs with electrodes made without SWNTs (Figure 6B). Once again redox waves were observed at 300 mV vs SCE and the peak currents increased with the number of bilayers.

There were however a couple of differences in the electrochemical behavior between films made with and without SWNTs. The first difference is in the magnitude of the peak current densities. LBL films made with GOX-SWNT conjugates had peak currents that were 2–3 times greater than films made with GOX alone. This increase in the electrochemical response with SWNTs is similar to our previous report.⁴² The other noticeable difference is that, in the CVs for the four- and six-bilayer films made with GOX-SWNTs, the current never returns to zero at the limits of the potential scans. This nonzero current (i.e., “diffusional tail”) occurs when charge transport through the film becomes limiting

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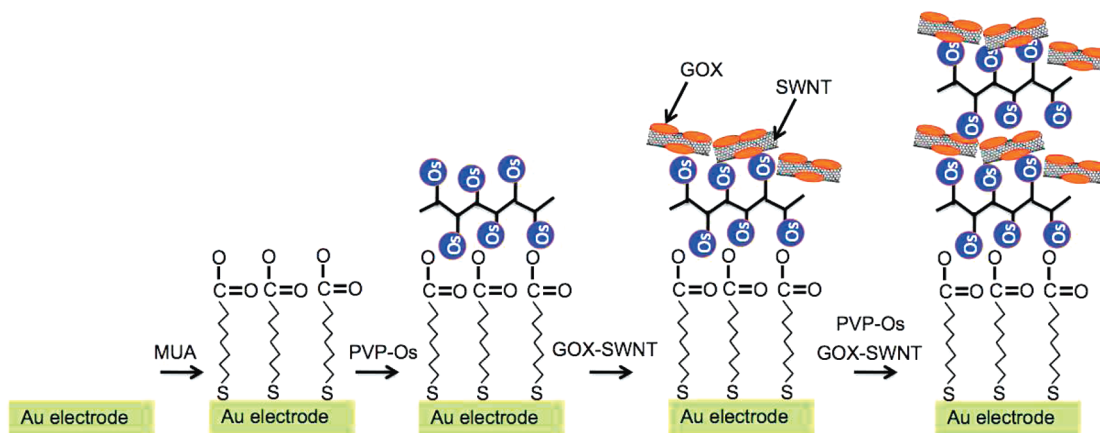


Figure 4. Schematic of the LBL process. Gold electrodes were initially functionalized with the negatively charged thiol, 11-mercaptoundecanoic acid (MUA), and then alternatively incubated in solutions of the positively charged redox polymer (PVP-Os) and negatively charged GOX-SWNT conjugates.

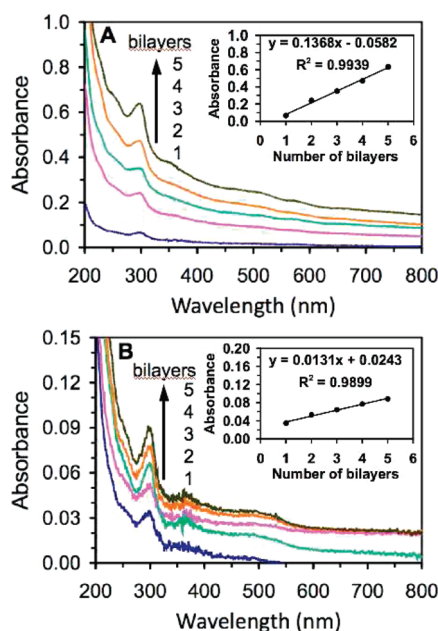


Figure 5. UV-vis absorption spectra of films with different number of bilayers. Films made (A) with SWNTs (i.e., PVP-Os/GOX-SWNTs), and (B) without SWNTs (i.e., PVP-Os/GOX) on quartz slides. The inserts show the absorbance at 300 nm vs the number of bilayers.

and not all of the redox centers in the film are completely oxidized or reduced. The presence of the diffusional tail is characteristic of “thick” films and suggests semi-infinite linear diffusion. In contrast, the current of films made without SWNTs returned to zero at both potential limits indicating that all of the redox centers were exhaustively oxidized and reduced. To confirm these observations of a diffusion or surface-controlled process, the scan rate dependence of the redox peak currents was investigated. For films made with GOX alone a linear relationship between peak current and scan rate (SI Figure S2A and B) was observed which indicates a surface-controlled redox process. In contrast the peak current in CVs for films made with GOX-SWNTs (SI Figure S3A) varied linearly with the square root of the scan rate (SI Figure S3B), which indicated a diffusion-controlled redox process.

As mentioned above both the UV-vis and CV data suggest that more redox polymer is deposited during the LBL process

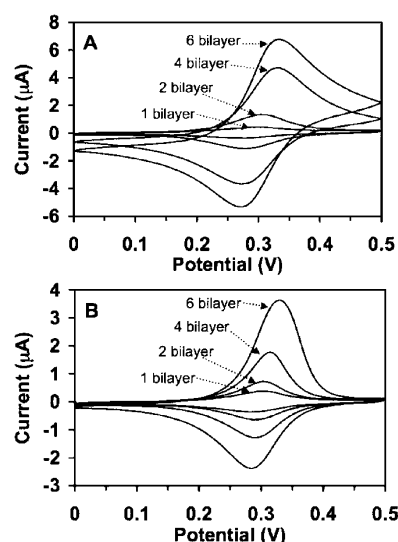


Figure 6. Effect of multilayers on the electrochemical response of the LBL films. CVs of gold electrodes modified with one, two, four, and six bilayers (A) with SWNTs (PVP-Os/GOX-SWNT) and (B) without SWNTs (PVP-Os/GOX). Scan rate = 50 mV/s in PBS (pH 7.4) at 25 °C.

when SWNTs are present. Although the exact mechanism is unknown at this time, we suspect that the presence of the SWNTs in the GOX-SWNT layer influences the geometry and smoothness of this layer. In contrast to the idealized schematic of Figure 4, where all the SWNTs lie in the same plane and parallel to the surface, we envision that the orientation of the SWNTs with respect to the electrode surface is heterogeneous, with some GOX-SWNTs oriented other than parallel to the surface. Such a heterogeneous layer would increase the surface roughness/surface area and promote more redox polymer to be adsorbed. In support of this hypothesis is the recent work of Lee et al.,⁵¹ who demonstrated that LBL assembly of multi wall carbon nanotubes (MWNTs) led to a random orientation of the nanotubes and that the root-mean-squared (rms) roughness of the MWNT films increased with the number of bilayers.

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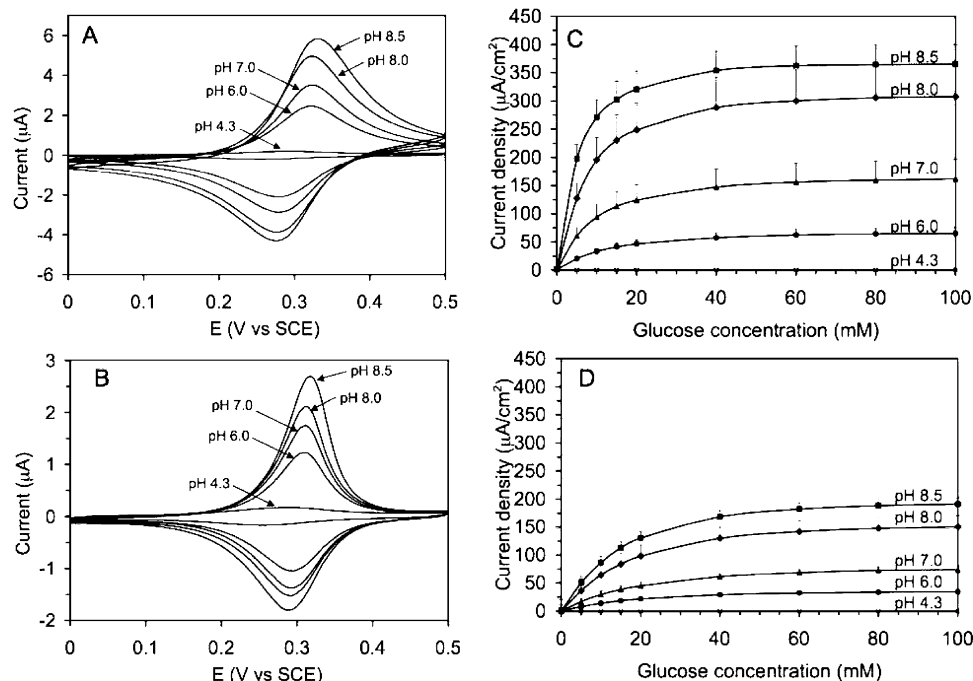


Figure 7. Effect of pH on electrochemical and enzymatic response. CVs of gold electrodes modified with four bilayers (PVP-Os/GOX) with SWNTs (A) and without SWNTs (B) as a function of the pH of the redox polymer assembly solution and tested in a PBS pH 7.4 solution. (Scan rate = 50 mV/s, 25 °C). Calibration curves of gold electrodes modified with four bilayers (PVP-Os/GOX) with SWNTs (C) and without SWNTs (D) as a function of redox polymer solution pH ($E = 0.4$ V vs SCE, PBS at 25 °C).

Effect of Redox Polymer Solution pH on Electrochemical and Enzymatic Response. Recently it has been reported that the film thickness, redox site concentration, enzyme loading, and catalytic response of LBL films fabricated with GOX and an osmium-derivatized poly(allylamine) redox polymer (PAH-Os) can be controlled by varying the pH of the redox polymer solution during the LBL adsorption process.¹⁹ The hypothesis is that at low pH, the redox polymer adopts an extended rod conformation due to a high linear charge density, whereas at high pH, the linear charge density is reduced and the polymer adopts a coiled conformation.^{19,52} This difference in redox polymer conformation leads to an increased film thickness, enzyme loading, and redox charge when the adsorption solution pH is high (~pH 8). To determine what effect the pH of the redox polymer solution would have on the assembly of LBL films made with the PVP-Os redox polymer and GOX-coated SWNTs, we systematically varied the pH of the redox polymer solution from pH 4.3 to 8.5.

Increasing the redox polymer solution pH from 4.3 to 8.5 had a dramatic effect on the electrochemical response of films built with or without SWNTs. For films made with SWNTs (Figure 7A) and without SWNTs (Figure 7B) the peak current and the area under the curves increased with pH, indicating an increase in the amount of redox polymer absorbed. Similar to the multilayer results in Figure 6, the response of films with SWNTs was 2–3 times greater than those without for pH > 4.3. In contrast, the oxidation and reduction peak potentials remained relatively constant for pH > 4.3 (Table 1), with the peak separation for films with SWNTs ($\Delta E = 43$ –54 mV) being roughly twice that of films without SWNTs ($\Delta E = 21$ –30 mV).

Table 1. Effect of Redox Polymer Solution pH on the Electrochemical and Enzymatic Response of Four-Bilayer Films^a

pH of redox polymer solution	$E_{p,A}$ (mV)	$E_{p,C}$ (mV)	ΔE (mV)	sensitivity ($\mu A/mM \cdot cm^2$)	K_m (mM)	J_{max} ($\mu A/cm^2$)
4.3	288	253	35	0.03	6.2	0.2
6.0	311	290	21	1.6	20.7	34
7.0	312	292	20	3.4	21.5	74
8.0	311	292	19	7.3	19.0	150
8.5	317	287	30	10.1	16.8	191
4.3 (with SWNTs)	291	252	39	0.03	4.5	0.5
6.0 (with SWNTs)	321	278	43	4.2	12.6	65
7.0 (with SWNTs)	324	278	46	12.2	9.4	162
8.0 (with SWNTs)	321	277	44	25.4	7.8	308
8.5 (with SWNTs)	330	276	54	39.5	4.6	365

^a $E_{p,A}$ and $E_{p,C}$ are the anodic and cathodic peak potentials, respectively. ΔE is the peak separation potential. J_{max} is the maximum current obtained experimentally at saturating glucose concentrations. K_m was determined graphically from an Eadie–Hofstee plot. Sensitivity was determined from the experimental current response at 5 mM glucose concentration.

Figure 7 also shows a significant effect of pH on the enzymatic response as well. At pH 4.3 there was little enzymatic response to glucose in either film, but as the pH was increased from 6.0 to 8.5 the sensitivity to glucose increased from 6-fold (without SWNTs) to 9-fold (with SWNTs), and the maximum current density increased 5.5-fold (Table 1). Surprisingly the K_m for films made with SWNTs was ~2–3 times lower than those without SWNTs, and there was a negative correlation between K_m and J_{max} in both cases (Figure 8). The fact that the K_m for films without SWNTs were 2–3 times lower than those with SWNTs would suggest that films without SWNTs were more compact and limited the binding of glucose to the enzyme and thus K_m was increased. This interpretation would agree with the UV–vis

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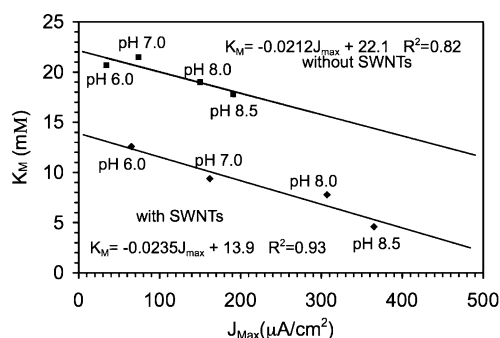


Figure 8. Effect of the maximum current density (J_{\max}) on the apparent Michaelis–Menten constant (K_M). A plot of the K_M and J_{\max} values from Figure 7 and Table 1 for four-bilayer films made with and without SWNTs.

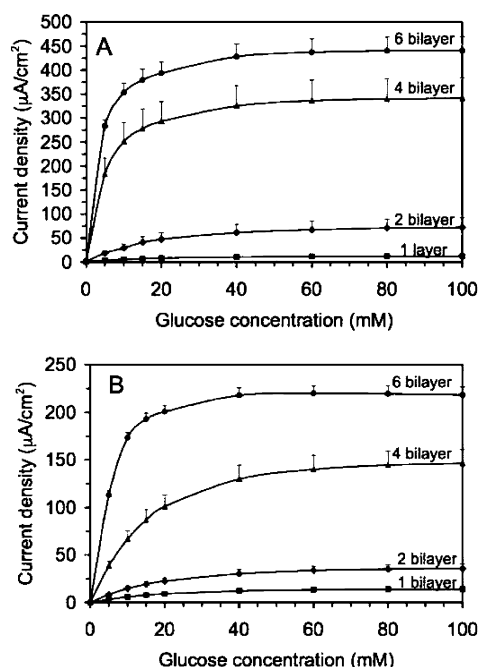


Figure 9. Effect of multilayers on enzymatic response. Calibration curves of gold electrodes modified with one, two, four, and six bilayers (PVP-Os/GOX) with SWNTs (A) and without SWNTs (B). $E = 0.4$ V vs SCE, in PBS at 25 °C.

absorption results (Figure 5) which also suggested thinner films in the absence of SWNTs. Similarly the decrease in K_M and increase of J_{\max} with pH would also support the idea that as the pH of the redox polymer is increased the film is more porous and allowed more of the glucose to bind to GOX and thus decrease K_M . Taken together these results are in agreement with the results of Flexar¹⁹ and demonstrate that the pH of the redox polymer solution is an important control parameter in fabricating LBL films.

Effect of Bilayer Number on Enzymatic Response. After determining that a redox polymer solution pH of 8.5 produced the highest enzymatic response, we then investigated the effect of the number of bilayers on the sensor's enzymatic response. Figure 9 shows that the response of the films to glucose increased with the number of bilayers. Similar to our previous results⁴² and those in Figure 7, the response with SWNTs was 2–3-fold higher than those without SWNTs. It should be noted that the limiting current density of 440 $\mu\text{A}/\text{cm}^2$ and sensitivity of 56 $\mu\text{A}/\text{mM} \cdot \text{cm}^2$

at 5 mM glucose for the six-bilayer films with SWNTs are among the highest ever reported for LBL type glucose sensors and compare favorably with those incorporating multiwalled carbon nanotubes (400 $\mu\text{A}/\text{cm}^2$ and 30 $\mu\text{A}/\text{mM} \cdot \text{cm}^2$)⁴¹ as well as non-LBL sensor designs. These results demonstrate that enzymatic response of LBL films can be controlled by the number of bilayers.

Effect of SWNT Dispersion on Sensor Response. The limiting current densities in Figure 9 are 5 times higher than our previous measurements (90 $\mu\text{A}/\text{cm}^2$) with LBL films containing SWNTs.⁴² Although the primary factor for this increase appears to be related to the higher pH of the redox polymer solution, there are other parameters that were different as well. In our previous study the enzyme GOX was coupled to SWNTs by dissolving GOX in a concentrated SWNT solution (6.9 mg/mL) and incubating overnight at 4 °C. This solution was not well dispersed and contained both individual and bundles of SWNTs; thus, the sensitivity enhancement in the present study may also be related to the quality of the SWNT dispersion. In order to investigate the role of SWNT dispersion independent of redox polymer pH, we fabricated LBL films with three different types of enzyme solutions (Figure 10A): (i) well-dispersed GOX-SWNT conjugates produced by the dialysis method of Figure 1; (ii) GOX-SWNT conjugates produced by simply incubating GOX with SWNTs for 24 h; and (iii) a GOX solution without SWNTs. As shown in Figure 10A, the GOX-SWNT solution produced by the dialysis methods was well dispersed and homogeneous, suggestive of individual SWNTs. In contrast, the dispersion made by simple incubation of GOX with SWNTs was not stable and within 30 min after sonication clusters of SWNTs formed at the top and bottom of the solution (arrows in Figure 10A) and is suggestive of large SWNT bundles and aggregates. It should be noted that the same amount of SWNTs was used in both solutions.

Figure 10B shows the electrochemical behavior of four-bilayer films made with these three enzyme solutions. Incorporation of the SWNTs into the LBL films either by the dialysis method or simple incubation method led to a significant increase in the peak current and the area under the curves as compared to the films without SWNTs. Although the peak currents, and peak current redox potentials were not significantly different in the two films containing SWNTs, the films made with the GOX-SWNT dialysis method did appear to exhibit a diffusional tail, whereas the GOX-SWNT incubation method did not. These results would suggest that the mere presence of SWNTs, and not necessarily the dispersion state, was responsible for the enhanced electrochemical response.

In contrast to the electrochemical results, Figure 10C shows that the enzymatic response of the LBL films was highly dependent upon the quality of the GOX-SWNT dispersion. Films made with the GOX-SWNT incubation method produced a limiting current of 146 $\mu\text{A}/\text{cm}^2$ (~30% higher than control films) and a sensitivity of 20 $\mu\text{A}/\text{mM} \cdot \text{cm}^2$. Films made with the GOX-SWNT dialysis method produced limiting current of 341 $\mu\text{A}/\text{cm}^2$ and a sensitivity of 37 $\mu\text{A}/\text{mM} \cdot \text{cm}^2$.

Taken together, the results of Figure 10 provide some insight into the nature of the increased electrochemical and enzymatic response of the films fabricated with the GOX-SWNT (dialysis)

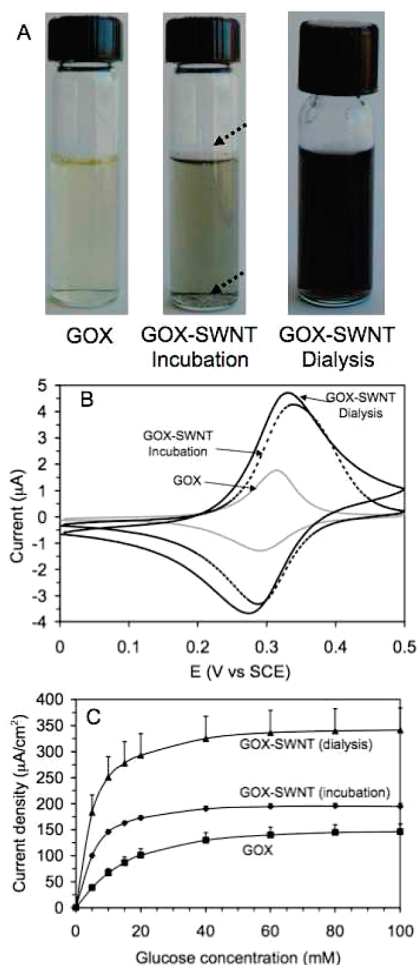


Figure 10. Effect of SWNT dispersion method on electrochemical and enzymatic response. (A) Optical images of a GOX solution, a GOX-SWNT incubation suspension, and a GOX-SWNT dialysis suspension. (B) CVs of gold electrodes modified with four bilayers (PVP-OS/GOX) without SWNTs or with GOX-SWNTs conjugates produced by the incubation or the dialysis method. (C) Calibration curves of gold electrodes modified with four bilayers (PVP-OS/GOX) without SWNTs or with GOX-SWNTs conjugates produced by the incubation or the dialysis method. $E = 0.4$ V vs SCE, in PBS at 25 °C.

conjugates. In redox polymer bioelectrocatalysis, the enzymatic current is dependent upon the flow of electrons between the substrate and the electrode surface and involves five steps: (1) transport of the substrate to the film; (2) transfer of electrons from the substrate to the enzyme; (3) transfer of electrons from the enzyme to the redox polymer; (4) transport of electrons through the redox polymer film; and (5) transfer of the electron from the redox polymer to the electrode surface. Depending upon the system design, any of these transduction steps may limit the overall reaction rate and consequently the signal output. A recent report study, in which MWNTs were modified with a ferrocene redox polymer and combined with GOX in a LBL films, reported that the presence of MWNTs led to a 10-fold increase in the electron diffusion through the films (step 4) and a 12-fold increase in wiring efficiency of the enzyme (step 3). In addition, other studies have demonstrated that both SWNTs⁴³ or MWNTs^{54,55} can increase a sensor response by providing a larger electrochemical area (step 5).

The increased electrochemical response of both the GOX-SWNT(dialysis) and GOX-SWNT (incubation) conjugates in Figure 10 may be explained by an increase in the electrochemical surface area and/or enhanced electron transport through the films. Experiments are currently under investigation in our lab to determine the relative importance of these two mechanisms. However given the fact that the GOX-SWNT(dialysis) conjugates provided a higher enzymatic response than the GOX-SWNT(incubation) conjugates suggests that other factor(s) are involved. We hypothesize that the GOX-SWNT(dialysis) conjugates increase the wiring efficiency relative to both the GOX-SWNT(incubation) and GOX cases by affecting the molecular orientation of the enzyme in the films. However, additional experiments are needed to confirm this.

CONCLUSIONS

In this study, we demonstrated that GOX-SWNT conjugates produced by the sodium cholate suspension-dialysis method were highly dispersed, remained stable for 30 days, and retained a significant amount (75%) of native enzymatic activity. We also demonstrate that these GOX-SWNT conjugates can be combined with a cationic redox polymer (PVP-OS) by the LBL self-assembly technique to fabricate highly sensitive sensors to glucose. The electrochemical and enzymatic properties of these films were dependent upon the adsorption pH of the redox polymer solution and the dispersion quality of SWNTs.

These results are significant and noteworthy for a variety of reasons. First, several recent studies have highlighted the fact that retention of protein structure and activity upon attachment to SWNTs is dependent upon the type of protein,^{56,57} the amount of protein adsorbed,⁵⁸ and the method of attachment.⁵⁹ Thus demonstrating that the sodium cholate suspension-dialysis method could be applied to GOX and produce highly active GOX-SWNT conjugates is novel. In addition the enzyme loadings obtained are ~4 times higher than the loadings (~5.3 mg GOX/SWNT) achieved by an ultrasonic processing method.⁶⁰ A second important aspect of this work is that we report the resonance ratio, which is related to the fraction of individual SWNTs,⁴⁸ of our GOX-SWNT dispersions. A persistent challenge in the SWNT field is quantitatively comparing the quality of SWNT dispersions produced by different methods. Our measurements of the resonance ratio provide useful information for comparison as new methods of SWNT solubilization are developed. Moreover the resonance ratio of 0.129 compares favorably with values measured for surfactant dispersed SWNTs. A final significant aspect of this work is the high biocatalytic response of the sensors (limiting current density of 440 $\mu\text{A}/\text{cm}^2$ and sensitivity of 56 $\mu\text{A}/$

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mM·cm²). These values are among the highest ever reported for LBL biosensors based on GOX, and should allow for miniaturization in biosensor development and for increased power output in biofuel cell applications.

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SUPPORTING INFORMATION AVAILABLE

UV–vis spectra of different concentrations of SWNTs dispersed by sodium cholate. Effect of scan rate on LBL films made with and without SWNTs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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