BIOFUNCTIONAL INTERFACES FOR BIOSENSING APPLICATIONS

Saroja Mantha[1], Virginia Davis[2], Bryan Chin[1] and Aleksandr Simonian[1]
[1]*Departments of Materials Research and Education Center, Auburn University, Auburn, AL 36849, U.S.A.*
[2]*Department of Chemical Engineering, Auburn University, Auburn, AL 36849, U.S.A.*

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Abstract: Layer-by-layer assembled CNTs customized with biopolymers has recently attracted a great attention as a simple, robust and inexpensive method for creating nanocomposite thin films with a high degree of control that may provide potentially powerful interfaces for multiple applications, including but not limited to biomedicine and biosensing. Intercalation of oppositely charged polymers and catalytically active proteins on the CNT surface allow assembling of unique nanointerfaces with the ability to detect single or multiple analytes (Hitzky et al., 2005; Kumar and Swetha, 2010; Dujardin and Mann, 2002; Palin et al., 2005; Geetha et al., 2006; Yan et al., 2010; Riccardi et al., 2006; Darder et al., 2005; Liu et al., 2004; Raravikar et al., 2005; Du et al., 2004; Katz and Willner, 2004; Wang, 2005; Allen et al., 2007; Ghindilis et al., 1997; Joshi et al., 2005; Chikkaveeraiah et al., 2009; Wang et al., 2006). The aim of this study is to design of multifunctional systems for the detection of numerous compounds, such as glucose and OP neurotoxins, in one platform using nanocomposite interface. A redox enzyme glucose oxidase (GOX) and organophosphate hydrolase (OPH), a phosphotriesterase catalyzing degradation of phosphorus-containing toxins and pesticides, were covalently immobilized on the multiwalled carbon nanotube (MWNT) surface using EDC/NHS chemistry. Layer-by-layer assembly (LBL) of oppositely charged CNTs customized with different biopolymers were examined on several substrates including glass or silicon slides and glassy carbon electrode. The interface assembly were characterized using Thermogravimetric analysis, Raman spectroscopy, Fourier Transform Infrared Spectroscopy, and scanning electron microscopy (SEM). The catalytic activity of the biopolymer layers were characterized using absorption spectroscopy and electrochemical analysis. Experimental results show that this approach yields an easily fabricated catalytic multilayer with well-defined structures and properties for biosensing applications whose interface can be reactivated via a simple procedure.

1 EXPERIMENTAL

1.1 Enzyme Immobilization

Enzyme immobilization on carboxylated MWNTs was performed using carbodiimide chemistry. A dispersed solution that was optically homogeneous to the naked eye was obtained by mixing 2 mg of MWNT in 5 ml of de-ionized water and sonicating the mixture for 1 hr. Under fast stirring condition EDC (20 mM) was then added to initiate the coupling of NHS to the carboxylic groups on the oxidized nanotubes and the mixture was stirred at 400 rpm for 30 min. The activated nanotube solution was then filtered through a 200 nm polycarbonate membrane and rinsed thoroughly with MES buffer to remove excess EDC and NHS and re-dispersed in protein solution (Pedrosa et al., 2010). After incubating the mixture on a platform shaker at 4°C for 9.5 h, the nanotube suspension was centrifuged at 13200 rpm and rinsed with MES buffer solution three times to remove unbound protein. The protein–nanotube conjugate was finally suspended in CHES buffer solution. Oxidized MWNT were separately dispersed in PEI (1 mg/ml) by sonicating for 1 hr in ultra sonication; excess polymer was then removed by centrifugation at 13200 rpm for about 30 min. Similarly 0.1 wt% of MWNT was dispersed in 0.1 wt% of DNA by sonication for 1 hr in ultra sonicator bath followed by centrifugation at 13200 rpm for about 30 min to remove unbound DNA.

1.2 Instruments

The samples of raw MWNT and oxidized MWNT were analyzed by thermogravimetric analysis (TGA)

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using TGA Q500 (TA Instrument, USA) instrument in air atmosphere over a temperature range from 30 to 800 °C at a heating rate of 10 °C/min. Raman spectroscopy was performed using 785 nm laser excitation (model SDL-8530, SDL Inc.) on Reinshaw inVia Raman microcope system. FT-IR measurements were taken for raw, oxidized MWNT and MWNT-OPH. The samples were ground with potassium bromide (KBr) to form a very fine powder using a mortar and pestle. This powder was then compressed into a thin and transparent pellet and was placed into the sample holder for analysis. Analysis was performed using a Shimadzu (Thermo-Electron Corp., Waltham, MA) bench machine with 32 scans. A drop of MWNT-OPH solution was placed on the glass slide, allowed to spread uniformly, and dried over night. The slide was examined by field emission scanning electron microscopy equipped with an energy dispersive X-ray analyzer (JEOL USA, Inc., Peabody, MA).

Cyclic voltammetric and amperometric measurements were performed using a CV-50 potentiostat (BAS USA) connected to a personal computer. A three-electrode configuration was employed, consisting of modified/glassy carbon (GC) electrode (3-mm diameter) serving as a working electrode, whereas Ag/AgCl (3 M KCl) and platinum wire served as the reference and counter electrodes respectively. Batch electrochemical experiments were carried out in a 2 ml voltammetric cell at room temperature (25 °C).

2 LAYER-BY-LAYER ASSEMBLY OF MWNT THIN FILMS

2.1 Slides

Glass or silicon slides were cleaned in concentrated H_2SO_4/30% H_2O_2 (3:1). The negatively charged slides were alternately immersed in aqueous dispersion of MWNT-PEI and MWNT-DNA. The adsorption time of 15 min was considered sufficient for the formation of MWNT monolayer. After each layer deposition, the substrate was rapidly dried using 50 psi air from a nozzle for 30 seconds. On top of these cushioning layers, alternate layers of MWNT-OPH and MWNT-DNA were deposited by immersing the slide in aqueous solutions of MWNT-OPH and MWNT-DNA for 15 min. The surface was renewed by immersing the slides in MWNT-OPH solution for 15 minutes. These solutions appear stable even after a year.

2.2 Glassy Carbon Electrode

The glassy carbon electrode (GCE) was polished with 0.10 and 0.05 μm alumina slurries and then ultrasonically cleaned in water for 15 min. The GC electrode was put into 1 M NaOH solution for 5 min and potential of 1.2 V was applied to introduce negative charges on the surface; this was followed by two washings steps with distilled water. The positively charged MWNT-PEI was adsorbed by dipping the negatively charged GC electrode in an aqueous solution of MWNT-PEI for 15 minutes, and the MWNT-PEI/GC electrode was dried in nitrogen. Using the same procedure, a layer of negatively charged MWNT-DNA was adsorbed. Following that, MWNT-OPH layer was adsorbed on the $(MWNT\text{-}DNA/MWNT\text{-}PEI)_4$/GC electrode by dipping in MWNT-OPH solution, and further bilayers were formed in the same way. The modified electrode was stored at refrigerated conditions until use. All the electrochemical measurements were performed at room temperature. A three electrode system containing platinum as auxiliary electrode, an LbL modified glassy carbon working electrode and a saturated Ag/AgCl reference electrode was used. The buffer solution was 50 mM PBS (pH 7.54). The regeneration of the biosensor interface was realized by immersing the sensor in a fresh solution of MWNT-OPH for 15 min.

3 RESULTS AND DISCUSSION

The objective of this work was to design the hybrid catalytic interfaces based on the interaction of anionic/cationic biomolecular layers structured with MWNTs (Figure 1). The initial step requires assembling of supporting bilayer of oppositely charged MWNT- polyethyleneimine (PEI) and MWNT-DNA. This allows for further adsorption of positively charged complex of MWNT-protein which adsorbs better on this cushioning support rather than adsorbing directly on a solid support.

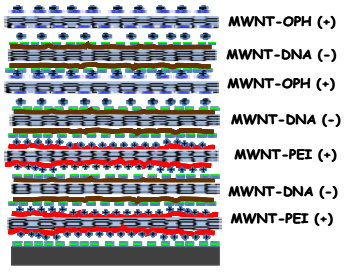

Figure 1: LbL interface design (not in scale): the initial layers of MWNT-PEI and MWNT-DNA provide support for subsequent layers of MWNT-OPH and MWNT-DNA.

Confirmation of nanotube functionalization was provided by Raman spectroscopy and FTIR analysis. It is obvious from Figure 2a that the intensity of characteristic peaks of MWNTs, namely, the D band at 1305 cm^{-1} and the G band at 1580 cm^{-1} have changed after functionalization. The D/G ratios of as received MWNTs and oxidizes MWNTs were found around 1.36 and 1.7 respectively, and the increased D/G ratio corresponds to the increased degree of functionalization.

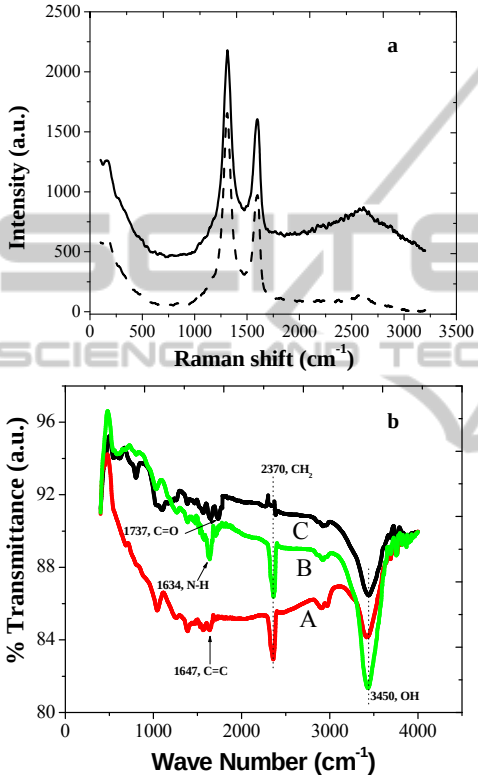

Figure 2: (a) Raman spectra of intact MWNT (—) and oxidized MWMT (---) at 785 nm; (b) FT-IR spectra of (A) intact MWNT, (B) MWNT-OPH and (C) oxidized MWNT.

In order to get direct affirmation for the added functional groups on the nanotube surfaces and immobilization of OPH on oxidized MWNT, FT-IR analysis was performed on as received MWNTs, oxidized MWNT and MWNT-OPH samples. Figure 2b shows spectra of as received MWNT (A), oxidized MWNT (B) and MWNT-OPH (C) respectively. The oxidation of CNT by the combination of H_2SO_4 and HNO_3 results in the formation of hydrophilic groups at defect sides and ends, −COOH, −C=O and −OH. All spectra for the oxidized CNT displayed a peak at 1734 cm^{-1} corresponding to –COOH and 1650 cm^{-1} which corresponds to $–COO^-$. These results are consistent with previous work on CNT oxidation; the exact peak positions are the result of the extent of oxidation. In addition, the increased in intensity of 3434 cm^{-1} peak clearly confirms introduction of more –OH groups after acid treatment. The introduction of OPH results in the peaks at 3434 cm^{-1} and 2857 cm^{-1} are respectively attributed to symmetric and asymmetric $–CH_2$ stretching. However, after the enzyme immobilization on oxidized MWNT, the 1734 cm^{-1} peak disappeared and a new peak at ~1634 cm^{-1} was observed. This can be attributed to the in-plane N–H molecular vibrations of the amine group. It is believed that a substitution reaction occurs and a –NH group replaces the –OH group of the carboxylated MWNTs after amide functionalization to form the –CO–NH functional group.

The aim of this study is to design of multifunctional systems for the detection of umerous compounds in one platform. To demonstrate catalytic properties of nanocomposite interface for single analyte, organophosphate hydrolase (OPH) enzyme was used in multilayer assembly. It is well known that OPH hydrolyzes the phosphotriester bond of the model OP paraoxon (λ_{max} = 274 nm), releasing the hydrolysis products *p*-nitrophenol (PNP) (λ_{max} = 405 nm) (Wang, 2005). Absorption spectra show two peaks, one corresponding to paraoxon at 274 nm and the peak at 405 nm corresponds to PNP, the hydrolysis product formed after exposure of the paraoxon solution to the slide with MWNT-OPH interface. Presumably, the activity of layers with catalytically active biopolymer should be different from that for non-catalytic layer. Slides with different number of layers ending with MWNT-OPH or MWNT-DNA were exposed to 0.1 μM paraoxon for 10 min and the absorption spectrum was recorded. As shown in Figure 3 the absorption at 405 nm increased with the number of layers with an MWNT-OPH ending, indicating a raise in the enzymatic activity proportional to the number of enzyme layers. Contrary to that, absorbance was much lower for layers ending with MWNT-DNA. These results indicate that assembled multilayers are relatively permeable for paraoxon, which penetrate into the deeper layers and react with OPH. More detailed investigation of layers density and deepness of substrate penetration and permeability in such interfaces are following.

Electrochemical studies of LBL on glassy carbon electrode showed increase in response with the subsequent addition of paraoxon and glucose.

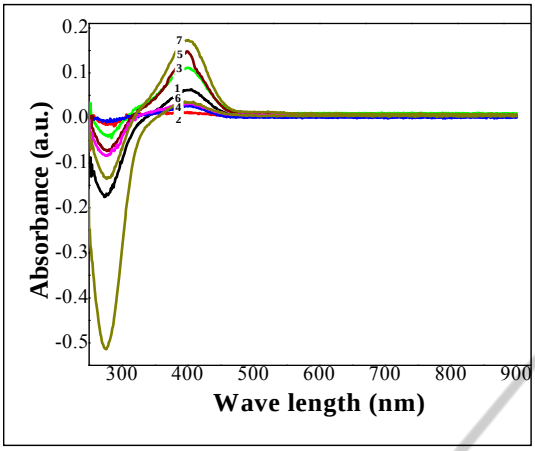

Figure 3: UV-Vis absorption spectra of LbL assembly for different number of layers by exposing the surface to 0.1 mM Paraoxon for 10 minutes.

Figure 4, a shows flow-injection calibration data for paraoxon over the concentration ranges of 0.5-10 µM, and the inset shows that current linearly increased with the concentration in the range from 0 to 10 µM. The system showed excellent sensitivities ($y = -8x10^{-10} + 0.074$ A/µM cm^{-2} calculated from the slopes of the linear part of the calibration curve). In addition, based on an estimated signal-to-noise (S/N) ratio of 3, the sensor has a detection limit of 77 nM paraoxon.

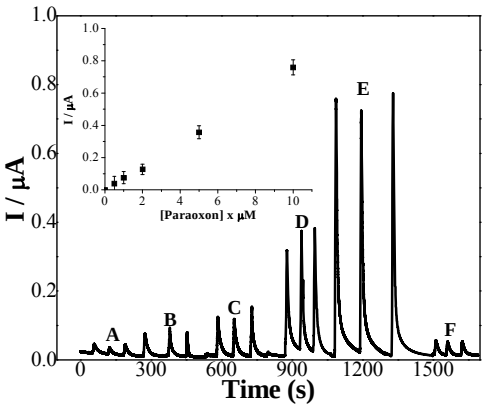

Figure 4: Amperometry for LBL on GCE injecting paraoxon only in sequence at 850 mV using 10 mM PBS buffer.

To demonstrate catalytic properties of nanocomposite interface for multiple analytes, two enzymes, glucose oxidase (GOX) and organophosphate hydrolase (OPH) were used in multilayer assembly. Since OPH and GOX are oppositely charged, it was possible to use them in alternating layers for LbL assembly. As shown in Figure 5, the biosensor generating an adequate responses on the sequential injection of glucose and paraoxon.

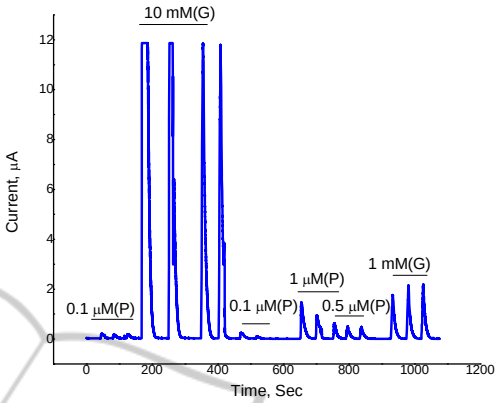

Figure 5: Amperometry for LBL on GCE injecting paraoxon and glucose in sequence at 850 mV using 10 mM PBS buffer. For glucose detection a redox mediator Ferrocene methanol was added.

Since the catalytic activity of the interface is decreasing in time, it is desirable to regenerate it after significant reduction of the activity (Dumas et al., 1989). In experiments with OPH the electrode surface was renewed after 6 months of usage, when the electrode response dropped to 45% of its original value. We found a very easy way to restore up to 95% of the original response level by immersing the electrode in the MWNT-OPH solution for 15 minutes. The most probable reason for gradually reducing activity of the electrode might be that the upper MWNT-OPH layer is depleting. Taking into account that the activity of the surface ending with MWNT-DNA is about 50% less than for MWNT-OPH ending layer, we may conclude that after 6 months of use the electrode surface is losing completely its MWNT-OPH upper layer, and the negatively charged MWNT-DNA layer is exposing to the solution. Then, immersing the electrode in the MWNT-OPH solution allows restoration of active MWNT-OPH layer and restoration of initial activity. Thus, this simple procedure of interface re-activation brings a significant advantage of the LbL assembly over other technologies and allows using the biosensor for very long time.

In conclusion, this approach to the generation of multifunctional LbL biopolymer nanocomposite interfaces is relatively simple, does not require complex synthesis, and yields excellent catalytically active interfaces appropriate for biosensing and other applications.

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