Layer-by-Layer Assembly of Carbon Nanotubes Modified with Invertase/Glucose Dehydrogenase Cascade forSucrose/O₂ Biofuel Cell

Yuanyuan Zhang,a,** Mary A. Arugula,a,* Shannon T. Williams,a Shelley D. Minteer,b,** and Aleksandr L. Simoniana***,z

aDepartment of Materials Engineering, Auburn University, Auburn, Alabama 36832, USA
bDepartment of Chemistry, University of Utah, Salt Lake City, Utah 84112, USA

A layer-by-layer (LbL) assembly technique was employed to modify glassy carbon electrodes (GCEs) and screen printed electrodes (SPEs) utilizing multiwalled carbon nanotubes (MWCNTs)/polyelectrolyte binary composites and an enzyme cascade to facilitate efficient electron transfer in sucroseeO₂ biofuel cell. In this study, MWCNTs immobilized invertase (INV) and glucose dehydrogenase (GDH) were alternatively assembled upon polyethylenimine (PEI) and DNA nanocomposites to construct a bioanode. [Ni(phendion)(phen)Cl₂ complex and methylene green (MG) were investigated as mediator for electrocatalytic oxidation of NADH at reduced overpotentials. The LbL architecture showed advantages for sequential enzymatic reaction that favored the efficient penetration of substrate and products in a cascade system while MWCNTs facilitated the efficient electron transfer from sucrose oxidation and enhancement of the current density. With a GCE bioanode modified with a MG or Ni complex, the biofuel cell produced a higher power density (μW/cm²) with 145.8% and 130.11% enhancement comparing to a SPE bioanode, respectively. Moreover, the Ni complex modified GCEs and SPEs produced power densities (μW/cm²) 93.7% and 107.0% higher compared to MG modified bioanodes, respectively. The maximum current density of 1400 ± 46 μA/cm² was obtained with the Ni complex on GCE at an OCP of 604 ± 17 mV with a maximum power density of 405 ± 6 μW/cm². The LbL assembly showed great feasibility as a simple and efficient way to construct controlled MWCNT-multi-enzyme modified electrodes for biofuel cell applications.

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Biofuel cells convert the chemical energy of biofuels into electric energy via the enzymatically catalyzed oxidation and reduction reactions. One of the most important factors affecting the performance of fuel cell is the fabrication of bioanode which has great influence in generation of electrical power outputs. Therefore developing effective bioanodes remains critical. Till date, several reports have been published on various approaches to assemble enzymes on the anodes in BFCs utilizing carbon based materials.1,2 Amongst those layer-by-layer (LbL) assembly technique via electrostatic interactions of oppositely charged species has emerged as a very attractive way to construct multilayer films of polyelectrolytes, biomolecules, organic materials offering advantages in various fields, such as surface functionalization,9,10 drug delivery,11,12 and biosensing.13,14 Its application was considered plausible in the development of amperometric biosensors initiating vast research activities on biosensors comprised of LbL organized multilayers. LbL nanostructures decorated with multi-enzymes were proven to be one of the successful strategies to establish high electrical performance, long-term stability and long lifetime in bioelectronics devices.14,16–18 For example, LbL structures consisting of Au nanoparticles (AuNPs), thiol-functionalized polyaniline and glucose oxidase (GOX) were fabricated for glucose biosensing by Komathi et al.9 Similarly, Wu et.al and Cui et.al used LbL assembly of carbon nanotubes (CNTs), AuNPs and an insulating polymer to fabricate a glucose sensor.20,21 Our group has reported extensively on LbL based single and bi-enzyme biosensing systems incorporating multi-walled carbon nanotubes (MWCNTs) immobilized with organophosphorus hydrolase (OPH) and acetylcholinesterase (AChE), for discriminative detection between organophosphorus and non-organophosphorus pesticides.11,12 In these organized LbL nanostructures, a homogenous and stable CNT-based assembly of multi-enzyme interfaces with desired architecture provides control over the position of the polyelectrolyte and the enzyme molecules compared to random hydrogels.22,23 Further, more, spatially organized multilayers with close proximity could be very advantageous for sequential enzymatic reactions with single and bi-enzyme cascade systems that favors efficient substrate/product penetration, molecular recognition, redox mediation and efficient electron transfer.24,25 To the best of our knowledge, no study on the fabrication of bioanode utilizing an enzyme cascade by layer by layer assembly method and investigation corresponding to its biofuel cell performance has been explored.

On the other hand, the most extensively investigated biofuel resources include saccharides such as glucose and alcohols.26–28 Since the current challenges for biofuel cell development lie in deeper oxidation and improved energy density,29 recent reports have been mainly focused on disaccharides or polysaccharide based biofuel cells.30 Hickey et al.31 reported an enzyme cascade system employing invertase, fructose dehydrogenase and glucose oxidase immobilized in ferrocene-modified linear poly(ethyleneimine) (Fc-C₆-LPEI) hydrogel, which was then drop casted onto carbon electrode for catalyzing sucrose oxidation in a biofuel cell. This generated 42 ± 15 μW/cm² in 100 mmol/L sucrose. Several other reports include trehalose,32 celllobiose,33 and starch34 based fuel cells obtaining maximum current density of 0.1 mA/cm², 1.9 μW/cm² and 8.15 μW/cm², respectively. Handa et al.35 fabricated a carbon-felt based inverase, fructose dehydrogenase and glucose oxidase immobilized bioanode mediated by tetrathiafulvalene and bilirubin oxidase immobilized biocathode with ABTS as mediator for sucrose biofuel cell. A maximum power density of 2.9 μW/cm² was obtained in 50 mmol/L sucrose. It should be noted that the enzyme cascades so far reported for disaccharides and polysaccharides are based on randomly built hydrogels immobilized on the electrode surfaces.

Herein, we demonstrate a simple strategy via LbL assembly method by alternate assembling oppositely charged CNT-PEI (positive charge) and CNT-DNA (negative charge) for cushion structure for further binding CNT-enzymes invertase (INV) and glucose dehydrogenase (GDH). The aim of this work is to investigate whether the LbL based bionanostructures via layer by layer assembly utilizing small surface area conventional electrodes immobilized with enzyme cascade system is an effective approach to promote electricity generation. A schematic illustration showing the construction of the LbL assembled bioanode is presented in Fig. 1.
Materials and chemicals.—Glassy carbon electrodes (GCE, 3 mm in diameter) and screen printed electrodes (SPE, 4±5 mm) were obtained from CH Instruments (Austin, Texas) and Pine Instrument (Grove city, PA), respectively, and used as anode electrodes. Carboxylated multiwalled carbon nanotubes (purity 95%, length 1–5 μm, diameter 30 ± 10 nm) were obtained from Nanolab Technologies (Milpitas, CA). Invertase from baker’s yeast (INV, EC 3.2.1.26, 200–300 U/mg solids), glucose dehydrogenase from Pseudomonas sp. (GDH, EC 1.1.1.47, ≥200 U/mg), dihydronicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD⁺), methylene green (MG), 5-amino-1,10-phenanthroline (phen), sucrose, D-glucose, lyophilized salmon sperm DNA (1,10-phenanthroline-5,6-dione (phendion)), glutaraldehyde, and Nafion were all obtained from Sigma-Aldrich (St. Louis, MO). 1,10-phenanthroline-5,6-dione (phenidion) and NiCl₂ were from Acros Organics. Mutarotase (1800 U/mg solids) was obtained from CALZYME laboratories (San Luis Obispo, CA). Synthesis for [Ni(phenidion)Cl₂] complex was followed using the procedures reported by Korani et al. with slight modification. Briefly, 5 μL of carboxylated MWCNTs (1 mg/mL in ethanol) was drop cast onto the electrode surface followed by 5 μL of 5-amino-1,10-phenanthroline (phen) (10 mM in ethanol) and 5 μL of EDC (30 mM in ethanol). The electrode was kept at 4°C for 24 hrs for covalent attachment of phen onto the electrode surface. The carboxylated MWCNT/phen modified electrode was further incubated in a 10 mM [Ni(phenidion)Cl₂] complex at 45°C for 24 hrs. Since [Ni(phenidion)(phen)₂]Cl₂ creates a positive charge on the electrode surface, post drying, 20 μL of negatively charged MWCNT-DNA solution was first deposited and subjected to further drying- rinsing-drying to remove the unbound MWCNT-DNA. Using similar procedures another layer of MWCNT-PEI/DNA was deposited, dried and stored in 4°C before use. The schematic of preparation of CNTs-polymer/enzyme anode assembled in LbL fashion was shown in Fig. 1.

Preparation of CNT-biomolecules.—Preparation of MWCNT-PEI and MWCNT-DNA was reported in our previous article. INV was immobilized onto carboxylated MWCNTs via the NHS/EDC crosslinking process and the protocol was used as previously described with minor modifications. For enzyme immobilization, 2.5 mg of INV was dissolved in phosphate/nitrate buffer (100 mM, pH 7.0) dispersed with EDC/NHS activated MWCNTs and was allowed to incubate overnight at 4°C. Approximately 1.25 mg of GDH enzyme was weighed and dissolved in 70 μL phosphate/nitrate buffer (100 mM, pH 7.0) followed by the addition of 20 μL carboxylated MWCNTs (10 mg/mL solution) and 10 μL glutaraldehyde (0.5%v/v). The mixture was sonicated for 30 s followed by vortex for 1 hr and further 30s sonication. The final mixture was stored in the refrigerator at 4°C for further use.

Nafion/MWCNTs/MG bioanode.—MWCNTs (1 mg/mL) were prepared in 10 μL ethanol and Nafion (final concentration 0.1%) and sonicated for 1 hr to obtain homogeneous suspension. Later, 500 μL of MWCNTs solution was transferred into a micro-centrifuge tube and diluted with 500 μL DI water followed by addition of 3.5 μL MG (100 mM). This mixture was vortexed immediately for 5 s and sonicated for 30 s. Subsequently, 20 μL of Nafion/MWCNTs/MG suspension was drop cast onto electrode surface and stored in the refrigerator at 4°C overnight. Nafion is negatively charged, therefore, 20 μL of positively charged MWCNT-PEI solution was deposited followed by 15 min drying-rinsing and drying process described above. Following bilayers of MWCNT-DNA/PEI and MWCNT-GDH, MWCNT-PEI and negatively charged MWCNT-INV layers were consecutively constructed on the electrode surfaces.
**UV-Vis/Electrochemical characterization of LbL assembled bioanode.**—All optical measurements were carried out using a UV-Vis spectrophotometer (Amer sham Biosciences Ultrospec 2100 pro) with 1 mL PMMA cuvettes. An electrochemical analyzer CHI 760E (CH Instruments, Austin, TX) potentiostat connected to a computer with the CHI 760E software package was used for all electrochemical measurements. A conventional three-electrode system with 3 M Ag/AgCl reference electrode and platinum counter electrode were employed for all measurements. Cyclic voltammetric experiments at various scan rates (0.005, 0.01, 0.05, 0.1 V/s) were carried out on mediator modified electrode surfaces for surface characterization. Phosphate/nitrate buffer (100 mM, pH 7.0) and phosphate/sodium chloride buffer (100 mM, pH 7.0) were used for GCE and SPE, respectively. All experiments were performed at room temperature (25 ± 2°C).

Sucrose/O2 biofuel cell assembly and characterization.—The biofuel cell consists of an “I shape” glass chamber separated by the carbon cloth cathode coated with Nafion polymer electrolyte membrane (Fig. 1). The fuel solution was added to the upper glass chamber, and the bottom chamber was left open for air breathing allowing O2 to reach the cathode. The cathode was prepared by hot pressing Pt/C cloth onto Nafion membrane. The Nafion side of the cathode was soaked in concentrated sulfuric acid overnight before use. For the GCE anode, the fuel solution consisted of 200 mM sucrose, 10 mM NAD+, and 60 μL mutarotase (2000 U/300 μL) with a total volume of 6 mL in phosphate/nitrate buffer (100 mM, pH 7.0), while phosphate/sodium chloride buffer (100 mM, pH 7.0) was used for SPE anode.

Results and Discussion

Sucrose cascade determination.—We investigated the activities of the MWCNT-INV/GDH layers by a spectrophotometric method based on the reduction of the cofactor NAD+ to NADH which can be observed at 340 nm. The increase in absorbance resulting due to catalytic reaction of INV and GDH was found to be slow initially (>5hrs) as shown in Fig S1. This might be due to the delayed utilization of α-D-glucose by the GDH, which is produced as a product of sucrose hydrolysis. To fasten the NADH production, the substrate and cofactor were increased which reduced the time to 2 hrs. Mutarotase that can convert α-D-glucose to β-D-glucose was incorporated into the enzyme cascade. In our strategy, optimization of conditions, such as sucrose concentration, amount of enzyme/cofactor NAD+, and addition of mutarotase was investigated to expedite the enzymatic cascade reaction (Fig. S1 in Supplementary Information). The results indicate that the DNA, PEI coated surfaces had electrostatically interacted with GDH and PEI and facilitated in binding of INV. The increase in NADH absorbance indicated that the cascade system worked with GDH and PEI coated surfaces had electrostatically interacted with the DNA, PEI coated surfaces had electrostatically interacted with INV. The increase of oxidation peak and a decrease in reduction peak with both mediators on SPE and GCE. The recorded onset potentials for NADH oxidation were ~0.35 V and ~0.34 V with MG on GCE and SPE, respectively and with carboxylated MWCNT/Ni(phenidion)(phen)Cl2 were ~0.20 V and lower than ~0.30 V, respectively. The increase of oxidation peak and a decrease in reduction peak on SPE was slightly higher when compared to GCE due to a relatively larger working area of SPE. We found that the modified SPE electrodes redox peak currents with and without NADH showed higher change than the GCE. With GCE the change in peak currents in the presence and absence of NADH, was ~10 μA and SPE ~13 μA. Upon comparison of mediators, the NADH oxidation potentials at the [Ni(phenidion)(phen)]Cl2 modified electrodes were more negative than the MG modified electrode, demonstrating [Ni(phenidion)(phen)]Cl2 has a better mediation potential toward the oxidation of NADH compared with MG.

Performance of LbL assembled bioanode.—To verify the performance of the LbL assembled bioanode, the nanostructure complex of NADH oxidase with the Ni/Fe bioanode layers was first assembled on carboxylated MWCNT[Ni(phenidion)(phen)]Cl2 and Nafion/MWCNTs/MG modified electrodes. The cascade involves the hydrolysis of sucrose by INV to glucose and GDH further converting glucose to glucoalactone by reduction of NAD+ to NADH (Eq. 1–3). The LbL architecture with close proximity in molecular scale within enzyme layers favors the efficient penetration of substrate and products, showing great advantage for the cascade reaction. The bioanode was immersed into 6 mL of PB buffer containing 200 mM sucrose, 3 mM NAD+, and 60 μL mutarotase. After 3 hrs incubation, cyclic voltammetry was performed to monitor the NADH oxidation. Fig. 3 shows the electrocatalytic oxidation of enzymatically produced NADH. The NADH oxidation commenced at 250 and 300 mV at MG modified GCE and SPE, respectively, while the oxidation started at ~300 and ~350 mV on carboxylated MWCNT[Ni(phenidion)(phen)]Cl2 modified GCE and SPE. The change in peak currents at oxidative potentials was ~200 μA and ~220 μA for Ni complex modified GCE and SPE, respectively (Figs. 3A & 3B). ~300 μA and ~350 μA for MG/Nafion/MWCNTs modified GCE and SPE, respectively (Figs. 3C & 3D). To verify whether the current was due to the enzymatic reduction of NADH, a control experiment in the absence of CNT-enzyme layers were carried out for modified GCE and SPE. This shows that the layer adsorption is diffusional limited process for SPE and GCE. The difference in peak potentials (ΔEp = Ep −Ep0) at 100 mVs scan rate were 102 and 162 mV for Ni complex on GCE and SPE, respectively; 352 and 348 mV for MG on GCE and SPE, respectively. The cathodic and anodic peaks shifted non-symmetrically, suggesting that the ΔEp corresponds to quasi reversible diffusional reaction. The average peak potential Ep and Ep0 for Ni complex on GCE and SPE were ~0.018 and ~0.093 V versus Ag/AgCl, respectively; ~0.139 and ~0.348 V versus Ag/AgCl for MG on GCE and SPE, respectively. The potential values obtained as formal potentials were found to be very close to the MG and Ni complex peaks as shown in previous literature, proving that the enzymes in cascade maintained their activities even when wrapped with CNTs or intermingled with biopolymers. Previously, we have successfully demonstrated the increase of LbL assembly process between MWCNT dispersed PEI/DNA and enzymes via electrostatic interactions using surface plasma resonance (SPR) real time monitoring and electrochemical characterization. Meanwhile optimization of the LbL nanostructure in terms of layer density, number of layers, and position of enzyme layers was also investigated.22–25 The charged CNT-PEI (positive charge) and CNT-DNA (negative charge) with sufficient opposite charges were demonstrated to serve as a strong cushion for firmly binding of above CNT-enzyme layers.

**NADH electrocatalysis with mediator modified electrode.**—Various electrocatalysts have been reported as efficient electron-transfer mediators for NADH oxidation.39–41 Characteristic voltammograms for 1 mM NADH mediation on GCEs and SPEs were shown in Fig. 2. The presence of NADH demonstrated increase in the oxidation peak and a decrease in reduction peak with both mediators on SPE and GCE. The recorded onset potentials for NADH oxidation were ~0.17 V and ~0.34 V with MG on GCE and SPE, respectively and with carboxylated MWCNT/Ni(phenidion)(phen)Cl2 were ~0.20 V and lower than ~0.30 V, respectively. The increase of oxidation peak and a decrease in reduction peak on SPE was slightly higher when compared to GCE due to a relatively larger working area of SPE. We found that the modified SPE electrodes redox peak currents with and without NADH showed higher change than the GCE. With GCE the change in peak currents in the presence and absence of NADH, was ~10 μA and SPE ~13 μA. Upon comparison of mediators, the NADH oxidation potentials at the [Ni(phenidion)(phen)]Cl2 modified electrodes were more negative than the MG modified electrode, demonstrating [Ni(phenidion)(phen)]Cl2 has a better mediation potential toward the oxidation of NADH compared with MG.
Figure 2. NADH Electrocatalysis with Ni complex on GCE (A) and SPE (B), MG/Nafion/MWCNTs on GCE (C) and SPE electrode (D), in the presence of 1 mM NADH, scan rate 5 mV/s.

Figure 3. Electrocatalytic effect of Ni complex on enzymatically produced NADH with GCE (A) and SPE (B), MG/Nafion/MWCNTs on GCE (C) and SPE electrode (D), using 200 mM sucrose, 3 mM NAD$^+$ at scan rate 5 mV/s.
Figure 4. Representative polarization (red line) and power curve (blue line) sucrose/O₂ biofuel cell composed of LbL assembled bioanode (solid line) and control (dashed line). (A) Ni complex with GCE, (B) Ni complex with SPE, (C) MG with GCE and (D) MG with SPE. The power density was calculated from the current density and voltage.

under the same condition which resulted in reduced electrocatalytic activity (partial data shown in Fig. S3 supplementary information). Therefore, the results implied that all the elements in LbL assembled enzyme cascade bioanode are indispensable for full electrocatalytic oxidation of sucrose, showing great potential for application of the proposed bioanode for biofuel cell development.

Sucrose/O₂ biofuel cell operation.—The open circuit potential (OCP) was measured and allowed to reach steady state. Linear sweep voltammetry (LSV) from just above the measured OCP potential to 0 mV was subsequently employed to obtain polarization and power curves at 1 mV/s and the data is presented as current and power densities. Control experiments were performed by substituting the top MWCNT-INV enzyme layer with a MWCNT-DNA layer. To examine the performance of LbL assembled sucrose bioanodes, the “I-shape” sucrose/O₂ enzymatic biofuel cell was assembled with an air breathing Pt membrane serving as cathode, as shown in schematic illustration (Fig. 1). The biofuel cells with different mediators were characterized using LSV sweeping from their corresponding OCP to 0 V. The representative polarization curves as well as calculated power curves were displayed in Fig. 4. A summary of the biofuel cells performances showing OCP, maximum power density, maximum current density were concluded in Table I.

The full LbL assembled sucrose bioanode demonstrates significant enhancement in current and power density compared with the control, demonstrating the efficiency of LbL assembled sucrose enzyme cascade bioanode with both mediators in a biofuel cell. Upon

Table I. The performance comparison of proposed biofuel cells with currently reported disaccharides and polysaccharides systems.

| Substrate concentration (mM) | OCP (mV) | Max. curr. Dens. (μA/cm²) | Max. power dens. (μW/cm²) | Ref. |
|-----------------------------|---------|--------------------------|--------------------------|------|
| Sucrose/O₂-MG/SPE           | 200     | 686 ± 21                 | 412 ± 36                 | 85 ± 6 | This work |
| Sucrose/O₂-MG/GCE           | 200     | 692 ± 9                  | 823 ± 32                 | 209 ± 3 | This work |
| Sucrose/O₂-Ni complex/SPE   | 200     | 619 ± 25                 | 635 ± 73                 | 176 ± 11 | This work |
| Sucrose/O₂-Ni complex/GCE   | 200     | 604 ± 17                 | 1400 ±46                 | 405 ± 6 | This work |
| Sucrose/O₂/carbon felt      | 50      | /                        | 12000                    | 2900  | 36 |
| Succrose/O₂/Toray paper     | 100     | /                        | 344                      | 42    | 32 |
| Lactose/disk graphite       | 34      | /                        | 13                       | 19    | 34 |
| Trehalose/carbon cloth      | 32      | /                        | 100                      | /     | 33 |
| Starch/GCE                  | 0.5%    | 530                      | /                        | 8.15  | 35 |
comparison of GCE and SPE electrode, the biofuel cell with MG and [Ni(phenidion)(phen)$_2$]Cl$_2$ modified GCE bioanode, produced power densities ($\mu$W/cm$^2$) that were 145.8% and 130.1% higher than that on SPE electrode, respectively. While comparing Ni complex and MG mediator, the biofuel cell with Ni complex modified GCE and SPE produced power densities ($\mu$W/cm$^2$) 93.7% and 107.0% higher than that with MG modified GCE and SPE bioanode, respectively. The maximum power density from MG and [Ni(phenidion)(phen)$_2$]Cl$_2$ were 209 $\pm$ 3 $\mu$W/cm$^2$ and 405 $\pm$ 6 $\mu$W/cm$^2$ on GCE. It is noteworthy to observe that Handa et.al has obtained 2.9 mW/cm$^2$ power density to observe that Handa et.al has obtained 2.9 mW/cm$^2$ power density when directly comparing two different approaches for biofuel cell performance. Therefore, experimental condition and setup should be considered obvious higher currents. We have implemented our system for longer power or current densities. Handa et.al uses carbon felt, multiple cascades, longer incubation times, and bilirubin cathode which led to obvious higher currents. We have implemented our system for longer incubation periods which actually led to 50% increase of current signal. Therefore, experimental condition and setup should be considered when directly comparing two different approaches for biofuel cell performance. Since this is first of its kind of cascade biofuel cell via LbL, we anticipate obtaining higher energy output with further addition of sophisticated carbon materials and biocathode in near future.

Nevertheless, the maximum power density obtained in our study showed great performance, succeeding most of other reported disaccharides biofuel cells in literature.

Conclusions

In this study, LbL assembly was utilized for construction of an enzyme cascade bioanode for use in a biofuel cell. The high current/power density obtained showed that LbL assembly is of great advantage in enzyme cascade bioanode fabrication and could be used in applications such as small electronic devices, microfluidic devices etc. Further, LbL assembly showed its excellent adaptability in fabrication of controllable nanocomposite in various substrate surfaces. Therefore, LbL assembly was demonstrated to be a simple, reliable and efficient approach in development of multi-enzyme system for multifunctional applications. In future studies, investigation of the biocathode, other types of electrode surface, number of assembled enzyme layers, etc. can be studied to achieve further improvement of the performance of LbL assembled enzyme-cascade system.

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