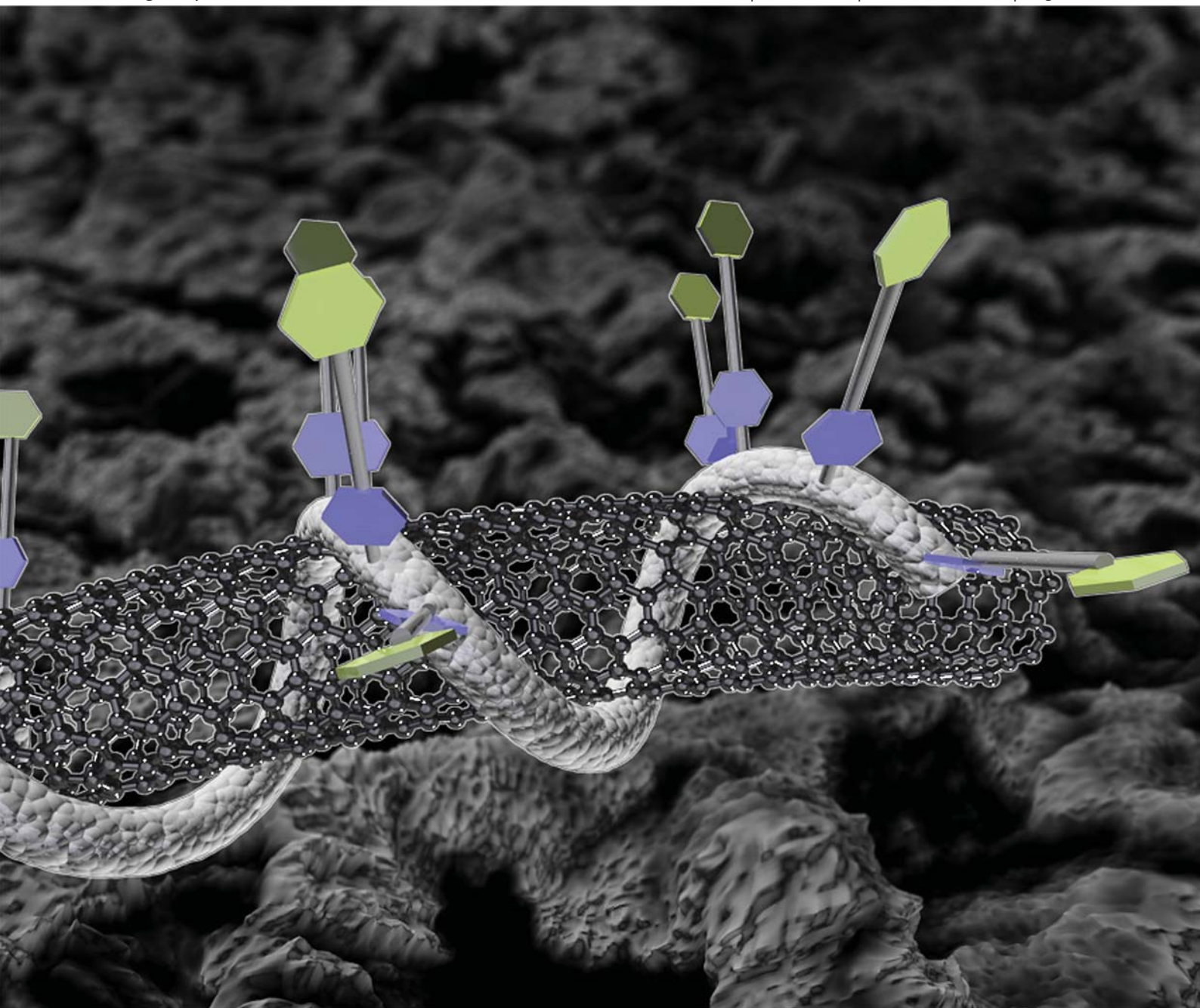


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PAPER

Microbiosensors based on DNA modified single-walled carbon nanotube and Pt black nanocomposites

Jin Shi,^{abc} Tae-Gon Cha,^{bf} Jonathan C. Claussen,^{abd} Alfred R. Diggs,^{abd} Jong Hyun Choi^{bf} and D. Marshall Porterfield^{*abcde}

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Glucose and ATP biosensors have important applications in diagnostics and research. Biosensors based on conventional materials suffer from low sensitivity and low spatial resolution. Our previous work has shown that combining single-walled carbon nanotubes (SWCNTs) with Pt nanoparticles can significantly enhance the performance of electrochemical biosensors. The immobilization of SWCNTs on biosensors remains challenging due to the aqueous insolubility originating from van der Waals forces. In this study, we used single-stranded DNA (ssDNA) to modify SWCNTs to increase solubility in water. This allowed us to explore new schemes of combining ssDNA-SWCNT and Pt black in aqueous media systems. The result is a nanocomposite with enhanced biosensor performance. The surface morphology, electroactive surface area, and electrocatalytic performance of different fabrication protocols were studied and compared. The ssDNA-SWCNT/Pt black nanocomposite constructed by a layered scheme proved most effective in terms of biosensor activity. The key feature of this protocol is the exploitation of ssDNA-SWCNTs as molecular templates for Pt black electrodeposition. The glucose and ATP microbiosensors fabricated on this platform exhibited high sensitivity (817.3 nA/mM and 45.6 nA/mM, respectively), wide linear range (up to 7 mM and 510 μ M), low limit of detection (1 μ M and 2 μ M) and desirable selectivity. This work is significant to biosensor development because this is the first demonstration of ssDNA-SWCNT/Pt black nanocomposite as a platform for constructing both single-enzyme and multi-enzyme biosensors for physiological applications.

Introduction

Electrochemical biosensors are highly effective in measuring biomolecule concentrations due to the high sensitivity, real-time monitoring capabilities and low cost. This contrasts with conventional measurement techniques including radioisotope tracing,^{1–4} NMR spectroscopy,⁵ and microfluorometry assays,^{6,7} which are complex and expensive, and severely limited in terms of spatial and temporal resolution. However, biosensors based on conventional materials are constrained in terms of total

sensitivity, which in turn limits the potential for miniaturization. This is due to restrictions in mass transport, enzyme loading, and electrochemical coupling. These sensitivity issues not only impact the limit of detection, but also the signal-to-noise ratio in measuring very small changes in concentration over time, while some small changes are key to exploring important physiological phenomena, such as β cell glucose consumption during insulin secretion.⁸ To overcome these limitations, nanomaterials with good biocompatibility and electrocatalytic activities have been widely incorporated in biosensors.^{9–14}

Carbon nanotubes (CNT) and metal nanomaterials are the most commonly used nanomaterials in biosensor construction.^{10–22,48} CNTs are allotrope of carbon. The carbon atoms at tube ends or at tube defect sites possess the catalytic capability for electrochemical reactions.^{23–25}

The application of CNTs to biosensing requires immobilization. The major obstacle for CNT immobilization is the fact that CNTs are minimally soluble in aqueous media due to van der Waals aggregation. Most CNT immobilization approaches such as chemical vapor deposition, abrasion or polymer immobilization do not change the solubility,^{13,25–31} while biochemical modification of CNTs (e.g. glucosamine³² and single-stranded

^aPhysiological Sensing Facility, Purdue University, 1203 W. State Street, West Lafayette, IN, 47907-2057. E-mail: porterf@purdue.edu; Fax: +765-496-1115; Tel: +765-494-1190

^bBirk Nanotechnology Center and Bindley Bioscience Center, Purdue University, 1203 W. State Street, West Lafayette, IN, 47907-2057

^cWeldon School of Biomedical Engineering, Purdue University, 1203 W. State Street, West Lafayette, IN, 47907-2057

^dDepartment of Agricultural and Biological Engineering, Purdue University, 1203 W. State Street, West Lafayette, IN, 47907-2057

^eDepartment of Horticulture & Landscape Architecture, Purdue University, 1203 W. State Street, West Lafayette, IN, 47907-2057

^fSchool of Mechanical Engineering, Purdue University, 1203 W. State Street, West Lafayette, IN, 47907-2057

DNA (ssDNA)³³) significantly increases the solubility in water, thus opening up technical approaches for applying CNTs that can be mediated in aqueous media. This greatly enhances the application of CNTs to biosensing. For example, abrasive immobilization is not possible with microscale devices, while the aqueous solution of ssDNA modified CNT can be applied on micro electrodes by drop-coating. The main problem with polymers and linking agent immobilization is that residual materials remain on the biosensor after CNT immobilization, which limit mass analyte transport and sensor sensitivity. By adopting ssDNA modification approaches, there is potential to apply CNTs to microbiosensors while preserving the effective surface area. Compared with CVD, ssDNA modification is much easier and cheaper. ssDNA modified single-walled CNTs (ssDNA-SWCNT) have been demonstrated to dramatically increase the electroanalytical current output,³⁴ while decreasing the redox overpotential³⁵ for electrochemistry, and have been used as a catalyst for redox reactions.³⁶ This is explained by virtue of a systematic change in SWCNT valence energy levels due to DNA wrapping.³⁷ Sensors incorporating ssDNA-SWCNT without enzymes exhibited increased sensitivity towards the electrochemical measurement of dopamine *via* direct oxidation.^{38,39} However, so far, despite the many reports on applications of ssDNA-SWCNTs,^{35,38,39} none have incorporated ssDNA-SWCNT with enzymes to enhance biosensing.

Platinum black (Pt black) is a metal layer formed by electrodepositing amorphous clusters of Pt nano-particles.⁴⁰ Pt black has been used to enhance biosensor sensitivity^{12–14,28,41} due to the catalytic activity of Pt nano-particles, and the ease of attaching enzymes to Pt by glutaraldehyde *via* Schiff bases.^{12,42,49}

Our previous work has shown that the combination of SWCNTs and Pt nanoparticles can significantly enhance the performance of electrochemical biosensors.¹⁰ Considering the advantages of ssDNA-SWCNT over existing CNT immobilization approaches, incorporating ssDNA-SWCNT with Pt black may greatly enhance biosensing. Since ssDNA-SWCNTs are conductive/semiconductive, they can be used as molecular templates for Pt black electrodeposition, and as electrical contacts between Pt black and electrode.¹⁰ Thus, possible aqueous media based schemes of combining ssDNA-SWCNT and Pt black are: 1. immobilize ssDNA-SWCNT on the micro electrode by drop-coating, and electrodeposit Pt black over ssDNA-SWCNT (“layered” scheme), 2. mix ssDNA-SWCNT with the electrodeposition media for Pt black (chloroplatinic acid/lead acetate solution), and co-electrodeposit both materials (“co-deposition” scheme) and 3. immobilize ssDNA-SWCNT by drop-coating, mix ssDNA-SWCNT with the electrodeposition media, and co-electrodeposit both materials, which essentially combines the layered scheme and the co-deposition scheme (“combination” scheme).

Glucose is one of the most important biochemical substrates for cellular catabolism. Glucose biosensors measure glucose based on enzymatic recognition of glucose by glucose oxidase (GOx) and have been widely used in diagnostics (*e.g.* diabetes monitoring⁴³) and research (*e.g.* β cell physiology study^{13,28}).

ATP is the fundamental unit of currency in cellular energetics, and also an important extracellular messenger in eukaryotic systems.⁴⁴ Biosensors for ATP measurement have shown great advantages over conventional techniques.^{45,46} The main

difference between ATP biosensors and glucose biosensors is that the former are based on a multi-enzyme approach to convert ATP into an electro-oxidative species. ATP can be measured by combining hexokinase and GOx. One major drawback with this scheme is that the linear range is up to 200 nM,⁴⁵ which is lower than human plasma ATP concentration (up to 11 μ M⁴⁶) and limits its physiological applications. A second measurement scheme using the bienzyme system of glycerol kinase (GK) and glycerol-3-phosphate oxidase (G3POx) is adopted in this study, because it has been reported to have a wider linear range (up to 50 μ M⁴⁷) compared with the first scheme,⁴⁵ which is important for physiological applications. Additionally, it has been applied *in vivo* to study ATP signaling during spinal motor activity.⁴⁷ Although ATP biosensors possess the potential to be an extremely useful tool for cell physiology, reports on electrochemical ATP biosensors are still quite limited.

This work for the first time explores possible ways of combining ssDNA-SWCNT and Pt black for microbiosensor enhancement. Microelectrodes modified with different schemes are characterized with SEM, cyclic voltammetry and DC potential amperometry. For different schemes, effective surface area and electrocatalytic activities on the oxidation of H₂O₂ are studied and compared. By attaching the *de facto* enzyme GOx, and the bienzyme system of GK and G3POx to modified electrodes using glutaraldehyde, single-enzyme and multi-enzyme microbiosensors are constructed and compared to determine the most effective scheme for both glucose and ATP microbiosensors. Major parameters for biosensors based on the most effective scheme are characterized to demonstrate that they can be used in a wide variety of physiological sensing applications.

Experimental

Chemicals and reagents

All solutions, if not specified, were prepared in deionized water (DI) of resistivity 18.2 M Ω cm (Milli Q). Glucose oxidase (E.C.1.1.3.4, 100,000–250,000 units/g, from *Aspergillus niger*), D-Glucose, glycerol kinase (E.C.2.7.1.30, 25–75 units/mg, from *Cellulomonas* sp), glycerol 3-phosphate oxidase (E.C.1.1.3.21, ≥ 10 units/mg solid, from *Streptococcus thermophilus*), adenosine-5-phosphate (disodium salt hydrate), glycerol ($\geq 99\%$), potassium chloride (KCl, 99%), potassium ferricyanide (K₃Fe(CN)₆), chloroplatinic acid solution (8% *w/w*), lead acetate (reagent grade, 95%), sodium chloride (NaCl), magnesium chloride ($\geq 99\%$), Tris(hydroxymethyl)aminomethane, hydrochloric acid (HCl) ($\geq 30\%$), bovine serum albumin, glutaraldehyde (Grade II, 25% Aqueous Solution), ascorbic acid, uric acid and acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO). Sodium phosphate (Na₂HPO₄·7H₂O) and potassium phosphate (KH₂PO₄, monobasic) were purchased from Fisher chemicals (Pittsburg, PA). RPMI 1640 medium with 10% FBS and 1% Pen-Strep were purchased from GIBCO. PBS solution (0.01M) was prepared by dissolving 8.0g NaCl, 1.2g Na₂HPO₄, 0.2g KCl and 0.2g KH₂PO₄ in 1.0L DI. Tris-HCl buffer was prepared by dissolving 1 M Tris(hydroxymethyl)aminomethane and adjusting the pH to 8.3 with HCl. Bulmer's buffer was prepared by dissolving 100 mM NaCl, 2 mM glycerol and 1mM MgCl₂ in 2 mM Na₂HPO₄ buffer.

ssDNA-SWCNT sample preparation

HiPco SWCNTs (Unidym, Sunnyvale, CA) were dispersed in aqueous solution using single-stranded, 30 base-long poly T oligonucleotides (Integrated DNA Technologies, Coralville, IA).³³ Briefly, raw nanotube powder with DNA in solution was bath-sonicated for 1 h and centrifuged at 15,000 rpm for 150 min. After centrifugation, the supernatant was carefully decanted to separately obtain DNA-coated SWCNTs from denser catalyst particles, bundles, and impurities. The concentration of SWCNTs was estimated to be approximately 23.8 mg/L.

Construction of microbiosensors

All micro sensors were constructed on a Pt/Ir wire microelectrode (PI20033.0A10, 51 mm length, 0.256 mm shaft diameter, 1–2 μ m tip diameter). The design schemes that used nanomaterials are:

Pt black only scheme. The microelectrode was connected to a potentiostat (cathode) against a bare Pt wire (0.5 mm in diameter; Alfa Aesar, Ward Hill, MA) (anode). Pt black was electrodeposited using a potentiostat (Applicable Electronics) in a solution of 0.36% chloroplatinic acid and 0.0005% lead acetate (10 V for 1 min).

Layered scheme. 2 μ l ssDNA-SWCNT was cast on the microelectrode and air-dried for 30 min. Pt black was then electrodeposited following the previous protocol.

Co-deposition scheme. Pt black was electrodeposited following the protocol for “Pt black only” except that the solution contained 23.8 mg/L ssDNA-SWCNT in addition to 0.36% chloroplatinic acid and 0.0005% lead acetate.

Combination scheme. 2 μ l ssDNA-SWCNT was cast on the microelectrode and air-dried for 30 min. Pt black was then electrodeposited following the protocol for “co-deposition”. “Combination” here meant combining “layered” and “co-deposition”.

Glucose biosensor. 60 μ l 50 mg GOx/ml PBS was mixed with 40 μ l 25 mg BSA/ml PBS and 20 μ l 2.5% glutaraldehyde. 2 μ l mixture was cast on the micro electrode and air-dried for 30 min.

ATP biosensor. 8 μ l 60 mg glycerol-3-phosphate oxidase/ml Tris-HCl was mixed with 8 μ l 60 mg glycerol kinase/ml Tris-HCl, 8 μ l 1.6 M glycerol and 8 μ l 2.5% glutaraldehyde. 2 μ l mixture was cast on the micro electrode and air-dried for 30 min.

Sensor calibration

DC potential amperometry was conducted with a 3 electrode electrochemical (C-3) cell stand (BASi, West Lafayette, IN) at a working potential of +500 mV *versus* a Ag/AgCl reference electrode with a sampling rate of 1 kHz following previous works.^{10,50} Reference electrodes (Ag/AgCl) and auxiliary electrodes were purchased from BASi. Amperometric sensitivity towards glucose was determined by measuring current at a constant working potential while sequentially adding glucose to mixed solutions (all solutions stirred at 300 rpm). Following

each glucose addition, measured current signal was allowed to reach steady state (defined as less than a 3% fluctuation for 10 s). Average current values represented the arithmetic mean of observed current ($n = 10,000$ data points). Response time (t_{95}) was calculated as the time for the sensor to reach 95% of its maximum amperometric response following addition of 100 μ M glucose. The same calibration protocol applied to ATP except that 3 mM glycerol was added into the cell before test because glycerol was required in the reaction cascade that measured ATP. Cyclic voltammetry (CV) was performed with a 3 electrode electrochemical (C-3) cell stand (BASi, West Lafayette, IN) in 4 mM $\text{Fe}(\text{CN})_6^{3-}/1 \text{ M KNO}_3$. A sweep range of 0 to +650 mV was used with a 10 s quiet time.

Optical measurement

The excitation and emission spectra of DNA-coated SWCNTs were measured with Horiba Jobin Yvon spectrofluorometer with a N_2 -cooled InGaAs detector (Edison, NJ). The spectral resolutions in excitation and emission measurements were 4 nm and 3 nm, respectively. The optical absorption spectrum was measured by a Perkin Elmer Lambda 950 spectrophotometer (Waltham, MA). The Raman spectra were recorded using a Renishaw inVia Raman microscope with 633 nm excitation.

FESEM

All field emission scanning electron microscopy (FESEM) biosensor images were obtained using a Hitachi S-4800 microscope with a power setting of 5.0 kV and magnification settings of 3.5k and 25k (no additional preprocessing).

Results and discussion

Optical characterization and SEM

SWCNTs dispersed in aqueous solution using 30 base-long poly T DNA (ssDNA-SWCNT) showed discrete optical transitions at characteristic wavelengths ranging from visible to near-infrared. The signatures of optical absorption in the visible and photoluminescence in the near-infrared were shown in Fig. 1a, and assigned to specific nanotube species based upon chiral vectors, (n, m), which indicated the diameter and chirality of each nanotube.^{51,52} The nucleobases of the oligonucleotide strands non-covalently interacted with the graphitic lattice of nanotubes *via* π – π stacking, while negatively charged DNA backbone rendered ssDNA-SWCNT hybrids soluble in aqueous solution *via* entropic repulsion.^{33,53,54} Typical carbon nanotube synthesis methods produced a mixture of semiconducting and metallic species, and the fraction varied depending on synthesis conditions. We used carbon nanotubes grown by the high-pressure CO disproportionation method or the so-called HiPco method, which typically included approximately two-thirds of semiconductors and a third of metallic species.⁵⁵ These carbon nanotube mixtures have been demonstrated to be electrochemically active materials.^{56,57} The photoluminescence excitation spectra of ssDNA-SWCNTs in Fig. 1a presented the population of semiconducting species, while the optical absorption in Fig. 1b showed a fraction of metallic species in the spectral region from

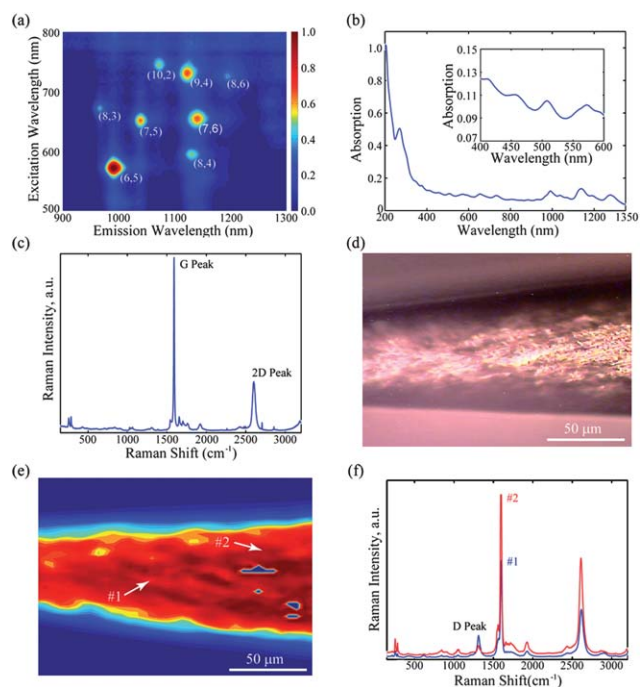


Fig. 1 (a) Photoluminescence excitation profile of ssDNA-SWCNTs showing distinct near-IR (NIR) fluorescence. (b) The corresponding absorption spectrum. Inset showed a fraction of metallic species. (c) Raman spectrum of ssDNA-SWCNTs with a HeNe laser excitation at 633 nm. (d) Visible image and (e) Raman image reconstructed using G band intensity ($\nu \approx 1590 \text{ cm}^{-1}$) of ssDNA-SWCNTs deposited on the electrode. (f) Raman spectra detected at the marked positions 1 and 2 in (e).

400 to 600 nm.⁵⁸ The discrete signatures of the spectra also indicated that the ssDNA-SWCNTs were well dispersed in aqueous solution; otherwise, it would be difficult to see measurable optical transition signatures.^{33,56} The Raman signatures of ssDNA-SWCNTs dispersed in the aqueous solution were probed using resonant Raman spectroscopy with 633 nm laser excitation (Fig. 1c). The Raman spectra included a sharp tangential stretching mode at $\sim 1590 \text{ cm}^{-1}$ (G peak) and a broader band around 2600 cm^{-1} (2D peak) of carbon nanotubes.⁵⁹ After the deposition of ssDNA-SWCNTs on the electrode, we recorded the visible image of the electrode (Fig. 1d) and the corresponding, raster-scanned Raman image of SWCNTs based on the intensity of G band (Fig. 1e). The Raman image appeared to be similar to the visible image of the electrode, suggesting that nanotubes were well distributed on the electrode. Fig. 1f presented the Raman spectra measured at the two marked positions shown in Fig. 1e, where the G and 2D peaks were prominent. In contrast to the solution-phase SWCNTs in Fig. 1c, the Raman spectra of ssDNA-SWCNTs on the electrode showed a discrete D peak or so-called a disorder peak at $\sim 1310 \text{ cm}^{-1}$ (Fig. 1f), indicating some defects in SWCNTs. The D peak have been observed in typical deposited nanotubes,⁶⁰ and we would show in the following sections that the defects did not significantly affect the electrochemical activities of SWCNTs for biosensor fabrication and biomolecular detection.

SEM images of micro electrodes modified with different schemes were shown in Fig. 2a–h (the inset of Fig. 2a showed

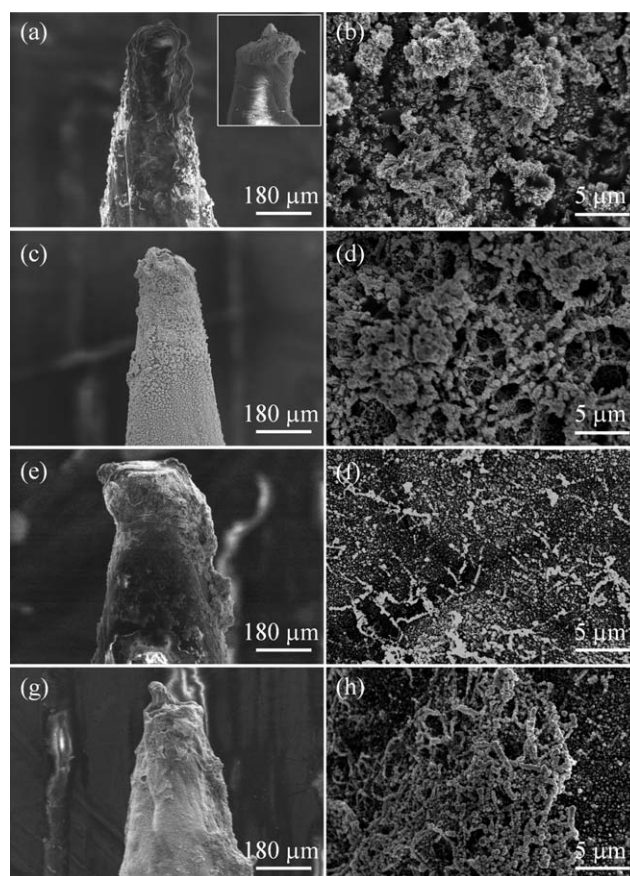


Fig. 2 SEM images of micro electrodes modified with (a) Pt black only (inset: unmodified electrode) and (b) magnified image of (a), (c) layered and (d) magnified image of (c), (e) co-deposition and (f) magnified image of (e), (g) combination schemes and (h) magnified image of (g).

an unmodified electrode). The densities of Pt black for the layered (Fig. 2c) and the combination electrodes (Fig. 2g) were significantly higher than the Pt black only (Fig. 2a) and the co-deposition electrodes (Fig. 2e). Pt black exhibited the typical structure of a film consisting of amorphous clusters of Pt nanoparticles (Fig. 2b), while the layered (Fig. 2d), the co-deposition (Fig. 2f) and the combination schemes (Fig. 2h) showed both cluster structure and line structure, indicating that Pt black was growing along ssDNA-SWCNTs, while technologies such as energy-dispersive X-ray spectroscopy (EDX) could be used to further verify the composition of the nanocomposites. Magnified image (Fig. 2d) showed that Pt clusters were deposited at defect sites of ssDNA-SWCNTs to form the lines according to previous studies.¹¹ Compared with the more flat structure of Pt black and co-deposition electrodes, the structure of the layered and the combination electrodes were more three-dimensional with a higher density of Pt nanoparticles, suggesting that the immobilized ssDNA-SWCNTs promoted the three-dimensional growth of Pt black. Since surface expansion of the electrodes was determined by the vertical growth of metal nanomaterials,⁴⁰ the more three-dimensional structure should increase the effective surface area and the electro-oxidation of H_2O_2 , which is the intermediate for glucose and ATP biosensors.

Electrochemical characterization

Cyclic voltammetry in $\text{Fe}(\text{CN})_6^{3-}$ for the unmodified microelectrodes exhibited typical sigmoid curves with steady state diffusion limited currents (Fig. 3a). The CV data for Pt black electrodes exhibited redox peaks with characteristics of macroelectrodes (Fig. 3a), demonstrating that the surface expansion by Pt black was significant. For the layered electrodes the CV analysis showed even larger redox peak currents than Pt black only, indicating a larger effective surface area associated with this surface modification. Peak separation for the layered electrodes did not increase as peak currents increased, suggesting that the incorporation of ssDNA-SWCNT did not affect the electrochemical reversibility of electrodes. Effective surface area for modified electrodes can be quantified from CV data with the Randles-Sevcik equation:⁶¹

$$i_p = (2.69 \times 10^5) n^{3/2} D^{1/2} C A \nu^{1/2} \quad (1)$$

where: i_p is the reduction peak current (A), n is the number of transferred electrons for the redox reaction of $\text{Fe}(\text{CN})_6^{3-}$, D is the diffusion coefficient ($6.70 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$), C is the molar concentration of ferricyanide (4 mM), A is the electroactive surface area (cm^2), and ν is the scan rate (V sec^{-1}). After carrying out CVs at different scan rates ($\nu = 20, 50, 100, 125, 150$ and 200 mV/s), effective surface areas of the modified electrodes were determined from the slope of linear regression between i_p and $\nu^{1/2}$. Histogram for the average effective surface area of micro electrodes based on different schemes was shown in Fig. 3b. Pt black electrodes ($4.7 \times 10^{-4} \pm 1.3 \times 10^{-4} \text{ cm}^2$) had an average surface area more than ten thousand times that of bare electrodes (specified as $1.8 \times 10^{-8} \text{ cm}^2$), and the layered

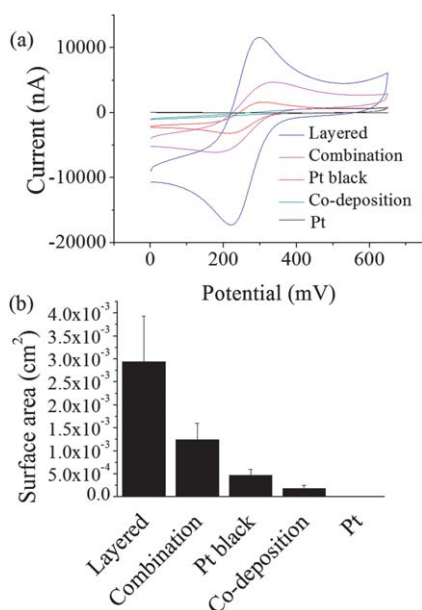


Fig. 3 (a) CV in $\text{Fe}(\text{CN})_6^{3-}$ for a bare electrode and electrodes modified with Pt black only, layered, co-deposition and combination schemes at scan rate 100 mV/s . (b) Histogram for the average electroactive surface area of micro electrodes based on different schemes (error bars represented standard error of the mean ($n = 3$)).

electrodes ($2.9 \times 10^{-3} \pm 9.9 \times 10^{-4} \text{ cm}^2$) showed an even larger surface area compared with Pt black only. As previously mentioned, expansion of surface area may be related to the vertical growth of Pt black. This data (Fig. 3b) demonstrated that the use of ssDNA-SWCNTs as molecular templates for electrodeposition Pt black can further enhance the expansion of surface area over Pt black only. This result agreed well with SEM images, where the layered electrodes were more three-dimensional than Pt black only (Fig. 2d and Fig. 2b). ssDNA-SWCNT alone drop-coated on the micro electrodes could dissolve back off when the electrodes were dipped in aqueous solution over time. However, the layered electrode showed a significantly increased surface area, indicating that the ssDNA-SWCNTs were entrapped on the electrodes during the 1 min Pt black electrodeposition process, which followed ssDNA-SWCNT drop-coating. Our data (Fig. 3b) also showed that the surface area of the co-deposition electrodes ($1.9 \times 10^{-4} \pm 6.8 \times 10^{-5} \text{ cm}^2$) was smaller compared with Pt black, and that the combination ($1.2 \times 10^{-3} \pm 3.7 \times 10^{-4} \text{ cm}^2$) was smaller than the layered. This result suggested that adding ssDNA-SWCNT into the electrodeposition media did not enhance the surface expansion, probably because most of the ssDNA-SWCNTs were dispersed in the solution rather than being attached to the electrode surface, so electrical contact was not adequately established between SWCNTs and the electrode. This result indicated that the effective use of ssDNA-SWCNTs as templates for electrodeposition Pt black required the immobilization of ssDNA-SWCNTs (e.g., via drop-coating).

H_2O_2 is the electro-oxidative intermediate for enzyme based biosensors. To examine the enhancement in electrocatalytic activities by nanomaterials towards H_2O_2 oxidation, DC potential amperometry at $+500 \text{ mV}$ was carried out for micro electrodes modified with different schemes. All the electrodes showed well-defined amperometric response to H_2O_2 (Fig. 4a). For different schemes, a trend similar to surface area was observed (Fig. 4b). The Pt black electrode ($375.7 \pm 140.1 \text{ nA/mM}$) had a significantly increased H_2O_2 sensitivity than the bare electrode ($1.3 \pm 0.1 \text{ nA/mM}$), and the sensitivity for the layered electrode ($3129.9 \pm 483.2 \text{ nA/mM}$) was even higher, demonstrating that ssDNA-SWCNT further enhanced the electrocatalytic activities of Pt black. We also showed (Fig. 4b) that the sensitivity for co-deposition ($317.0 \pm 68.0 \text{ nA/mM}$) was not significantly different from Pt black only, and that combination ($2375.1 \pm 497.2 \text{ nA/mM}$) was not significantly different from layered ($\alpha = 0.05$), indicating that adding ssDNA-SWCNTs to the electrodeposition media did not increase the electrocatalytic activities of Pt black.

Biosensing

Glucose oxidase (GOx), the *de facto* enzyme, was immobilized on the micro electrodes via the covalent linker glutaraldehyde to construct glucose biosensors. Glucose was electrochemically measured in two steps:

Step 1. GOx converted glucose and O_2 into gluconic acid and H_2O_2 ,



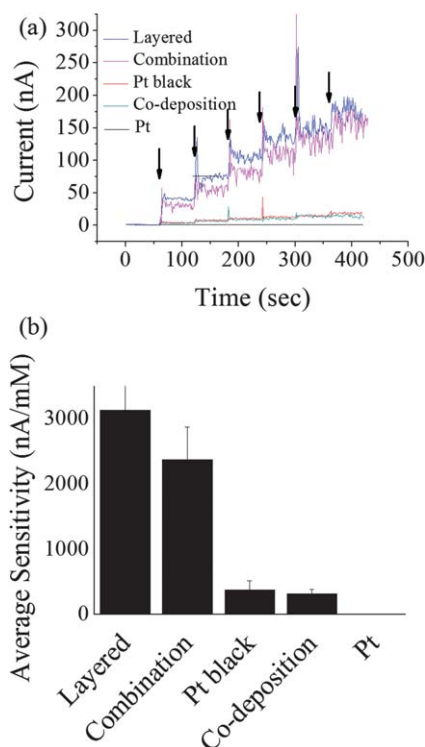
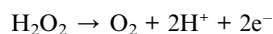


Fig. 4 (a) Amperometric response to H₂O₂ for a bare electrode and electrodes modified with Pt black only, layered, co-deposition and combination schemes. Arrows represented additions of 10 μM H₂O₂. (b) Histogram for the average sensitivity of micro electrodes based on different schemes (error bars represented stand error of the mean (*n* = 3)).

Step 2. H₂O₂ was electrochemically oxidized in the proximity of biosensor at +500 mV,



The measured current was therefore proportional to the glucose concentration. Well-defined amperometric response was observed for biosensors based on each design (including a Pt/GOx biosensor design fabricated by linking GOx to a bare micro electrode) (Fig. 5a). Similar to H₂O₂ sensitivity, the Pt black/GOx biosensors possessed significantly increased glucose sensitivity (18.3 ± 5.7 nA/mM) than Pt/GOx biosensors (0.8 ± 0.2 nA/mM) (Fig. 5b). The enhancement was even more significant for the layered biosensors (817.3 ± 185.8 nA/mM) when ssDNA-SWCNT was incorporated as the template for the electrodeposition of Pt black. For the 2 designs (co-deposition and combination) with mixed ssDNA-SWCNT/electro-deposition media, the sensitivity for co-deposition biosensors (102.7 ± 43.9 nA/mM) was higher than Pt black only, and that for the combination (651.6 ± 127.1 nA/mM) was lower than the layered. In a word, among all the schemes in this study, results proved the layered approach the most effective for constructing single-enzyme glucose biosensor.

For the layered biosensors, linear response range was up to 7 mM (*R* = 0.99), limit of detection was 1 μM and response time (*t*₉₅) was ~5 s (Fig. 5). The linear range covered the glucose range in normal human blood (~5 mM) and in many cell culture media

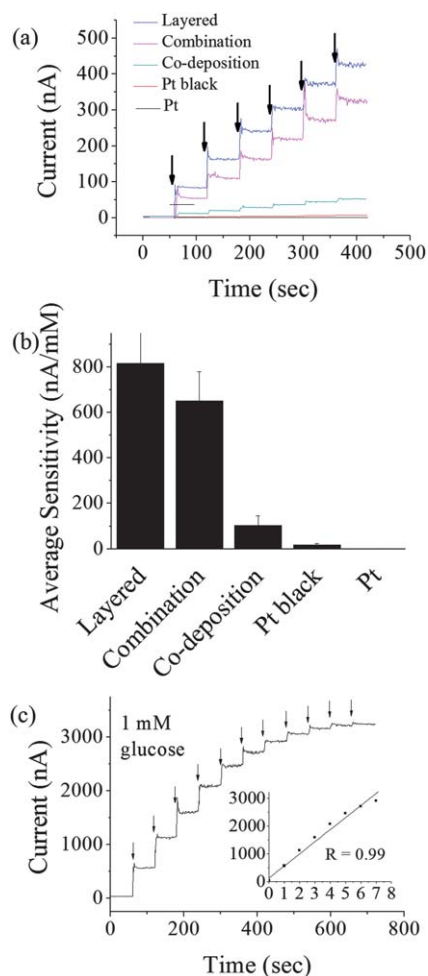


Fig. 5 (a) Representative amperometric response to glucose for biosensors based on a bare electrode and electrodes modified with Pt black only, layered, co-deposition and combination schemes. Arrows represented additions of 100 μM glucose. (b) Histogram for the average sensitivity of biosensors based on different schemes (error bars represented stand error of the mean (*n* = 3)). (c) Representative amperometric response of a glucose micro biosensor based on layered design when glucose was changed from 0 to 11 mM. (Inset) Linear regression for the linear range in (c).

(e.g., 5.5 mM in mesenchymal stem cell basal medium⁶²), demonstrating the wide potential applications of glucose biosensors based on the layered design.

Selectivity tests were carried out with the common interferences in blood (ascorbic acid, uric acid and acetaminophen) for the layered biosensors following a previous work⁹ (Fig. 6a). Ascorbic acid and uric acid of physiological concentrations (125 μM and 330 μM, respectively), and acetaminophen of therapeutic concentration (130 μM) were added, followed by 5 mM glucose (physiological concentration) addition. The response to ascorbic acid was 7.9% that of glucose, while to uric acid and acetaminophen were 13.7% and 7.2%, respectively. The selectivity of the layered biosensors could be further improved by applying additional anion repellant layers such as Nafion.⁶³ The capability of the layered biosensors towards measuring glucose in complex samples was evaluated with RPMI 1640 media containing 10%

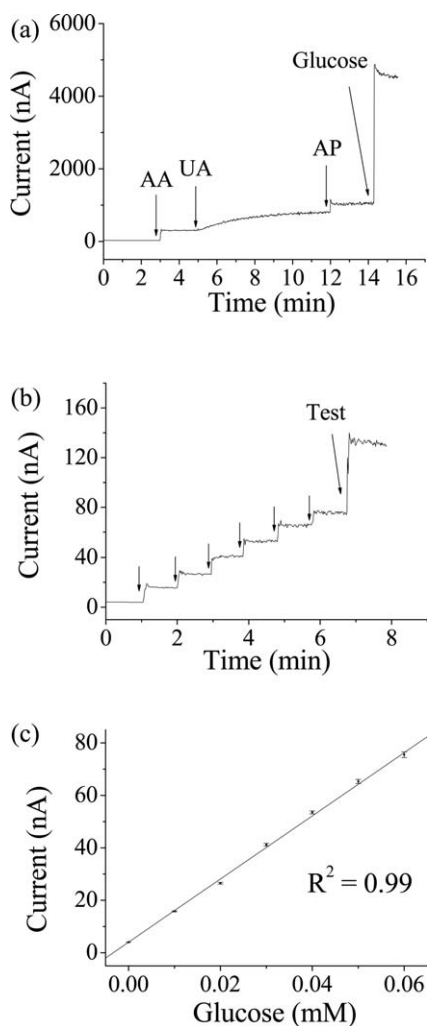
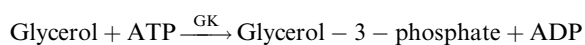


Fig. 6 (a) Representative amperometric response to 125 μM ascorbic acid (AA), 330 μM uric acid (UA), 130 μM acetaminophen (AP) and 5 mM glucose (Glucose) for a glucose biosensor based on the layered scheme. (b) Representative amperometric response to RPMI 1640 media containing glucose (arrows indicated 6 additions of 10 μM glucose as a calibration), followed by a recovery test (Test). (c) Linear regression for the calibration in (b).

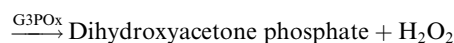
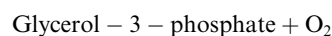
fetal bovine serum (Fig. 6b). 6 additions of the media containing 10 μM glucose were added for calibration, followed by one addition of the media containing 55.5 μM glucose as a recovery test. Excellent linearity was observed with the calibration ($R^2 = 0.99$) (Fig. 6c), and the accuracy of the recovery test was 96.4%. The results demonstrated that the layered biosensors could be readily used for research and clinical applications.

ATP micro biosensors were constructed by attaching the bienzyme system of GK and G3POx to electrodes *via* glutaraldehyde. ATP was measured in three steps:

Step 1. GK transferred one phosphate from ATP to glycerol,



Step 2. glycerol-3-phosphate was oxidized by G3POx, and H_2O_2 was generated,



Step 3. The current from oxidizing H_2O_2 was measured, as Step 2 in glucose sensing.

Bare micro electrodes with enzymes attached showed no observable response to ATP, while all the nanomaterial modified electrodes showed well-defined linear amperometric response, demonstrating the great advantage of nanomaterial modified biosensors over biosensors based on conventional materials (Fig. 7a). The sensitivity of biosensors based on the layered scheme ($45.6 \pm 10.8 \text{ nA/mM}$) was 216 times that of Pt black only

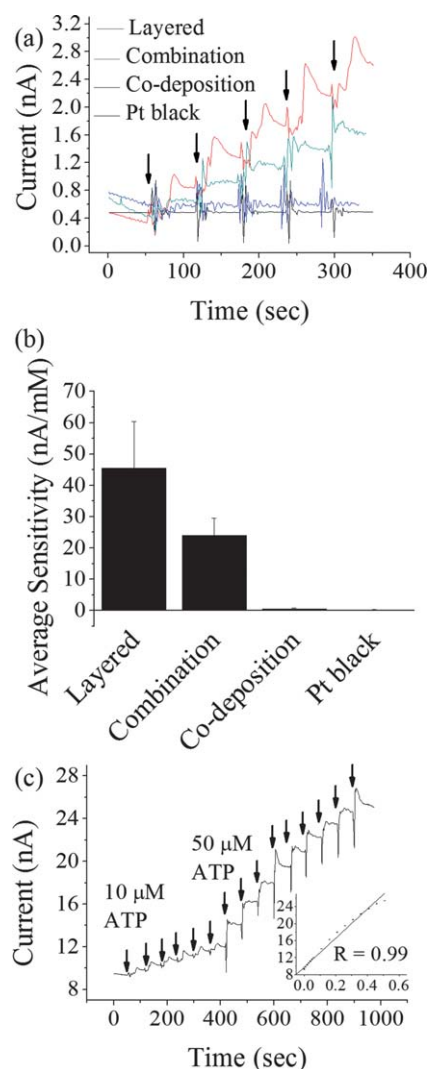


Fig. 7 (a) Representative amperometric response to ATP for biosensors based on electrodes modified with Pt black only, layered, co-deposition and combination schemes. Arrows represented additions of 10 μM ATP. (b) Histogram for the average sensitivity of biosensors based on different schemes (error bars represented stand error of the mean ($n = 3$)). (c) Representative amperometric response of an ATP micro biosensor based on layered design in the whole linear range. (Inset) Linear regression for (c).

(0.21 ± 0.08 nA/mM), justifying the effectiveness of incorporating ssDNA-SWCNTs with Pt black. The sensitivity of the co-deposition biosensors (0.57 ± 0.29 nA/mM) was higher than Pt black only, and the combination (23.9 ± 5.7 nA/mM) was lower than the layered, although the differences were not significant *via* ANOVA tests ($\alpha = 0.05$) (Fig. 7b). Similar to glucose biosensors, the layered design has been validated as the most effective for constructing multi-enzyme ATP biosensor. ATP biosensors based on the layered design showed a detection limit of 2 μ M, a linear range up to 510 μ M and a response time of ~ 8 s (Fig. 7c). The linear detection range covered human plasma ATP concentration range (up to 11 μ M⁴⁶) and was wider than a previous work (up to 50 μ M⁴⁷), suggesting potential applications as physiological sensors. The enhanced performance of the layered scheme exhibited for single-enzyme and multi-enzyme biosensors justified that this scheme was an effective way of combining ssDNA-SWCNT and Pt black, which provided an excellent platform to construct biosensors for a wide variety of physiological applications.

Conclusions

This work explores possible schemes of combining ssDNA-SWCNT and Pt black to enhance enzyme based biosensors. Modification of SWCNTs with ssDNA overcomes the insolubility issue with CNT immobilization, and makes possible the simple aqueous media based approaches for utilizing CNT for biosensing. Optical absorption and photoluminescence profiles demonstrated that ssDNA-SWCNTs were well dispersed in DI water. SEM images and CV analyses showed that for the “layered scheme”, where ssDNA-SWCNTs were immobilized on the electrode followed by electrodeposition of Pt black, the expansion of effective surface area was more significant compared with Pt black only. This is due to nanostructured three-dimensional growth of Pt black by ssDNA-SWCNTs as a template. This result was supported by electrocatalytic activity characterization, where the layered electrodes showed the highest H₂O₂ sensitivity (3129.9 ± 483.2 nA/mM) among all schemes. Single-enzyme glucose micro biosensors and multi-enzyme ATP micro biosensors based on the layered design exhibited high sensitivity (817.3 ± 185.8 nA/mM and 45.6 ± 10.8 nA/mM, respectively), wide linear range (up to 7 mM and 510 μ M) and low limit of detection (1 μ M and 2 μ M). For the first time, this work presents a novel ssDNA-SWCNT/Pt black nanocomposite based on the layered scheme that effectively combines the advantages of two important nanomaterials (ssDNA-SWCNT and Pt black). The nanocomposite is an excellent platform for enhancing enzyme based biosensors and holds great promise for versatile sensing applications.

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