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ORIGINAL ARTICLES

Electrochemical Glucose Biosensor of Platinum Nanospheres Connected by Carbon Nanotubes

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Abstract

Background:

Glucose biosensors comprised of nanomaterials such as carbon nanotubes (CNTs) and metallic nanoparticles offer enhanced electrochemical performance that produces highly sensitive glucose sensing. This article presents a facile biosensor fabrication and biofunctionalization procedure that utilizes CNTs electrochemically decorated with platinum (Pt) nanospheres to sense glucose amperometrically with high sensitivity.

Method:

Carbon nanotubes are grown *in situ* by microwave plasma chemical vapor deposition (MPCVD) and electrochemically decorated with Pt nanospheres to form a CNT/Pt nanosphere composite biosensor. Carbon nanotube electrodes are immobilized with fluorescently labeled bovine serum albumin (BSA) and analyzed with fluorescence microscopy to demonstrate their biocompatibility. The enzyme glucose oxidase (GO_X) is immobilized onto the CNT/Pt nanosphere biosensor by a simple drop-coat method for amperometric glucose sensing.

Results:

Fluorescence microscopy demonstrates the biofunctionalization capability of the sensor by portraying adsorption of fluorescently labeled BSA unto MPCVD-grown CNT electrodes. The subsequent GO_X–CNT/Pt nanosphere biosensor demonstrates a high sensitivity toward H_2O_2 (7.4 μA/mM/cm^2) and glucose (70 μA/mM/cm^2), with a glucose detection limit and response time of 380 nM (signal-to-noise ratio = 3) and 8 s ($t_{90\%}$), respectively. The apparent Michaelis–Menten constant (0.64 mM) of the biosensor also reflects the improved sensitivity of the immobilized GO_X/nanomaterial complexes.

Conclusions:

The GO_X–CNT/Pt nanosphere biosensor outperforms similar CNT, metallic nanoparticle, and more conventional carbon-based biosensors in terms of glucose sensitivity and detection limit. The biosensor fabrication and biofunctionalization scheme can easily be scaled and adapted for microsensors for physiological research applications that require highly sensitive glucose sensing.

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Abbreviations: (BSA) bovine serum albumin, (CNT) carbon nanotube, (FITC) fluourescein isothiocyanate, (GO_X) glucose oxidase, (H_2O_2) hydrogen peroxide, (MPCVD) microwave plasma chemical vapor deposition, (NADH) nicotinamide adenine dinucleotide, (PAA) porous anodic alumina, (PBS) phosphate buffered saline, (Pt) platinum

Keywords: carbon nanotubes, fluorescence, glucose biosensor, platinum nanospheres

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Introduction

Diabetes mellitus is a metabolic disease marked by high levels of blood glucose that can lead to serious complications, including kidney failure, blindness, cardiovascular disease, and premature death.[1] An active glucose monitoring and control regime is required of all diabetes patients to maintain a healthy lifestyle and prevent diabetes-related complications. Furthermore, tightly controlling glucose levels in critically ill patients with diabetes and even without diabetes has shown to reduce numerous medical complications and premature death.[2,3] A highly sensitive glucose biosensor could improve the sensitivity and accuracy of current blood glucose monitoring technologies since reports by the Food and Drug Administration suggest raising standards in home glucose monitors, including glucose testing strips and meters, to improve their glucose detection accuracy.[4] Therefore, a glucose monitoring technology with increased glucose sensitivity and accuracy could substantially improve the prognosis of diabetes and critically ill patients and subsequently reduce their associated health care expenses. Thus the focus of glucose monitoring technologies has been on treating diabetes instead of elucidating the fundamental mechanisms behind abnormal insulin secretion in pancreatic islets and β cells.

Insulin production within pancreatic islets and β cells is dependent on proper glucose uptake and metabolism,[5–7] and therefore, highly sensitive glucose biosensors that monitor cellular glucose transport processes would be of great benefit to fundamental diabetes research. The signaling pathway controlling insulin release is a target for elucidating the mechanistic model of diabetes, and this starts with glucose uptake. While measurements of cellular/islet glucose transport have been demonstrated in fundamental research using biosensors, the sensitivity and reliability have limited broad application of such an important and powerful tool in the fundamental research domain. Nanotechnology-inspired biosensors have shown promising results—displaying the potential necessary for highly sensitive glucose detection. In the cellular research domain, critical performance criteria are sensitivity and limit of detection.

Nanomaterials such as carbon nanotubes (CNTs) and metallic nanoparticles exhibit impressive results in electrochemical glucose biosensing.[8,9] Nano-inspired electrochemical biosensors are advantageous because of their low power requirements (<1 V), fast response times (<10 s), and high sensitivities (<1 mM).[10] Carbon nanotubes in particular boast exceptional electrochemical glucose sensing capabilities through their inherent ability to catalyze a variety of redox reactions with hydrogen peroxide (H_2O_2) and nicotinamide adenine dinucleotide (NADH),[11] the two chemical products typically measured during enzymatic glucose sensing. Conventional electrode materials such as platinum (Pt),[12] palladium,[13] and gold[14] have been scaled down and combined with CNT-based glucose biosensors to enhance electrochemical performance and facilitate enzyme immobilization. CNT/metallic nanoparticle composite glucose biosensors have displayed impressive results—demonstrating high sensitivities and low detection limits necessary for highly accurate and precise blood glucose sensing. Despite these promising results, several technical barriers, including complex biofunctionalization strategies, prevent nanotechnology-inspired biosensors from developing into autonomous glucose monitoring systems.

In this article, we present a CNT/nanoparticle composite glucose biosensor that is shown to facilitate sensitive glucose sensing while avoiding the complex fabrication schemes that have hindered the development of nano-inspired glucose biosensors. We introduce a bottom-up approach to CNT growth combined with metallic nanoparticle deposition and a simple drop-coat bio-functionalization scheme for glucose monitoring—methods that can be easily scaled for commercial production. Results from this new CNT-based biosensor are discussed and correlated to the enhanced electrocatalytic, mass-transport, and enzyme-coupling characteristics of the developed biosensor.

Methods

The CNT biosensor is fabricated *in situ* from an oxidized silicon wafer [P <100> Si (5 μm), SiO_2 (500 nm)] upon which a porous anodic alumina (PAA) template is created for subsequent CNT growth by a method previously reported.[14,15] This PAA template is created by consecutively evaporating a thin metal film stack consisting of titanium (100 nm), aluminum (100 nm), iron (1 nm), and aluminum (400 nm). The metalized silicon wafer is anodized by biasing the substrate to 40 V relative to a Pt auxiliary electrode within an oxalic bath (0.3 M, 5 °C). This anodization process not only forms semi-ordered pores that extend through the aluminum and iron layers to the underlying titanium layer but

also creates a corrosion-resistant surface that decreases the extent of chemical fouling during electrochemical biosensing. A portion of the substrate is left unanodized, leaving an electrically addressable metal contact pad for subsequent electrochemical processing and for possible electronic integration with portable glucose monitoring systems.

Carbon nanotube synthesis initiates within individual PAA pores by a microwave plasma chemical vapor deposition (MPCVD) process (SEKI A_X5200S). Within the MPCVD reaction chamber, the anodized substrate is heated to 900 °C under a 10 torr hydrogen ambient by a 3.5 kW radio-frequency power supply. A 300 W unbiased hydrogen plasma is subsequently formed over the substrate, and methane gas is introduced to accomplish CNT growth. The CNTs grow from the iron catalyst layer embedded within the pores of the PAA and extend horizontally 3–10 μm in length along the surface of the substrate. Platinum from a 15 mM H_2PtCl_6 salt solution is subsequently electrodeposited onto the substrate through a three-electrode electrochemical setup (BASi Epsilon Three-Electrode Cell Stand), where the CNT/PAA substrate acts as the working electrode, platinum gauze as the auxiliary electrode, and Ag/AgCl (3 M NaCl) as the reference electrode. Through this electrodeposition process, platinum first partially fills the pores of the PAA, creating an electrical contact between the CNTs and conducting titanium bottom layer. Once the Pt wires reach the bases of the CNTs within the pores, the contacted CNTs become part of the electrode, allowing Pt nanospheres (approximately 200 nm in diameter, see **Figure 1**) to form concentrically at defect sites on the portions of the CNTs extending out of the pores.

In order to verify the biofunctionalization compatibility via physical adsorption, a CNT electrode is first checked for autofluorescence and then exposed to fluourescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA);(Sigma-Aldrich, Catalog # A-9771) and fluorescently analyzed again by a Nikon Labophot fluorescence microscope with a FITC filer and an Optronics 479T charge-coupled device camera. The FITC-labeled BSA is exposed to the CNT electrode by first immersing the electrode with phosphate buffer saline (PBS) within an individual polystyrene well. A 5 μl solution of FITC-labeled BSA (2 mg/ml in PBS) is injected into the well and allowed to incubate at room temperature by placing the well on a rotary shaker (100 rpm for 30 min). The CNT electrode is then washed three consecutive times by injecting the well with fresh PBS and agitating it with the rotary shaker (100 rpm for 5 min).

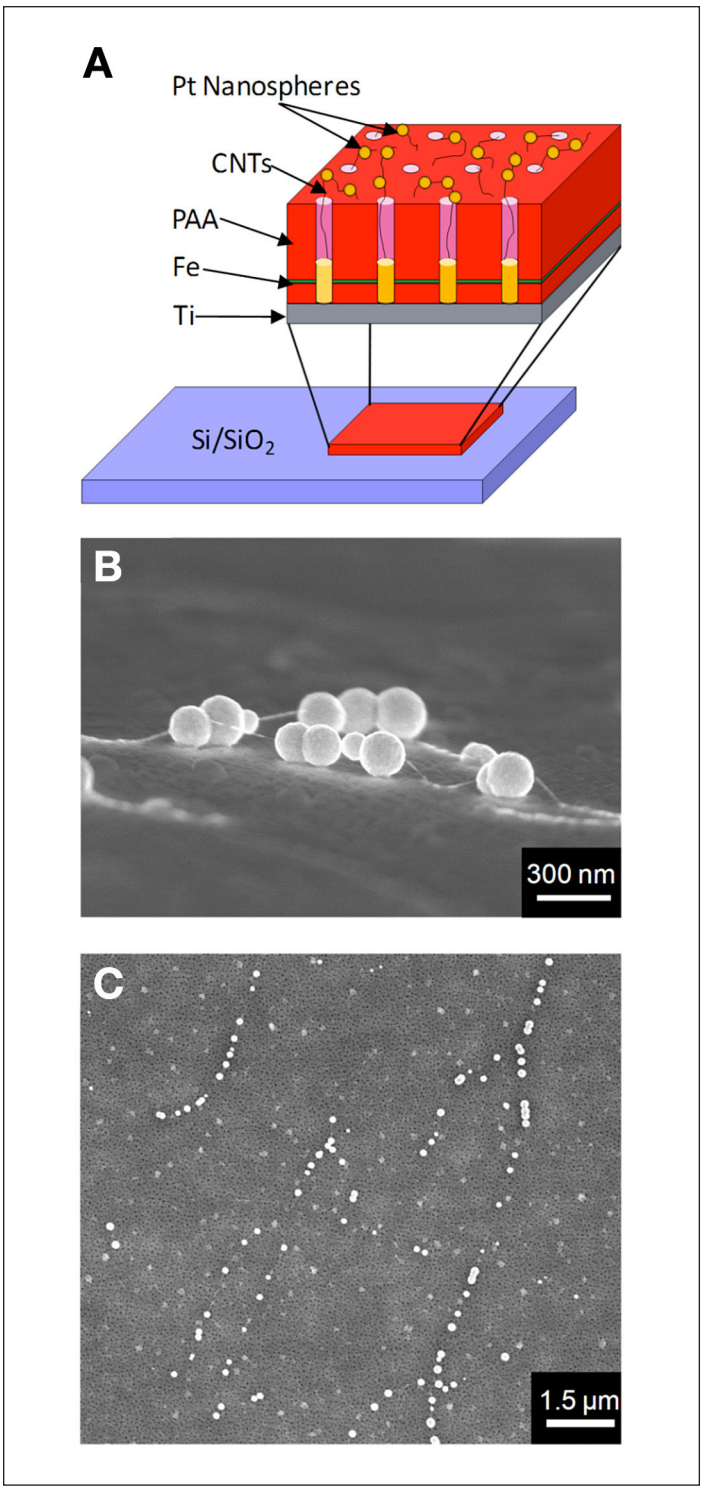

Figure 1. Cross-sectional schematic portraying **(A)** the templated *in situ* growth of CNTs from the PAA decorated with Pt nanospheres to form the CNT/Pt nanosphere electrode on an oxidized silicon wafer. Field emission electron microscopy micrographs show **(B)** a high magnified side view and **(C)** low magnified top view of Pt nanospheres (~200 nm diameter) electrically wired by CNTs (~1–3 nm diameter).

This rigorous washing scheme helps to minimize nonspecific binding by eliminating any unbound FITC-labeled BSA from the CNT surface. The washed CNT

electrode is analyzed with fluorescence microscopy by placing the electrode into a transparent PBS-filled chamber that is created by sandwiching a silicone gasket between two glass microscope slides. Adsorbed FITC-labeled BSA is observed on the biofunctionalized CNT electrode via fluorescence microscopy with exposure times of 500 ms and 1 s, respectively.

Using the localization techniques similar to those employed in the foregoing fluorescently marked BSA adsorption experiments, the CNT/Pt nanosphere electrode is converted into a glucose biosensor by immobilizing the enzyme glucose oxidase (GO_X) to the electroactive surface of the biosensor via a cross-linking matrix of BSA and glutaraldehyde. Glucose oxidase solution is prepared from a 130,000 U/gm stock (Sigma Aldrich, St. Louis, MO) by dissolving 25 mg of GO_X in 1 ml of PBS (pH 7.4). The enzyme matrix is then created by mixing GO_X solution with 2.5% BSA and 2.5 % glutaraldehyde. The immobilization scheme is based on covalent linking of the enzyme with the aldehyde groups of glutaraldehyde through formation of Schiff bases. Bovine serum albumin serves to block the excess aldehyde sites that can denature the enzyme and aids in maximizing enzyme activity. A 5 µl drop of this enzyme matrix solution (16.25 U GO_X) is uniformly pipetted onto the sensor surface and allowed to air dry for 1 h before electrochemical testing. When not in use, the biosensor is stored in PBS (pH 7.4) at 4 °C.

Amperometric H_2O_2 and glucose biosensing is carried out through a three-electrode arrangement in which the GO_X–CNT/Pt nanosphere biosensor acts as the working electrode, a Pt wire as the auxiliary, and Ag/AgCl as the reference. The three electrodes are immersed in a vial containing 20 ml of PBS (pH 7.4) that is continuously stirred with a 1 cm (length) magnetic bar that rotates at 500 rpm, while successive concentration increases of H_2O_2 or D glucose are injected into the test vial at a working potential of 350 mV. The enzymatic reaction of GO_X and D-glucose produces H_2O_2 [see **Equations (1)** and **(2)**], which is subsequently oxidized at the electrochemically active CNT/Pt nanosphere surface—producing a measurable electrical signal.

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{GO_X} \text{D-gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (2)$$

Results

Fluorescence Microscopy

In order to demonstrate the biofunctionalization capability of nonchemically altered MPCVD-grown CNT electrodes, fluorescence microscopy is employed to analyze CNT protein adsorption. Fluorescence microscopy results (see **Figure 2**) reveal the relative intensity peaks of the CNT/PAA electrodes with and without immobilized FITC-labeled BSA protein.

The fluorescence intensity with immobilized BSA protein (~0.5 k) is five times greater in intensity than the intensity peak associated with the CNT-only electrode (~0.1 k). The fluorescence micrograph (inset) portrays the binding of the FITC-labeled BSA protein to the CNT array biosensor. These results are consistent with the scanning electron microscope image patterns of CNT/Pt distribution, a view that is corroborated by previous reports of spontaneous adsorption of protein on CNTs,[16] where surface acidic groups located at CNT defect sites enhance the binding of proteins to the CNT arrays.[17,18] Thus this experiment suggests that the MPCVD-grown CNTs possess an adequate quantity of defect sites created during the high-temperature hydrogen plasma (H_2–CH_4) CNT growth conditions[19] for subsequent noncovalent CNT/enzyme functionalization and biosensing experimentation.

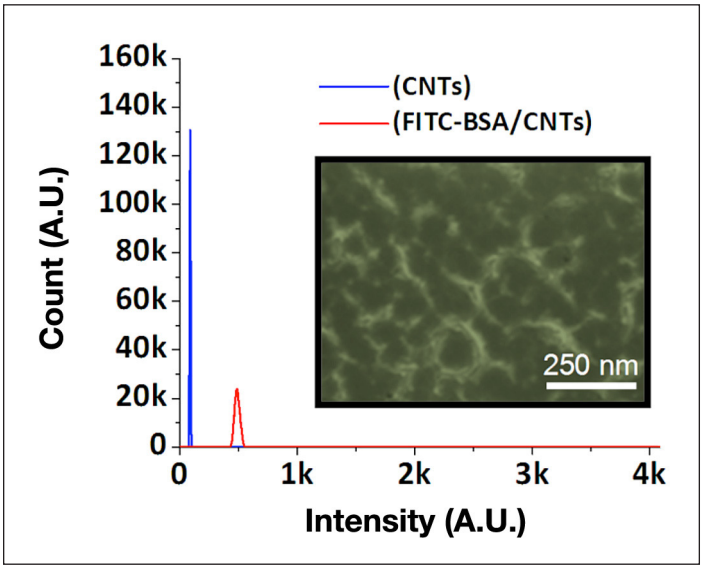

Figure 2. Fluorescence intensity results (exposure time = 1 s) for MPCVD-grown CNT electrodes with and without immobilized FITC-labeled BSA. The inset shows a fluorescence micrograph demonstrating the capture of FITC-labeled BSA protein to the CNT array biosensor.

Hydrogen Peroxide Sensing

Amperometric H_2O_2 testing (see **Figure 3**) is performed in order to test the electroactive nature of the of the CNT/Pt nanosphere biosensor toward oxidation of H_2O_2. The CNT/Pt nanosphere biosensor exhibits a high sensitivity (7.4 μA/mM/cm^2) toward the oxidation of H_2O_2. This sensitivity is important, as H_2O_2 is the electrochemical transducer between the GO_X enzyme and the biosensor surface.

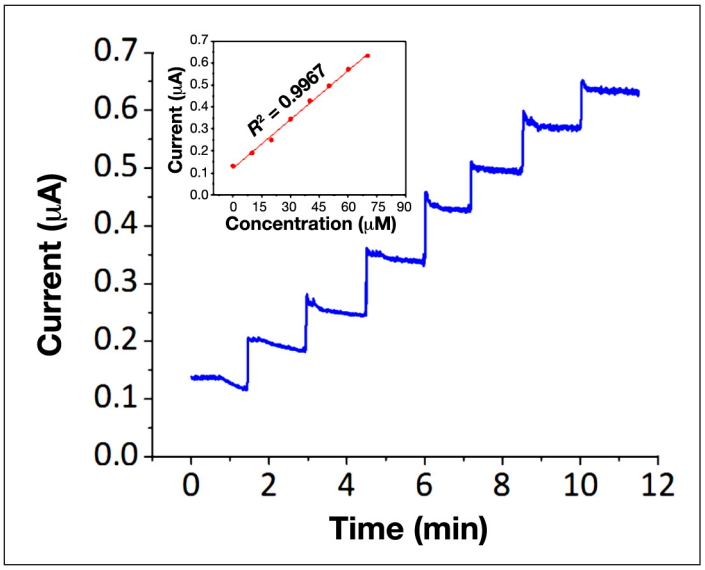

Figure 3. Amperometric H_2O_2-sensing experiment for the CNT/Pt nanosphere biosensor. The current response to seven successive concentration increases of 10 μM H_2O_2 is displayed, while the inset shows the linear regression analysis of the current versus concentration plot.

Glucose Sensing

The GO_X biofunctionalized CNT/Pt nanosphere biosensor was subsequently tested with increasing concentrations of D glucose following the same three-electrode experimental protocol established during H_2O_2 sensing (see **Figure 4**).

The micromolar sensitivity of the biosensor is 70 μA/mM/cm^2, while the response time is 8 s ($t_{90\%}$). In terms of glucose sensitivity and detection limit, the CNT/Pt nanosphere biosensor outperforms CNT/Pt nanoparticle,[20–24] gold nanoparticle,[25] CNT,[26,27] porous carbon,[28] and modified glassy carbon[29–31] amperometric glucose biosensors (see **Table 1**). Furthermore, the GO_X–CNT/Pt nanosphere biosensor demonstrates a glucose detection limit of 380 nM at a signal-to-noise ratio of 3, with linear sensing region from 1 μM to 0.75 mM (see **Figure 5**).

In order to evaluate the biological activity of immobilized GO_X enzyme on biosensor surfaces, the effective or apparent Michaelis–Menten constant (K'_M) is often determined amperometrically from the Lineweaver–Burk-type equation,[32]

$$\frac{1}{i_{ss}} = \left(\frac{K'_M}{i_{max}}\right)\left(\frac{1}{C}\right) + \left(\frac{1}{i_{max}}\right) \quad (3)$$

where i_{ss} is the steady state current associated with a given concentration (C) and i_{max} is the measured current during analyte saturation. The apparent

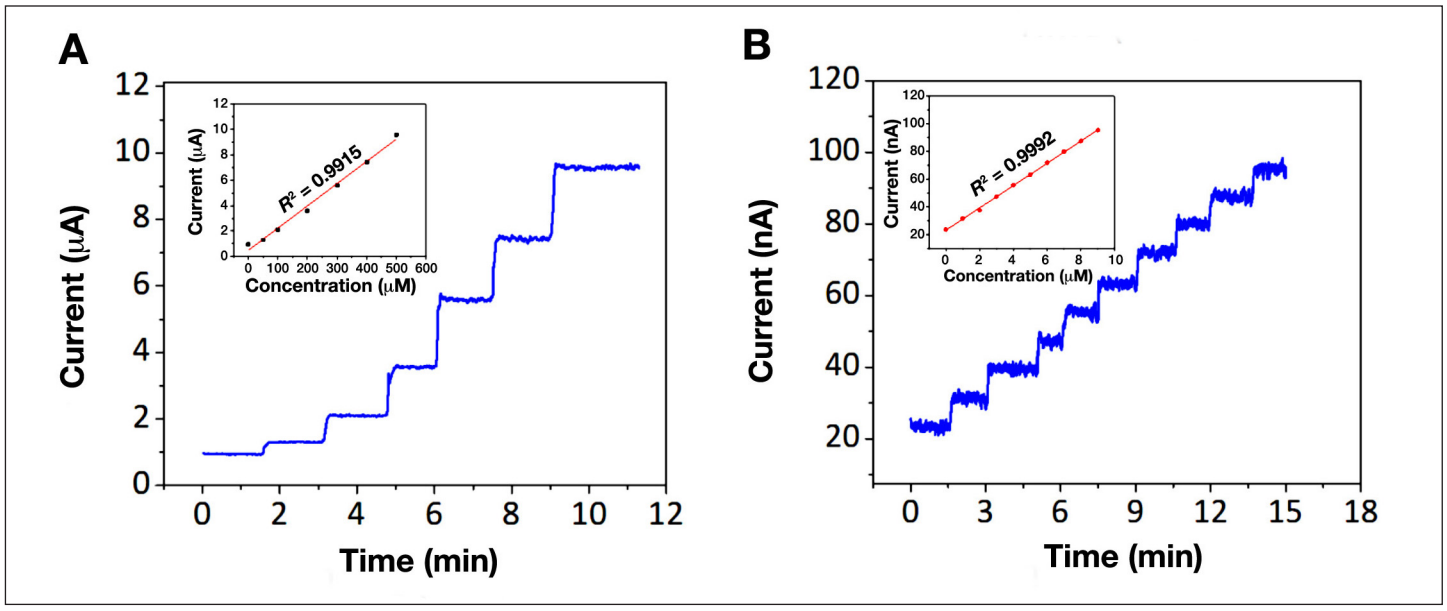

Figure 4. Amperometric glucose calibration experiments for the CNT/Pt nanosphere biosensor. The micromolar linear range calibration plot **(A)** portrays the current response to concentration increases of 50, 100, 200, 300, 400, and 500 μM, while the experimental detection limit graph **(B)** portrays the current response to eight successive concentration increases of 1 μM, respectively. Insets display the linear regression analysis of the respective current versus concentration plots.

Michaelis–Menten constant of the CNT/Pt nanosphere biosensor is calculated to be 0.64 mM by analyzing the slope and intercept of the Lineweaver–Burk equation plot (see **Figure 5**). This apparent Michaelis–Menten constant is lower than 2.6 mM reported for a similar CNT/Pt-nanoparticle-based biosensor,[22] 8.2 mM of a CNT-based biosensor,[27] 22 and 23 mM reported by more traditional GO_X/glucose biosensors derived from solgel and glassy carbon materials,[31,33,34] and lower than 33 mM reported for GO_X in solution phase.[35] Assuming that the rate of the enzymatic reaction is catalysis controlled,[32] the lower apparent Michaelis–Menten constant does correlate with our sensitivity and limit of detection, suggesting that the CNT/Pt nanosphere biosensor creates a unique nanoenvironment for enzymatic biosensing.

Conclusions

The advantages of utilizing CNTs and Pt nanoparticles over conventional materials in electrochemical glucose biosensing include increased effective surface area, enhanced mass transport of target analytes to what is effectively functional as nanoelectrodes arrays, and improved catalysis as redox transducers.[9,36] The GO_X–CNT/Pt nanosphere biosensor reported here achieved a glucose detection limit (380 nM) that was significantly lower than the glucose detection limit (1.3 μM) of our previous CNT-based biosensor that utilized gold-coated palladium nanocubes connected through CNT networks.[14] We hypothesize that the low detection limit and high glucose sensitivity of the GO_X–CNT/Pt nanosphere biosensor is related to the electrocatalytic properties of the CNT/Pt nanosphere networks, the high GO_X enzyme activity, and the enhanced electroanalytical mass transport created by individual CNT/Pt nanoelectrodes arrays.

We believe that the key to the enhanced mass transport to the GO_X–CNT/Pt nanosphere biosensor is related to the unique nanoenvironment created by the electrically connected networks of CNTs and Pt nanospheres. The Pt-nanosphere-decorated arrays of CNTs are spatially distanced by approximately 2–20 μm on the surface of the electrically insulating PAA (see **Figure 1**). This average spatial distance of at least 10 times the diameter of the individual Pt nanospheres ensures radial diffusion of glucose to the conducting CNT/Pt nanosphere networks on the planar biosensor surface.[37] Radial diffusion to arrays of individual conducting nanoelectrodes situated on planar biosensor surfaces significantly enhance the sensitivity and signal-to-noise ratio during electrochemical biosensing.[38,39]

Table 1.
Glucose-Sensing Performance Comparison between Carbon- and Metallic-Nanoparticle-Based Biosensors[a]

Biosensor Description	Sensitivity (μA/mM/cm²)	Detection Limit (μM)	Reference
GO_X-glutaraldehyde/ Pt-SWCNTs/PAA	70	0.38	this work
GO_X/PtNW-CNTs-chitosan/GCE	30	3	21
GO_X/Pt-CNTs/TiO_2	0.24	6	20
GO_X-glutaraldehyde/ Pt-MWCNTs/GCE	52.7	30	23
GO_X-Pt-(solgel)/ MWCTNs/CPE	0.98	—	24
GO_X-Nafion-Pt-CNTs/GCE	—	55	22
Nafion/GO_X-GNPs/GCE	6.5	34	25
GO_X/ASWCNTs/Si	—	80	26
GO_X/CNTs-chitosan/GCE	0.52[b]	—	27
Poly-electrolyte-GO_X/PCE	4.39[b]	10	28
GO_X-silica-(solgel)/CE	2.42[b]	40	30
GO_X-iridium/GCE	0.46[b]	370	31

[a] The dashes signify that the value was not reported in the corresponding reference. SWCNT, single-walled carbon nanotube; PtNW, platinum nanowire; GCE, glassy carbon electrode; MWCNT, multi-walled carbon nanotube; CPE, carbon paste electrode; GNP, gold nanoparticle; ASWCNT, aligned single-walled carbon nanotubes; PCE, porous carbon electrode; CE, carbon electrode.
[b] The sensitivity was not normalized on a per-unit-area basis.

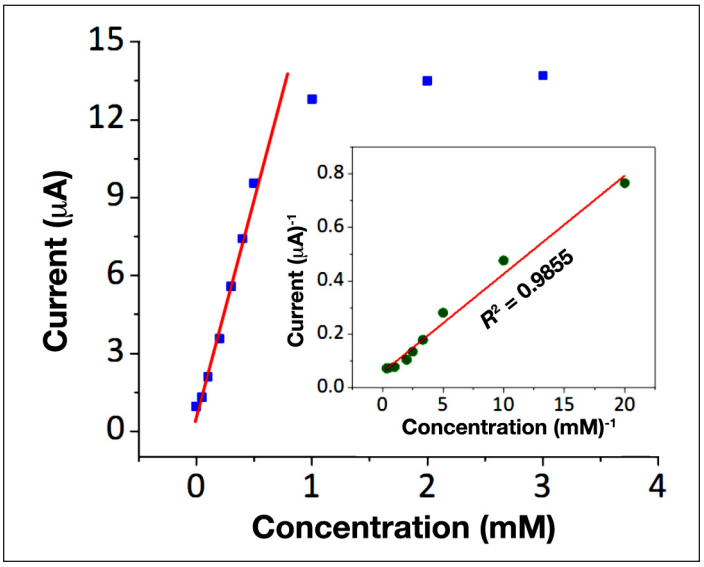

Figure 5. Amperometric glucose calibration plot of the CNT/Pt nanosphere biosensor. The inset portrays the plot of the Lineweaver–Burk equation with linear regression analysis.

This enhanced mass transport of glucose to the biosensor surface is due to the three-dimensional spherical growth of the diffusion boundary layer around the individual Pt nanospheres, which are separated by nonconducting oxide on the planar surface. This is in contrast with conventional planar-surface-based biosensors, which are limited to two-dimensional planar diffusion boundary layer growth that impedes the mass transfer of glucose to the biosensor surface. This interpretation is based on an extension of Bard and Faulkner's fundamental model[40] that explains the enhanced performance of microelectrodes over planar macroelectrodes.

Additional characteristics that enhance biosensor performance are the electrocatalytic properties and high surface area of the nanostructured Pt nanospheres and CNTs. These biosensor attributes potentially enhance the charge transport characteristics of the biosensor. We believe that the unique nanoenvironment of the nanostructured surface of the biosensor is well suited to preserve the tertiary structure of the GO_X enzyme—thus permitting a high enzyme activity. We hypothesize that all these characteristics, including the mass transport, electroactive, and enzymatic properties of the GO_X–CNT/Pt nanospheres, combine synergistically to create a biosensor capable of sensing glucose at submicromolar concentrations.

One significant area of emphasis in our program is on the development of tools for fundamental physiology research. For these applications, the submicromolar glucose sensitivity of the GO_X–CNT/Pt nanosphere biosensor is well suited for monitoring the glucose transport process in pancreatic β cells, via a self-referencing microbiosensor.[41] In these cell physiology research applications, high glucose sensitivity significantly enhances the spatial and temporal resolution of the biosensor[41,42] at the levels of the cellular/subcellular domain. Because of the mechanistic relationship between glucose uptake, cell signaling, and insulin secretion, measuring glucose flux across cellular membranes is crucial to understanding diabetes. Ultimately, regulation of insulin release from pancreatic islets and β cells depends in part on glucose uptake.[7,43]

In summary, the GO_X–CNT/Pt nanosphere biosensor outperforms similar Pt nanoparticle/CNT biosensors in terms of glucose sensitivity and detection limit. The CNT/Pt nanosphere biosensor demonstrates a glucose sensitivity of 70 $\mu A/mM/cm^2$, low detection limit 380 nM (signal-to-noise ratio = 3), and linear sensing region from 1 μM to 0.75 mM. Although the GO_X–CNT/Pt nanosphere biosensor is currently well suited for cell physiology research, we also believe it can be utilized for blood glucose sensing. Based on this platform and our experience from previous work,[14] we believe that altercation of the enzyme immobilization scheme will allow us to engineer the sensor for linear glucose sensing within the physiological range for blood glucose (3.8–6.1 mM).[31] The high enzyme activity of the immobilized GO_X enzyme, the enhanced mass transport (glucose and/or H_2O_2), and the efficient redox activities associated with the CNT/Pt nanospheres are key reasons for the glucose sensing results reported here.

In terms of commercial production, the *in situ* fabrication protocol of the biosensor eliminates the need for exhaustive CNT and Pt nanoparticle processing steps. Thus the facile biosensor fabrication and biofunctionalization scheme of the GO_X–CNT/Pt nanosphere biosensor can be scaled easily for mass production of research-grade microsensors or future integration into commercial glucose monitoring systems that require highly sensitive and accurate glucose sensing.

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