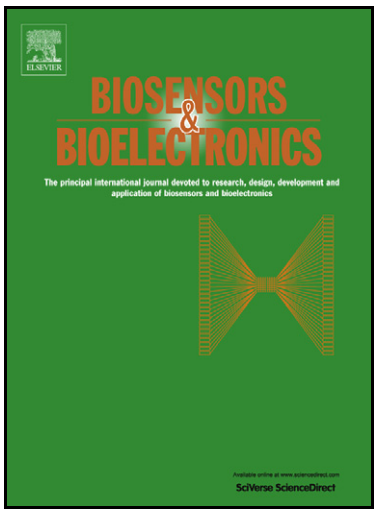

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Immobilization of glucose oxidase into a nanoporous TiO_2 film layered on metallophthalocyanine modified vertically-aligned carbon nanotubes for efficient direct electron transfer

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Abstract:

Glucose oxidase (GOD) was adsorbed into a nanoporous TiO_2 film layered on the surface of an iron phthalocyanine (FePc)- vertically-aligned carbon nanotube (CNT) modified electrode. A Nafion film was then dropcast on the electrode's surface to improve operational and storage stability of the GOD-based electrode. Scanning electron microscopy (SEM) micrographs revealed the formation of FePc and nanoporous TiO_2 nanoparticles along the sidewall and the tip of CNTs. Cyclic voltammograms of the GOD electrode in neutral PBS exhibited a pair of well-defined redox peaks, attesting the direct electron transfer of GOD ($FAD/FADH_2$) with the underlying electrode. The potential of glucose electro-oxidation under nitrogen was ~ +0.12 V with an oxidation current density of 65.3 μA cm^{-2} at +0.77 V. Voltammetric and amperometric responses were virtually unaffected by oxygen, illustrating an efficient and fast direct electron transfer. The modification of the CNT surface with FePc resulted in a biosensor with remarkable detection sensitivity with

oxygen-independent bioelectrocatalysis. In deaerated PBS, the biosensor displayed an average response time of 12 s, linearity from 50 μM to 4 mM, and a detection limit of 30 μM (S/N = 3) for glucose.

Keywords: Glucose oxidase, Direct electron transfer, Vertically-aligned carbon nanotubes, Iron phthalocyanine, Bioelectrocatalytic, Nanoporous TiO_2.

1. Introduction

Effective and stable immobilization of glucose oxidase (GOD), a flavin enzyme, has been exploited for the development of glucose biosensors and biofuel cells. GOD has two identical polypeptide chains, each with a flavin adenine dinucleotide (FAD) redox. This enzyme requires oxygen for the oxidation of glucose to *δ*-gluconolactone with H_2O_2 as co-byproduct (Eq. 1). Oxygen consumption or H_2O_2 formation can be detected and equated to the corresponding glucose level (Heller and Feldman, 2008; Wang, 2008). However, the requirement of oxygen or an artificial mediator for the reoxidation of the reduced enzyme ($FADH_2$) prevents its applicability in implantable biosensors and biofuel cells (named 'the third generation biosensors and biofuel cells'). The third generation biosensors and biofuel cells are a striking example of the continued development of redox-enzyme electrodes (Alkire, et al., 2011), where a direct electron transfer (DET) between GOD and the supporting electrode is a prerequisite (Eq. 2). DET for GOD is extremely difficult and inefficient because the FAD active redox center of GOD is deeply embedded within a protective protein shell. Early work on the direct electrochemistry of redox proteins often suffers from poor stability of proteins at the electrode surface (Alkire, et al., 2011).

$$\text{GOD(FAD)} + \text{glucose} + O_2 \rightleftharpoons \delta\text{-gluconolactone} + H_2O_2 + \text{GOD}(FADH_2) \quad \text{Eq. (1)}$$

$$\text{GOD(FAD)} + 2H^+ + 2e^- \rightleftharpoons \text{GOD}(FADH_2) \quad \text{Eq. (2)}$$

To date, DET of GOD has been realized by using nanomaterials as supports (Cai and Chen, 2004; Wang et al., 2011) or additives of matrices (Liu et al., 2005) for GOD immobilization. Nanoscale conductive materials could penetrate the protein shell of GOD to a distance for realizing electron tunneling. Since their discovery (Iijima, 1991), carbon nanotubes (CNTs) have emerged as an attractive electrode support for enzyme immobilization (Chen et al., 2003; Zhang et al., 2009), especially for the fabrication of biosensors and biofuel cells (Lee et al., 2010a, 2010b). CNTs have been advocated for such applications because of their high electrical conductivity, biocompatibility, inertness and other desired properties. DET of GOD can also be realized by the incorporation of CNTs into enzyme electrode constructions (Cai and Chen, 2004; Gao and Zheng, 2009; Janegitz et al., 2011; Lee et al., 2010b; Liu et al., 2005; Wang et al., 2011). However, enzyme electrodes prepared by casting CNTs or CNT composites on substrates still suffer from mechanical instability during repeated and prolonged detection, thus limiting their practical uses. High-density vertically-aligned CNTs on a large area of substrates could overcome this drawback (Zhang et al., 2002a, 2002b). Well-aligned multi-walled CNTs (MWCNTs) synthesized on a Ta substrate also exhibit stable and excellent electrical contact with the underlying substrate. The Ta supported MWCNTs can be functionalized with iron phthalocyanine (FePc) to serve as a carrier for enzyme loading in a nonconductive polymer film (Ye et al., 2005), resulting in remarkable detection sensitivity. Metallophthalocyanines are known to exhibit electrocatalytic activity for H_2O_2 (Ye et al., 2005) and thiols (Ozoemena et al., 2003). They can be modified at different electrode substrates including CNTs to improve detection sensitivity (Cao et al., 2003). To our knowledge, DET of GOD using FePc modified CNTs as an enzyme immobilization carrier has not been reported.

Nanostructured TiO_2, especially a nanoporous TiO_2 film as a protein immobilization matrix, has attracted considerable interest due to its large specific surface area, low-cost, non-toxicity, good mechanical, thermal and chemical stability, and excellent biocompatibility (Topoglidis et al., 1998; Yu and Ju, 2002; Zhang et al., 2011a, 2011b). Stable and bioactive protein immobilization could be readily achieved

by adsorption of a target protein to nanoporous TiO_2 films (Topoglidis et al., 1998; Topoglidis et al., 2001a). However, TiO_2 adsorbed redox enzymes often show irreversible bioelectrocatalysis, i.e., a stumbling block, owing to low electrical conductivity of TiO_2 (Liu and Chen, 2005; Topoglidis et al., 2001a, 2001b). Doping Cu (II) into nanoporous TiO_2 could improve the conductivity of TiO_2 (Zhang et al. 2011a). Other approaches involve the modification of a TiO_2-MWCNT-chitosan composite with Prussian blue and poly(diallyldimethylammonium) chloride functionalized gold nanoparticles (Zhang et al., 2011b).

This paper describes a simple approach for the immobilization of GOD into a nanoporous TiO_2 film layered on the surface of FePc- well-aligned MWCNTs (denoted as FePc-CNT), which is attached on a glassy carbon (GC) electrode. A Nafion film is cast on the surface of the as-prepared GOD@TiO_2/FePc-CNT electrode to improve enzyme stability and detection selectivity. Bioelectrocatalysis of the GOD electrode is conducted to assess its analytical performance with respect to detection sensitivity, response time, linearity and stability.

2. Experimental

2.1. Materials and reagents

GOD from *Aspergillus niger*, 100-250 kU/g) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nafion (5 wt% solution) and FePc (purity: 96%) were purchased from Alfa Aesar (Ward Hill, MA, USA). Other remaining chemicals were obtained from Sinopharm Chemical Reagent (Shanghai, China). All chemicals were of analytical grade and used as received. The phosphate buffer solution (PBS) contains: 8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4 and 0.24 g l^{-1} KH_2PO_4 with pH 7.4. A stock solution of glucose was prepared and allowed to mutarotate at room temperature overnight. Deionized (DI) water obtained from a Millipore water system was used throughout the experiment. Purified nitrogen with purity of 99.99% was obtained from Zhengzhou Keyi Industrial Gas (Zhengzhou, China).

2.2. Apparatus and measurement

Pristine and modified MWCNTs were probed by scanning electron microscopy (SEM, JEOL JSM 6700F, operated at 5 kV or 15 KV). All electrochemical measurements were performed at room temperature (~25°C) using a CHI-660C electrochemical workstation (Shanghai Chenhua Instrument, CHI, China) in a three-electrode arrangement consisting of a working electrode (pristine or modified MWCNTs), a Pt counter electrode and an Ag|AgCl|KCl (3 M) reference electrode.

2.3. Synthesis of TiO_2 sol-gel solution

Nanoporous TiO_2 was synthesized from a TiO_2 sol-gel solution as described in Supplementary Material. The resulting TiO_2 precursor solution was sealed and stored at 4 ºC, ready for use. If exposed to air, a thin layer of the TiO_2 precursor solution would congeal in minutes to form a TiO_2 gel.

2.4. Synthesis of well-aligned MWCNTs and the construction of MWCNT electrodes

Well-aligned MWCNTs were synthesized on small Ta plates (3 mm × 3 mm) by chemical vapor deposition with ethylenediamine as a precursor according to Zhang et al. (2002a, 2002b). A Ta plate with MWCNTs was connected to the surface of a copper rod electrode using conductive silver paint (Structure Probe, USA). The edges of the Ta plate and the copper rod electrode were insulated by pasting with nail enamel (Maybelline, NY, USA).

2.5. Modification of MWCNT electrodes

The Ta plate with MWCNTs grown-on was soaked in a saturated FePc/toluene solution for 4 h before connected to the copper rod electrode. The FePc-CNT (or pristine CNT) electrode was dipped in the TiO_2 sol-gel solution for ~ 2 s. The electrodes were then placed in air at room temperature overnight for the completion of TiO_2 gel formation. The as-prepared TiO_2/FePc-CNT electrode was soaked in PBS 3 times (30 min each time) to remove entrapped acetic acid followed by vacuum-drying

for 3 h to remove entrapped water. The electrode was then soaked in PBS containing GOD (1 kU ml^{-1}) at 4℃ for >10 h to adsorb GOD. After immersed in neutral PBS to remove loosely absorbed GOD, the GOD$^{@}TiO_2$/FePc-CNT electrode was produced. The GOD-based electrode was air-dried and coated with a Nafion film (denoted as Nafion/GOD$^{@}TiO_2$/FePc-CNTs) by dropping 4 μl 0.5 wt% Nafion solution on the electrode surface. After air-drying, the enzyme electrode was stored at 4 °C in sterilized neutral PBS.

2.6. Electrochemical characterization of the modified MWCNT electrodes

The effect of FePc modification was investigated by cyclic voltammetry (CV) in deaerated PBS without and with H_2O_2. The electron-transfer properties of the modified CNT electrodes were investigated by CV and electrochemical impedance spectroscopy (EIS) experiments. The CVs were run from -0.1 V to +0.6 V at various scan rates in 5 mM $K_3[Fe(CN)_6]$ supported by 1 M KCl. The EIS experiments were performed in 0.1 M KCl solution containing equimolar $[Fe(CN)_6]^{3-/4-}$ (0.01 M/0.01 M) with AC from 0.1 Hz to 100 KHz.

2.7. Biocatalysis of the GOD electrode

CV was cycled between +0.8 and -0.8 V at 0.01 V s^{-1} in PBS with or without glucose (in air or under N_2). Signal responses were recorded at an applied potential of +0.5 V in PBS (in air or under N_2) successively by injection of glucose solution. The signal response to glucose was measured as the catalytic current (I_{cat}). The catalytic current density (J_{cat}) was then calculated from I_{cat} and the effective surface area (ESA) of the corresponding FePc-CNT (or pristine CNT) electrode support. The ESA was estimated according to the classic method (Bard and Faulkner, 2001) and described in Supplementary Material. The 'in air' condition meant in the atmosphere environment, while the 'under N_2' condition was performed by purging N_2 into the solution for 20 min and maintaining a nitrogen blanket over the solution in the cell.

3. Results and discussions

3.1. Morphological characterization of modified MWCNT electrodes

FePc nanoparticles were formed along the sidewalls as well as the tips of well-aligned MWCNTs (Fig. 1A-B). About 3.05% of Fe at the CNT tip position was determined by energy dispersive X-ray spectroscopy (EDS). The adsorption of FePc on CNTs could be attributed to the π-π interaction between phthalocyanine and CNTs whereas the π-π interaction between FePc molecules per se contributed to the formation of FePc nanoparticles. The aggregation of metallophthalocyanine can occur in solution due to the π-π interaction between phthalocyanine molecules (Nyokong, 1993). Therefore, the aggregation of FePc nanoparticles at the tips of CNTs was due to easy access of FePc to the tip position, and/or facile adsorption of FePc to the circular structure of the tip. After modification by TiO_2 (C, D in Fig. 1), more nanoparticles were coated on the surface of CNTs, indicating the formation of a TiO_2 film on the FePc-CNT surface. EDS measurements estimated an element content of 4.22% Ti and 1.09% Fe at one position, and 3.49% Ti without Fe at another position. TiO_2 nanoparticles at the tips of CNTs were nanoporous with a cauliflower-like shape. They also appeared tinier and more uniform compared to FePc nanoparticles.

3.2. Electrochemical characterization of modified MWCNTs electrodes

3.2.1. Electrocatalytic activity of FePc-CNT electrodes to H_2O_2

CNTs exhibit strong electrocatalytic activity towards dissolved oxygen and H_2O_2 (Cui et al., 2005; Wang et al., 2003; Ye et al., 2005). As shown in Fig. 2, the CV at the FePc-CNT electrode in blank PBS featured a pronounced anodic peak with a peak potential of E_{pa} = +0.46 V, corresponding to the electro-oxidation of Fe(II)Pc. A very small cathodic peak at E_{pc} = +0.19 V corresponding to the paired reduction of Fe(III)Pc also appeared in the CV. The asymmetry of the redox peaks indicated that the electrochemical reaction of FePc adsorbed on the CNT surface was virtually irreversible. The potential for H_2O_2 electroreduction at the FePc-CNT electrode and the pristine CNT electrode was +0.21 V (cathodic peak current I_{pc} = 55.8 μA) and

+0.09 V (I_{pc} of 27.1 μA), respectively, with a 0.12 V positive shift after FePc modification. The electro-oxidation of H_2O_2 at the FePc-CNT electrode and the pristine CNT electrode commenced at -0.1 V (anodic peak current I_{pa} = 9.1 μA) and +0.09 V (with I_{pa} of 8.8 μA), respectively, showing a 0.19 V negative shift upon FePc modification. Thus, the modification by FePc on the surface of CNTs enhanced the electrocatalytic activity of CNTs towards the redox reactions of H_2O_2. Interestingly, two anodic peaks with E_{pa} of ~ +0.12 V and +0.57 V were observed at the FePc-CNT electrode in the H_2O_2 solution (d). The peak with E_{pa} of +0.12 V was doubtlessly attributed to the electro-oxidation of H_2O_2 whereas the peak with the E_{pa} of +0.57 V resulted from the electro-oxidation of Fe(II)Pc. This anodic oxidation profile (on curve d) resembled that of the Nafion/GOD$^{@}$$TiO_2$/FePc-CNT electrode in glucose solution in air as discussed below.

3.2.2. Effect of modification on electron-transfer properties at CNTs electrodes

For all electrodes, a symmetric pair of current responses with a formal potential ($E^{0'}$) of ~+0.26 V from the redox reactions of $[Fe(CN)_6]^{3-}$ were observed (Fig. 3). The corresponding peak potential separations ($\Delta E_p = E_{pa} - E_{pc}$) under various scan rates are shown in the Inset of Fig. 3. For the pristine CNT electrode, ΔE_p was ~59 mV at all sweeping rates, suggesting reversible redox reaction of $[Fe(CN)_6]^{3-}$. Upon the modification with FePc, ΔE_p was still ~ 59 mV at 0.01 V s^{-1}, but decreased gradually to 44 mV as the scan rate increased to 0.20 V s^{-1}. Such behavior suggested that the $[Fe(CN)_6]^{3-}$ probe in solution was slowly adsorbed to FePc molecules on CNTs as the CV experiment proceeded from low to high scan rates. After a valley stage at 44 mV, the value of ΔE_p successively attained ~ 59 mV with increasing scan rate to 1.00 V s^{-1}. Apparently, the adsorption of FePc displayed no obvious adverse effect on the electron transfer rate at the CNT electrode, in agreement with Cao et al. (2003) and Wang et al. (2002). The adsorption of $[Fe(CN)_6]^{3-}$ on the FePc surface should be due to van der Waals and π-π interactions. Increased ESA upon FePc modification on CNTs, was confirmed by their SEM micrographs (Fig. 1) and by electrochemical area determination (data not shown), contributing to the enhanced $[Fe(CN)_6]^{3-}$ adsorption.

In contrast, the ΔE_p vs. scan rate plot of the TiO_2/FePc-CNT electrode displayed an increase of ΔE_p with increasing scan rates from 0.01 to 0.07 V s^{-1}. Further increases in the scan rate resulted in a gradual decrease of ΔE_p. A slight increment of ΔE_p suggested that the TiO_2 film slightly impeded the electron-transfer rate at the modified CNT electrode, owing to the low electrical conductivity of TiO_2. The decrease of ΔE_p suggested that surface-confined $[Fe(CN)_6]^{3-}$ molecules dominated the electrode redox reaction, when the experiment proceeded to a time point that sufficient $[Fe(CN)_6]^{3-}$ molecules were adsorbed onto the electrode interface. This result inferred that the TiO_2/FePc-CNT electrode possessed a high surface area for adsorption, an advantageous property for enzyme immobilization. The nanoporous structure of TiO_2, as illustrated by SEM micrographs (Fig. 1), could enhance the surface area of the modified CNTs.

The Nyquist plot shows a semicircle related to the redox probe $Fe(CN)_6^{3-/4-}$ followed by a Warburg line in the low frequency region which corresponds to the diffusion step of the overall process for both the FePc-CNT and TiO_2/FePc-CNT electrodes (Fig. S-1, Supplementary Material). However, the incorporation of non-conducting TiO_2 to the FePc-CNT surface blocked the diffusion and passage of the redox probe to the active area of the detecting electrode. The charge transfer resistance for the TiO_2/FePc-CNT electrode was estimated to be 296 Ω compared with 20 Ω for the FePc-CNT electrode whereas changes in both electrolyte resistance and double-layer capacitance were not significant (Supplementary Material, Table S-1). These results were consistent with those obtained from the above-mentioned CV experiments.

3.2.3. Electrochemical redox reactions of GOD at the enzyme electrodes

The CVs at the Nafion/GOD$^{@}TiO_2$/FePc-CNT electrode in blank PBS exhibited a pair of redox peaks with $E^{0'}$ of -0.483 V, indicating DET of the FAD center of GOD (Fig. 4 and Fig. S-2 of Supplementary Material) was achieved. The E_{pa} and E_{pc} peak of the GOD electrode reaction was -0.460 V and -0.505 V, respectively (ΔE_p = 45 mV). The cathodic and anodic peak currents were equal and nearly symmetrical,

indicating quasi-reversibility of the electrochemical reaction of GOD. Redox enzymes such as cytochrome c immobilized in nanoporous TiO_2 only exhibit an irreversible reaction due to low electrical conductivity of TiO_2 (Liu et al., 2005; Topoglidis et al., 2001a, 2001b). For GOD, the FAD redox center is deeply embedded within a protective protein shell; therefore DET of GOD is more difficult than the above reported redox enzymes. The pronounced DET of GOD at the Nafion/GOD$^{@}$$TiO_2$/FePc-CNT electrode could be attributed to a concerted effect contributed by the CNT electrode support and the FePc modification. The Nafion/GOD$^{@}$$TiO_2$/FePc-GC or Nafion/GOD$^{@}$$TiO_2$/CNT electrode displayed no redox reaction of GOD (Fig. S-3, Supplementary Material). Doubtlessly, the CNT electrode support facilitated the GOD immobilized in the TiO_2 matrix to attain DET, considering the excellent electrical conductivity and electrocatalytic activity of CNTs. The attachment of FePc on the surface of CNTs also played an important role to effect the electrochemical reaction of GOD. Conductive FePc nanoparticles were capable of penetrating into the GOD protein shell, facilitating electron tunneling between the FAD redox center and the underlying electrode. I_{pa} and I_{pc} were proportional to the scan rate (ν) ranging from 0.01 to 0.50 V s^{-1} [I_{pc} (μA) = -0.49 - 12.69 ν (V s^{-1}), R = 0.999; I_{pa} (μA) = 0.29 + 11.96 ν (V s^{-1}), R = 0.999], indicating a surface-confined electrode process (Fig. 4).

3.3. Biocatalysis of GOD at Nafion/GOD$^{@}$$TiO_2$/FePc-CNTs electrodes

3.3.1. Investigation by CV

The O_2 reduction peak with E_{pc} ~ +0.07 V in PBS in air disappeared in the presence of both glucose and air (Fig. 5), indicating that O_2 was consumed as expected from the biocatalytic oxidation of glucose (Eq. 1). Such a result confirmed that GOD at the Nafion/GOD$^{@}$$TiO_2$/FePc-CNT electrode retained its original biocatalytic activity. Bioelectrocatalysis of the electrical-contacted GOD was then investigated by comparing the anodic sweeping segments of CVs in glucose solution to those in blank PBS. Obvious electro-oxidation current was observed in the

presence of glucose in both aerated and deaerated PBS solutions at ~ +0.12 V. In addition, the glucose electro-oxidation current at the potential of > +0.77 V was similar, ~5.35 μA (current density of 65.3 μA cm^{-2}), illustrating strong bioelectrocatalysis of the electrical-contacted GOD for glucose, which was also unperturbed by O_2. In the presence of glucose in air, two small anodic peaks with E_{pa} of +0.23 V and +0.58 V appeared at the shoulder of the glucose electro-oxidation peak. This anodic oxidation profile was similar to that at the FePc-CNT electrode in deaerated PBS containing H_2O_2 as shown in Fig. 2. The E_{pa} peak at +0.23 V was unambiguously from the electro-oxidation of H_2O_2, a co-product of biocatalytic oxidation of glucose in the presence of O_2 (Eq. 1). Accordingly, the anodic peak with the E_{pa} of +0.58 V was assigned to the catalytic electro-oxidation of Fe(II)Pc by H_2O_2 as shown in Eqs. 3,4 .

$$\text{Fe(II)Pc} - e \rightleftharpoons \text{Fe(III)Pc} \quad \text{(Electrochemical reaction)} \qquad \text{Eq. (3)}$$

$$2\text{Fe(III)Pc} + H_2O_2 \rightarrow 2\text{Fe(II)Pc} + O_2 \quad \text{(Chemical reaction)} \qquad \text{Eq. (4)}$$

The electrochemical redox reaction of FePc at the FePc-CNT electrodes was almost irreversible (Eq. 3). Upon modification with a Nafion/GOD$^{@}$$TiO_2$ layer, the redox reaction of FePc at the CNT electrode was hampered, resulting in a negligible FePc oxidation peak (Fig. 5). In the presence of glucose and O_2, H_2O_2, a co-product of the GOD biocatalytic oxidation of glucose, reacted with the electro-oxidation product [i.e. Fe(III)Pc] of FePc at the electrode surface, producing Fe(II)Pc (Eq. 4). Repetitive alternating between the electrochemical and chemical reactions resulted in a catalytic electro-oxidation of Fe(II)Pc, contributing to the enhanced anodic peak of E_{pa} ~ +0.58 V.

3.3.2. Amperometric response

The Nafion/GOD$^{@}$$TiO_2$/FePc-CNT electrode responded to the increase of glucose concentration ($C_{glucose}$) within 1 s, reaching 95% of steady-state current within 12 s (Fig. 6). The rapid response was contributed by the MWCNT support as well as

the FePc modification layer on the CNT surface. The Nafion/GOD@TiO_2/CNT electrode in PBS in air showed an average response time, reaching 95% of steady-state current in 190 s (Fig. S-4 in Supplementary Material), i.e., it was much slower than that at the Nafion/GOD@TiO_2/FePc-CNT electrode. Apparently, the enhanced glucose sensing response was attributed to the realization of DET of GOD, and enhanced conductivity as well as electrocatalytic activity of the GOD immobilization matrix. In general, the turn-over rate of electrical-contacted GOD is usually faster than that of GOD with O_2 as electron acceptor (Xiao et al., 2003). The TiO_2 matrix dip-coated on the surface of FePc-CNTs should actually form a TiO_2-FePc nanocomposite, considering the nanoparticle morphology of FePc on the CNT surface. The TiO_2-FePc nanocomposite exhibits higher conductivity and electrocatalytic activity than the TiO_2 matrix. It was observed that the amperometric spectra showed an obvious background noise, which came from the magnetic stirring or the magnetic field during the amperometric experiment. The background noise was not smoothed but presented in reality to reflect practicality of data collection in Fig. 6. The inset in Fig. 6 shows that the current response was linear in the range of 50 μM-4 mM (R = 0.997) for glucose with a limit of detection of 30 μM (S/N=3). The glucose detection sensitivity in the linear range was 8.25 μA cm^{-2} mM^{-1} in air, and 7.11 μA cm^{-2} mM^{-1} under N_2 (86% of the value in air). This detection sensitivity was ~9-fold higher than the value obtained for the Nafion/GOD@TiO_2/CNT electrode (1.11 μA cm^{-2} mM^{-1} in air) (Fig. S-4, Supplementary Material). The modification of FePc on the CNT surface drastically improved glucose sensing sensitivity, perhaps owing to a higher amount of GOD loading, and enhanced biocatalysis/bioelectrocatalysis of GOD in the TiO_2-FePc matrix compared to the GOD loading in the TiO_2 matrix on pristine CNTs. With modification of FePc nanoparticles, the roughness factor and the ESA of the CNT surface increased, resulting in an increased TiO_2 dipping amount, i.e., a higher amount of enzyme loading. The enzyme reaction to glucose at the Nafion/GOD@TiO_2/FePc-CNT electrode in both aerated and deaerated conditions follows Michaelis–Menten kinetics. A linear $1/I_{cat}$ vs. $1/C_{glucose}$ plot was obtained with a slope of 1.64 (R = 0.998) in air and 1.91 (R = 0.993) under N_2, corresponding to a

maximum current density of 55.09 μA cm^{-2} and 49.32 μA cm^{-2}, respectively. The apparent Michaelis–Menten constant was 7.39 mM and 7.71 mM, respectively, for the immobilized enzyme in air and under N_2. Thus, the immobilization procedure and the absence of air did not alter the Michaelis–Menten type kinetics of GOD. The average sensitivity of glucose response at different enzyme electrode preparations is 8.07± 0.85 (mean±S.D) μA cm^{-2} mM^{-1} (n=3), indicating that the enzyme electrode is quite reproducible.

The operational stability of the enzyme electrodes for repetitive detection of glucose was evaluated by injecting different amounts of glucose into PBS up to a final concentration of 4 mM. There was no significant change in amperometric response sensitivity from detection to detection [7.71±0.54 (mean±S.D) μA cm^{-2} mM^{-1}, n=3]. The response sensitivity for the second and third detection was 96% and 87%, respectively, of the value for the first detection, indicating negligible enzyme leaching. Strong chelating of carboxylate groups of protein to Ti^{4+} centers on the oxide surface (O'Regan and Grätzel, 1991) and the Nafion film cast on the enzyme electrode surface contributed to this stability. The storage stability of the enzyme electrodes was tested at 4 °C in sterilized PBS for 60 days. During the storage period, the response of the enzyme electrode to glucose was tested every 7-8 days during the first 30 days and then at the 60^{th} day. The response sensitivity gradually decayed to ~ 60% of the original value after 30 days and then remained constant. Mainly a slight leaching and a gradual denaturation of the enzyme during storage should contribute to the slow decay. The TiO_2 gel matrix apparently provided a biocompatible micro-environment for the immobilized GOD. The unique deaerated sensing ability accompanying with high sensing stability and sensitivity might provide this Nafion/GOD$^{@}TiO_2$/FePc-CNT electrode as an ideal third generation glucose biosensor.

4. Conclusions

DET of GOD immobilized into the TiO_2 gel matrix layered on the surface of FePc-well-aligned CNT electrodes was realized. Modification of FePc nanoparticles on the surface of CNTs contributed to high enzyme loading, resulting in an Nafion/GOD$^{@}TiO_2$/FePc-CNT electrode with strong bioelectrocatalysis for glucose. Its voltammetric and amperometric response signals were unaffected by O_2. Such important features of the GOD-based electrodes are two key prerequisites for the development of third generation biosensors and enzyme biofuel cells.

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Figure Captions

Fig. 1. Top view SEM images of Ta-supported vertically-aligned pristine CNTs (A), FePc modified CNTs (FePc-CNTs) (B), and FePc-CNTs modified with TiO_2 (TiO_2/FePc-CNTs) (C, D). D is a higher magnification image of the enclosed area in C.

Fig. 2. CVs at a pristine CNT (a, c) and a FePc-CNT (b, d) electrode in deaerated PBS containing 0 mM (a, b) and 1 mM (c, d) H_2O_2 at a scan rate of 0.05 V s^{-1}. The two electrodes have a similar ESA of ~ 0.10 cm^2.

Fig. 3. CVs in 5 mM $K_3[Fe(CN)_6]$ supported by 1 M KCl, at (a) pristine CNT, (b) FePc-CNT, and (c) TiO_2/FePc-CNT (c) at 0.10 V s^{-1}. **Inset:** Redox peak potential separations (ΔEp) vs. scan rates (0.01 to 1.00 V s^{-1}) at the CNT electrodes without and with modification.

Fig. 4. CVs at the Nafion/GOD$^{@}TiO_2$/FePc-CNT electrode in deaerated PBS at various scan rates. **Inset**: Plots of I_{pa} and I_{pc} of the GOD electro-redox reactions vs. scan rates. ESA of the corresponding FePc-CNT electrode: 0.082 cm^2.

Fig. 5. CVs at a Nafion/GOD@TiO_2/FePc-CNT electrode in PBS under N_2 (dotted line, dash line) or PBS in air (dash-dotted-dotted line, solid line) containing 0 mM (dotted line, dash-dotted-dotted line) and 5 mM (dash line, solid line) glucose at 0.01 V s^{-1}.

Fig. 6. Amperometric responses of the Nafion/GOD@TiO_2/FePc-CNT electrode to successive step increases of $C_{glucose}$ in PBS under N_2 or PBS in air at +0.50 V. The arrow symbols indicate the injection time position of 0.05, 0.1, 0.2, 0.5, and 1 mM glucose, respectively, leading to a group of different concentration steps. **Inset**: Calibration plots of J_{cat} vs. $C_{glucose}$. The ESA of the FePc-CNT electrode was 0.082 cm^2.

Figure 1

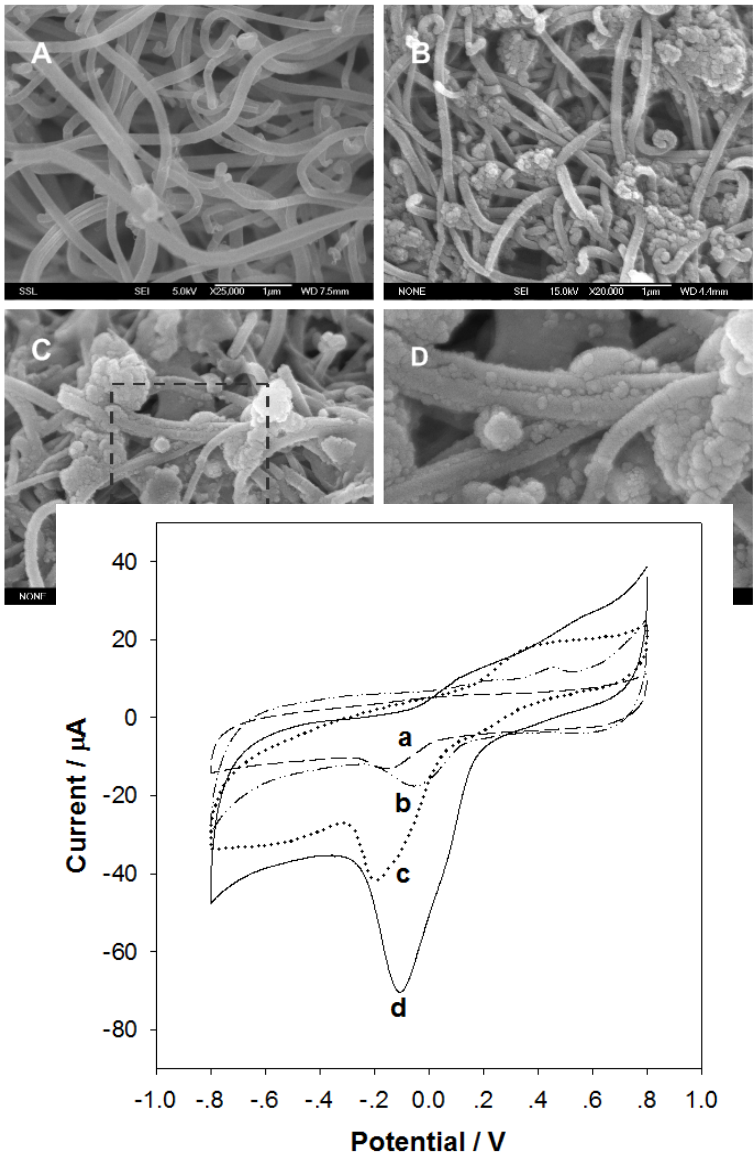


Figure 2

Figure 3

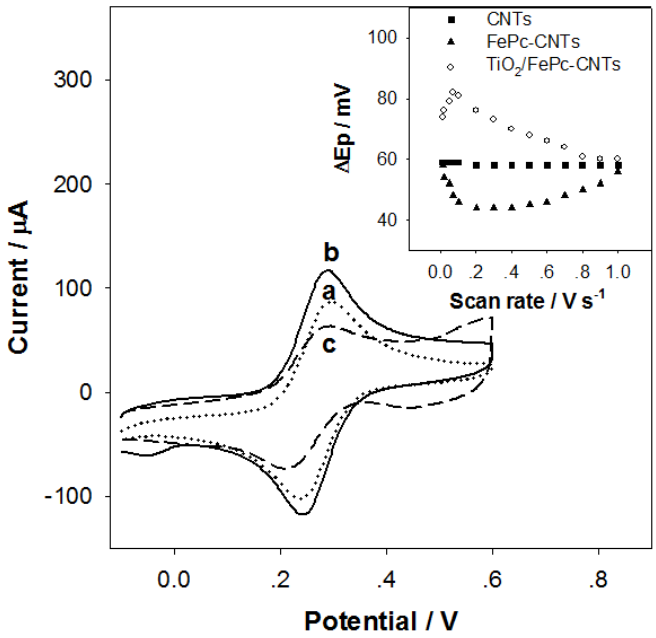


Figure 4

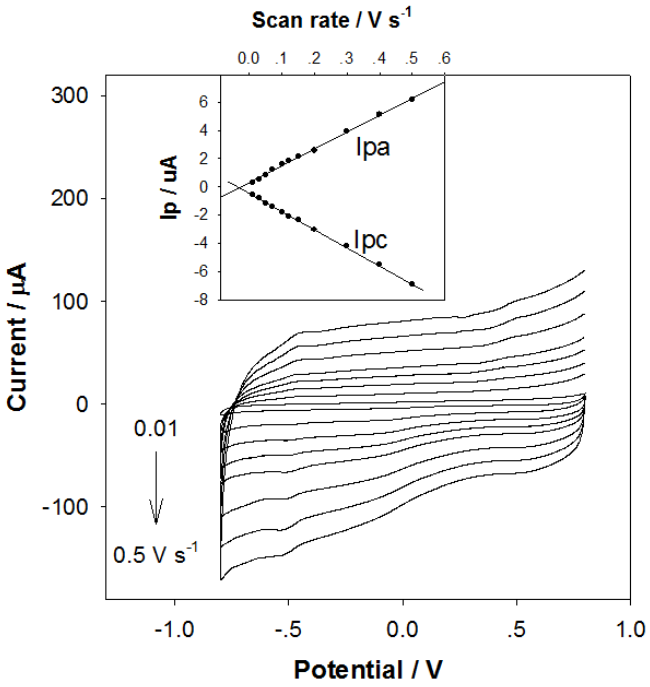


Figure 5

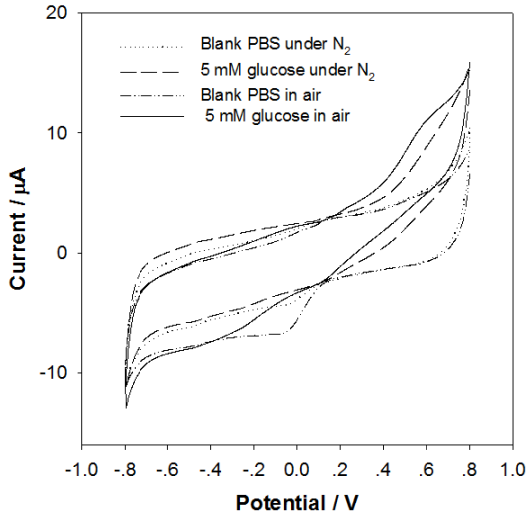
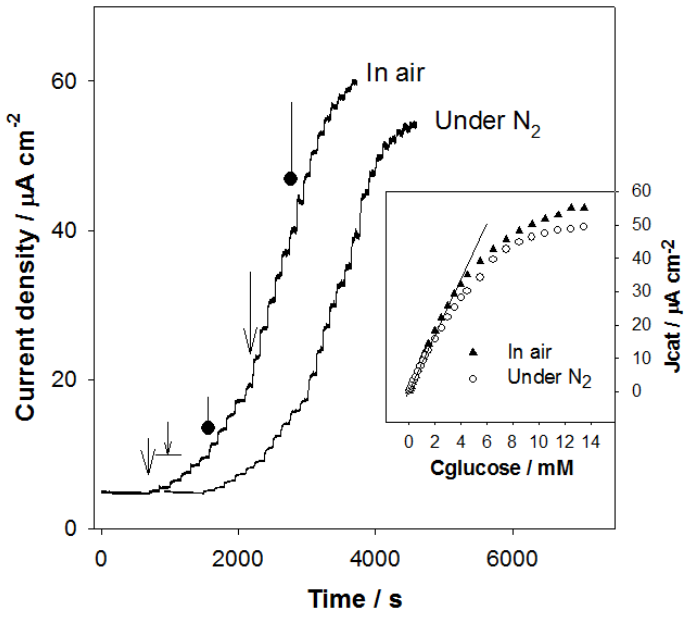


Figure 6



Highlights

- Glucose oxidase (GOD) was adsorbed into a nanoporous TiO_2 film.
- The TiO_2 film was layered on iron phthalocyanine (FePc)- vertically-aligned carbon nanotubes.
- The GOD electrode in neutral PBS exhibited a pair of well-defined redox peaks.
- FePc nanoparticles contributed to high enzyme loading and bioelectrocatalysis for glucose.
- Voltammetric and amperometric responses of the GOD electrode to glucose were unaffected by O_2.