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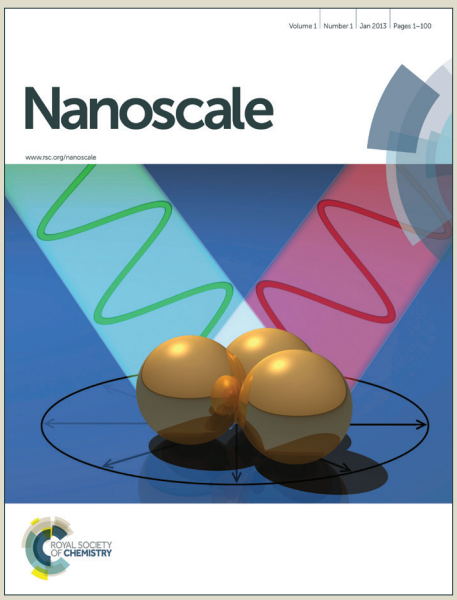

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Article

Novel ferrocene-anchored ZnO nanoparticles/carbon nanotube assembly for glucose oxidase wiring: application to a glucose/air fuel cell

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ABSTRACT

Glucose oxidase (GOx) is immobilized on ZnO nanoparticle-modified electrodes. The immobilized glucose oxidase shows efficient mediated electron transfer with ZnO nanoparticles to which the ferrocenyl moiety was π-stacked into a supramolecular architecture. The constructed ZnO-Fc/CNTs modified electrode exhibit high ferrocene surface coverage, preventing from any leakage of π-stacked ferrocene from the newly described ZnO hybrid nanoparticles. The use of the new architecture of ZnO supported electron mediators to shuttle electrons from the redox centre of the enzyme to the surface of the working electrode can effectively provide successful glucose oxidation. These modified electrodes evaluated as high efficient architecture providing a catalytic current for glucose oxidation and are integrated in a specially designed glucose/air fuel cell prototype using a conventional platinum-carbon (Pt/C) cathode at physiological pH (7.0). The obtained architecture leads to peak power densities of 53 μW cm^{-2} at 300 mV for the Nafion® based biofuel cell at "air breathing" conditions at room temperature.

1. Introduction

Electrochemical wiring of most redox enzymes on bare electrodes is difficult to achieve because of the instability of the biological matrix upon interaction with the electrode surface and the often deeply embedded prosthetic groups in the protein polypeptide shell [1]. Efficient enzyme wiring also often requires a redox mediator to shuttle the electrons between enzyme cofactor and electrode. The immobilization of both the enzyme and the redox mediator need a subtle combination of parameters (flexibility, hydrophilicity, stability,…) to achieve bioelectrocatalysis [2]. In order to overcome this drawback, recent advances in nanomaterial synthesis and characterization have provided nano-scaled support for the efficient combination of enzymes and redox mediators [3, 4]. Nanomaterials provide high surface areas for enzyme and redox mediator loadings and afford a favourable microenvironment which helps the enzyme to retain its bioactivity. In addition, nanomaterials can improve electron transfer between the enzyme active site, the redox mediator of interest, and therefore the electrode surface. Many efforts have been initiated to immobilize redox mediators on nanostructured materials. In this respect, multiwalled carbon nanotubes (MWCNTs) have attracted considerable interests owing to their low electrical resistance, high accessible surface area, good mechanical strength, and excellent chemical stability [3]. Possessing many unique properties such as high electrocatalytic effect, strong adsorption ability, and excellent biocompatibility, CNTs are often used for improving the electron transfer in biosensors and biofuel cells [5]. Furthermore, CNT sidewalls can be functionalized by covalent and non-covalent techniques. In particular, CNT-based electrodes have proven to be efficient support for redox mediators such as osmium complexes or ferrocene for the wiring of glucose oxidases in glucose biofuel cells [6]. To date, ferrocene has been one of the most successful redox mediators for glucose oxidase due to its well-balanced electrochemical properties and suitable redox potential. The glucose oxidase (GOx) is one of most widely employed enzyme for oxidizing glucose in biosensors and biofuel cells due to its stability and high selectivity towards glucose. In this respect, ferrocene has been extensively immobilized on CNTs for GOx wiring by different techniques such as π-stacking of pyrene-modified ferrocene [7], amide coupling with CNT defects [8] and covalent cycloaddition reactions [9]. The functionalization of nanoparticles has also been investigated. In particular, the electrochemistry of grafted ferrocenes were investigated on fullerenes [10], silica NPs [11]... ZnO nanoparticle is a typical inorganic semiconductor material presenting a wide band gap (Eg = 3.37 eV) and a large excitation binding ability (60 meV) [12]. Due to its nontoxicity, biological compatibility, high catalytic efficiency, strong adsorption

ability, fast electron transfer rate and easy preparation, ZnO nanoparticles have become a favourable material for biomolecules immobilization [13–16]. The high isoelectric point (IP: 9.5) of the ZnO nanoparticle makes it suitable for electrostatically-driven adsorption of glucose oxidase (GOx) with low IP of 4.2.

Consequently the modification of CNTs with other nanomaterials such as semiconductor nanoparticles can lead to composite materials that not only possess properties of the individual components but can also demonstrated outstanding synergistic effects [4].

In this context and with the aim to develop a new strategy offering an optimum electron transfer between the glucose oxidase active centre and the electrode surface, we report the synthesis of ferrocene (Fc)-functionalized ZnO nanoparticles and their combination with MWCNT-based electrodes. This new synthetic procedure is based on simple and efficient steps resulting in surface modified ZnO nanoparticles exhibiting good dispersibility in aqueous media as well as electrochemical redox activity due to the binding of ferrocene moieties. Hence the remarkable synergistic effects of the designed ZnO nanoparticles and the MWCNTs resulted in the high surface coverage of ferrocene and excellent mediated electrocatalytic oxidation of glucose. These bioanodes were employed in a glucose fuel cell set-up, using a platinum-based gas-diffusion cathode (**figure 1**).

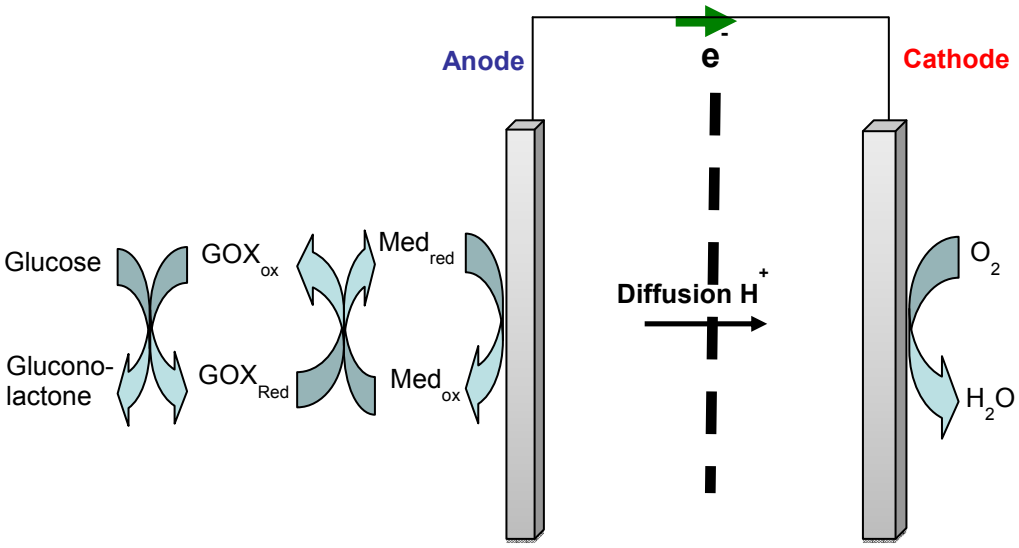


Figure 1: Principle of the bio-fuel cell described in the present manuscript

2. Experimental

2.1. Reagents and apparatus

MWCNTs (>95% purity) were purchased from NANOCYL™ (NC 7000) and oxidized by fluxing in concentrated nitric acid for 3 h at 110 °C prior to use. The carbon black/Pt catalyst was purchased from Johnson Matthey Catalysts. All other chemicals were from Sigma Aldrich and were used without further purification. Phosphate buffer solutions (PBS) at neutral pH were prepared using 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4. Glucose stock solution was kept at least 24 h after preparation for mutarotation. Glucose oxidase solution was prepared with PBS and stored at 4 °C when not in use.

Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA) including a SynergyPak® unit. Water achieved resistivity was 18 MΩ.cm at 25 °C. A Heraeus Multifuge X3R laboratory centrifuge (Thermo Fisher Scientific, France) and a SONOREX DIGITEC sonification water-bath (Roth, France) were used.

TEM images were acquired and processed using the Gatan Digital Micrograph environment. To prepare the sample for TEM analyses, a droplet of 3 µl containing the nanoparticles in aqueous solution was deposed on an amorphous ultrathin carbon film supported on a 400 mesh copper grid. The sample was analyzed using a Tecnai OSIRIS microscope operating at 200 kV. Elemental mapping of isolated particles was performed by energy dispersive X-ray spectroscopy (XEDS) to localize elements in the nanoparticles. The EDS spectra were recorded in STEM HAADF mode, using a new XEDS system (super X, FEI Company). Spectra and mapping EDS were performed with the software "ESPRIT".

The electrochemical measurements were performed on a VMP3 BioLogic potentiostat using the EC-Lab V 9.55 software. The conventional three-electrode system included a functionalized graphite electrode as working electrode, a saturated calomel as reference electrode (SCE) (obtained from Radiometer Analytical reference 421), and a platinum wire as auxiliary electrode and was used to characterize the ZnO-Fc nanoparticles.

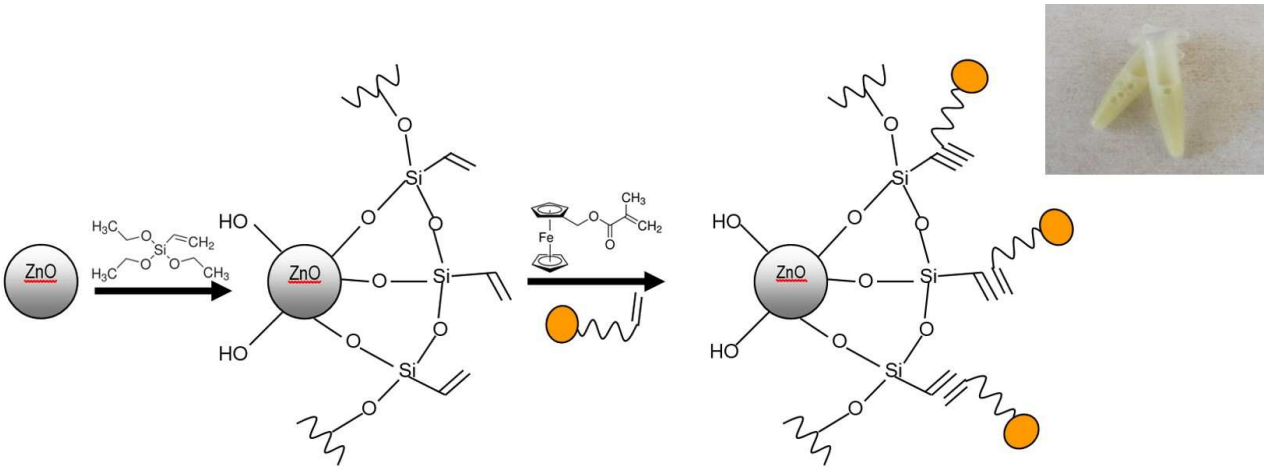
2.2. Synthesis and surface modification of ZnO nanoparticles

The synthesis of pure ZnO nanoparticles by co-precipitation has been previously described in the literature [17]. The synthetic path of the modified ZnO nanoparticles presents 3 steps:

ZnO nanoparticles were first prepared by reacting zinc nitrate hydrate with sodium carbonate. In order to prepare a large batch of ZnO nanoparticles (*e.g.* 10 g), an aqueous solution of $Zn(NO_3)_2.6H_2O$ (36.50 g dissolved in distilled water, 0.2 M) was added dropwise to an aqueous solution of sodium carbonate (13.78 g dissolved also in distilled water, 0.5 M) under vigorous stirring at room temperature. The resulting solution was stirred for 2 hours at room temperature. The precipitate was filtered using a Buchner funnel. The obtained powder was dried overnight at 100 °C in a drying oven and then calcinated in air (100 Lh^{-1}) at 400 °C for 4 hours.

In order to functionalize the ZnO nanoparticles, 1 g of the synthesized nanoparticles were grounded in a mortar and then dispersed in isopropanol (500 mL) under vigorous stirring at room temperature. The basic hydrolyzing agent, an aqueous ammonia solution (61 mL, 30%w) was added, followed by a solution of triethoxyvinylsilane (0.323 g, 1.7 mmol) prepared in isopropanol (50 mL). The resulting mixture was stirred at room temperature for 24 h. Afterwards, the ZnO nanoparticles were recovered by centrifugation (8000 rpm for 10 min), and washed thoroughly three times with ethanol and once with water. Ultrasonification was used to disperse nanoparticles aggregated into the washing solvent and to increase the desorption rate of the remaining by-products from the surface of the synthesised nanoparticles. Finally, the surface modified ZnO nanoparticles were dried in an oven at 50 °C for 4 h.

We present an original way for the ZnO functionalization by the ferrocene. The obtained surface modified ZnO nanoparticles (0.5 g, 5 %w) were then dispersed in an aqueous solution containing PVA (0.1 g, 1%w) followed by the addition of ferrocenylmethylmethacrylate (0.5 g, 1.76 mmol, 5 %w) under vigorous stirring. The mixture was stirred for 4 days at room temperature in order to obtain a homogeneous solution. The resulting dispersion was dip-frozen dropwise into liquid nitrogen, filtered and then freeze-dried for 48 hours. The obtained ferrocene functionalized ZnO nanoparticles (ZnO-Fc) are formulated as beads having between 2 and 3 mm diameter (**Figure 2**). Theses beads are easily dispersed in water as shown in the insert of the **Scheme 1**. **Scheme 1** also shows the ZnO functionalization procedure by the ferrocene mediator.



Scheme 1: Mechanism for the ZnO nanoparticles functionalization by the ferrocene redox mediator; (inset): image of the ZnO-Fc based dispersion in water

2.3. Fabrication of the modified electrodes

Glassy carbon electrode (GCE) was polished carefully with 2 µm diamond paste on polishing cloth to obtain mirror like surface. Then it was rinsed with distilled water and followed by successive ultrasonic bath to remove the physically adsorbed substance. Oxidized MWCNTs were dispersed in distilled water by using an ultrasonic water-bath to obtain a MWCNTs suspension (2 mg mL^{-1}). A suspension of the ferrocene modified ZnO nanoparticles (30 mg mL^{-1}) and glucose oxidase (3.5 mg mL^{-1}) was prepared by using an ultrasonic bath. 500 µL of the latter dispersion was drop-casted on the 500 µL of the MWCNT suspension under vigorous agitation. 20 µL of a Nafion ® 117 solution was added (5% in a mixture of lower aliphatic alcohols and water). Then, a droplet of 10 µL of the obtained suspension was dropped onto the GCE surface and left to dry for 4 hours at 4 °C to obtain the ZnO-Fc/GOx/MWCNTs/Nafion modified glassy carbon electrode. The modified electrode was then rinsed three times in distilled water to remove the non-fixed enzyme. When not in use, the electrode was stored at 4 °C in a refrigerator.

2.4. Preparation of the fuel cell

For the glucose fuel cell experiments, 500 µL of the catalytic ZnO-Fc/GOx/MWCNTs/Nafion ink prepared as described in **Section 2.3** was deposited onto the anodic compartment of a Nafion® membrane (DuPont, USA). At the cathodic compartment of the fuel cell, an

ink based on carbon particles (Vulcan XC-72) covered with platinum nanoparticles (60% Pt) was used. The carbon black/Pt nanoparticles ink (C/Pt) was sprayed with a 600 μg cm^{-2} Pt coverage. One layer of the deposited (C/Pt) consists of 13 μL of ink per square centimetre. The electrodes were sandwiched by pressing at 1 ton for 2 min at room temperature. The fuel cell components were firmly clamped together in a polycarbonate holder (jet-cut from 5 mm Makrolon, Bayer Sheet Europe GmbH, Darmstadt, Germany), using silicone rubber gaskets (jet-cut from 2 mm thick silicone elastomer foil, VWR, Darmstadt, Germany) and four screws. The silicon rubber gaskets were used to avoid a crossover reaction. A copper grid was used as electron collector. This grid was designed so that the oxygen required at the cathodic side can be directly picked up from the atmospheric air (Air breathing cathode). The electric connection was made through the small copper strips left from the cell. Both cathode and anode have active electrode dimensions of 1.5 cm x 1.5 cm. **Figure 8** depicts the construction of the complete fuel cell. The anodic side of the biofuel cell was grooved in serpentine geometry with a flow channel width of 2 mm and a depth of 0.5 mm and was supplied with the phosphate buffer solution (pH 7) containing 50 mM glucose under constant flow using a peristaltic pump. The fuel cells performances were tested under ambient conditions at 20 °C. Positive potentials were applied linearly using a linear sweep voltammetry to the cathode at a sweep rate of 1 mV s^{-1} and the oxidation current was monitored, with an anode as both counter and reference electrodes.

Figure 2: Obtained ferrocene functionalized ZnO nanoparticles formulated as beads

3. Results and discussion

3.1. Synthesis and characterization of the ZnO-Fc/MWCNTs electrode

An original way of synthesis of modified ZnO nanoparticles by sol-gel process within 3 steps is presented in this work. First the classical combination of co-precipitation of the appropriate precursors combined to calcination at 400 °C gave the original ZnO cores [17]. Further surface modification using an unsaturated siloxane in a basic medium provided the desired intermediate nanometric species to interact additionally with a ferrocene acrylate derivative via π-π stacking greatly emphasized in the aqueous medium. Finally, consolidation of the aromatic shell was performed (in the presence of a slight amount of PVA) by dip-freezing in liquid nitrogen. The obtained composite was then subjected to freeze-drying resulting in the desired ferrocenyl surface modified ZnO nanoparticles.
Transmission Electron Microscopy (TEM) analysis was carried out on isolated nanoparticles (**Figure 3**). TEM experiments give evidence for agglomerated nanoparticles of ZnO, with a spherical morphology and a mean size of 90 nm. At a higher magnification, a coating could be observed at the surface of ZnO nanoparticles, associated with a difference in chemical composition.

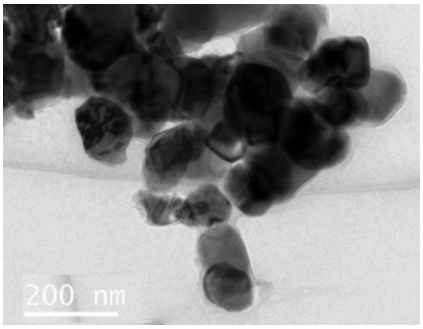


Figure 3: TEM micrograph of the ZnO-Fc nanoparticles

In order to demonstrate the presence of ferrocene molecules on the surface of ZnO nanoparticles, several nanoparticles were analysed using XEDS. EDS Mapping was performed at the edge of iron and zinc. As observed in **Figure 4**, EDS mapping reveals a homogenous coverage of iron atoms (in blue) surrounding ZnO nanoparticles (in red). TEM investigations were also performed on the ZnO-Fc nanoparticles adsorbed on MWCNTs. The TEM micrograph clearly shows the dispersion of ZnO-Fc particles on MWCNTs. Likewise,

XEDS analyses give also evidence of the close vicinity of Fe atoms (in green) to Zn atoms (in red), and equally the close vicinity of ZnO particles and MWCNTs (in blue). The homogeneous distribution of Fe atoms confirms the anchoring of the ferrocene molecules to the surface of the ZnO nanoparticles. The linescan extracted from this hypermap allows a vouching of the localization of iron on the surface of ZnO nanoparticles, the main supported on carbon nanotubes.

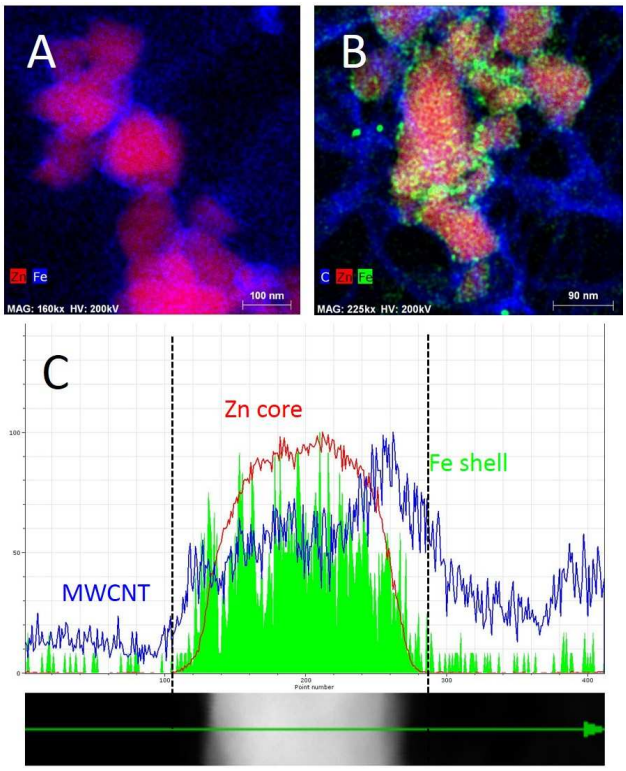


Figure 4: XEDS analysis of ferrocene-modified ZnO nanoparticles both (A) isolated and (B) supported on carbon nanotubes. (C) Linescan reveals the abundance of ferrocene on the surface

3.2. Electrochemical characterization of the ferrocene cored ZnO nanoparticles

The cyclic voltammograms (CVs) of the ZnO-Fc/CNT modified glassy carbon electrode in 0.1 M PBS (pH 7.0) was investigated. As shown in **Figure 5**, very small redox peaks appeared at ZnO-Fc/GCE (curve a). After immobilizing ZnO-Fc on the CNT sidewalls, stable, well-defined and reversible redox peaks were observed, corresponding to the ferrocene/ferrocenium redox couple (curve b). Peak currents were greatly increased when the ZnO-Fc nanoparticles were assembled onto the CNT-modified electrode compared to GCE.

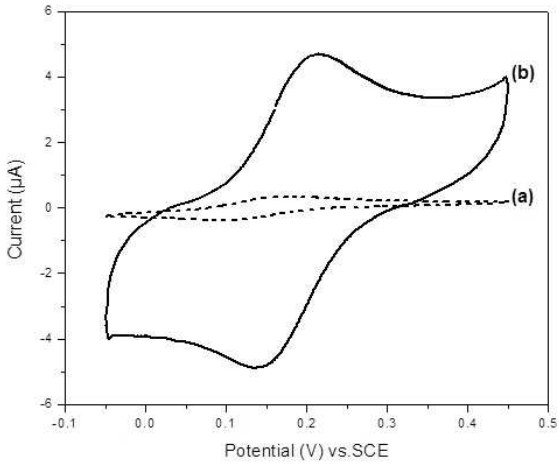


Figure 5: Cyclic voltammogram of (a) ZnO-Fc and (b) ZnO-Fc/CNT on glassy carbon electrode in 0.1 M phosphate buffer solution (pH 7) at 50 mV s^{-1} Scan rate

Calculated from the average value of anodic and cathodic peak potentials, the formal potential ($E^{0\prime}$) of the Fc/Fc^+ redox couple was 175 mV. This value is very close to the ferrocenylmethanol redox potential [18], confirming that the redox peak pair, which appears at the modified GCE, corresponds to the redox mediator attached to the ZnO nanoparticles. However, the separation of anodic to cathodic peak potentials (ΔEp = 25 m V at slow scan rates) is higher than expected for a surface-bounded redox specie (ΔE_{TH} = 0 mV), which can be attributed to the increase of the distance between the redox mediator and the electrode surface. The ratio of anodic to cathodic peak current is about one.

CVs at different scan rates were recorded in 0.1 M PBS (pH 7.0). As shown in **Figure 6**, both the anodic and the cathodic peak currents were linearly proportional to the scan rate in the range of 20 to 400 mV s^{-1}, indicating a surface-controlled electrochemical oxidation/reduction of the Fc/Fc^+.

The linear regression equation is Ipa = 30.65 υ (V s^{-1}) + 3.6494, R^2 = 0.99989;

Ipc = -29.69 υ (V s^{-1}) - 3.9374, R^2 = 0.9997

This indicates that the electron transfer process for the ZnO-Fc/CNT/GOx/GCE is a surface-controlled mechanism in the range mentioned above.

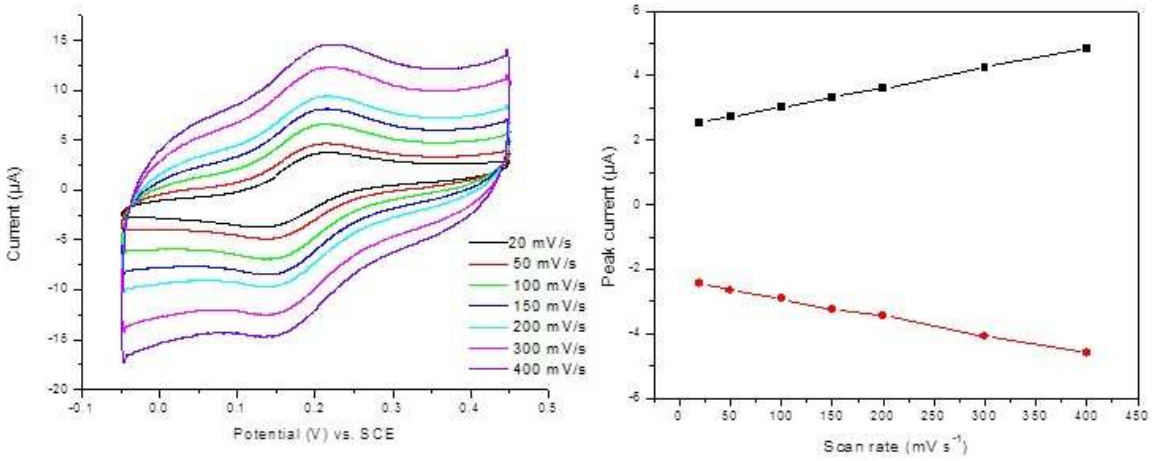


Figure 6: Left: Cyclic voltammogram of ZnO-Fc/CNT on glassy carbon electrode in 0.1 M phosphate buffer solution (pH 7) at 20, 5, 100, 150, 200, 300 and 400 mV s^{-1} scan rate. Right: Anodic and cathodic peak current *versus* the scan rate for ZnO-Fc/CNT on glassy carbon electrode

The surface coverage (Γ) of the electroactive ferrocene was investigated based on the following Laviron equation [19] $Q = nFA\Gamma$ where F is the Faraday constant, Q is the total amount of charges, n and A stand for electron transfer number and the electroactive surface area of the electrode, respectively. The total amount of charge (Q) passed through the electrode for reduction or oxidation of electroactive species can be calculated through integration of CV peak [20].

The value of Γ for the ZnO-Fc/CNTs/GCE was estimated to be 6.49 $\times 10^{-10}$ mol cm^{-2}, which is almost two-order-of-magnitude higher than the value (2.86 $\times 10^{-12}$ mol cm^{-2}) for the ZnO-Fc monolayer deposited on the bare-electrode, underlining the fact that both CNT electrode and ZnO nanoparticles provide a large and effective surface-area for ferrocene immobilization.

3.3. Biolectrocatalytic glucose oxidation by a glucose oxidase/ZnO-Fc/MWCNT electrode

The electrocatalytic behaviour of a glucose oxidase/ZnO-Fc MWCNT electrode was evaluated by cyclic voltammetry. **Figure 7** shows the cyclic voltammograms of the modified electrode in phosphate buffer solution (PBS) at pH 7 (a) in the absence and (b) presence of glucose.

In the absence of glucose, only the cyclic voltammogram of ZnO-Fc nanoparticles was observed. When glucose solution was added into the electrochemical cell before measurement, an electrocatalytic response appeared with an increase of the oxidation current.

This result strongly indicates the presence of an enzyme-dependent catalytic oxidation of glucose, which originated from the GOx reaction mediated by a ferrocene. These experiments confirm that ferrocene-grafted ZnO nanoparticles immobilized on MWCNT electrodes efficiently shuttle electrons from the electrode surface to the FAD redox centre of the glucose oxidase.

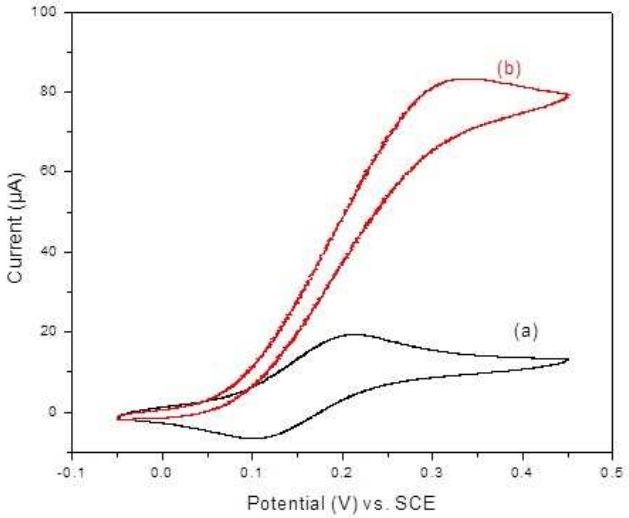


Figure 7: Cyclic voltammogram of ZnO-Fc/CNT/GOx on glassy carbon electrode in 0.1 M phosphate buffer solution (pH 7) (a) in absence and (b) in presence of glucose. Scan rate: 50 mV s^{-1}.

3.4. Fuel cell characterization

For the glucose/oxygen fuel cell device fabrication (**figure 8**), the bio anode was integrated in a chamber and operates in liquid phase supplied by a flow-through design. The continuous flow rate of 5 ml min^{-1} diminishes substrate depletion, by-product accumulation, and allows mass transport investigations of the anode under fuel cell conditions. The cathode operates in gas phase (ambient air) that reduces the overcoming limitations associated with the low oxygen solubility in aqueous media. The active area of the fuel cell based on the Nafion® membrane is of 5.76 cm^2. The outer area is of 53.72 mm^2. Glucose is oxidized at the bio anode and oxygen is reduced at the cathode side.

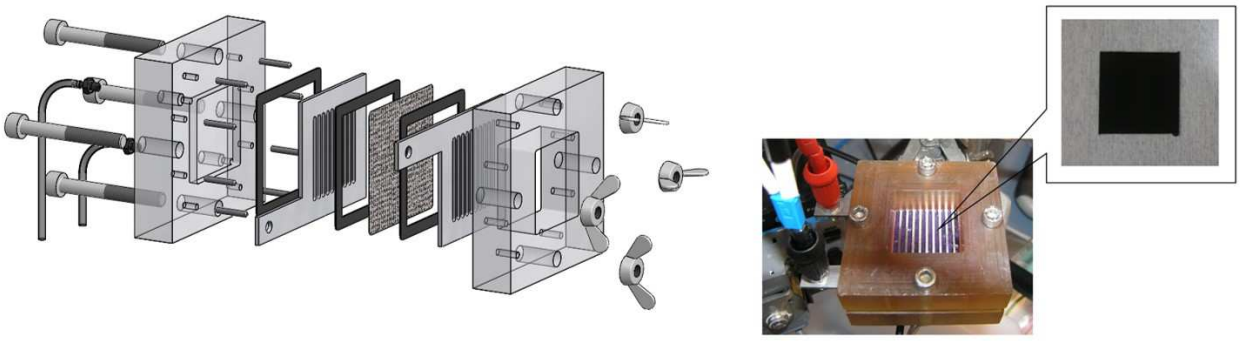


Figure 8: Schematic representation and image of the glucose/air fuel cell

Typical bell shaped curve showing the performances of the biofuel cell based on ZnO-Fc/ CNTs/GOx ink for the bio anodes and a carbon black/Pt for the cathode is presented in **Figure 9**.

The measured OCV (Open Circuit Voltage) is about 600 mV for the biofuel cell. The power peak value is around 53 μW cm^{-2} at 300 mV for the Nafion® based biofuel cell. The biofuel cell based on Fc/CNTs/GOx ink, gives a maximum power density of 13 μW cm^{-2} at 200 mV with an open circuit potential of 430 mV.

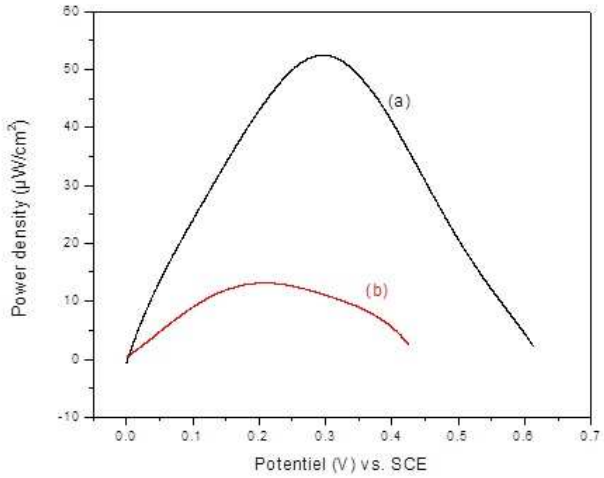


Figure 9: Power density plots of a glucose/air fuel cell using C/Pt as cathode catalyst and (a) ZnO-Fc/CNTs/GOx or (b) Fc/CNTs/GOx as anode catalyst. The flow rate of glucose is of 5 mL min^{-1} and the cathode operates under air conditions

4. Conclusion

Glucose oxidase (GOx) can be effectively immobilized on ZnO-Fc nanoparticles modified electrode. The immobilized glucose oxidase maintained its bioactivity, showing efficient mediated electron transfer with ferrocene/ZnO hybrid nanoparticles. The constructed ZnO-Fc/CNTs modified electrode exhibit high ferrocene surface coverage. Due to the design of the newly prepared ZnO-Fc nanoparticles, leakage of ferrocene was prevented from the novel supramolecular architecture, and the efficiency of the π-stacking was exhibited in aqueous media. From the cyclic voltammetry measurements, the grafting of the ferrocenyl moieties was demonstrated to be redox active and function as electron shuttling agent between glucose oxidase and the electrode. The use of the new architecture of ZnO supported electron mediators to shuttle electrons from the redox centre of the enzyme to the surface of the working electrode can effectively provide successful glucose oxidation. This new way of nanoparticles decoration with mediators can be used for the development of three dimensional architectures using different mediators suitable for the redox potential of the used enzyme.

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Notes

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