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A novel ferrocene-tagged peptide nanowire for enhanced electrochemical glucose biosensing

Jianxiu Wang, Ding Li, Minghui Yang* and Yi Zhang*

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Ferrocene (Fc)-tagged peptide nanowires (Fc-PNWs) were synthesized *via* the self-assembly of Fc-coupled diphenylalanine (Phe-Phe, FF) and then used as supporting matrix for immobilization of glucose oxidase (GOx). Scanning electron microscopy (SEM) characterization indicated that the Fc-PNWs were twisted together with the diameter around 50 nm. The GOx-functionalized Fc-PNWs contained both mediator Fc and GOx for the electrochemical detection of glucose. Thus, by simply dropping the biocomposite onto the electrode surface in a single step, the resulting biosensor displays high sensitivity, wide linear range and good stability towards glucose detection. The good performance of the biosensor originated from the large amount of Fc moieties contained in the nanowire and the facile electron transfer between Fc and GOx. For real sample analysis, the glucose contents in blood samples determined by the biosensor were in good agreement with those obtained using the glucose detection kit. The simplicity of the biosensor preparation process enables mass production of the biosensor with broad potential commercial applications. The synthesized Fc-PNWs can also be used in diverse sensing and biosensing fields.

1. Introduction

Since the pioneering work of Clark and Lyons that integrated enzymes onto electrode surfaces,[1] enzyme-based electrochemical biosensors have undergone tremendous development.[2–4] Redox centers in most enzymes are located deep inside the protein shell, making them electrically inaccessible. Consequently, redox mediators are introduced to "wire" the enzyme's active centers to ensure efficient electron transfer between the enzyme and electrode. Heller's group[5] developed such "wired" enzyme electrodes based on immobilization of enzymes in redox hydrogels or polymers. Hydrogels or polymers were made mostly by attaching redox functions to poly(4-vinylpyridine) (PVP) or poly(*N*-vinylimidazole) (PVI) and the redox functions are usually complexes of $Os^{2+/3+}$. Examples include redox hydrogel formed by cross-linking PVI complexed to Os-(4,4′-dimethylbpy)$_2$Cl (termed PVI_{15}-dmeOs) with poly(ethylene glycol) diglycidyl ether (PEG), and redox polymers based on cross-linkable PVP complex of $[Os\text{-}(bpy)_2C1]^{+/2+}$.[6] However, the synthesis of such polymers is rather complex and time-consuming.

Besides the abovementioned $Os^{2+/3+}$, ferrocene (Fc) and its derivatives are another type of widely used redox mediator due to favorable chemical and electrochemical properties.[7,8] Ferrocenyl residues can be covalently conjugated onto enzymes.[9] However, such conjugation results in partial denaturing of the enzymes. Most often, Fc and enzymes are immobilized separately onto the electrode surface. With the advancement of nanotechnology in recent years, various Fc-based nanomaterials have been reported, such as Fc functionalized dendrimer and gold nanoparticles.[10–12] If a single biocomposite that contains both Fc and enzyme can be prepared, it would significantly simplify the biosensor fabrication process by simply immobilizing the biocomposite onto the electrode.

Recently, peptide-based nanomaterials have attracted much attention due to their good biocompatibility, simple self-assembly process and flexibility in functionalization. Among the diverse peptide molecules reported, phenylalanine (Phe, F) and its derivatives have been widely researched for production of various nanomaterials.[13–15] The unique properties of peptide-based nanomaterials have been exploited for application in drug delivery and biosensing.[14,16,17] Recently, our group discovered that Fc-modified phenylalanine (Fc-F) could aggregate in water *via* a rapid self-assembly mechanism to form stable hydrogels,[18] while Fc-functionalized diphenylalanine monomers (Fc-FF) could self-assemble into nanowire structures.[19] In this work, we report the use of synthesized Fc-tagged peptide nanowires (Fc-PNWs) for enhanced electrochemical glucose biosensing. With the immobilization of glucose oxidase (GOx) onto the Fc-PNW surface, the resulting biocomposite displays high sensitivity towards glucose detection.

College of Chemistry and Chemical Engineering, Central South University, Changsha, China, 410083. E-mail: yangminghui@csu.edu.cn; yzhangcsu@csu.edu.cn

2. Experimental methods

2.1. Reagents and apparatus

Glucose oxidase (GOx, from *Aspergillus niger*, EC 1.1.3.4.150000 units per g), D(+)-glucose, and chitosan (CS, 75% deacetylation) were purchased from Sigma-Aldrich (St Louis, MO, USA). Dichloromethane (DCM, ACS grade) was stored with molecular sieves, dried over CaH_2 and distilled right before synthesis. *N*-Hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Highfine Biotech Co. (Suzhou, China). Boc-Phe-OH and H-Phe-OMe·HCl were purchased from GL Biochem (Shanghai, China). For thin-layer chromatography (TLC), glass plates coated with silica gel (60 GF_{254}) were used. For column chromatography, 18–22 cm of 200–300 mesh silica gel (Silicylcye, 230–240 mesh) were packed into a 2.7 cm wide and 45 cm long glass tube. Phosphate buffered saline (PBS, pH 7.4) was used as the electrolyte for all electrochemical measurements. Other reagents were of analytical grade.

Electrochemical measurements were performed on a CHI-650D electrochemical workstation (Shanghai CH Instruments Co., China). A conventional three-electrode system was used with a glassy carbon (3 mm in diameter) as the working electrode, an Ag/AgCl electrode as the reference electrode and a platinum wire as the auxiliary electrode. Scanning electron microscopy (SEM) images were obtained on a Nova Nano-SEM230 (FEI, USA).

2.2. Synthesis of Fc-Phe-Phe-COOH

The synthesis of Fc-Phe-Phe-COOH was executed as described in a previous report with a minor difference.[19] Initially, Boc-Phe-COOH (4 mM) and HBTU/HOBt (4.4 mM) were dissolved in DCM (50 mL). Et_3N was then added dropwise to activate the carboxyl group for 1 h at 0 °C. H-Phe-OMe·HCl (4.4 mL) was added and the reaction mixture was stirred overnight. This was followed by washing with saturated aqueous solutions of $NaHCO_3$, HCl (10%), and water, and drying over Na_2SO_4 under reduced pressure. The crude product was purified by flash column chromatography (DCM–EtOAc = 2 : 1, v/v), and then evaporated under reduced pressure in a rotovap to white oil. The oil was dissolved in DMSO and dried overnight in a freeze dryer, producing white crystals at the end, which is Boc-Phe-Phe-OMe.

Boc-Phe-Phe-OMe (2 mM) was dissolved in the mixture of DCM (20 mL) and trifluoroacetic acid (10 mL). The reaction mixture was stirred for 30 min, and the resulting H-Phe-Phe-OMe was treated with Et_3N in CH_2Cl_2 (2 mL, pH ~ 8). The solution was diluted in DCM (20 mL) and mixed with Fc-OBt (2.2 mM), which was obtained by the standard HBTU/HOBt method in solution of activated Fc-OH. The reaction mixture was stirred for 1 h and purified by flash column chromatography (DCM–EtOAc = 3 : 1, v/v) to obtain Fc-Phe-Phe-OMe. Fc-Phe-Phe-OMe (1 mM) was then dissolved in trifluorofuran (12 mL) and mixed with LiOH (6 mL, 2.5 mM). The reaction mixture was then stirred for 2 h, and then purified again by flash column chromatography (DCM–EtOAc–MeOH = 9 : 3 : 1, v/v/v) to obtain Fc-Phe-Phe-COOH.

2.3. Self-assembly of Fc-PNWs and conjugation of GOx onto Fc-PNWs

Hexafluoroisopropanol was used to prepare the stock Fc-Phe-Phe-COOH solution (100 mg mL^{-1}), which was diluted with MeOH to 2 mg mL^{-1}. During the solvent evaporation, Fc-Phe-Phe-COOH molecules self-assemble into Fc-PNWs. Fc-PNWs were dispersed into chitosan (CS) solution (1%, w/w) to reach a final concentration of 1 mg mL^{-1}. Upon stirring for 2 h and centrifugation, a mixture of 0.25% glutaraldehyde (v/v) and 10 mg mL^{-1} GOx was added into the solution. Free GOx remaining in the solution was separated *via* centrifuging, and the final Fc-PNW-GOx biocomposite was washed with PBS and stored at 4 °C before use.

2.4. Construction of Fc-PNW-based glucose biosensor

To prepare the electrochemical glucose sensor, 5 μL of the Fc-PNW-GOx bioconjugate were cast onto each glassy carbon electrode. After drying the electrode surface in the ambient environment and washing the electrode with PBS, the Fc-PNW-GOx-modified electrode was used for glucose determination.

3. Results and discussion

The preparation of Fc-PNWs was simple and efficient. Fc was first attached onto diphenylalamines (Fig. 1). Then, the resultant Fc-Phe-Phe-COOH was dissolved in hexafluoroisopropanol. Finally, after diluting the solution with MeOH and allowing the solvent to evaporate, nanowires were formed. The synthesized Fc-PNWs incorporated a significant number of Fc moieties that could serve as mediators for GOx. After the conjugation of GOx onto Fc-PNW, the final Fc-PNW-GOx biocomposite contained both the mediator and GOx, which means that the biocomposite can be utilized for electrochemical glucose detection.

Fig. 2 shows the SEM image of the synthesized Fc-PNWs. It can be seen that the nanowires are twisted or intertwined

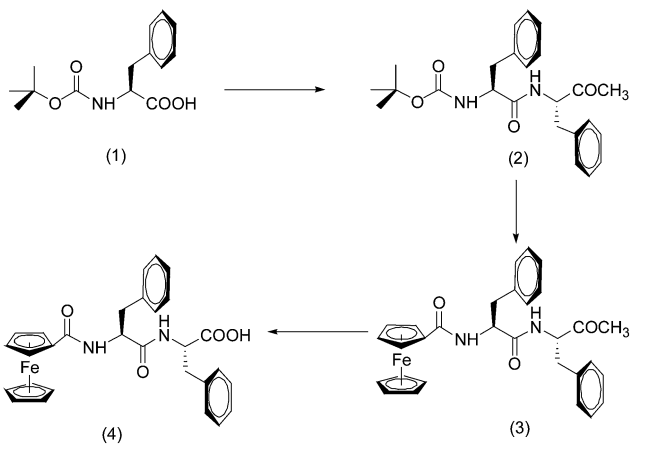

Fig. 1 Synthetic route for producing Fc-Phe-Phe-COOH.

together and display a porous 3D structure. The diameters of the nanowire are around 50 nm, while the length can extend to a micrometer. The morphology and self-assembly process appear to be highly comparable to those of the Phe-Phe dipeptide.[20] Our previous work reported the self-assembly of Fc-PNWs from Fc-Phe-Phe-OMe.[19] It was discovered that Fc-Phe-Phe-OMe generated straight and rigid nanowire, while Fc-Phe-Phe-COOH generated soft and twisted nanowire.

To render the Fc-PNWs water-dispersible, chitosan (CS) was adsorbed onto the Fc-PNW surface *via* electrostatic attraction between negatively charged carboxylic groups on the Fc-PNW and positively charged amino groups on CS. With multiple amino groups on the CS-coated Fc-PNWs, many biomolecules can be easily conjugated to the resultant nanocomposite. For example, we found that GOx can be conveniently attached to the CS-coated Fc-PNWs using glutaraldehyde as the cross-linking reagent. Thus, the network of twisted and intertwined GOx-conjugated Fc-PNWs not only serves as a matrix rich in pores for diffusion of glucose and its oxidation products, but also provides a myriad of redox mediators for facile electron transfer.

We then characterized the Fc-PNW-based glucose sensor with cyclic voltammetry (CV). Fig. 3A shows successive CV scans acquired at the as-prepared electrode. It can be seen that the electrode displays a reversible wave with the anodic and cathodic peak potentials at 0.42 V and 0.48 V, respectively. The good reversibility can be attributed to the short distance between the Fc tags on the nanowires and the underlying electrode.[21–23] In addition, the narrow separation between the anodic and cathodic peaks indicated good conductivity of Fc-PNW, which is in accordance with literature reports about the good conductivity of diphenylalanine peptide self-assembled nanomaterials.[15,24] Furthermore, the large number of Fc tags produces easily discernible currents, amplifying otherwise smaller currents and enhancing the sensitivity of the sensor. During successive CV scans, no obvious peak current change was observed, suggesting that the Fc-PNWs are firmly immobilized onto the electrode.

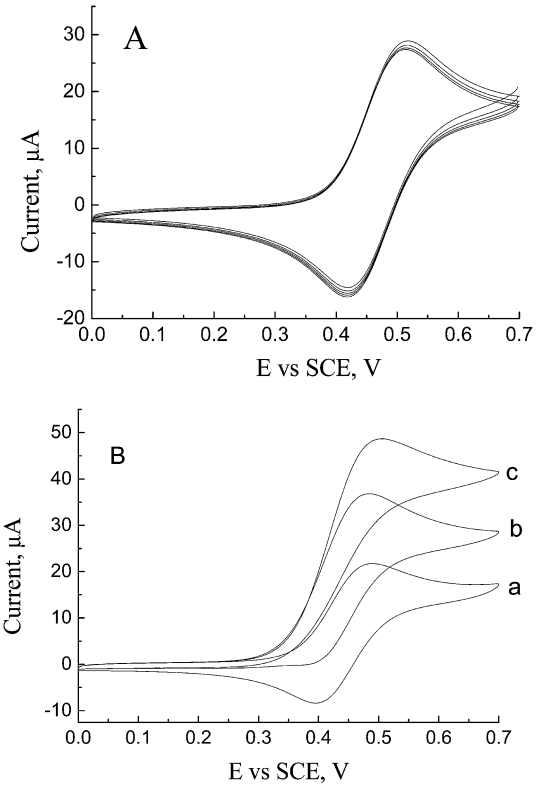

Fig. 3 (A) Five successive CV scans at Fc-PNWs modified glassy carbon electrode. (B) CVs of the Fc-PNW-based glucose biosensor in the absence (a) and presence of 10 (b) and 20 mM (c) glucose. Scan rate, 0.1 V s^{-1}.

After glucose addition, the Fc oxidation peak increases at the expense of the ferrocenium (Fc^{+}) reduction peak, a response typical of GOx-catalyzed electrode reaction (Fig. 3B).[8,25,26] To gauge the sensitivity and linear range of the biosensor towards glucose detection, we recorded amperometric curves at the Fc-PNW-based glucose sensor by continuously adding glucose into the solution. The effect of detection potential on the sensitivity of the glucose detection was studied. As seen in Fig. 4, with the increase of detection potential, the sensitivity of the biosensor

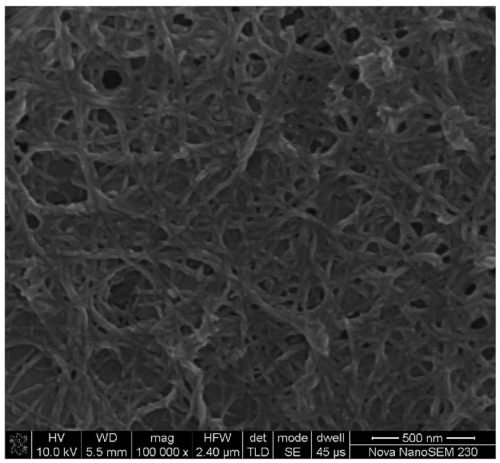

Fig. 2 SEM image of the synthesized Fc-PNW.

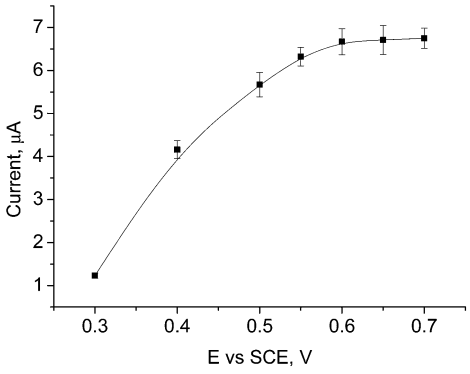

Fig. 4 Effect of detection potential on the sensitivity of the biosensor to 5 mM glucose. Error bar = SD (n = 3).

to 5 mM glucose was increased. When the potential reached 0.6 V, further increase of the detection potential resulted in no improvement of sensitivity. On the other hand, when the detection potential was too high, the biosensor was susceptible to interference of certain compounds that are often present in serum samples, such as ascorbic acid (AA), uric acid (UC) and acetaminophen. So a detection potential of 0.6 V was selected.

As seen in Fig. 5A, the Fc-PNW-based glucose sensor has a fast response (reaching each steady state within 5 s). The fast response is again indicative of a facile electron-transfer process between the reduced form of GOx and the electrogenerated Fc^+ moieties at the nanowires. Since the Fc tags are not affixed to the nanowires with a flexible chain or bond but are in close vicinity of the GOx molecules, the electron transfer pathway between GOx and Fc is shorted, and consequently detection sensitivity is enhanced. The linear range of the Fc-PNW-based glucose sensor (Fig. 5B) spans from 0.01 to 10 mM (sensitivity of 21.9 μA cm^{-2} mM^{-1}), with a detection limit of 5 μM based on $S/N = 3$. The performance of the proposed biosensor for glucose detection was compared with other kinds of glucose sensors including non-enzymatic glucose sensors and enzymatic glucose sensors. As shown in Table 1, the performance of our biosensor is comparable to or even better than that of other reported glucose sensors.

To study the repeatability of the proposed biosensor, a single electrode was examined for successive detection of 5 mM glucose. A relative standard deviation (RSD) of 6.2% was obtained for 10 successive detections. The reproducibility of the biosensor was also studied. Six biosensors were prepared independently, and an RSD of 7.3% was obtained using the prepared biosensors for detection of 5 mM glucose.

The amenability of the Fc-PNW-based glucose sensor for real sample analysis was assessed by determining glucose concentrations in blood samples donated by healthy and diabetic persons. Results were compared with those determined by the colorimetric method using glucose detection kit. As seen in Table 2, glucose contents determined by the two methods are quite similar. These results also demonstrated the good selectivity of the biosensor, indicating AA, UC and acetaminophen in the serum did not interfere with glucose detection. The stability of the Fc-PNW-based glucose sensor was also investigated. When not in use, electrodes were stored at 4 °C in phosphate buffer, which showed little signal degradation even after 1 month (around 90% of the initial sensitivity remained). The observation that GOx is not denatured and remained active can be attributed to biocompatibility of the peptidic constituents of Fc-tagged nanowires. These results demonstrate the applicability of the Fc-PNW-based glucose sensor for clinical assays.

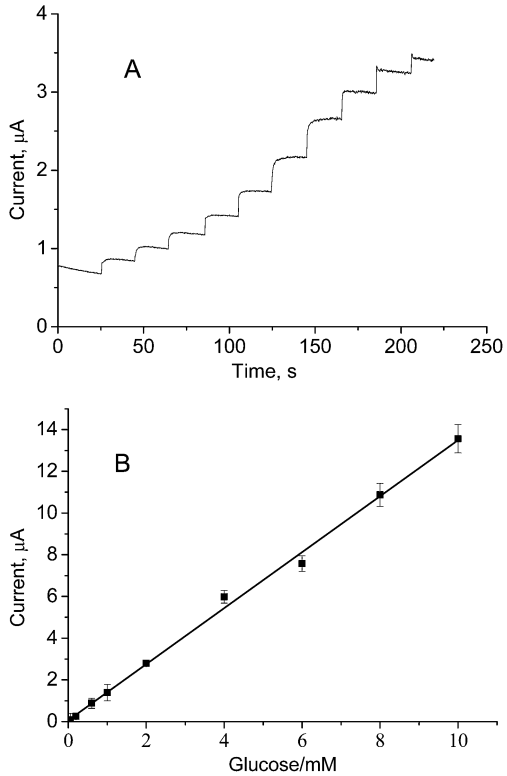

Fig. 5 (A) Amperometric response of the Fc-PNW-based glucose biosensor showing incremental additions of 0.2 mM glucose. The electrode was held at 0.6 V. (B) Calibration curve of the Fc-PNW-based glucose sensor to different concentrations of glucose. Error bar = SD ($n = 3$).

Table 2 Glucose concentrations in healthy and diabetic donors

Sample number	1	2	3
Fc-PNW-based sensor (mM)	5.52 ± 0.27	6.27 ± 0.30	9.37 ± 0.42
Colorimetric method (mM)	5.60 ± 0.23	6.30 ± 0.24	9.30 ± 0.34
Relative deviation (%)	−1.4	−0.5	0.8

Table 1 Comparison of analytical performance of fabricated glucose sensor and other kinds of glucose sensors

Sensors	Sensitivity (μA (cm^2 mM^{-1}))	Detection limit (mM)	Linear range (mM)	Detection potential (V)	Reference
PAMAM-Fc dendrimers	6.5	0.48	1–22	0.25	27
Carbon nanotubes and Fc-incorporated cryogel	7.8	0.01	0.01–30	0.175	28
Nanoporous PtPb	10.89		1–16	0.4	29
Ni/Al-layered double hydroxide	24.45	0.005	0.005–10	0.7	30
Ti/TiO_2 nanotube arrays/Ni	200	0.004	1–9	0.6	31
Fc-PNW	21.9	0.005	0.01–10	0.6	Our work

4. Conclusion

In summary, we have synthesized redox-tagged peptidic nanowire and straightforwardly cross-linked enzyme (GOx) molecules onto these nanowires. A one-step casting of the GOx-coated Fc-PNWs onto an electrode produces a glucose sensor with excellent stability, reproducibility, and sensitivity. The as-prepared sensor contains large numbers of the electron mediator (Fc) and functional groups for enzyme immobilization, affording high redox activity and versatility for fabrication of various types of electrochemical biosensors. The biocompatibility inherent in the peptide nanowires is also attractive for construction of durable biosensors for clinical samples or samples of complex matrices.

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