Analytical Methods

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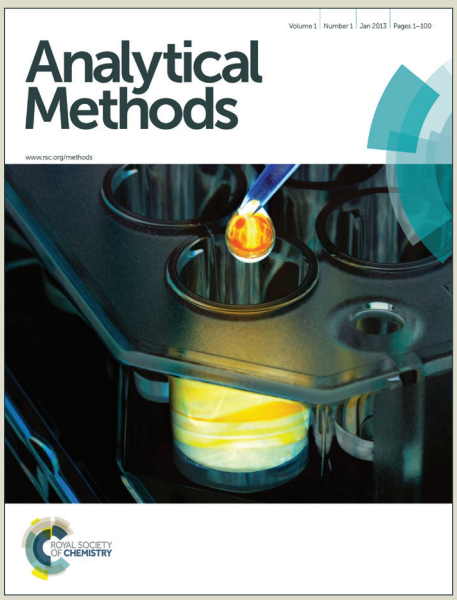

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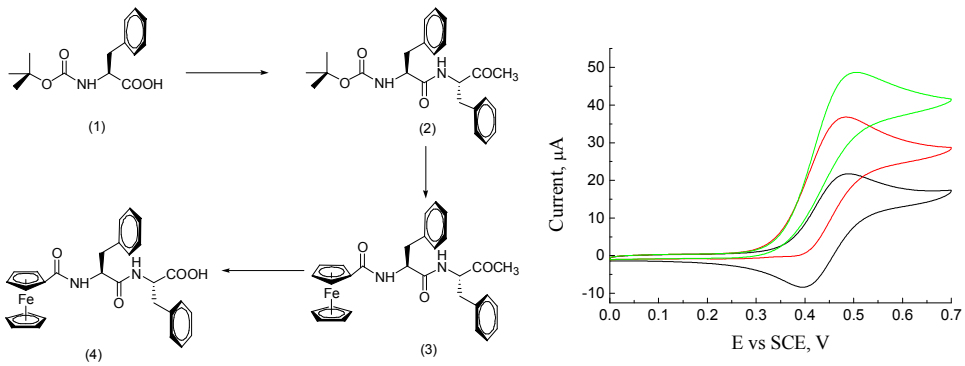
Graphical abstract



Synthetic route for producing Fc coupled diphenylalanine and the response of the resulted glucose biosensor to glucose.

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

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Abstract Ferrocene (Fc) tagged peptide nanowires (Fc-PNWs) were synthesized *via* the self-assemble of Fc coupled diphenylalanine (Phe-Phe, FF) and then utilized as supporting matrix for the immobilization of glucose oxidase (GOx). Scanning electron microscopy (SEM) characterization indicates the Fc-PNWs were twisted together with diameter around 50 nm. The GOx functionalized Fc-PNWs contains both mediator Fc and GOx that necessary for the electrochemical detection of glucose. So, with simply dropping the biocomposite onto electrode surface in one step, the resulting biosensor displays high sensitivity, wide linear range and good stability towards glucose detection. The good performance of the biosensor was originated from the great 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 was 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 wide potential commercial applications. The synthesized Fc-PNWs can also be used in different sensing and biosensing fields.

Keywords: Electrochemical; Ferrocene; Peptide nanowire; Glucose oxidase; Glucose

1. Introduction

Since the pioneering work of Clark and Lyons that integrate enzyme onto electrode surface,[1] enzyme based electrochemical biosensors have undergone a tremendous development.[2-4] For most of the enzymes, their redox centers are located deep inside the protein shell to be electrically inaccessible. So redox mediators are introduced to "wire" the enzyme's active centers to assure efficient electron transfer between enzyme and the electrode. Heller's group developed such "wired" enzyme electrodes based on the 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 $Os^{2+/3+}$. For example, 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),[5] 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 above mentioned $Os^{2+/3+}$, ferrocene (Fc) and its derivatives are another kind of widely used redox mediators due to their favorable chemical and electrochemical properties.[7, 8] The ferrocenyl residues can be covalently conjugated onto enzyme.[9] However, such conjugation will result in partial denaturing of the enzymes. Most often, Fc and enzymes are immobilized separately onto the electrode surface. With the advancement of nanotechnology during the past years, different Fc-based nanomaterials have been reported, such as Fc functionalized dendrimer and

gold nanoparticles.[10-12] If we can prepare one biocomposite that containing both Fc and enzyme, it will significantly simplify the biosensor fabrication process with just immobilizing the biocomposite onto the electrode.

Recently, peptide-based nanomaterials have received great interests due to their good biocompatibility, simple self-assembly process and flexibility in functionalization. Among the different peptide molecules reported, phenylalanine (Phe, F) and its derivatives have been widely researched for producing various nanomaterials.[13-15] The unique properties of the peptide-based nanomaterials were 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 structure.[19] Here, in this work, we report the use of the 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.

2. Experimental methods

2.1. Reagents and apparatus

Glucose oxidase (GOx, from *Aspergillus niger*, EC 1.1.3.4.150000 units g^{-1}), 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 the synthesis.

N-hydroxybenzotriazole (HOBt) and 2-(1H-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 Nova NanoSEM230 (FEI, USA).

2.2 Synthesis of Fc-Phe-Phe-COOH

The synthesis of Fc-Phe-Phe-COOH was according to our previous report with minor revision.[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. Into the mixture, 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), 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 with 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 mixtured 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 centrifuge, a mixture of 0.25% glutaraldehyde (v/v) and 10 mg mL^{-1} GOx was added into the solution. Free GOx remained in the solution was separated via centrifuging, and the final Fc-PNW-GOx biocomposite was

washed with PBS and stored at 4 ^{0}C before use.

2.4 Construction of the Fc-PNW-based glucose biosensor

To prepare electrochemical glucose sensor, 5 μL of the Fc-PNW-GOx bioconjugate was cast onto each glassy electrode. After drying the electrode surface in ambient 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 (Figure 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 can be served as mediator for GOx. After the conjugation of GOx onto Fc-PNW, the final Fc-PNW-GOx biocomposite contains both of the mediator and GOx, which means the biocomposite can be utilized for electrochemical glucose detection.

Figure 2 shows the SEM image of the synthesized Fc-PNWs. It can be seen the nanowires are twisted or intertwined together and display a porous 3D structure. The diameters of the nanowire are around 50 nm, while the length can extend to micrometer-long. The morphology and self-assembly process appears 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 Fc-Phe-Phe-OMe generate nanowire that is straight and rigid, while for

Fc-Phe-Phe-COOH, the obtained nanowire is soft and twisted.

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). Figure 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 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 peak separation between the anodic and cathodic peak indicated the 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 the 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.

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 (Figure 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 can be seen from Figure 4, with the increase of detection potential, the sensitivity of the biosensor to 5 mM glucose was increased. When the potential reached 0.6 V, further increase of the detection potential resulted in no improvement of the sensitivity. On the other hands, when the detection potential was too high, the biosensor was susceptible to the interference of some compounds that commonly present in the serum samples, such as ascorbic acid (AA), uric acid (UC) and acetaminophen. So a detection potential of 0.6 V was selected.

From Figure 5A, it can be seen that 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 in result enhance the detection sensitivity. The linear range of the Fc-PNW-based glucose sensor

(Figure 5B) spans from 0.01 to 10 mM (sensitivity of 21.9 μA/cm^2 mM), 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 sensor and enzymatic glucose sensors. As shown in Table 2, it can be seen the performance of our biosensor is comparable or even better that other reported glucose sensors.

To study the repeatability of the proposed biosensor, one 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 a RSD of 7.3% was obtained using the prepared biosensors for the 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 colorimetric method using glucose detection kit. As can be seen from Table 1, glucose contents determined by the two methods agree well and the glucose level in the diabetic patient is significantly higher than those in healthy donors. The good results also demonstrated the good selectivity of the biosensor, indicating AA, UC and acetaminophen in the serum will not interfere the glucose detection. The stability of the Fc-PNW-based glucose sensor was also investigated. When not in use, electrodes were stored at 4 ^{o}C in phosphate buffer, which showed little signal degradation even

after one month (around 90% of the initial sensitivity was still obtained). That GOx is not denatured and remains active can be attributed to the biocompatibility of the peptidic constituents of Fc-tagged nanowires. These results demonstrate the applicability of the Fc-PNW-based glucose sensor for clinical assays.

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.

Acknowledgments

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Figure Captions:

Figure 1 Synthetic route for producing Fc-Phe-Phe-COOH

Figure 2 SEM image of the synthesized Fc-PNW

Figure 3 (A) Five successive CV scans at a 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.

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

Figure 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 1. 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 2. Comparison of the analytical performance of the fabricated glucose sensor with other kinds of glucose sensors

Sensors	Sensitivity ($\mu A/(cm^2\ mM)$)	Detection limit (mM)	Linear range (mM)	Detection potential (V)	References
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

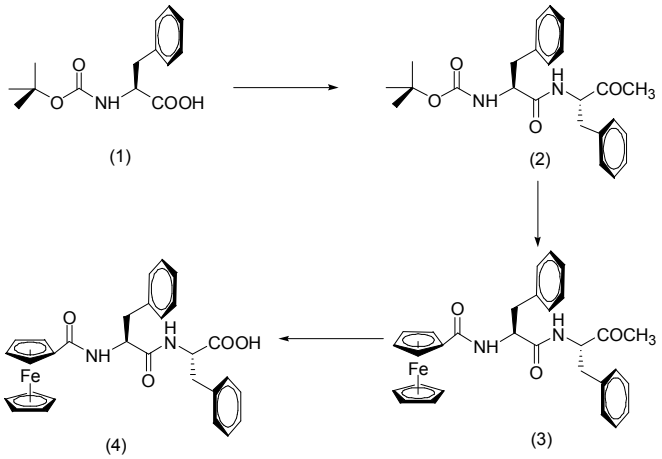


Figure 1

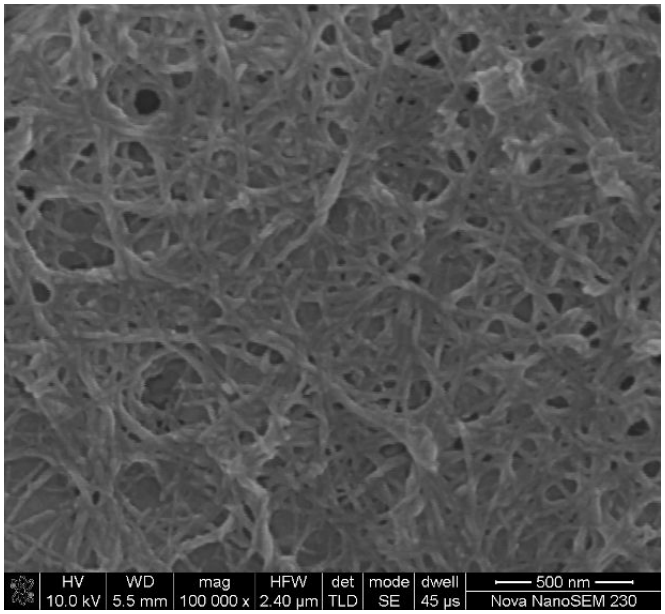
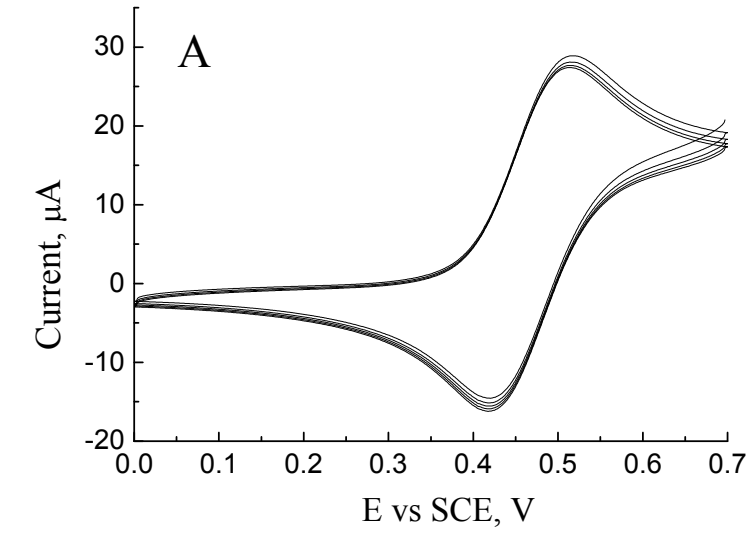


Figure 2



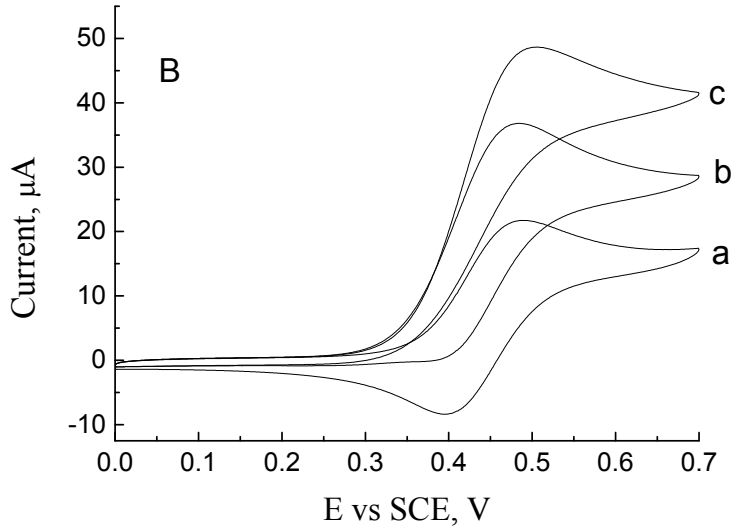


Figure 3

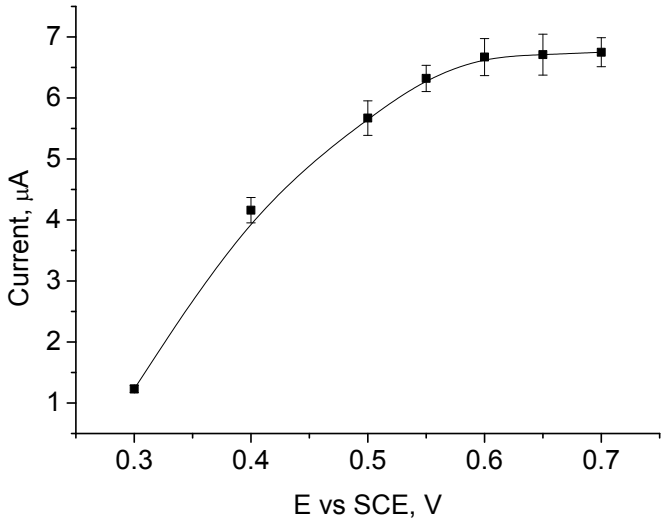
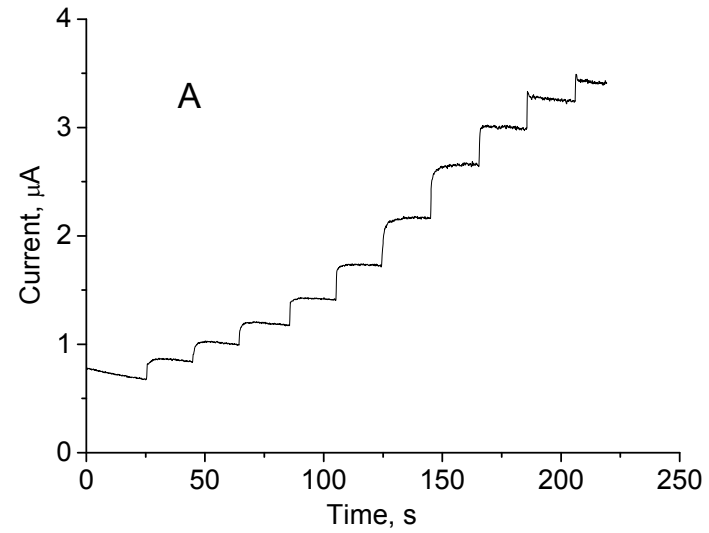


Figure 4



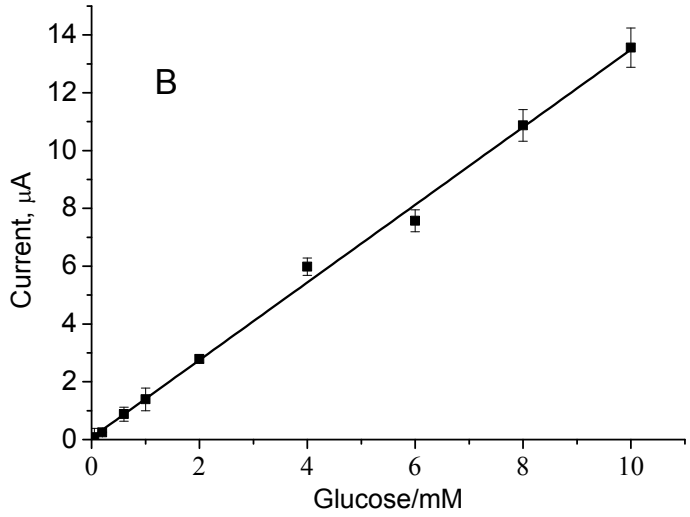


Figure 5