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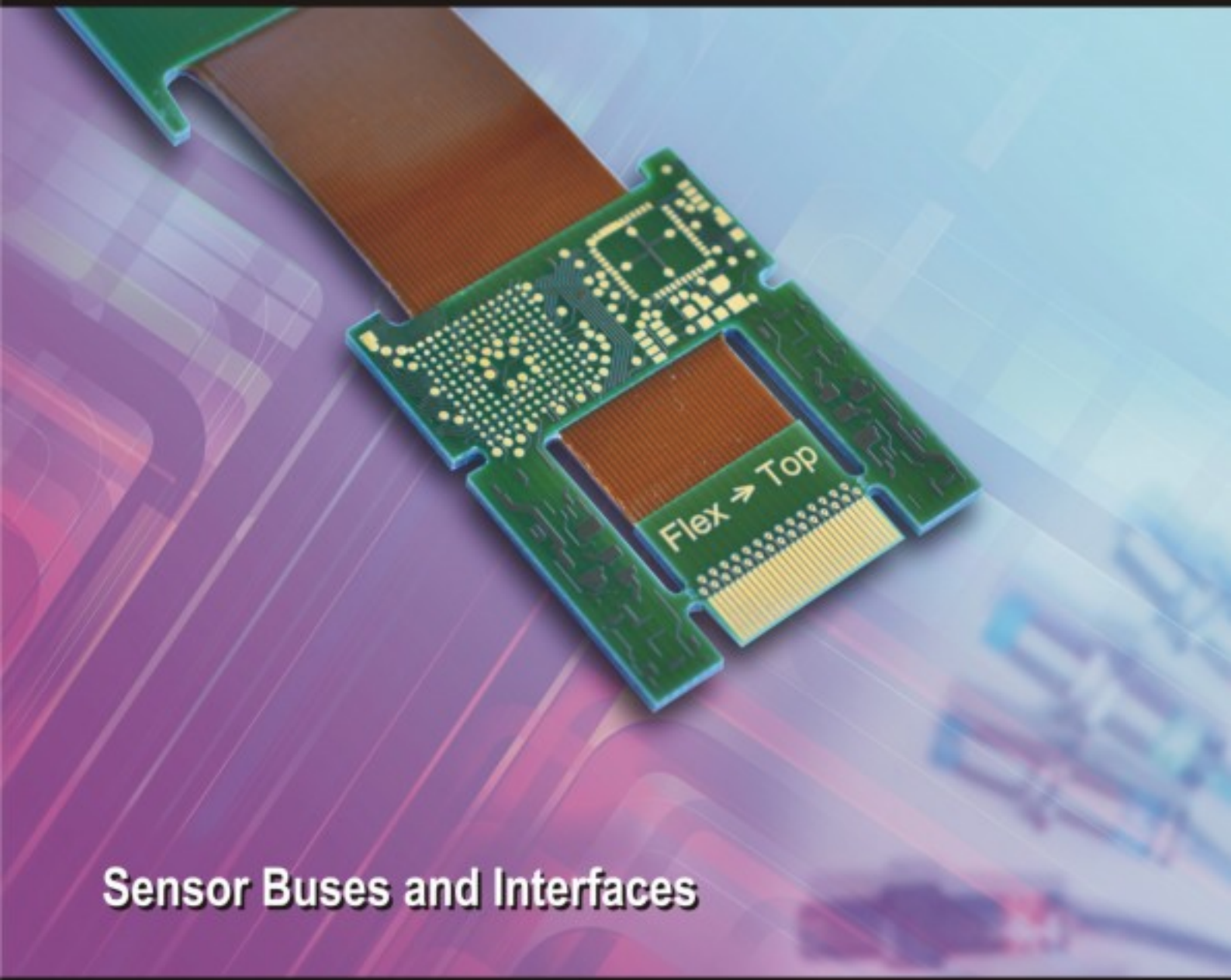

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A Glucose Sensor Based on Glucose Oxidase Immobilized by Electrospinning Nanofibrous Polymer Membranes Modified with Carbon Nanotubes

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Abstract: A glucose biosensor based on glucose oxidase immobilized by electrospinning nanofibrous membranes has been developed. Nanofibrous membranes were electrospun from the solution of poly(acrylonitrile-co-acrylic acid) containing carbon nanotubes suspension and directly deposited on Pt electrodes for immobilizing glucose oxidase. The morphologies and structure of the nanofibrous membranes with or without carbon nanotubes were characterized by scanning electron microscopy. The fabrication parameters of nanofibers were optimized such as thickness of the nanofibrous membranes and mass ration of carbon nanotubes. The biosensor showed the relationship with a concentration range of 0.1–10 mM and response time was 60 s. The sensitivity of carbon nanotubes modified biosensors was two times larger than which of no carbon nanotubes modified ones. The pH effect, interference and lifetime of biosensors were discussed. *Copyright © 2013 IFSA.*

Keywords: Electrospinning, Glucose biosensor, Enzyme immobilization, Carbon nanotubes.

1. Introduction

The glucose biosensors are regarded as the most successful electrochemical sensors so far. They are widely applied in human blood glucose monitoring and other analytical detections due to their advantages of rapid analysis, high selectivity, ease of operation and portability [1-3]. In order to detect the concentration of glucose in biological samples repeatedly or continuously, it is necessary to immobilize enzymes on the sensing area [4]. The technique used to immobilize the enzyme is one of the key factors in developing a reliable biosensor. Various enzyme immobilization approaches have been employed to date, including covalent binding [5] and entrapment in gels or polymer matrices [6, 7]. Nanofibrous membranes are characterized by large surface area-to-volume ratio, high porosity and interconnectivity. These features make them to be

optimal candidates as carrier material in enzyme immobilization [8, 9].

Electrospinning was first reported by A. Formhals in 1934 [10] and P. K. Baumgarten fabricated nanofibers successfully using electrospinning technique in 1971 [11]. However, this technique applied in nanofibers fabrication did not attract scientists' attention broadly until 1990's when the research on nano-materials began to bloom. Electrospinning produces polymer filaments using an electrostatic force. A polymer solution (e.g. cellulose acetate), is fed through the spinneret (e.g. a pipette tip) under an external electric field of high voltage power, between the spinneret and the grounded collecting metal plate, a suspended conical droplet is formed. Once the applied electric field is strong enough to overcome the surface tension, a tiny jet is ejected from the surface of the droplet and drawn toward the collecting plate. Meanwhile the solvent in the jet stream evaporates and a non-woven fiber with diameters ranging from tens of nanometers to microns is produced [9, 12]. Electrospinning has been discovered as a unique technique and an easy method for generating non-woven fibrous articles from a rich variety of materials [13].

Electrospun nanofibrous membranes possess an extremely high surface-to-volume ratio, tunable porosity, and malleability to conform over a wide variety of sizes and shapes [9]. In addition, the membranes composition can be controlled to achieve desired properties and functionality. Due to these advantages, electrospun nanofibers have been demonstrated as suitable substrates for immobilized enzymes. Compared to other immobilizing technologies, electrospinning boasts several advantages in producing polymer nanofibers, such as larger surface area for enzymes or bioactive components to attach to; much bigger mass-transfer rate of the substrate to the active site of an enzyme; more durable for repeating usage and can be conveniently recovered from a reaction solution when used as supports for biocatalysts [14]. At present, materials such as polyvinylpyrrolidone (PVP), poly (vinyl alcohol) (PVA), polyacrylonitrile (PAN) and chitosan have been used for electrospinning due to their good properties in forming fibers and membranes. An electrospun PVP membrane was prepared to immobilize urease and to develop a urea detector [15]. PVA was widely used by electrospinning technology to immobilize glucose oxidase (GOx) [16, 17], cellulase [18], luciferase [19] and lipase [20]. PAN nanofibers could be fabricated by electrospinning with fiber diameter in the range of 150–300 nm, providing huge surface area for lipase immobilization [21].

Carbon nanotubes (CNTs) have been recognized as one of the most promising electrode material since the first electrode application in the oxidation of dopamine in 1996 [22], because of their excellent properties such as superb electrical conductivity and remarkable mechanical strength and modules. The similarity in length scales between nanotubes and redox enzymes suggests interactions that may be favorable for biosensor electrode applications [23]. In view of the advantage of CNTs, it is expected that CNTs filling electrospun nanofibers can enhance the activity of the immobilized redox enzymes and to increase the sensitivity of biosensors potentially.

In this work, a reactive group containing copolymer, poly(acrylonitrile-*co*-acrylic acid) (PANCAA) was electrospun on Pt electrode to immobilize GOx covalently on the nanofibrous membrane. Multi-walled carbon nanotubes (MWCNTs) were co-electrospun with PANCAA and experiments data indicated that MWCNTs filling increased the current of GOx electrode obviously. Its advancements over prior technology are faster response time, lower detection limit of glucose and a more versatile design.

2. Experimental

2.1. Materials

The copolymer poly(acrylonitrile-*co*-acrylic acid) (PANCAA) was synthesized by a water phase precipitation copolymerization process [24]. The viscosity averaged molecular weight (M_v) was 8.32×10^4 g/mol. The molar content of acrylic acid in this copolymer was about 10%. MWCNTs prepared by a chemical vapor deposition process were purchased from Shenzhen Nanotech Port Co., Ltd. (China). *N*-(3-dimethylaminopropyl)-*N*'-ethyl-carbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Biochemical Technology Development Co., Ltd. (China), both of which were HPLC grade. GOx (EC 1.1.3.4, from *Aspergillus niger*) and *D*-glucose anhydrous ($C_6H_{12}O_6$, AR) was obtained from Sigma (USA) and Sinopharm Chemical Reagent Co., Ltd. (China), respectively. Other chemicals including *N*,*N*'-dimethylformamide, phosphate and borate, sodium chloride (NaCl) and calcium chloride ($CaCl_2$) were of analytical-reagent grade without further purification. Double-distilled water was used in all experiments.

Glucose standard solutions of different concentrations were prepared one day prior to use, carried out in a 50 mM borate-phosphate buffer solution (BPBS, pH 6.0) and stored at 4 °C.

2.2. Apparatus

The morphology of the PANCAA nanofibrous membranes was evaluated by field emission scanning electron microscopy (FEI, SIRION-100, USA). Before analysis, the samples were sputtered with gold using Ion sputter JFC-1100. Amperometric measurements were performed by a CHI760B Electrochemical Work Station (CH Instrument Inc., USA).

2.3. Electrode Preparation

Before electrospinning, the surface of Pt electrode was polished thoroughly with 0.03 micron Al_2O_3 power, rinsed with 5.0 wt.% sulphuric acid and distilled water in turn.

MWCNTs were treated with a mixture of concentrated sulfuric and nitric acids (3:1, 98 % and 70 %, respectively) at 40°C, in order to uniformly disperse MWCNTs in the copolymer matrix. PANCAA was dissolved in DMF with 5 wt.% concentration at 100°C and different concentration of MWCNTs (0, 5, 15, 25 and 35 wt.%) was suspended in the PANCAA solution for optimization. Electrospinning was worked out using a syringe with a 1.2 mm diameter stainless steel spinneret at an applied electrical potential difference of 14 kV over 15 cm gap between the spinneret and the Pt electrode surface which was connected to a 12×12 cm aluminum foil. The aluminum was connected to the ground to help provide a stable electric field. A microinfusion pump was set to deliver the solution at a flow rate of 1.0 mL/h using a 20 mL syringe. The electrospinning setup is shown in Fig. 1. During electrospinning the temperature and relative humidity was kept at 20-25 °C and 35-45 %, respectively.

GOx was covalently immobilized onto the fibrous membrane deposited electrode with the EDC/NHS activation procedure. After rinsed by BPBS (pH 6.0), the pretreated electrode surface covered by the fibrous membranes was submerged into an EDC/NHS solution (10 mg/mL in BPBS, the molar ratio of EDC to NHS = 1:1) and shaken gently for 2 h at 4 °C. Then, the activated surface was washed several times with BPBS, and immersed into GOx solution (4.0 mg/mL in BPBS). GOx immobilization was conducted at 4 °C for 24 h. Finally, the PANCAA and PANCAA/MWCNTs fibrous membranes with GOx immobilized were washed and rinsed in BPBS to remove the unfixed enzyme and then were dried at room temperature.

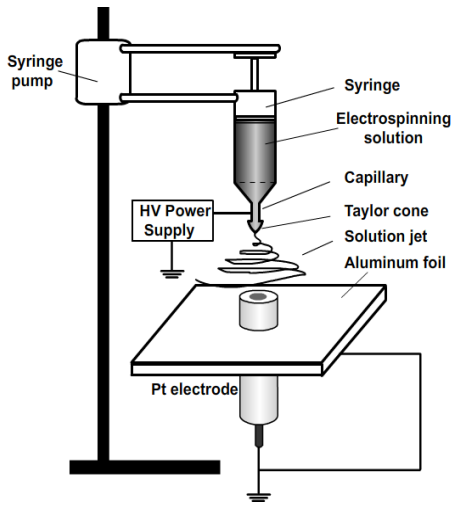


Fig. 1. Schematic illustration of electrospinning PANCAA on Pt electrode.

2.4. Measurements

Amperometric measurements were carried out using a CHI760B Electrochemical Work Station at room temperature at about 22 °C. The enzyme modified electrode was used as working electrode while a bare platinum disk and Ag/AgCl were used as counter and reference electrodes, respectively. The working potential was 0.8 V versus the Ag/AgCl reference electrode. All electrodes were immerged in 5 ml glucose standard solution to measure the biosensor response. Current–time curves of the amperometry were recorded using an IBM PC compatible computer via a RS232 series port communicating to the Electrochemical Work Station. The response time of the sensors was 60 s. The calibration curve was obtained by testing samples of different glucose concentrations to investigate the characteristics of the biosensor to determine the glucose concentration.

3. Results and Discussion

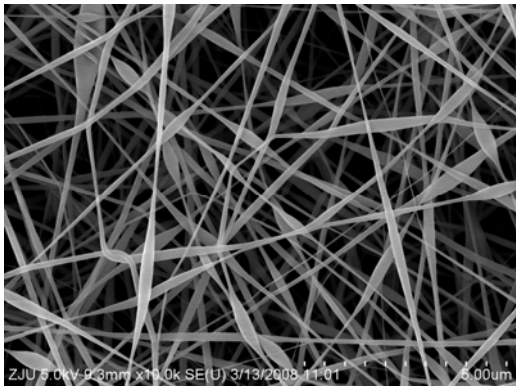
3.1. Field Emission Scanning Electron Microscopy

Field emission scanning electron microscopy was employed to evaluate the morphology and fibrous membranes. As shown in Fig. 1, the fibrous membranes were deposited onto Pt surface of the electrode using the special electrospinning setup. Then GOx was directly immobilized onto the membrane through EDC/NHS activation procedure. Fig. 2 shows the images of pure PANCAA and MWCNTs filled PANCAA fibrous membranes. The latter one was interspersed with many beads, but fewer beads in pure PANCAA nanofibrous membranes. The beads were very common nanofiber defects. Their formation was dependent on some parameters, such as the spinning solution and the relative humidity of the surroundings. According to our study, MWCNTs filling has little effect on the enzyme loading.

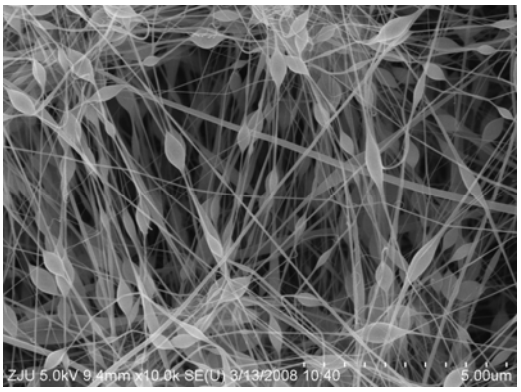
3.2. Optimization of Membrane Amounts and PANCAA/MWCNTs Mass Ration

The amounts of the PANCAA fibers film were determined by the flow rate of the spinneret and the electrospinning time. A microinfusion pump was set to deliver the solution at a flow rate of 1.0 mL/h. So with the electrospinning time increased, the fibers membrane amount increased considering the rate was constant. Generally, the thicker the nanofibrous membranes were, the more enzymes could be covalently immobilized, but the analyte diffusion would be retarded. Our experiment results are shown in Fig. 3. When the electrospinning membrane amount was less than 1.6 mg/cm^2, the response

current was not high enough because little enzyme was covalently immobilized. When the membrane amount was more than 1.6 mg/cm^2, the response current increased slightly. In our experiments, the optimal membrane amount was 1.6 mg/cm^2 and electrospinning time was 4 hours.



(a)



(b)

Fig. 2. SEM images of electrospun PANCAA fibers (a) and PANCAA-MWCNTs fibers (b).

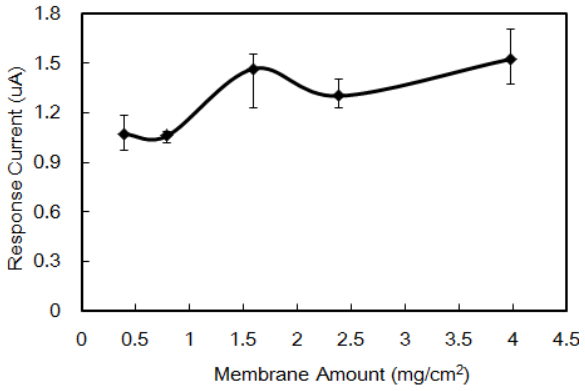


Fig. 3. Effect of electrospinning time on response current to 5 mM glucose.

The response of different amounts (0, 5, 15, 25 and 35 wt.%) MWCNTs filled PANCAA nanofibrous membrane with GOx immobilized to 5 mM glucose was compared in experiments. It can be demonstrated that the current increases until the ratio rises up to 25 wt.% according the experiment data. This can be attributed to the formation of percolating network among MWCNTs and to much more MWCNTs protruded out of the nanofibers, which enhance the electrical conductivity of the nanofibrous membrane and increase the opportunity for GOx to interact with MWCNTs. However, the current changes little as the MWCNT/PANCAA mass ratio increases from 25 to 35 wt.%. A MWCNTs/ PANCAA mass ratio of 25 wt.% was chose as the optimal and final recipe of the biosensor in the following experiments.

3.3. Sensor Characteristics

The calibration curve of the biosensor modified by PANCAA/GOx and PANCAA/MWCNTs/GOx is shown in Fig. 4.

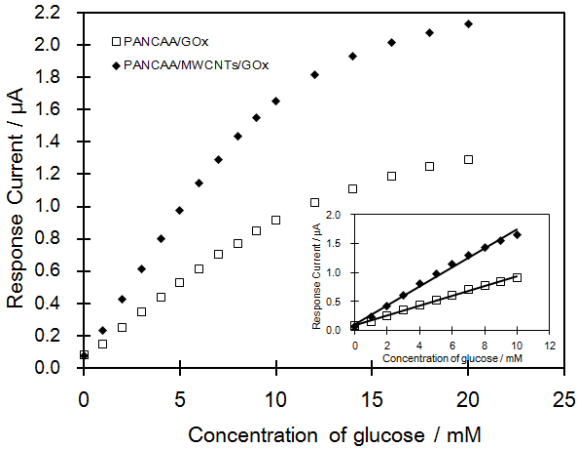


Fig. 4. Calibration curves of the PANCAA/GOx and PANCAA/MWCNTs/GOx sensors.

Sixteen different glucose concentrations were measured using the electrodes and each concentration was measured 5 times. The calibration curve indicated that the linear range of the glucose electrode response was from 0.1 to 10 mM, and the limit of detection was 5.5 μM (calculated as three times the signal-to-noise ratio). The apparent Michaelis constants and the maximum current density for these electrodes were calculated to the electrochemical version of the Michaelis-Menten Equation

$$j = j_{\max}[S]/(K_M^{app} + [S]) \quad (1)$$

where K_M^{app} is the Michaelis constant and $j_{\max}$ is the maximum current density.

These parameters were recorded in Table 1.

Table 1. The electrochemical parameters of PANCAA/GOx and PANCAA/MWCNTs/GOx sensors.

Electrode	PANCAA/GOx	PANCAA/MWCNTs/GOx
Calibration Equation	I(μA) = 0.0856C(mM) + +0.0855	I(μA) = 0.1626C(mM) + +0.1142
R^2	0.9972	0.9927
Sensitivity	2.73 μA mM^{-1} cm^{-2}	5.18 μA mM^{-1} cm^{-2}
R.S.D (n=5)	4.59 %	3.33 %
K_M^{app}	10.38 mM	21.48 mM
j_{max}	52.9 μA cm^{-2}	163.9 μA cm^{-2}

3.4. pH Effect

The response of the glucose biosensor depends on the activity of immobilized GOx, which is related to the pH of the solution. The pH influence was investigated by amperometric measurement of 5 mM glucose in BPBS of different pH values between 4.0 and 8.0. As shown in Fig. 5, the maximum response current was observed at pH 6.0.

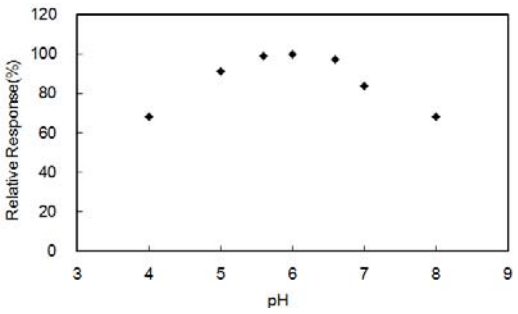


Fig. 5. Effect of pH on the response current to 5 mM glucose.

3.5. Interference Study

In order to demonstrate the selectivity of the PANCAA/MWCNTs/GOx biosensor, the interferences of electroactive compounds to the glucose response were examined. Some electroactive compounds, such as ascorbic acid (AA) and uric acid (UA), may interfere in the detection of glucose. The normal levels of AA and UA in human blood are 0.023–0.034 mM (0.40–0.59 mg dl^{-1}) and 0.12–0.42 mM (2.0–7.0 mg dl^{-1}), respectively. When the patient is taking vitamin C, the concentration of AA in his or her blood will increases. So the responses of the sensors to 0.17 mM (3.0 mg dl^{-1}) AA, 0.48 mM (8.0 mg dl^{-1}) UA with glucose concentration at 5 mM were measured (Table 2). It can be considered that there is no difference among the responses of glucose with AA or with UA and that of glucose alone ($P > 0.05$ by paired t-test, $n = 4$). The interference of AA and uric acid UA to the glucose response was effectively eliminated by the PANCAA fibers.

Table 2. Influence of electroactive interferents on the response of glucose biosensors.

Substance	Response Current (μA)	R.S.D.	i_{G+I}/i_G
Glucose(5 mM)	1.067	5.86 %	1
Glucose(5 mM) +AA(0.17 mM)	1.010	6.02 %	0.97
Glucose(5 mM) +UA(0.48 mM)	1.174	2.99 %	1.06

3.6. Sensor Lifetime

The operational stability was examined by measuring the response to 5 mM glucose. The PANCAA/MWCNTs/GOx sensors were stored at 5 °C in the dark for 4 weeks, while measurements were conducted every 2 days during the first week and then once a week subsequently. The response remained 57.9 % of the initial value in 2 weeks and 40.0 % in 4 weeks. The experimental data about the lifetime of the sensor are shown in Fig. 6.

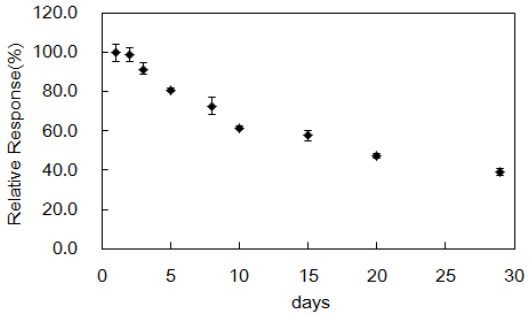


Fig. 6. The lifetime of PANCAA/MWCNTs/Gox.

4. Conclusions

In summary, the electrospun MWCNTs-filled PANCAA nanofibrous membranes were applied to immobilize glucose oxidase for fabricating glucose biosensors, which could be used for multi-time measurements. The reactive groups EDC/NHS possessed by the membranes were used for the covalent immobilization of GOx. The parameter of nanofibrous membranes fabrication was optimized such as the amount and the mass ration of MWCNTs vs. PANCAA. The results from chronoamperometric measurements showed that sensitivity of MWCNTs modified biosensors was ca. two times larger than which of no MWCNTs modified ones. However, there are several problems need to resolve, such as improving the adhesion between nanofibrous membrane and the Pt electrode surface, extending the lifetime and decreasing the response time of MWCNTs modified biosensors. Therefore, clarifying the interactions of embedded MWCNTs with enzyme will be necessary in future study.

Acknowledgements

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ADVANCES IN SENSORS: REVIEWS 2

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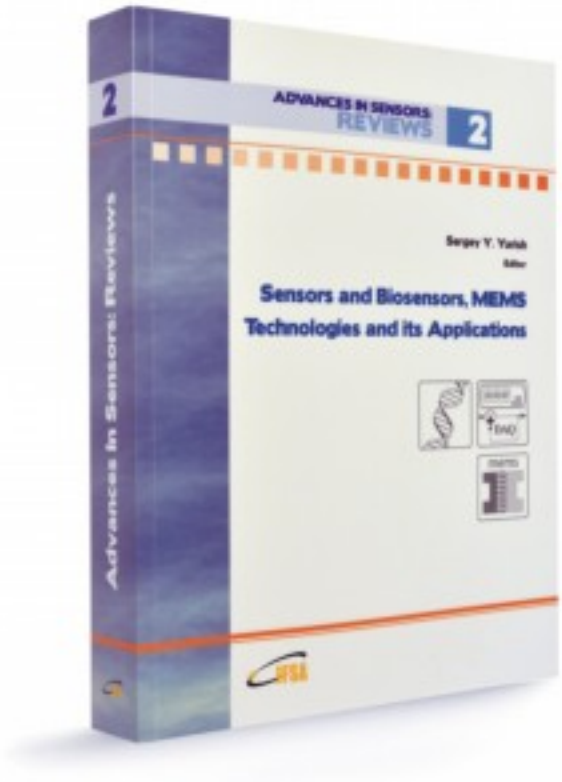

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