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"Ready-to-use" hollow nanofiber membrane-based glucose testing strips†

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A novel "ready-to-use" glucose test strip based on a polyurethane hollow nanofiber membrane was fabricated through facile co-axial electrospinning. By utilizing glucose oxidase and horseradish peroxidase in the core-phase solution, and a chromogenic agent either in the core solution (in which case 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was used) or in the shell-phase solution (in which case *o*-dianisidine was used) for co-axial electrospinning, *in situ* co-encapsulation of the two enzymes within the hollow nano-chamber and incorporation of chromogenic agents either inside the nano-chamber or in the shell of the hollow nanofibers was realized. Such unique "all-in-one" feature enabled the prepared hollow nanofiber membrane-based test strips to be applied either as colorimetric sensors in solution or as an optical biosensor operated in the "dip-and-read" mode. When used as a colorimetric biosensor in solution, the test strip with *o*-dianisidine as chromogenic agent shows an excellent linear response range between 0.01 mM to 20 mM and a high apparent lumped activity recovery of 62.1% as compared to the reaction rate of the free bi-enzyme system. While the activity recovery of the test strip with ABTS as chromogenic agent is only 18.0%, and the test strip is found to be unstable due to spontaneous-oxidation of the ABTS. The *o*-dianisidine test strip was also applied as an optical biosensor, visible rufous color was quickly developed on the surface of the membrane upon dropping 10 μL of glucose sample, and an excellent correlation between differential diffusive reflectance of the test strip at 440 nm and glucose concentration was obtained in the range of 0.5–50 mM. The test strips also exhibited excellent long-term storage stability with a half-life at 25 °C as long as four months.

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1. Introduction

Multifarious biological substances, such as enzymes, antibodies, antigens, microorganisms and cells, are used as the biorecognition elements of biosensors for a wide range of applications from detecting hazardous chemicals in the atmosphere to measuring glucose in the human body.[1–5] Enzymes are the most selective and specific biosensors that enable more precise detection.[6–8] One of the biggest obstacles in developing enzyme-based biosensors is the loss of activity in environments slightly outside of biocompatible regimes. With the development of nano technologies, the applications of a variety of nanomaterials including nanoparticles[9–12] and nanofibers[13–16] in fabricating enzyme-based biosensors have been proven to afford the biosensors high sensitivity and much enhanced stability. In particular, nanofibrous membranes as enzyme carriers have been shown to have more advantages over other nanostructured materials due to their high porosity, high surface-area-to-volume ratio, interconnectivity, and ease of preparation *via* a facile electrospinning technique; therefore electrospun nanofibrous membranes have been widely used for fabricating biosensors for sensitive and quick detection.[17–20]

Another challenge for biosensor fabrication is that many of the detection systems need the involvement of multienzymes and coenzymes, or chromogenic agents,[21,22] whose incorporation into the bio-recognition element by a traditional enzyme immobilizing process is very difficult because coenzymes and chromogenic agents could not be retained in most types of carriers. This problem has not yet been well solved by using nanofibers. Currently, most nanofiber enzyme-based biosensors have been fabricated through adsorbing enzymes onto the surface of the pre-prepared nanofibers,[13,15,16] which cannot adsorb coenzyme or chromogenic agents efficiently.[23] Therefore, developing a "ready to use" type biosensor by efficiently immobilizing all of these required elements in appropriate carrier materials is a promising but challenging task.

In one of our previous works, novel hollow nanofiber-based artificial cells were fabricated through facile co-axial electrospinning by utilizing two enzymes and the shared coenzyme in

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the core-phase electrospinning solution, so that *in situ* co-immobilization of the multienzyme system was realized.[24] This brand-new design of multienzyme artificial cells has been proven to afford the multi-step biotransformation a high efficiency and much enhanced enzyme stability. What is more important, the unique advantage of the *in situ* co-immobilization strategy provides us opportunities to fabricate "ready to use" type biosensors by incorporating all the required elements in the hollow nanofibers, so that the limitation of traditional nanofiber-based biosensors can be greatly overcome.

The huge market size and the simplicity of the sensing reaction chemistry make diabetes a model disease for developing new biosensing concepts. Accurate and rapid determination of glucose is a crucial issue in clinical diagnostics, the food industry and biotechnology. Miscellaneous techniques such as electrochemical or non-enzymatic methods,[25–29] and enzymatic methods, including enzyme-amperometric and enzyme-colorimetric methods,[30–35] have been applied in glucose determination. Enzymatic methods, especially in colorimetric sensors, have been proven to be a powerful approach and have attracted much attention, on account of their simplicity and practicality, in that the testing result even can be easily read by the naked eye without the aid of any expensive and sophisticated instruments.[36] For colorimetric sensors, there are many blood glucose test kits and test strips available on the market. Even though good selectivity and high sensitivity are obtained with these enzymatic sensors, inevitable drawbacks such as chemical and thermal instabilities originating from the intrinsic nature of enzymes as well as tedious fabrication procedures still limit their analytical applications.[36] Up to now, the design and preparation of glucose sensors meeting all these demands still remain a significant challenge.

In this paper, glucose oxidase (GOD), horseradish peroxidase (HRP) and chromogenic agents were co-immobilized *in situ* in the hollow chamber or shell of PU hollow nanofibers by a coaxial electrospinning technique to fabricate a "ready-to-use" biosensor for quick and sensitive glucose detection. This bioactive nanofiber membrane can be used either as a colorimetric biosensor to detect glucose in solution, or using as an optical biosensor in the "dip-and-read" mode.

2. Experimental section

2.1. Reagents and chemicals

Polyurethane A85E (PU) pellets with bulk density about 700 kg m^{-3} were supplied by Xiamen Jinyouju Chemical Agent Co (Xiamen, China). Glucose oxidase (GOD, EC 1.8.1.4) and horseradish peroxidase (HRP, EC 1.11.1.7) were supplied by Beijing Apis Biotechnology Co. (Beijing, China). 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), *o*-dianisidine, glycerol, and *N*,*N*-dimethylacetylamide (DMAc) were purchased from Sigma.

2.2. Instruments and characterization

The morphologies of the hollow nanofiber membranes were characterized by scanning electronic microscope (SEM, JSM-6700F, JEOL, Japan) and transmission electron microscope (TEM, JEM-2100UHR, JEOL, Japan). To view the cross-section of the hollow nanofibers, the collected nanofibrous membranes were frozen in liquid nitrogen, and then cut perpendicular to the axis of the fibers to expose their hollow structure.

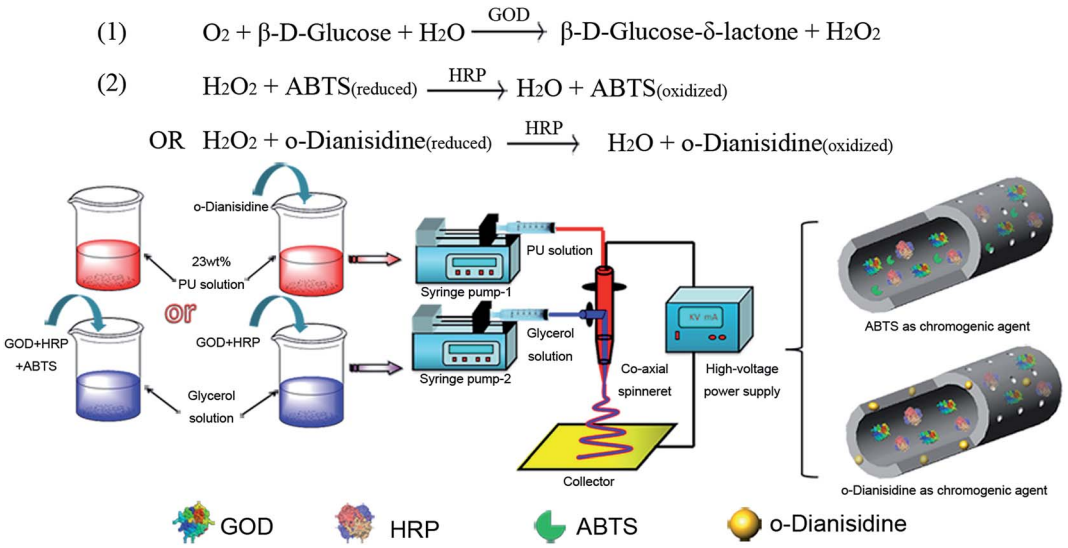
2.3. Preparation of hollow nanofiber based testing strip for glucose measurement

The bi-enzyme system for glucose colorimetric detection includes GOD, HRP and chromogenic agents. In the present work, two commonly used chromogenic agents, ABTS and *o*-dianisidine, were selected. A co-axial electrospinning technology, which has been well described in our previous work,[24] was used to prepare the hollow nanofiber membrane based testing strips for glucose measurement. A schematic illustration of the set-up for co-axial electrospinning and the reaction mechanism of the bi-enzyme system for glucose detection is shown in Scheme 1. Briefly, the shell-phase solution was prepared by dissolving PU in DMAc to get a final polymer concentration of 23 wt%, the core-phase solution was prepared by mixing well 800 μL glycerol with 200 μL of an ultra-pure water solution containing 1.0 mg GOD, and 1.0 mg HRP. To realize simultaneous *in situ* immobilization of the chromogenic agent in the electrospun hollow nanofibers, either 30 mg mL^{-1} water-soluble ABTS was utilized in the core-phase solution for co-axial electrospinning or 8 mg mL^{-1} water-insoluble *o*-dianisidine was utilized in the shell-phase solution.

The spinneret contains two coaxial stainless steel needles, whose inner and outer diameters of the core nozzles are 0.42 and 0.72 mm, and those of shell nozzles are 0.92 and 1.28 mm, respectively. The core solution and shell solution were fed at a constant rate of 0.07 mL h^{-1} and 0.5 mL h^{-1}, respectively, through two syringe pumps. A positive voltage of 15–17 kV was applied and an aluminium sheet was used as the collector at a distance of 25 cm away from the nozzle tip. Electrospinning was carried out at 25 ± 3 °C, humidity 10–15%. After a stable electrospinning operation was obtained, nanofibrous membrane was collected every 30 min. The collected nanofiber membrane was placed overnight in a laminar hood at room temperature to evaporate residual solvent, and then was sealed in a plastic bag and stored in a dry place.

2.4. Colorimetric detection of glucose using the testing strip in solution

About 3 mg of as-spun hollow nanofiber membrane with co-immobilized GOD, HRP and chromogenic agent (ABTS or *o*-dianisidine) was immersed into 1 mL glucose solution at different concentrations prepared in PBS buffer (pH 7.0, 50 mM). All assays were conducted at 37 °C, and the generated colour in solution after 5 min reaction as a function of glucose concentration was measured using a Unico 2800 spectrophotometer at a wavelength of 420 nm ($A_{420\ nm}$ for ABTS as chromogenic agent) or 440 nm ($A_{440\ nm}$ for *o*-dianisidine as chromogenic agent). A control test was carried out by using blank hollow nanofibers containing no enzymes and chromogenic agent. When the measurement was carried out using the



Scheme 1 Schematic illustrations of the bi-enzyme reaction for glucose measurement and the setup for co-axial electrospinning to prepare hollow nanofiber membrane-based glucose testing strips. During co-axial electrospinning, GOD, HRP, and chromogenic agent (ABTS or *o*-dianisidine) were simultaneously immobilized *in situ* in the hollow nanofiber membrane.

bi-enzyme free system, the concentrations of GOD, HRP and chromogenic agent were all adjusted to the same values as that of the above hollow nanofiber-based detecting system.

2.5. Optical detection of glucose by measuring the color intensity changes on the testing strip

The as-spun hollow nanofiber membrane containing GOD, HRP, and *o*-dianisidine was cut into little round-shaped test strips with diameter of 10 mm. Then 10 μL of glucose solutions of various concentrations were dropped onto the prepared test strips. A circular rufous spot was formed on the test strip in about 30 seconds. The color intensity of the rufous spot was determined by measuring its diffusive reflectance at 440 nm with an X-rite 500 spectrodensitometer. The lower the diffusive reflectance at 440 nm, the stronger the intensity of the rufous spot on the test strips. For each sample, three replicates were conducted and the average result was reported. The change in reflectance was calculated using eqn (1):

$$\Delta R = R_1 - R_0 \quad (1)$$

where R_0 and R_1 are the reflectance of the paper test strip before and after reaction for 30 seconds with glucose sample solution.

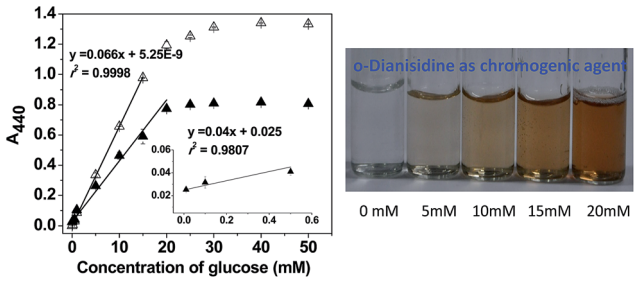


Fig. 2 Calibration curves for the glucose assay obtained by using (△) free and (▲) hollow nanofiber membrane based GOD–HRP bi-enzyme systems with *o*-dianisidine as chromogenic agents. Inset figure shows the data points for 0.01, 0.1, and 0.5 mM glucose solution measured with *o*-dianisidine-test strip. Concentrations of enzymes and chromogenic agents for the free system were adjusted to the same as that of the immobilized system. The picture on the right shows a photograph of the detection of different concentration of glucose obtained by digital camera after removal of the *o*-dianisidine-test strips.

To illustrate the feasibility of the hollow nanofiber membrane-based glucose test strip, it was employed to measure glucose concentration in human blood serum. Five fresh

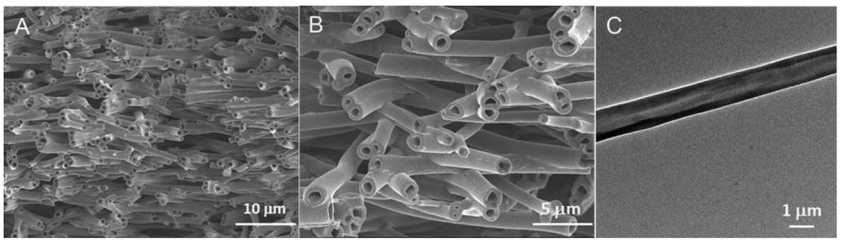


Fig. 1 Characterization of hollow nanofibers prepared by co-axial electrospinning. (A and B) Cross-sectional SEM images, (C) TEM images.

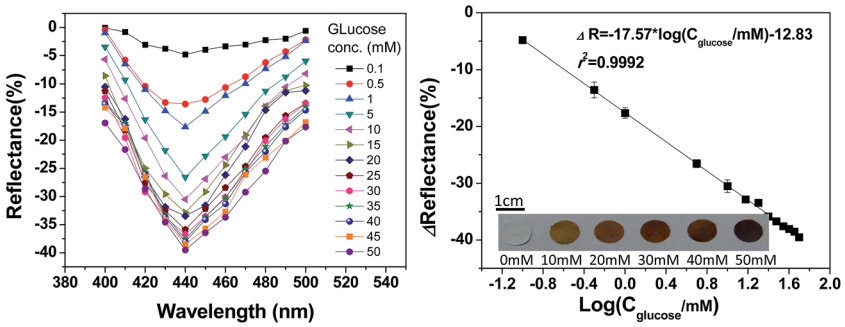


Fig. 3 Optimal detection of glucose by measuring the color intensity changes on the testing strip with *o*-dianisidine as chromogenic agent. (A) Differential reflectance spectra of the membrane test strips upon reaction with glucose solutions of different concentrations for 30 s. (B) Correlation between the ΔR of test strips and the log of glucose concentration. The inset picture demonstrates the visual color change in response to the change in glucose concentration of the *o*-dianisidine-test strip. The diameter of the membrane is 1 cm.

human blood serum samples, provided by a local hospital, were determined by dropping 10 μL of the sample onto the prepared test strips; thereafter the color intensity of the developed rufous spot was measured and the glucose concentration was calculated from the standard curve. For comparison, glucose concentrations were also measured using a commercial glucose test kit (Beijing Leadman Biochemistry Co. LTD, Beijing), which is used for clinical blood glucose determination in hospitals.

2.6. Stability of the glucose testing strip

Hollow nanofiber membrane-based glucose testing strips were sealed in plastic Ziploc bags and stored at 4 °C or 25 °C. The stability of the testing strips was examined by monitoring changes in reaction rate as a function of storage time with the reaction rate of freshly prepared testing strip defined as 100%.

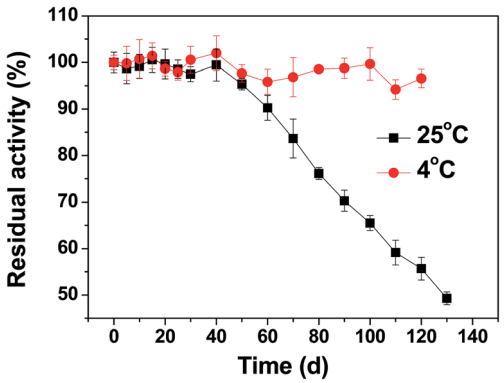


Fig. 4 Stability of the hollow nanofiber membrane test strips with *o*-dianisidine as chromogenic agent.

3. Results and discussion

3.1. Fabrication of hollow nanofiber membrane-based testing strip

Due to their simplicity and practicality, enzyme-colorimetric methods for glucose analysis[21,33,36] based on the GOD–HRP bi-enzyme reaction are most frequently employed in the clinic. The measurement could be performed either in solution using a standard test kit in which GOD, HRP and chromogenic agent solutions are usually separately packed thus a pre-mixing operation is required during measurement, or in "dip-and-read" mode using a test strip where the GOD, HRP and chromogenic agent are loaded on the surface of the testing zone by simple adsorption or spraying. In the present work, the novel hollow-nanofiber membrane-based glucose testing strip was prepared using a facile co-axial electrospinning technique, with GOD, HRP and chromogenic agent all co-immobilized *in situ* in the hollow nanofiber (Scheme 1).

Fig. 1 shows the SEM and TEM images of the as-electrospun hollow nanofibers with the *in situ* immobilized GOD–HRP bi-

Table 1 Determination of glucose in blood serum samples using a commercial test kit and the hollow nanofiber membrane-based test strip with *o*-dianisidine as chromogenic agent

Blood serum sample	Glucose concentration[a] (mM)	Glucose concentration[b] (mM)	Bias[c] (%)
1	3.80 ± 0.13	3.85 ± 0.22	1.3
2	4.37 ± 0.09	4.41 ± 0.24	0.9
3	4.46 ± 0.15	4.48 ± 0.19	0.9
4	4.77 ± 0.21	4.81 ± 0.25	0.8
5	5.89 ± 0.26	5.93 ± 0.31	1.0

[a] Determined by commercial glucose test kit. [b] Determined by hollow nanofiber membrane-based test strip with *o*-dianisidine as chromogenic agent. [c] Calculated by dividing the difference between values of glucose concentration determined from these two methods with the values determined by commercial glucose test kit.

enzyme system inside the hollow chamber of the fibers. On average the fibers have an outer diameter ranging from 800 to 1000 nm, an inner diameter of about 500 nm, and the thickness of the shell was about 400 nm.

It has been suggested that the identification of an appropriate chromogenic agent is one of the key factors in the development of test strips for glucose analysis.[33] In the present work, water soluble ABTS and non-polar *o*-dianisidine were chosen as chromogenic agents, whose *in situ* co-immobilization were therefore realized by involving the ABTS in the core-phase solution for co-axial electrospinning or involving the *o*-dianisidine in the shell-phase solution. Correspondingly, two types of hollow nanofiber membrane-based test strips were fabricated, the first one with GOD, HRP and ABTS all inside the hollow chamber of the fibers (referred to as ABTS-test strip); while for the second one, the two enzymes were inside the hollow chamber, but *o*-dianisidine was incorporated within the shell of the fibers (referred to as *o*-dianisidine-test strip). This unique 'all-in-one' feature will enable the prepared test strips to be applied either in solution as colorimetric sensors or as optical biosensor that can be operated in the "dip-and-read" mode.

3.2. Colorimetric detection of glucose using the testing strip in solution

The hollow nanofiber membrane-based testing strips were firstly evaluated for colorimetric detection of glucose in solution. Fig. 2 shows that when 3 mg of as-prepared hollow nanofiber membrane-based *o*-dianisidine-test strip was immersed into glucose solution, the coupled reaction by GOD and HRP was initiated and a brown color was developed in the glucose solution due to conversion of the colorless reduced *o*-dianisidine to its corresponding oxidized products, whose maximal absorbance wavelength are at 440 nm. During 5 min measurement, the absorbance increased linearly with time and upon removal of the testing strip by simply picking up with forceps, the reaction stopped, indicating that there was no leakage of immobilized enzymes. By measuring the absorbance of the solution at 5 min over a range of glucose concentration, a standard curve for glucose assay was developed with a linear range for glucose assays from 0.01 mM to 20 mM, and a correlation coefficient r of 0.990. The limit of detection was estimated to be 0.05 μM at a signal-to-noise ratio (S/N) of 3.

By comparing the reaction rates of *o*-dianisidine oxidization catalyzed by immobilized and free formulations of the bi-enzyme system at a glucose concentration of 20 mM, the apparent lumped activity recovery of the immobilized bi-enzyme system was calculated approximately to be 62.1%. The high activity of the *o*-dianisidine-test strip system is attributed to the following two reasons: firstly, the GOD and HRP molecules retained inside the hollow chamber of nanofibers remained in free formation, and the cascade reaction could even be enhanced due the spatial confining effects from the nano-scaled hollow chamber; secondly, the chromogenic agent *o*-dianisidine was embedded in the wall of the hollow fiber, therefore its oxidized product could be quickly released into solution. The normal concentration of glucose in human serum ranges from 3.9 to 6.4 mM;[37] therefore the testing strips can potentially be used as a colorimetric biosensor to detect the glucose concentration in sera.

When the ABTS-test strip was used for glucose measurement, a green color was developed in the glucose solution due to conversion of the colorless reduced ABTS to its corresponding oxidized products, whose maximal absorbance wavelength is at 420 nm (Fig. S1†). The standard curve for glucose assay was developed with a linear range for glucose assays from 0.01 mM to 25 mM, a correlation coefficient r of 0.998, and a limit of detection of 0.1 μM estimated at a signal-to-noise ratio (S/N) of 3. Compared with the high apparent lumped activity recovery of the *o*-dianisidine-test strip (62.1%), the value for the ABTS-test strip was calculated to be only 18.0%. This much lower apparent lumped activity recovery is mainly attributed to encapsulation of ABTS inside the chamber of the hollow fiber, the oxidized product of encapsulated ABTS has to diffuse across the fiber wall to color the solution. In contrast, in the *o*-dianisidine-test strip, the chromogenic agent was embedded in the wall of the hollow fiber thus enabling its oxidized product a quick release to solution. Furthermore, it was found that the ABTS-test strip is unstable. Fig. S2† shows that ABTS-test strip turns to green gradually in air due to significant spontaneous-oxidation of the ABTS, while the color of the nanofibrous membrane with *o*-dianisidine remains unchanged. Therefore, the following study was focused on the evaluation of *o*-dianisidine-test strip.

3.3. Evaluation of hollow nanofiber membrane-based glucose test strip as optical biosensor

Using the hollow nanofiber membrane-based test strip in solution as a colorimetric biosensor has shown excellent sensitivity and a broad detection range. The "all in one" feature of the test strip also makes it possible to be operated in the "dip-and-read" mode as an optical biosensor. Fig. 3 shows that in response to 10 μL glucose samples of different concentration, a visible rufous color spot was quickly developed on the surface of the *o*-dianisidine-test strip and the intensity of color reached a maximum in about 30 s, and thereafter kept stable for about 10 min. More importantly, the color intensity increased with increasing glucose concentration.

By measuring the diffusive reflectance of the rufous spot and plotting the differential reflectance (ΔR) against the logarithm of the glucose concentration, a calibration curve for glucose measurements could be developed. The results shown in Fig. 3(B) indicated that within the tested glucose concentration range (0.1–50 mM), an excellent correlation between the ΔR and the glucose concentration was obtained, with a regression correlation coefficient of 0.999.

To illustrate the feasibility of using the hollow nanofiber membrane based *o*-dianisidine-test strip in potential clinical diagnoses, it was employed to measure glucose in human blood serum. Five fresh human blood samples were analyzed by both the commercial glucose test kit based on the spectrophotometric method and the *o*-dianisidine-test strip. The results are summarized in Table 1. As can be seen, the glucose

concentrations determined by the hollow nanofiber membrane-based test strips were satisfactory and in good agreement with values measured using the spectrophotometric method. Thus, these results indicated the suitability of the hollow nanofiber membrane-based glucose test strips for practical applications.

The *o*-dianisidine-test strip also showed excellent storage stability. As shown in Fig. 4, when the test strips were stored at 4 °C, no loss of activity was found for a 4 month period. Even at 25 °C, about 50% of the original activity was retained after storage for four months. Because GOD and HRP were confined inside the nano-chamber of the hollow fibers, it was considered that the spatial confining effect provided the enzymes with a unique stabilizing mechanism. Such excellent storage stability makes the prepared test strips attractive for potential clinic applications.

4. Conclusions

In this paper, we proposed a simple strategy to fabricate hollow nanofiber membrane-based test strips for glucose measurement. Using a co-axial electrospinning technique, both GOD and HRP were encapsulated *in situ* inside the chamber of the hollow fiber, at the same time chromogenic agent, *o*-dianisidine or ABTS, was also simultaneously immobilized in the hollow fiber. Such unique "all-in-one" feature of the hollow fiber membrane enabled the prepared test strips to be applied either in solution as colorimetric sensors or as optical biosensors operated in "dip-and-read" mode. Owing to good stability and high activity, the strips with *o*-dianisidine as chromogenic agent showed an excellent linear response range between 0.01 mM to 20 mM when was used as colorimetric biosensors for glucose in solution. As an optical biosensor, visible rufous color was quickly developed on the surface of the membrane by dropping 10 μL of glucose sample onto it, and an excellent correlation between ΔR and the glucose concentration was obtained over a glucose concentration range from 0.1–50 mM. The test strips also exhibited excellent long-term storage stability. The broad detection range and excellent stability make the prepared test strips suitable for practical clinic application. Moreover, the facile preparation process for the hollow nanofiber membrane and the advantages of the simultaneous *in situ* co-immobilization approach for multiple substances are expected to promote the development of a great variety of biosensors that need multienzymes and coenzymes or chromogenic agents for measurement.

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