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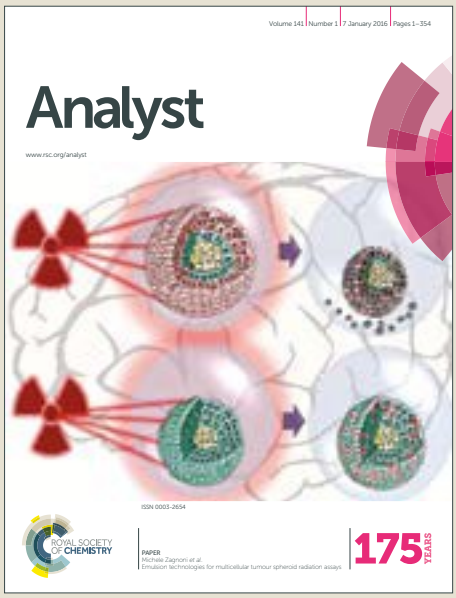

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Temperature-induced amperometric glucose biosensor based on a poly (N-vinylcaprolactam)/graphene oxide composited film

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Abstract

A temperature-induced sensing film consisting of poly (N-vinylcaprolactam) (PVCL), graphene oxide (GO) and glucose oxidase (GOD) was fabricated and used to modify a glassy carbon electrode (GCE). The PVCL/GO/GOD/GCE composited film was characterized by the electrochemical impedance spectroscopy (EIS). The morphological properties of the composite were investigated by scanning electron microscopy (SEM). The direct electron transfer (DET) of GOD was achieved. GOD at PVCL / GO / GOD / GCE exhibited a couple of well-defined redox peaks with a formal potential of −0.432 V (vs. Ag/AgCl). The composited film showed temperature-induced catalytic activity toward glucose. Large peak currents were observed by amperometry at -0.39V (vs. Ag/AgCl) when the temperature was above the lower critical solution temperature (LCST) of PVCL, and then disappeared when it's below LCST. The modified electrode displayed excellent electrocatalytic response to the glucose. The detection of glucose with the composited film ranged from 0.1 to 1.7 mmol·L^{-1} above 35°C.The constructed biosensor also possesses good stability, reproducibility and anti-interference ability.

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1. Introduction

In recent years, the controllable or "signal-triggered" bioelectrocatalysis has aroused great interest and demonstrated great significance not only in the development of "stimuli-responsive" biosensors, but also in the development of biofuel cells, bioelectronics elements, signal transduction and amplification, information storage and processing.[1-4] Most of the stimuli-responsive materials are used in sensors are polymers, such as hydrogel polymer or biopolymer.[5, 6] These polymers may provide a suitable microenvironment for enzymes to retain their biological activity. They also enhances the direct electron exchange between the enzymes and underlying electrodes. So incorporating redox enzymes into stimuli-responsive polymer films has shown great potential in biosensor field.[7,8]. In all stimuli-responsive polymers, temperature-responsive polymers as one of the largest classes, with a characteristic property of having a lower critical solution temperature (LCST), have opened new horizons for biosensor development.

Several groups[9-13] used stimuli-responsive polymers to modify electrodes for biosensing studies. Dou[14] constructed a layered double hydroxides (LDH)/ poly (N-isopropylacrylamide) (PNIPAm) composited membrane to control the glucose oxidation process through regulating the temperature. Zhou[15] employed poly (N-isopropylacrylamide)-b-poly (2-acrylamidoethyl benzoate), graphene oxide and hemoglobin to construct a temperature-responsive amperometric H_2O_2 biosensor. These biosensors showed switchable interfacial electron transporting or electro-catalysis properties by controlling external temperature. But it remains a challenge to construct biosensors with high flexibility and enzyme activity.

Poly (N-vinylcaprolactam) (PVCL) is one of the several nonionic water-soluble polymers that undergo temperature-induced phase separation in water. With the advantages of little interacting with biomolecules and low toxicity, it is suitably used in cell, protein carrier, bio-separation and other fields. PVCL also has a LCST around 35°C .[16, 17] which is in favor of electrochemical measurements at room temperature.

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Graphene oxide (GO), a new class of two-dimensional carbon nanostructure, with some containing oxygen functional groups such as epoxy group, −OH and –COOH on its edges and plane. GO having a large specific surface area and abundant functional groups, provides an ideal substrate for the study of enzyme immobilization.[18, 19] At the same time, it helps to modify the surface of the electrode by regulating the number of oxygen-containing functional groups. These unique properties can provide a friendly microenvironment for attached redox proteins.

In the present paper, temperature-sensitive polymers PVCL with GO surfaces are coupled with glucose oxidase (GOD) through π–π stacking interactions to constitute a thermo-sensitive composited membrane, which improves the stability of the composited film.[20] To the best of our knowledge, the investigation of the temperature-responsive PVCL / GO / GOD composite film has not been reported. On one hand， the temperature-induced glucose biosensors can improve the selectivity of glucose biosensors. On the other hand, it provides a facile and reliable approach for designing ordered command interface for continuous monitoring of glucose.

Mixing PVCL polymer with GO and GOD can obtain a composite film with temperature-tunable interfacial resistance property. When temperatures are below LCST, the polymer PVCL chain is in a contracted state, and the GOD is entrapped so that it cannot be in contact with the electrode surface, direct electron transfer is prohibited, and the redox process cannot proceed. When the temperature are higher than the LCST, the PVCL chain of the polymer is stretched, the GOD can reach the electrode surface, the direct electron transfer process is realized, and the electrocatalytic reaction of glucose is obtained. GOD entrapped in this composite film possessed temperature-tunable electrochemical performance and catalytic activity toward glucose (Scheme 1).

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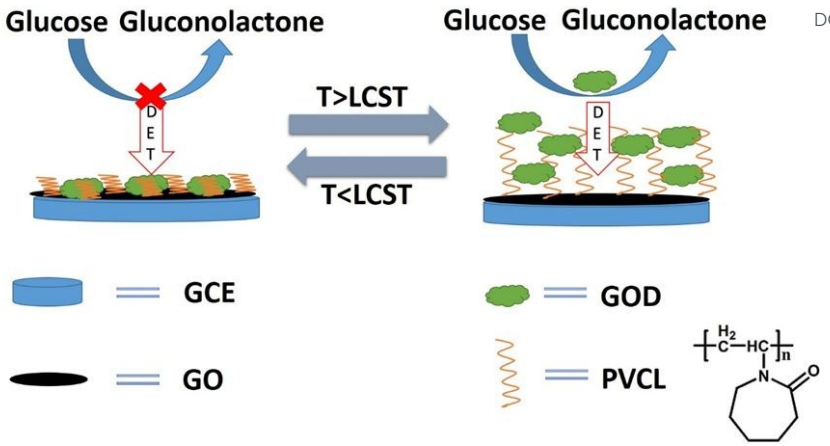

Scheme 1. Schematic illustration of PVCL / GO / GOD film with a temperature-induced "contraction-expansion" property

2. Experimental

2.1. Reagents and Materials

GOD was purchased from Sigma-Aldrich, and used without further purification. GO was prepared according to literature procedures.[21] Vinyl caprolactam (VCL) was purchased from Sigma-Aldrich, and used via recrystallization further purification. PVCL was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization according to literature procedures, and its LCST is around 35 °C.[22] All the other chemicals were of analytical grade and all aqueous solutions prepared by deionized water (DW, 18.25MΩ/cm) were used in the experiments throughout. The buffer, which was used as supporting electrolyte, was 0.1 mol·L^{-1} phosphate solution.

2.2. Fabrication of the Modified Electrodes

A glassy carbon electrode (GCE) was carefully polished with 0.5-μm alumina on a polish cloth, followed by sequential cleaning for 5 min with ethanol and deionized water in an ultrasonic bath, and then dried in the air before use.

3 μL 4 mg·mL^{-1} PVCL was mixed with 2 μL 4 mg·mL^{-1} GO solution, and ultrasonicated for 10 min. then added to 8 μL 4 mg·mL^{-1} GOD solution, and shaken constantly. 6 μL the mixed solution was dropped on the surface of clean GCE, and dried at room temperature to form a composited film on GCE.

2.3. Electrochemical Measurements

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Electrochemical experiments were performed at a CHI 630 electrochemical workstation with a conventional three-electrode cell. A modified electrode was used as the working electrode, a platinum wire was employed as the auxiliary electrode and Ag/AgCl electrode was served as the reference electrode. During the electrochemical measurements, electrolyte solutions were purged with high purity nitrogen or oxygen for at least 20 min, and then maintained at nitrogen or oxygen atmosphere. Zahner Zennium electrochemical workstation (ZAHNER-ELEKTRIK GmbH & Co. KG) was employed for the electrochemical impedance spectra (EIS) measurement. Cyclic voltammetry (CV) measurement was carried out in 0.1 mol·L^{-1} phosphate buffer, ranging from -0.8 V to 0 V at a scan rate of 50 mV·s^{-1}.

3. Results and Discussion

3.1. SEM characterization

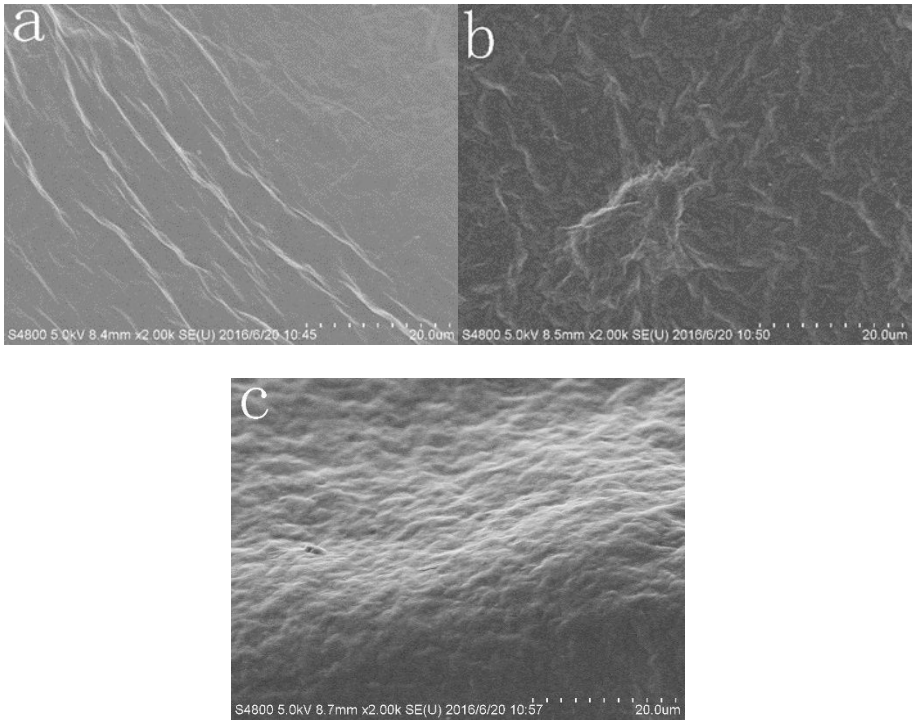

Fig.1 SEM images of (a) GO (b) PVCL / GO (c) PVCL / GO / GOD.

Scanning electron microscopy (SEM) was used to characterize the surface morphology of the modified material. As seen in Fig. 1a, GO is a uniform film-like structure. Compared with Fig.1a, PVCL / GO (Fig. 1b) shows wrinkled flakes, demonstrating that PVCL successfully functionalized the GO. Under the same conditions, PVCL / GO / GOD

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(Fig. 1c) showed aggregated lumps, indicating that there was mutual interaction between GOD and PVCL / GO.

3.2. EIS study of Different Electrodes and EIS Temperature Effect of the composited Electrode

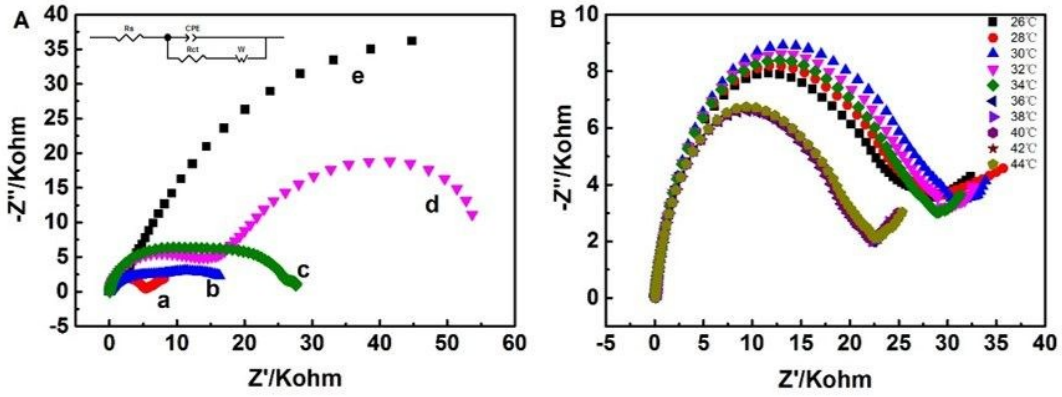

Fig.2 (A) Electrochemical impedance spectra obtained at different modified electrodes in supporting electrolyte at 35°C. (a) GCE (b) PVCL / GO / GOD / GCE (c) PVCL / GCE (d) PVCL /GOD / GCE (e) GO / GCE (B) Electrochemical impedance spectra obtained at the PVCL / GO / GOD / GCE upon stepwise heating from 28 to 42°C. Frequency range: 0.1 Hz to 100 KHz; Supporting electrolyte: 0.1 M KCl containing 5 mM K_3 [Fe (CN) ^{6}]/K_4 [Fe (CN) ^{6}] (1:1).

EIS provide information about the impedance changes of modified electrodes. The high frequency part of semicircle in the EIS spectrum indicates the electron transfer process of speed, and diameter size is closely related to the electron transfer resistance (Rct). The low frequency part of straight line indicates diffusion control process. According to the Rct of different modified electrodes, we can obtain the interfacial impedance change of these modified electrodes, which is related to some interface properties such as permeability and conductivity. Fig.2A shows the EIS results of different modified electrodes toward a soluble redox probes $[Fe(CN)_6]^{3-/4-}$. It is observed that the Rct at GO / GCE is smaller than other modified electrodes. Because the moderate conductivity of GO enhances the interfacial electron transfer at GO / GCE (Fig.2A curve e). The Rct of PVCL / GCE (Fig. 2A curve c) is found to be the biggest, mainly because PVCL is a large molecular weight polymer, which results in putting off electron transfer. When GOD is embedded in PVCL / GO / GCE film, the Rct decreases dramatically (Fig.2A curve b), which indicates PVCL / GO / GOD / GCE hybrid film can act as electron conducting tunnel, and increase the electron transfer rate between $[Fe(CN)_6]^{3-}$/ $[Fe(CN)_6]^{4-}$ probe and underlying electrode. The result also proves

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GOD is successfully embedded in PVCL / GO / GCE film.

The temperature-dependent EIS experiments of PVCL / GO / GOD / GCE (Fig.2B) is conducted after reaching a steady state through several repetitive EIS test in 1 mmol·L^{-1} K_3 [Fe (CN) $_6$]/K_4 [Fe (CN) $_6$] at 28°C. Upon increasing the solution temperature from 28 to 42°C, the semicircle diameter basically remained unchanged at 28 to 34°C. However, heating above 35°C, a progressive decrease in the semicircle diameter is observed at 36 to 42°C. The results demonstrated an enhanced interfacial electron transfer of PVCL / GO / GOD / GCE above 35°C and a decreased one below 35°C.

3.3. Electrochemical properties and temperature effect on cyclic voltammetry (CV)

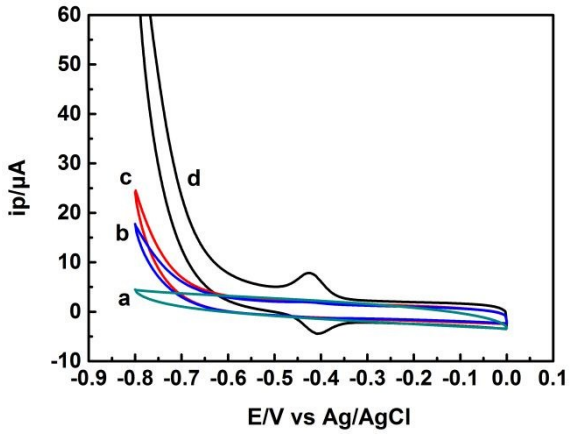

Fig. 3. Cyclic voltammetrics of (a) GCE (b) PVCL / GO / GCE (c) PVCL / GOD / GCE (d) PVCL / GO / GOD / GCE.

The electrochemical behaviors of different GOD electrodes in N_2-saturated pH 7.0 PBS were studied by cyclic voltammetry. As shown in Fig.3，there was no obvious voltammetric signal at the GCE (curve a) , PVCL / GO / GCE (curve b) and PVCL / GOD / GCE(curve c)，which indicates that these modified electrodes can't provide a favorable electrochemical environment for GOD for direct electron exchange with the underlying electrode. However, a couple of well-defined and symmetrical redox peaks are observed at PVCL / GO / GOD / GCE (curve d), which indicated that the composited film can provide a more friendly environment for GOD. The anodic peak potential (Ep_a) and the cathodic peak potential (Ep_c) are located at about -0.414V and -0.449 V respectively, with a formal potential E^{θ} at -0.432 V, which revealed the presence of redox active center ($FAD/FADH_2$) in the GOD. And

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the peak separation in potential (ΔE_p) is 35 mV. The smaller ΔE_p shows a rapid direct electron transfer that occurs on the composited modified electrode. Also, the anodic and cathodic peak currents are almost equal, implying that GOD undergoes an aquasi-reversible electrochemical reaction.

Upon increasing scan rate from 20 to 300 mV·s^{-1} (Fig. S1), the anodic and cathodic peak potentials remain the same, while the currents (ip_a and ip_c) increased linearly. These indicated that the electrochemical reactions of GOD at PVCL / GO / GOD / GCE obey a thin layer electrochemical characteristic and the reaction is a typical surface-controlled quasi-reversible process.

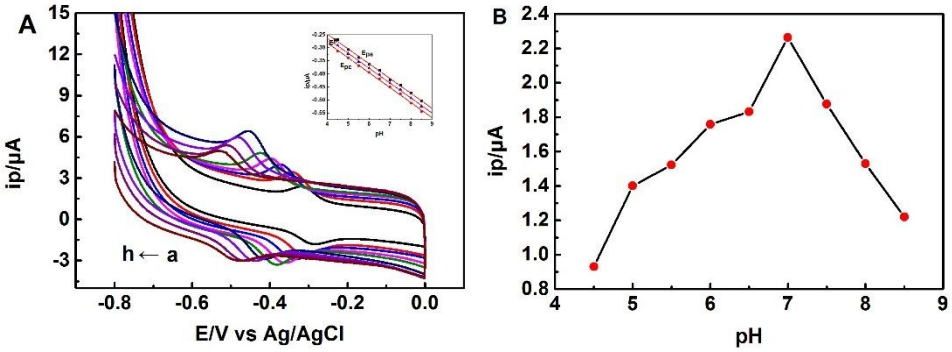

Fig. 4 (A) CV plots of PVCL / GO / GOD / GCE at various pH from 4.5 to 8.5; Scan rate: 50 mV·s^{-1}; (B) The plot of i_{pa} vs. pH

According to Faraday's equation, Q=nFAΓ*, Where n represents the number of electron transfer, A (cm^2) is 0.0872cm^2 of the effective area of the modified electrode, Q (C) is the quantity of charge, and F is Faraday's constant, Γ*is the surface concentration of electroactive GOD. Here, the Γ*is calculated to be (4.8±0.05) ×10^{-12} mol/cm^2, which was slightly larger than the theoretical GOD monolayer coverage (2.86 × 10^{-12} mol/cm^2) .[23] It indicates that increasing electroactive surface area on the electrode. When $n\Delta Ep<200$ mV, the electron transfer rate constant k_s of electrode reaction can be analyzed by using the model of Laviron.[24] The average k_s value of GOD at PVCL / GO / GOD / GCE is calculated to be 2.15±0.05 s^{-1} at the scan rates ranging from 20 mV·s^{-1} to 300 mV·s^{-1}. The pH of the buffer is a prominent factor that affects the electrochemical behavior of the biosensor because the pH value has a great influence on the activity of the enzyme. So the influence of solution pH toward PVCL / GO / GOD / GCE is studied by the cyclic voltammetric at the scan rate of 50 mV·s^{-1}(Fig. 4A). When solution pH changes from 4.5 to

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8.5, the maximum peak current response was observed at pH 7.0(Fig. 4B), hence the optimum pH value was 7.0, which was employed in the following experiments. In addition, the cathodic potential (Ep_a), anodic peak potential (Ep_c) and formal potential (E^{θ}) negatively shift with increase in pH value with a slope of -56.6, -57.1 and -56.9 mV / pH respectively, close to the theoretical value (-59 mV/pH).[25] These indicate that the electron transfer between GOD in the composited film is an equal number of protons and electrons process.

To further reveal the temperature effect on electrochemistry behavior of PVCL / GO / GOD / GCE by the cyclic voltammetric with the scan rate of 50 $mV \cdot s^{-1}$(Fig. S2), the temperature increased from 22 to 42°C, an obvious change of anodic and cathodic peak current are observed at the phase transition temperature of PVCL. A high anodic and cathodic peak current are observed in the swollen state of PVCL above 36 °C and a low one is observed in the shrunken state. Because the expansion-to-contraction transition of PVCL / GO / GOD / GCE film is induced by the phase transition of PVCL upon temperature change. When the temperature is higher than 36°C, the anodic and cathodic peak current increase with the temperature increasing.

3.4. Electrocatalytic activity and temperature effect on electrocatalytic activity

Firstly, the electrocatalytic activity of GOD at PVCL / GO / GOD / GCE film was explored by the cyclic voltammetric with the scan rate of 50 $mV \cdot s^{-1}$.As shown in Fig.S3, a couple of well-defined and symmetrical redox peaks are observed at PVCL / GO / GOD / GCE in the N_2-saturated environment (curve a). However, the composited film at O_2-saturated environment (curve b), the CV plots is obviously different. Compared with the curve a, the anodic peak current is reduced, and the cathodic peak current is increased. And added glucose into the oxygen saturation in the PBS, as shown in figure curve c, compared with curve b, the cathodic peak current curve c is reduced, and with the increase of the concentration of glucose, cathodic peak current decrease (curve d and e). This process is called as a typical electrocatalysis of glucose by the GOD in the presence of oxygen saturated PBS. It is well known that the DET of GOD is a two-electron along with two-proton reaction that the enzymes react with glucose

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occurred as follows[26]:

$$GOD - FAD + 2e^- + 2H^+ \leftrightarrow GOD - FADH_2 \quad I$$

$$GOD - FADH_2 + O_2 \rightarrow GOD - FAD + H_2O_2 \quad II$$

$$Glucose + GOD - FAD \rightarrow GOD - FADH_2 + glucose\ acid \quad III$$

Moreover, the amperometric response is most widely used in electrochemical sensor research, in this work, amperometric response toward glucose was carried out in a well-stirred solution. Fig.S4 shows the amperometric response effect of PVCL / GO / GOD / GCE in catalyzing glucose at different potentials. When the potential increases from -0.22V to -0.39 V, the reduction currents increased. Below the -0.39V, the reduction currents decrease. Hence, the optimum detection potential for glucose is -0.39V.

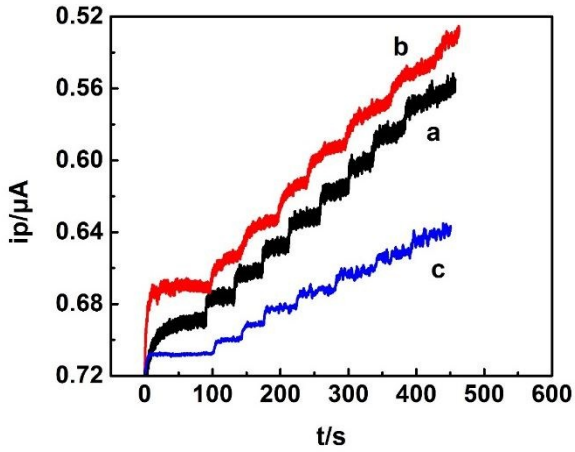

Fig. 5. Amperometric response diagram for glucose reduction catalyzed by different modified electrodes :(a) PVCL / GO / GOD / GCE (b) PVCL / GOD / GCE(c) GO / GOD / GCE.

Successive additions of glucose were performed every 40 s at -0.39 V in 0.1 M oxygen saturated PBS (pH=7) solution around 35°C (Fig.S6). It is clear that with increasing the concentration of glucose, the reduction currents increase fast. Although some unstable current responses are observed, the differences between each two steps are basically consistent. The linear detection range at the composited film is from 0.1 to 1.7 mmol·L^{-1} with a correlation coefficient of 0.999(n=17) .The detection limit is 0.066 mM (S/N=3). However, a same composited film was carried out in accordance with the same steps for ampere detection at 25°C. It can be seen clearly there is no obvious electrocatalytic activity at 25 °C (Fig. S6). The result is, in most cases, lower than the other GOD modified electrodes (Table 1),[27-30] which indicate that the excellent conductivity and good biocompatibility of the PVCL / GO / GOD /

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GCE modified electrode. Moreover, compared with the modified electrodes PVCL / GOD / GCE and GO / GOD / GCE, PVCL / GO / GOD / GCE shows the best amperometric responses in catalyzing glucose (Fig. 5).

Table 1 Comparison of the analytical performance of the other glucose biosensors

Modified electrode	Linear range (mM)	Detection limit (mM)	Comments	Ref.
Graphene-chitosan nanocomposite/GOD	0.08–12	0.02	A favorable microenvironment of the enzyme, but poor dispersion	[27]
Gelatin-multiwalled carbon nanotube/GOD	6.30–20.09	-	Good biocompatibility, excellent selectivity, but poor LODs	[28]
Poly(2,6-diaminopyridine)-carbon nanotube electrode/GOD	0.00042–8.0	0.00013	Convenient preparation, excellent properties and nanomole detection limit, but poor anti-interference ability	[29]
Ag@MWCNT-IL-Fe_3O_4/GOD	0.006–2.0	0.00212	High sensitivity, stability, but complicated modified procedures of electrodes.	[30]
Au–ZnO/GOD /GCE	1-20	0.02	Facile and low cost procedure, but poor anti-interference ability	[31]
PVCL/GO/GOD	0.1-1.7	0.066	Temperature-induced electrochemical behaviors and highly sensitive	This work

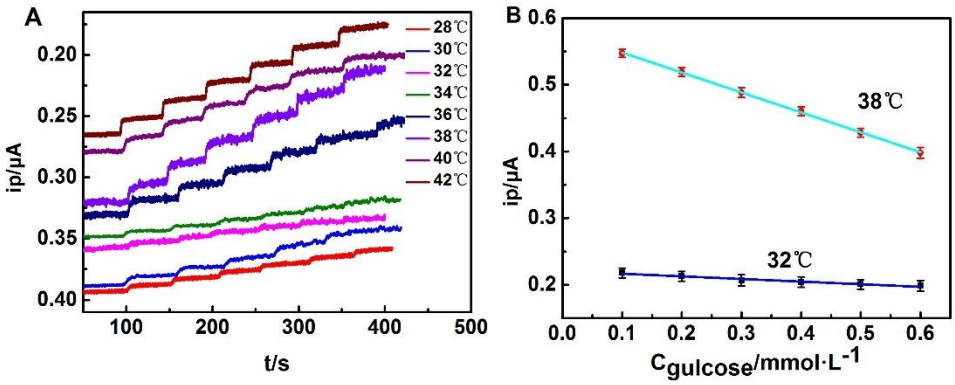

Fig. 6. (A)Influence of temperatures on amperometric response of PVCL / GO / GOD / GCE with a successive injection of glucose in 0.1 mol·L^{-1} phosphate buffer (pH 7.0) saturated with O_2 at −0.39 V.(B) Linear relation of amperometric response vs glucose concentration at 32 ℃ and 38 ℃ (the numbers of experiments calculating standard deviations is 3)

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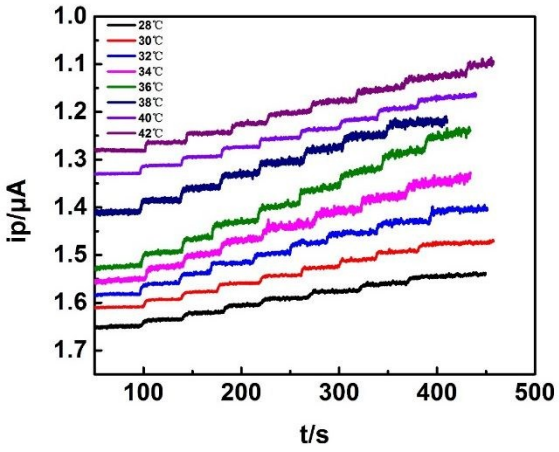

Fig. 7. Influence of temperatures on amperometric response of GO / GOD / GCE with a successive injection of glucose in 0.1 mol·L^{-1} phosphate buffer (pH 7.0) saturated with O_2 at −0.39 V.

Fig. 6A shows the amperometric response of PVCL / GO / GOD / GCE in catalyzing glucose at different temperatures under the same optimal experimental conditions. Upon successive additions of 0.1 mM glucose to phosphate buffer (pH 7.0), gradual increases in the amperometric signal are observed from 28 to 42°C. But, a more obvious increase in electrocatalytic current as well as sensitivity was obtained at the temperature above 35°C, compared to the temperature below 35 °C, which clearly indicates a better electrocatalytic activity exists above 35 °C. For comparison, the amperometric responses toward glucose at GO / GOD / GCE were conducted in the same experiment condition. There is no obvious electrocatalytic activity difference upon heating from 28 to 42 °C (Fig. 7).This result is consistent with the temperature-dependent EIS spectrum (Fig.2B). So the changes of electrocatalytic activity at PVCL / GO / GOD / GCE film above 35°C and below 35°C should be attributed to the temperature-responsive phase transition behavior of PVCL. From 25 to 35°C, PVCL undergoes dehydration/hydration transition and induces "contraction-to-expansion" of PVCL / GO / GOD / GCE film. This leads to two different electrocatalytic activity states of PVCL / GO / GOD / GCE toward glucose. The amperometric response currents at 32°C and 38°C were plotted against glucose concentration to obtain a linear plot of current versus concentration (Fig. 6B). The linear equation were ip(10^{-7}A)=-0.936$C_{glucose}$+2.21 and ip(10^{-7}A)=-2.99 $C_{glucose}$+5.78, which showed a linear slope at 38°C, much greater than the value at 32°C.

3.5. Stability, Reproducibility and Anti-interference Ability

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The stability of PVCL / GO / GOD / GCE had been investigated by repeated CV scanning for 15 cycles at 50 $mV \cdot s^{-1}$, the direct electrochemical responses remained constant. The modified electrode was stored in 4 °C for 7 days, the current retained 94 % of its initial current response. These results indicate that this biosensor possesses high stability. PVCL / GO / GOD / GCE were used for amperometric response to 0.1 $mmol \cdot L^{-1}$glucose in 0.1 $mol \cdot L^{-1}$ phosphate buffer (pH 7.0). The relative standard deviation (RSD) for 10 successive assays is 4.63% at 35 °C and 5.82% at 25 °C. These imply that the biosensor possesses good reproducibility.

In order to study the selectivity of the biosensor, anti-interference experiments were carried out. The results turned out that 2 times glycine; 2 times Uric Acid; 3 times D-galactose; 5 times L-tyrosine; 2 times dopamine and 2 times ascorbic acid many as glucose co-existing in the sample matrix don't affect the determination of glucose. (Fig.S5)

3.6 Analysis of glucose in serum samples

To illustrate accuracy and feasibility of the biosensor for real sample analysis， the PVCL / GO / GOD / GCE was used for detection of glucose in serum samples. The serum samples were obtained from the school hospital, and the serum samples were not pretreated except for the dilution step with PBS (pH 7.0). In addition, the amount of glucose in the serum samples was also measured photometrically in the hospital. Comparison results are listed in Table 2. The measured value of the biosensor is very close to the data provided by the hospital, and the relative error is less than 3%, which confirmed that the prepared PVCL / GO / GOD / GCE can serve as a stable and effective sensor for analyzing real samples.

Table 2 Measurement results of glucose in serum samples (n=3)

Serum Sample	Photometric method (mM)	Proposed method (mM)	Relative error (%)
A	6.75	6.83	+1.2
B	6.37	6.21	-2.5

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C	4.74	4.78	+0.8

4. Conclusion

The PVCL / GO composited film with a temperature-induced property was applied to immobilize GOD on GCE. Its redox peak current shows two different current states around the critical temperature of LCST. Also, it exhibits two different electrocatalytic activities toward glucose in this temperature range. When the temperature is above the LCST of PVCL, the composite exhibited an excellent electro-catalytic activity. In contrast, it hardly showed electro-catalytic activity at the temperature below LCST. The temperature-responsive CV and amperometric behavior of the composite film are attributed to the contraction−expansion transition of PVCL. The novel strategy presented in this work can be effectively useful for the fabrication of stimuli-responsive composite with prospective applications in switchable sensors.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Temperature-induced amperometric glucose biosensor based on a poly (N-vinylcaprolactam)/graphene oxide composited film

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Abstract

A temperature-induced sensing film consisting of poly (N-vinylcaprolactam) (PVCL), graphene oxide (GO) and glucose oxidase (GOD) was fabricated and used to modify a glassy carbon electrode (GCE). The PVCL/GO/GOD/GCE composited film was characterized by the electrochemical impedance spectroscopy (EIS). The morphological properties of the composite were investigated by scanning electron microscopy (SEM). The direct electron transfer (DET) of GOD was achieved. GOD at PVCL / GO / GOD / GCE exhibited a couple of well-defined redox peaks with a formal potential of −0.432 V (vs. Ag/AgCl). The composited film showed temperature-induced catalytic activity toward glucose. Large peak currents were observed by amperometry at -0.39V (vs. Ag/AgCl) when the temperature was above the lower critical solution temperature (LCST) of PVCL, and then disappeared when it's below LCST. The modified electrode displayed excellent electrocatalytic response to the glucose. The detection of glucose with the composited film ranged from 0.1 to 1.7 mmol·L^{-1} above 35°C.The constructed biosensor also possesses good stability, reproducibility and anti-interference ability.

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1. Introduction

In recent years, the controllable or "signal-triggered" bioelectrocatalysis has aroused great interest and demonstrated great significance not only in the development of "stimuli-responsive" biosensors, but also in the development of biofuel cells, bioelectronics elements, signal transduction and amplification, information storage and processing.[1-4] Most of the stimuli-responsive materials are used in sensors are polymers, such as hydrogel polymer or biopolymer.[5, 6] These polymers may provide a suitable microenvironment for enzymes to retain their biological activity. They also enhances the direct electron exchange between the enzymes and underlying electrodes. So incorporating redox enzymes into stimuli-responsive polymer films has shown great potential in biosensor field.[7,8]. In all stimuli-responsive polymers, temperature-responsive polymers as one of the largest classes, with a characteristic property of having a lower critical solution temperature (LCST), have opened new horizons for biosensor development.

Several groups[9-13] used stimuli-responsive polymers to modify electrodes for biosensing studies. Dou[14] constructed a layered double hydroxides (LDH)/ poly (N-isopropylacrylamide) (PNIPAm) composited membrane to control the glucose oxidation process through regulating the temperature. Zhou[15] employed poly (N-isopropylacrylamide)-b-poly (2-acrylamidoethyl benzoate), graphene oxide and hemoglobin to construct a temperature-responsive amperometric H_2O_2 biosensor. These biosensors showed switchable interfacial electron transporting or electro-catalysis properties by controlling external temperature. But it remains a challenge to construct biosensors with high flexibility and enzyme activity.

Poly (N-vinylcaprolactam) (PVCL) is one of the several nonionic water-soluble polymers that undergo temperature-induced phase separation in water. With the advantages of little interacting with biomolecules and low toxicity, it is suitably used in cell, protein carrier, bio-separation and other fields. PVCL also has a LCST around 35°C .[16, 17] which is in favor of electrochemical measurements at room temperature.

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Graphene oxide (GO), a new class of two-dimensional carbon nanostructure, with some containing oxygen functional groups such as epoxy group, −OH and –COOH on its edges and plane. GO having a large specific surface area and abundant functional groups, provides an ideal substrate for the study of enzyme immobilization.[18, 19] At the same time, it helps to modify the surface of the electrode by regulating the number of oxygen-containing functional groups. These unique properties can provide a friendly microenvironment for attached redox proteins.

In the present paper, temperature-sensitive polymers PVCL with GO surfaces are coupled with glucose oxidase (GOD) through π–π stacking interactions to constitute a thermo-sensitive composited membrane, which improves the stability of the composited film.[20] To the best of our knowledge, the investigation of the temperature-responsive PVCL / GO / GOD composite film has not been reported. On one hand， the temperature-induced glucose biosensors can improve the selectivity of glucose biosensors. On the other hand, it provides a facile and reliable approach for designing ordered command interface for continuous monitoring of glucose.

Mixing PVCL polymer with GO and GOD can obtain a composite film with temperature-tunable interfacial resistance property. When temperatures are below LCST, the polymer PVCL chain is in a contracted state, and the GOD is entrapped so that it cannot be in contact with the electrode surface, direct electron transfer is prohibited, and the redox process cannot proceed. When the temperature are higher than the LCST, the PVCL chain of the polymer is stretched, the GOD can reach the electrode surface, the direct electron transfer process is realized, and the electrocatalytic reaction of glucose is obtained. GOD entrapped in this composite film possessed temperature-tunable electrochemical performance and catalytic activity toward glucose (Scheme 1).

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Scheme 1. Schematic illustration of PVCL / GO / GOD film with a temperature-induced "contraction-expansion" property

2. Experimental

2.1. Reagents and Materials

GOD was purchased from Sigma-Aldrich, and used without further purification. GO was prepared according to literature procedures.[21] Vinyl caprolactam (VCL) was purchased from Sigma-Aldrich, and used via recrystallization further purification. PVCL was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization according to literature procedures, and its LCST is around 35 °C.[22] All the other chemicals were of analytical grade and all aqueous solutions prepared by deionized water (DW, 18.25MΩ/cm) were used in the experiments throughout. The buffer, which was used as supporting electrolyte, was 0.1 mol·L^{-1} phosphate solution.

2.2. Fabrication of the Modified Electrodes

A glassy carbon electrode (GCE) was carefully polished with 0.5-μm alumina on a polish cloth, followed by sequential cleaning for 5 min with ethanol and deionized water in an ultrasonic bath, and then dried in the air before use.

3 μL 4 mg·mL^{-1} PVCL was mixed with 2 μL 4 mg·mL^{-1} GO solution, and ultrasonicated for 10 min. then added to 8 μL 4 mg·mL^{-1} GOD solution, and shaken constantly. 6 μL the mixed solution was dropped on the surface of clean GCE, and dried at room temperature to form a composited film on GCE.

2.3. Electrochemical Measurements

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Electrochemical experiments were performed at a CHI 630 electrochemical workstation with a conventional three-electrode cell. A modified electrode was used as the working electrode, a platinum wire was employed as the auxiliary electrode and Ag/AgCl electrode was served as the reference electrode. During the electrochemical measurements, electrolyte solutions were purged with high purity nitrogen or oxygen for at least 20 min, and then maintained at nitrogen or oxygen atmosphere. Zahner Zennium electrochemical workstation (ZAHNER-ELEKTRIK GmbH & Co. KG) was employed for the electrochemical impedance spectra (EIS) measurement. Cyclic voltammetry (CV) measurement was carried out in 0.1 mol·L^{-1} phosphate buffer, ranging from -0.8 V to 0 V at a scan rate of 50 mV·s^{-1}.

3. Results and Discussion

3.1. SEM characterization

Fig.1 SEM images of (a) GO (b) PVCL / GO (c) PVCL / GO / GOD.

Scanning electron microscopy (SEM) was used to characterize the surface morphology of the modified material. As seen in Fig. 1a, GO is a uniform film-like structure. Compared with Fig.1a, PVCL / GO (Fig. 1b) shows wrinkled flakes, demonstrating that PVCL successfully functionalized the GO. Under the same conditions, PVCL / GO / GOD

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(Fig. 1c) showed aggregated lumps, indicating that there was mutual interaction between GOD and PVCL / GO.

3.2. EIS study of Different Electrodes and EIS Temperature Effect of the composited Electrode

Fig.2 (A) Electrochemical impedance spectra obtained at different modified electrodes in supporting electrolyte at 35°C. (a) GCE (b) PVCL / GO / GOD / GCE (c) PVCL / GCE (d) PVCL /GOD / GCE (e) GO / GCE (B) Electrochemical impedance spectra obtained at the PVCL / GO / GOD / GCE upon stepwise heating from 28 to 42°C. Frequency range: 0.1 Hz to 100 KHz; Supporting electrolyte: 0.1 M KCl containing 5 mM K_3 [Fe (CN) ^{6}]/K_4 [Fe (CN) ^{6}] (1:1).

EIS provide information about the impedance changes of modified electrodes. The high frequency part of semicircle in the EIS spectrum indicates the electron transfer process of speed, and diameter size is closely related to the electron transfer resistance (Rct). The low frequency part of straight line indicates diffusion control process. According to the Rct of different modified electrodes, we can obtain the interfacial impedance change of these modified electrodes, which is related to some interface properties such as permeability and conductivity. Fig.2A shows the EIS results of different modified electrodes toward a soluble redox probes $[Fe(CN)_6]^{3-/4-}$. It is observed that the Rct at GO / GCE is smaller than other modified electrodes. Because the moderate conductivity of GO enhances the interfacial electron transfer at GO / GCE (Fig.2A curve e). The Rct of PVCL / GCE (Fig. 2A curve c) is found to be the biggest, mainly because PVCL is a large molecular weight polymer, which results in putting off electron transfer. When GOD is embedded in PVCL / GO / GCE film, the Rct decreases dramatically (Fig.2A curve b), which indicates PVCL / GO / GOD / GCE hybrid film can act as electron conducting tunnel, and increase the electron transfer rate between $[Fe(CN)_6]^{3-}$/ $[Fe(CN)_6]^{4-}$ probe and underlying electrode. The result also proves

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GOD is successfully embedded in PVCL / GO / GCE film.

The temperature-dependent EIS experiments of PVCL / GO / GOD / GCE (Fig.2B) is conducted after reaching a steady state through several repetitive EIS test in 1 mmol·L^{-1} K_3 [Fe (CN) $_6$]/K_4 [Fe (CN) $_6$] at 28°C. Upon increasing the solution temperature from 28 to 42°C, the semicircle diameter basically remained unchanged at 28 to 34°C. However, heating above 35°C, a progressive decrease in the semicircle diameter is observed at 36 to 42°C. The results demonstrated an enhanced interfacial electron transfer of PVCL / GO / GOD / GCE above 35°C and a decreased one below 35°C.

3.3. Electrochemical properties and temperature effect on cyclic voltammetry (CV)

Fig. 3. Cyclic voltammetrics of (a) GCE (b) PVCL / GO / GCE (c) PVCL / GOD / GCE (d) PVCL / GO / GOD / GCE.

The electrochemical behaviors of different GOD electrodes in N_2-saturated pH 7.0 PBS were studied by cyclic voltammetry. As shown in Fig.3，there was no obvious voltammetric signal at the GCE (curve a) , PVCL / GO / GCE (curve b) and PVCL / GOD / GCE(curve c)，which indicates that these modified electrodes can't provide a favorable electrochemical environment for GOD for direct electron exchange with the underlying electrode. However, a couple of well-defined and symmetrical redox peaks are observed at PVCL / GO / GOD / GCE (curve d), which indicated that the composited film can provide a more friendly environment for GOD. The anodic peak potential (Ep_a) and the cathodic peak potential (Ep_c) are located at about -0.414V and -0.449 V respectively, with a formal potential E^{θ} at -0.432 V, which revealed the presence of redox active center ($FAD/FADH_2$) in the GOD. And

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the peak separation in potential (ΔE_p) is 35 mV. The smaller ΔE_p shows a rapid direct electron transfer that occurs on the composited modified electrode. Also, the anodic and cathodic peak currents are almost equal, implying that GOD undergoes an aquasi-reversible electrochemical reaction.

Upon increasing scan rate from 20 to 300 $mV \cdot s^{-1}$ (Fig. S1), the anodic and cathodic peak potentials remain the same, while the currents (ip_a and ip_c) increased linearly. These indicated that the electrochemical reactions of GOD at PVCL / GO / GOD / GCE obey a thin layer electrochemical characteristic and the reaction is a typical surface-controlled quasi-reversible process.

Fig. 4 (A) CV plots of PVCL / GO / GOD / GCE at various pH from 4.5 to 8.5; Scan rate: 50 $mV \cdot s^{-1}$; (B) The plot of i_{pa} vs. pH

According to Faraday's equation, Q=nFAΓ*, Where n represents the number of electron transfer, A (cm^2) is 0.0872cm^2 of the effective area of the modified electrode, Q (C) is the quantity of charge, and F is Faraday's constant，Γ*is the surface concentration of electroactive GOD. Here, the Γ*is calculated to be (4.8±0.05) $\times 10^{-12}$ mol/cm^2, which was slightly larger than the theoretical GOD monolayer coverage (2.86×10^{-12} mol/cm^2) .[23] It indicates that increasing electroactive surface area on the electrode. When nΔEp<200 mV, the electron transfer rate constant k_s of electrode reaction can be analyzed by using the model of Laviron.[24] The average k_s value of GOD at PVCL / GO / GOD / GCE is calculated to be 2.15±0.05 s^{-1} at the scan rates ranging from 20 $mV \cdot s^{-1}$ to 300 $mV \cdot s^{-1}$. The pH of the buffer is a prominent factor that affects the electrochemical behavior of the biosensor because the pH value has a great influence on the activity of the enzyme. So the influence of solution pH toward PVCL / GO / GOD / GCE is studied by the cyclic voltammetric at the scan rate of 50 $mV \cdot s^{-1}$(Fig. 4A). When solution pH changes from 4.5 to

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8.5, the maximum peak current response was observed at pH 7.0(Fig. 4B), hence the optimum pH value was 7.0, which was employed in the following experiments. In addition, the cathodic potential (Ep_a), anodic peak potential (Ep_c) and formal potential (E^{θ}) negatively shift with increase in pH value with a slope of -56.6, -57.1 and -56.9 mV / pH respectively, close to the theoretical value (-59 mV/pH).[25] These indicate that the electron transfer between GOD in the composited film is an equal number of protons and electrons process.

To further reveal the temperature effect on electrochemistry behavior of PVCL / GO / GOD / GCE by the cyclic voltammetric with the scan rate of 50 $mV \cdot s^{-1}$(Fig. S2), the temperature increased from 22 to 42°C, an obvious change of anodic and cathodic peak current are observed at the phase transition temperature of PVCL. A high anodic and cathodic peak current are observed in the swollen state of PVCL above 36 °C and a low one is observed in the shrunken state. Because the expansion-to-contraction transition of PVCL / GO / GOD / GCE film is induced by the phase transition of PVCL upon temperature change. When the temperature is higher than 36°C, the anodic and cathodic peak current increase with the temperature increasing.

3.4. Electrocatalytic activity and temperature effect on electrocatalytic activity

Firstly, the electrocatalytic activity of GOD at PVCL / GO / GOD / GCE film was explored by the cyclic voltammetric with the scan rate of 50 $mV \cdot s^{-1}$.As shown in Fig.S3, a couple of well-defined and symmetrical redox peaks are observed at PVCL / GO / GOD / GCE in the N_2-saturated environment (curve a). However, the composited film at O_2-saturated environment (curve b), the CV plots is obviously different. Compared with the curve a, the anodic peak current is reduced, and the cathodic peak current is increased. And added glucose into the oxygen saturation in the PBS, as shown in figure curve c, compared with curve b, the cathodic peak current curve c is reduced, and with the increase of the concentration of glucose, cathodic peak current decrease (curve d and e). This process is called as a typical electrocatalysis of glucose by the GOD in the presence of oxygen saturated PBS. It is well known that the DET of GOD is a two-electron along with two-proton reaction that the enzymes react with glucose

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occurred as follows[26]:

$$GOD - FAD + 2e^- + 2H^+ \leftrightarrow GOD - FADH_2 \quad I$$

$$GOD - FADH_2 + O_2 \rightarrow GOD - FAD + H_2O_2 \quad II$$

$$Glucose + GOD - FAD \rightarrow GOD - FADH_2 + glucose\ acid \quad III$$

Moreover, the amperometric response is most widely used in electrochemical sensor research, in this work, amperometric response toward glucose was carried out in a well-stirred solution. Fig.S4 shows the amperometric response effect of PVCL / GO / GOD / GCE in catalyzing glucose at different potentials. When the potential increases from -0.22V to -0.39 V, the reduction currents increased. Below the -0.39V, the reduction currents decrease. Hence, the optimum detection potential for glucose is -0.39V.

Fig. 5. Amperometric response diagram for glucose reduction catalyzed by different modified electrodes :(a) PVCL / GO / GOD / GCE (b) PVCL / GOD / GCE(c) GO / GOD / GCE.

Successive additions of glucose were performed every 40 s at -0.39 V in 0.1 M oxygen saturated PBS (pH=7) solution around 35°C (Fig.S6). It is clear that with increasing the concentration of glucose, the reduction currents increase fast. Although some unstable current responses are observed, the differences between each two steps are basically consistent. The linear detection range at the composited film is from 0.1 to 1.7 mmol·L^{-1} with a correlation coefficient of 0.999(n=17) .The detection limit is 0.066 mM (S/N=3). However, a same composited film was carried out in accordance with the same steps for ampere detection at 25°C. It can be seen clearly there is no obvious electrocatalytic activity at 25 °C (Fig. S6). The result is, in most cases, lower than the other GOD modified electrodes (Table 1),[27-30] which indicate that the excellent conductivity and good biocompatibility of the PVCL / GO / GOD /

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GCE modified electrode. Moreover, compared with the modified electrodes PVCL / GOD / GCE and GO / GOD / GCE, PVCL / GO / GOD / GCE shows the best amperometric responses in catalyzing glucose (Fig. 5).

Table 1 Comparison of the analytical performance of the other glucose biosensors

Modified electrode	Linear range (mM)	Detection limit (mM)	Comments	Ref.
Graphene-chitosan nanocomposite/GOD	0.08–12	0.02	A favorable microenvironment of the enzyme, but poor dispersion	[27]
Gelatin-multiwalled carbon nanotube/GOD	6.30–20.09	-	Good biocompatibility, excellent selectivity, but poor LODs	[28]
Poly(2,6-diaminopyridine)-carbon nanotube electrode/GOD	0.00042–8.0	0.00013	Convenient preparation, excellent properties and nanomole detection limit, but poor anti-interference ability	[29]
Ag@MWCNT-IL-Fe_3O_4/GOD	0.006–2.0	0.00212	High sensitivity, stability, but complicated modified procedures of electrodes.	[30]
Au–ZnO/GOD /GCE	1-20	0.02	Facile and low cost procedure, but poor anti-interference ability	[31]
PVCL/GO/GOD	0.1-1.7	0.066	Temperature-induced electrochemical behaviors and highly sensitive	This work

Fig. 6. (A)Influence of temperatures on amperometric response of PVCL / GO / GOD / GCE with a successive injection of glucose in 0.1 mol·L^{-1} phosphate buffer (pH 7.0) saturated with O_2 at −0.39 V.(B) Linear relation of amperometric response vs glucose concentration at 32 ℃ and 38 ℃ (the numbers of experiments calculating standard deviations is 3)

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Fig. 7. Influence of temperatures on amperometric response of GO / GOD / GCE with a successive injection of glucose in 0.1 mol·L^{-1} phosphate buffer (pH 7.0) saturated with O_2 at −0.39 V.

Fig. 6A shows the amperometric response of PVCL / GO / GOD / GCE in catalyzing glucose at different temperatures under the same optimal experimental conditions. Upon successive additions of 0.1 mM glucose to phosphate buffer (pH 7.0), gradual increases in the amperometric signal are observed from 28 to 42°C. But, a more obvious increase in electrocatalytic current as well as sensitivity was obtained at the temperature above 35°C, compared to the temperature below 35 °C, which clearly indicates a better electrocatalytic activity exists above 35 °C. For comparison, the amperometric responses toward glucose at GO / GOD / GCE were conducted in the same experiment condition. There is no obvious electrocatalytic activity difference upon heating from 28 to 42 °C (Fig. 7).This result is consistent with the temperature-dependent EIS spectrum (Fig.2B). So the changes of electrocatalytic activity at PVCL / GO / GOD / GCE film above 35°C and below 35°C should be attributed to the temperature-responsive phase transition behavior of PVCL. From 25 to 35°C, PVCL undergoes dehydration/hydration transition and induces "contraction-to-expansion" of PVCL / GO / GOD / GCE film. This leads to two different electrocatalytic activity states of PVCL / GO / GOD / GCE toward glucose. The amperometric response currents at 32°C and 38°C were plotted against glucose concentration to obtain a linear plot of current versus concentration (Fig. 6B). The linear equation were ip(10^{-7}A)=-0.936$C_{glucose}$+2.21 and ip(10^{-7}A)=-2.99 $C_{glucose}$+5.78, which showed a linear slope at 38°C, much greater than the value at 32°C.

3.5. Stability, Reproducibility and Anti-interference Ability

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The stability of PVCL / GO / GOD / GCE had been investigated by repeated CV scanning for 15 cycles at 50 $mV \cdot s^{-1}$, the direct electrochemical responses remained constant. The modified electrode was stored in 4 °C for 7 days, the current retained 94 % of its initial current response. These results indicate that this biosensor possesses high stability. PVCL / GO / GOD / GCE were used for amperometric response to 0.1 $mmol \cdot L^{-1}$glucose in 0.1 $mol \cdot L^{-1}$ phosphate buffer (pH 7.0). The relative standard deviation (RSD) for 10 successive assays is 4.63% at 35 °C and 5.82% at 25 °C. These imply that the biosensor possesses good reproducibility.

In order to study the selectivity of the biosensor, anti-interference experiments were carried out. The results turned out that 2 times glycine; 2 times Uric Acid; 3 times D-galactose; 5 times L-tyrosine; 2 times dopamine and 2 times ascorbic acid many as glucose co-existing in the sample matrix don't affect the determination of glucose. (Fig.S5)

3.6 Analysis of glucose in serum samples

To illustrate accuracy and feasibility of the biosensor for real sample analysis，the PVCL / GO / GOD / GCE was used for detection of glucose in serum samples. The serum samples were obtained from the affiliated Hospital of Xiangtan University, and the serum samples were not pretreated except for the dilution step with PBS (pH 7.0). In addition, the amount of glucose in the serum samples was also measured photometrically in the hospital. Comparison results are listed in Table 2. The measured value of the biosensor is very close to the data provided by the hospital, and the relative error is less than 3%, which confirmed that the prepared PVCL / GO / GOD / GCE can serve as a stable and effective sensor for analyzing real samples.

Table 2 Measurement results of glucose in serum samples (n=3)

Serum Sample	Photometric method (mM)	Proposed method (mM)	Relative error (%)
A	6.75	6.83	+1.2
B	6.37	6.21	-2.5

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C	4.74	4.78	+0.8

4. Conclusion

The PVCL / GO composited film with a temperature-induced property was applied to immobilize GOD on GCE. Its redox peak current shows two different current states around the critical temperature of LCST. Also, it exhibits two different electrocatalytic activities toward glucose in this temperature range. When the temperature is above the LCST of PVCL, the composite exhibited an excellent electro-catalytic activity. In contrast, it hardly showed electro-catalytic activity at the temperature below LCST. The temperature-responsive CV and amperometric behavior of the composite film are attributed to the contraction−expansion transition of PVCL. The novel strategy presented in this work can be effectively useful for the fabrication of stimuli-responsive composite with prospective applications in switchable sensors.

Conflicts of interest

There are no conflicts to declare.

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Temperature-induced amperometric glucose biosensor based on a poly (N-vinylcaprolactam)/graphene oxide composited film

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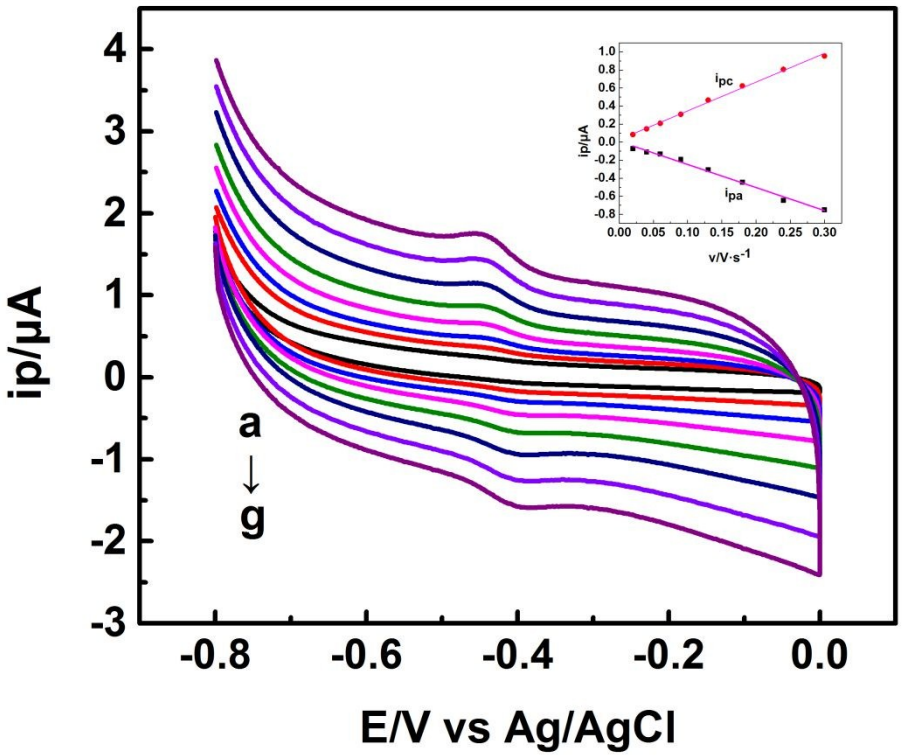

Fig. S1. Cyclic voltammetrics of PVCL / GO / GOD / GCE at various scan rates from 20, 40, 60, 90, 130, 180, and 240 to 300 $mV \cdot s^{-1}$ respectively (from a to g).Inset: plot of peak current (ip) vs scan rate. Supporting electrolyte: 0.1 $mol \cdot L^{-1}$phosphate buffer (pH 7.0)

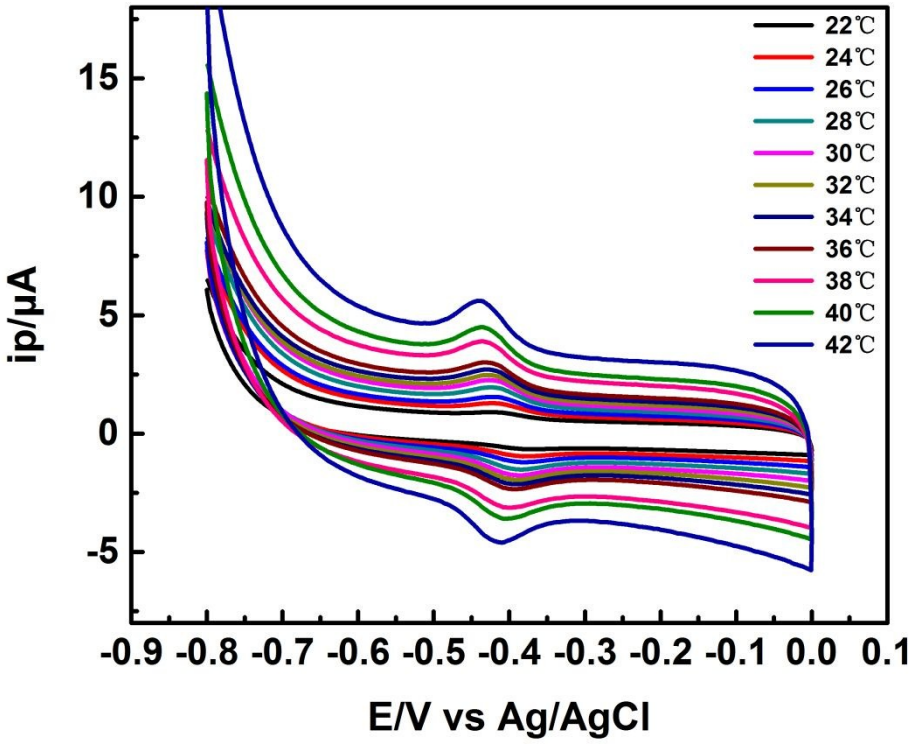

Fig. S2. CV plots of PVCL / GO / GOD / GCE at various temperatures from 22 to 42 °C; Scan

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rate: 50 mV·s^{-1}; Supporting electrolyte: 0.1 mol ·L^{-1} phosphate buffer (pH 7.0).

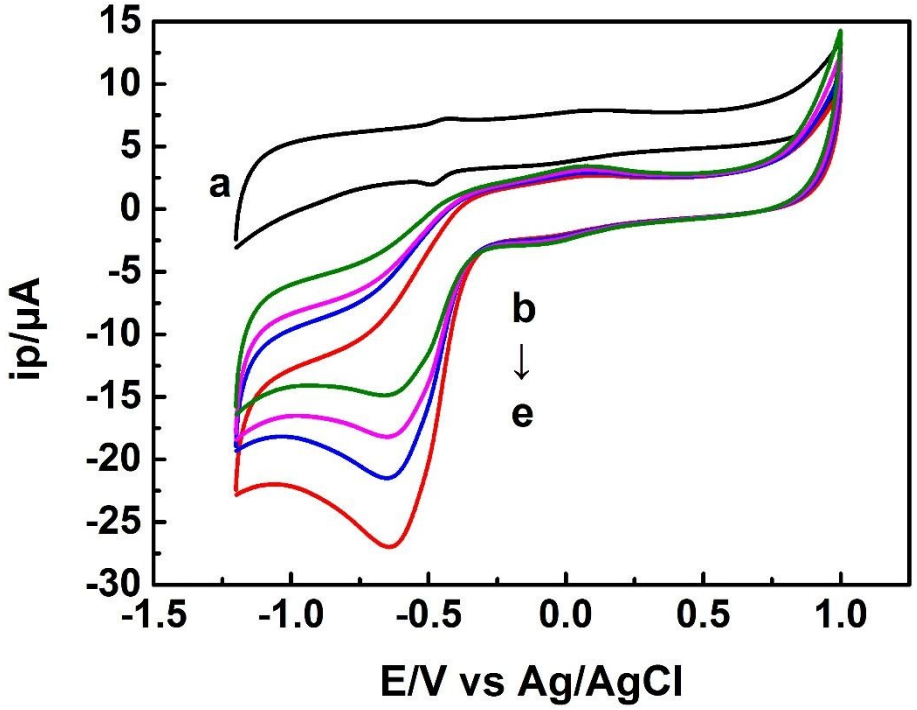

Fig. S3 CV plots of PVCL / GO / GOD / GCE at (a) N_2-saturated and at (b)(c)(d)(e)O_2-saturated with 0,0.3,0.5,0.6 mM glucose Scan rate:50 mV·s^{-1};Supporting electrolyte: 0.1 mol ·L^{-1}phosphate buffer (pH 7.0)

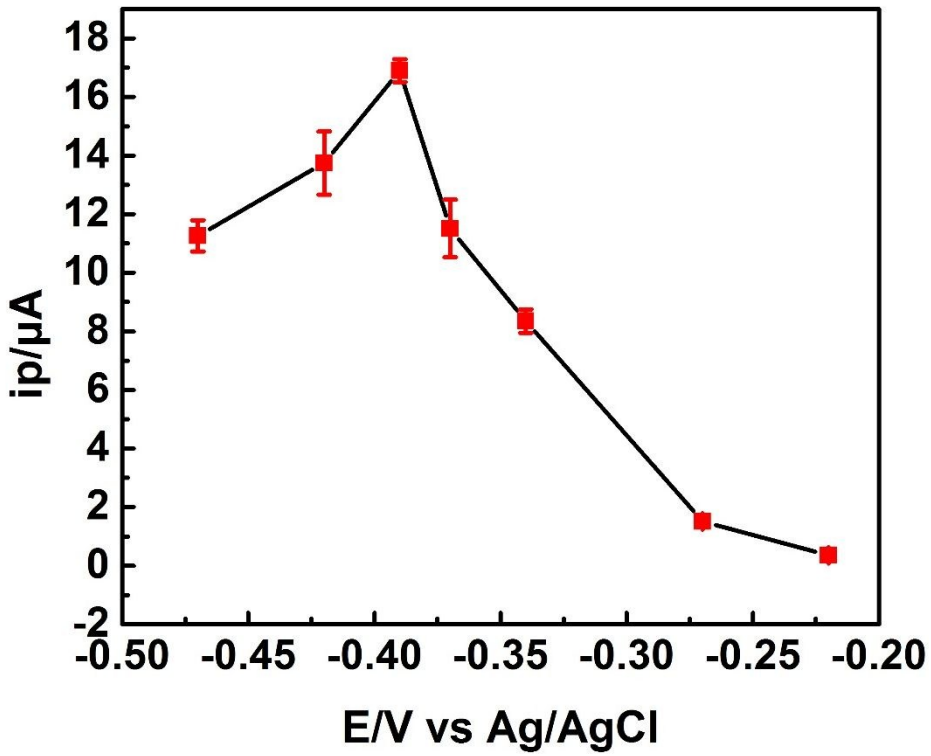

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Fig.S4 under different potential ampere of glucose catalysis

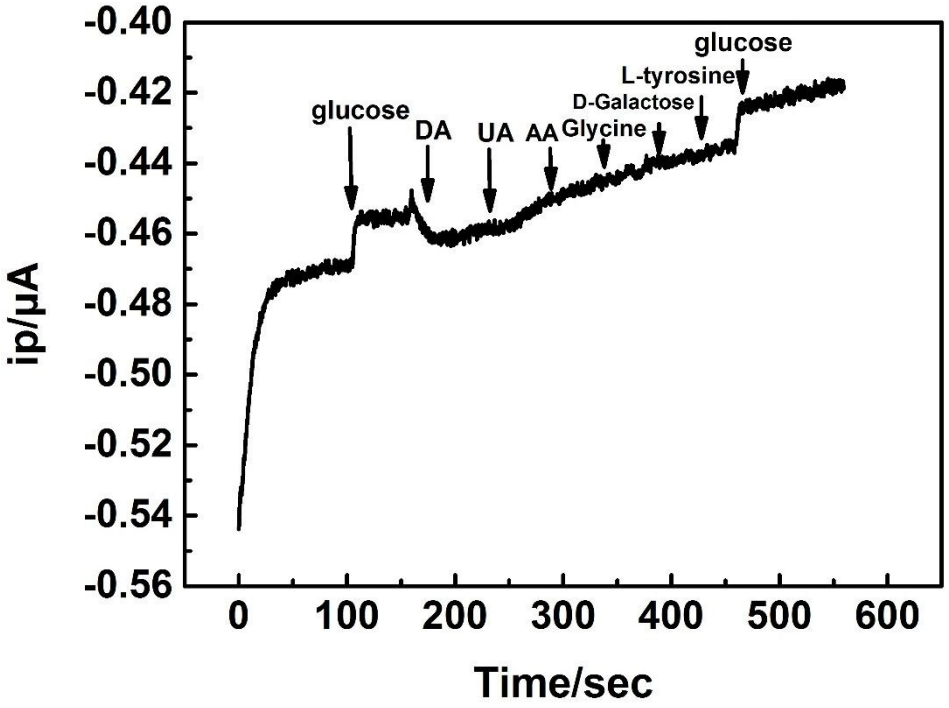

Fig. S5 Amperometric response of PVCL / GO / GOD / GCE at different interference

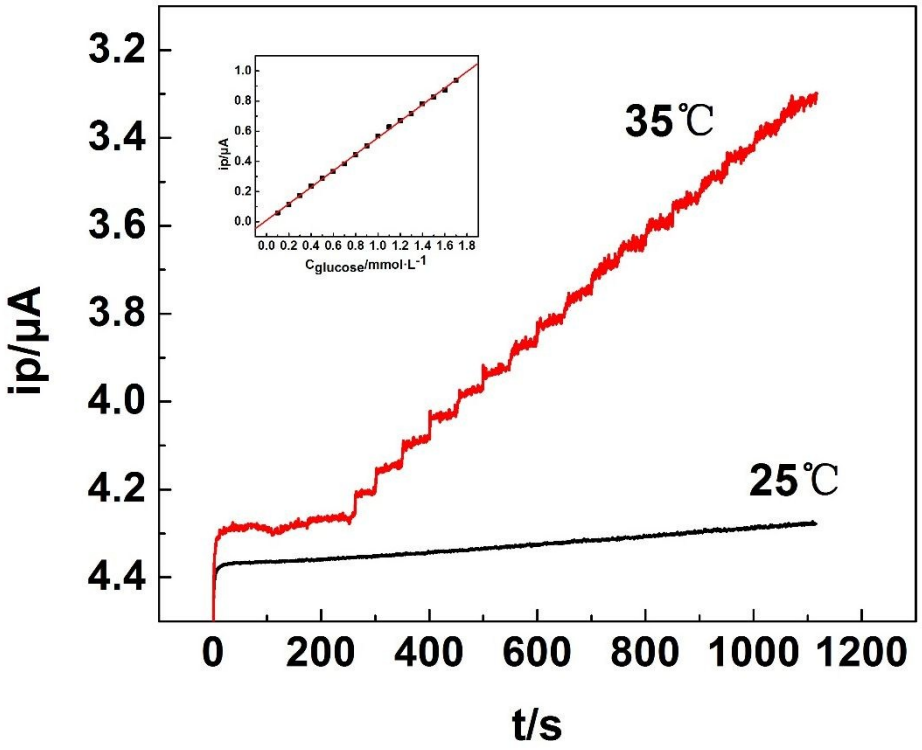

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Fig.S6 Amperometric response of PVCL / GO/ GOD / GCE to glucose compare 25°C to 35°C.the inset: the current was proportional to the concentration of glucose at 35°C.