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Biosensing platform based on graphene oxide via self-assembly induced by synergic interactions

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ABSTRACT

A novel self-assembled glucose biosensor based on graphene oxide (GO) was constructed by using 1-pyrenebutyric acid–*N*-hydroxysuccinimide ester (PANHS) as linking molecular. The stepwise self-assembly process was performed for PANHS anchoring in *N*,*N*-dimethylformamide (DMF) solvent and the further glucose oxidase (GOD) binding in aqueous solution, respectively. The molecular interactions and the morphologic properties were characterized by Fourier transform infrared spectroscopy (FTIR), field emission scanning electronic microscopy (FESEM), and atomic force microscopy (AFM). In addition, the quantitative loadings of anchored PANHS and GOD were well elucidated by surface plasmon resonance (SPR) measurements. The obtained novel glucose sensor exhibited satisfactory analytical performance to glucose: wide linear range (4.0×10^{-6} to 4.4×10^{-3} M), fast response (10 s), high sensitivity (40.5 ± 0.4 mA M^{-1} cm^{-2}), and low detection limit (2 μM, S/N = 3). Furthermore, the biosensor exhibited excellent long-term stability and satisfactory reproducibility.

Rapid detection and monitoring in clinical diagnosis, environmental analysis, and food quality control have paved the way for the elaboration of biosensors, alternative bioanalytical devices combining the recognition properties of biological macromolecules with the sensitivity of electrochemical, optical, thermal, or gravimetric sensors [1]. However, the low output signal and the poor lifetime are regarded as "bottleneck" problems limiting the development of a biosensor.

Intensive efforts have been devoted to the development of retention of the biological activity of immobilized biomolecules and enlarging the signal via selected matrix via appropriate methods [2–4]. Graphene became most popular in 2010 when Geim and Novoselov were awarded a Nobel Prize in physics [5]. Graphene, along with its chemical precursor, graphene oxide (GO),[1] has been pursued in biological systems due to its extraordinary electrical, mechanical, and chemical properties, such as the large specific surface area, rich π conjugation structure, long-term stability, and friendly biocompatibility [6,7]. Abundant oxygen-containing functional groups on its basal planes and edges enable the suspension of GO in aqueous solution [8,9]. The major limitation of a biosensor based on GO is the exfoliation of the biofilms from the electrode.

Driven by different noncovalent bonding forces, the aggregate exhibits different properties [10]. Encouraged by the self-assembly features in different polar solvents, we developed a facile and efficient method for biosensor construction based on noncovalent functionalized GO as illustrated in Scheme 1. 1-Pyrenebutyric acid–*N*-hydroxysuccinimide ester (PANHS) was used as a scaffold molecule comprising an anchor group and a terminal group. The hydrophobic π system group, pyreny moiety, has strong affinity with the basal plane of GO via π-stacking [11], and a succinimidyl ester group is highly reactive to nucleophilic substitution by primary and secondary amines that exist on the surface of proteins [12]. Glucose oxidase (GOD) from *Aspergillus niger* (EC 1.1.3.4) was taken as a model redox protein in this present work, because of its high stability, high catalytic ability, commercial availability, and moderate cost [13].

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[1] *Abbreviations used:* AFM, atomic force microscopy; DMF, *N*,*N*-dimethylformamide; FESEM, field emission scanning electronic microscopy; FTIR, Fourier transform infrared spectroscopy; GO, graphene oxide; GOD, glucose oxidase; PANHS, 1-pyrenebutyric acid–*N*-hydroxysuccinimide ester; PBS, phosphate buffer solution; SPR, surface plasmon resonance.

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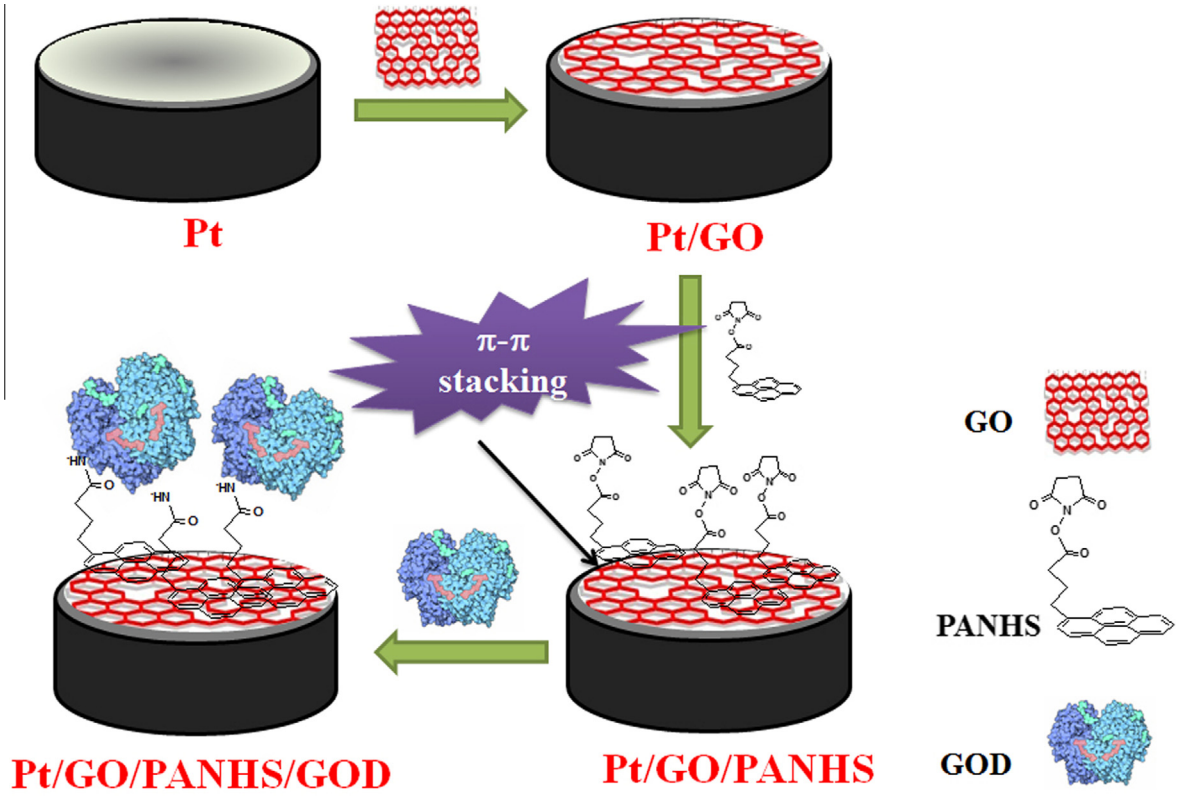


Scheme 1. Schematic Illustration of stepwise self-assembly procedures of GO/PANHS/GOD-modified Pt electrode.

Experimental

Reagents and materials

Glucose oxidase (EC 1.1.3.4, > 100 U/mg, from *A. niger*) was purchased from Amresco. 1-Pyrenebutyric acid *N*-hydroxysuccinimide ester was obtained from Sigma-Aldrich. Graphene oxide was made from natural flake graphite. All other chemicals were of analytical grade and used without further purification. Phosphate buffer solution (PBS) was 0.1 M K_2HPO_4 and KH_2PO_4 and its pH was adjusted with H_3PO_4 or KOH solutions. Twice-distilled water was used throughout the experiment. Stock glucose solution was prepared daily and stored overnight to reach mutarotational equilibrium before use.

Measurements and apparatus

Fourier transform infrared (FTIR) spectra of the samples were measured employing a Tensor 27 spectrometer. Field-emission scanning electron microscopy (FE-SEM) was carried out with a XL-30 E scanning electron microscope operated at an accelerating voltage of 15 kV. Atomic force microscopy (AFM) images were obtained with a multimode digital instrument (Vecco-DI). Surface plasmon resonance (SPR) measurements were performed using a single channel, Autolab E-SPR Springle instrument (Eco Chemie, The Netherlands) and a standard sensor disk gold-coated glass (BK-7 Eco Chemie). The sensor disk was mounted on a hemicylindrical lens through index-matching oil to form the base of a cuvette. Sample solutions were injected manually into the cuvette. The measurements were carried out under batch conditions at 25 °C. The SPR angle shifts ($\Delta\theta$ were converted into mass uptake using a sensitivity factor of 120 m° corresponding to 100 ng cm^{-2} of molecular loading, and the MW of each immobilized molecule [14].

Electrochemical measurements were performed on CHI 660 D electrochemical workstation with a three-electrode system composed of a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference electrode, and a platinum disk electrode (Pt) with a diameter of 2 mm as working electrode. The working electrode was polished carefully with 0.05 mm alumina particles on silk followed by rinsing with distilled water and drying in air before use. All measurements were carried out in a thermostated cell at 25 °C, containing phosphate buffer solution.

Electrode preparation

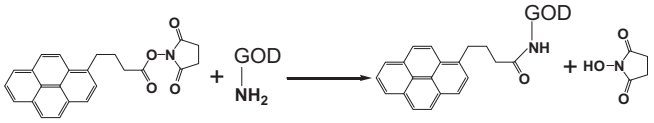
GOD was dissolved in 0.1 M PBS (pH 7.0) with a concentration of 4 mg ml^{-1}. GO was dispersed in deionized water under sonication with a concentration of 1 mg ml^{-1}. PANHS was dissolved in DMF with a concentration of 6 mM. The defined amount of GO suspension (i.e., 10 ml) was spread on the surface of a clean Pt electrode and was dried under ambient conditions. As-prepared GO-modified Pt electrode (Pt/GO) was immersed into 6 mM PANHS solution for 2 h and washed thrice to remove the free PANHS molecules. The obtained Pt/GO/PANHS was then immersed into 4 mg ml^{-1} GOD solution for 14 h at 25 °C and washed carefully with 0.1 M PBS to remove the enzyme not firmly immobilized. The resulting electrode was denoted as Pt/GO/PANHS/GOD.

Results

Molecular interaction

In this work, FTIR spectrometry was performed to investigate the molecular interactions during the self-assembly procedure

for GO/PANHS/GOD. FTIR spectra of GOD, GO, GO/PANHS, and GO/PANHS/GOD films on silicon wafers in the range of 4000–500 cm^{-1} were obtained via a grazing-angle attenuated total reflection method (Fig. 1). The FTIR spectrum of GO exhibits stretching-vibration bands at 1712, 1623, 1203, and 1039 cm^{-1}, which are attributed to C–O stretching in carbonyl groups, C=C of aromatic ring, C–O bond, and C–O–C, respectively, as reported in the literature [15]. For the self-assembled GO/PANHS film, the vibrations located at 1741, 1656, 1440, 1257, 1226, and 672 cm^{-1} arise for the C=O stretching in ester groups, C=O in amide groups (carbonyl groups), C–N in amide groups, C–O bond, C–O–N bond, and C–H bending in aromatic groups, respectively. It implies the successful self-assembly of PANHS on the GO surface. Moreover, the newly emerged peaks at 2381 and 2347 cm^{-1} indicate the conjugation of a polycyclic aromatic group, which may be due to π–π stacking. The GOD binding was realized via the following reaction:



Thus, the FTIR spectrum for the self-assembled GO/PANHS/GOD shows vibrations similar to those of GO/PANHS except for an additional shoulder at 1610 cm^{-1} (amide group) and the nearly disappeared peaks for the succinimidyl ester group at 1440 cm^{-1} and 1226 cm^{-1}. The results confirm the GOD assembly via nucleophilic substitution of succinimidyl ester groups. Furthermore, it is noteworthy that the vibration at 1740 cm^{-1} representing the C=O stretching in ester group remains, demonstrating the residue of PANHS in the self-assembled system.

Quantitative analysis by SPR

SPR has becoming one of the more powerful tools for monitoring the construction and recognition event of a biosensing platform in real-time, allowing one to obtain a label-free quantitative analysis [16]. This technique is based on changes of SPR angle (SPR reflectivity) due to the specific molecular bonding and exhibits a high sensitivity, making possible the discrimination between bound and unbound molecules [17,18]. Therefore, in this work, the SPR technique was used for quantitative analysis of the anchored PANHS and GOD. Fig. 2A reveals the SPR angular scan taken before and after PANHS equilibrium adsorption on the SPR sensor modified by GO. The anchoring of PANHS presents a binding

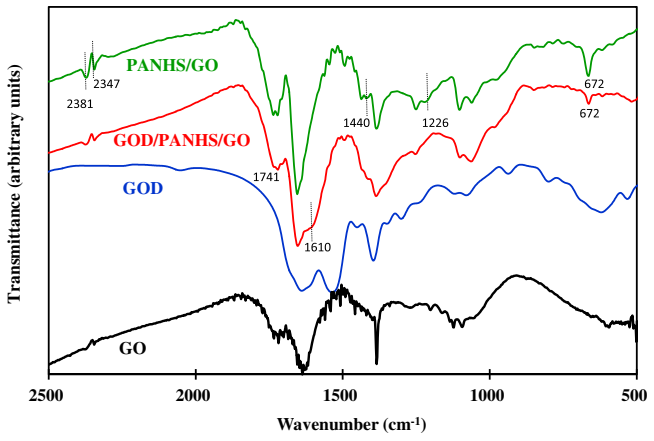


Fig.1. FTIR spectra of GO, GO/PANHS, GO/PANHS/GOD, and GOD.

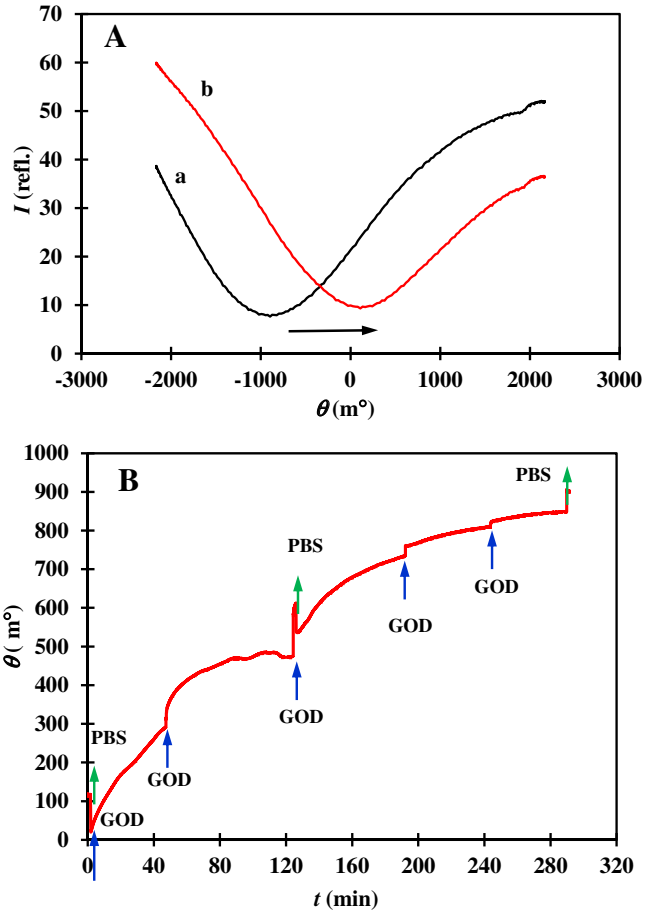


Fig.2. (A) The SPR angular scan curves for the GO before (a) and after equilibrium PANHS anchoring. (B) Real-time monitoring of the assembly of GOD on Au-coated SPR sensor surface modified by GO/PANHS. The down arrow (↓) indicates the point in time when GOD solution (4 mg ml^{-1}) was introduced into the measurement cell and the up arrow (↑) represents the point in time at which the measurement cell was rinsed with 0.1 M PBS buffer (pH 7.0).

signal of 904.4 m° degrees that corresponds to 7.54 ng mm^{-2} (1.96×10^{-11} mol mm^{-2}). The stepwise SPR curve in a 5 h measurement window demonstrates that the GOD binding to self-assembled film of GO/PANHS dramatically increases in 1 h and achieves equilibrium in 4 h (Fig. 2B). The equilibrium GOD immobilization results in an identical binding signal of 785 m°, which corresponding to 6.54 ng mm^{-2} (4.10×10^{-14} mol mm^{-2}). The tremendous ratio of 478 is obtained for PANHS/GOD.

Morphologic characterization

FESEM and AFM techniques were used to obtain direct visualization of the self-assembly procedure. The surface of pure GO-modified GCE is smooth with a few thin wrinkles and ridge structure (Fig. 3A). To our surprise, the morphology of the final self-assembled GO/PANHS/GOD reveals an obviously different characterization as portrayed in Fig. 3B. The surface becomes more porous and heterogeneous, randomly distributed with dense cotton-like bulges and many small amorphous aggregates. As shown in Fig. 3C, the surface topography image of the GO thin film reveals a characteristic surface with domains of ridges and valleys formed by the deposition and the imperfect stacking of GO. The thickness of this thin GO film is 6 nm. As one can see, a surface topography image of the self-assembled GO/PANHS/GOD film changes a lot with thickness of 31.5 nm (Fig. 3D).

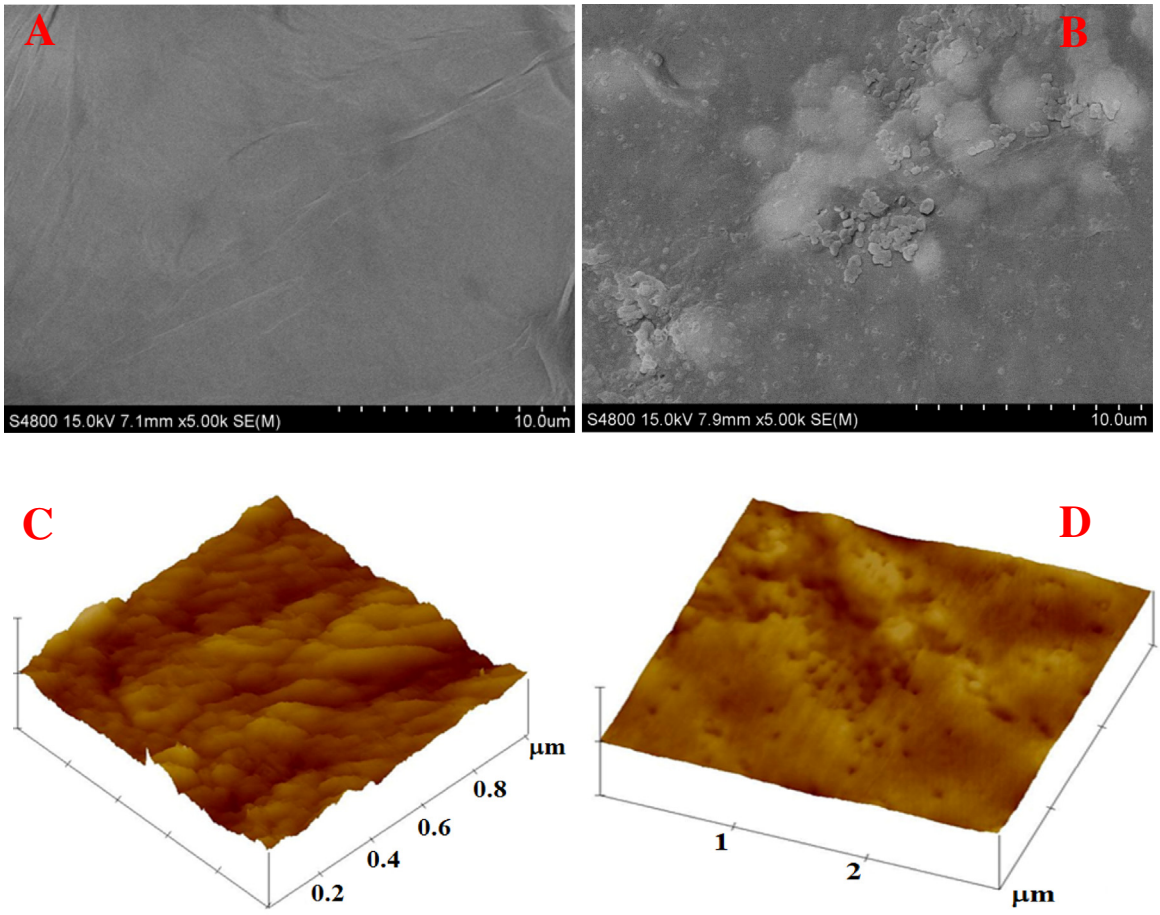


Fig.3. FE-SEM and tapping mode AFM topography images for on GO (A,C) and the self-assembled GO/PANHS/GOD films (B,D).

Analytical performance of the Pt/GO/PANHS/GOD

GOD catalyzes, in the presence of molecular oxygen, the oxidation of β-D-glucose into gluconic acid and hydrogen peroxide. As a consequence, the amperometric detection of glucose was assayed by potentiostating the Pt/GO/PANHS/GOD at 0.6 V vs SCE to oxidize the enzymatically generated hydrogen peroxide (inset A of Fig. 4). Fig. 4 shows typical response curves for Pt/GO/PANHS/GOD on successive injections of glucose to the stirring 0.1 M PBS (pH 7.0). Inset B in Fig. 4 depicts the corresponding calibration curve. The proposed self-assembled electrode achieves 95% of the steady-state current within 10 s. The response to glucose is linear in the range from 4.0×10^{-6} to 4.4×10^{-3} M, and with a linear regression equation for I (mA) = 1.214 [glucose] (mM) + 0.045, $R^2 = 0.9959$ ($n = 26$). The sensitivity is calculated to be 40.5 ± 0.4 mA M^{-1} cm^{-2}. A low detection limit of 2×10^{-6} M is determined at a signal-to-noise ratio of 3. The Michaelis–Menten constant (K_M^{app}), which is a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constant, is calculated to be 14.3 mM according to the Lineweaver–Burk equation.

The reproducibility of the analytical response obtained from different electrodes constructed by the same procedure was analyzed. Five different electrodes were tested independently for these amperometric responses to 1 mM glucose. The result reveals that the RSD value for all five electrodes is 4.8%. The long-term stability of the self-assembled Pt/GO/PANHS/GOD stored at 4 °C was evaluated periodically using the same PBS containing 1 mM glucose (inset C of Fig. 4). The biosensor exhibits excellent stability for 25 days; no loss of activity of the immobilized enzyme is observed. It still retains about 83% of its original response after 65 days of storage.

The analytical performances of the proposed Pt/GO/PANHS/GOD were compared with other glucose-sensing systems based on the self-assembly of GOD, as summarized in Table 1. We can conclude that the self-assembled Pt/GO/PANHS/GOD exhibited excellent performance in terms of a wide linear range, low detection limit, and particularly satisfactory long-term stability for glucose.

Discussion

The properties of the self-assembled systems, such as morphology and the analytical performance of the biosensing platform, are highly controlled by the molecular interactions [24]. Moreover, self-assembly behaviors at the solid–liquid interface may be more complicated than those in solution and involve possible noncovalent bonding forces such as ionic bridges, hydrogen bonding, and π–π stacking [25]. In this work, PANHS was anchored on the surface of a GO-modified electrode in DMF solvent, one of the widely used aprotic solvents. GO contains abundant epoxy, hydroxyl, carbonyl, and carboxyl groups. The driving force for the self-assembly of PANHS on a GO-modified electrode is not only π–π stacking of pyrene groups but also the hydrogen bonding of –OH··O. These coexisting bonding forces result in the large amount of PANHS attached, which further plays a significant role in governing the assembled morphology. Generally, the pyrene ring of PANHS could be spontaneously attracted to the surface of GO through π–π stacking with its long axis parallel to the GO axis. Nevertheless,

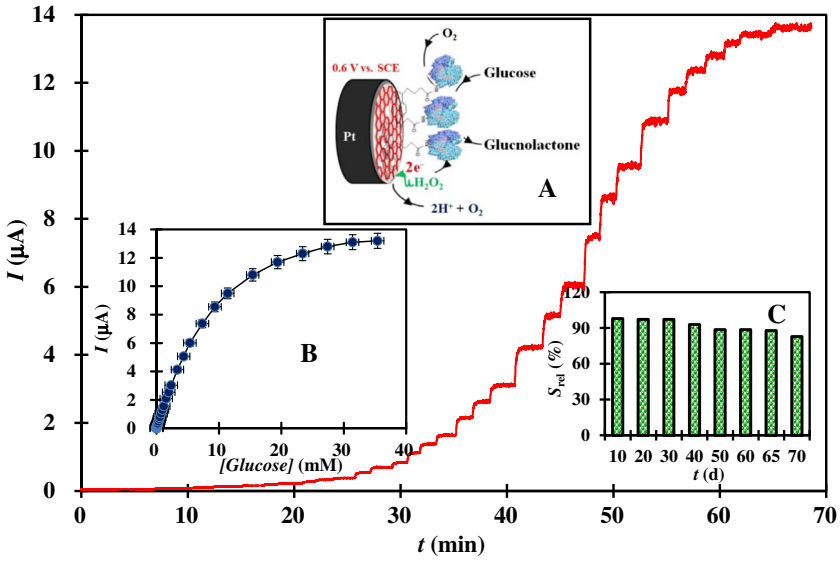


Fig.4. Typical steady-state responses of the self-assembled GO/PANHS/GOD-modified Pt electrode on successive injections of glucose into 10 ml of stirring 0.1 M PBS (pH 7). Applied potential: 0.6 V vs SCE. Inset A: Schematic description of the reaction mechanisms of the self-assembled Pt/GO/PANHS/GOD. Inset B: Calibration curve of the self-assembled GO/PANHS/GOD-modified Pt for glucose. Inset C: The long-term stability of the self-assembled GO/PANHS/GOD-modified Pt.

Table 1
Comparison analytical performance of other self-assemble GOD electrode.

Electrode	Linear range (M)	Sensitivity (mA M^{-1})	DL (M)	Stability	Reference
Pt/GO/PANHS/GOD	4.0×10^{-6}–4.4×10^{-3}	1.21	2.0×10^{-6}	83%/65 days	This work
GCE/(MWCNT-Polyhis/GOD)$_5$/Nafion	2.5×10^{-4}–5×10^{-3}	1.94	2.2×10^{-6}	90%/15 days	[19]
Graphite/GP/(5.4 pyrene-GOD)$_4$	2.0×10^{-4}–4×10^{-2}	–	1.54×10^{-4}	82.2%/28 days	[20]
GOD-(H30-SO_3H)/Au	2.0×10^{-4}–2×10^{-2}	0.053	1.2×10^{-5}	–	[21]
GOD-CNDs-rGO/GCE	4.0×10^{-5}–2×10^{-2}	–	4.0×10^{-5}	81%/5 days	[22]
{IL-RGO/S-RGO}$_n$/GOD/Nafion	1.0×10^{-5}–5×10^{-4}	0.072	3.33×10^{-6}	83%/14 days	[23]

the random existence of hydroxyl, carbonyl, and carboxyl groups in GO causes the adsorbed PANHS to have a random orientation.

When the Pt/GO/PANHS electrode is immersed into GOD aqueous solution for GOD binding, π–π stacking works as a dominate influence factor. The strengthened π–π stacking interactions between PANHS and GO in an aqueous environment resulted in the enhanced adsorption energy [26,27], the firm immobilization of GO/PANHS/GOD on the electrode surface, and finally the satisfactory stability obtained. Simultaneously, preanchored PANHS molecules by hydrogen bonding may aggregate into a special structure for the favored π–π stacking between pyrene moieties. Due to the competition of H_2O, the "bonded" succinimidyl ester groups are "released" and available for GOD binding in 3-D space.

Since GOD molecules are immobilized though one or more amine groups with a random orientation, it is not surprising that the amount of PANHS is larger than that of GOD molecules under the working conditions. FTIR results reveal that the residue of PANHS, which may be attributed to steric hindrance for the accessibility of protein, and only parts of PANHS molecules are available to target capture.

The above hypothesis on forming a special architecture during the GOD binding was further confirmed by AFM and SPR observations. The dimensional size of GOD molecule was reported to be $6.0 \times 5.2 \times 7.7$ nm [13]. An optical method, SPR, provides a more reliable method for quantifying "net" mass change at the surface. According to the SPR result, the GOD layer immobilized is about 1.14 by compact ranking GOD molecules one by one. The maximum increased thickness is then calculated to be about 9 nm, which is significantly different from our AFM observation (≈25 nm). The latter may arise from the GOD binding in the 3-D architecture of GO/PANHS.

Conclusion

In summary, we have successfully elaborated a novel biosensing platform based on the self-assembly of GO/PANHS/GOD through the cooperative interactions of π–π stacking and hydrogen bonding in different polar solvents. Owing to the synergic effect, the linking agent, PANHS molecules could be largely anchored on the GO surface and randomly aggregated with a 3-D architecture formed simultaneously in the process of GOD binding in aqueous solution. Since the π–π stacking interaction plays a dominate force in an aqueous environment and the pyrene group of PANHS is hydrophobic, the adherence of GO/PANHS/GOD can be highly improved, which is a benefit for the enhancement of stability. This proposed strategy is simple, scalable, controllable, impurity free, and economic and will stimulate the development of highly efficient electrochemical sensors and advanced biosensing systems.

Acknowledgments

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