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A general strategy to prepare homogeneous and reagentless GO/lucigenin&enzyme biosensors for detection of small biomolecules

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ABSTRACT

In this work, a novel biosensor was developed for the detection of glucose based on glucose oxidase (GOD) functionalized graphene oxide (GO)/lucigenin nanocomposite. In this sensing strategy, GO/lucigenin composite was first prepared by vigorously stirring GO with lucigenin. Then the functionalization of GOD was achieved by simply storing GOD with GO/lucigenin at 4 °C overnight to form GO/lucigenin&GOD composite. When glucose was incubated with GO/lucigenin&GOD composite for 50 min to generate H_2O_2, followed by the injection of 0.2 M NaOH, CL signal was detected due to the reaction of lucigenin with H_2O_2. Glucose could be determined in the range of 1.0×10^{-6}–5.0×10^{-3} g mL^{-1} with a detection limit of 9.9×10^{-7} g mL^{-1}. The present biosensor has been successfully applied for the detection of glucose in human serum samples. Compared with previously reported methods, this sensing strategy is homogeneous and reagentless and avoids complicated assembly procedure and pretreatment of serum sample, showing good stability, repeatability, high selectivity and simplicity. Moreover, this strategy has been demonstrated to be a general strategy by replacing GOD with other enzymes such as uricase and choline oxidase for the detection of small molecules such as uric acid and choline. The proposed biosensors may find future applications in the fields such as disease diagnosis and biomedicine.

1. Introduction

Graphene oxide (GO) has attracted considerable attention in recent years because of its unique characteristics such as high mechanical strength (Dikin et al., 2007), good water dispersibility (Liu et al., 2008), photoluminescence (Sun et al., 2008) and particularly its facile surface functionalization (Mohanty and Berry, 2008; Zhou et al., 2009; Zuo et al., 2009; Tao et al., 2013a, 2013b). By covalent or non-covalent interaction, GO could be functionalized with a broad range of functional materials such as nanoparticles, organic compounds, polymers and biomaterials. The functionalized GO materials demonstrate excellent catalytic activity, enhancement of mass transport, highly effective surface area, photoactivity, electroactivity, chemical stability and improved dispersion, which render them a more versatile precursor for a wide range of applications including enzyme-based biosensing, immunosensing and DNA sensing (Chen et al., 2012; Dreyer et al., 2010).

Recently, attention has been paid to chemiluminescence (CL) functionalized GO, including GO functionalized by lucigenin, luminol and its analogs (He and Cui, 2012a; Shen et al., 2012). All these materials have exhibited excellent CL activity and have been applied to sensitive bioassays (He and Cui, 2012c). On the other hand, much effort has also been made for enzyme functionalized GO, which is mainly applied to electrochemical detections (Liu et al., 2010). The immobilized enzymes possess various advantages such as enhanced stability, repeated or continuous use, easy separation from the reaction mixture and possible modulation of catalytic properties (Dyal et al., 2003; Rocchietti et al., 2002). However, GO bifunctionalized by enzyme and CL reagent as a sensing platform has not been explored, to the best of our knowledge.

Small biomolecules such as glucose, uric acid and choline play important roles in biomedicine of most living cells. The detection of these molecules is very important in clinical diagnosis, disease treatment and drug discovery. Up to date, various methods have been documented for the determination of these small biomolecules, including separation-based methods, enzyme-based methods and nonenzymatic methods (Pomfret et al., 1989 Kang et al., 2007; Shao et al., 2010; Yuan et al., 2005). Generally, enzyme-based methods are superior to separation-based methods in simplicity, rapidity and cost and to nonenzymatic methods in selectivity (Song et al., 2005). Various enzyme-based sensors have been reported, including mainly electrocatalytic sensors (Cao et al., 2008), fluorescent sensors (Pickup

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et al., 2005; Hu et al., 2012) and phosphorescent sensors (Wu et al., 2010). Although some of the sensors show low detection limit and wide linear range, most of these sensors either require expensive instrumentation or complicated assembly procedure. Therefore, it is important to develop an accurate, rapid, simple, low-cost and general method for the determination of small biomolecules in biological samples. In this work, we report a novel strategy to fabricate GO/lucigenin&enzyme CL biosensor for the determination of small molecules by using GO bifunctionalized by glucose oxidase (GOD) and CL reagent lucigenin as the sensing platform and using glucose as a model compound. The assembly of GO/lucigenin&GOD composites was studied by high-resolution transmission electron microscopy (HRTEM), atomic force microscopy (AFM) and circular dichroism (CD). The CL property of GO/lucigenin&GOD composites was exploited by a static injection method. Moreover, the detection conditions of the biosensor were optimized. The analytical performance and selectivity of biosensor for the detection of glucose were examined. The applicability of biosensor in real samples was also explored by determining glucose level in human serum. Finally, the generalization of the proposed strategy was studied by replacing glucose oxidase with other enzymes such as uricase and choline oxidase to detect the corresponding small biomolecules uric acid and choline.

2. Experimental

2.1. Materials and chemicals

GOD and uricase were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Choline oxidase and uric acid were obtained from Sigma-Aldrich (USA). GO was obtained from XFNANO Materials Tech Co. Ltd. (Nanjing, China). Lucigenin was purchased from TCI Co. Ltd. (Tokyo, Japan). Glucose and choline were purchased from Shanghai Reagent Company (Shanghai, China). 50-fold-diluted Britton–Robinson (B–R) buffer solution was used to dissolve enzymes, substrates and GO/lucigenin&enzyme composites. All other reagents were of analytical grade. Ultra-pure water was prepared by a Millipore Milli-Q system and used throughout.

2.2. Apparatus

CL was measured with a centro LB 960 microplate luminometer (Berthold, Germany). A PST-60 HL plus thermoshaker (Biosan, Latvia) was used to control the temperature of the reactions. UV–visible spectra were obtained from an Agilent 8453 UV–visible spectrophotometer (USA). CD spectra were recorded with a Jasco-810 CD spectrometer (Japan). AFM (Multimode V, Veeco) and HRTEM (JEM-2010, Hitachi, Japan) were used to characterize the morphology of the as-synthesized GO/lucigenin&enzyme composite.

2.3. Synthesis of GO/lucigenin&enzyme composite

GO/lucigenin&enzyme composite was prepared as follows: 100 mL of the homogeneous graphene oxide dispersion was ultrasonicated for 5 min followed by the addition of 100 μL of lucigenin (5 mM) aqueous solution. The mixture of reactions was stirred fiercely at room temperature for 24 h (He and Cui, 2012b). The yellow solution was centrifuged and washed two times and redispersed in diluted B–R buffer. The pH of B–R buffer depended on the enzyme used (glucose oxidase: pH=4.5, uricase: pH=8.5, and choline oxidase: pH=8.0). After adding the corresponding enzyme (100 U mL^{-1}), the above redispersed solution was stored at 4 °C overnight. The composite materials were obtained by centrifuging and redispersing in B–R buffer to remove residual enzyme.

2.4. CL detection of enzyme substrate

In a typical assay, 100 μL of as-prepared composite materials and 50 μL of enzyme substrate such as glucose (buffer pH=4.5) were mixed and incubated at 37 °C for 50 min. Glucose reacted with the dissolved oxygen under the catalysis of GOD to generate H_2O_2. While injecting 50 μL of NaOH (0.2 M) into the system, CL signal was produced and recorded.

3. Result and discussion

3.1. Construction and characteristics of GO/lucigenin& enzyme composite

Fig. 1 illustrates the strategy to synthesize GO/lucigenin&GOD composite materials. First, lucigenin was attached to GO via π–π interaction by reacting graphene oxide aqueous dispersion with lucigenin aqueous solution for 24 h as described previously (He and Cui, 2012b). Subsequently, the as-prepared GO/lucigenin composite dispersed in B–R buffer solution was mixed with GOD in B–R buffer solution at 4 °C overnight so that the enzyme could be functionalized to GO sheets to form GO/lucigenin&GOD composite.

To confirm the successful preparation of GO/lucigenin&GOD composite, the morphology and thickness of GO/lucigenin&GOD composite were studied by HRTEM and AFM images. The specimen of HRTEM was prepared by dropping the sample onto a copper net covered by a carbon film. As the solvent water evaporates, wrinkles appeared under surface tension effect because GO/lucigenin was hydrophilic while carbon film was hydrophobic. After the immobilization of GOD as shown in Fig. S1, GO/lucigenin material spreads easily on the carbon film, demonstrating the change in surface characteristics of the composite. Fig. S2 indicates that the thickness of GO/lucigenin sheet is about 1 nm (Eda et al., 2008; Shen et al., 2012), while the height of the functional GO/lucigenin&GOD composite shows two increments of 0.7 nm and 1.4 nm, corresponding to one and two layers of GOD attachment, respectively. The size of graphene oxide was also analyzed by HRTEM and AFM images (see Figs. S1 and S2). The statistical size of graphene oxide ranges from 0.50 μm to 1.25 μm.

CD refers to the differential absorption of left and right circularly polarized light (Berova et al., 2007). The absorption bands exhibit in CD spectrum of optically active chiral molecules (Li et al., 2004). Generally, most of the biomacromolecules are chiral. However, GO is a kind of two-dimensional material without chirality and hence cannot be observed in CD spectrum. Therefore, CD spectroscopy can be a well assistant method to ascertain the functionalization of GOD on the surface of GO. The image of CD spectra (Fig. 2) demonstrated that GOD and GO/lucigenin&GOD composite had optically active band in CD spectrum except GO, revealing successful assembly of GOD on the surface of GO/lucigenin.

The assemble mechanism of GOD on the surface of GO/lucigenin is further discussed. In the first step, the large π-conjugated structure of GO interacted with lucigenin having aromatic rings through π–π stacking to form GO/lucigenin as reported in our previous work (He and Cui, 2012a). In the second step, GOD was positively charged in the buffer solution with a pH range from 4.0 to 4.9 (GOD isoelectric point=4.9; active pH range of 4.0–7.0), while GO sheets were negatively charged with a pH range from 4.0 to 11.0 (Li et al., 2008). Hence, glucose oxidase was functionalized to GO/lucigenin by electrostatic interaction. In addition, hydrogen bonding between oxygen-containing functionalities of GO and surface amino acid residues of GOD might also contribute to GO–GOD interaction (Zhang et al., 2010). In order to

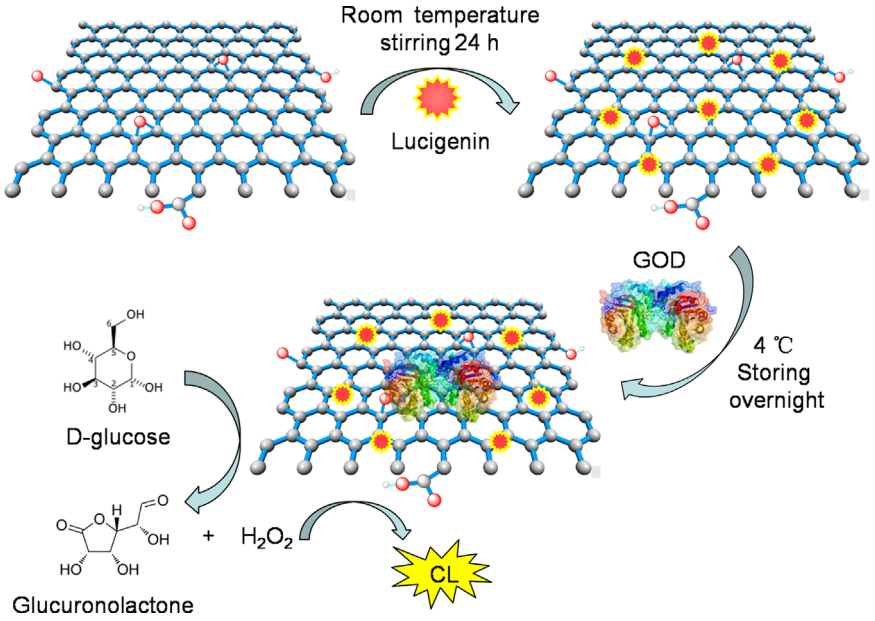

Fig. 1. Schematic illustration for fabrication and detection of GO/lucigenin&GOD biosensor.

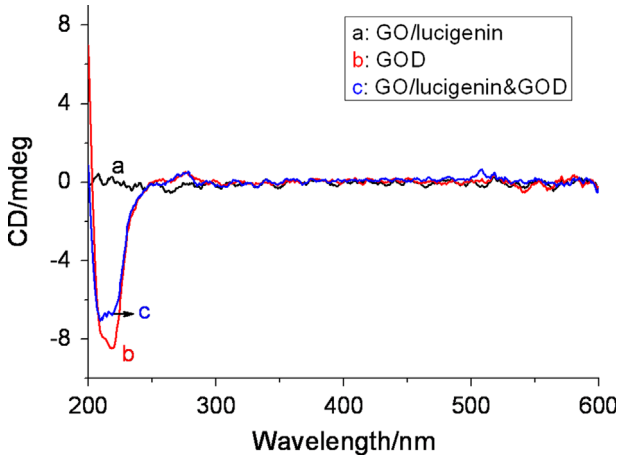

Fig. 2. CD spectra of GO/lucigenin, GOD, and GO/lucigenin&GOD.

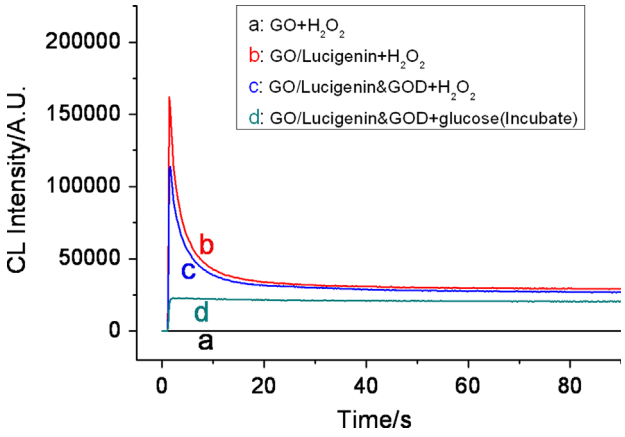

Fig. 3. CL kinetic curves of (a) GO, (b) GO/lucigenin, and (c) GO/lucigenin&GOD when 50 μL of 5 mM H_2O_2 containing 0.2 M NaOH solution is injected into composite solution. CL kinetic curves of (d) GO/lucigenin&GOD incubated with glucose (50 μL, 5 mM) when 50 μL of 0.2 M NaOH solution is injected into composite solution. Condition: composite solution, 100 μL.

determine whether or not there is an interaction between GOD and lucigenin, UV–visible spectroscopy is used to study the interaction as shown in Fig. S3. The absorption intensity of the mixed system's absorption spectra was almost equal to the sum of two individuals GOD and lucigenin, indicating that there was no significant interaction between GOD and lucigenin.

3.2. CL property of GO/lucigenin&GOD composite

The CL behavior of various as-prepared composites including GO, GO/lucigenin, GO/lucigenin&GOD was studied by static injection on the microplate luminometer. As shown in Fig. 3, when 50 μL of 5 mM H_2O_2 containing 0.2 M NaOH solution is injected into 100 μL of composites dispersed in B–R buffer solution, no CL emission is observed for GO, whereas strong CL signal is observed for GO/lucigenin and GO/lucigenin&GOD. In the latter two cases, the CL intensity first increased to a maximum, then decreased to a certain value and finally reached a constant intensity to form a platform. GOD immobilized on the surface of GO/lucigenin led to a decrease in peak value but had no significant effect on the platform. When the mixture of 100 μL of GO/lucigenin&GOD composite solution and 50 μL of 5 mM glucose was incubated at 37 °C for 50 min and 50 μL of 0.2 M NaOH was injected into the composite solution, strong and stable CL signal was obtained. This is due to that glucose reacted with the dissolved oxygen under the catalysis of GOD during incubation to yield H_2O_2, which was followed by the reaction with lucigenin on the surface of GO/lucigenin composite in alkaline solution to produce CL emission. The result indicated that graphene oxide immobilized with lucigenin and enzyme GOD (GO/lucigenin&GOD composite) could be functionalized as a sensing platform for recognition and CL detection of glucose. The steady CL signal is advantageous for good repeatability of this biosensor.

3.3. Optimization of experimental conditions

In order to obtain the optimal analytical performance, the concentration of NaOH, incubation time, pH of the buffer for the assembly of GOD with GO/lucigenin (step one) and pH of the buffer for enzyme reaction (step two) were investigated. As shown

in Fig. S4 (A), the CL intensity rises sharply (with 1 mg/mL glucose) with increment of NaOH till the concentration reaches 0.2 M. After that, further increment of NaOH concentration resulted in a slight increase in CL signal, but high concentration of NaOH solution could corrode the channel of the instrument. Thus, 0.2 M NaOH was chosen for subsequent experiments. The incubation time also affected the enzyme reaction as well as the CL intensity. The CL intensity increased quickly from 10 min to 50 min. When the incubation time was over 50 min, the CL signal increased slowly (Fig. S4 (B)). Thus, 50 min incubation time was employed for subsequent experiments in order to shorten the analytical time. The effect of pH of B–R buffer solution on the assembly of GOD with GO/lucigenin and enzyme reaction was examined in the range of 4.0–5.5. In both cases, the highest CL signal is obtained at pH 4.5 as shown in Fig. S4 (C, and D), which is chosen for further experiments.

3.4. Analytical performance for detecting glucose

Under the optimized experimental conditions, the working curve of the proposed biosensor was assessed using different concentrations of glucose. The CL signal intensity increased linearly with increase in glucose concentration over the range from 1.0×10^{-6} to 5.0×10^{-3} g mL^{-1} (Fig. 4 (a)). The regression equation is $\log I = 7.106 + 0.873\log C$ with a regression coefficient of 0.9951, where I is CL intensity and C is the concentration of glucose (g mL^{-1}). The limit of detection (LOD), at a signal to noise ratio of three ($S/N=3$), is 9.9×10^{-7} g mL^{-1}. The physiological level of glucose in human serum is 7.9×10^{-4}–1.1×10^{-3} g mL^{-1} (American Diabetes Association, 1998). This detection limit and the linear range were sufficient to monitor physiological levels of glucose in human serum. The relative standard deviations (RSDs) of seven replicate detections of 1.0×10^{-6}, 1.0×10^{-5}, and 1.0×10^{-4} glucose were 5.206%, 1.145%, and 3.144% ($n=7$), indicating good repeatability of the proposed biosensor. Batch-to-batch variations for preparing GO/lucigenin&GOD were also studied. Five batches of GO/lucigenin & GOD (100 μL) were mixed with glucose (5 mM, and 50 μL) respectively. As shown in Fig. S5, every batch of the nanocomposite showed good repeatability and batch-to-batch variation is tiny (RSD=6.361%).

The CL biosensor was assumed to be highly selective towards glucose due to specific enzyme reaction. Thus, control experiments were carried out to explore the specificity of the glucose biosensor. Some glucose analogs such as galactose, fructose, sucrose, lactose, maltose and starch were used instead of glucose in the sensing process. The results (Fig. 5) showed that none of them could

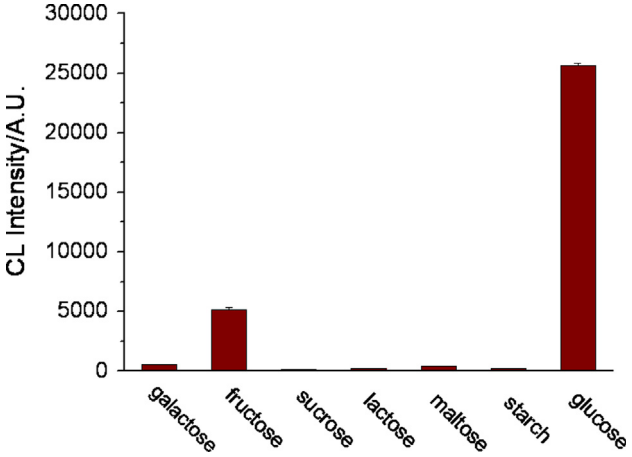

Fig. 5. Selectivity of the proposed CL biosensor.

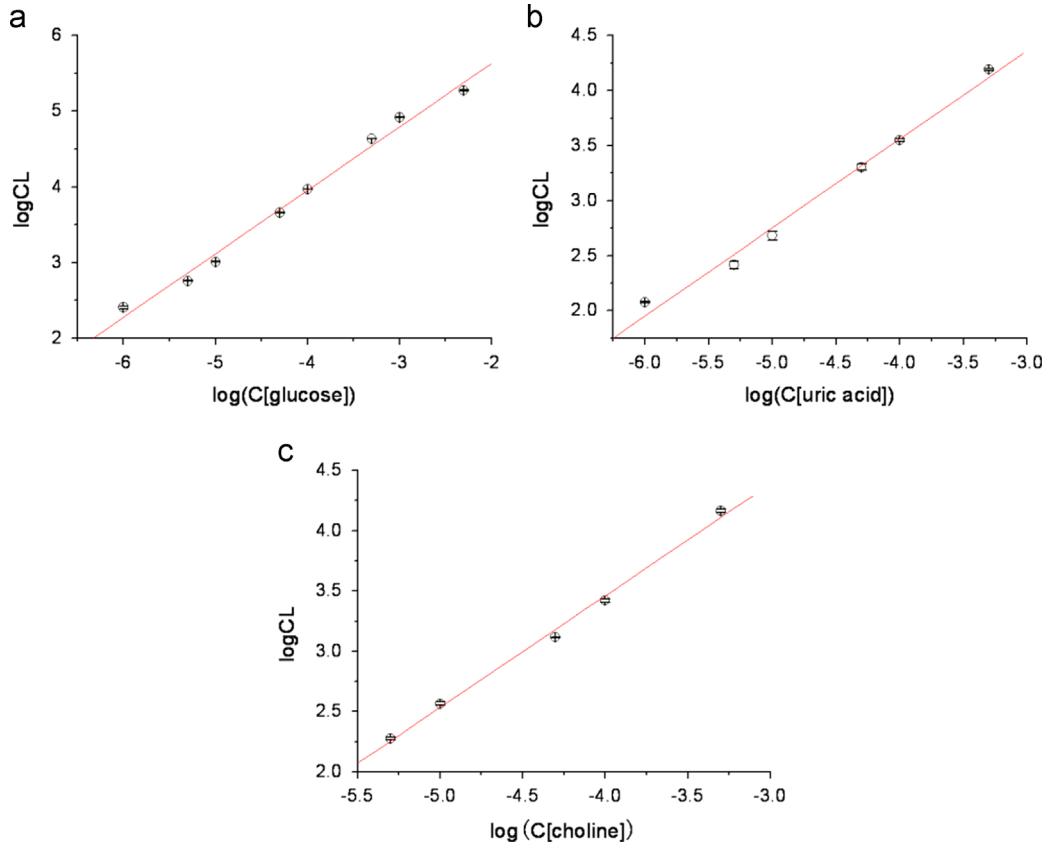

Fig. 4. (a) Calibration curve for glucose concentration. (b) Calibration curve for uric acid concentration. (c) Calibration curve for choline concentration.

Table 1
Determination of glucose in human serum samples.

Human serum	Reference result (mg/mL)	Found (mg/mL)	RD (%)	Added (mg/mL)	Recovered (mg/mL)	Recovery (%)
1	0.875	0.887 ± 0.017	1.371	1.000	0.892 ± 0.019	89.2
2	1.069	1.097 ± 0.000	2.619	1.000	0.939 ± 0.005	93.9
3	1.211	1.220 ± 0.000	0.743	1.000	1.020 ± 0.024	102.0

influence the CL as glucose did. Thus, the present biosensor is highly specific towards glucose.

The practical applicability of the biosensor was also investigated by detecting glucose level in human serum (obtained from the University of Science and Technology of China Hospital, Hefei, China). Before the test, the samples were diluted appropriately step by step to be in the linear range of the proposed method. The reference results of each corresponding serum sample were obtained from the Control Laboratory of the University of Science and Technology of China Hospital for a comparison. Table 1 shows that the results obtained by the proposed biosensor are comparable with reference results achieved by the clinic method (relative deviation < 3%). Recovery experiments were also conducted (Table 1). It was found that the recoveries were in the range of 89.2–102.0%. Therefore, the developed biosensor is applicable for the determination of glucose in real samples.

A comparison of the proposed biosensor with previously reported biosensors for the determination of glucose is made as shown in Table S1. Compared with most of previously reported biosensors, the proposed biosensor shows wider linear range and comparable detection limit. Although the biosensor proposed by Zhou and coworkers (Zhou et al., 2013) is superior to our biosensor in sensitivity, the sensitivity of the proposed biosensor is good enough to determine physiological levels of glucose in human serum. Most importantly, the biosensor is reagentless, homogeneous and convenient, simple, rapid and of low-cost, which renders it more potential to be applied in practical applications.

3.5. Generalization of the CL strategy for other enzyme substrates

Motivated by the consideration that whether the CL strategy provides a general and effective method for the determination of multiple substrates that can produce H_2O_2 under the catalysis of enzyme, other enzymes including uricase and choline oxidase were used to fabricate GO/lucigenin&enzyme biosensors for the determination of uric acid and choline. Fig. 4 (b), and (c) shows working curves for the determination of uric acid and choline based on GO/lucigenin composite functionalized with uricase (GO/lucigenin&uricase) and choline oxidase (GO/lucigenin & choline oxidase), respectively. The regression equation is $\log I = 6.780 + 0.805 \log C$ ($R = 0.9942$, LOD = 1.1×10^{-6} g mL^{-1}) for uric acid and $\log I = 7.168 + 0.927 \log C$ ($R = 0.9976$, LOD = 7.5×10^{-6} g mL^{-1}) for choline, respectively. The linear range is from 1.0×10^{-6} to 1.0×10^{-3} g mL^{-1} for uric acid and from 5.0×10^{-6} to 5.0×10^{-4} g mL^{-1} for choline. The results indicate that the CL strategy could be used for the detection of uric acid and choline and may be extended to the detection of other enzyme substrates.

4. Conclusions

In summary, we have demonstrated a facile and general strategy to fabricate a series of reagentless biosensors for detecting small biological molecules including glucose, uric acid and choline based on an assembly strategy of GO with lucigenin and enzymes for the first time. During the process, lucigenin as CL reagent and enzymes as catalyst have been immobilized on the surface of GO through non-covalent bond including π–π interaction, electrostatic attraction and hydrogen bonding. In the presence of target molecules, H_2O_2 was generated and strong CL signal was obtained. By taking glucose as a model compound, the proposed biosensor shows good selectivity, repeatability and applicability in real samples. Compared with previously reported biosensors, the present biosensors are homogeneous and reagentless, avoiding complicated assembly procedure and pretreatment of sample, which could be an easier and simpler sensing approach for automation in disease diagnosis and biomedicine. This work reveals that GO bifunctionalized by recognition element enzyme and CL reagent is an ideal sensing platform for the detection of small biological molecules. Due to its brilliant repeatability, broad linear range and good practical applicability, the proposed biosensors may find future applications in the fields such as clinical diagnosis and biomedicine.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.01.057.

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