Glucose biosensor using fluorescence quenching with chitosan-modified graphene oxide

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A novel graphene oxide (GO) nanomaterial, which was rapidly and simply synthesised and conjugated with low-molecular-weight chitosan (CS), was used to develop a sensitive GO biosensor system for glucose detection by fluorescence resonance energy transfer. The GO-CS was used to detect low concentrations of glucose based on competitive binding with maltose-binding protein (MBP). The α-subunit of recombinant phycocyanin (rPC), which emits far-red fluorescence, was used to label MBP (MBP-rPC). The rPC emission was quenched by binding between GO-CS and MBP. However, in the presence of glucose, GO-CS was out-competed for binding to MBP, leading to rPC fluorescence. Glucose was sensitively and selectively detected with this biosensor, with a linear detection range of 0.1–1 mg glucose/ml. The limit of detection for glucose was ~0.05 mg/ml.

1. Introduction: Glucose, which is a major carbon and energy source, plays a significant role in our everyday life. Lack of glucose in organisms inhibits cellular growth and division, and in industrial food bioprocessing, it can limit product yields [1]. However, excessive glucose is also detrimental because it can induce lactate formation via the glycolytic pathway and is a clinical indicator of diabetes [2–5]. Numerous glucose sensors have been developed, all with the long-term goal of finding the ‘ideal’ glucose sensor [6]. However, these systems have intrinsic problems such as signal reversibility, low reproducibility of sensor fabrication, and low-biocompatibility, and low signal stability. Reliable glucose sensors for monitoring physiological glucose levels are therefore still needed.

Fluorescence resonance energy transfer (FRET) is widely used for the detection of targets and molecular interaction studies [7]. Graphene oxide (GO), which is a novel fluorescence quencher and substitute for carbon nanotubes, has received increasing attention [8–10]. There are a large number of hydroxyl groups, carboxyl groups, and epoxy groups on the GO surface, therefore GO dissolves easily in water and can be simply covalently bonded to other molecules such as the low-molecular-weight chitosan (CS) [11–13]. Because of the large adsorption surface area and the potential for non-radioactive excitation energy transfer from a fluorophore to GO [14, 15], GO has also been used to construct FRET biosensors [16].

Phycocyanin (PC), which is a protein complex with excellent fluorescence characteristics, has been used to develop a magnetic molecularly imprinted microsensor [17], and Escherichia coli maltose-binding protein (MBP) is an excellent model for prototyping biosensors [18–21]. The α-subunit of recombinant PC (rPC), which is a fluorescent protein with phycocyanobilin chromophores, has been used to label MBP (MBP-rPC). rPC has excellent biocompatibility, long-term stability, and near-infrared (NIR) emission [22–24]. Its fluorescence emission at 640 nm has considerable advantages because absorbance in the NIR by tissue chromophores is low, therefore the signal can propagate through two or more centimetres of tissue. This provides deeper tissue monitoring with highly sensitive detection techniques [23, 25].

We present a nanoscale biosensor system based on MBP-rPC and GO-CS for the rapid and sensitive monitoring of glucose concentrations. In the absence of glucose, the binding interaction between CS and MBP quenches MBP-rPC fluorescence in vitro [21, 26]. However, in the presence of glucose, because the MBP binding affinity for glucose is stronger than that for CS, MBP binds preferentially with glucose, and the fluorescence signal of MBP-rPC is detected (see Fig. 1).

2. Materials and methods
2.1. Synthesis of GO and GO-CS: GO was prepared by a slightly modified version of Hummers’ method [27]. MBP-rPC was prepared according to the methods described in our previous study, with slight modifications [23, 28].

CS modified GO was synthesised according to methods described in related works [29–32]. Low-molecular-weight CS (100 mg) was dissolved in a 1% (v/v) aqueous solution of acetic acid (10 ml). The mixture was stirred until the CS was completely dissolved, to obtain a 10 mg CS/ml solution. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 3 mg) and N-hydroxysuccinimide (NHS, 3.6 mg) were added to a GO solution (0.25 mg GO/ml, 12 ml). The mixture was stirred at room temperature for 1 h. The CS solution (10 mg/ml, 1 ml) was added and the mixture was allowed to react overnight. Semipermeable membranes

Fig. 1 Schematic diagram of glucose detection with biosensor based on MBP-rPC and GO-CS
were used to remove excess molecules, i.e. EDC, NHS, and low-molecular-weight CS.

2.2. Absorbance spectroscopy, atomic force microscopy (AFM), and fluorescence spectroscopy: GO, CS, and GO-CS were dissolved in ultrapure water, and the absorbance spectra of each solution were recorded with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). AFM was used to characterise the GO and GO-CS. MBP-rPC was dissolved in 0.01 M phosphate-buffered saline (PBS) and fluorescence spectra were recorded, with excitation at 600 nm.

2.3. Glucose sensing: Glucose standards (1, 1.5, 2.5, 5, 8, 10, 20, 40, and 80 mg glucose/ml) were prepared with ultrapure water. Each glucose standard solution (0.1 ml) was added to MBP-rPC (30 µg MBP-rPC/ml, 0.1 ml) and PBS buffer (50 mM, pH 7) was added to a final volume of 0.9 ml. These solutions were incubated for 15 min. Then GO-CS (400 µg GO-CS/ml, 1 ml) was added and the solution was thoroughly mixed prior to incubation for 5 min. Fluorescence emissions were measured with excitation at 600 nm. The relative fluorescence unit (RFU) values of GO-CS and MBP-rPC with 0 mg glucose/ml ($F_0$) were determined for comparison.

2.4. Specificity: The specificity of the glucose sensor was tested in the presence of KCl, MgCl$_2$, vitamin B$_1$, and human serum albumin in the sensing system, using the procedure described above. These data were compared with data obtained using a solution of concentration 0.5 mg glucose/ml.

3. Results and discussion

3.1. Characterisation of biosensor materials: GO was conjugated with a CS and formation of a GO-CS complex was verified by AFM. We obtained images and detection data within a scan area of 500 nm × 500 nm. The GO sheets were 1 nm in height and 20 nm in diameter (Fig. 2a). When GO was conjugated with CS, the height of the sheet increased to 2 nm, indicating successful conjugation of CS to the GO surface (Fig. 2b).

CS, GO, and GO-CS were also characterised by absorbance spectroscopy (Fig. 3). The absorbance of CS across the spectrum was low, but a peak at 240 nm was observed in the absorbance spectrum of GO. However, after CS modification of GO, a red shift to 255 nm occurred because of formation of CO–NH bonds. This indicates that CS was successfully conjugated to the GO nanosheet.

We reported the genetic engineering of an E. coli strain that efficiently expressed an MBP-rPC fusion protein [23]. In this study, the same bacterial strain was used to overexpress high yields of MBP-rPC protein. High-purity MBP-rPC was obtained by nickel affinity chromatography and fast protein liquid chromatography, and SDS-PAGE was used to confirm purification. Fig. 4 shows that the purified MBP-rPC had only one subunit and a molecular weight of about 60 kDa.

We also examined the absorbance and fluorescence spectra of the purified protein, and a peak corresponding to pure MBP-rPC was observed at 620 nm. Excitation at 600 nm produced a significant fluorescence peak at 640 nm (Fig. 5).

3.2. Optimisation of experimental conditions: After MBP-rPC specifically binds to glucose, it can no longer move close to GO-CS and the fluorescence will no longer be quenched. The RFU value of the system after glucose addition ($F$) is, therefore, higher than that of the system before glucose addition ($F_0$), and $(F - F_0)/F_0$ indirectly reflects the glucose level. The difference between the intensities before and after fluorescence quenching is affected by factors such as the MBP-rPC concentration, GO-CS concentration, reaction temperature, solution pH, and incubation time. These factors not only affect the conformation of the MBP-rPC protein, resulting in changes in the RFU, but may also affect the adsorption of glucose, GO-CS, and MBP-rPC.
The RFU of the MBP-rPC solution was determined. Based on the range of effective detection monitoring possible, a final MBP-rPC concentration of 3 µg/ml was chosen for all experiments.

GO-CS solutions (0.1 ml) of various concentrations (100, 200, 400, 600, 800, and 1000 µg/ml) were added to 0.1 ml MBP-rPC (30 µg MBP-rPC/ml, 0.1 ml), and PBS buffer (50 mM, pH 7) was added to a final volume of 1 ml. After incubation for 15 min, the addition of GO-CS decreased the signal from MBP-rPC, indicating a high binding affinity between these complexes. In the absence of GO-CS, the fluorescence signal reached 2,712,120 RFU. However, after the addition of 10 µg GO-CS/ml (final concentration), this signal decreased by almost 40% to 1,670,740 RFU. After the addition of more GO-CS, the fluorescence peaks from 40, 60, 80, and 100 µg GO-CS/ml decreased further (Fig. 6a). The fluorescence signals dropped significantly over the concentration range of 0–40 µg GO-CS/ml, and 40 µg GO-CS/ml was sufficient to achieve the minimum RFU. The optimum concentration of GO-CS in the system was therefore 40 µg/ml, and this concentration was used in subsequent experiments.

PBS buffer solutions of pH 2–11 were used to evaluate the effects of pH on the response of MBP-rPC to GO-CS. At pH 7, the fluorescence signal reached a maximum, i.e. 2.5. At pH 4–7, the ratio decreased slightly, and at pH <4, the ratio decreased greatly. The (F − F₀)/F₀ values gradually decreased at pH >7 (Fig. 6b). This indicates that the pH affects the conformation of MBP-rPC in the system, and this greatly affects the sensitivity and accuracy of the final detection system. The optimum pH was therefore 7, and this was used in subsequent experiments.

The responses to 0.5 mg/ml of glucose at 20–40°C were determined to evaluate the effects of temperature. Although (F − F₀)/F₀ gradually decreased with increasing incubation temperature from 20 to 40°C, the temperature had little effect on the results (Fig. 6c). For simplicity and convenience of operation, room temperature was regarded as the optimum temperature for the system.

GO-CS can non-specifically absorb MBP-rPC, which leads to fluorescence quenching. If glucose is present in the system, MBP-rPC will specifically adsorb glucose, resulting in separation of MBP-rPC from GO-CS and fluorescence recovery. The glucose content can then be quantitatively determined indirectly. When MBP-rPC, glucose, and GO-CS are mixed, the following reactions occur: (i) specific binding of MBP-rPC and glucose to form a MBP-rPC–glucose complex; (ii) non-specific binding of GO-CS and MBP-rPC for to form a GO-CS–MBP-rPC complex; and (iii) a competitive reaction between glucose and GO-CS, which converts the GO-CS–MBP-rPC complex to the MBP-rPC–glucose complex. When MBP-rPC, glucose, and GO-CS were mixed, the RFU of the system took hours to reach steady state. This is because the molecular weight of glucose is much smaller than that of GO-CS. To decrease the time consumed by the third reaction, we used a two-step method. First, MBP-rPC and glucose solution were mixed to form the MBP-rPC–glucose complex. Then GO-CS was added to allow GO-CS to fully adsorb the free MBP-rPC, causing fluorescence quenching. In the two-step process, MBP-rPC–glucose complex formation is the rate-limiting reaction. Solutions were prepared by adding MBP-rPC (30 µg MBP-rPC/ml, 0.1 ml) to 5 mg glucose/ml (0.1 ml), followed by addition of PBS buffer (50 mM, pH 7) to a final volume of 0.9 ml. These solutions were incubated for 1–25 min. GO-CS solution (400 µg/ml, 0.1 ml) was then added. The solutions were thoroughly mixed and then incubated for 5 min. The fluorescence emission with excitation at 600 nm was determined. The results show that for the mixture of MBP-rPC with glucose, (F − F₀)/F₀ increased rapidly with increasing incubation time up to 10 min, but after 10 min, (F − F₀)/F₀ increased slowly. When the reaction time exceeded 20 min, (F − F₀)/F₀ decreased slightly (Fig. 6d). For simplicity, the convenience of operation, and the accuracy of detection, 15 min was chosen as the optimum incubation time for the assay.

3.3. Analytical performance: The optimum parameters were used for glucose detection. A series of glucose solutions of different concentrations were added and the RFU values of the reaction systems were recorded. Glucose addition produced a significant increase in fluorescence. This indicates that the quenching effect of GO-CS on MBP-rPC is suppressed by glucose because of glucose out-competes GO-CS for binding to MBP-rPC. The signal increased from 365,260 to 2,101,320 RFU in the presence of 0.5 mg glucose/ml (Fig. 7a). Addition of glucose at levels...
above 4 mg glucose/ml did not yield higher fluorescence values (Fig. 7b). A linear relationship was observed between the concentration of glucose \( \chi \) and the ratio \( Y \) [where \( Y = (F - F_0)/F_0 \), \( F \) is the final RFU after glucose addition and \( F_0 \) is the initial RFU] for glucose concentrations ranging from 0.1 to 1 mg glucose/ml. The equation \( Y = 4.9728X - 0.0871 \) was fitted with a linearity correlation coefficient of \( R^2 = 0.9954 \). The limit of detection for glucose was 0.05 mg glucose/ml, based on the lowest signal greater than the background signal plus three times the standard derivation.

3.4. Specificity of GO-CS sensor for glucose: Blood serum is a common clinical sample for glucose detection. It is a complex mixture of proteins, antibodies, electrolytes, hormones, certain vitamins, and antigens. To investigate interference by these components, we tested the effects of several typical substances on glucose detection with the GO-CS sensor. KCl, MgCl\(_2\), and vitamin B1 had no obvious effects, even at micromolar concentrations. However, 0.5 mg human serum albumin/ml in the solution did slightly affect the assay (Fig. 8). This may have been because of non-specific adsorption of some albumin on GO-CS during the assay. GO-CS would then be unable to continue to adsorb MBP-rPC, and this would lead to the generation of a small number of false-positive results.

4. Conclusion: In summary, in this work, we developed a simple and rapid biosensor system, based on FRET and GO-CS, for monitoring glucose concentrations. Detailed synthetic methods for the production of the GO-CS nanomaterial and rPC-labelled MBP are described. In this biosensor system, GO-CS was observed to bind with, and significantly decrease the RFU of, MBP-rPC. In contrast, the fluorescence signal significantly increased in the presence of glucose because of competitive binding to MBP-rPC, which blocked the quenching effect of GO-CS. This enabled accurate detection of glucose at concentrations as low as 0.05 mg/ml. This FRET-based biosensor easily targeted glucose and rapid biosensor system, based on FRET and GO-CS, for one-step detection of bisphenol A ligands based on graphene oxide sheets.

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6 References

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