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A New Route to the Considerable Enhancement of Glucose Oxidase (GOx) Activity: The Simple Assembly of a Complex from CdTe Quantum Dots and GOx, and Its Glucose Sensing

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Abstract: A new complex consisting of CdTe quantum dots (QDs) and glucose oxidase (GOx) has been facilely assembled to achieve considerably enhanced enzymatic activity and a wide active temperature range of GOx; these characteristics are attributed to the conformational changes of GOx during assembly. The obtained complex can be simultaneously used as a nanosensor for the detection of glucose with high sensitivity. A mechanism is put forward based on the fluorescence quenching of CdTe QDs, which is caused by the hydrogen peroxide (H_2O_2) that is produced from the GOx-catalyzed oxidation of glucose. When H_2O_2 gets to the surface of the CdTe QDs, the electron-transfer reaction happens immediately and H_2O_2 is reduced to O_2, which lies in electron hole traps on CdTe QDs and can be used as a good acceptor, thus forming the nonfluorescent CdTe QDs anion. The produced O_2 can further participate in the catalyzed reaction of GOx, forming a cyclic electron-transfer mechanism of glucose oxidation, which is favorable for the whole reaction system. The value of the Michaelis–Menton constant of GOx is estimated to be 0.45 mM L^{-1}, which shows the considerably enhanced enzymatic activity measured by far. In addition, the GOx enzyme conjugated on the CdTe QDs possesses better thermal stability at 20–80 °C and keeps the maximum activity in the wide range of 40–50 °C. Moreover, the simply assembled complex as a nanosensor can sensitively determine glucose in the wide concentration range from micro- to millimolar with the detection limit of 0.10 μM, which could be used for the direct detection of low levels of glucose in biological systems. Therefore, the established method could provide an approach for the assembly of CdTe QDs with other redox enzymes, to realize enhanced enzymatic activity, and to further the design of novel nanosensors applied in biological systems in the future.

Keywords: enzymes · glucose oxidase · nanomaterials · quantum dots · sensors

Introduction

In recent years, the assembly of nanomaterials and biomolecular complexes as nanosensors for biological analyses and applications has become a hot research field with the development of inorganic–biological hybrids such as nanostructure-conjugated DNA, proteins, and enzymes.[1–9] The biological properties of biomolecules conjugated on nanomaterials may be regulated by the way of assembly. In particular, enzymes, as important biomacromolecules, play a prominent role in biological reaction systems. Thus, the research on the improvement of enzymatic properties including activity, stability, and active temperature range has attracted increasing attention.[1,2]

Nanomaterials, with their interesting properties such as large surface-to-volume ratio, high catalytic efficiency, and high surface reaction activity have been favorably adopted as potential materials to play a catalytic role in enzyme-based biosensing systems.[10–12] The increased surface area of nanoparticles can provide a better matrix for the immobilization of enzymes, and then enables larger amounts of enzymes to immobilize on the particles. The multipoint combination of enzyme molecules to nanoparticle surfaces can improve the enzyme–substrate interaction effectively by avoiding the potential aggregation of free enzymes, which would

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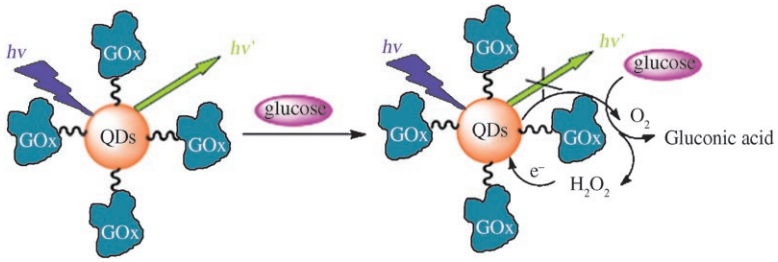
lead to the enhancement of enzymatic activity.[13–15] In addition, the enzyme attached on nanomaterial surfaces can reduce protein unfolding and turbulence, and thus ensure the enhanced stability of enzymes in solution.[16] So, much effort has been made to develop different materials to be used as assembled matrices for enzymes, including magnetite nanoparticles, gold nanoparticles (AuNPs), titania sol–gel membranes, carbon nanotubes, and other oxides such as ZrO_2.[17–21] Of these, the materials widely used for investigation of the activity and stability of enzymes are AuNPs and magnetite nanoparticles. At present, AuNPs have been identified as a suitable biocompatible material and have been used to improve the enzymatic activity favorably in enzyme-based analytical systems.[22] The studies of different enzymes assembled onto magnetite nanoparticle surfaces have also demonstrated the potential of enhancing the enzymatic activity.[23,24] However, the investigation of the enhanced activity, stability, and active temperature range of enzymes is still limited to an initial stage.

Glucose, an important bioactive substance, plays a prominent role in the natural growth of cells. Therefore, it is necessary to regulate the glucose levels in biological systems.[25] At present, glucose oxidase (GOx) has been widely employed in the determination of glucose based on the enzyme-catalyzed oxidation mechanism using optical and electrical methods.[26–28] However, the direct sensing of glucose with high sensitivity accompanied by the considerably enhanced activity of GOx has not yet been realized. Quantum dots (QDs), owing to their good optical characteristics, high catalytic effects, and high electron-transfer efficiency, have been used as a preferable material in enzyme-based biological analyses and applications.[29,30] For example, QDs have been reported to monitor biocatalytic transformations and to probe the activities of polymerase, telomerase, and tyrosinase.[31,32] Herein, we have made a new assembly of CdTe QDs and the GOx complex, and the obtained CdTe QDs–GOx can be used as a nanosensor for simultaneous assay of GOx enzymatic activity, thermal stability, and glucose sensing. Our results indicate that the lower value of the Michaelis–Menton constant of GOx is estimated to be 0.45 mM L^{-1}, which shows the considerably enhanced enzymatic activity measured by far. In addition, the GOx enzyme conjugated on the CdTe QDs possesses better thermal stability at 20–80 °C and keeps the maximum activity in the wide range of 40–50 °C. The conformation of GOx has been demonstrated to change greatly during the assembly process. Moreover, the simply assembled nanosensor can sensitively determine glucose over a wide concentration range from micro- to millimolar with a detection limit of 0.10 μM, which could be used for the direct detection of lower levels of glucose in biological systems. Therefore, the established method could provide an approach for the assembly of CdTe QDs with other redox enzymes, to realize enhanced enzymatic activity, and to further the design of novel nanosensors applied in biological systems in the future.

Results and Discussion

Design of the CdTe QDs–GOx complex: Our strategy for designing the nanocomplex is based on the facile covalent conjugation between CdTe QDs and GOx. The obtained CdTe QDs–GOx can be used as a nanosensor for simultaneous assay of GOx enzymatic activity, thermal stability, and glucose sensing. A mechanism is put forward based on the fluorescence quenching of CdTe QDs, which is caused by the hydrogen peroxide (H_2O_2) that is produced from the GOx-catalyzed oxidation of glucose. When H_2O_2 gets to the surface of the CdTe QDs, the electron-transfer reaction happens immediately and H_2O_2 is reduced to O_2, which lies in electron hole traps on CdTe QDs and can be used as a good acceptor, thus forming the nonfluorescent CdTe QDs anion. The produced O_2 can further participate in the catalyzed reaction of GOx, forming a cyclic electron-transfer mechanism of glucose oxidation, which is favorable for the whole reaction system, as is shown in Scheme 1.



Scheme 1. The structure of the new assembled CdTe QDs–GOx complex and a schematic illustration of its glucose sensing principles.

Characterization of the CdTe QDs–GOx complex: In our study, the CdTe QDs–GOx complex was assembled by using coupling reagents, followed by purification with an ultrafiltration membrane, and was characterized by excitation and fluorescence spectra, and TEM and confocal fluorescence images, which are shown in Figures 1, 2, and 3.

From the experimental results in Figure 1, the maximum excitation peak of the CdTe QDs redshifts from $\lambda = 336$ to 340 nm and the maximum fluorescence emission peak redshifts from 520 to 525 nm after its conjugation with GOx, which is attributed to the increase of the final size of the CdTe QDs–GOx complex after surface modification of GOx with the larger molecular weight.[9] In addition, as can be seen from the TEM (Figure 2) and confocal fluorescence images (Figure 3) of CdTe QDs before and after conjugation

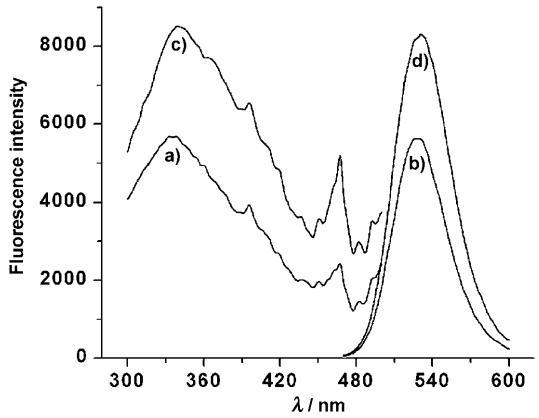


Figure 1. The excitation spectra and fluorescence spectra observed from CdTe QDs (a and b, respectively) and the CdTe QDs–GOx complex (c and d, respectively). Solutions were prepared in PBS buffer (10 mM, pH 7.4).

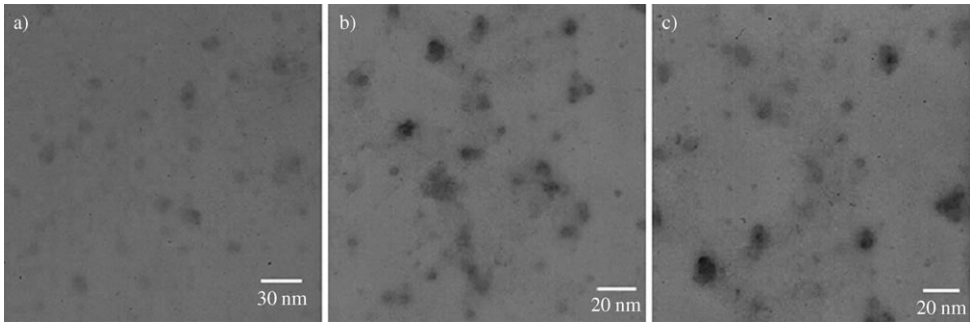


Figure 2. TEM images of a) CdTe QDs, b) the CdTe QDs–GOx complex, and c) after addition of glucose to (b).

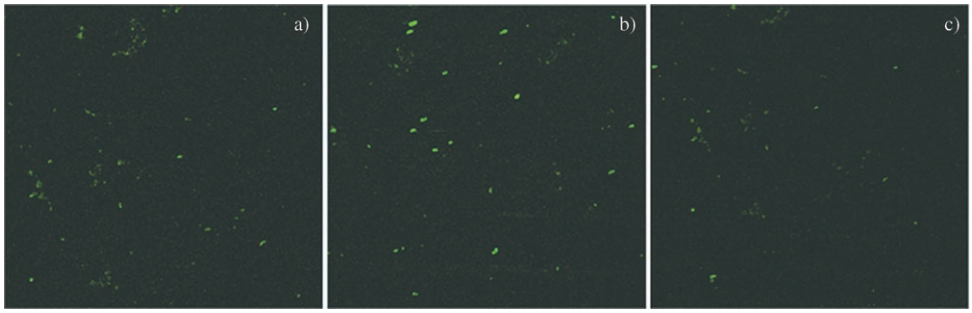


Figure 3. Confocal fluorescence images of a) CdTe QDs, b) the CdTe QDs–GOx complex, and c) after addition of glucose to (b).

with GOx, the average diameter of the CdTe QDs increases clearly, showing that the GOx has been well conjugated on the CdTe QDs. Therefore, the conjugation between carboxyl group-coated CdTe QDs and GOx, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as coupling reagents, is feasible.

Biological activity of the CdTe QDs–GOx complex: The biological activity of the assembled CdTe QDs–GOx complex was confirmed by circular dichroism (CD) spectra. As can be seen from Figure 4, the CD spectra of GOx and the CdTe QDs–GOx complex are similar to each other, indicating that GOx retains its biological activity after conjugation. In addition, the CD spectral peak of CdTe QDs–GOx becomes weaker because of removing excess GOx in the conjugation. And it also proves that ultrafiltration is a practical way of purifying the CdTe QDs–GOx complex. The data from the CD spectra reveal that the tertiary structure of GOx remains mostly intact.[33]

Confirmation of the mechanism based on the fluorescence quenching of CdTe QDs: It is well known that D-glucose can be oxidized quickly to gluconic acid and H_2O_2 in the presence of GOx.[26–28] To illustrate the mechanism of the fluorescence quenching of the CdTe QDs–GOx nanosensor after the introduction of glucose, catalase, which is a scavenger of H_2O_2, was used in the reactive system. Figure 5 shows that the addition of catalase to the nanosensor solution before glucose sensing blocks the decrease in the fluorescence and does not produce any influence on the fluorescence of the nanosensor. In addition, control experiments indicate that D-glucose by itself does not affect the fluorescence of the CdTe QDs. These results demonstrate that H_2O_2 generated by biocatalysis of GOx indeed results in fluorescence quenching. The precise mechanism that stimulates the decrease in the fluorescence of CdTe QDs–GOx is based on the electron-transfer reaction that occurs on the surface of CdTe QDs. When H_2O_2 gets to the surface of the CdTe QDs, it is reduced to O_2 immediately, which lies in electron hole traps on the CdTe QDs and can be used as a good acceptor, thus forming the nonfluorescent CdTe QDs anion.[34–37] The produced O_2 can further participate in the catalyzed reaction of GOx, forming a cyclic mechanism of glucose oxidation, which is favorable for the whole reaction system. The catalytic kinetic assay on glucose sensing revealed that the fluorescence decreases with reaction time, which also suggests a cyclic mechanism (Figure 6). Furthermore, the TEM and confocal fluorescence images of CdTe QDs–GOx (Figures 2 and 3) indicate that the morphology of the nanosensor is not destroyed and remains intact after the addition of glucose, confirming that the fluorescence quenching is not caused by

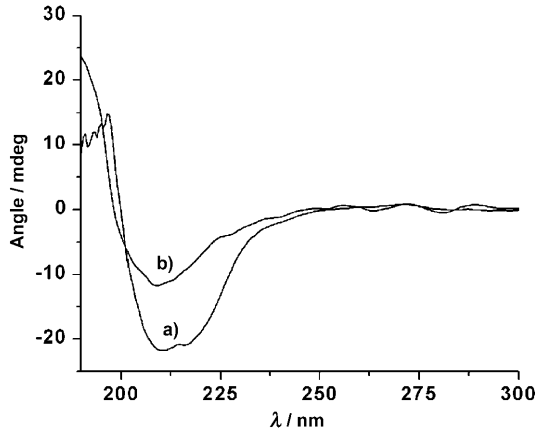


Figure 4. CD spectra of a) free GOx and b) GOx conjugated on the CdTe QDs. Samples were dissolved in PBS (10 mM, pH 7.4). The initial concentration of the GOx solution was 1.00 mg mL^{-1}.

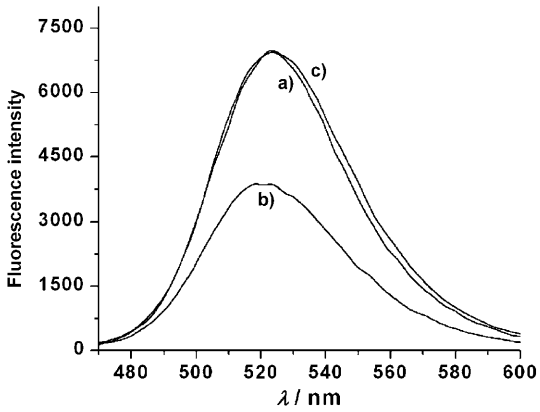


Figure 5. The fluorescence spectra of a) the CdTe QDs–GOx nanosensor, b) after addition of glucose to (a), and c) the catalase introduced before glucose sensing.

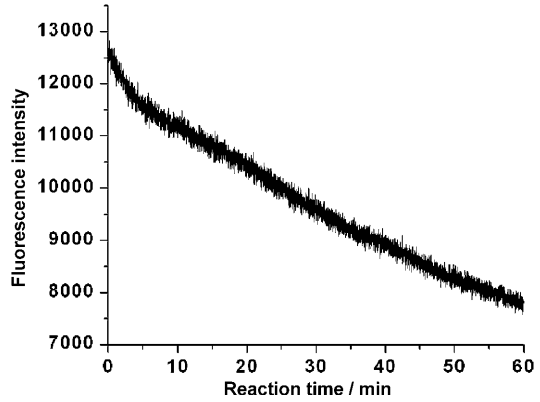


Figure 6. The change of fluorescence intensity with the sensing reaction time.

surface oxidation of CdTe QDs, but is an electron-transfer mechanism. Therefore, the glucose sensing mechanism using the new assembled CdTe QDs–GOx nanosensor is based on formation of the cyclic electron-transfer mechanism between CdTe QDs and GOx during the catalyzed oxidation of glucose, which leads to the fluorescence quenching of CdTe QDs. And, thus, CdTe QDs can sensitively respond to H_2O_2, in accord with the recently reported CdSe/ZnS QDs.[38]

The enzymatic activity assay on GOx: The enzymatic activity of GOx assembled on the CdTe QDs was investigated by monitoring the decrease in fluorescence intensity during oxidation of glucose. Because GOx can catalyze the oxidation of glucose to produce H_2O_2, it is expected that for a given enzymatic activity, the rate of H_2O_2 production would increase as the glucose concentration increases. So the extent of fluorescence quenching embodies the changes of GOx enzymatic activity. In addition, as the biocatalytic reaction proceeds, the luminescence quenching of CdTe QDs–GOx is enhanced, in accord with the theory of the catalytic kinetic study and the proposed cyclic mechanism. In the experiment, we carried out the assays upon interaction of glucose with the sensing system for a fixed reaction time of ten minutes (Figure 6). As can be seen from Figure 7a, the relative fluorescence intensity increases with the increasing glucose concentration up to 6.0 mM, after which it reaches saturation. According to the theory of kinetics of enzyme-catalyzed reactions, the Michaelis–Menton parameter (K_m) can

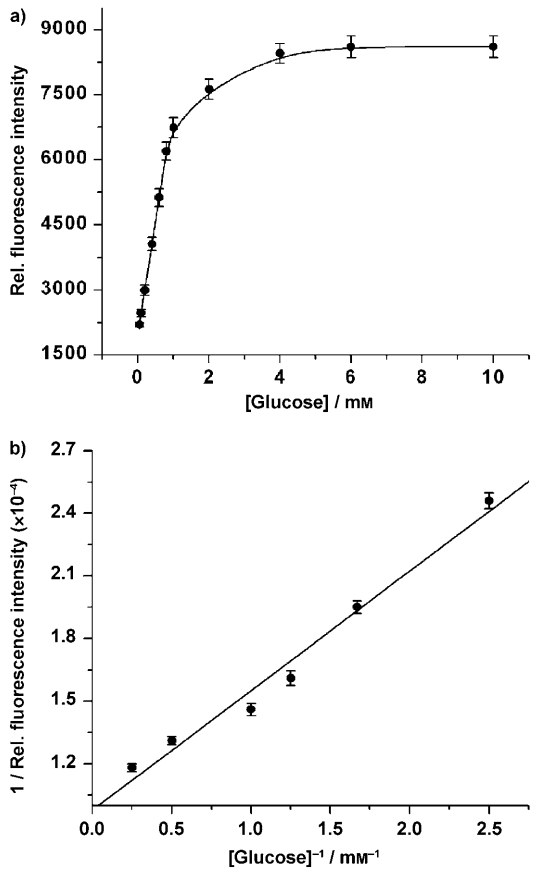


Figure 7. a) The relative fluorescence intensity changes after addition of different concentrations of glucose to the sensing solution. b) Lineweaver–Burke plots of the GOx enzyme–glucose reaction.

be determined by analysis of enzyme–substrate reactions. In our present assay system, the parameter was estimated by using the Lineweaver–Burke plot[39] (Figure 7b). The value of K_m was calculated to be 0.45 mM L^{-1}, indicating the considerably enhanced activity of GOx for glucose after its conjugation with CdTe QDs relative to the value of 5.85 mM L^{-1} for free GOx and 3.74 mM L^{-1} for GOx immobilized on the gold nanoparticles that had the lowest value reported.[22]

The secondary and tertiary structures of the enzyme are known to play an important role in the enzymatic activity,[40,41] so the conformational changes of GOx were studied by CD and fluorescence spectral experiments. As can be seen from Figure 4, the CD spectrum of free GOx has characteristic peaks at $\lambda = 210$ and 220 nm, which are retained in the native conformation. Whereas, GOx conjugated on the CdTe QDs shows a CD minimum peak at $\lambda = 208$ nm, demonstrating that the precise enzymatic conformation, including α helix, β sheet, β turn, and random coil, changes greatly compared with the free GOx. The conformational changes can also be illustrated by fluorescence measurements on GOx, which are shown in Figure 8. The fluorescent amino

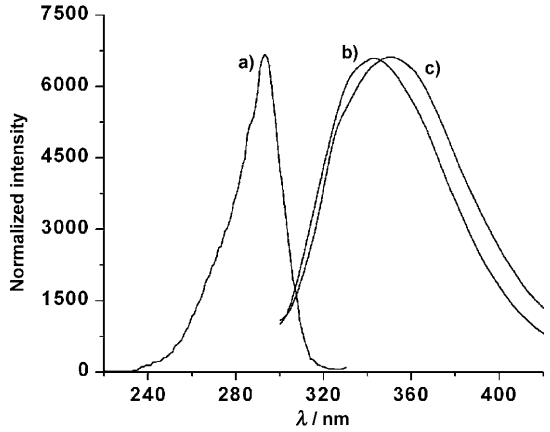


Figure 8. The excitation spectrum of a) GOx, and the fluorescence spectra of b) GOx and c) GOx conjugated on the CdTe QDs.

acid residues in proteins are a useful indicator of the local and overall conformation.[42] And the maximum emission of proteins is highly dependent on the environment around it. In our study, the native GOx has a maximum fluorescence peak at $\lambda = 343$ nm when excited at the maximum excitation wavelength of 293 nm. A redshift to 350 nm is observed in GOx after its conjugation with CdTe QDs, which indicates that the conformation of GOx changes in the process of assembly. Therefore, the considerably enhanced activity of GOx by the new assembly of GOx and CdTe QDs is due to the favorable changes of its conformation.

Thermal stability assay on the GOx enzyme: The thermal stability of covalently conjugated GOx was investigated in the range of 20–80 °C, as can be shown in Figure 9. Our results indicate that the conjugated GOx remains active over a wide range of temperature. It is observed that GOx has

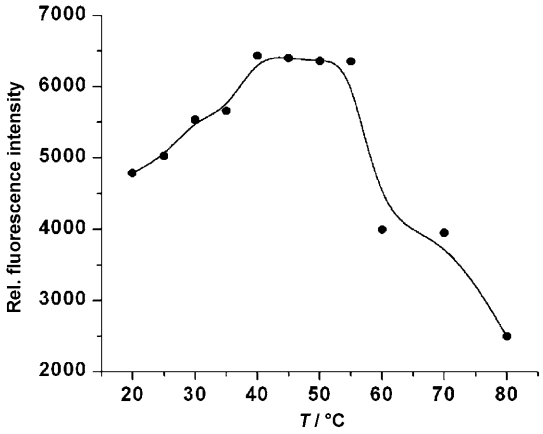


Figure 9. The changes of fluorescence intensity in glucose sensing at the temperature of 20–80 °C.

the maximum activity at 40–50 °C and a sharp activity reduction occurs when the temperature is above 50 °C. Although the activity of GOx decreases at higher temperature, the relative activity of GOx still remains at 80 °C. Our results show that the conjugated GOx enzyme exhibits a wider active temperature range than the free GOx enzyme, the thermal stability of which has been reported frequently in the literature.[22,43–45] The reason behind the thermal stability enhancement of GOx may lie in the large surface areas on CdTe QDs, which reduce enzyme unfolding and turbulence, leading to the conformational changes of GOx, and thus ensure the enhanced stability of enzymes in solution.[16]

Glucose sensing based on the CdTe QDs–GOx complex: On the basis of the considerably enhanced GOx enzymatic activity, we performed the sensitive determination of glucose by using the assembled CdTe QDs–GOx nanosensor. The experimental conditions including the pH of the solution and the amounts of the sensing solution were optimized (see the Supporting Information). Under optimal conditions, as can be seen from Figure 10, the fluorescence intensity grad-

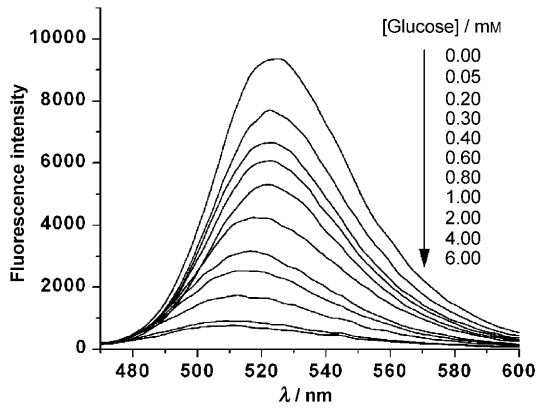


Figure 10. The changes in fluorescence intensity of the CdTe QDs–GOx nanosensor at different concentrations of glucose for a fixed reaction time of 10 min under optimal experimental conditions.

ually decreases as the concentration of glucose increases. In addition, the decrease of fluorescence intensity is directly proportional to the concentration of glucose in the range of 5.0 μM to 1.0 mM (Figure 11). Statistical analysis gave us a

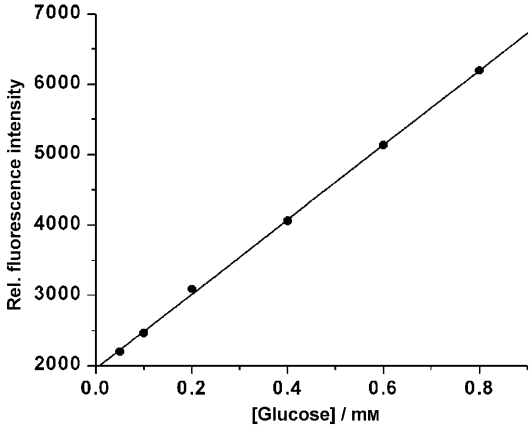


Figure 11. Linear plot of relative fluorescence intensity as a function of glucose concentration.

value for the detection limit as low as 0.10 μM towards the concentration of glucose, thus the sensitivity of this method is a clear improvement on other GOx-based glucose detection methods.[26–28] Therefore, our new assembled CdTe QDs–GOx nanosensor can be applied to the ratiometric detection of glucose with high sensitivity and simplicity.

Conclusion

In summary, we have assembled a new CdTe QDs–GOx complex, and thus achieved considerably enhanced enzymatic activity and widened the active temperature range of GOx. The obtained complex can be used as a nanosensor for simultaneous assay on GOx enzymatic activity, thermal stability, and glucose analysis. A mechanism is put forward based on the fluorescence quenching of CdTe QDs, which is caused by the H_2O_2 that is produced from the GOx-catalyzed oxidation of glucose. When H_2O_2 reaches the surface of the CdTe QDs, the electron-transfer reaction occurs immediately and H_2O_2 is reduced to O_2, which lies in electron hole traps on CdTe QDs and can be used as a good acceptor, thus forming the nonfluorescent CdTe QDs anion. The produced O_2 can further participate in the catalyzed reaction of GOx, forming a cyclic electron-transfer mechanism on glucose oxidation, which is favorable for the whole reaction system. The lower value of the Michaelis–Menton constant is estimated to be 0.45 mM L^{-1}, which shows the considerable enhanced enzymatic activity measured by far. In addition, the GOx enzyme conjugated on the CdTe QDs obtains better thermal stability at 20–80 °C than free GOx and keeps the maximum activity in the wide range of 40–50 °C. The conformational changes of GOx are attributed to the above-enhanced enzymatic activity and the wide active temperature range. The assembly of GOx to CdTe QDs is facile, and the assembled nanosensor has good stability, which ensures its potential for bioanalytical applications. Moreover, the simply assembled nanosensor can sensitively determine glucose over a wide concentration range from micro- to millimolar with the detection limit of 0.10 μM, which could be used for the direct detection of lower levels of glucose in complicated biological systems. These results indicate that CdTe QDs with good luminescence, high catalytic effects, and electron-transfer efficiency may become a promising nanomaterial for the enhancement of enzymatic activity. In addition, the new method of assembly provide an approach for the assembly of CdTe QDs with other redox enzymes, to realize enhanced enzymatic activity, and to further the design of novel nanosensors applied in biological systems in the future.

Experimental Section

Materials: Glucose oxidase (GOx, 200 U mg^{-1}), catalase (1340 units per mg solid, from bovine liver), thioglycolic acid (TGA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma (Aldrich). D-Glucose was purchased from Amresco Corporation. Tellurium powder (99%) and sodium hydrogen boride (99%) were obtained from China Medicine Group Shanghai Chemical Reagent Corporation. All chemicals were used without further purification. Ultrapure water used in the experiment was purified with a Milli-Q (electric resistivity 18.2 MΩ cm^{-1}) water purification system. A 100K Nanosep filter (Pall Corporation, USA) and Microcon YM-30-30000 NMWL (Millipore, USA) were used as the ultrapurification instrumentation.

Physical instrumentation and methods: Excitation and fluorescence spectra were carried out with an Edinburgh FLS920 spectrofluorimeter (Edinburgh Instruments, Scotland) equipped with a xenon lamp and a quartz cuvette (1.0 cm optical path). Spectrometer slits were set to 2.0 nm. TEM images were performed on a Hitachi Model H-800 instrument (Japan). CD spectra were obtained on a circular dichroism spectrometer (J-810, JASCO, Japan); a JASCO cell of path length 0.10 cm was used. Confocal fluorescence microscopy images were obtained on a LSM510 confocal laser-scanning microscope (Carl Zeiss). Centrifugation was carried out on a Sigma 3K 15 centrifuge.

Preparation and purification of water-soluble CdTe QDs: CdTe QDs were prepared by using the reaction between Cd^{2+} and a NaHTe solution in the presence of thioglycolic acid (TGA) as a stabilizer according to the literature.[46] To remove excess thioglycolic acid, the as-prepared QDs were precipitated with an equivalent amount of 2-propanol, and then redispersed in ultrapure water and precipitated with 2-propanol twice. The pellet of purified QDs was dried overnight at room temperature under vacuum, and the final product in the powder form could be redissolved in ultrapure water (100 mL). The aggregated nanoparticles that appeared during the process of redissolving were removed by ultrafiltration using a 100K Nanosep filter under centrifugation (12000 rpm, 5 min). The upper phase was discarded. The obtained homogeneous QDs were in the lower phase and used as the stock solution.

Assembly of the CdTe QDs–GOx complex: Glucose oxidase (GOx) was dissolved in phosphate-buffered saline solution (PBS, 10 mM, pH 7.4) to obtain a solution (1.0 mg mL^{-1}) that was stored at 4 °C. The conjugation proceeds by NHS and EDC forming active esters to conjugate the carboxyl groups of QDs to the primary amine groups of GOx. Briefly, EDC (0.50 mg) and NHS (0.25 mg) were added to the CdTe QDs stock solution (1.00 mL) to activate the QDs in PBS (10 mM, pH 7.4), and it was then incubated for 30 min at RT with continuous gentle mixing. Next, the activated QDs and GOx solution (100 μL) were incubated at room tem-

perature for another 2 h with continuous gentle mixing, and then stored at 4 °C overnight. The GOx-conjugated QDs were separated from the solution by removing free GOx as well as other small molecules through ultrafiltration under centrifugation (12000 rpm, 5 min) with a YM-30K ultrafilter. The final concentrated CdTe QDs–GOx complex was diluted to 1.00 mL with ultrapure water for use in the following fluorescence assays. The obtained CdTe QDs–GOx complex as a nanosensor was characterized by excitation and fluorescence spectra, TEM images, confocal fluorescence images, and circular dichroism (CD) spectra.

The scavenging of H_2O_2 by the catalase enzyme: Catalase was dissolved in PBS (10 mM, pH 7.4) to obtain a solution (1.0 mg mL^{-1}) that was stored at 4 °C. The scavenging experiment was as follows: catalase (50 μL) was added to the CdTe QDs–GOx sensing solution and glucose (0.50 mM) was introduced afterwards; this was followed by the fluorescence determination.

Choosing of reaction time by the kinetic assay method: Because the luminescence quenching of CdTe QDs is gradually enhanced as the biocatalytic reaction proceeds, we carried out the kinetic assay upon interaction of glucose in the sensing system. As can be seen from Figure 6, the fluorescence decreases greatly in the initial five minutes, after which it decreases at a steady rate. To do the determination easily, we chose to fix the reaction time at 10 min.

Activity and thermal stability assays of the GOx enzyme: According to the analytical method of catalytic kinetic fluorimetry, the activity of GOx after its conjugation with the CdTe QDs was measured based on the change of fluorescence intensity in the CdTe QDs–GOx nanosensor. From the theoretical analysis, the slope of the fitted line to the recorded fluorescence-intensity change was proportional to the enzymatic activity in a certain amount of enzyme. The thermal stability measurement was performed as follows: the CdTe QDs–GOx sensing solution was incubated at a certain temperature for 15 min in a water bath; it was then cooled to room temperature. Then the treated sensing solution was introduced to glucose and left to react for 10 min. The fluorescence changes reflected the change of GOx enzymatic activity at a certain temperature.

Glucose sensing by fluorescence detection: Typically, the obtained CdTe QDs–GOx nanosensor solution (200 μL), PBS buffer solution (100 μL, 10 mM, pH 7.4), and different concentrations of glucose were added to the colorimetric tube (1.50 mL), respectively, and each sample solution was diluted with ultrapure water to a final volume of 1.00 mL. After reaction for 10 min at the optimal temperature of 40 °C, the fluorescence spectra were obtained in the spectral range from $\lambda = 470$ to 600 nm by use of the maximal excitation wavelength at 340 nm.

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