Anal. Chem. **2010,** *82,* 1427–1433

Conjugation of Glucose Oxidase onto Mn-Doped ZnS Quantum Dots for Phosphorescent Sensing of Glucose in Biological Fluids

Peng Wu, Yu He, He-Fang Wang, and Xiu-Ping Yan*

Research Center for Analytical Sciences, College of Chemistry, Nankai University, 94 Weijin Road, Tianjin 300071, China

Integrating various enzymes with nanomaterials provides various nanohybrids with new possibilities in biosensor applications. Furthermore, the enzymatic activity and stability are also improved due to the large surface area of nanomaterials. Here we report the conjugation of glucose oxidase (GOD) onto phosphorescent Mn-doped ZnS quantum dots (QDs) using 1-ethyl-3-(3-dimethylaminopropy)carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) as coupling reagents for glucose biosensing based on the effective quenching of the room temperature phosphorescence (RTP) of Mn-doped ZnS QDs by the H_2O_2 generated from GOD-catalyzed oxidation of glucose. The obtained bioconjugate not only provided improved enzymatic performance with Michaelis−Menten constant of 0.70 mM but also favored biological applications because the phosphorescent detection mode avoided the interference from autofluorescence and scattering light from the biological matrix. In addition, the GOD-conjugated Mn-doped ZnS QDs showed better thermal stability in the temperature range of 20−80 °C. The GOD−Mn-doped ZnS QDs based RTP sensor for glucose gave a detection limit of 3 μM and two linear ranges from 10 μM to 0.1 mM and from 0.1 to 1 mM. The developed biosensor was successfully applied to the determination of glucose in real serum samples without the need for any complicated sample pretreatments.

Enzymes are an important type of biomacromolecules with high catalytic efficiency and high substrate-specificity and play prominent roles in biological systems. However, they tend to suffer from instability and are easily denatured. Immobilization of enzymes onto a solid support provides an effective way for improving enzymatic performance.[1] In recent years, nanostructured materials have received great attention for enzyme immobilization because of their intriguing properties such as large surface-to-volume ratio, high catalytic efficiency, and high surface reaction activity.[2,3] Attachment of enzymes onto the surface of nanomaterials can reduce protein unfolding and turbulence and thereby improves enzyme stability. Besides, the multipoint combination of enzyme molecules with the surface of nanomaterials can effectively improve the enzyme−substrate interaction by avoiding the potential aggregation of free enzymes and thereby enhances enzymatic activity.[4−10] Among various functional nanomaterials, semiconductor quantum dots (QDs) are of considerable interest due to their size-dependent optical and physical properties.[11−13] The size of QDs allows them to function effectively both as a fluorophore and as a multifunctional nanoscaffold for the attachment of biomolecules or other moieties. Immobilization of enzymes onto QDs has also been reported based on the fruitful surface chemistry as well as large surface area of QDs.[14−16] Exploring QDs-enzyme hybrid systems can provide dual functions of enzyme immobilization and biosensing as the biocatalytic processes induced by enzymes can be readily read out with the fluorescence change of QDs.

Most of previous publications on QDs−enzyme nanohybrids for biosensing have focused on the use of the fluorescence of QDs.[11−13] However, the short-lived autofluorescence and scattering light from biological matrixes can make the use of fluorescent QDs problematic. Biocongugated phosphorescent QDs not only preserve the promising features of QDs−biomolecule hybrids but also eliminate the drawbacks of fluorescence detection because

* Corresponding author. Fax: (86)22-23506075. E-mail: xpyan@nankai.edu.cn.

(1) Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2003**, *42*, 3336–3337.
(2) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547–1562.
(3) Katz, E.; Willner, I. *Angew. Chem., Int. Ed.* **2004**, *43*, 6042–6108.
(4) Rossi, L. M.; Quach, A. D.; Rosenzweig, Z. *Anal. Bioanal. Chem.* **2004**, *380*, 606–613.
(5) Lin, Y. H.; Lu, F.; Tu, Y.; Ren, Z. F. *Nano Lett.* **2004**, *4*, 191–195.
(6) Pandey, P.; Singh, S. P.; Arya, S. K.; Gupta, V.; Datta, M.; Singh, S.; Malhotra, B. D. *Langmuir* **2007**, *23*, 3333–3337.
(7) Yang, Y. H.; Yang, H. F.; Yang, M. H.; Liu, Y. L.; Shen, G. L.; Yu, R. Q. *Anal. Chim. Acta* **2004**, *525*, 213–220.
(8) Scodeller, P.; Flexer, V.; Szamocki, R.; Calvo, E. J.; Tognalli, N.; Troiani, H.; Fainstein, A. *J. Am. Chem. Soc.* **2008**, *130*, 12690–12697.
(9) Wei, A.; Sun, X. W.; Wang, J. X.; Lei, Y.; Cai, X. P.; Li, C. M.; Dong, Z. L.; Huang, W. *Appl. Phys. Lett.* **2006**, *89*, 123902.
(10) Samanta, B.; Yang, X. C.; Ofir, Y.; Park, M. H.; Patra, D.; Agasti, S. S.; Miranda, O. R.; Mo, Z. H.; Rotello, V. M. *Angew. Chem., Int. Ed.* **2009**, *48*, 5341–5344.
(11) Gill, R.; Zayats, M.; Willner, I. *Angew. Chem., Int. Ed.* **2008**, *47*, 7602–7625.
(12) Costa-Fernández, J. M.; Pereiro, R.; Sanz-Medel, A. *Trends Anal. Chem.* **2006**, *25*, 207–218.
(13) Sapsford, K. E.; Pons, T.; Medintz, I. L.; Mattoussi, H. *Sensors* **2006**, *6*, 925–953.
(14) Narayanan, S. S.; Sarkar, R.; Pal, S. K. *J. Phys. Chem. C* **2007**, *111*, 11539–11543.
(15) Cao, L. H.; Ye, J.; Tong, L. L.; Tang, B. *Chem.—Eur. J.* **2008**, *14*, 9633–9640.
(16) Vastarella, W.; Nicastri, R. *Talanta* **2005**, *66*, 627–633.

10.1021/ac902531g
Published on Web 01/21/2010

the long lifetime of phosphorescence allows an appropriate delay time to avoid the interferences from autofluorescence and scattering light.[12] It has been reported that Mn-doped ZnS QDs exhibited promising phosphorescence emission.[17] Energy transfer from the band gap of ZnS to Mn^{2+} dopant and subsequent transition from the triplet state ($^4T_1$) to the ground state ($^6A_1$) of the Mn^{2+} incorporated into the ZnS host lattice results in an orange phosphorescence emission (about 590 nm).[18] The use of phosphorescent QDs for optical sensing is only on its infant stage but has proven to be very promising.[17,19−21] To the best of our knowledge, the anchoring of biomacromolecules onto phosphorescent QDs for bioassays has not been reported so far.

Glucose is the major energy source in cellular metabolism and plays an important role in the natural growth of cells. Glucose deficiency or excess can produce detrimental influence on cellular functions. The glucose level in blood is usually used as a clinical indicator of diabetes, so rapid and accurate determination of glucose in human blood and urine is essential in the diagnosis and management of diabetes, which is affecting about 150 million people in the world.[22] Millions of diabetics need to test their blood glucose levels daily, making glucose the most commonly tested analyte. Glucose oxidase (GOD) has been widely employed for optical and electrochemical determination of glucose based on the enzyme-catalyzed oxidation mechanism,[23−28] but phosphorescence-based detection for blood glucose is not so common.[29−31]

Here we report the conjugation of GOD onto Mn-doped ZnS QDs for phosphorescent sensing of glucose in aqueous solution. The obtained biosensor was evaluated for enzyme activity, thermal stability, and glucose sensing in serum samples.

EXPERIMENTAL SECTION

Materials and Chemicals. Glucose oxidase (GOD, > 100 U mg^{-1}, Shanghai Sangon, Shanghai, China), D-glucose and H_2O_2 (Tianjing Guangfu Chemical Co., Tianjing, China), 1-ethyl-3-(3-dimethylaminopropy)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (Shanghai Medpep Co. Ltd., Shanghai, China) were used as received. Mercaptopropionic acid (MPA) (Aladin, Shanghai, China), $ZnSO_4 \cdot 7H_2O$, $Mn(Ac)_2 \cdot 4H_2O$, and $Na_2S \cdot 9H_2O$ (Tianjing Guangfu Chemical Co., Tianjing, China) were used for the preparation of Mn-doped ZnS QDs. Ultrapure water (18.2 MΩ cm) was obtained from a WaterPro water purification system (Labconco Corporation, Kansas City, MO). Millipore Microcon YM-30 ultrafilter was used for ultrapurification.

Apparatus. The morphology and microstructure of the QDs were characterized by high-resolution transmission electron microscopy (HRTEM) on a Philips Tecnai G2 F20 microscope (Philips, Holland) operating at a 200 kV accelerating voltage. Circular dichroism (CD) spectra were recorded with a J-715 circular dichroism spectrometer (JASCO, Japan) with a JASCO cell of 0.1 cm. The phosphorescence measurements were performed on an F-4500 spectrofluorometer (Hitachi, Japan) equipped with a plotter unit and a quartz cell (1 cm × 1 cm) in the phosphorescence mode. The slit width was 10 and 20 nm for excitation and emission, respectively. The photomultiplier tube (PMT) voltage was set at 950 V. Absorption spectra were recorded on a UV-3600 UV−vis-NIR spectrophotometer (Shimadzu, Japan).

Synthesis of the Mn-Doped ZnS QDs. MPA capped Mn-doped ZnS QDs was synthesized in aqueous solution based on a previous publication with minor modifications.[32] Briefly, to a three-necked flask, aqueous solutions of $ZnSO_4$ (5 mL, 0.1 M), $Mn(Ac)_2$ (0.2 mL, 0.1 M), and MPA (0.17 mL) were added and the final volume of the mixture was 50 mL. The pH of the mixed solution was adjusted to 11 with 1 M NaOH. After removal of air with argon bubbling for 30 min at room temperature, 5 mL of 0.1 M Na_2S was quickly injected into the solution. The mixture was stirred for 20 min, and then the solution was aged at 50 °C under open air for 2 h to form MPA capped Mn-doped ZnS QDs. For purification, the obtained QDs were precipitated with ethanol, separated by centrifuging, washed with ethanol, and dried in vacuum. The prepared QDs powder is highly soluble in water.

Conjugating GOD with Mn-Doped ZnS QDs. The conjugation of GOD with MPA-capped Mn-doped ZnS QDs was based on the approach for constructing an antigen/antibody immunocomplex with CdTe QDs using the EDC/NHS method via the formation of amide between the carboxyl groups of QDs and the primary amine groups of GOD.[33] GOD was dissolved in phosphate-buffered saline solution (PBS, pH 7.4, 10 mM) to obtain a solution of 5.0 mg mL^{-1} and was stored at 4 °C. For the bioconjugation process, MPA-capped Mn-doped ZnS QDs (5 mg), EDC (2 mg), and NHS (1 mg) were mixed in PBS buffer (pH 7.4, 10 mM, 0.5 mL) and the mixture was incubated at room temperature for 30 min with continuous gentle mixing to activate the QDs. Then, the GOD solution (0.5 mL) was added to the above mixture. The activated QDs and GOD were incubated for another 2 h at room temperature under gentle stirring. This mixture was stored at 4 °C for overnight to allow the unreacted EDC to hydrolyze and lose its activity. After ultrafiltration under centrifugation (12 000 rpm, 10 min) with a YM-30 ultrafilter, the GOD−QD bioconjugate was redissolved with 2 mL of PBS buffer (pH 7.4, 10 mM) for phosphorescent assay.

Assay of the Enzymatic Activity and Thermal Stability. The enzymatic activity of GOD immobilized onto Mn-doped ZnS QDs

(17) Thakar, R.; Chen, Y. C.; Snee, P. T. *Nano Lett.* **2007**, *7*, 3429–3432.
(18) Chung, J. H.; Ah, C. S.; Jang, D. J. *J. Phys. Chem. B* **2001**, *105*, 4128–4132.
(19) He, Y.; Wang, H.-F.; Yan, X.-P. *Anal. Chem.* **2008**, *80*, 3832–3837.
(20) He, Y.; Wang, H.-F.; Yan, X.-P. *Chem.—Eur. J.* **2009**, *15*, 5436–5440.
(21) Wang, H.-F.; He, Y.; Ji, T.-R.; Yan, X.-P. *Anal. Chem.* **2009**, *81*, 1615–1621.
(22) Wang, J. *Chem. Rev.* **2008**, *108*, 814–825.
(23) Zhang, T. Z.; Anslyn, E. V. *Org. Lett.* **2007**, *9*, 1627–1629.
(24) Vamvakaki, V.; Tsagaraki, K.; Chaniotakis, N. *Anal. Chem.* **2006**, *78*, 5538–5542.
(25) Shiang, Y. C.; Huang, C. C.; Chang, H. T. *Chem. Commun.* **2009**, 3437–3439.
(26) Barone, P. W.; Parker, R. S.; Strano, M. S. *Anal. Chem.* **2005**, *77*, 7556–7562.
(27) Pickup, J. C.; Hussain, F.; Evans, N. D.; Rolinski, O. J.; Birch, D. J. S. *Biosens. Bioelectron.* **2005**, *20*, 2555–2565.
(28) Tsai, T. W.; Heckert, G.; Neves, L. F.; Tan, Y. Q.; Kao, D. Y.; Harrison, R. G.; Resasco, D. E.; Schmidtke, D. W. *Anal. Chem.* **2009**, *81*, 7917–7925.
(29) Li, Y.; Liu, X. Y.; Yuan, H. Y.; Xiao, D. *Biosens. Bioelectron.* **2009**, *24*, 3706–3710.
(30) Valencia-González, M. J.; Liu, Y. M.; Díaz-García, M. E.; Sanz-Medel, A. *Anal. Chim. Acta* **1993**, *283*, 439–446.
(31) Papkovsky, D. B.; Olah, J.; Kurochkin, I. N. *Sens. Actuators, B* **1993**, *11*, 525–530.
(32) Zhuang, J. Q.; Zhang, X. D.; Wang, G.; Li, D. M.; Yang, W. S.; Li, T. J. *J. Mater. Chem.* **2003**, *13*, 1853–1857.
(33) Wang, S. P.; Mamedova, N.; Kotov, N. A.; Chen, W.; Studer, J. *Nano Lett.* **2002**, *2*, 817–822.

was evaluated based on a previous publication.[15] Generally, the enzymatic activity was measured based on the change of RTP intensity in the GOD-conjugated Mn-doped ZnS QDs biosensor. Theoretically, the slope of the fitted line to the changed RTP intensity is proportional to the enzymatic activity in a certain amount of enzyme. The enzymatic activity was calculated with a Lineweaver−Burke plot. The thermal stability of GOD immobilized onto Mn-doped ZnS QDs was evaluated as follows: the GOD-conjugated Mn-doped ZnS QDs biosensor solution was incubated at a certain temperature for 15 min in a water bath, then 100 μM glucose was introduced and the mixture was allowed to react for 10 min. The thermal stability of GOD was monitored by the RTP change. For comparison, free GOD was also treated with the same procedure and then subjected to the assay of the thermal stability.

Sample Collection and Pretreatment. Nine human serum samples were donated by a local hospital. All samples were subjected to a 100-fold dilution before analysis and no other pretreatments were necessary.

Measurement Procedures. To a 5 mL calibrated test tube, PBS buffer (0.1 M, 0.50 mL), GOD−Mn-doped ZnS QD bioconjugate (5 mg mL^{-1} as QDs, 25 μL), and serum sample (50 μL) or certain amounts of glucose standard solution were sequentially added. The mixture was diluted to volume with ultrapure water, mixed thoroughly, and incubated in a water bath (35−40 °C) for 10 min. Then, the mixture was taken out from the water bath and allowed to cool to room temperature for 5 min for phosphorescence measurements at an excitation wavelength of 290 nm and an emission wavelength of 595 nm.

RESULTS AND DISCUSSION

Characterization of the GOD−Mn-Doped ZnS QD Bioconjugate. In this study, the conjugation of GOD with MPA-capped Mn-doped ZnS QDs was performed via the aid of coupling reagents, EDC and NHS, followed by purification with an ultrafilter membrane. As shown in Figure 1A, the peak shape of excitation and emission spectra of Mn-doped ZnS QDs remained almost unchanged after conjugation, but the intensity decreased somewhat. Besides, the maximum excitation peak blue-shifted about 5 nm, but the emission peak position remained unchanged. This may be explained by the excitation and emission mechanisms of Mn-doped ZnS QDs (the inset in Figure 1A). The room temperature phosphorescence (RTP) emission of Mn-doped ZnS QDs is the triplet transition of Mn^{2+} ($^4T_1-^6A_1$) incorporated into the ZnS host lattice, with a long lifetime of about 2.5 ms (Figure 1B). The conjugation process might generate new surface defects and thus the maximum excitation shift and emission intensity of QDs decreased after conjugation. While the luminescence center, Mn^{2+}, was still in the lattice of ZnS host, the RTP emission position was unchanged.

The successful conjugation of GOD onto Mn-doped ZnS QDs was also revealed from the TEM and HRTEM images of QDs before and after conjugation. As shown in Figure 2, the prepared Mn-doped ZnS QDs was nearly monodispersed and had an average diameter of 3 nm. However, the QDs were aggregated after conjugation but still showed obvious crystal lattice. The isoelectric point of GOD and the pK_{COOH} of MPA are 4.2 and 4.3, respectively. So both the enzyme and MPA capped Mn-doped ZnS QDs were negatively charged at pH 7.4, and the

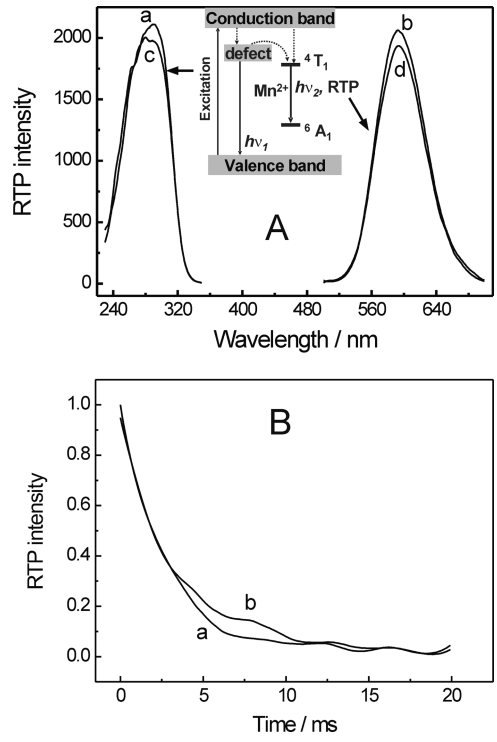


Figure 1. (A) The excitation (curves a, c) and RTP emission (curves b, d) spectra of Mn-doped ZnS QDs (25 μg mL^{-1}; curves a, b), and the GOD-conjugated Mn-doped ZnS QDs (25 μg mL^{-1}; curves c, d). Inset: schematic illustration of electronic transition involved in the RTP emission from Mn-doped ZnS QDs. (B) The decay curve of phosphorescent emission of Mn-doped ZnS QDs (curve a) and GOD-conjugated Mn-doped ZnS QDs (curve b). Solutions were prepared in PBS buffer (10 mM, pH 7.4).

electrostatic interaction between MPA capped Mn-doped ZnS QDs and GOD could be minimal. Possibly, the GOD acted as a bridge between QDs.

Mechanism of the GOD−Mn-Doped ZnS QD Bioconjugate for Glucose Sensing. Enzyme-involved biocatalytic process can be monitored by QDs using fluorescence resonance energy transfer (FRET) processes[34] or electron-transfer quenching.[35] In all of these QDs-based assays for monitoring enzyme activities, it is mandatory to include a quencher (energy or electron-transfer quencher) in the analyzed samples as a reporter unit. H_2O_2 is not only an effective quencher for CdSe@ZnS or CdTe QDs[15,36−40] but also is the product of many enzymatic processes such as GOD, lysine oxidase, and chlorine oxidase. Coupling QDs with these enzymes provides promising biosensing scaffolds for a series of oxidase enzyme substrates without extra quenching units for QDs. In particular, the QDs−GOD hybrid system has been readily employed for glucose sensing.[15,36−40]

(34) Shi, L. F.; De Paoli, V.; Rosenzweig, N.; Rosenzweig, Z. *J. Am. Chem. Soc.* **2006**, *128*, 10378–10379.
(35) Gill, R.; Freeman, R.; Xu, J. P.; Willner, I.; Winograd, S.; Shweky, I.; Banin, U. *J. Am. Chem. Soc.* **2006**, *128*, 15376–15377.
(36) Gill, R.; Bahshi, L.; Freeman, R.; Willner, I. *Angew. Chem., Int. Ed.* **2008**, *47*, 1676–1679.
(37) Bahshi, L.; Freeman, R.; Gill, R.; Willner, I. *Small* **2009**, *5*, 676–680.
(38) Yuan, J. P.; Guo, W. W.; Yin, J. Y.; Wang, E. K. *Talanta* **2009**, *77*, 1858–1863.
(39) Li, X. Y.; Zhou, Y. L.; Zheng, Z. Z.; Yue, X. L.; Dai, Z. F.; Liu, S. Q.; Tang, Z. Y. *Langmuir* **2009**, *25*, 6580–6586.
(40) Yuan, J. P.; Guo, W. W.; Wang, E. K. *Biosens. Bioelectron.* **2008**, *23*, 1567–1571.

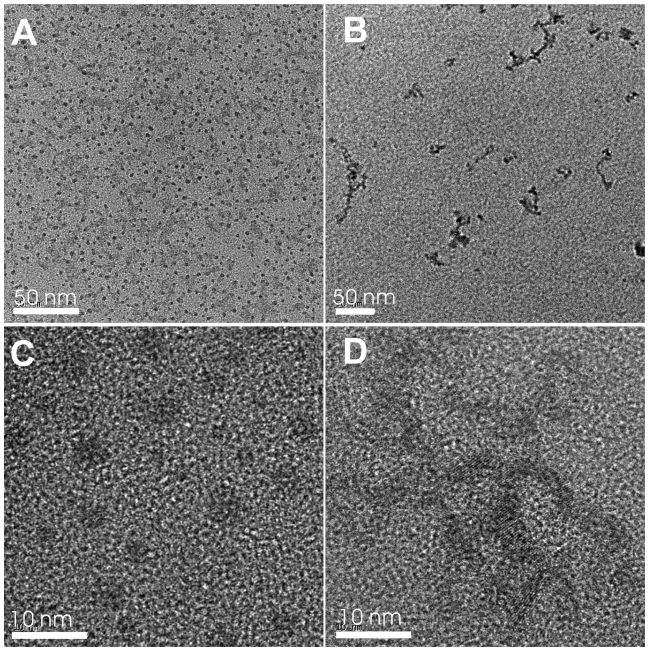


Figure 2. TEM (A, B) and HRTEM (C, D) images of Mn-doped ZnS QDs (A, C) and GOD−Mn-doped ZnS QD bioconjugate (B, D).

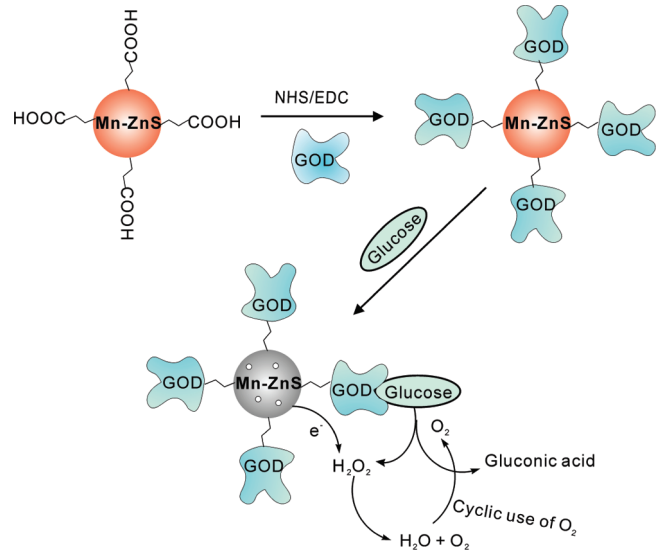
In this work, we found that H_2O_2 could also effectively quench the RTP of Mn-doped ZnS QDs (Figure S1a in the Supporting Information). In principle, the emission of RTP from Mn-doped ZnS QDs has similarities with fluorescence emission by CdSe@ZnS or CdTe QDs, so it is reasonable to infer that the RTP quenching by H_2O_2 toward Mn-doped ZnS QDs should be also through an electron-transfer mechanism:[15] electrons at the conduction band of Mn-doped ZnS QDs can be captured by H_2O_2, and subsequently the radiative recombination of the photoinduced electrons and holes is inhibited. Control experiments showed that glucose itself caused negligible influence on the RTP of Mn-doped ZnS QDs (Figure S1b in the Supporting Information), but H_2O_2 significantly quenched the RTP of GOD−Mn-doped ZnS QD bioconjugate (Figure S1a in the Supporting Information). Therefore, the mechanism of the GOD−Mn-doped ZnS QD bioconjugate for glucose sensing can be ascribed to the H_2O_2 (generated from oxidation of glucose)-induced quenching of the RTP of Mn-doped ZnS QDs. The produced O_2 from the reduction of H_2O_2 by the QDs could reparticipate in the oxidation of glucose because of its easy availability to glucose and GOD in solution (Scheme 1), resulting in the cyclic use of O_2, which is favorable for the biosensor system as it avoided the potential quenching effect of O_2 on the phosphorescence of Mn-doped ZnS QDs.[41]

GOD Enzymatic Activity after Conjugation. In the presence of glucose oxidase, glucose can be catalytically oxidized to produce gluconic acid and H_2O_2:[39]

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

Thus, the enzymatic activity can be evaluated based on the method proposed by Cao et al.:[15] for a given enzymatic activity, the rate

Scheme 1. Schematic Diagram for the Preparation and Application of GOD−Mn-Doped ZnS QD-Bioconjugate for Glucose Sensing



of H_2O_2 production increases with the concentration of glucose. Since H_2O_2 is the quenching unit in this assay, the extent of RTP quenching actually embodies the changes of GOD enzymatic activity. As can be seen from Figure 3A, the RTP change almost leveled off when the glucose concentration increased to 6.0 mM in a fixed time interval of 15 min. Michaelis−Menten kinetics parameters (K_m and V_{max}), which indicated the enzyme−substrate kinetics, were determined by the analysis of the slope of enzymatic reactions. The K_m value for an enzymatic reaction determined the affinity of the enzyme for the substrate, whereas the value of V_{max} provided the

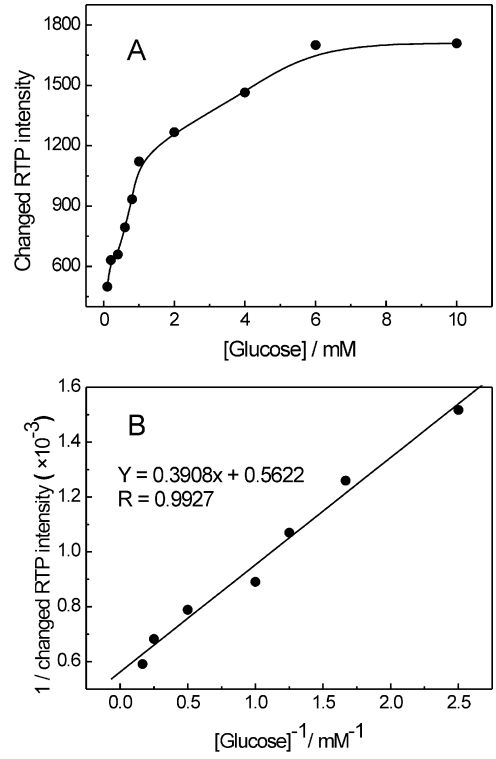


Figure 3. (A) Glucose concentration dependent RTP intensity of the GOD-conjugated Mn-doped ZnS QDs solution in a fixed time of 15 min; (B) Lineweaver−Burke plots of the GOD−glucose reaction.

(41) Sánchez-Barragan, I.; Costa-Fernández, J. M.; Valledor, M.; Campo, J. C.; Sanz-Medel, A. *Trends Anal. Chem.* **2006**, *25*, 958–967.

Table 1. Comparison of the Performance of Immobilized GOD onto Various Nanomaterials for Glucose Sensing

nanomaterials	enzyme immobilization	detection scheme	K_m(mM)	LOD (μM)	refs
AuNPs	covalent conjugation	spectrophotometry	3.74		6
CdTe QDs	covalent conjugation	fluorescence	0.45	0.1	15
ZrO_2/chistosan	physical adsorption	amperometry	3.14	10	7
CNT	covalent conjugation	amperometry		80	5
Fe_3O_4	covalent conjugation	fluorescence	6.8		4
CdTe/CNT	physical adsorption	amperometry	0.651		42
ZnO nanorod	electrostatic interaction	amperometry	2.9	10	9
Mn-doped ZnS QDs	covalent conjugation	phosphorescence	0.70	3	this work

maximum rate of enzyme reaction when the enzyme was saturated by the substrate. These parameters were estimated using the Lineweaver−Burke plot:[6]

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

The Michaelis−Menten constant was determined to be 0.70 mM from the Lineweaver−Burk plot (Figure 3B), indicating that considerably enhanced enzyme activity of GOD after its conjugation with Mn-doped ZnS QDs (5.85 mM for free GOD[6]). For comparison, Table 1 summarizes the key performance of the present glucose biosensor and those of previous biosensors making use of various nanomaterials for immobilization of GOD. It can be seen that Mn-doped ZnS QDs is an attractive support for GOD immobilization since it provides small K_m (comparable to CdTe QDs[15] and CdTe/CNT (carbon nanotube) composite,[42] smaller than CNT,[5] magnetic Fe_3O_4 nanoparticles,[4] ZnO nanorod,[9] and Au nanoparticles (AuNPs)[6]).

The enzymatic activity is known to be directly related with the second and tertiary structures of the enzyme.[43] As shown in Figure 4, the CD spectrum of free GOD showed characteristic bands at $\lambda = 208$ and 220 nm, which confirmed the native conformation of GOD. However, GOD-immobilized Mn-doped ZnS QDs gave new CD peaks at $\lambda = 209$ and 213 nm, demonstrating that the precise enzymatic conformation, including the α helix, β sheet, β turn, and random coil, changed greatly in comparison with free GOD (Table S1 in the Supporting Information). So, the improved enzymatic activity can be attributed to the enzyme conformation change after immobilization, the high GOD loading owing to the large surface area of Mn-doped ZnS QDs and subsequently more availability and high affinity of GOD to glucose, the high catalytic effect of Mn-doped ZnS QDs, and better matrix of Mn-doped ZnS QDs for GOD immobilization.

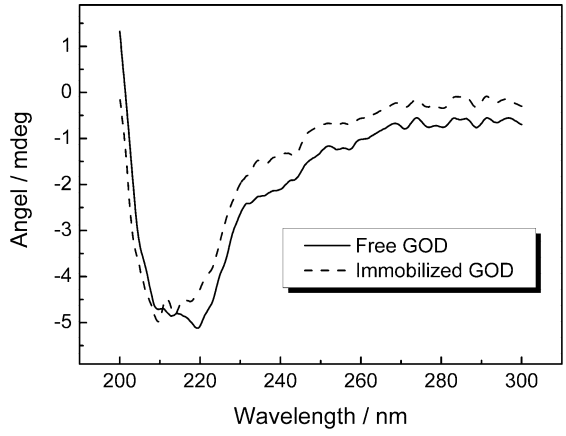


Figure 4. CD spectra of free GOD and GOD immobilized onto Mn-doped ZnS QDs. Samples were dissolved in PBS (10 mM, pH 7.4), and the initial concentration of GOD was 0.17 mg mL^{-1}.

Factors Affecting the RTP Sensing of Glucose Based on the GOD−Mn-Doped ZnS QD Bioconjugate. To achieve sensitive detection of glucose, several enzymatic factors that may influence the enzyme−substrate interactions were studied. To further verify the effect of enzyme immobilization by Mn-doped ZnS QDs, we optimized both GOD−Mn-doped ZnS QD bioconjugate and the GOD and Mn-doped ZnS QDs mixture (without conjugation) for glucose detection. Increasing the GOD concentration enhanced the quenching effects of 0.1 mM glucose on the RTP of both the GOD−Mn-doped ZnS QD bioconjugate and the mixture of GOD and Mn-doped ZnS QDs (Figure 5A), but the GOD−Mn-doped ZnS QD bioconjugate required less GOD to reach the maximum quenching. Besides, at low GOD concentrations (≤20 mg L^{-1}), GOD after conjugation was more effective for catalyzing glucose oxidation than free GOD, indicating the significance of the conjugation of GOD and Mn-doped ZnS QDs. For the glucose biosensing application, the initial GOD concentration of 15 mg L^{-1} was selected for preparing the GOD−Mn-doped ZnS QD bioconjugate.

The thermal stability of GOD before and after conjugation onto Mn-doped ZnS QDs was investigated in the range of 20−80 °C. As shown in Figure 5B, the GOD−Mn-doped ZnS QD bioconjugate gave a wider optimal temperature range (35−55 °C) in comparison with the mixture of GOD and Mn-doped ZnS QDs. The maximum activity of GOD was obtained in the range of 35−55 °C for the bioconjugate and 30−40 °C for free GOD. A sharp activity reduction occurred at temperatures above 55 °C, but the bioconjugate retained relatively higher enzymatic activity in the range of 55−65 °C than the free GOD. Enzymes tended to be denatured at high temperatures, but the immobilization of enzymes onto Mn-doped ZnS QDs prevented enzyme unfolding and turbulence to some extent due to the large surface area of QDs and thus enhanced the enzyme stability. In biosensor applications, we chose a water bath temperature of 40 °C for glucose sensing.

The pH dependence of glucose-induced RTP quenching was investigated in the pH range of 4.5−9.1. As shown in Figure 5C, free GOD worked best in the pH range of 6.2−7.8. Conjugation of GOD onto Mn-doped ZnS QDs gave the optimal pH range of

(42) Liu, Q.; Lu, X. B.; Li, J.; Yao, X.; Li, J. H. *Biosens. Bioelectron.* **2007**, *22*, 3203–3209.
(43) Pollegioni, L.; Wels, G.; Pilone, M. S.; Ghisla, S. *Eur. J. Biochem.* **1999**, *264*, 140–151.

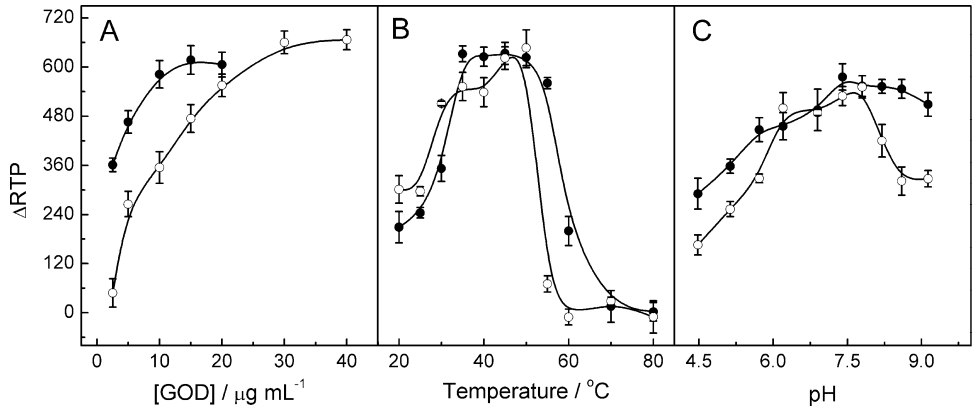


Figure 5. Effects of GOD concentration (A), temperature (B), and pH (C) on the quenched RTP intensity of the GOD−Mn-doped ZnS QD bioconjugate (●) and the mixture of GOD and Mn-doped ZnS QDs (○) in the presence of 100 μM glucose.

7.4−8.6. In the following investigations, 0.01 M PBS buffer (pH 7.4) was selected as the biosensor media.

Analytical Performance of the GOD−Mn-Doped ZnS QD Bioconjugate for RTP Sensing of Glucose. Under the optimal conditions, the RTP intensity of the GOD−Mn-doped ZnS QD bioconjugate gradually decreased as the concentration of glucose increased (Figure 6A). Statistical analysis of the quenched RTP intensity versus the glucose concentration revealed two linear ranges for glucose sensing (Figure 6B, inset). The quenched RTP intensity (ΔRTP) linearly increased with glucose concentration from 10 to 100 μM with a calibration function of $\Delta RTP = 5.64C_{glucose} + 27.4$ ($R = 0.996$) and from 100 μM to 1 mM with a calibration function of $\Delta RTP = 0.97C_{glucose} + 488$ ($R = 0.997$).

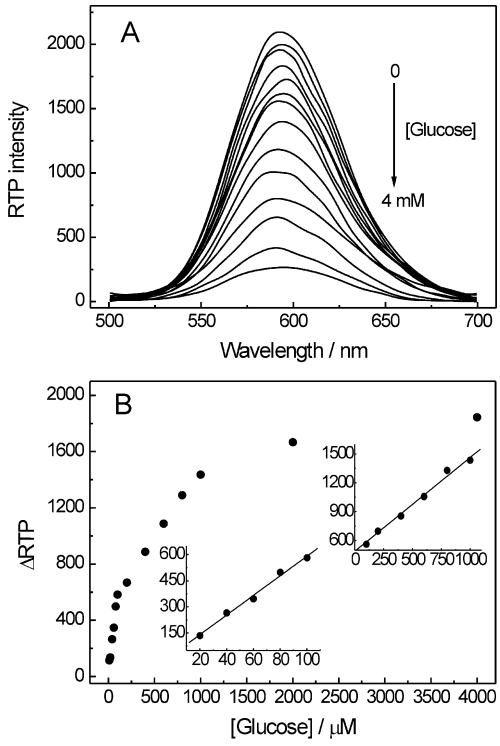


Figure 6. (A) Effect of the concentration of glucose on the phosphorescence spectra of THE GOD−Mn-doped ZnS QD biosensor; (B) plots of the quenched RTP intensity as a function of glucose concentration, showing two linear ranges. Buffer, 0.01 M PBS (pH 7.4); GOD−Mn-doped ZnS QD bioconjugate, 25 mg L^{-1}; temperature, 35 °C.

Similar two linear ranges were also reported in a previous work for glucose sensing with CdTe QDs.[40] This phenomenon probably resulted from the integration of the enzymatic reaction and the H_2O_2 induced quenching course as the data were recorded at a certain time of the enzymatic reaction course. However, the exact reasons are still not clear at the present stage and need further investigation. The limit of detection (LOD) (3*s*) of the present RTP biosensor for glucose was 3 μM, while the precision for 11 replicate detections of 100 μM glucose was 3.2% (relative standard deviation, RSD). As shown in Table 1, the LOD of the proposed RTP biosensor is better than those obtained by the amperometric biosensors based on the GOD−ZnO nanorod, ZrO_2/chistosan, and carbon nanotube conjugates but worse than that of the GOD−CdTe QD conjugate based fluorescence biosensor.

To assess the selectivity of the developed GOD-conjugated Mn-doped ZnS biosensor for glucose detection, we studied the effect of main relevant metal ions and other molecules in biological fluids on the determination of glucose (100 μM). An error of ±10.0% in the relative RTP intensity was considered tolerable (Table S2 in the Supporting Information). Because of the high specificity of GOD, several glucose analogues, including maltose, sucrose, fructose, mannose, xylose, and galactose, exhibited little influence on glucose detection even at concentrations 100 times higher than that of glucose. Typical amino acids showed minimal interference on glucose determination at millimolar levels except L-cysteine and glutathione. Considering the average concentrations of these biothiols (10×−100× μM in plasma)[44] and the natural levels of glucose in human blood serum (4.4 to 6.6 mM),[29] such interferences may be ignored. A concentration of 20 mg L^{-1} of human serum albumin (HSA), 25 μM of ascorbic acid and citrate, and 1000 μM of oxalic acid caused less than 10% interference with the detection of glucose. The 100 μM glucose-induced RTP quenching of GOD-conjugated Mn-doped ZnS QDs was not affected by a 1000-fold excess of K^+, 4000-fold excess of Na^+, 5-fold of Ca^{2+}, and 7.5-fold of Mg^{2+}. Physiologically relevant transition metal ions caused severe interferences for glucose analysis. However, their average levels are typically far lower than that of glucose. Besides, the normal glucose concentration in vivo is at the millimolar level (4.4−6.6 mM),[29] while the working range of the proposed method is in the micromolar

(44) Zhang, Y.; Li, Y.; Yan, X.-P. *Anal. Chem.* **2009**, *81*, 5001–5007.

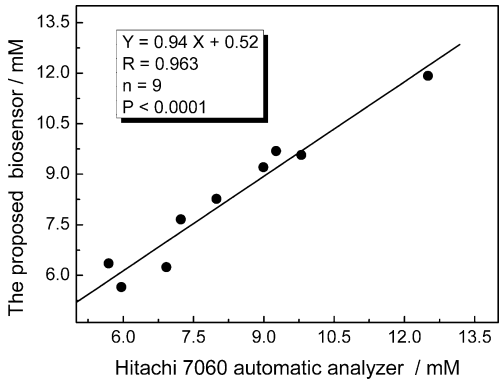


Figure 7. Comparison of the analytical results of serum samples determined with the proposed biosensor and a commercial blood glucose analyzer.

range. Thus, the designed biosensor can work with small amounts of serum samples, and most potential interferences can be largely eliminated by simple dilution.

Determination of Glucose in Serum Samples. The developed biosensor was applied to the determination of glucose in nine serum samples. No other pretreatments for the serum samples except dilution were employed for the determination of glucose by the developed biosensor. As shown in Figure S2 in the Supporting Information, no RTP background from serum was observed although the fluorescence background was significant. Thus, complicated pretreatments such as deproteinization and centrifugation typically required for the analysis of biological fluids were avoided. After a 100-fold dilution, the addition of serum samples caused negligible changes in the RTP intensity of Mn-doped ZnS QDs (Figure S3 in the Supporting Information, trace a cf. trace b) but obviously quenched the RTP of GOD-conjugated Mn-doped ZnS QDs (Figure S3 in the Supporting Information, trace c cf. traces d−f). These results demonstrated that the diluted serum matrix did not interfere with the RTP of sole Mn-doped ZnS QDs, and the RTP quenching of GOD-conjugated Mn-doped ZnS QDs should be ascribed to the glucose in the serum samples.

The analytical results for the nine serum samples determined by the developed method were compared with those determined by a Hitachi 7060 automatic analyzer. As shown in Figure 7, there is a good agreement between the results obtained by two independent methods.

CONCLUSIONS

In summary, we have assembled a new GOD−Mn-doped ZnS QD bioconjugate as a RTP biosensor using EDC/NHS chemistry for detecting glucose. The immobilized GOD resulted in enhanced enzymatic activity and better thermal stability in comparison with free GOD. The developed RTP biosensor favored biological applications since the interference from autofluorescence and scattering light was greatly eliminated. The developed sensor displays relative high sensitivity and good selectivity, but it is difficult to be re-engineered, and the interaction of H_2O_2 with QDs requires a relatively long time (15 min). The phosphorescent Mn-doped ZnS QDs can also be employed as a robust support for other redox enzymes to develop bisosensors for other biomolecues such as lysine or choline. Works on this direction is in progress in our laboratory.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (Grants 20935001 and 20977049) and the National Basic Research Program of China (Grant 2006CB705703).

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review November 5, 2009. Accepted December 31, 2009.

AC902531G