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Paper

Glucose Oxidase-Directed, Instant Synthesis of Mn-doped ZnS Quantum Dots in Neutral Media with Retained Enzymatic Activity: Mechanistic Study and Biosensing Application

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Protein-direct synthesis of quantum dots (QDs) is a "greener" alternative to the current high-temperature and aqueous synthetic protocols, which provide water-soluble, biocompatible protein-functionalized QDs in one-pot. However, the protein activity in such synthetic schemes is a critical issue, since the synthetic conditions (for instance, high pH of the precursors, long time of synthesis, and disruption of disulfide bonds) are not suitable for its retainment (especially for enzymes). Herein, we presented a facile and instant glucose oxidase (GOD)-directed strategy for the preparation of highly luminescent phosphorescent Mn-doped ZnS (Mn-ZnS) QDs in one-step at room temperature and neutral aqueous media. With such mild synthetic condition, the enzymatic activity of GOD was totally retained. Further, we also carried out GOD-direct synthesis of QDs with several other conditions that reported in the literature. It turned out that the GOD enzymatic activity with these synthetic conditions was lower than that of the proposed protocol, indicating that the mild synthetic condition is the prerequisite for retaining the enzymatic activity. Importantly, the as-prepared GOD-mediated Mn-ZnS QDs exhibited high photostability, high salt tolerance and colloidal stability, which can be stored for months under 4 °C or 25 °C without changing their phosphorescent intensity and enzymatic activity. Via selective chemical modification, the exact functional groups (amino acid residues) of GOD in directing the synthesis of Mn-ZnS QDs were studied in detail. It turned out to be imidazole in histidine residues but not thiol in cysteine residues that directed the formation of Mn-ZnS QDs, and this was further confirmed with several other proteins for synthesis of Mn-ZnS QDs. The as-prepared GOD-capped Mn-ZnS QDs were employed as a phosphorescent probe for background-free sensing of glucose in serum samples.

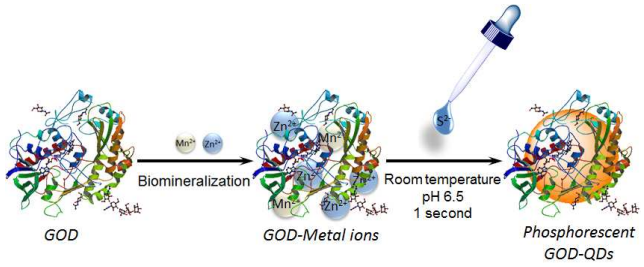
Introduction

Quantum dots (QDs) are superior luminophores with increased brightness, photostability, and large Stokes shifts over conventional organic dyes, which make them appealing candidates for various applications in chemo-/bio-sensing and bioimaging.[1] Currently, the state-of-the-art synthetic strategies of high-quality QDs are majorly high-temperature organic routes (usually performed in the temperature range of 250-350 °C in organic solvents using fairly toxic and expensive compounds) and small thiols-assisted aqueous methods. For biological applications, organically synthesized QDs must be first made hydrophilic through ligand exchange, silica coating, or polymer encapsulation.[2] Then, the water-soluble QDs are further bioconjugated with enzymes, antibodies or other targeting biomolecules to endow them with specific recognition functionality and biocompatibility.[3] From the synthetic point of view, the fabrication of bioconjugated QDs is a laborious multistep process, the synthetic complexity of which would probably prohibit the end-users from gaining direct access to customizable QD probes in an easy and efficient manner.

Proteins have a key role in controlling the formation of crystalline materials *in vivo*, and several specific bio-recognition processes are involved in crystal nucleation, growth regulation and growth arrest. Learning from the nature, exploration of proteins for the direct nanocrystal synthesis via biomineralization is therefore proposed, which provide a "greener" alternative to synthetic chemistry for obtaining various protein-functionalized QDs in one-pot.[4] When tracing the pace of protein-mediated synthesis, one can see that the synthetic strategies are mostly referenced from the conventional thiol-assisted aqueous methods. In other words, the role of proteins is similar to that of thiols, which direct the growth of QDs as capping ligands. When preparing the synthetic precursors, sometimes proteins are first treated with $NaBH_4$ to release thiol groups from disulfide bonds,[5] and then strong basic media (typically pH > 9) are often chosen to guarantee sufficient coordination of proteins with metal ions.[6] Without these stringent conditions, large sized nanocrystals (larger than the Bohr radius) would be obtained and accordingly,

losing the quantum confinement of QDs.[7] Although protein-conjugated QDs are successfully synthesized via the above mentioned strategies, the protein activity is often lost or at least largely reduced due to the unmatched synthetic conditions and those required for protein activity retainment. This is in fact against the initial desire of protein-mediated synthesis to obtain protein-functionalized QDs with both features of QDs and proteins. It should be noted that there have been several reports of protein-directed synthesis of QDs with retained protein activity, but the required synthesis time was relatively long (up to several days).[8] Ma *et al*. presented a new general facile strategy for preparation of protein-functionalized QDs at ambient conditions, but the process needed auxiliary thiols and high pH in some cases.[9]

Enzymes play prominent roles in biological metabolism systems owing to their high substrate-specificity and high catalytic efficiency. Therefore, coupling the excellent substrate selectivity of enzymes with the superior optical properties of QDs is one of the common strategies for biosensors fabrication. However, enzymes also prone to be denatured when working in non-physiological conditions and lose their enzymatic activity. Using enzymes to direct the growth of QDs is not as popular as those of other proteins,[4a, 10] probably because of the considerations on possible enzymatic activity loss during synthesis. To obtain enzyme-functionalized QDs in one-pot with enzyme-directed synthesis, mild synthetic conditions that permit the formation of high-quality QDs and retaminment of enzymatic activity are required.



Scheme 1. Schematic illustration of one-step, glucose oxidase-directed instant synthesis of phosphorescent Mn-doped ZnS QDs.

Therefore, in this work, we studied the enzyme-guided growth of phosphorescent Mn-doped ZnS (Mn-ZnS) QDs[11] with glucose oxidase (GOD) as a model enzyme (Scheme 1). Phosphorescent Mn-doped ZnS QDs have been a unique model for biosensing applications ranging from metal ions,[12] small molecules,[13] and biomacromolecules.[14] GOD is one of the most frequently used enzymes in enzymatic biofuel cells and glucose biosensors, in part due to its stability under physiological conditions (37 °C and neutral pH). We found that highly luminescent Mn-ZnS QDs can be readily obtained in one second and in neutral aqueous media at room temperature (25 °C), which is uncommon for aqueous synthesis of QDs. Most importantly, the activity of GOD was not decreased but even increased somewhat after the synthesis. The role of various amino acids in guiding the growth of Mn-ZnS QDs was studied in detail. The as-prepared GOD-capped Mn-ZnS QDs exhibited high photostability, high salt tolerance and colloidal stability, which could be stored for months under the 4 °C without changing the phosphorescence intensity of QDs and the enzymatic activity of GOD. Taking the advantage of phosphorescence detection and H_2O_2 (produced from GOD enzymatic reaction)-induced phosphorescence quenching,[15] the as-prepared GOD-capped Mn-ZnS QDs were explored as a biosensor for glucose detection in biological samples.

Results and discussion

Characterization of GOD-capped Mn-doped ZnS QDs

GOD-directed, instant synthesis of Mn-ZnS QDs was based on first biomineralization of GOD with Zn^{2+} and Mn^{2+}, and then crystallized with S^{2-} in pH 6.5 Tris-HCl media at room temperature in just one second (Scheme 1, see ESI† for details). To confirm the successful synthesis, Mn-ZnS QDs biofabricated with 2.5% Mn^{2+} (molar ration, $Mn^{2+}/Mn^{2+} + Zn^{2+}$, Fig. S1 and Fig. S2, ESI†) were selected for detailed characterization. The absorption edges of doped and undoped ZnS QDs were located at

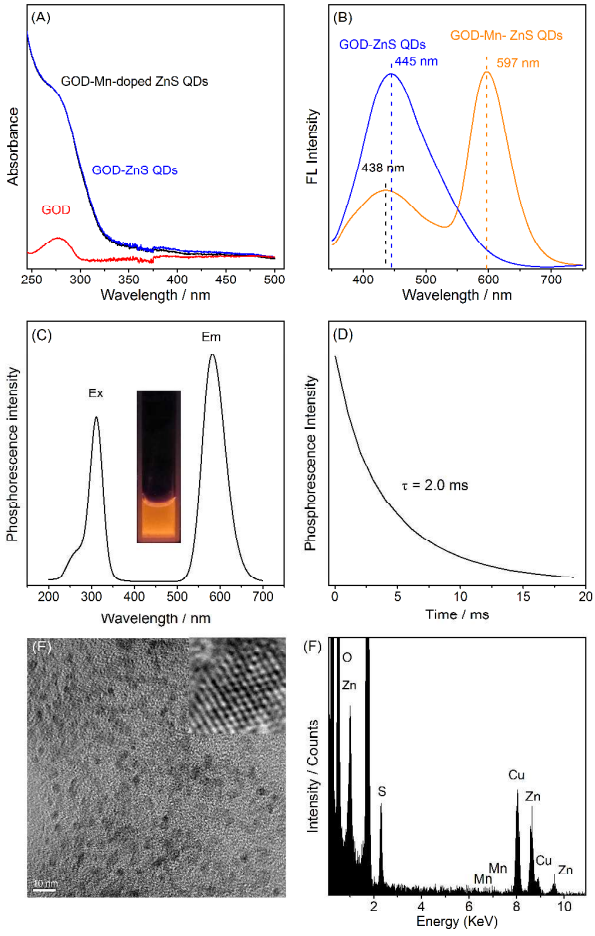


Fig. 1 Characterization of the as-prepared Mn-doped ZnS QDs with with 2.5% Mn^{2+}: (A) UV-vis absorption spectra of GOD, GOD-ZnS QDs, and GOD-Mn-ZnS QDs; (B) fluorescence emission spectra (Ex: 310 nm) of GOD-ZnS and GOD-Mn-ZnS QDs; (C) phosphorescent excitation and emission spectra of GOD-Mn-ZnS QDs; (D) phosphorescent decay curve of GOD-Mn-ZnS QDs (Ex: 310 nm); (E) TEM image of the Mn-doped ZnS QDs, with high-magnification TEM image shown in inset; and (F) the EDX of the Mn-doped ZnS QDs.

330 nm (Fig. 1A), which were similar to our previously reported BSA- and cytochrome C-capped Mn-ZnS QDs[5a, 6d] and also other previous reports.[5b, 8a] Besides, the characteristic absorption of GOD protein at about 260 nm was also retained (Fig. 1A and Fig. S3 ESI†). Emission spectra revealed that Mn doping resulted in blue-shifting of the defect-related emission of ZnS from 445 to 438 nm. Meanwhile, the intensity of such defect emission was significantly quenched upon Mn doping to the benefit of a new emission peak centered at 597 nm (Fig. 1B). This is fully consistent with doping of Mn^{2+} into the ZnS lattice and energy transfer from the conduction band of ZnS to the Mn^{2+} $^4T_1 \rightarrow {}^6A_1$ transition.[11a-e] The maximum emission wavelength showed about 10 nm-red shift as compared to our previous publications,[5a, 5d, 6d] probably because of different lattice strain of the QDs.[16] Because of the triplet nature, the Mn^{2+} dopant emission can be observed in phosphorescent detection mode (Fig. 1C) with a decay time of about 2.0 ms (Fig. 1D), whereas the defect emission could not. These spectroscopic evidences indicated successful synthesis of phosphorescent Mn-ZnS QDs.

The shape and sizes of the resultant Mn-ZnS QDs were characterized by high-resolution transmission electron microscopy (HRTEM). Nearly spherical and monodispersed Mn-doped ZnS QDs were seen from the TEM images (Fig. 1E and Fig. S5 in ESI†), with an average diameter of about 2.7 ± 0.2 nm. Clear lattice fringes of Mn-ZnS QDs were seen from the HRTEM image. Energy dispersive X-ray analysis (EDX) confirmed that Zn, Mn, and S were all present (Fig. 1F, in which Cu is from the copper grids). The distinct fringe spacing and the corresponding selected-area electron diffraction (SAED) pattern (Fig. S5, ESI†) reveal the good crystallinity of the as-prepared Mn-ZnS QDs.

Enzymatic activity of GOD in GOD-capped Mn-doped ZnS QDs

The enzymatic activity is a critical issue in enzyme-directed synthesis of QDs. To investigate the enzymatic activity change of GOD during synthesis, the enzymatic reaction processes were studied and regulated with Michaelis-Menten equation. Principally, GOD can catalyze the oxidation of glucose by dissolved oxygen to yield H_2O_2, horseradish peroxidase (HRP) can then catalyze the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by H_2O_2 and produce colored oxidized TMB. Therefore, the enzymatic activity of GOD can be measured using TMB as a probe via monitoring the absorbance of oxidized TMB (@652 nm) with UV-vis spectroscopy (Fig. 2A and Fig. S6 in ESI†) and derivated with the Lineweaver-Bruke plot (Fig. 2B-2E):[17]

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

$$V_{max} = k_{cat}[E]_0$$

Here, K_m is the Michaelis-Menton constant describing the affinity of the enzyme for the substrate; V_{max} is the maxim velocity of the enzymatic reaction; k_{cat} is the turnover number that showing the maximum number of substrate molecules (glucose) converted to product (H_2O_2) per enzyme molecule per second; and $[E]_0$ is the concentration of GOD. The constant k_{cat}/K_m is a measure of how efficiently an enzyme (GOD) converts a substrate (glucose) into its product (H_2O_2).

To illustrate the advantage of this method in retaining enzymatic activity, we also performed the GOD-directed synthesis of Mn-ZnS QDs with several other conditions reported in the literature, including 37 °C, 4 days;[8a] 50 °C, 3 h;[5b, 5d] 60 °C, 3 h; 70 °C, 3 h.[5a] Also, several conditions that typically employed in conventional thiols-assisted aqueous methods were included for better comparison, namely pH from 6 to 11 (25 °C, instant). For all these synthetic conditions, the concentrations of precursors were set at the same. The GOD enzymatic activity after these synthetic conditions was evaluated with the above mentioned method (Table S1, Fig. S7-S16, ESI†).

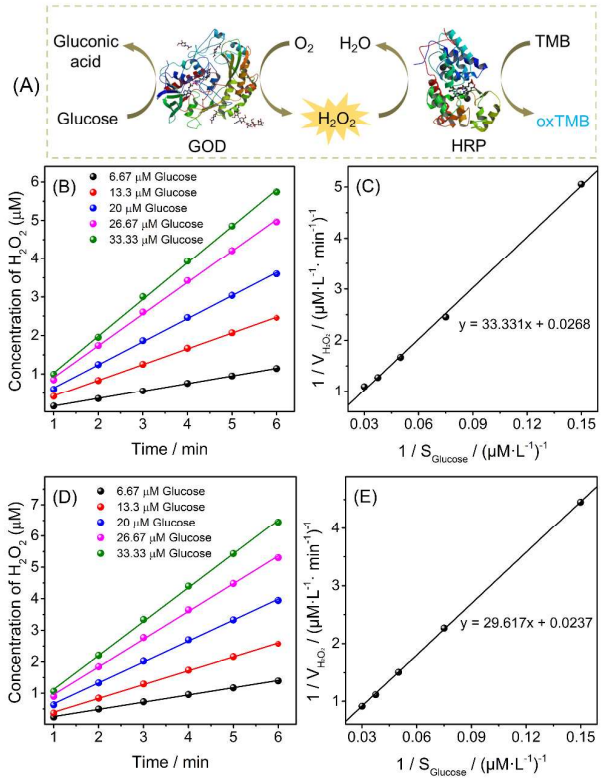


Fig. 2 Evaluation of the enzymatic activity of free GOD and GOD after directing the synthesis of QDs: (A) schematic illustration of the method principle for measuring the enzymatic activity; (B) the generation velocity of H_2O_2 in the presence of different amounts of glucose catalyzed by free GOD; (C) the corresponding Lineweaver-Burk plot of free GOD; (D) the generation velocity of H_2O_2 in the presence of different amounts of glucose catalyzed by free GOD after directing the synthesis of Mn-ZnS QDs (instant, pH 6.5, 25 °C); and (E) the corresponding Lineweaver-Burk plot of GOD in GOD-capped Mn-ZnS QDs. The experimental conditions were given in the "Enzymatic activity assay" Section of the ESI†.

As shown in Fig. 3, the k_{cat}/K_m values of free GOD and GOD after directing the synthesis of Mn-ZnS QDs in this work were determined to be 1.97×10^4 and 2.28×10^4 1/M·s, respectively, indicating that the GOD enzymatic activity was not reduced but rather increased somewhat after directing the synthesis of Mn-ZnS QDs in one second and in neutral aqueous media at room temperature. Besides, such retained activity was not lost or reduced even after one-month of storage at 4 °C or 25 °C that typically used for storing of GOD and QDs, respectively (Fig. S17, ESI†). For other synthetic conditions, the enzymatic activity

was varied, but was always lower than that of GOD in the current synthetic conditions (25 °C, instant). From Fig. 3, it is also clear that the prerequisite for retainment of enzymatic activity is the match of synthetic conditions with the normal working conditions of enzymes.

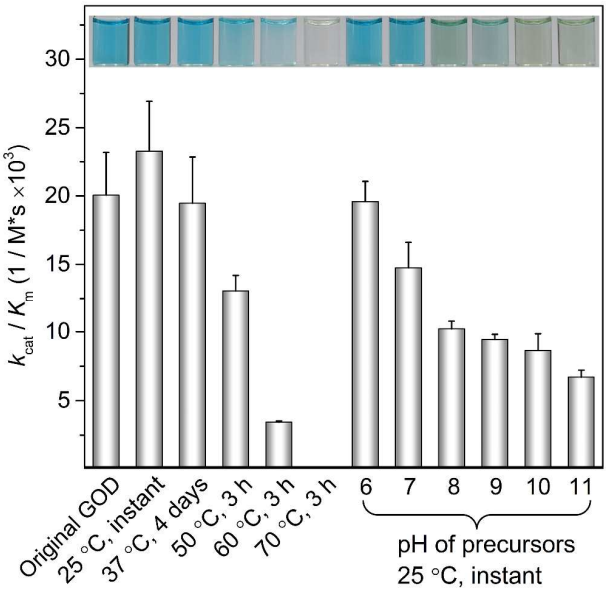


Fig. 3 k_{cat}/K_m of free GOD and GOD after directing the synthesis of QDs. The inset is the photographs of the solution concluding HPR, TMB, glucose and the corresponding kinds of GOD. The experimental conditions were given in the "Enzymatic activity assay" Section of the ESI†.

The enzymatic activity is known to be directly related with the second and tertiary structures of the enzyme. Therefore, the conformational information of GOD was investigated with circular dichroism (CD) and FT-IR spectroscopy. CD is observed when molecules absorb left and right circularly polarized light differently. As shown in Fig. 4, the CD spectra of free GOD showed characteristic protein bands at λ = 210 and 219 nm. Upon biomineralization with Zn^{2+} and Mn^{2+}, the CD profile of GOD was largely retained. After directing the synthesis of Mn-ZnS QDs with the current method (pH 6.5, instant, room temperature) and that of pH 6.5, 37 °C, 4 days, slight changes were observed in the CD profiles of GOD demonstrating that the precise enzymatic conformation, including α helix, β sheet, β turn, polypro II helix and random coil, changed a little in comparison with free GOD (Table S2, ESI†), which may result in slightly increased enzymatic activity. However, after directing the synthesis at 50 °C or pH 11, the conformation of GOD underwent significant changes, leading to largely reduced enzymatic activity. The experimental results of FT-IR were generally in accordance with those CD spectra (Fig. S18, ESI†).

After directing the synthesis of Mn-ZnS QDs, the optimal enzymatic working conditions of GOD in Mn-ZnS QDs and free GOD are generally the same. As shown in Fig. 5, the catalytic activity of both types of GOD is pH- and temperature-dependent. The optimal pH of both is approximately pH 5. For temperature, free GOD worked best at about 40 °C, while GOD in Mn-ZnS QDs was 35 °C. Besides, the thermal stability of free GOD was slightly better than that of GOD in Mn-ZnS QDs, probably because of minimal structure change as revealed by CD spectroscopy.

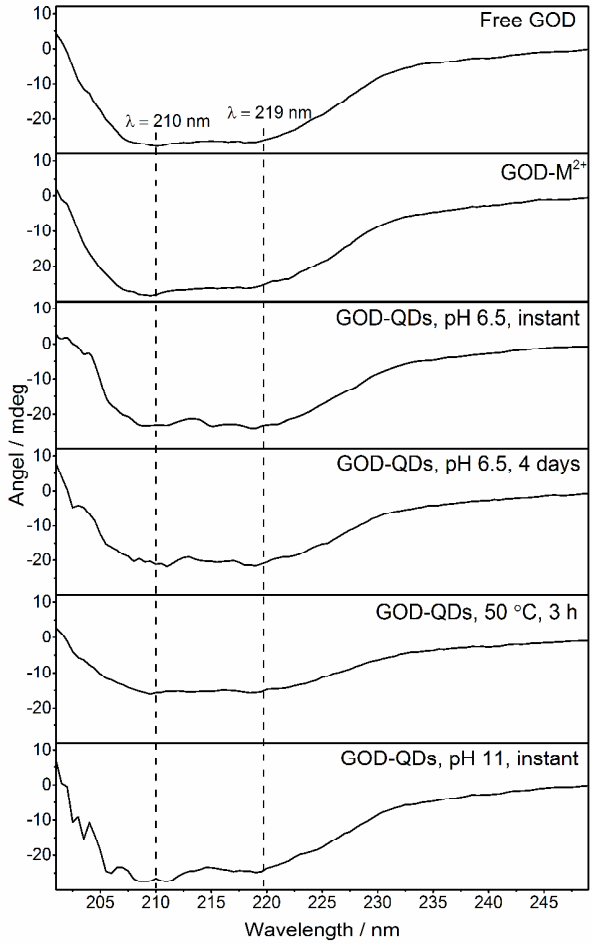


Fig. 4 CD spectra of free GOD; GOD-Zn^{2+}/Mn^{2+} complex; GOD-QDs obtained with the current instant approach at pH 6.5; GOD-QDs obtained at pH 6.5, 37 °C, 4 days; GOD-QDs obtained at pH 6.5, 50 °C, 3 h; and GOD-QDs obtained at pH 11, 25 °C, instant. Samples were dissolved in Tris-HCl (10 mM, pH 6.5). Evaluation about the change of α helix, β sheet, β turn, polypro II helix and random coil was given in Table S2.

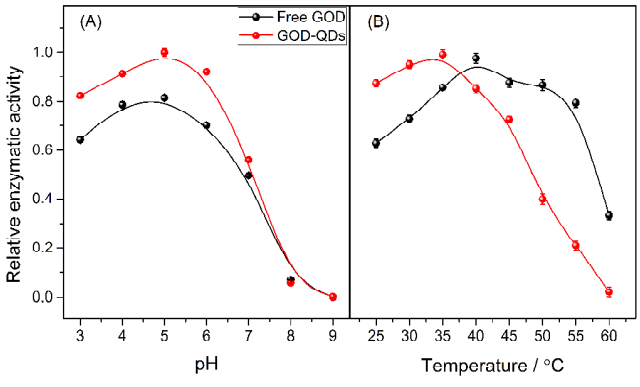


Fig. 5 Comparison of the optimal working conditions of free GOD and GOD in Mn-ZnS QDs: (A) pH and (B) temperature. The concentrations of GOD, GOD-capped Mn-ZnS QDs, HRP, and TMB were the same as those in Fig. 2, and the glucose concentration was 26.7 μM.

Besides enzymatic activity, the synthetic conditions also had great influence on the optical properties of Mn-ZnS QDs. As shown in Fig. 6, increasing the pH of synthetic precursor media of the proposed instant approach, the phosphorescence intensity of Mn-ZnS QDs underwent stepwise decreased, indicating the interaction of GOD with Mn-ZnS QDs is different from that of thiols in conventional thiols-assisted aqueous methods. For the synthetic conditions reported in the literature with temperature higher than 37 °C, although the phosphorescence intensity is similar to the that of the proposed instant approach, the as-obtained solution of QDs possessed appreciable amounts of precipitations and thus not suitable for further biological applications (Fig. 6A and 6B). Besides, at high synthetic temperature (60 and 70 °C), the defect-related emission of ZnS host increased significantly (Fig. S19), resulting in green-yellow fluorescent color of the solution. Therefore, the proposed GOD-directed synthetic approach permits both high quality of Mn-ZnS QDs and well-retained GOD enzymatic activity.

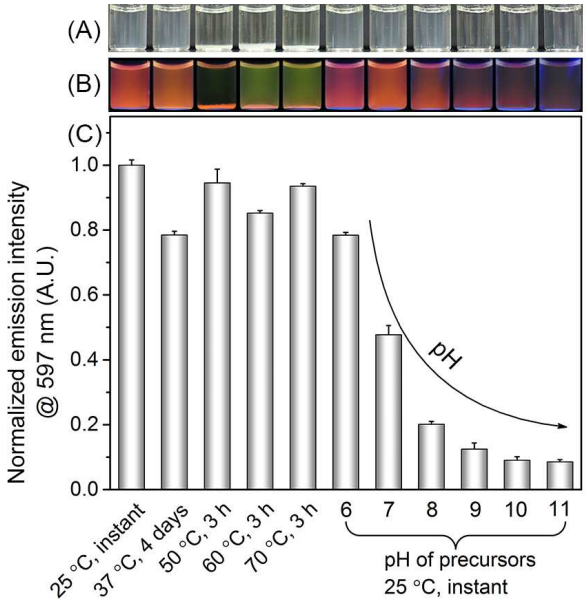


Fig. 6 Normalized phosphorescence intensity and photographs (the inset) of GOD-mediated Mn-doped ZnS QDs under various synthetic conditions. The experimental conditions were the same as those in Fig. 2.

Stability of GOD-capped Mn-doped ZnS QDs

The stability of the proposed GOD-capped Mn-ZnS QDs was evaluated against salinity, pH, UV radiation, and storage time. As shown in Fig. S20, when challenging with high salinity of 1 M NaCl, the phosphorescence intensity of Mn-ZnS QDs still remain almost unchanged, while conventional thiol-capped Mn-ZnS QDs can only stand up to 50 mM NaCl.[5a] Investigation of pH stability indicated that GOD-capped Mn-ZnS QDs were stable in a broad pH range (3-11, Fig. S20, ESI†), even better than other protein-capped Mn-ZnS QDs.[5a, 8a] After stored at 4 °C for one month, the emission intensity of Mn-ZnS QDs also remained stable, along with unchanged GOD enzymatic activity.

The photostability of the QDs was also reasonably good. Upon continuously irradiated with 310 nm UV light for 1 h, slight increase of phosphorescence intensity of Mn-ZnS QDs were observed (Fig. S21, ESI†), possibly because of annihilation of surface defects during UV irradiation.[18] Overall, from the above evidences, it can be concluded that the stability of the proposed GOD-capped Mn-ZnS QDs is excellent.

Investigation on the role of GOD in one-step instant synthesis of Mn-doped ZnS QDs

To deconvolute the role of GOD in the one-step instant synthesis of Mn-ZnS QDs, we first studied the effect of the amounts of GOD on the optical performance of Mn-ZnS QDs. In this QDs synthesis regime, the GOD molecule was used as the only ligand to generate protein-functionalized QDs. The cation precursors (Zn^{2+} and Mn^{2+}) were first biomineralized with GOD to form the protein-metal complexes in about 5 min (Fig. S22, EIS†), during which time the cations could coordinate with a variety of amino acid residues (e.g., imidazole, hydroxyl groups, carboxyl groups, amine groups, and thiol groups).[9, 19] Especially Zn^{2+}, which is well-known for its role in "zinc-finger motifs", can coordinate with imidazole ring in His.[20] Subsequent addition of Na_2S triggered the formation of Mn-ZnS QDs in place of these previous complexes (Scheme 1). As shown in Fig. 7, the critical concentration for obtaining a transparent QDs solution was 0.6 mg/mL. Below such a critical concentration, precipitation of QDs was obtained, indicating inefficient ligands for solubilization of QDs. Besides, the phosphorescence intensity increased almost linearly with the amount of GOD.

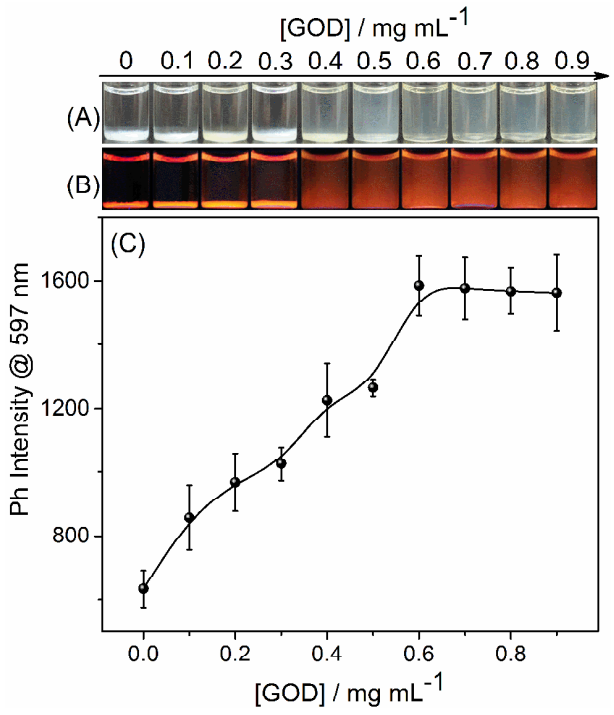


Fig. 7 Effect of the GOD amount on the optical performance of Mn-ZnS QDs: (A) photographs of Mn-ZnS QDs mediated with increasing concentrations of GOD; (B) the UV-excited fluorescent photographs (λ_{ex} = 302 nm) corresponding to (A); and (C) phosphorescence intensity of the resultant Mn-ZnS QDs (@ 597 nm, λ_{ex} = 310 nm). The concentrations of Zn^{2+}, Mn^{2+}, and S^{2-} were given in the "Experimental" Section of the ESI†.

To confirm that all GOD was functionalized onto the surface of Mn-ZnS QDs at the GOD critical concentration of 0.6 mg/mL, polyacrylamide gel electrophoresis (PAGE) was employed to monitor the protein anchoring. As shown in Fig. S23, GOD-capped Mn-ZnS QDs could be localized into a single band, indicating no excess GOD existed and confirming the experimental results in Fig. 7. It should be noted that the mobility difference of free GOD and GOD-capped Mn-ZnS QDs was rather small. Therefore, the relative molecular weight of such QDs (taken as a molecule) is negligible in comparison with GOD (MW = 160 KDa), which is in good accordance with the small particle size observed by TEM (2.7 nm).

Table 1. The characteristics of GOD.

Molecular weight (MW)	160 KDa	
Isoelectric point (p*I*)	4.7	
Amino acid residues	Number	Percentage (%)
His (H, imidazole)	32	2.6
Asp / Glu (D / E, -COOH)	64 / 58	10
Lys (K, -NH_2)	46	3.7
Tyr / Ser / Thr (Y / S / T, -OH)	38 / 92 / 66	15.9
Cys (C, -SH)	16	1.3

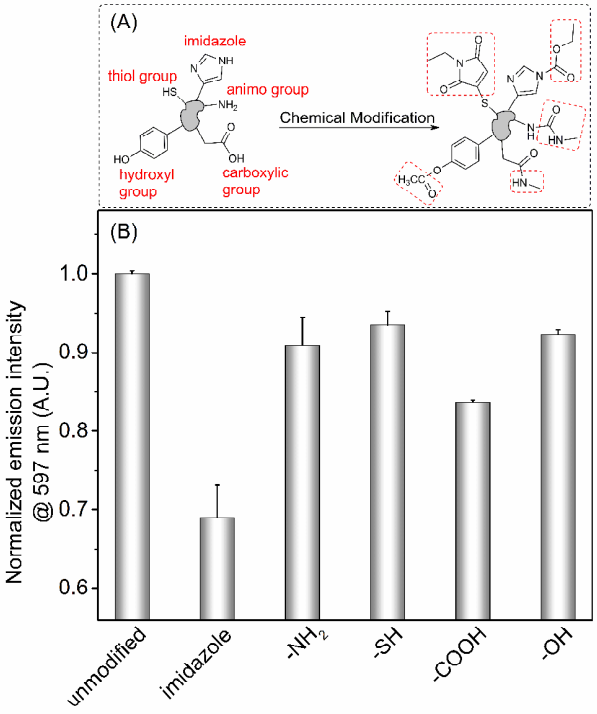


Fig. 8 Investigation on the functional groups of GOD in directing the synthesis of Mn-ZnS QDs: (A) the schematic illustration of the selective chemical modification of the functional groups of amino acids; and (B) the normalized phosphorescence intensity of Mn-ZnS QDs after chemical modification of GOD.

On the basis of the functional groups in GOD, five categories can be identified, namely imidazole in His, hydroxyl groups in Tyr/Ser/Thr, carboxyl groups in Asp/Glu, amine groups in Lys, and thiol groups in Cys (Table 1). To qualitatively evaluate the effect of these residues in directing the synthesis of Mn-ZnS QDs, selective chemical modification of each type of residues was performed to block their coordination with QDs (Fig. 8A, Fig. S24, ESI†).[9] Then, the chemically modified GOD was explored for the proposed on-step instant synthesis of Mn-ZnS QDs. As shown in Fig. 8B, all these functional groups contributed to ligands to the QDs. However, in contrast to the experiences of conventional thiols-assisted aqueous synthetic methods, thiol groups in GOD were not the decisive ligands for directing the synthesis of QDs. It turned out to be the imidazole groups that played the most predominant role. In fact, direct use of histidine or histidine-tagged peptides/proteins as ligands for synthesis of ZnS QDs have already been reported.[21] The fluorescence intensity of histidine-capped ZnS QDs was reported to be higher than cysteine-capped ones.[21a] All these evidences supported the role of histidine residues in GOD for directing the synthesis of Mn-ZnS QDs.

In order to further verify the role of these amino acid residues in directing the synthesis of Mn-ZnS QDs, four other proteins of different size (MW) and amino acids contents, namely bovine serum albumin (BSA), pepsin, papain and lysozyme, were investigated for directing the synthesis of Mn-ZnS QDs under the proposed synthetic conditions. The characteristics of the above four proteins, including protein sizes (MW) and contents of key amino acid residues were given in Table S2 and S3. To compare with GOD, six sets of experiments were performed for synthesis of Mn-ZnS QDs: (1) the same molar amounts of protein as GOD; (2) the same molar amounts of His (imidazole) in proteins as that in GOD; (3) the same molar amounts of Asp/Glu (-COOH) in proteins as that in GOD; (4) the same molar amounts of Lys (-NH_2) in proteins as that in GOD; (5) the same molar amounts of Tyr/Ser/Thr (-OH) in proteins as that in GOD; and (6) the same molar amounts of Cys (-SH) in proteins as that in GOD. Meanwhile, Mn-ZnS QDs synthesized with GOD and without ligand were taken as the control experiments.

The phosphorescence performance of the obtained Mn-ZnS QDs were given in Fig. S27. Among the five proteins investigated, GOD gave the best performance in directing the synthesis of Mn-ZnS QDs (evaluated through the phosphorescence intensity). With equal molar amount as that of GOD, the phosphorescence of BSA-directed Mn-ZnS QDs was higher than those of pepsin-, papain-, and lysozyme-directed QDs, probably because of the similar contents of amino acid residues (particularly His) in BSA as that of GOD. Moreover, BSA possesses the highest amounts of Cys residue amount all the five proteins investigated, but the phosphorescence intensity of Mn-ZnS QDs was still lower than that of GOD, again indicating thiols were not the decisive groups for liganding Mn-ZnS QDs. For lysozome, although the content of His is similar to that in GOD, the protein size is much smaller than that of GOD (14 kDa of lysozyme vs. 160 kDa of GOD), the phosphorescence of the as-synthesized Mn-ZnS QDs was also much lower. Therefore, the balance of five types of functional amine acid residues was critical for one-pot instant synthesis of Mn-Zns QDs.

Besides the amino acid residues, the isoelectric point (p*I*) of proteins also played an important role in controlling the performance of Mn-ZnS QDs. When the p*I* of the protein was lower than the pH of the precursor solution, proteins would be negatively charged and advantageous for adsorption of positively charged Zn^{2+} and Mn^{2+}. It is thus expected to obtain QDs with

better phosphorescence performance. To test such a hypothesis, the synthesis with papain (p*I* = 8.8) and lysozyme (p*I* = 11.4) was carried out at pH 12. As shown in Fig. S28, the phosphorescence intensity of Mn-ZnS QDs with such conditions was far better than those in neutral media. For GOD (p*I* = 4.9) and BSA (p*I* = 4.7), it was desirable to obtain QDs at pH higher than 5, thus could be carried out in neutral media.

From the above investigations, the role of GOD in the current instant directing the synthesis of Mn-ZnS QDs could be roughly uncovered. (1) In neutral aqueous media, negatively charged GOD first interact with positively charged Zn^{2+} and Mn^{2+} via electrostatic adsorption. (2) Due to the well-known role of "zinc-finger motifs",[20, 22] biomineralization of Zn^{2+} (together with Mn^{2+}) occurs through localization to the imidazole rings and other functional groups of GOD backbone. (3) Addition of S^{2-} precursor triggers the formation of Mn-ZnS QDs at the locations of previous complex formation. Of all these three steps, the second step is the most critical one. The protein size (MW) and the relative contents of functional groups (amino acid residues, especially histidine contents) determined the stability and phosphorescence performance of Mn-ZnS QDs.

Analytical performance of GOD-Mn-ZnS QDs for glucose sensing

Glucose is the major energy source in cellular metabolism and plays an important role in the natural growth of cells. The blood glucose level is usually taken as a clinical indicator of diabetes mellitus. Therefore, rapid and accurate determination of glucose in human blood and urine is essential in the diagnosis and management of diabetes.[23] H_2O_2 produced by GOD-catalyzed oxidation of glucose has been identified an effective electron transfer-based phosphorescence quencher for Mn-ZnS QDs.[15] Therefore, the as-prepared GOD-capped Mn-ZnS QDs were explored as a phosphorescent biosensor for glucose (Fig. 9A).

First, factors that affect the phosphorescence quenching induced by glucose, including the temperature and time for incubation of GOD-capped Mn-ZnS QDs and glucose, were investigated. As shown in Fig. S29, the enzymatic-generation of H_2O_2 should be carried out at 40 °C for 20 min, which were similar to that of free GOD. Under the optimal conditions, the phosphorescence intensity of the Mn-ZnS QDs decreased gradually as the concentration of glucose increased (Fig. 9B), and this can be used for the quantification of glucose. Statistical analysis of the quenched phosphorescence intensity versus the glucose concentration revealed two linear ranges for glucose sensing (Fig. 9C). In the concentration range of 20 to 100 μM, the quenched phosphorescence intensity was linearly increased with a calibration function of $\Delta Ph = 8.36C_{glucose} + 98.6$ ($R = 0.996$); and in the concentration range of 100 to 800 μM, the calibration function was $\Delta Ph = 3.86C_{glucose} + 430.2$ ($R = 0.997$). Therefore, the evolution of phosphorescence intensity is suitable for the determination of glucose within a wide range of 20 to 800 μM. The limit of detection (LOD, 3σ) of the present biosensor was 7 μM, and the precision for 11 replicate detections of 200 μM glucose was 0.5% (relative standard deviation, RSD).

Due to the selective enzyme-substrate reaction, the developed phosphorescent biosensor exhibited excellent selectivity toward glucose. As shown in Table S4, main relevant metal ions and other small biomolecules potentially existed in serum samples did not cause appreciable interferences to glucose detection. Besides, phosphorescence detection could also efficiently eliminate fluorescence background typically encountered in biological samples.

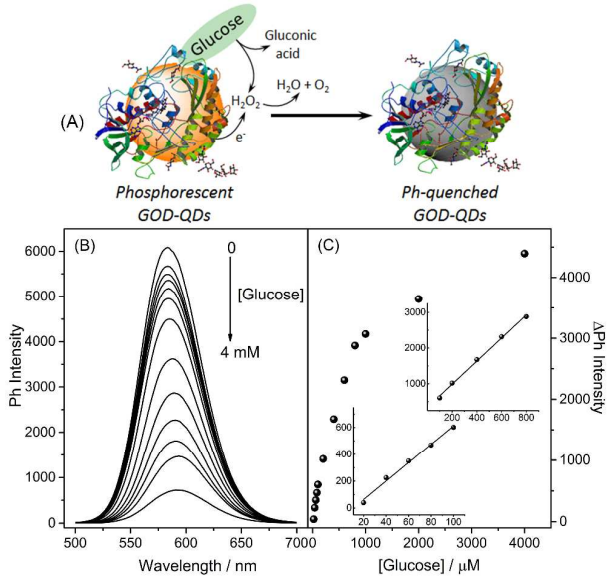


Fig. 9 Exploration of the GOD-Mn-ZnS QDs for phosphorescence-quenching detection of glucose: (A) Effect of the concentration of glucose on the phosphorescence spectra of the GOD-mediated Mn-doped ZnS QDs biosensor. (B) Plots of the quenched RTP intensity as a function of glucose concentration, showing two linear ranges. Buffer, 0.01 Tris-HCl (pH, 6.5); incubation time, 20 min; and temperature, 40 °C.

Table 2. Analytical results of glucose in serum samples with the proposed biosensor.

Samples	Determined (mM)	Test in hospital (mM)	Recovery (%)
Serum 1	4.28 ± 0.09	4.20	98
Serum 2	4.86 ± 0.36	4.60	95
Serum 3	3.86 ± 0.09	3.80	103
Serum 4	4.54 ± 0.45	4.40	104
Serum 5	4.38 ± 0.44	4.60	98

The developed biosensor was then applied for determination of glucose in serum samples collected from a local hospital. The normal glucose level in blood of healthy people is in the range of 4.4-6.6 mM.[24] Therefore, the proposed biosensor can work with a very small amounts of serum and most potential interferences can be largely eliminated by simple dilution. Before analysis, serum samples were first ultrafiltrated (8000 rmp, 10 min with a YM-10 ultrafilter) to remove insoluble substances and then subjected to analysis. As shown in Table 2, the analytical results for glucose in five serum samples were in good agreement with the values tested in the hospital (with a Hitachi Analyzer). The quantitative spike-recoveries for 20 μM of glucose ranged from 95% to 104%. These data demonstrated the analytical potential of the proposed

phosphorescent biosensor in real biological sample analysis.

Conclusions

In summary, we found the protein activity could be effectively retained in protein-directed synthesis of QDs. Taking GOD as a model enzyme, a GOD-directed, instant approach for synthesis of highly phosphorescent Mn-doped ZnS QDs in neutral media was realized readily. The enzymatic activity of GOD was unchanged after directing the synthesis of QDs. Besides, the retained enzymatic activity and the phosphorescence intensity of Mn-ZnS QDs can be stable for months. It turns out to be that the histidine residues (imidazole) played a decisive role during the synthesis of QDs. Besides, the balance of functional groups contents in GOD (imidazole, amine, carboxyl, hydroxyl, and thiol) was found also to be critical for the formation of highly luminescent QDs. Such synthetic protocol can also be extended to other doped QDs, for example Cu-doped ZnS QDs (Fig. S30). The as-prepared GOD-capped Mn-ZnS QDs were successfully explored for sensitive phosphorescent detection of glucose in serum samples. It is feasible to obtain protein-functionalized QDs in one-pot, featuring both high luminescence for QDs and retained activity for proteins.

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