

Fluorescent cholesterol sensing using enzyme-modified CdSe/ZnS quantum dots

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Abstract CdSe/ZnS quantum dot nanocrystals with wurtzite structure were synthesized using trioctylphosphine oxide (TOPO) templates. For biological applications, the capping surfactants, TOPO were replaced with mercaptoacetic acid (MAA). The carboxylic groups in MAA were activated by esterification of *n*-hydroxysulfo-succinimide (sulfo-NHS) catalyzed by water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), namely through the EDC/NHS coupling reaction. MAA not only provided water solubility to CdSe/ZnS quantum dots but also acted as a linker between cholesterol oxidase (COx) and the quantum dots due to its carboxyl group. The CdSe/ZnS–COx bioconjugates showed sensitive and linear decrease in the photoluminescence (PL) peak intensity with cholesterol concentration up to 9 mM. The PL intensity variation was elucidated based upon collisional quenching by hydrogen peroxide generated from the enzymatic oxidation reaction between COx and cholesterol. This collisional quenching mechanism was confirmed by monitoring the response of

bovine serum albumin-modified CdSe/ZnS bioconjugates to cholesterol molecules. Furthermore, the bioconjugates showed specificity to cholesterol molecules due to selective enzymatic oxidation reaction by COx. A simple quantum dot-based optical biosensor is proposed for precision cholesterol detection.

Keywords CdSe/ZnS quantum dots · Ligand exchange · Enzyme · Cholesterol · Photoluminescence · Optical biosensing

Introduction

Recently, many people are facing the risk of sudden cardiovascular diseases such as heart attack and stroke in part due to improper diet and exercise. These diseases are fatal and the second most common reason for death after cancer. Thus, early diagnosis and prevention of arteriosclerosis is of high importance. Together with early diagnosis and prevention, monitoring blood cholesterol level is critically important especially for aged people. Numerous cholesterol sensing methods have been developed owing to the high demand in sensitive and selective detection (Tan et al. 2005; Tatsuma and Watanabe 1991; Ram et al. 2001; Yao and Takashima 1998; Li et al. 2005; Motonaka and Faulkner 1993; Dey and Raj 2010; Ahmad et al. 2010). Likewise glucose sensors, most of these cholesterol sensors are based on electrochemical

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detection. However, electrochemical sensors in general require a complex structure consisting of three electrodes, working, reference, and counter electrodes. As an alternative to electrochemical sensing, many researchers are exploring fluorescent methods for biological sensing. Fluorescence sensing relies on the interaction between fluorophores and analytes that causes the change in the optical signals of fluorophores. The use of fluorescent indicators can provide sensitive and selective detection in liquid media which has led to widespread use in biological analysis (Arata et al. 2006; Kim et al. 2009; Kim et al. 2012). Among various fluorescent sensing, approaches based on enzymatic reaction show many advantages. Primarily, enzyme-based sensing is not limited by irreversible binding of biomolecules such as glucose and cholesterol, since the target biomolecules are decomposed by catalytic oxidation reaction, which can endow reusability to the biosensors. However, concerns with enzyme-based biosensors include limitations in the loading capacity and bioactivity of enzymes. To solve these problems, nano-structured materials have been introduced as a strategy to efficiently immobilize enzymes (Cortz et al. 2011; Johnson et al. 2008). Nano-scale materials can provide large surface area for more enzyme loading and a compatible microenvironment helping enzyme retain bioactivity. Among nanostructures, quantum dot nanocrystals have attracted much attention due to unique optical and electrical properties (Klostranec and Chan 2006; Bruchez et al. 1998; Lee et al. 2008). Currently, organic fluorophores have been replaced with inorganic semiconductor and metal nanoparticles, especially quantum dots, because of their high resistance to photo-bleaching and intense light emission by high quantum efficiency. Also, tuning the color of light emission can be achieved simply by changing the particle size due to quantum confinement effect (Dabbousi et al. 1997; Peng et al. 2000). Through careful control for size distribution, narrow color emission is also possible by the small full-width at half maximum (FWHM) in photoluminescence (PL) (Sung et al. 2007; Murray et al. 1993). Massive and cheap wet chemical processes were already developed and commercialized for the quantum dots. However, compared to fluorescent glucose biosensors, fluorescent cholesterol biosensors are highly limited (Prasad et al. 2011). Moreover, quantum dot-based enzyme sensors for cholesterol detection have not been reported so far.

In this study, an effective design of fluorescent cholesterol sensing was suggested by using enzyme-immobilized CdSe/ZnS quantum dots. Overcoating quantum dots with another semiconductor having a higher energy band gap can considerably enhance PL intensity by passivating the surface of quantum dots (Dabbousi et al. 1997). After replacing the capping agents, trioctyl phosphine oxide (TOPO) with mercaptoacetic acid (MAA), cholesterol oxidase (COx) molecules were immobilized to CdSe/ZnS quantum dots. The change in the PL intensity of CdSe/ZnS-MAA-COx bioconjugates was monitored according to cholesterol concentration and their specificity against glucose was investigated. The optical cholesterol detection mechanism was proposed for the bioconjugates.

Experimental

Synthesis of CdSe/ZnS quantum dots

CdSe/ZnS core/shell nanoparticles were synthesized following the previous reports by Huang et al. (2007) and Peng and Peng (2001). CdO which is the Cd-precursor, stearic acid (SA), trioctyl phosphine oxide (TOPO), and hexa-decyl amine (HDA) were loaded in a three-necked flask and heated to 310 °C. Then Se/tri-*n*-butylphosphine (TBP) solution was quickly injected into the flask to nucleate CdSe quantum dots. To overcoat CdSe with ZnS, $(\text{ZnCH}_3)_2$, $(\text{TMS})_2\text{S}$, and TBP were mixed and the mixed solution was dropped into the flask as slowly as possible after the temperature was cooled to 230 °C. The washing was conducted by repeating separation/purification process.

Analysis of CdSe/ZnS quantum dots

Powder x-ray diffraction (XRD: Rigaku Ultima 2000, Tokyo, Japan) using Cu-K α radiation at 40 kV and 100 mA was performed for analysis of structure of CdSe/ZnS quantum dots. The morphology and crystallinity of the core-shell quantum dots were investigated by high-resolution transmission electron microscopy (HRTEM: TECNAI G2 F20, FEI, Amsterdam, The Netherlands). UV-visible spectroscopy (JASCO UV-visible Spectrophotometer: V530, Tokyo, Japan) and photoluminescence spectroscopy

(PL: Hitachi F-4500, Tokyo, Japan) were performed to analyze optical property of quantum dots.

Ligand exchange of quantum dots and bioconjugation

TOPO used for CdSe/ZnS synthesis was replaced with MAA to make them soluble in phosphate buffer solution (PBS). Typically, 30 ml of the MAA was poured into 3 ml of the CdSe/ZnS quantum dot-containing solution and stirred for 5 h. The quantum dots were rinsed and centrifuged several times for the removal of MAA remnants. MAA-capped quantum dots were reacted with a mixture of freshly prepared 50 mM sulfo-NHS and 20 mM EDC solution for 30 min to induce coupling reaction. Sulfo-NHS-terminated CdSe/ZnS quantum dots were isolated by centrifugation and re-dispersed in the buffer solution and then, cholesterol oxidase was added. After incubating for 12 h, the quantum dots in a solution were obtained by centrifugation and these particles were rinsed and redispersed in PBS. Finally, bioconjugation of CdSe/ZnS nanoparticles with COx was completed, and they were stored at 4 °C in a refrigerator. In order to confirm the biosensing mechanism, another globular protein, bovine serum albumin (BSA) was employed and immobilized to CdSe/ZnS quantum dots in this study. BSA was added to PBS containing NHS-terminated CdSe/ZnS quantum dots. After incubating and centrifuging BSA-modified CdSe/ZnS quantum dot bioconjugates were obtained and they were re-dispersed in PBS.

Preparation of cholesterol solution and PL measurements

For cholesterol solution preparation, the method of Ram et al. (2001) and Trettnek et al. (1993) was used. 125 mg of cholesterol was dissolved in 3.75 g of Triton X-100, a detergent at 65 °C and hot water was added to obtain 25 ml solution. A standard solution of 10 mM of cholesterol with Triton X-100 (15 % w/v) was used for the experiments, and the standard solution was stored at 4 °C. The PL measurement was conducted by adding the standard solution into 10 ml of 0.1 M PBS with pH 6.3, containing 1 % Triton X-100. The cholesterol concentration was varied from 0.484 to 9.02 mM. The cholesterol solution was added to PBS in the cuvette and after 5 min, the PL emission from CdSe/ZnS was measured.

Successive cholesterol addition and PL detection was repeated. Also, cholesterol solution was added to the cuvette containing BSA-modified CdSe/ZnS dispersed PBS and the experimental condition was identical to that of COx-modified CdSe/ZnS. The PL variation by the cholesterol molecules was monitored with this system.

Results and discussion

Structure and crystallinity of quantum dots

Figure 1a shows XRD patterns of CdSe/ZnS semiconductor quantum dots. They showed wurtzite structure with high crystallinity. In XRD data of CdSe/ZnS, XRD peaks were shifted to high 2θ angles compared with the reference JCPDS card (#02-0330).

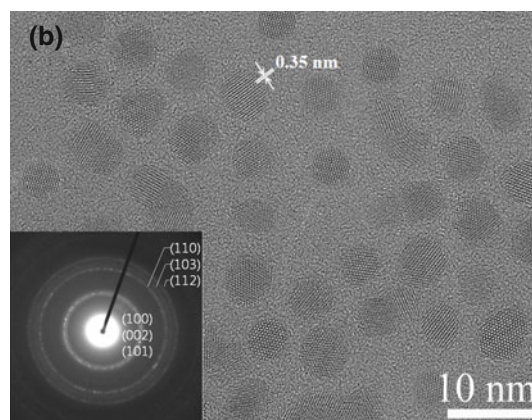
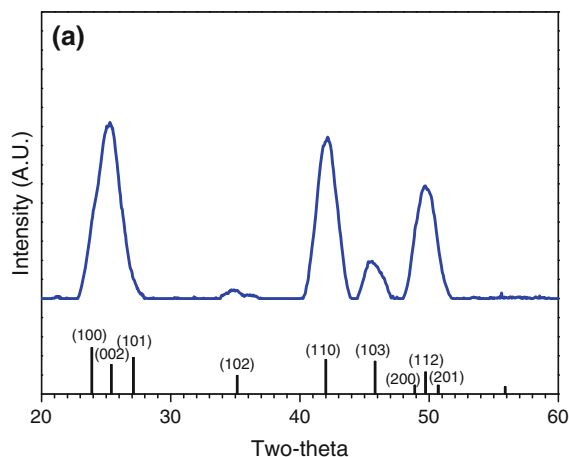


Fig. 1 **a** XRD patterns and **b** TEM image of CdSe/ZnS quantum dots. *Inset* is SAED pattern

This peak shift occurred due to the compressive strain of CdSe core (lattice parameters, $a = 4.299 \text{ \AA}$, $c = 7.010 \text{ \AA}$) coming from the ZnS shell (lattice parameters, $a = 3.777 \text{ \AA}$, $c = 6.188 \text{ \AA}$) having smaller lattice parameters. This is an evidence of ZnS shell formation (Lee et al. 2006). Figure 1b shows HRTEM image of CdSe/ZnS quantum dots. Nanoparticles are spherical in shape with the average size of $\sim 5\text{--}6 \text{ nm}$, and they showed narrow size distribution. The inset image of Fig. 1b displays selected area electron diffraction (SAED) ring patterns which clearly show the crystallinity and wurtzite phase of CdSe.

Optical properties of CdSe/ZnS quantum dots

Figure 2 shows UV–visible light absorption and PL spectra from CdSe/ZnS core/shell quantum dots. Due

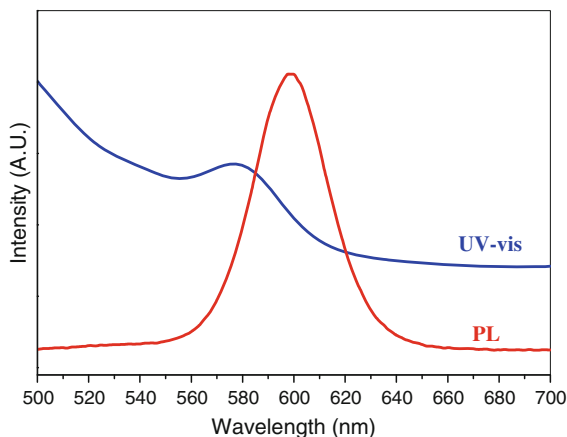


Fig. 2 UV–visible absorption and PL spectra of CdSe/ZnS quantum dots

to the passivation of surface dangling bonds, the quantum dots showed strong light emission near 690 nm. The difference in the peak position of UV–visible and PL spectra comes from “Stokes shift (Dabbousi et al. 1997).” Both absorption and emission spectra show blue shift of the peaks considering the energy band gap of CdSe (1.74 eV) corresponding to $\sim 713 \text{ nm}$. This blue shift originates from quantum confinement effect that takes place when the size of a semiconductor becomes down below the so-called Bohr’s radius. The Bohr’s radius of CdSe is about 5 nm and the average size of our nanoparticles was $\sim 5\text{--}6 \text{ nm}$.

Ligand exchange and bioconjugation of CdSe/ZnS quantum dots

CdSe/ZnS quantum dots were synthesized using a non-polar surfactant TOPO. Thus, surface modification to resolve non-water solubility and non-biocompatibility of quantum dots is needed, and it can be done by replacing TOPO with water-soluble ligands. MAA was used for this purpose. Figure 3 shows the process of replacing capping agent, TOPO with MAA and bioconjugating of quantum dots with COx. The ligand exchange for quantum dots is possible due to the two functional groups that are mercapto groups and carboxylic acid groups. The thiolate group ($-\text{SH}$) is very highly nucleophilic, and it has a strong affinity to the zinc in the ZnS shell, and thus surface TOPO can be easily replaced with MAA. Carboxylic acid groups in the end of MAA chains show very excellent hydrophilic property needed for water solubility, and they also provide reactive functional groups for

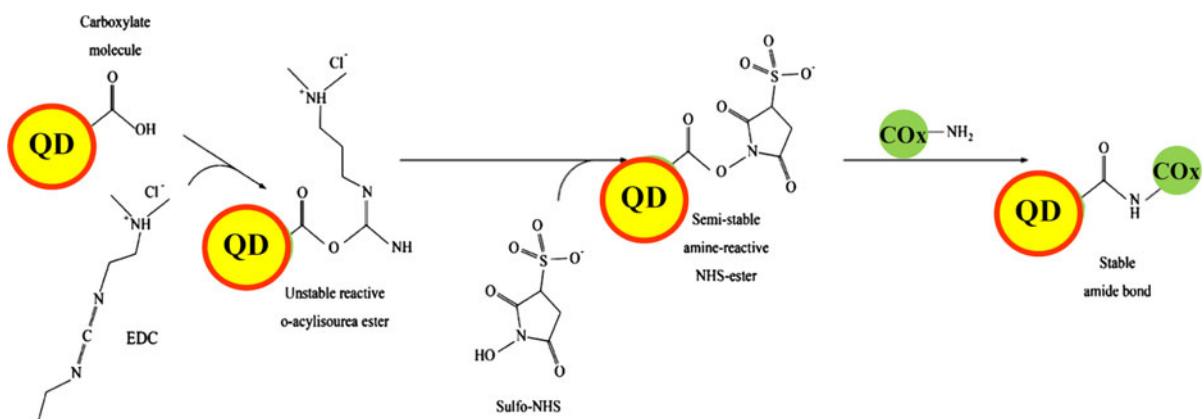
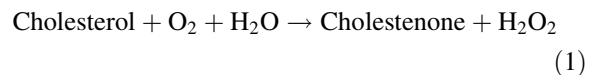


Fig. 3 The ligand exchange and bioconjugation procedure of CdSe/ZnS quantum dots

immobilization of COx. After TOPO stabilized CdSe/ZnS were modified by MAA, peripheral carboxylic groups in MAA were activated by esterification of *n*-hydroxysulfo-succinimide (sulfo-NHS) catalyzed by water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), namely through the EDC/NHS coupling reaction. The NHS-terminated CdSe/ZnS quantum dots were mixed with enzyme, COx in a PBS and then, NHS groups at the surface of quantum dots were replaced with the side chains of amino groups in the enzyme molecules (Patel et al. 1997; Li et al. 2007). Finally, COx could be immobilized onto the CdSe/ZnS at the surface of CdSe/ZnS nanocrystals by covalent bonding with the organic molecules. CdSe/ZnS-MAA-COx bioconjugates are ready to detect cholesterol molecules.

Detection of cholesterol using CdSe/ZnS-MAA-COx bioconjugates

After washing, the CdSe/ZnS-MAA-COx bioconjugates were dispersed into a quartz cuvette containing PBS and cholesterol solution was added. Figure 4 shows PL spectra variation of the solution containing CdS/ZnS-MAA-COx bioconjugates according to cholesterol concentration. It was noticed that the intensity gradually decreases as cholesterol concentration increases. The curve in Fig. 4 exhibits a strong linear fit up to 9.11 mM of cholesterol concentration. Carbon nanotube-based cholesterol sensors show the cholesterol detection range between 1.0 and 8.0 mM (Wisitorsaat et al. 2009). People can be diagnosed as high cholesterol when the cholesterol concentration is higher than about 6.2 mM (240 mg/dL). So cholesterol concentration range which was added in the solution up to 9.11 mM is reliable to diagnose hypercholesterolemia. Also, the detection limit of our cholesterol sensor was 0.01 mM although that of commercially used amperometric cholesterol sensors is ~0.025 mM. This high sensitivity could come from fluorescent-based cholesterol sensing using semiconductor quantum dots. Apparent quenching of PL intensity and wide range linearity can guarantee strong potential to be used as a promising cholesterol sensor. The enzymatic cholesterol oxidation reaction can be described as following;



The possible mechanism for fluorescence quenching by appearance of cholesterol molecules is related to the hydrogen peroxide generation. There are two basic types of quenching, static, and dynamic (collisional). Both types require an interaction between the fluorophore and quencher. In the case of collisional quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. In the case of static quenching, a complex forms between fluorophores and quenchers, and this complex is non-fluorescent. Especially, dynamic quenching occurs when the excited-state fluorophore is deactivated by contact with some other molecules in solution, which is called the quencher (Gruber and Leonard 1975; Cheng et al. 2006). In this study, decrease of PL intensity by adding cholesterol molecules could be related to collisional quenching. In this case,

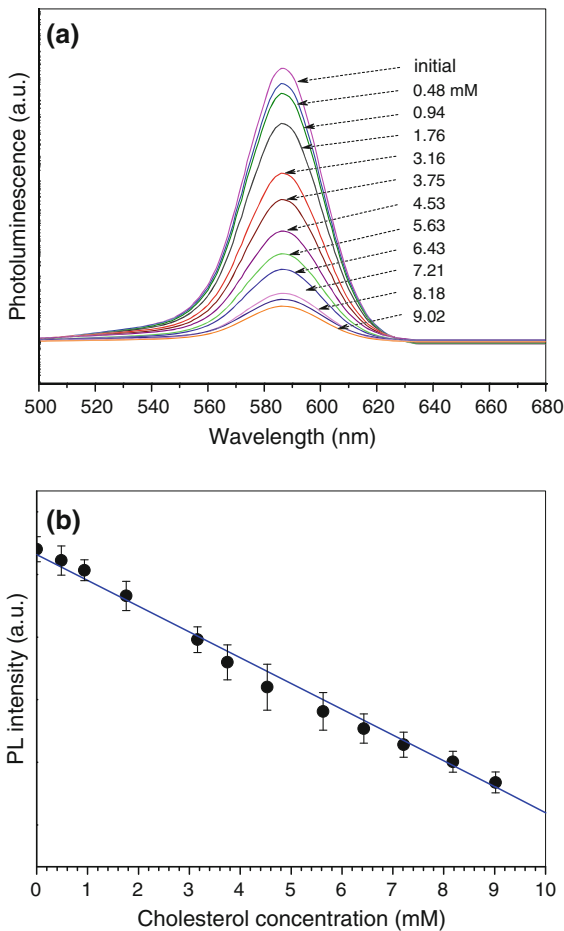
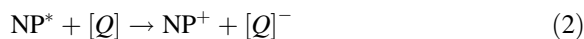


Fig. 4 PL variation of CdSe/ZnS-MAA-COx with cholesterol concentration

CdSe/ZnS and nanoparticles act as a fluorophore and hydrogen peroxide can be operated as a quencher. Various semiconductor quantum dots by nature have a high surface-to-volume ratio and many of their optoelectronic characteristics are related to the nature of the surface (Tolbert and Alivisatos 1994; Soloviev et al. 2000). Eichkorn and Ahlrichs (1998) have modeled the electronic structures of small sized nanoparticles and they confirmed larger confinement effects on the energy levels for clusters than for larger nanoparticles. It is verified that very small nanoparticles in the order of tens of atoms have more sensitive electronic, structural, and chemical properties than their larger counterparts. So many groups have studied using small nanoparticles to monitor the change in the optoelectronic properties when simple electron or hole acceptors are present. Weller group reported fluorescence quenching experiments of differently sized CdS nanoparticles with quenchers with different electron acceptor potentials. The fluorescence quenching of colloidal CdS is usually understood in terms of electron transfer reactions from the photoexcited particles to adsorbed electron acceptors (Hasselbarth et al. 1993). Recently, the El-Sayed group reported that using butylamine, a hole acceptor, causes emission quenching in CdSe nanoparticles without changing its decay characteristics. And they also suggested that CdSe nanoparticles exhibit molecular collisional quenching properties in the presence of butylamine (Landes et al. 2001). These reports suggest that electron or hole acceptors adsorbed at the surface of semiconductor nanoparticles can change their luminescence properties and quench the exciton emission by fast electron transfer. In our experiments, hydrogen peroxide can play an important role as an electron acceptor in the solution containing bioconjugates and biomolecules. Verduyn et al. (1991) reported that hydrogen peroxide may indeed function as an electron acceptor for mitochondrial respiration via the action of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Matsumi group studied kinetics of the collisional quenching of spin-orbitally excited atomic chlorine by H₂O, D₂O, and H₂O₂. They reported quenching process of Cl by H₂O₂ and the fast rate constant for quenching due to the local mode of O–O stretch in H₂O₂ (Kono et al. 2006). In light of these references, hydrogen peroxide could be expected as an electron acceptor in solution containing enzyme conjugated quantum dots. Photoexcited CdSe/ZnS quantum dots

that have multiple relaxation pathways can be expected to undergo decrease of PL intensity in the presence of hydrogen peroxide due to collisional quenching. The following reaction explains the quenching of quantum dot nanoparticles.



Here, NP* denotes quantum dot nanocrystals in a neutral state, and Q is the quencher (H₂O₂). If size of quantum dots is very small, the valence and conduction bands can be replaced by more widely spaced individual energy levels. It is possible then that excited-state electron transfer with hydrogen peroxide becomes more energetically favorable because of band gap increase. Thus, collisional quenching becomes a favorable relaxation pathway in these small nanoparticles. Figure 5a shows appearance of an alternate relaxation pathway. Through oxidation reactions catalyzed by enzymes conjugated to quantum dots, biomolecules are oxidized and hydrogen peroxide is produced. Electrons of excited quantum dots can transfer to H₂O₂ acting as an electron acceptor, which can cause the decrease in PL emission. Through change of PL intensity, biomolecules can be detected and this detection method could be applied to other biomolecules such as glucose and lipase. In order to confirm our proposed quenching mechanism by H₂O₂ produced by COx, we replaced COx with BSA. BSA-modified CdSe/ZnS quantum dots were dispersed in PBS and their PL response was monitored after 3.16 mM cholesterol solution was added as shown in Fig. 5b. The PL did not show any notable change after cholesterol was added. This result excludes the possibility that cholesterol molecules affect PL response of CdSe/ZnS quantum dots.

CdSe/ZnS–MAA–COx bioconjugates revealed specificity to cholesterol molecules. Figure 6 shows PL peak variation after addition of 3.16 mM cholesterol and glucose, respectively. PL peak intensity negligibly decreased after glucose addition, while that decreased by about 55 % after cholesterol addition. The CdSe/ZnS–MAA–COx bioconjugates showed apparent specificity to cholesterol molecules, which comes from selective oxidation reaction of an enzyme, COx.

In order to confirm our proposed collisional quenching by hydrogen peroxide, hydrogen peroxide was added to the CdSe/ZnS–MAA–COx bioconjugates. Figure 7 shows PL variation with hydrogen

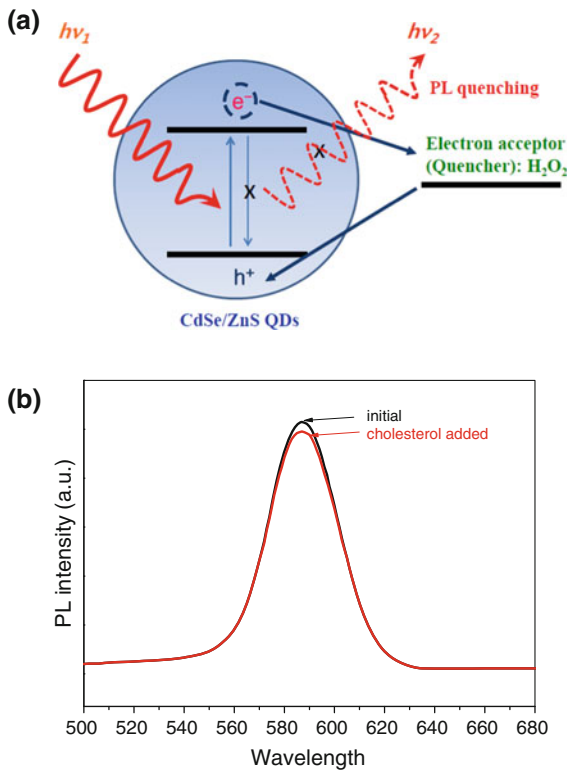


Fig. 5 **a** Schematic diagram showing luminescence quenching mechanism. **b** PL response of BSA-modified CdSe/ZnS quantum dots after 3.16 mM cholesterol was added

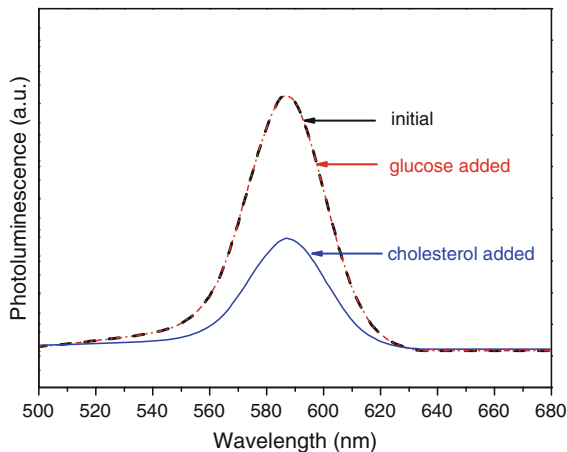


Fig. 6 Comparison of the PL spectra after addition of 3.16 mM cholesterol and glucose, respectively

peroxide concentration. The PL intensity almost linearly decreases according to the hydrogen peroxide which is very similar to Fig. 3. Therefore, it is certain that collisional quenching by hydrogen peroxide is the

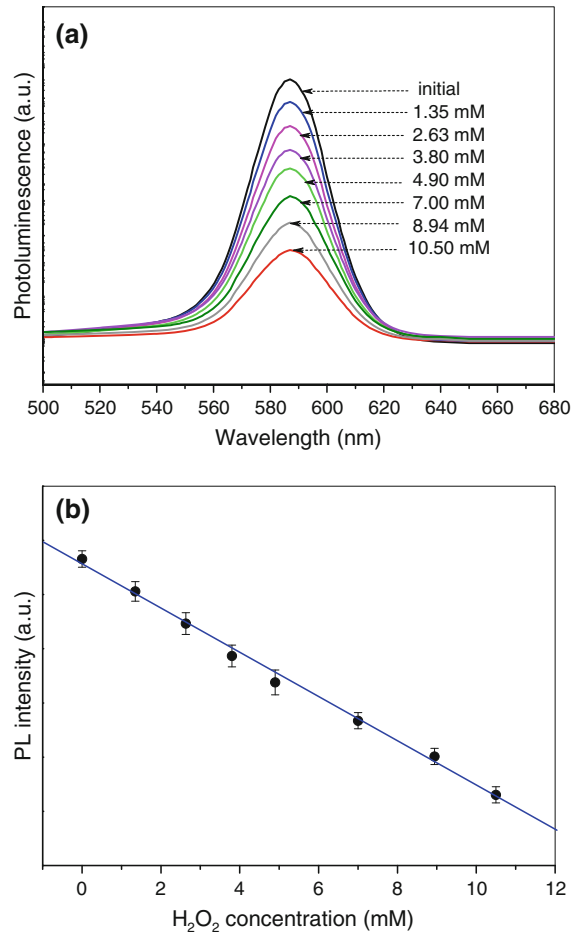


Fig. 7 **a** PL response of CdSe/ZnS-MAA-COx to the hydrogen peroxide. **b** PL variation according to hydrogen peroxide concentration

mechanism for the PL decrease in our bioconjugates. Also, BSA modified for the response time 3.80 mM cholesterol was added to CdSe/ZnS-MAA-COx bioconjugates as shown in Fig. 8. PL intensity rapidly dropped within 5 s and it showed saturation at 40 s. This response time of 5 s is comparable to or even shorter than that (5–10 s) of electrochemical biosensors (Wang et al. 2006). Due to magnified surface area by CdSe/ZnS nanocrystals, we could obtain sensitive and fast responding biosensing system.

Conclusions

To conclude, CdSe/ZnS core/shell nanoparticles with the wurtzite structure were successfully synthesized and their capping agent, TOPO was replaced with

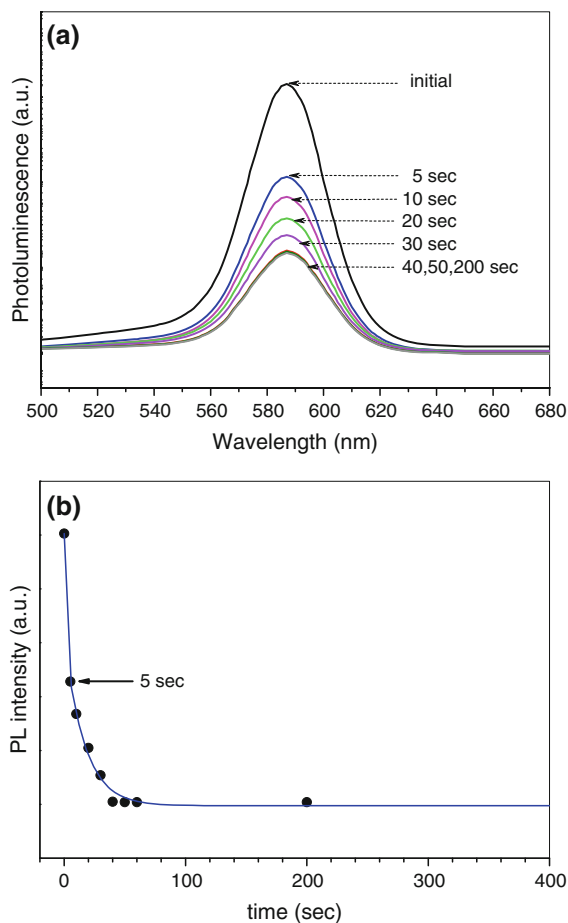


Fig. 8 **a** Time response of CdSe/ZnS-MAA-COx to 3.80 mM cholesterol. **b** PL variation according to time

MAA in order to obtain water solubility and biological compatibility. After the ligand exchange, an enzyme, COx was immobilized to the surface of CdSe/ZnS activated by EDC/NHS coupling reaction. The CdSe/ZnS-MAA-COx bioconjugates showed decrease in PL intensity in the presence of cholesterol molecules. The mechanism of PL intensity decrease was proposed based upon collisional quenching by hydrogen peroxide generated during enzymatic oxidation reaction. Hydrogen peroxide can act as an electron acceptor, and thus light emission from excited-state CdSe/ZnS quantum dots can be quenched. The CdSe/ZnS-MAA-COx bioconjugates showed specific sensing of cholesterol by selectivity of COx. Not only due to sensitive PL response and strong linearity in the PL intensity variation up to 9 mM but also due to specificity, CdSe/ZnS-MAA-COx bioconjugates are proposed as a candidate for precise cholesterol

sensing. They also showed fast response time of 5 s that is comparable to or even shorter than that of currently used electrochemical biosensors. These optical sensors using enzyme conjugated quantum dots have high sensitivity, specificity, fast response, and simple structure, and thus this approach can be extended to enzymatic sensing of other biomolecules.

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