Quantum Dots

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Optical Detection of Glucose and Acetylcholine Esterase Inhibitors by H_2O_2-Sensitive CdSe/ZnS Quantum Dots**

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There is a growing interest in using semiconductor quantum dots (QDs) as optical labels for biosensing events.[1] The size-controlled fluorescence properties of QDs,[2] the high fluorescence quantum yields of QDs,[3] and their stability against photobleaching[4] makes QDs superior optical labels for multiplexed analysis of antigen–antibody complexes,[5] nucleic acid–DNA hybrids,[6] and other biorecognition complexes.[7] QDs were also applied to monitor biocatalytic transformations using fluorescence resonance energy transfer (FRET) processes. FRET processes between CdSe/ZnS QDs and dye units incorporated into replicated DNA systems or into telomers were used to probe the activities of polymerase and telomerase, respectively.[8] Similarly, FRET reactions were used to monitor the biocatalytic cleavage of peptides by hydrolytic enzymes.[9] Alternatively, electron-transfer quenching of QDs by quinone-functionalized peptides was used to detect the activity of tyrosinase, and the hydrolytic cleavage of the quinone-modified peptide and the restoration of the fluorescence of the QDs were used to probe the activities of tyrosinase and thrombin, respectively.[10] In all of these QD 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. Also, for each of the enzymes, a specific assay needs to be developed.

Numerous oxidases generate hydrogen peroxide (H_2O_2) as a product. Thus, controlling the photophysical properties of QDs by H_2O_2 may provide a new and versatile method to develop QD-based sensors. In fact, the biocatalyzed generation of H_2O_2 by oxidases was used for the development of different electrochemical biosensors,[11] and recently for the development of optical biosensors using Au nanoparticles.[12] Herein we demonstrate that the fluorescence of CdSe/ZnS QDs is sensitive to H_2O_2. This sensitivity enables the use of the QDs as H_2O_2 sensors and provides a versatile fluorescent reporter for the activities of oxidases and for the detection of their substrates. This utility is exemplified herein for the analysis of glucose in the presence of glucose oxidase. Furthermore, we apply the fluorescent QDs as sensors that monitor the inhibition of acetylcholine esterase (AChE). AChE hydrolyzes acetylcholine to choline and, subsequently, choline oxidase (ChOx) oxidizes choline to betaine while generating H_2O_2. In the presence of an inhibitor, the hydrolytic cleavage of acetycholine by AChE is perturbed, and the inhibited formation of H_2O_2 is reflected by the fluorescence of the QDs. In addition to the broad application of the CdSe/ZnS for different sensing processes, we introduce the ratiometric fluorescent analysis of the different substrates. This analysis enables us to monitor the stability of the different sensors, and to correct for any precipitation events of the QDs that might cause an "apparent" decrease in the observed fluorescence intensities. We describe the use of the enzymes in solution or in immobilized forms on the QDs.

Figure 1a depicts the time-dependent luminescence changes upon the reaction of mercaptoundecanoic acid (MUA) capped CdSe/ZnS QDs with H_2O_2 (0.4 mM). The fluorescence of the QDs decreases with time, and addition of catalase to the system, which includes H_2O_2, blocks the decrease in the fluorescence, implying that H_2O_2 is, indeed, the component affecting the fluorescence. Figure 1b shows the fluorescence quenching of the QDs upon interaction with different concentrations of H_2O_2 for a fixed time interval of 10 minutes. Although the precise mechanism that stimulates the decrease in the fluorescence of the QDs is not fully

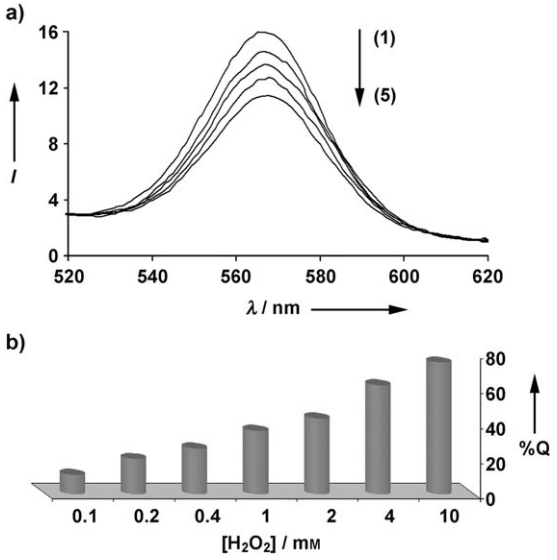

Figure 1. a) Time-dependent fluorescence changes of mercaptoundecanoic acid capped CdSe/ZnS QDs in the presence of 0.4 mM H_2O_2: (1) 0 min, (2) 2 min, (3) 5 min, (4) 10 min, (5) 15 min; b) Quenched fluorescence in the presence of variable concentrations of H_2O_2 upon interaction with the CdSe/ZnS QDs for a fixed time interval of 10 min. All measurements were performed in a 10 mM HEPES buffer solution (pH 7.2).

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understood, the oxidation of S^{2-} surface states presumably yields Zn^{2+} surface traps for the electrons.

The QDs were further applied to the ratiometric analysis of H_2O_2. Glutathione (GSH)-capped QDs were functionalized with fluorescein isothiocyanate (FITC)-modified avidin by the homobifunctional cross-linker bis(sulfosuccinimidyl) suberate (BS^3) (Figure 2a–c). The resulting avidin-capped QDs were used in all ratiometric analyses in the present study.

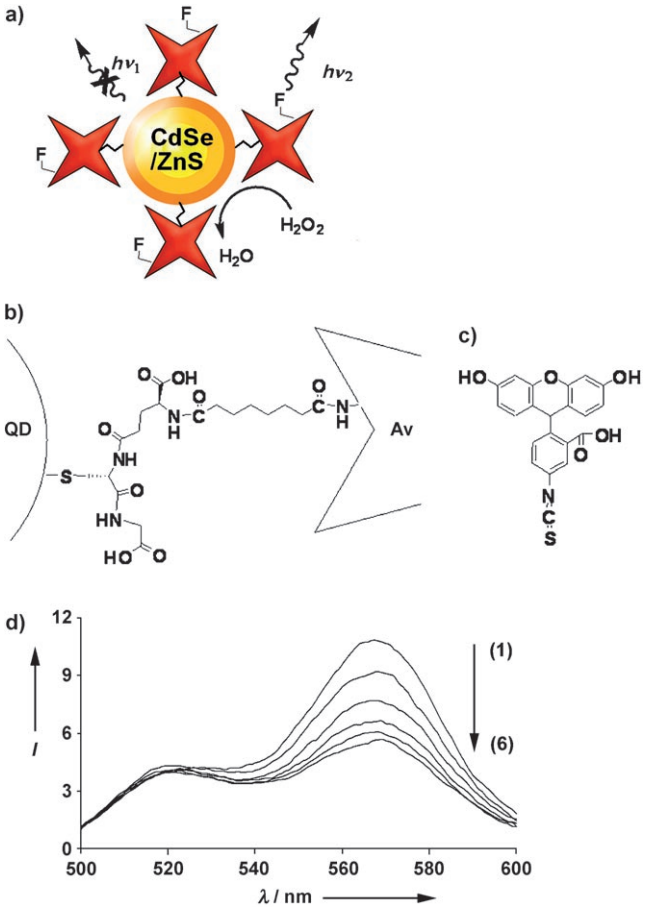

Figure 2. a) Ratiometric analysis of H_2O_2 by fluorophore-functionalized avidin-CdSe/ZnS QDs. b) Configuration of avidin-functionalized CdSe/ZnS QDs. c) Structure of the fluorophore that functionalizes avidin. d) Ratiometric time-dependent analysis of 4 mM H_2O_2 using the fluorophore-functionalized avidin-capped QDs; (1) 0 min, (2) 2 min, (3) 5 min, (4) 10 min, (5) 15 min, (6) 20 min. All measurements were performed in a 10 mM HEPES buffer solution (pH 7.2).

Figure 2d shows the ratiometric probing of the effect of H_2O_2 on the luminescence of the QDs. Whereas the luminescence of the QDs decreases, the fluorescence of the reference dye is unaffected. These results imply that the decrease in the fluorescence does not originate from a precipitation of the QDs or from a chemical surface etching process that degrades the QDs. Lifetime measurements indicate that when the fluorescence of the QDs decreases by 30%, the lifetime of the QDs is shortened from 2.69 ± 0.10 ns to 1.94 ± 0.05 ns. The detection limit for analyzing H_2O_2 corresponds to 0.1 mM.

Sensing of H_2O_2 by CdSe/ZnS QDs enabled us to assemble an intergrated ratiometric fluorescent nanosensor for the detection of glucose (Figure 3a). Biotin-modified glucose oxidase (B-GOx) was linked to FITC–avidin-modified QDs, and the biocatalyzed oxidation of glucose was monitored by following the effect of the generated H_2O_2 on the fluorescence of the QDs.

Figure 3b shows the ratiometric time-dependent fluorescence changes of the B-GOx-FITC–avidin-QDs assembly in

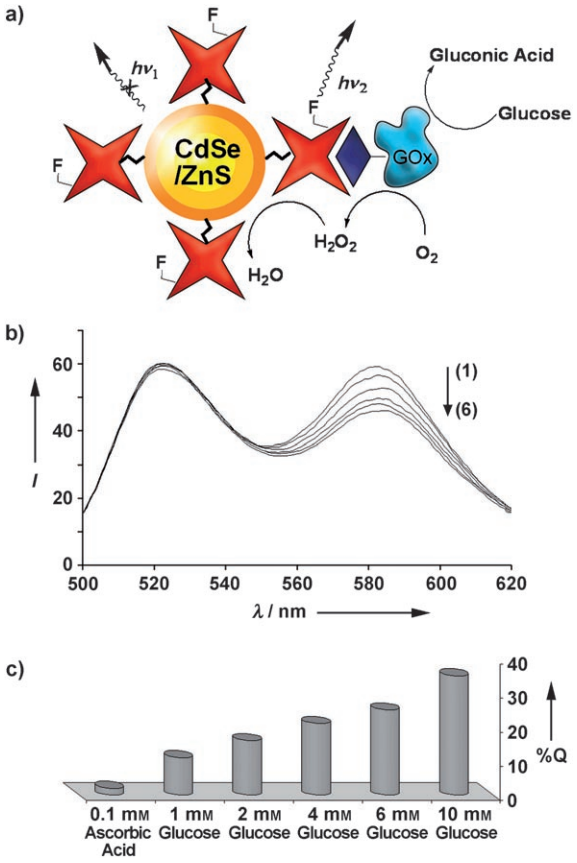

Figure 3. a) Ratiometric analysis of glucose by biotinylated GOx associated with the fluorophore-functionalized avidin bound to the CdSe/ZnS QDs. b) Time-dependent ratiometric fluorescence analysis of glucose (5 mM) by the GOx-modified QDs. (1) 0 min, (2) 2 min, (3) 5 min, (4) 10 min, (5) 15 min, (6) 20 min. c) Quenched fluorescence of the GOx-functionalized CdSe/ZnS QDs upon analyzing different concentrations of glucose for a fixed time interval of 10 min. All measurements were performed in a 10 mM HEPES buffer solution (pH 7.2).

the presence of 5 mM glucose. The enzyme GOx catalyzes the oxidation of D-glucose to gluconic acid with the concomitant formation of H_2O_2. As the biocatalytic reaction proceeds, the luminescence quenching of the QDs is enhanced. Control experiments indicate that D-glucose by itself does not affect the fluorescence of the QDs, and that substitution of D-glucose with L-glucose does not affect the fluorescence of the QDs, implying that the biocatalyzed generation of H_2O_2 leads to the luminescence quenching of the QDs. The ratiometric analysis of the quenching of the luminescence of the QDs, using FITC as a reference fluorophore, indicates that the fluorophore is unaffected by the generated H_2O_2 (or glucose),

and the modified QDs are stable within the analysis time interval. The quenching of the fluorescence of the QDs is controlled by the concentration of glucose (and the resulting H_2O_2; Figure 3c). Also, glucose sensing interference from ascorbic acid did not affect the fluorescence of the QDs, indicating that the analysis is selective for glucose.

The CdSe/ZnS QDs were also applied to the analysis of an inhibitor of AChE. Acetylcholine is a neurotransmitter that triggers neural responses, and its hydrolysis by AChE is the fundamental step in the path to controlling and regulating the neural responses. The inhibition of AChE leads to constant stimulation of the muscles by acetylcholine and to the fatal consequences of chemical warfare agents.[13] Recent reports described the use of nanoparticles to probe the activity and the inhibition of AChE; the AChE-functionalized CdS nanoparticles associated with electrodes were used to probe the enzyme activity and its inhibition by the generation of photocurrents.[14] Additionally, the growth of Au nanoparticles upon hydrolyzing acetylthiocholine by using AChE, and the inhibition of the enlargement of the nanoparticles by the respective inhibitors, were used to optically probe the enzyme activity and its inhibitors.[15]

AChE hydrolyzes acetylcholine to choline, whereas ChOx oxidizes choline to betaine with the concomitant generation of H_2O_2 (Figure 4a). Accordingly, the enzyme-generated H_2O_2 quenches the luminescence of the QDs. Thus, in the presence of inhibitors for AChE, the quenching of the fluorescence of the QDs should be eliminated, and the fluorescence intensities of the QDs should be controlled by the concentrations of the inhibitor. Figure 4c depicts the ratiometric detection of the activity of the AChE by the analysis of H_2O_2 generated by the AChE–ChOx cascade. As the biocatalytic cascade is prolonged the fluorescence of the QDs decreases, a result that is consistent with the generation of higher amounts of H_2O_2. Figure 4d shows the time-dependent decrease of the fluorescence intensities of the QDs upon interaction with the AChE–ChOx biocatalytic cascade in the presence of different concentrations of neostigmine (Figure 4b), a known inhibitor for AChE.[16] As the concentration of the inhibitor increases, the fluorescence quenching decreases, and the rate of luminescence quenching is slower. This result is consistent with the fact that increasing the inhibitor concentration lowers the content of enzyme-generated H_2O_2 and, therefore, decreases the quenching rates of the QDs. The inhibition effect on the luminescence quenching of the QDs can be quantitatively analyzed by using the Cheng–Prusoff equation[17] [(1)], in which K_i is the

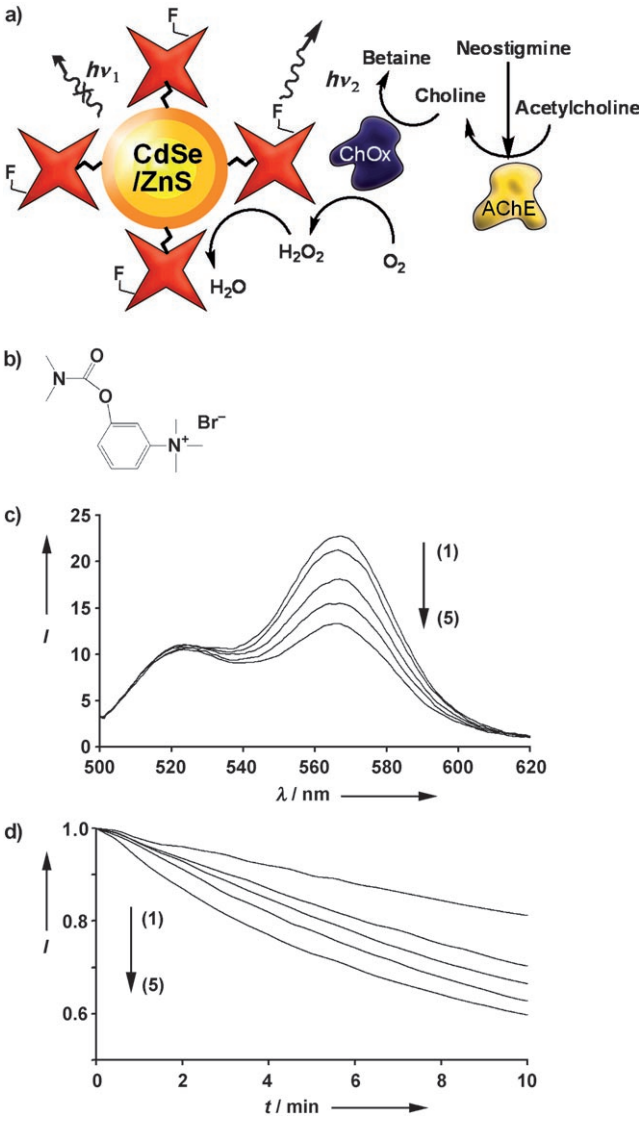

Figure 4. a) Ratiometric analysis of the activity of acetycholine esterase by the fluorophore-modified avidin-capped CdSe/ZnS QDs, and its inhibition by neostigmine. b) Structure of neostigmine. c) Time-dependent fluorescence changes upon interaction with acetylcholine esterase (2 U), choline oxidase (0.3 U), and acetylcholine (10 mM). (1) 0 min, (2) 2 min, (3) 5 min, (4) 10 min, (5) 15 min. d) Time-dependent fluorescence changes of the CdSe/ZnS QDs upon interaction with AChE (2 U), ChOx (0.3 U), and acetylcholine (10 mM), in the presence of different concentrations of neostigmine: (1) 100 μM, (2) 50 μM, (3) 25 μM, (4) 15 μM, (5) without neostigmine. All measurements were performed in a 10 mM HEPES buffer solution (pH 8.0).

$$K_i = IC_{50}/(1 + [S]/K_m) \quad (1)$$

inhibition constant, IC_{50} is the inhibitor concentration that yields 50% inhibition, K_m is the Michaelis–Menten constant of the enzyme towards the substrate, and $[S]$ is the substrate concentration used for the IC_{50} calculation.

The derived K_i value corresponds to 5×10^{-6} M, a value that is in good agreement with the reported literature value of 4.4×10^{-6} M determined by other methods.[18] The GSH-capped QDs exhibited high stability against aggregation for three weeks (concentration >1 μM). The binding of the protein to the capping layer was found, however, to destabilize the particles, and approximately 5% of the particles precipitated within two hours.

To conclude, the present study has introduced an optical method to analyze H_2O_2 by the luminescence quenching of CdSe/ZnS QDs. The QDs enable the assembly of an integrated nanoscale hybrid sensor that includes the biocatalyst and the fluorophore marker. The fluorescence quantum yield of the QDs (ca. 20%) and their stability towards photobleaching are two advantages of the sensing system. The method has enabled the application of the QDs to analyze the

activity of H_2O_2-generating oxidases. Specifically, we demonstrated the use of the QDs for the optical detection of glucose, in the presence of glucose oxidase, and for the sensing of an acetylcholine esterase inhibitor, using the AChE–ChOx cascade. We also emphasize the use of the fluorophore-labeled capping layers of the QDs for the ratiometric detection of the different analytes. In view of the moderate stabilities of the QDs, the ratiometric analysis may provide a normalization procedure for any partial instability of the sensing systems.[19] At this level of the research, the integrated sensing QD systems represent single cycle sensors. We are examining experiments to recycle the fluorescence features of the QDs by the reductive depletion of the surface traps.

Experimental Section

Materials: Ultrapure water from NANOpure Diamond (Barnstead Int., Dubuque, IA) source was used throughout the experiments. Hops Yellow Core Shell EviDots, CdSe/ZnS quantum dots in toluene were purchased from Evident Technologies. Bis(sulfosuccinimidyl) suberate (BS^3) was purchased from Pierce Biotechnologies. Biotinylated glucose oxidase was purchased from Vector Laboratories Inc. All other reagents were purchased from Sigma–Aldrich Inc.

Preparation of mercaptoundecanoic acid (MUA) capped QDs: QDs were precipitated from the toluene solution by addition of methanol (2 mL) to QDs (0.5 mL) in toluene and subsequent centrifugation for 5 min at 3000 rpm. The resulting precipitate was dissolved in 1 mL chloroform. A 200 μL MUA solution (containing 100 mg MUA and 100 mg KOH in 5 mL methanol) was added, and the mixture was then shaken for 1 min. After the addition of 1 mL of 0.001M NaOH solution in water, all the particles were transferred to the water phase and the chloroform became clear. The QDs solution was separated from the chloroform by centrifugation for 1 min. The excess of MUA was removed by two successive precipitation steps of QDs, using NaCl and methanol and subsequent centrifugation. The resulting QDs were dissolved in 1 mL of a 10 mM HEPES buffer (HEPES = *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.2).

Preparation of GSH-capped QDs: The GSH-capped particles were prepared in the same way as the MUA particles, except that at the second stage, the MUA solution was replaced by a GSH solution (containing 0.139 g GSH and 60 mg KOH in 1 mL methanol). After purification, the particles were dissolved in 1 mL of a 10 mM HEPES buffer (pH 8).

Preparation of FITC–Avidin-capped QDs: A BS^3 stock solution (20 μL, 1 mg mL^{-1} in 10 mM HEPES buffer, pH 8) was added to a solution of the GSH-capped QDs in HEPES buffer (150 μL) and the mixure was shaken for 15 min. The QDs were purified by precipitation, and the particles were subsequently dissolved in 10 mM HEPES buffer (pH 7.2). FITC–avidin stock solution (60 μL, 10 mg mL^{-1}) was added to the particle solution and the solution was shaken for 1 h. The excess of FITC–avidin was removed by two successive precipitation steps of QDs, and the purified particles were dissolved in 10 mM HEPES buffer (0.5 mL, pH 8).

Preparation of GOx-capped QDs: Biotin-GOx solution (150 μL) was added to the FITC–avidin capped QDs solution (300 μL), and the mixture was subsequently shaken for 1 h. The excess Biotin-GOx was cleaned by two consecutive precipitation steps of the QDs. After cleaning, the particles were dissolved in 10 mM HEPES buffer (0.5 mL, pH 7.2).

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