Labeled avidin bound to water-soluble nanocrystals by electrostatic interactions

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Semiconducting nanocrystals of three different sizes capped with 3-mercaptopropionic acid were synthesized in aqueous solutions. They can efficiently bind to an avidin biomolecule by the electrostatic attraction. The conjugation of avidin leads to a red shift and a decrease in the intensity of the fluorescence emission spectra of the nanocrystals. Moreover, the red shift of the fluorescence spectra of the bioconjugates depends strongly on the pH, ionic strength, quantity of avidin, and nanocrystal size.

Key words: semiconducting nanocrystals, avidin, labeling, electrostatic attraction.

Avidin, being a glycoprotein found in the egg white and tissues of birds, reptiles, and amphibia, is a positively charged homotetramer (isoelectric point 10—10.5) with a molecular weight of 67000—68000 Da.[1,2] Each of its subunit can bind one biotin molecule. Due to the very high affinity ($K_a = 10^{15}$ L mol^{-1})[3] and, as a consequence, high stability of this noncovalent interaction, the avidin—biotin complex finds wide use in many bioanalytical methods. It is used for the isolation and determination of localization of biomolecules, various immunoassays, drug delivery, technology of DNA probes, *etc*.

Organic dyes, including fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), and rhodamine B200, are among the probes commonly used for the avidin—biotin system. However, the characteristics of these dyes limit their efficient use in biology and medicine. For example, the narrow excitation spectrum and broad emission band with a long red "tail" impede the simultaneous detection of several probes because of spectral overlap.[4] Furthermore, organic fluorophores are prone to photobleaching and chemical degradation.

Colloidal semiconducting nanocrystals, *viz.*, quantum dots (QDs), can surmount disadvantages restricting the use of organic dyes for fluorescent tagging, because they possess unique size-dependent optical and electronic properties.[5] Contrary to dye molecules, QDs have a broad continuous excitation spectrum and narrow size-tunable symmetric emission spectrum, which make it possible to excite QDs of different size using a single-wavelength excitation source. In addition, the photochemical stability and resistance to chemical degradation of QDs are much higher than those of organic dyes. Earlier, the QDs used for fluorescent labeling were commonly prepared in organic solvents.[6—19] In order to obtain water-soluble QDs suitable for biological systems, the surface-capping hydrophobic ligands, such as trioctylphosphine (TOP) or trioctylphosphine oxide (TOPO), were replaced by siloxane,[6—8] an amphiphilic polymer shell,[9,10] or thiols.[11—18] These procedures are not only time-consuming but also necessarily use hazardous organic ligands. Recently,[20—23] water-soluble semiconducting nanoparticles, which were directly synthesized in aqueous solutions using thiols as stabilizing agents, have been exploited to label biomolecules. In these cases, however, biomolecules are linked to QDs mainly by covalent interactions.

In this paper, we report a different procedure for the preparation of bioconjugates of negatively charged water-soluble CdTe-based QDs of different size with highly positively charged avidin by the electrostatic attraction. In particular, the effects of the pH, ionic strength, quantity of avidin, and size of nanocrystals on the fluorescence characteristics of the CdTe—avidin bioconjugates were studied.

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Experimental

Absorption spectra in the visible and UV regions were recorded on a Shimadzu 3100 UV—Vis—near-IR spectrophotometer. Fluorescence spectra were obtained on a Shimadzu RF-5301 PC spectrofluorimeter (quartz cell 1 cm thick). Measurements were carried out at room temperature.

Analytical-purity grade reagents and twice-distilled water (>18 MOhm cm) were used to prepare all solutions. 3-Mercaptopropionic acid (MPA, 99+%), tellurium powder (~200 mesh, 99.8%), $CdCl_2$ (99+%), $NaBH_4$ (99%, Aldrich), and lyophilized avidin (Rockland Ltd, used as received) were used. A solution of the avidin powder in a saline solution containing a 2 m*M* phosphate buffer (PBS, pH 7.3) with a concentration of 10 mg mL^{-1} was stored at −20 °C, being diluted only prior to use.

Water-soluble CdTe nanocrystals. Water-soluble CdTe QDs modified by the MPA on the surface were obtained as described previously.[24—26] At the first stage, an aqueous solution of sodium hydrotelluride (NaHTe) was prepared by the reaction of sodium borohydride ($NaBH_4$) with the tellurium powder in a molar ratio of 2 : 1. At the second stage, the freshly prepared oxygen-free solution of NaHTe was added to a nitrogen-saturated $1.25 \cdot 10^{-3}$ *M* aqueous solution of $CdCl_2$ at pH 11.4 in the presence of MPA as a stabilizer. The molar ratio of Cd^{2+} : stabilizer : HTe^- was 1 : 2.4 : 0.5. The resulting mixture was refluxed to obtain CdTe nanocrystals of certain size. The luminescence quantum yield for the CdTe nanoparticles at room temperature was ~25% (compared to the emission of rhodamine 6G).[27] Quantum dots with emission maxima at 526 (particle size 2.8 nm), 540 (3.0 nm), and 566 nm (3.2 nm) were used in experiments (Fig. 1).

Conjugates QD—avidin. A mixture of dilute solutions of the protein and nanocrystals (3.0 nm, $8.4 \cdot 10^{-8}$ mol L^{-1}, calculated from the loading of the reactants and crystal structure[28]) in 2 m*M* PBS (2 mL, pH 7.3) was incubated for 5 min at room temperature. The resulting solution containing stable QD—avidin conjugates without obviously seen aggregates can be used for assay.

Dot blotting of affinity analysis. Two droplets of a biotin solution with a certain concentration were blotted on a nitrocellulose membrane and then incubated with QD and QD—avidin conjugates for 15 min at room temperature. The membranes were washed with PBS (3 times for 10 min each) to remove unbound QDs or QD—avidin conjugates. The resulting samples were observed under a UV lamp.

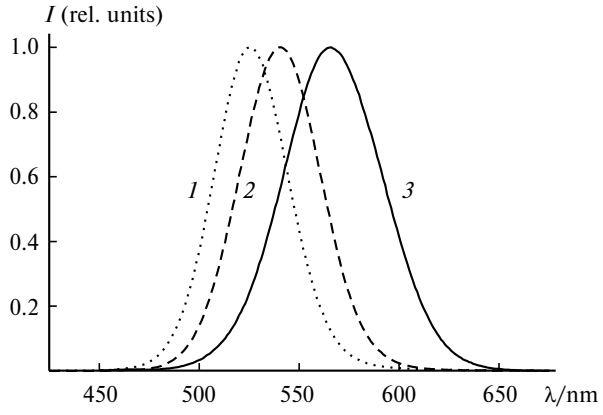

Fig. 1. Fluorescence spectra of the CdTe nanocrystals 2.8 (*1*), 3.0 (*2*), and 3.2 nm (*3*) in diameter with fluorescence maxima at 526, 540, and 566 nm, respectively. The excitation wavelength is 400 nm.

Results and Discussion

Fluorescence and absorption spectra. Figure 2 shows the excitation and fluorescence spectra of the CdTe nanocrystals and avidin in a PBS buffer solution. The maximal emission peak of the QDs appears at 540 nm, whereas that of avidin is at 340 nm. Avidin has no absorption at 400 nm and does not fluoresce when the excitation wavelength >400 nm is used and, thus, it has no effect on the fluorescent detection of QDs or QD—avidin conjugates in the wavelength range from 450 to 700 nm.

Both the absorption and fluorescence spectra change when nanocrystals are labeled to avidin (Fig. 3). The absorption spectrum of the QD—avidin bioconjugates is flatter than that of free QDs. However, the absolute absorbance values of the former are higher than those of the latter. Meanwhile, the photoluminescence peak position shifts from 540 to 549 nm when free QDs are conjugated

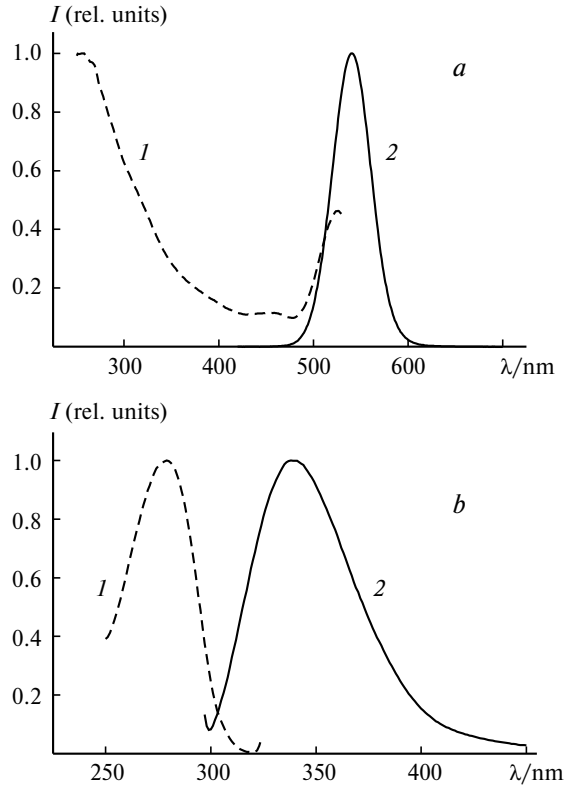

Fig. 2. Excitation (*1*) and fluorescence (*2*) spectra of the unbound CdTe nanocrystals (*a*) and avidin (*b*) in PBS. The excitation wavelengths are 400 (*a*) and 280 nm (*b*), and the emission peaks are 541 (*a*) and 340 nm (*b*).

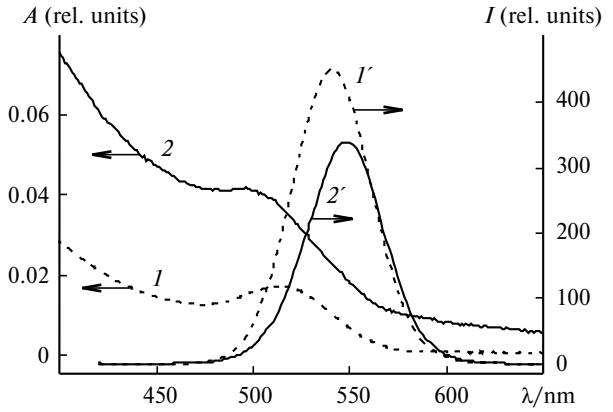

Fig. 3. Absorption (*1*, *2*) and fluorescence (*1′*, *2′*) spectra of solutions of the CdTe nanoparticles (*1*, *1′*) and CdTe—avidin conjugates (*2*, *2′*). The excitation wavelength is 400 nm.

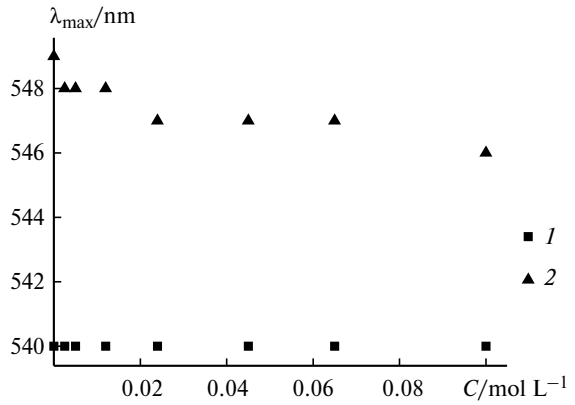

Fig. 4. Effect of an increase in the ionic strength on the position of the fluorescence maximum of the unbound CdTe (*1*) and CdTe—avidin conjugates (*2*). Several different volumes of 0.5 *M* NaCl were added to the CdTe and conjugated CdTe—avidin samples, and the concentration of NaCl (*C*) was calculated for the resulting solutions.

with avidin, whereas the intrinsic spectral width remains unchanged.

The distance between two QDs shortens due to the electrostatic attraction between the negatively charged CdTe quantum dots and positively charged protein, which enhances the dipole-dipole interactions between QDs and, hence, increases the Stokes loss, resulting in a red shift in the fluorescence spectrum.[29,30]

Effect of the pH of the buffer. It was shown experimentally that there was almost no change in the fluorescence spectrum of the CdTe—avidin system when the pH changed from 7 to 9. However, when the pH value of a PBS buffer solution was adjusted to a value (for example, 11) higher than the isoelectric point of avidin, the fluorescence spectrum of the CdTe—avidin solution was shown to be the same as that obtained with CdTe nanocrystals only. The reason for this phenomenon is that the charge of avidin displays negative when the pH of the solution is 11, which inhibits avidin from conjugation with the CdTe nanoparticles by the electrostatic attraction. This result indicates that avidin can electrostatically bind to the oppositely charged nanocrystals.

Ionic strength effect. It is well known that the conjugation *via* the electrostatic interaction is not stable enough and can easily be affected by environmental conditions, such as the pH and ionic strength. In the above studies, the analysis was carried out in a PBS buffer solution of low concentration (2 mmol L^{-1}) and low ionic strength. In order to examine the influence of the higher ionic strength on the interaction between QDs and avidin, the fluorescence spectra of free CdTe and a CdTe—avidin solution were recorded as a function of the increasing NaCl concentration (Fig. 4). The emission peak of the QD—avidin conjugates gradually shifts to shorter wavelengths, whereas that of free QDs shows no change upon salt addition, implying that the ionic strength has a great effect on the binding between QDs and avidin. The shift of the emission peak of QD—avidin arises from the counterion screening effect,[31] which decreases the binding affinity of QDs to avidin.

Effect of the avidin to QD (A : Q) molar ratio. To estimate an equivalent ratio between avidin and QDs, a series of solutions containing different quantities of avidin and the same amount of QDs were analyzed by fluorescence spectroscopy (Fig. 5). An interesting phenomenon was observed: with an increase in the quantity of avidin added, the red-shift value of the emission peak first reached a maximum (552 nm, maximum red shift 12 nm) and then moved back to a shorter wavelength (549 nm, final red shift 9 nm), whereas the intensity of the emission decreased. The changes observed can be explained by the lattice theory.[32,33] Quantum dots and avidin have multiple combining sites on their surfaces, resulting in the formation of a lattice arrangement of their molecules. The aggregates grow gradually if the A : Q ratio is lower than 3.15, after which the further increase in the A : Q ratio leads to a decrease in the size of aggregates on account of dissociation and re-equilibration. When the complexes gradually augment with excess QDs, the distance between QDs becomes shorter and shorter, which induces a gradual increase in the Stokes loss. Consequently, this results in a red shift of the fluorescence spectrum. The "equivalence point" of the QD—avidin conjugation corresponds to the maximum red shift in Fig. 5. However, when avidin is of sufficient excess (A : Q ratio higher than 3.6), lattice complexes dissolve gradually, and this weakens the dipole-dipole interaction owing to the elongating of the distance between QDs and, hence, reduces the Stokes loss. As a result, the emission peak returns to shorter wavelengths. Moreover, the decrease of the fluorescence intensity could be explained in terms of the concentration effect.[34]

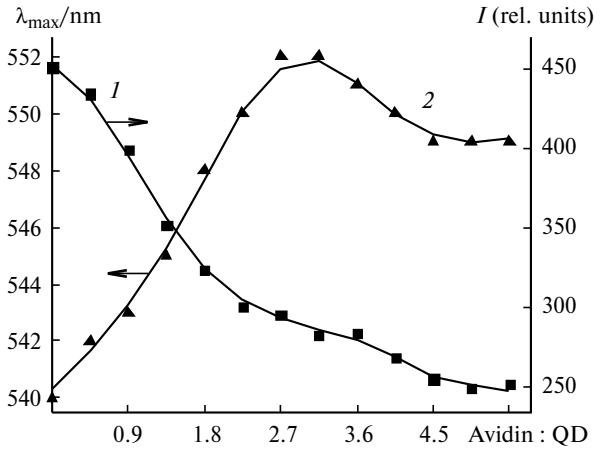

Fig. 5. Fluorescence intensity (*1*) and the position of the fluorescence peak (*2*) of the CdTe—avidin conjugates as functions of the avidin to QD molar ratio.

Effect of the QD size. Two other different sizes of QDs (2.8 and 3.2 nm) were utilized to conjugate with avidin in the same way. Similar changes in the fluorescence spectra were observed, *i. e.*, the red shift appears first and then the blue shift does (Fig. 6). Interestingly, the maximum red shift, as well as the final red shift of the QD—avidin conjugates, becomes more and more remarkable with an increase in the size of QDs. The reason for this is that the dipole-dipole interactions among the nanoparticles depend strongly on the particle volume.[30] As for larger QDs, the interaction between dipoles has a stronger effect on the Stokes loss. As a result, the phenomenon of the red shift of the emission peak is more and more prominent with increasing particle size.

Dot blotting of affinity analysis. To further confirm the conjugation between negatively charged CdTe QDs and highly positively charged avidin, we carried out a dot blotting of affinity analysis, which utilizes the high affinity of the avidin—biotin system. As can be seen from the data in Fig. 7, the fluorescence of dot *A* (biotin and QD—avidin) is more pronounced than that of dot *B* (biotin and QD). This result strongly supports our previous hypothesis on binding.

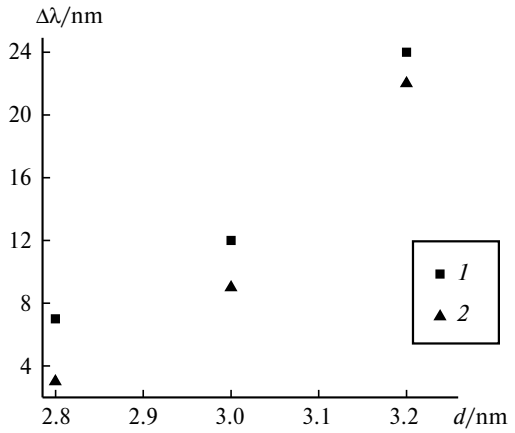

Fig. 6. Maximum red shift (*1*) and the final red shift (*2*) of the fluorescence peaks of CdTe upon the gradual addition of avidin *vs.* nanoparticle diameter (*d*).

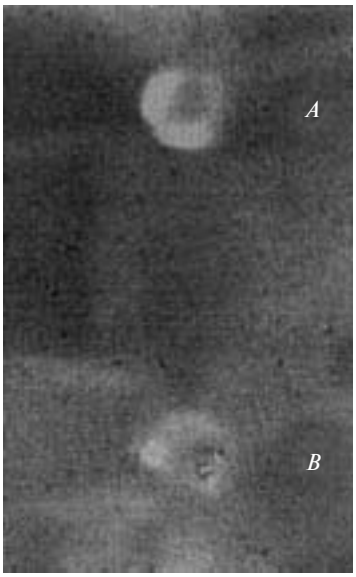

Fig. 7. Photograph of the nitrocellulose membrane in the dot blotting of affinity analysis: biotin and QD—avidin (*A*), biotin and QD (*B*).

In summary, CdTe semiconductor nanocrystals synthesized in an aqueous solution are efficiently conjugated with avidin *via* the Coulomb interaction. Labeling QDs to avidin makes it possible to attach these fluorescent inorganic particles to any biotin-modified antibody, protein, or nucleic acid. Being combined with a quantum dot and an avidin—biotin system, a new convenient method is exhibited to bioanalysts. Utilization of avidin-conjugated QDs in biological studies, such as to identify the specific antigen or acceptor on the surface of a cell or to detect an antibody or antigen in various samples, is our further work to be done.

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