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Colloidal CdSe and ZnSe/Mn quantum dots: Their cytotoxicity and effects on cell morphology

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Abstract

The CdSe and ZnSe:Mn colloidal quantum dots (QDs) have been synthesized in order to use them as a contrast agent for bioimaging. The synthesis of QDs was made in the aqueous solution. These compounds are fluorescent semiconductor nanoparticles and are held to be promising fluorophores which can be used as an important research tool in biology and medicine. They can be exploited to allocate the problematic biological tissues and individual cells. Their applicability to human examination was studied. For this purpose we investigated the morphological changes in the cells by reacting with the CdSe/l-Cys and ZnSe:Mn/MPA quantum dots. The cytotoxicity of CdSe/l-Cys in the line of breast carcinoma was examined using confocal microscopy. The results can be seen as encouraging.

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Keywords: Quantum dots; Fluorescence stability; Cytotoxicity; Confocal microscopy; Bioimaging.

Introduction

Fluorescent tagging methods (immunochemical tests, tissue imaging, drug delivery monitoring, etc.) have gained immense popularity in the last decade. Fluorophores have thus become one of the most important research tools. Fluorescent semiconducting nanoparticles, or quantum dots (QDs), are among the most promising [1,2].

QDs have some unique properties, e.g., a high absorptivity (10–100 times higher than that of organic dyes) and a high quantum yield (up to 90 %), which results in an exceptional luminescence intensity of these

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nanocrystals. QDs typically have relatively wide optical absorption spectra, narrow and symmetrical fluorescence spectra (peak width at half-height is about 25–40 nm), and also significantly large Stokes shifts. Since QDs may be excited by light of any wavelength less than their fluorescent wavelength, this wide excitation spectrum allows to excite various kinds of QDs by monochromatic light with a wavelength far removed (>100 nm) from their fluorescent wavelengths [3].

The cytotoxic effect of QDs can be mainly attributed to 4 factors:

- heavy metal ions making up their composition;
- capability of generating reactive oxygen species (ROS);
- colloidal instability;
- nonspecific interaction with biological molecules [4,5].

The QDs were experimentally shown to exhibit a relatively high toxicity [6–8] caused by the toxic elements (heavy metal ions) in their compositions. The problem of reducing the toxicity could be dealt with in one of two ways, which are selecting a material for the core and protecting its coating shell. The first case involves producing QDs based on wide-bandgap materials, i.e., zinc sulphide and zinc selenide (ZnS and ZnSe); annealing these allows to obtain a material that is fluorescent in the visible region of the spectrum. The second scenario entails blocking the surface by creating a dense inert coating (e.g. made from a silicon dioxide) around the particle [9]. QDs are non-toxic for human cells while covered with a protein or a biocompatible polymer coating. However, since QDs may possibly remain in the body for a long time and accumulate, numerous experiments must be carried out to thoroughly check they are safe to use [10,11].

A number of studies have revealed that there are currently many promising applications of QDs in biomedical research due to their unique properties (in particular, they are polychromatic when irradiated by a single laser beam, have a high luminescence intensity, and their properties remain stable in storage). For example, Rafalovskaya-Orlovskaya et al. [12] established that the obtained fluorescent nanoparticle–antibody conjugates retained the ability of staining slices for four months, while samples stained by these conjugates were fit for post hoc analysis for half a year. As for the other samples stained by the Alexa-Fluor 488-conjugated antibodies, these did not exhibit any fluorescence after a week.

The subjects and the methods of the study

The quantum dots studied were cadmium selenide nanoparticles coated with l-cysteine (CdSe/l-Cys) and manganese-doped zinc selenide nanoparticles coated with mercaptopropionic acid (ZnSe:Mn/MPA).

The CdSe/l-Cys nanoparticles were synthesized in an aqueous medium. The source of selenium was sodium hydrogen selenide NaHSe, synthesized directly before the experiment by the following reaction

$$4\,NaBH_4 + 2\,Se + 7\,H_2O \rightarrow 2\,NaHSe + Na_2B_4O_7 + 14\,H_2.$$

After the synthesis was completed, the hydrogen selenide solution was stored in a nitrogen atmosphere up until it was injected into a cadmium precursor solution. The source of cadmium was its chloride that had been saturated with sodium hydroxide to pH = 9 in order to deprotonate the l-cysteine. The molar ratio of the components was

$$L - Cys/Cd^{2+}/S^{2-} = 40 : 10 : 1.$$

The selenium source solution was injected into the cadmium precursor solution at room temperature and slowly heated to 95 °C, upon which it was exposed for half an hour. The nanoparticles were purified by centrifuging the initial solution with isopropanol at a 1:2 solution-to-isopropanol ratio. For the reduction of sulfur bonds, 5 mg of $NaBH_4$ was added to 1200 μl of the solution. The thus obtained samples exhibited orange–red luminescence.

To assess the stability of the CdSe/l-Cys quantum dots in buffer solutions we conducted experiments where QDs in a concentration of 17.5 μM/ml were added to the following solutions: a phosphate-buffered saline, pH = 7.5 (BIOLOT), a Wash buffer, pH = 8.0 (Dako), and a Tris buffer, pH = 5.0 (Sigma). Solution aliquots were selected after 60 min, placed on the slide, evaporated, and observed for fluorescence using an Olympus FV 1000 confocal laser scanning microscope (with a laser excitation wavelength of 405 nm).

The fluorescence intensity of the CdSe/l-Cys and ZnSe:Mn/MPA samples in comparison to organic fluorophores was assessed in the following way: a fluorophore was added to the slide in portions of 15 μl and mixed with a Fluorescence Mounting Medium (Dako) preventing the fluorophores from crystallizing and decolorizing, coverslipped and exposed to UV radiation (using a Mercury Vapor Short Arc 120 W lamp) of 25% intensity for 120 min. A Niba fluorescent filter (for the UV radiation) was used to observe luminescence. Samples were scanned each 15 min with the same laser beam

intensity. The QD sample was radiated with a 405-nm laser, the FITC sample with a 488-nm laser, and the Alexa Fluor sample with a 559–568-nm laser. Mean fluorescence intensity in relative units was then measured using the FluoView 10 software.

The morphological changes were traced on a normal human endometrial cell line. The cells were isolated from biopsies of human endometrium taken during exploratory laparoscopy. In early passages the object was a heterogeneous cell population consisting of fibroblast-like and epithelial cells. The cells were cultivated in a DMEM/F12 medium with 10% fetal calf serum (FCS), 2 mM glutamine, and an antibiotic mixture, at 37 °C in a moist atmosphere containing 5% of CO_2. The cells were grown to a 60–70% of monolayer confluence; then a QD with the highest concentration of 17.5 μM/ml was added, and the cells were incubated for 24 h, upon which they were washed with a phosphate-saline buffer, fixed with a 4% paraformaldehyde, and stained with hematoxylin–eosin- to study the morphology under an Olympys BX46 light microscope.

An assessment of the cytotoxic effect of cadmium selenide QDs

The cytotoxicity of the CdSe/L-Cys quantum dots was assessed in two cell cultures. The first one was a cell line, ZR-75-1 (breast carcinoma), and the second one normal human mononuclear lymphocytes (MNL).

The ZR-75-1 cell line was obtained from the Russian Cell Culture Collection (Institute of Cytology RAS). The MNLs were obtained from a healthy 29-year-old woman and separated in a Ficoll density gradient.

The cytotoxic effect of the cadmium selenide QD in the ZR-75-1 cell line was assessed in 1, 3, 18 and 24 h after the QD solution had been added, through determining the apoptosis induction by a fixed staining procedure with propidium iodide (PI) that stains cell nuclei with compromised cell membrane structures. Phosphate-buffered saline (PBS) was added as control to the cell cultures in the same quantities. Samples of quantum dots of 1, 10 and 100 μl for 1 ml of growth medium were injected to the nutrient medium, with the concentrations of 175 nM/ml, 1.75 μM/ml, and 17.5 μM/ml, respectively.

A similar method was used to assess the cytotoxic effect for the MNL culture; however, the QD concentration was 1.15 μM/μl, and incubation times of 1, 3, 18, 24 and 48 h were taken as control points.

The results of the study

Quantum dot stability: The observations carried out showed that after the QDs had been incubated in PBS solutions, liquid evaporated, and as the solution dried, a crystalline structure with orange-fluorescing quantum dots inside of it formed.

As a result of our studies, we established that the CdSe/L-Cys QDs remain stable in buffer solutions at room temperature. The acidity of a solution had no effect on the stability of a QD, since all three samples with pH values equal to 8.0, 7.5, and 5.0 exhibited fluorescence in an hour (Fig. 1).

Morphological changes in cells upon interaction with QDs: As noted above, CdSe/l-Cys and ZnSe:Mn/MPA QDs were studied. We compared the stability of these QDs with organic FITC fluorophores (FITC-conjugated rabbit anti-mouse immunoglobulins, Dako) and Alexa Fluor 568 (Alexa Fluor 568-conjugated donkey anti-mouse immunoglobulins, Abcam). We were interested in their optical properties and their possible applications in confocal microscopy. The comparison of QD fluorescence intensity and stability to those of Alexa Fluor 568 and FITC organic dyes is shown as a diagram in Fig. 2.

It can be concluded from analyzing the diagram that in the first 15 min the organic fluorophore FITC exhibited the maximum fluorescence intensity but after 15 min its intensity decreased (almost threefold). Another organic fluorophore, Alexa 568, was somewhat more stable than FITC but it also exhibited a decrease in fluorescence intensity over time. The CdSe/L-Cys QD fluorescence intensity has been shown to increase over time (from 230 to 1000 relative fluorescence units).

Further studies produced the following results: up to being UV-irradiated by a mercury lamp, the laser-excited fluorescence intensity in FITC and Alexa Fluor 568 was several times higher than that of the ZnSe:Mn/MPA QDs (the intensity in Alexa Fluor 568 was 4 times higher, and 12 times higher in FITC). The ZnSe:Mn/MPA QDs had a lower fluorescence intensity compared to the CdSe/l-Cys QDs, but it remained stable after prolonged excitation.

Due to their high photostablity the CdSe/L-Cys QDs may be used for observing fluorescence in objects for long periods of time and for staining tissue sections. A more intense fluorescence may be achieved by increasing the sizes of the ZnSe:Mn/MPA QDs, for example, by adding an extra shell; due to the low toxicity of QDs they may be used for long-term bioimaging studies.

To increase the fluorescence brightness of QDs, an extra shell may be used that would both increase the fluorescence brightness and reduce the cytotoxic effect [7,13].

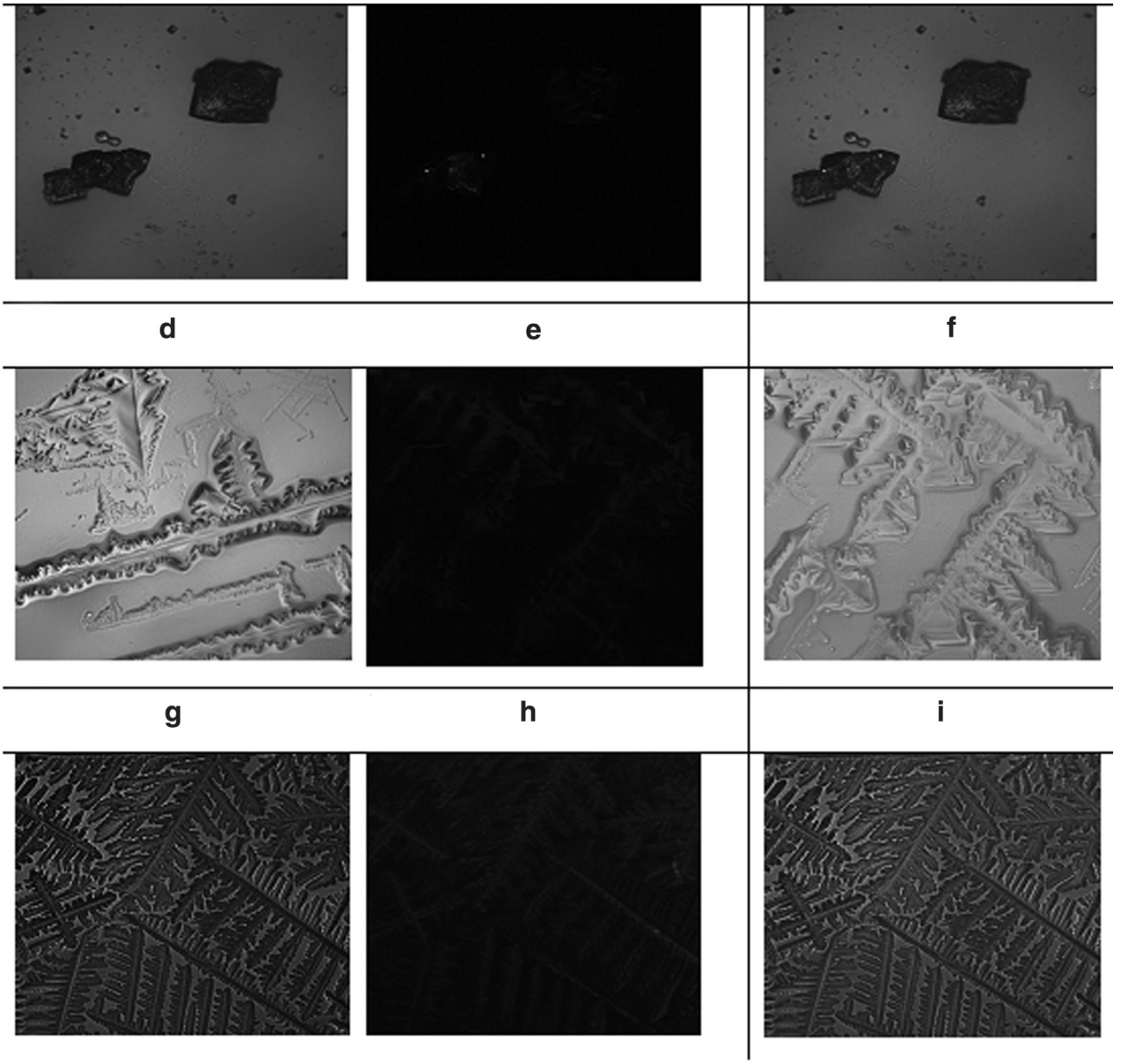

Fig. 1. Microphotographs of three CdSe/L-Cys QD samples with various pH values 1 h after preparation in various buffer solutions: PBS (a–c),Wash buffer (d–f) and Tris (g–i); the figure shows a light image (a, d, g), QD fluorescence (b, e, h), and a combination of these two images (c, f, i). The pH values: 8.0 (d–f), 7.5(a–c), 5.0 (g–i).

No morphological changes in cells indicating changes in their condition, such as nuclear vacuolation, enlargement or segmentation, were observed at prolonged QD incubation. Additionally, no cells were observed to detach from the substrate (Fig. 3).

The effect of the ZnSe:Mn/MPA QDs on the condition of the mononuclear cells (MNCs) isolated from peripheral blood (with an admixture of red blood cells) was assessed in the following way. The material was taken after 18, 24 and 48 h of QD-incubation. No morphological changes in the culture during these times of prolonged incubation with MPA QDs were revealed (Fig. 4). We may thus conclude that the studied QDs have no effect on cell morphology even in high concentrations.

The assessment of the QD cytotoxicity on the breast carcinoma line: The cytotoxicity of the CdSe/L-Cys quantum dots was assessed on the ZR-75-1 line. There is characteristically no cytotoxic effect for the time

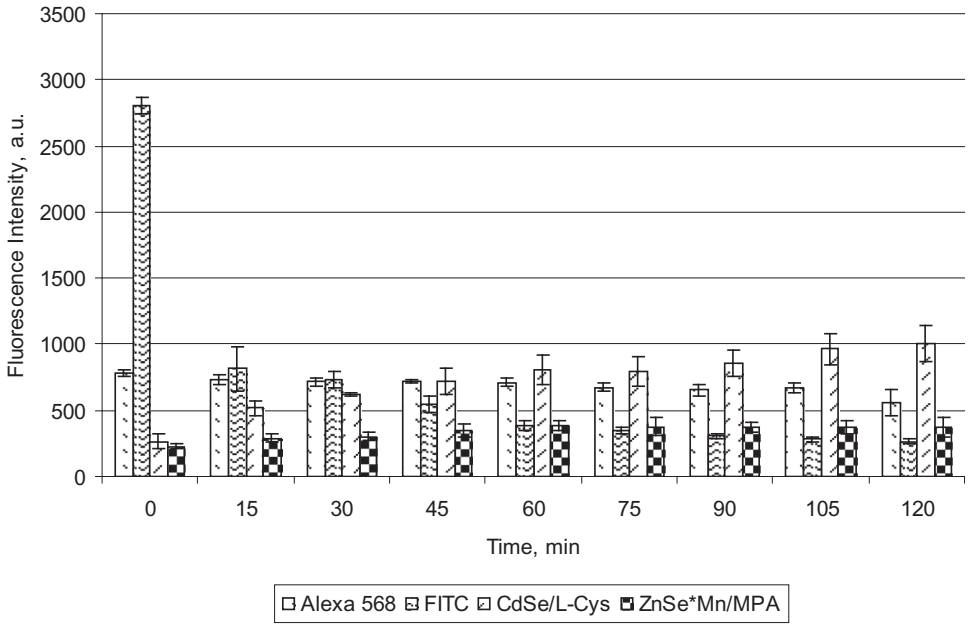

Fig. 2. A diagram showing the dynamic of fluorescence intensity of the CdSe/l-Cys and ZnSe:Mn/MPA QDs and the Alexa Fluor 568 and FITC organic dyes over time.

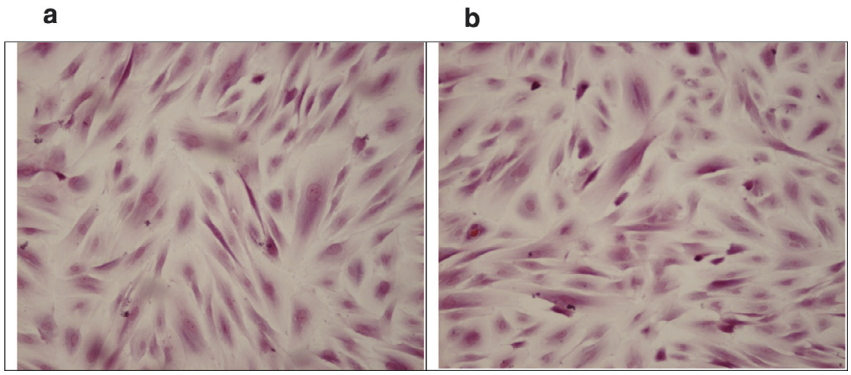

Fig. 3. The human endometrial cell culture stained with hematoxylin and eosin: (a) the control group, (b) the cells with QDs injected in a 17.5 μM/ml concentration. The image has been magnified by 400×.

intervals of 1, 3 and 18 h. After 24 h about 4 % of cells from the group injected with QDs (concentration of 17.5 μM/ml) accumulated PI, while for the two other groups these values were 3%. This result was not statistically different from the control group where the number of fluorescing cells was 2 for each 100 scanned. The number of dead cells in the ZR-75-1 culture did not exceed 3% in all control points in both the control and the experimental groups.

For a mixed MNC culture the following results were obtained: no cell apoptosis was observed in the first few hours; however, for prolonged (18 h and above) incubation of QDs with MNCs the cytotoxic effect appeared to increase, and after 48 h apoptosis reached 20%.

The increase in cytotoxicity may be connected to the destruction of the l-Cys coating in a cell culture medium. Besides, the MNCs were taken from peripheral blood where lymphocytes do not proliferate without appropriate conditions created. As a result, the cytotoxic effect for this culture may have been more pronounced than for the immortalized ZR-75-1 line.

In conclusion, the cytotoxicity was tested both on the more viable tumor cells, and on normal human cells,

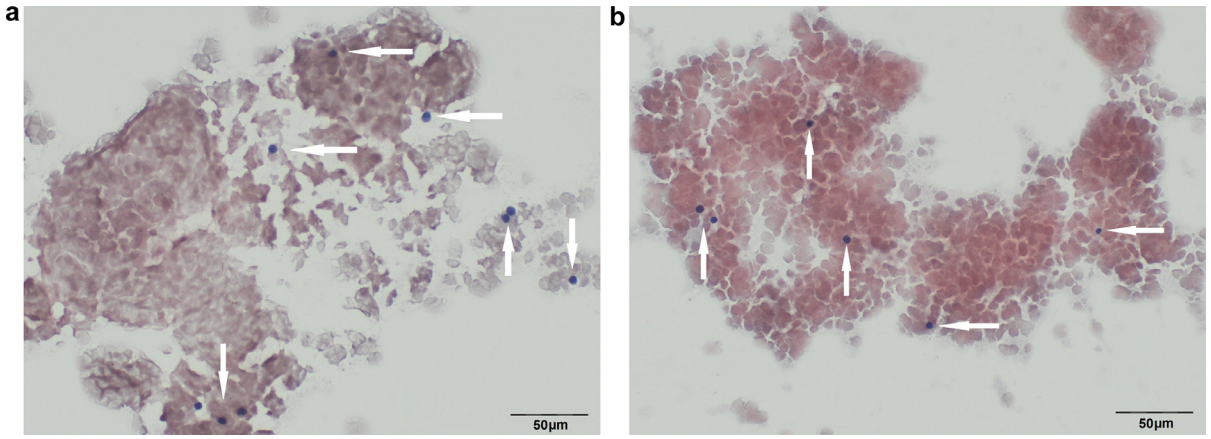

Fig. 4. The culture of human MNCs stained with hematoxylin and eosin: (a) the control group, (b) the cells with QDs injected in a 1.15 μM/ml concentration. Arrows mark the lymphocites. The image has been magnified by 100×.

which resulted in a cytotoxicity assessment approaching the in vivo conditions. In the future we plan to use this type of quantum dots to conjugate monoclonal antibodies and for applications in immunohistochemistry. All in all, the results may be considered encouraging.

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