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Original Article

Caveolae-dependent endocytosis mediates the cellular uptake of CdTe quantum dots in ovarian cancer cell lines

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Abstract

Background and purpose: Quantum dots (QDs) are semiconductor nanocrystals that are widely used in biology due to their good optical properties. QDs, especially cadmium-based QDs, play an important role in the diagnosis and treatment of cancer due to their intrinsic fluorescence. . The aim of the present study was the evaluation of the cellular uptake mechanisms of CdTe QDs in ovarian cancer cell lines.
Experimental approach: In this study, we used CdTe QDs coated with thioglycolic acid. The ovarian cancer cell lines SKOV3 and OVCAR3 were treated with different concentrations of QDs, triamterene, chlorpromazine, and nystatin, and cell viability was evaluated through the MTT test. To find the way of cellular uptake of CdTe QDs, we used the MTT test and interfering compounds in endocytic pathways. Intrinsic fluorescence and cellular internalization of CdTe QDs were assessed using flow cytometry and fluorescence microscopy imaging.
Findings / Results: The viability of CdTe QDs-treated cells dose-dependently decreased in comparison to untreated cells. To evaluate the cellular uptake pathways of CdTe QDs, in most cases, a significant difference was observed when the cells were pretreated with nystatin. The results of flow cytometry showed the cellular uptake of CdTe QDs was dose- and time-dependent.
Conclusion and implications: Nystatin had a measurable effect on the cellular uptake of CdTe QDs. This finding suggests that caveola-mediated endocytosis has a large portion on the internalization of CdTe QDs. According to the results of this study, CdTe QDs may have potential applications in cancer research and diagnosis.

Keywords: CdTe QDs; Cellular uptake; Endocytosis; Ovarian cancer.

INTRODUCTION

Nanoparticles (NPs) have many applications from agriculture to medicine. NPs can be used for targeted drug delivery, screening of disease, and tissue engineering (1,2). NPs include polymeric nanoparticles, liposomes, dendrimers, micelles, and inorganic nanoparticles (3). The effect of nanotechnology on medicine is important. NPs, improve diagnosis, treatment, and monitoring of biological systems. This application of nanotechnology in medicine is named nanomedicine (4). Quantum dots (QDs) are nanosized particles (2-10 nm) (5), that are often produced of atoms from groups II-VI or III-V elements in the periodic table (6). The properties of QDs include adjustable emission spectra, resistance to photobleaching, and high quantum yield (7). The unique optical properties of QDs make them a good choice for laboratory imaging (8). Cancer is the main cause of death worldwide (9). Ovarian cancer is one of the deadliest malignancies among gynecological diseases (10).

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The lack of proper screening methods and imaging techniques in the early stages of the disease are the main reasons for the late diagnosis of this cancer (11). Wang et al. utilized QDs with a maximum emission wavelength of 605 nm (QD 605) to discover CA125 in ovarian cancer specimens of different types (fixed cells, tissue sections, and xenograft tumors) with high specificity and sensitivity (12). To use QDs, the first issue is the internalization of these nanomaterials into cells and occurs through various mechanisms. When NPs reach the external plasma membrane of a cell, they can internalize the cell, predominantly *via* endocytosis (13). Clathrin-mediated endocytosis occurs in an area of the plasma membrane that has numerous clathrin (14). Caveolae-dependent endocytosis is an important process in many biological processes (*e.g.* cell signaling) and a variety of diseases (cancer, diabetes, etc.) (13). Caveolae is an area of the plasma membrane that is rich in cholesterol and is characterized by the presence of caveolin protein (15). Macropinocytosis is an actin-dependent process for extracellular fluid retrieval and is very similar to other endocytic mechanisms such as clathrin-mediated endocytosis. This pathway plays a dedicated role in normal cells, treatment of various diseases, and drug delivery (16-18).

Early diagnosis of cancer and targeted drug delivery are vital issues. The malignant tumors are often diagnosed at advanced stages when a high dose of chemotherapeutic drugs increases the risk of their side effects. Currently, most drugs are produced to bind to specific receptors, but they don't have selectivity for specific sites in the human body, i.e., specific cells, tissues, or organs, because the receptors may be expressed at various sites of the body. Nanoparticles are very promising for targeted drug delivery to a specific site in the human body because they can deliver the drug to specific places in the human body by having a targeting sequence (19). The aim of the present study was the evaluation of the cellular uptake mechanisms of cadmium telluride (CdTe) QDs in ovarian cancer cell lines.

MATERIALS AND METHODS

Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) dye and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co, Germany. Culture media and growth supplements were obtained from Gibco, Germany. Nystatin was obtained from Jaber Ebne Hayyan Pharmaceutical Co, chlorpromazine from Tehran Chemie Pharmaceutical Co., and triamterene from Tehran Darou Pharmaceutical Co. All drugs were pharmaceutically formulated.

Synthesis of CdTe QDs

In this study, we used CdTe QDs, coated with thioglycolic acid, prepared following our previously described protocols (20). Before using the QDs, we added acetone to them, then centrifuged, washed, and re-diffused them in the initial volume of deionized water (20).

Cell culture

Human ovarian cancer cell lines, SKOV3 and OVCAR, were purchased from the National Cell Bank of Iran (NCBI-Pasture Institute of Iran). The cells were cultured in RPMI-1640. Media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, and 100 µg/mL streptomycin and were incubated in a humidified atmosphere of %95 air and 5% CO_2 at 37 °C.

Cell viability assay

To assess the percentage of cell viability and the cytotoxic of CdTe QDs to the biological system, we seeded 6000 cells/well in 96-well culture plates and incubated them overnight for cell attachment. The cells were treated with different concentrations of CdTe QDs (0.01, 0.1, 1, 10, 50, and 100 µM) for 48 h. Then, 20 µL of MTT solution (5 mg/mL) was added and incubated for 4 more hours at 37 °C. Then, the supernatant was removed and 200 µL DMSO was added to each well. Absorbance was read by the microplate reader (Biotek, Synergy, USA) at 570 nm.

To assess the effect of nystatin, triamterene, and chlorpromazine on cell viability, the cells were treated with different concentrations of each drug separately (1, 5, 10, 15, 20, 25, and 50 µg/mL) for 24 h in the same way.

CdTe QDs uptake studies

To determine the mode of uptake, we seeded cells (6000 cells/well) in 96-well culture plates overnight to attach the cells to wells. Then, the

cells were pretreated with nystatin, triamterene, and chlorpromazine at different concentrations of each drug separately, 1 h before QDs treatment. In the next step, the cells were treated with QDs (0.1, 1, 10, and 50 µM) and were incubated for 24 h. Then, 20 µL MTT solution (5 mg/mL) was added to each well and the cells were incubated for 4 h at 37 °C. Then, the supernatant was removed and 200 µL DMSO was added to each well. Absorbance was read by the microplate reader at 570 nm.

CdTe QDs uptake evaluation by flow cytometry

Based on the fluorescence of CdTe QDs, the uptake of QDs in cells was measured by flow cytometry. To study the effect of QDs concentration and incubation time on the internalization of QDs in cells, the cells were seeded in 6-well culture plates (~ 600,000 cells/well) and were treated with different concentrations of CdTe QDs for 3, 6, 12, and 24 h. After incubation time, cells were washed with phosphate-buffered saline and were collected by trypsinization. Finally, cells were analyzed by a flow cytometry system (FACSCalibur, Biosciences, CA, USA).

Fluorescence microscopy imaging of CdTe QDs

Based on the fluorescence of CdTe QDs, fluorescence images were obtained by an inverted fluorescence microscope. To capture Fluorescence images, the cells (~ 500,000 cells/well) were seeded in 6-well plates and were treated at different concentrations of CdTe QDs for 0.5-15 h.

Statistical analysis

The one-way analysis of variance (ANOVA) followed by Tukey post hoc test was used to analyze the data by GraphPad Prism GraphPad software, Inc, La Jolla, CA. The results were represented as means ± SD and *P* values < 0.05 were regarded as statistically significant.

RESULTS

Characteristics of CdTe QDs

In this study, we used CdTe QDs coated with thioglycolic acid. As shown in Fig. 1A, the absorption edge occurs at ~ 540 nm. The spectrum of QDs is shown in Fig. 1B and the maximum emission peak occurs at ~ 570 nm.

Cytotoxic effects of CdTe QDs on the ovarian cancer cell lines

Both cell lines (SKOV3 and OVCAR3) were treated with different concentrations of CdTe QDs (0.01-100 µM) and the percentage of cell viability was calculated. Both cell lines showed a decrease in cell viability in a dose-dependent manner. As shown in Fig. 2, the percentage of SKOV3 and OVCAR3 cells' viability in presence of CdTe QDs at a concentration of 0.01 µM were approximately 96% and 98%, respectively. The cells' viability decreased significantly at concentrations more than 0.01 µM and reached about 25% and 20% in SKOV3 and OVCAR3 at the highest concentration of CdTe QDs (100 µM), respectively.

SKOV3 and OVCAR3 cells were treated with various concentrations (1, 5, 10, 15, 20, 25, and 50 µg/mL) of nystatin, triamterene, and chlorpromazine (separately), and then cell viability was measured.

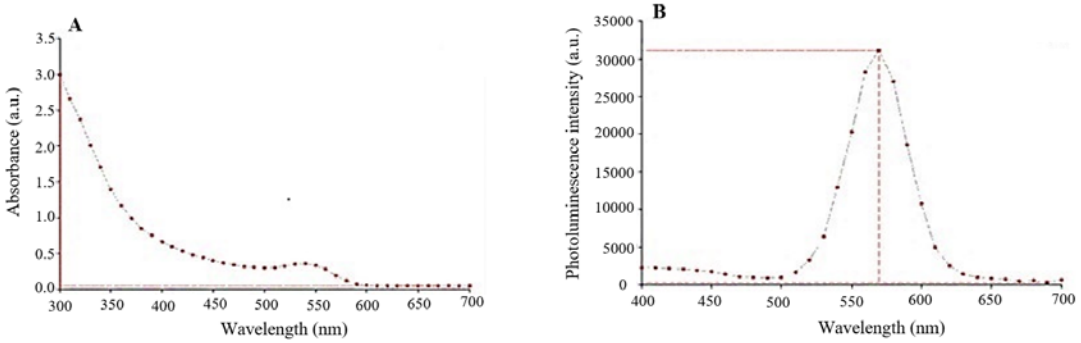

Fig. 1. (A) optical absorbance and (B) photoluminescence spectra of CdTe QDs. These results were obtained under excitation with ultraviolet light (360 nm). The absorption edge occurs at ~ 540 nm (A), and the maximum emission peak occurs at ~ 570 nm (B). CdTe, Cadmium telluride; QD, quantum dot.

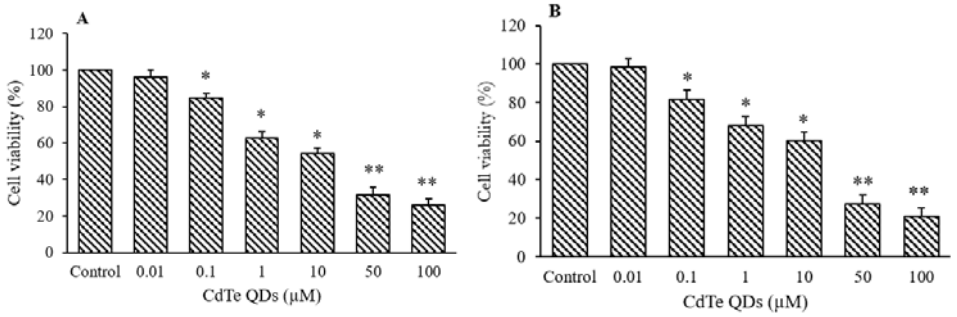

Fig. 2. Effects of CdTe QDs on the viability of (A) SKOV3 and (B) OVCAR3 cells were analyzed by MTT assay. Cell viability was measured as a percentage of untreated cells (control). Data are expressed as mean ± SD, each point represents four independent experiments performed in quadruplicate. *P < 0.05 and **P < 0.01 show a significant difference compared to the control group. CdTe, Cadmium telluride; QD, quantum dot.

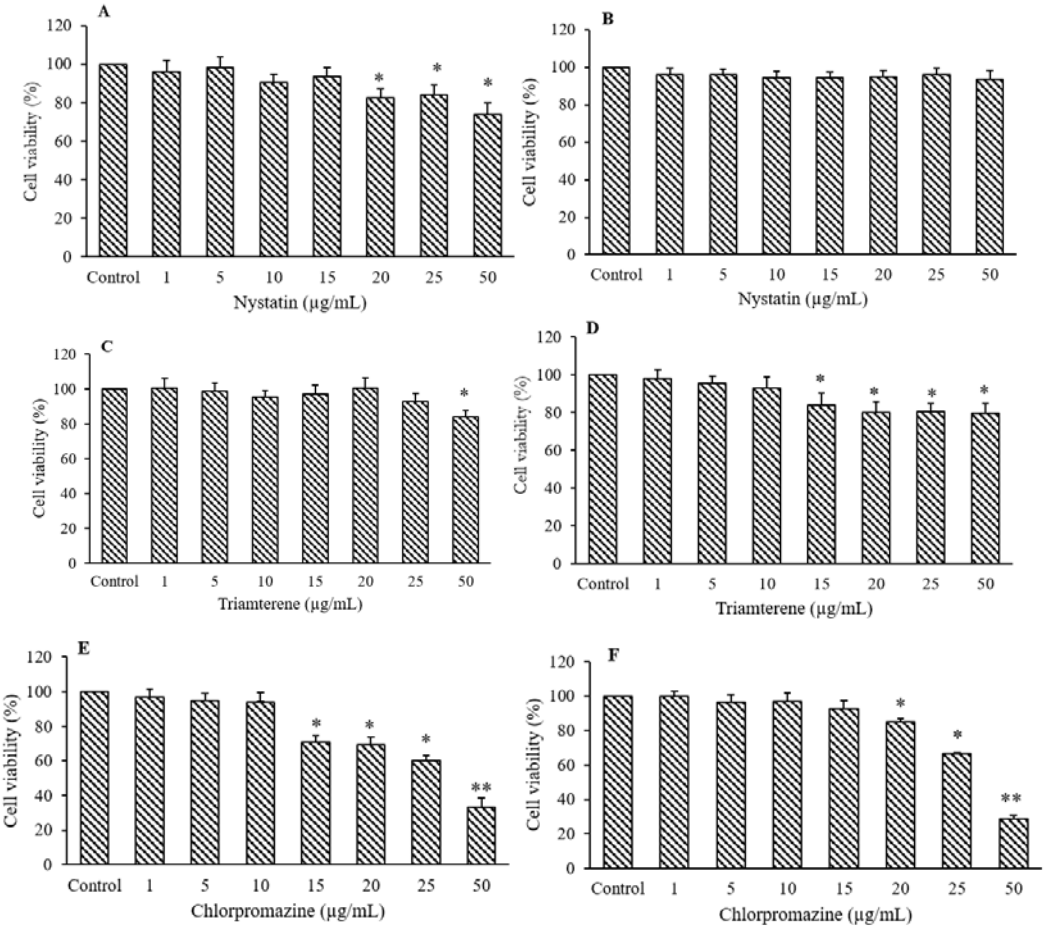

Fig. 3. Effects of nystatin, triamterene, and chlorpromazine on the viability of (A, C, E) SKOV3 and (B, D, F) OVCAR3 cells were assessed by MTT assay. Cell viability was calculated as a percentage of untreated cells (control). Data are expressed as mean ± SD, each point represents three independent experiments performed in triplicate. *P < 0.05 and **P < 0.01 show significant differences compared to the control group.

As shown in Fig. 3A, higher nystatin concentrations (20, 25, and 50 µg/mL) caused a significant decrease in SKOV3 cells' viability, however, the results showed that OVCAR3 cells exhibited more resistance to nystatin than SKOV3 cells (Fig. 3B). Also, Fig. 3C indicates that SKOV3 cells viability treated with triamterene remained unchanged below 50 µg/mL, but the viability of triamterene-exposed OVCAR3 cells decreased significantly from the concentration of 15 µg/mL of triamterene.

As shown in Fig. 3E and F, the viability inhibitory effect of 1, 5, and 10 µg/mL of chlorpromazine was not significant, however, the viability of cells decreased significantly at concentrations more than 10 µg/mL in both cell lines.

According to the results of the MTT test for the three substances mentioned above, we used two concentrations of 5 and 10 µg/mL of each of these substances to pretreat the cells in the next step.

Characterization of CdTe QDs cellular uptake

To determine which cellular uptake of QDs mediated by each of the endocytic pathways, we pretreated SKOV3 and OVCAR3 cells with chlorpromazine (clathrin-dependent endocytosis disrupter), nystatin (inhibitor of caveolin-dependent endocytosis), and triamterene (macropinocytosis blocker) and compared cell viability of QDS effects with relevant controls.

As shown in Fig. 4 pretreatment with nystatin reversed the dose-dependently significant cytotoxic effects of QDS in both cell lines compared to control. So, this finding shows nystatin reduced QDs uptake and indicates that a lipid raft-dependent process is involved in CdTe QDs internalization.

Figure 5 shows that the viability of both cell lines pretreated with triamterene was partially reversed by increasing the concentrations of CdTe QDs. Although at some concentrations of triamterene cell viability changed significantly, it was not dose-dependent. As shown in Fig. 6, chlorpromazine did not affect the entry of CdTe QDs into both cell lines.

However, we suggest that in rare conditions maybe macropinocytosis and caveolin-dependent endocytosis are also used for the internalization of QDs and needs more research.

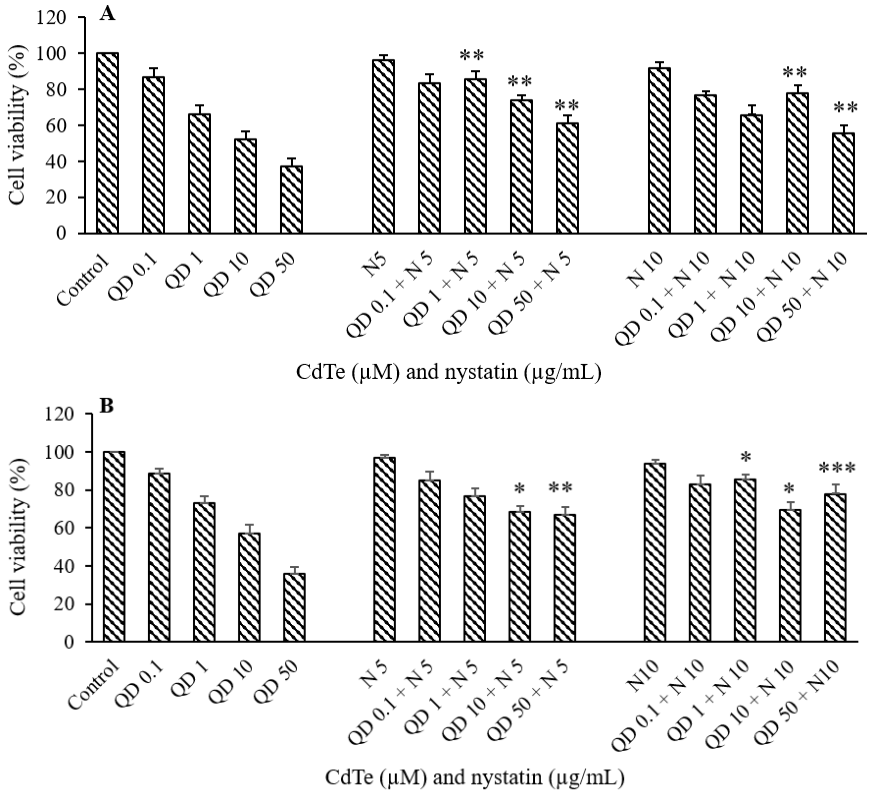

Fig. 4. Effects of nystatin (at 5 and 10 µg/mL) on the internalization of CdTe QDs (0.1, 1, 10, and 50 µM) into (A) SKOV3 and (B) OVCAR3 cells were assessed by MTT assay. Cell viability was calculated as a percentage of untreated control cells. Data are expressed as mean±SD, each point represents three independent experiments performed in triplicate. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences between the cells pretreated with nystatin + CdTe QDs with the cells pretreated with CdTe QDs alone (at the same concentration). CdTe, Cadmium telluride; QD, quantum dot; N, nystatin.

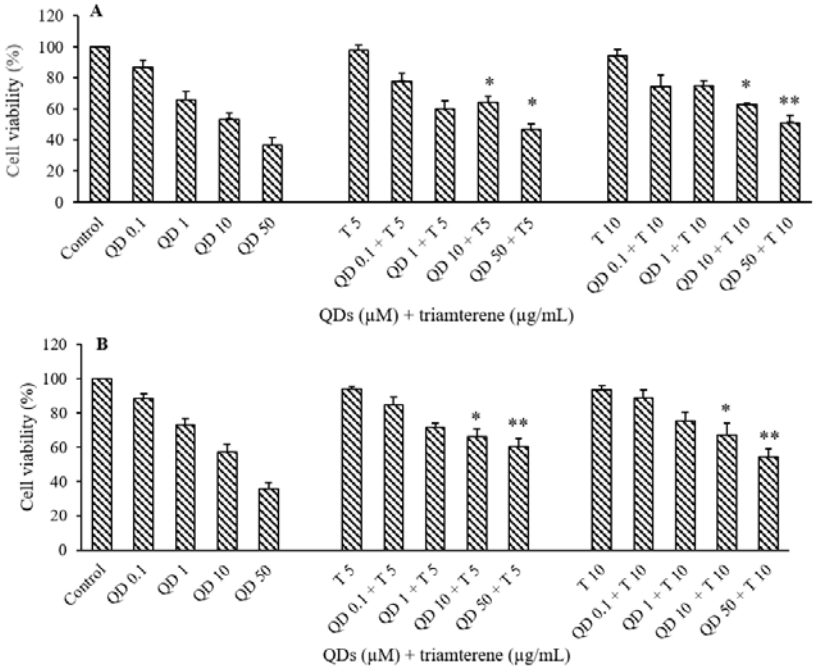

Fig. 5. Effects of triamterene (at 5 and 10 μg/mL) on the internalization of CdTe QDs (0.1, 1, 10, and 50 μM) into (A) SKOV3 and (B) OVCAR3 cells were assessed by MTT assay. Cell viability was calculated as a percentage of untreated cells (control). Data are expressed as mean ± SD, each point represents three independent experiments performed in triplicate. **P* < 0.05 and ***P* < 0.01 indicate significant differences between the cells pretreated with triamterene + CdTe QDs with the cells pretreated with CdTe QDs alone (at the same concentration). CdTe, Cadmium telluride; QD; quantum dot; T, triamterene.

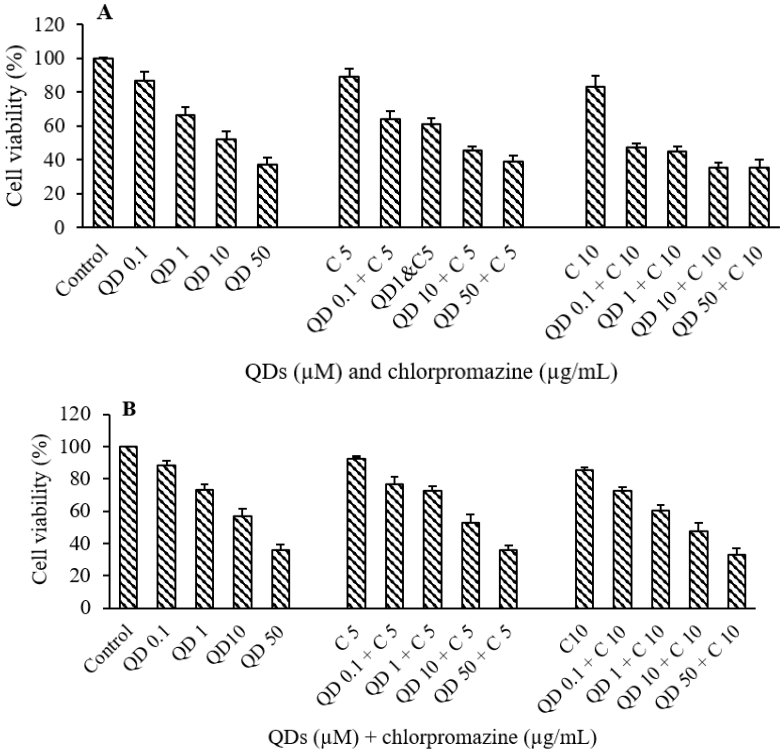

Fig. 6. Effects of chlorpromazine (at 5 and 10 μg/mL) on the internalization of CdTe QDs (0.1, 1, 10, and 50 μM) into (A) SKOV3 and (B) OVCAR3 cells were assessed by MTT assay. Cell viability was calculated as a percentage of untreated cells (control). Data are expressed as mean ± SD, each point represents three independent experiments performed in triplicate. CdTe, Cadmium telluride; QD; quantum dot; C, chlorpromazine.

Evaluation of cellular uptake of CdTe QDs

Based on the innate fluorescence of CdTe QDs, the uptake of QDs in cells was measured by flow cytometry to see the internalization by SKOV3 and OVCAR3 at different concentrations and incubation times. The flow cytometry results and analysis showed the increasing internalization of QDs in a concentration-dependent manner in SKOV3 (Fig. 7) and OVCAR3 (Fig. 9). Also, the internalization of QDs increased time-dependently. After 0, 3, 6, 12, and 24 h treatment with 100 µM QDs, the percentage of QDs-holding in SKOV3 cells was 0.37, 9.57, 27.56, 29.4, and 58.39, respectively (Fig. 8) and the percentage of QDs-holding OVCAR3 was 1.64, 10.96, 36.86, 54.7, and 73.36, respectively (Fig. 10).

Fluorescence imaging of CdTe QDs

Furthermore, we tested the internalization of different concentrations of CdTe QDs (20 and 50 µM) by ovarian cancer cell lines using live-cell fluorescence imaging in wide time intervals (1, 2, 15 h). Cellular imaging-fluorescence microscopy indicated that both cell lines uptake CdTe QDs (Figs. 11 and 12).

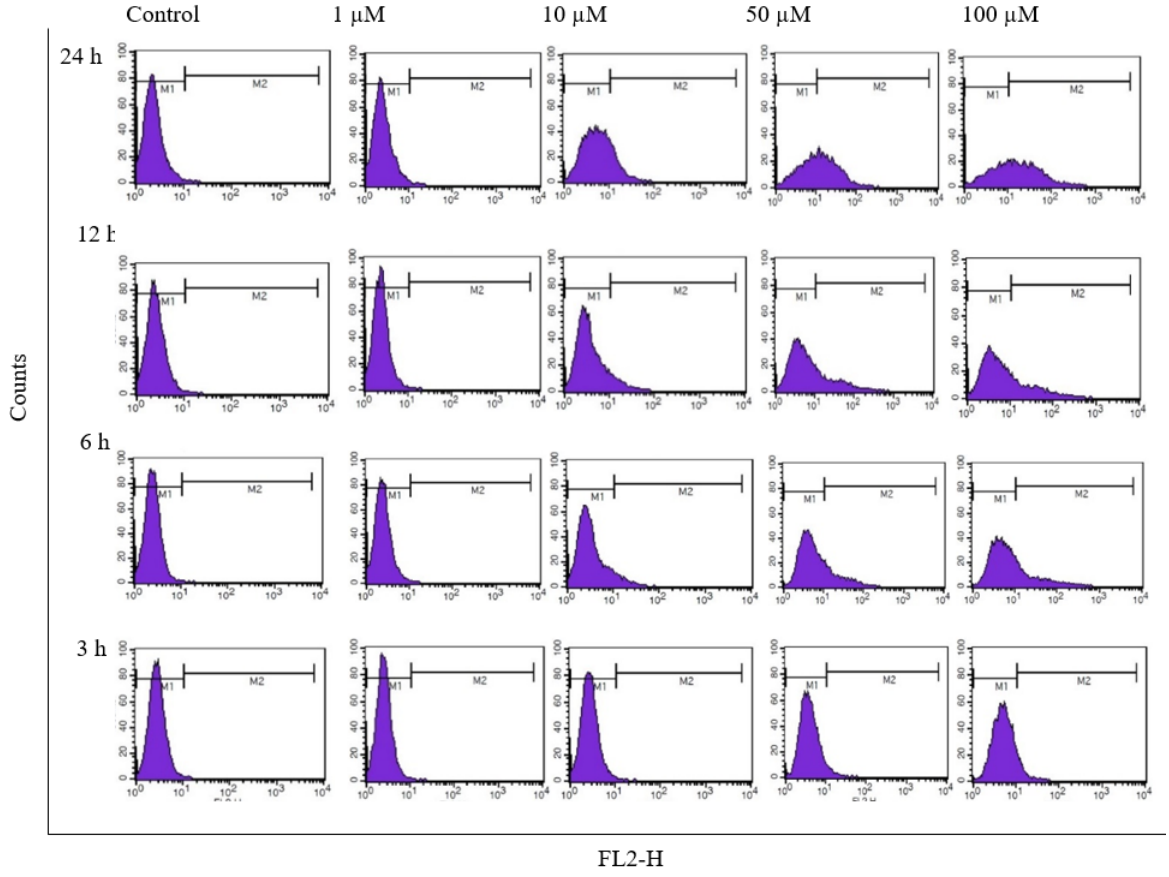

Fig. 7. Histograms of cellular internalization of CdTe QDs obtained *via* flow cytometry. The SKOV3 cells were treated with different concentrations of CdTe QDs (1, 10, 50, and 100 µM) for 3, 6, 12, and 24 h. These results were obtained on the FL-2 detector. CdTe, Cadmium telluride; QD; quantum dot.

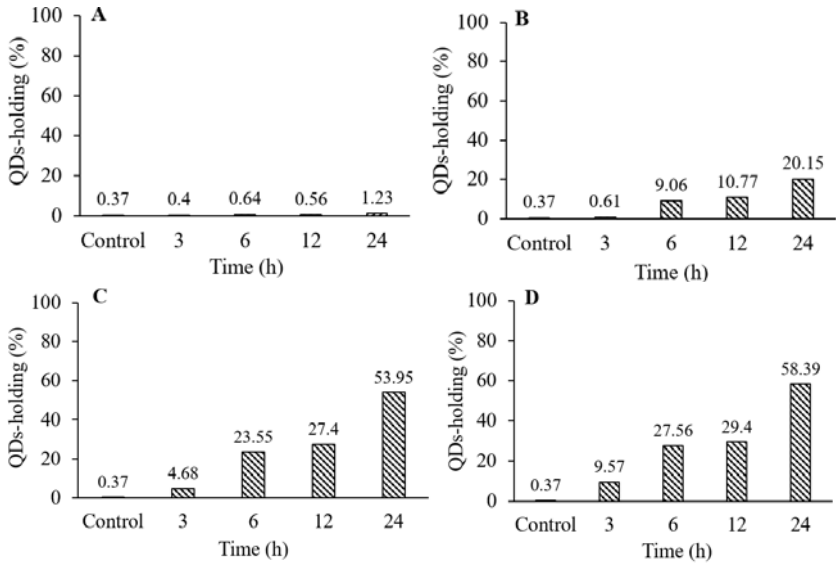

Fig. 8. Time-dependent internalization of CdTe QDs into the SKOV3 cells. The cells were treated with different concentrations of CdTe QDs: (A) 1 µM, (B) 10 µM, (C) 50 µM, and (D) 100 µM. The percentage of QDs-holding SKOV3 cells was measured on the FL-2 detector. CdTe, Cadmium telluride; QD; quantum dot.

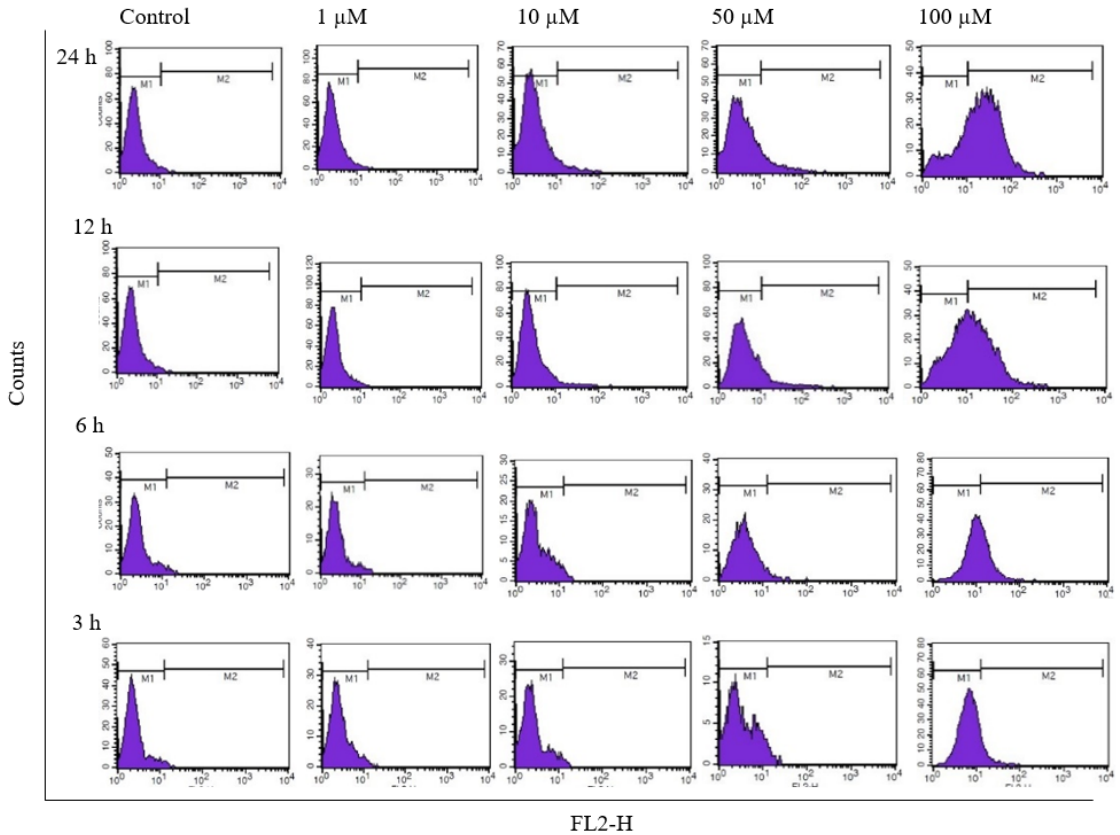

Fig. 9. Histograms of cellular internalization of CdTe QDs obtained *via* flow cytometry. The OVCAR3 cells were treated with different concentrations of CdTe QDs (1, 10, 50, and 100 µM) for 3, 6, 12, and 24 h. These results were obtained on the FL-2 detector. CdTe, Cadmium telluride; QD; quantum dot.

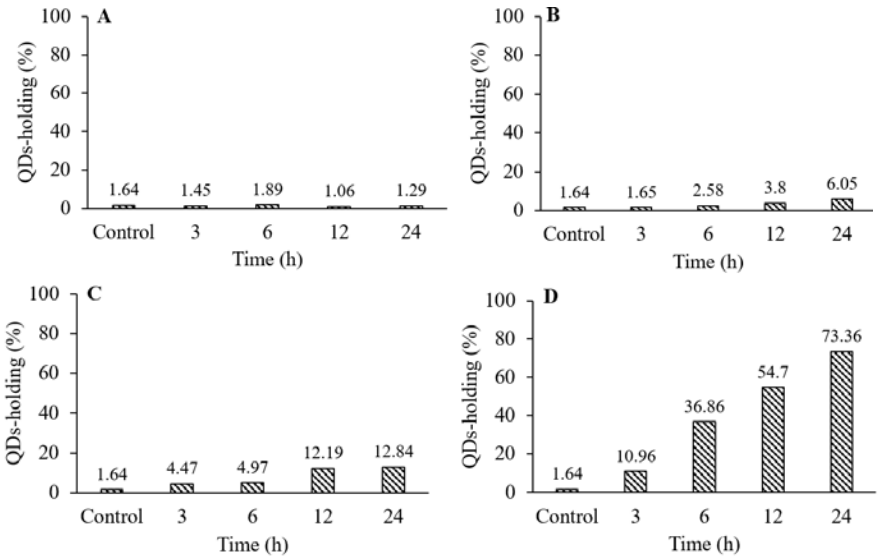

Fig. 10. Time-dependent internalization of CdTe QDs into the OVCAR3 cells. The cells were treated with different concentrations of CdTe QDs: (A) 1 µM, (B) 10 µM, (C) 50 µM, and (D) 100 µM. The percentage of QDs-holding OVCAR3 cells was measured on the FL-2 detector. CdTe, Cadmium telluride; QD; quantum dot.

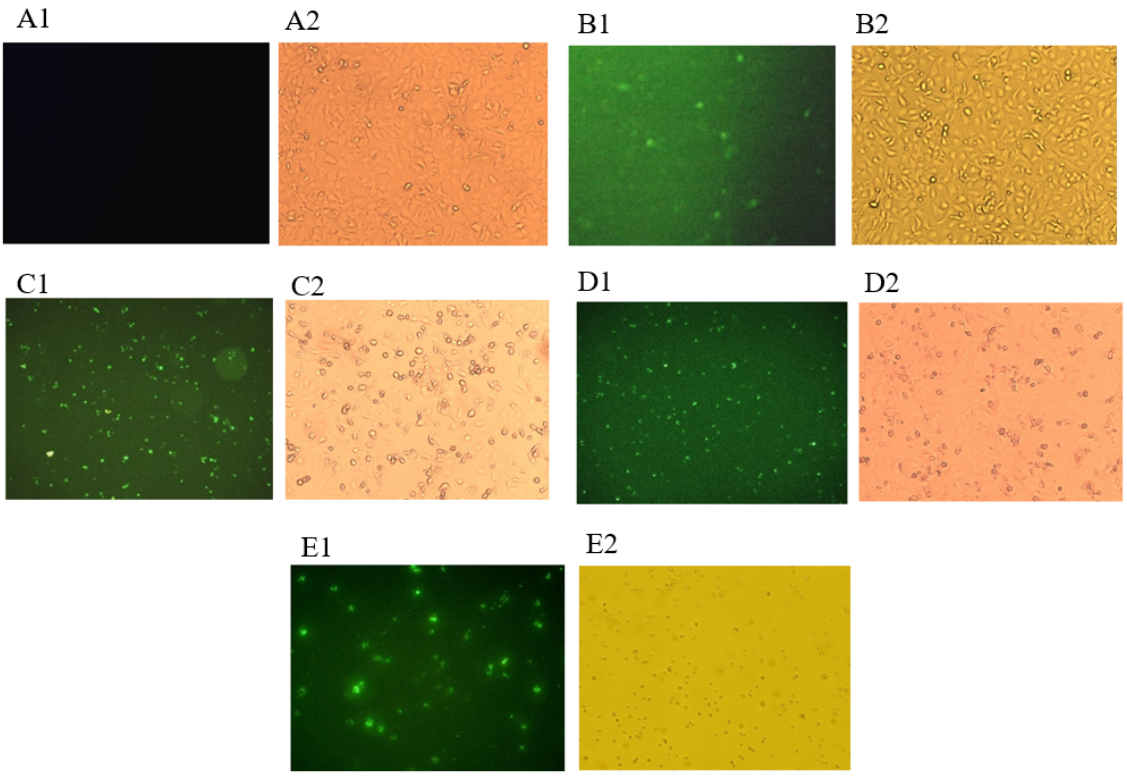

Fig. 11. Fluorescence (left) and optical (right) images. (A) Untreated SKOV3 cells as the negative control. SKOV3 cells treated with 20 µM of CdTe QDs for (B) 5 h and (C) 15 h. SKOV3 cells treated with 50 µM of CdTe QDs for (D) 5 h and (E) 15 h. CdTe, Cadmium telluride; QD; quantum dot.

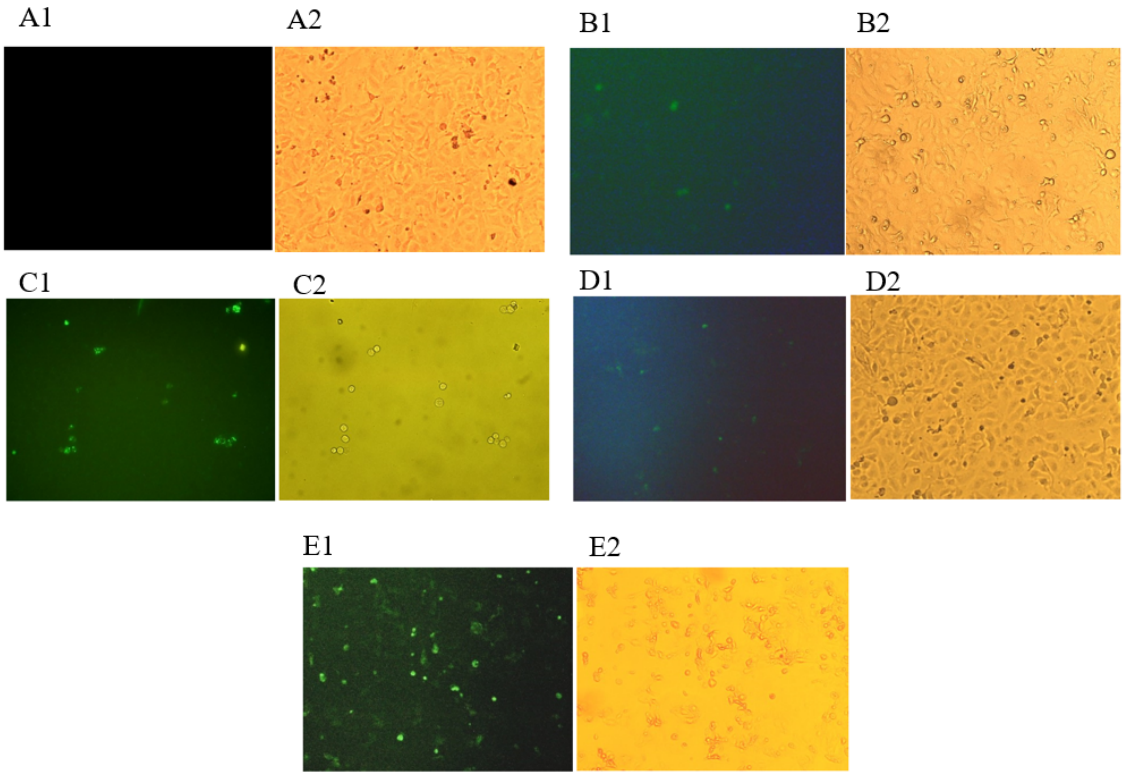

Fig. 12. Fluorescence (left) and optical (right) images. (A) Untreated OVCAR3 cells as a negative control. OVCAR3 cells treated with 20 µM of CdTe QDs for 5 h (B) 5 h and (C) 15 h. SKOV3 cells were treated with 50 µM of CdTe QDs for (D) 5 h and (E) 15 h. CdTe, Cadmium telluride; QD; quantum dot.

DISCUSSION

Due to the applications of QDs in cancer diagnosis and targeted drug delivery, specific internalization of QDs by cancer cells is desired and non-specific internalization by any cell type should be eschewed. Therefore, understanding the mechanism of QDs cellular uptake is vital to minimize unwanted non-specific cellular uptake of QDs. Unfortunately, the mechanism of cellular uptake of non-targeting QDs has been studied very little (21-23). In this study, we evaluated the cellular uptake of CdTe QDs in ovarian cancer cell lines (SKOV3 and OVCAR3) by MTT assay, fluorescence microscopy, and flow cytometry. The data showed the toxic effects of CdTe QDs on ovarian cancer cell lines, SKOV3 and OVCAR3, were in a dose-dependent manner. The MTT results are in line with previous studies. Su *et al*. evaluated the effect of CdTe QDs on human embryonic kidney cells (HEK293 cell) and realized that CdTe QDs caused cell growth inhibition dose-dependently (24). Chakraborty *et al*. studied the effect of bare CdTe QDs on lymphocytes. The MTT results showed that CdTe QDs caused dose-dependent cell death (25). Our previous study showed that CdTe QDs had an inhibitory effect on two human breast cancer cell lines MDA-MB468 and MCF-7 growth in a dose-dependent manner (26).

In this study, using ovarian cancer cell lines, we evaluated several endocytic pathways that CdTe QDs could probably be uptake into cells. Clathrin-mediated endocytosis was inhibited by chlorpromazine, caveolae-dependent endocytosis blocked by nystatin, and macropinocytosis prevented by triamterene. We observed statistically significant upon pretreatment of cells with nystatin and triamterene. Taken together, all of our data expressed that nystatin has a greater effect on the uptake of QDs into the SKOV3 and OVCAR3 cells than triamterene, however, chlorpromazine didn't inhibit the internalization of CdTe QDs into the SKOV3 and OVCAR3 cells. Thus caveolae-mediated

endocytosis and macropinocytosis play important role in the uptake of CdTe QDs into ovarian cancer cells.

Engelberg *et al*. reported that cellular uptake of QDs was a temperature-dependent process and found that the cells internalized QDs at 37 °C, but they didn't internalize them at 4 °C (27). The uptake of S15-APTs-decorated QDs into human non-small cell lung cancer A549 cells by using amiloride and filipin confirmed that pinocytosis and caveolae-mediated endocytosis didn't have a role in cellular uptake of QDs (27). To evaluate the clathrin-mediated endocytosis, they used other substances and observed that the cells didn't uptake the QDs and S15-APT QDs were internalized into A549 cells by clathrin-mediated endocytosis (Engelberg et al. studied). The PEGylated quantum dots internalization in MR90 fibroblast cells and CCD841CoN epithelial cells was reduced by monodansylcadaverine while nystatin did not affect significantly the internalization of QDs. So, the authors concluded that clathrin-dependent endocytosis is the primary mechanism for uptaking QDs (28).

To understand the mode of cadmium selenide (CdSe)/zinc sulfide (ZnS) core/shell QDs uptake by canine kidney MDCKII cells, Breus *et al*. used chlorpromazine, amiloride hydrochloride, and filipin III. They showed that the internalization of cysteamine-QDs pretreated with filipinIII was inhibited and concluded that a spontaneous uptake is responsible for the uptake of QDs (29). Also, Yan *et al.* showed the uptake of CdTe QDs into human umbilical vein endothelial cells is done by caveolae-mediated and clathrin-mediated endocytosis (30).

Here, we showed also that the internalization of CdTe QDs into the SKOV3 and OVCAR3 cells increased both concentrations- and time-dependently. Engelberg *et al*. found internalization of S15-APT QDs into A549 cells was a time-dependent process (27).

QDs are a good option for cancer imaging because of their adjustable optical properties including a long fluorescence lifetime, a large Stokes shift, narrow emission band, etc. (31).

The fluorescence images confirmed the uptake of CdTe QDs into cells and the CdTe QDs were found to be internalized by SKOV3 and OVCAR3 cells, thereby highlighting their potential to be used as an optical probe for biomedical diagnostics and labeling applications. Han *et al*. reported similar results. They treated the breast cancer cells (SK-BR3) with herceptin-conjugated CdSe/ZnS QDs and observed fluorescence after 1 h. Slightly, a higher fluorescence image was captured after 3 h. An intense fluorescence image was observed after 6 h (32). The QD-Her bound specifically to the membrane of SKBR3, which became almost saturated after 6 h incubation. This suggests that the growth signal of breast cancer cells is inhibited completely by the specific binding of herceptin to the Her-2 receptor of the SK-BR3 membrane, resulting in cell death (32).

To obtain high-contrast cancer imaging and labeling applications, Vibin *et al*. used silica-coated CdSe QDs. They obtained high-contrast images from the *in vitro* study on stem cells and cancer cells and *in vivo* study (33). Internalization of glutathione-CdSe/ZnS QD in BON cells evaluated by fluorescence microscopy after 4-24 h of incubation with 0.2 µM or 0.4 µM QDs, reflecting dose- and time-dependency of the QD-uptake (34).

Tang *et al*. used rapid chelator-free radiolabeling of QDs for *in vivo* imaging and reported that ZnS QDs can be rapidly radiolabeled with 68Ga or 64Cu through cation exchange without chelators. They obtained in vivo PET/CT images of 64Cu-QD-OCH3 and 64Cu-DOTA distribution in mice bearing 4T1 breast tumors. 64Cu-QD-OCH3 or 64Cu-DOTA were intravenously injected into tumor-bearing mice. Animals were then imaged by a PET/CT scanner under isoflurane anesthesia at 3 h and 24 h post-injection (35).

CONCLUSION

Our results based on cytotoxicity, fluorescence imaging, flow cytometry, and endocytosis inhibitors suggested that caveolae-mediated endocytosis has a large contribution on the internalization of CdTe QDs. These preliminary findings opened up new possibilities for overcoming the limitations that currently exist in the delivery of various nanomaterials to living cells. However, more studies are needed to understand the molecular mechanism underlying cell uptake.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

Z. Asadian performed the experiments and first manuscript writing; M. Panjehpour and M. Agahei supervised the project; H. Zare synthesized cadmium-telluride QDs and advised the project. The final version of the manuscript was approved by all authors.

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