BIOLOGICAL APPLICATIONS USING CDSES QUANTUM DOTS

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Abstract- We prepared a new CdSeS quantum dots (QDs) and modified them by poly(maleic anhydride-alt- 1-tetradecene) (QDs-COOH). QDs-COOH were functionalized with folic acid conjugation by EDC/NHS (QDs-FA). QDs were also linked with MPG by electrostatic interactions (QDs-MPG). QDs were firstly modified with Dimethylamino propylamine (QDs-modified) and then using for delivering siRNA into cancer cells (QDs-siRNA). The obtained QDs-FA performed significant affinity and specificity to cancer cells. Moreover, the competitive experiment also verified QDs-FA enter cancer cells through folate-receptor mediated endocytosis pathway. The QDs-MPG particles exhibit high penetration and stable, rapid enter into living cells. QDs-siRNA were able to rapidly penetrate cell membranes and slowly disrupt endosomal organelles because of multivalent amine groups coated on them. We envision that this nanocarrier will enable new developments in cancer cells targeting, living cells entering and siRNA delivery.

Index Terms- Quantum dots, Folic acid, Cell-penetrating peptide, siRNA delivery

I. INTRODUCTION

Semiconductor nanocrystals, also Known as quantum dots (QDs), have quickly grown to a new class of probes in biological and medical field [1]. Compared to conventional organic fluorophores, QDs are more stable that allowing long-time course studies without photobleaching, have narrow and symmetrical emission bands, broad and continuous absorption spectra; When using a single laser source, QDs can excite the fluorescence of nanocrystals emitting in different colors; Higher quantum yields, broad color tuning of the emission from the visible to the near infrared, et al [2].

The important development for QDs in biomedical applications is the selective targeting of QDs to tumor cells. Folic acid has emerged as an optimal targeting ligand for selective delivery of attached imaging and therapeutic agents to cancer tissues and sites because animal tumor cells lack key enzymes of the folate biosynthetic pathway depended on which they survival and proliferation [3]. Folic acid receptor (FR) overexpressed in several human cancer cells (e.g. ovary, breast, kidney, brain, endometrium, colon and liver) [4]. Exploration of the utility of the FR revealed its promising potential for targeting with folate-based fluorescent markers.

MPG peptide is a newly appeared peptide vector which is chemically stable, highly soluble in physiological buffers and rapidly transmembrance transport. Unlike lipid-derived and other deliver system, entry mechanism of MPG is independent of receptor-mediated and endocytic pathways [5]. The procedure of delivery involves MPG peptide interacts with QDs by the hydrophobic domain of MPG, QDs-MPG inserts into the lipid membrane of cells to create a pore-like structure, QDs-MPG particles internalized and QDs decoupled from the complex. MPG peptide can overcome the shortages such as sensitivity to cell culture media, cytoxicity, and avoids the endocytic pathway, thereby preventing endosomal or lysosomal degradation of QDs.

RNA interference (RNAi) is a post-transcriptional mechanism whereby short stretches less than 30 nucleotides of double-stranded RNA with sequences complementary to a gene sequence suppress the activity of the gene by bringing about the degradation of the corresponding messenger RNA, thus blocking the translation of the mRNA into proteins [6]. The immense therapeutic potential of RNAi has raised great research interest and efforts in the different strategies when deliver siRNA into mammalian cells. However, therapeutic applications of siRNAs have been hindered by lack of suitable vehicles. This remains a major hurdle for RNAi therapy. Therefore, there is an impetus to finding a safe, nonviral, highly efficient and tracking vehicle such as QDs for delivering siRNA into mammalian cells.

In this paper, we prepared water-soluble particles by entrapping hydrophobic TOPO-coated CdSeS QDs by poly(maleic anhydride-alt- 1-tetradecene) which we refer to as QDs-COOH. The QDs-COOH comprised a large fraction of carboxyl on the surface of QDs. QDs-COOH were functionalized with folic acid conjugation by using of EDC/NHS (QDs-FA). QDs were also linked with MPG by electrostatic interactions (QDs-MPG). QDs were firstly modified with Dimethylamino propylamine surface coatings (QDs-modified) and then using for delivering siRNA into cancer cells (QDs-siRNA). The obtained QDs-FA performed significant affinity and specificity to cancer cells when the QDs-COOH had no such efficacy. Moreover, the competitive experiment also verified QDs-FA enter cancer cells through folate-receptor

mediated endocytosis pathway. The QDs-MPG particles exhibit high penetration and stable, rapid enter into living cells. QDs-siRNA were able to rapidly penetrate cell membranes and slowly disrupt endosomal organelles because of multivalent amine groups coated on them. In this paper, we have demonstrated that QDs can be a useful fluorescent vector for cancer cells marking and anti-cancer drug delivery.

II. MATERIALS AND METHODS

Chemicals. All chemicals were used directly without further purification: Cadmium oxide (CdO, 99.0%, Shuanghuan Weiye Reagent Co.), sulfur powder (S, 99.5%, Sinopharm Chemical Reagent Co.), selenium powder (Se, 99.95%, Meixing Chemical Reagent Co.), oleic acid (OA, 97%, Kermel Chemical Reagent Co.), tri-n-octylphosphine (TOP, 99%, Fluka), tri-n-octylamine (TOA, 97%, Yuanyue Chemical Co.), folic acid ($>$ 96%, Acros),dimethyl sulfoxide (DMSO, 99%, Sigma-Aldrich), poly(maleic anhydride-alt-1- tetradecene) (PMA, Sigma-Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 98%, Amresco), chloroform and ethanol were purchased from Beijing Chemical Co. Ultrapure water (18.2 MΩ/cm) was used in the experiments.

Instruments. Absorption spectra were acquired with a TU-1901 UV-vis spectrophotometer (Beijing Puxi Tongyong Co.). PL spectra were recorded on an F-4500 fluorescence spectrophotometer (Hitachi). Differential interference contrast (DIC) and fluorescent imaging were performed on an IX 71 inverted microscope with a 60×oil immersion objective (NA 1.45; Olympus), equipped with an EM-CCD (iXon DV-887; Andor). The digital images were collected and analyzed by using IP Lab software (BD Biosciences Bioimaging).

Preparation of Water-soluble QDs. The CdSeS QDs were prepared by the method as described in the literature [1] with minor modifications. Typically, CdO(0.05 g), oleic acid(OA, 0.46 g), and tri-n-octylamine(TOA, 15 mL) were mixed in a three-necked flask and heated to 300 ^{o}C under an argon atmosphere to get a clear solution. A stock solution of Se (0.0021 g) and S (0.0124 g) in 1.0 mL TOP was swiftly injected into the hot solution of CdO/OA/TOA and allowed the reaction to proceed at 280 ^{o}C for 1 min . The CdSeS QDs (λ_{em}, 576 nm) were precipitated out of the reaction mixture by ethanol. Then 89.6mg of Poly (maleic anhydride alt-1-tetradecene) which was dissolved in 6 ml chloroform added to 1mg obtained CdSeS QDs and agitated gently for 1 hour in the dark. Rotary evaporated the mixed solution by Rotavapor equipment (BUCHI ROTAVAPOR R-205) to remove the chloroform and resuspend in 5ml TBE buffer containing 27mg Tris, 13.75mg Borate and 0.5×10^{-3}mM EDTA. The impurities were removed through volume exclusion column (Sephadex G75) and 2000g 10min ultrafiltration. Measure the UV-Visible absorption and fluorescent spectra before and after modifying QDs.

Preparation of QDs-FA. CdSeS quantum dots wrapped with a layer of Poly (maleic anhydride alt-1-tetradecene), so lots of free carboxyl groups are present on each quantum dot. To 1 mg/ml QDs-COOH (in 0.1 M PBS, pH 7.4) , 2 mM EDC solution (in 0.1 M PBS, pH 7.4) and 5 mM sulfo-NHS solution (in 0.1 M PBS, pH 7.4) were added and stirred for half an hour. Then, 10 mg/ml FA solution (in 0.1 M PBS, pH 7.4) was added and stirred for 2 hours at room temperature. After the reaction, the impurities were removed through volume exclusion column (Sephadex G75). Then centrifuged with 5000 g/min for 10min and then centrifuged with 50 KD ultrafiltration membrane 2000 g/min for 5 min. Then resuspended the QDs-FA solution into deionized water. Measure the particles diameters by DLS before and after linking QDs with FA.

Preparation of QDs-MPG. CdSeS quantum dots wrapped with a layer of Poly (maleic anhydride alt-1-tetradecene), so lots of free carboxyl groups are present on each quantum dot. Then added peptide with modified water-soluble quantum dots (QDs-COOH) (20:1 molar ratio) together. The mixed solution was stirred at room temperature for 2 hours. After the reaction, the mixed solution was centrifuged with 5000 r/min for 10min and then centrifuged with 50 KD ultrafiltration membrane 2000 r/min for 5 min. The solution above was ultrafiltration repeatedly to remove unreacted peptide. Then resuspended them in deionized water. Measure the UV-visible absorption and fluorescent spectra before and after linking QDs with peptide.

Agarose gel electrophoresis. QDs-COOH and QDs-MPG were electrophoresed on 1 % agarose gel for 60 min at 100 V. QDs' signals were visualized by UV excited. MPG peptide was dyed by Coomassie. Electrophoresis images of QDs-MPG, QDs-COOH and MPG were taken using digital cameras (Nikon).

Fluorescence titration. Fluorescence titration was performed on an F-4500 fluorescence spectrometer (Hitachi). The intrinsic tryptophan fluorescence of MPG was excited at 290 nm and the emission spectrum was recorded between 310 to 380 nm, with a spectral bandpass of 5 nm for both excitation and emission. The concentration of MPG is $1X10^{-6}$ M was titrated by QDs-COOH 50 nM at 25 ^{o}C in PBS (pH 7.4).

Preparation of QDs-siRNA. Water-soluble red fluorescent CdSeS QDs were synthesized as above method and then modified with Dimethylamino propylamine. To measure the conjugation efficiency of HER2 siRNA onto QDs, QDs was incubated with

different amounts of siRNA to achieve QDs/siRNA molar rations of 1:1, 1:5, 1:10, 1:20. Fluorescence imagings were used to measure conjugation efficiency.

In Vitro Cytotoxicity Measurement. Briefly, HeLa cells were trypsinized and resuspended in Dulbecco's modified Eagle's medium(DMEM) containing fetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%). The cells were seeded at a density of 10000 cells per well in a 96-well plate. After 24 h of incubation at 37 ℃ with 5% CO_2 (the following steps were carried out under the same conditions), the cells were washed with phosphate buffered saline (PBS, 0.01 mol/L, pH 7.4). The QDs-COOH, QDs-FA, QDs-MPG and QDs-modified solutions with different concentrations (10μL, 0.1-5.0 mg/mL) and DMEM (90μL) were added to the wells. After 24 h of incubation, the supernatant was removed, and the cells were washed with PBS for three times.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu m bromide) solution (10 μL, 0.5 mg/mL) and DMEM (90 μL) were then added to each well. After 4 h of incubation, the medium was discarded, and the intracellular water-insoluble formazan blue was collected by DMSO (100 μL). The optical absorbance was measured at 570 nm on a BioRad model 550 microplate reader. Each data point was collected by averaging that of six wells, and the untreated cells were used as controls. The data analyses were conducted by Student's t-test. Differences with P values < 0.05 were considered to be statistically significant.

III. RESULTS AND DISCUSSION

Folic acid-mediated CdSeS quantum dots for Lewis lung cancer cells imaging. In this work, we used the alloyed CdSeS QDs as the fluorescent cores, whose fluorescent emission could be precisely tuned in the visible range (from 450 nm to 620 nm) by changing the ratio of chemical components. The prepared CdSeS QDs were passivated by organic ligands such as TOPO and OA and thus well-dispersed in organic solvents. After cross linking the layer of poly (maleic anhydride-alt- 1-tetradecene), the prepared CdSeS (QDs-COOH) to transferred into aqueous solutions. The excessive EDC and NHS to the QDs-COOH forms an active ester leaving group, which can react subsequently with free amino group present in folic acid, as shown in figure 1, to form the resulting QDs-FA. The optical characteristics of the QDs and QDs-COOH were examined using absorption and photoluminescence. There was nearly no shift of absorbance peak of QDs-COOH compared to that of free QDs in chloroform. The photoluminescence emission peak at 576 nm of the QDs-COOH is close to that of the same QDs in chloroform. QDs-COOH displayed a symmetrical fluorescent spectrum (λ_{max}=576 nm) with full width at half maximum (FWHM) less than 30 nm (Fig. 1b). The QDs-COOH solution stored for 6 months at room temperature did not observe aggregation or any change in optical properties.

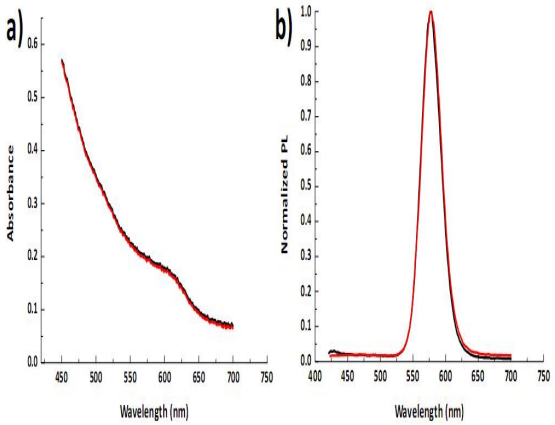


Fig. 1. Absorption (a) and emission spectra (b) of QDs (black line) and QDs-COOH (red line) in chloroform and water solution.

We incubated the Lewis Lung cancer (LLC) cells with an appropriate amount of QDs-FA at 37°C then washed the dishes carefully before imaging. The binding of QDs-FA to Lewis cancer cells was efficient and rapid occurring at the concentration of 50 nM at 37°C. No concentration- dependent increases uptake was noted with QDs-FA when added 100 nM and 200 nM QDs-FA into cell culture dishes. Figure 2 a-h shows the fluorescent images of the LLC cells after 1 h, 3 h, 9 h and 12 h of incubation with QDs-FA. QDs-FA attached to LLC cells immediately that fluorescence signals of QDs around cells were visualized clearly within 1 h. With the increase of time intervals, the uptake of the QDs-FA is gradually increasing as observed by a gradual increase of fluorescence intensity of QDs within the cells.

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The maximum increase in cell fluorescence was observed after the cells were incubated with QDs-FA at 9 h. To support the folate-receptor-mediated endocytosis of QDs-FA, control group that QDs-COOH lacking of FA (Fig. 2i), were imaged under similar conditions. The results showed nearly no uptake appeared suggesting that QDs binding were the result of specific binding to the folate receptor. We also performed experiment at 4°C to investigate FA-mediated endocytosis through the high-affinity folate receptor, which is inactive at this temperature because temperature may affect the binding of the receptor and the lateral mobility of QDs-FA complex. The results were disparity to those obtained at 37°C, but with nearly none in cell fluorescence. In the competition experiment, we treated the cells with 2

μM folic acid first to block receptor-mediated entry pathway which saturated with excess free FA and then incubated with QDs-FA as described above. No uptake of QDs-FA was detectable after 1 h of incubation is clearly seen in Fig. 2j suggesting the uptake of QDs-FA were entirely blocked by free FA in the medium.

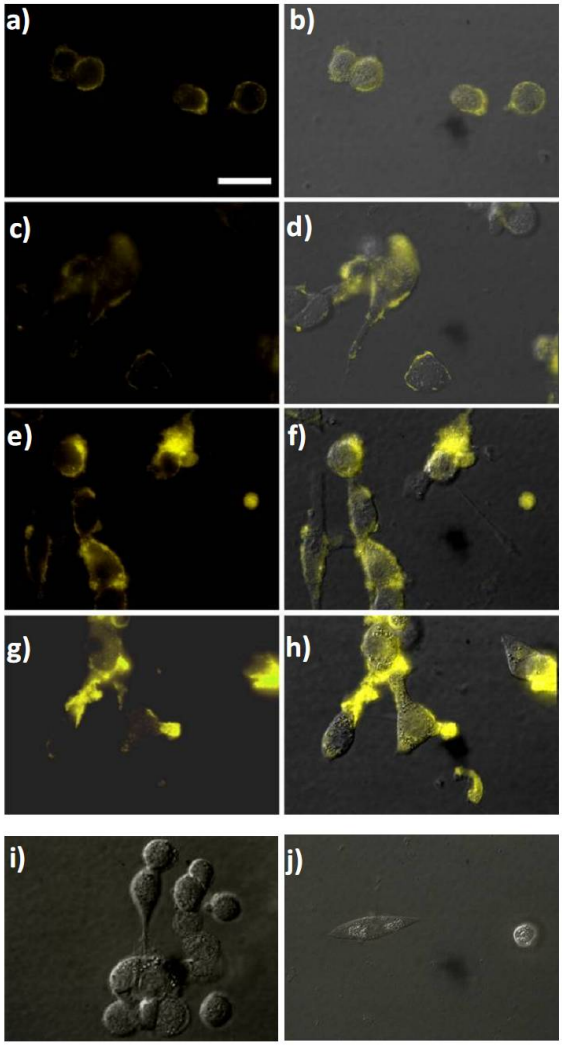


Fig. 2. Fluorescence microscopy analysis of Lewis cancer cells incubated with QDs-FA and QDs-COOH: cells were grown on glass slides incubated for different time intervals with QDs-FA preparation. 1 hour incubation with QDs-FA (fluorescence a and merge b); 3 hours incubation with QDs-FA (fluorescence c and merge d); 9 hours incubation with QDs-FA (fluorescence e and merge f); 12 hours incubation with QDs-FA (fluorescence g and merge h). Error bar=10 □m; (i) 3 hours incubation with QDs-COOH as control; (j) competition experiment. Fluorescent images were merged with bright images.

Intracellular Delivery of Quantum Dots with MPG peptide. We used Poly (maleic anhydride alt-1-tetradecene) modified QDs (QD-COOH) to link with MPG peptide. QDs' surface covered with –COOH group, so through electrostatic interactions they can be connected with MPG. In the reaction process MPG is excessive relative to the QDs (20 times), so the surface of each quantum dot has the opportunity to connect multiple MPG molecules. The entire connection process is simple, under mild conditions. Due to MPG is also water-soluble as modified QDs; the QDs-MPG is stable in water after the connection. From the result of gel electrophoresis we found the QDs-COOH run slower after linking with peptide (Fig.3a). And then we use Coomassie which is the indicator of peptide to dye the same gel, we found the MPG peptide was headed by QDs to the opposite direction (Fig. 3b). We also use the titration of intrinsic tryptophan fluorescence of MPG peptide to demonstrate it has linked with the QDs (Fig. 1c). We excited the solution at 290 nm. The emission spectrum recorded from 310nm to 390 nm indicates the tryptophan fluorescence has quenched dramatically after adding QDs. We evaluated it is because FRET has happened between peptide and QDs. Therefore, the results above fully showed that the peptide has successfully been linked with QDs-COOH.

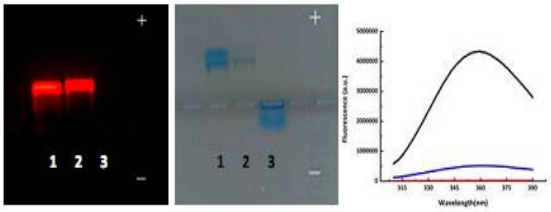


Fig. 3. (a) gel electrophoresis of QDs-MPG in lane 1, QDs-COOH in lane 2 and MPG in lane 3 under UV excitation; (b) the same gel dyed by Coomassie; (c) fluorescence titration of MPG peptide by QDs-COOH at 25^{o}C.

Under the same experimental conditions, comparing the cell membrane penetration levels of QDs-COOH, QDs-MPG on the MDA-MB-231, QDs-MPG was significantly increased. As shown in figure 4a, QDs-MPG was rapidly driven into cells after 30 min incubation. Complete delivery with more than 90% of cells achieved within 1 hour incubation. In the absence of MPG, QDs-COOH was taken up by the cells at very low levels (fig.4b). In order to understand the mechanism of cell delivery, similar experiments with peptide were performed at low temperature (4 ^{o}C) to block the endosomal pathway. As shown in Figure 4c, after incubate at 4 ^{o}C for 10 min QDs-MPG were also rapidly internalized into cells. This result suggests that the cellular uptake of QDs-MPG mediated by MPG does not require endosome derived pathway. We also use an early endosome marker to validate if they had co-localization with QDs-MPG. From confocal microscope images we found they were separated which demonstrated that QDs-MPG enter into living cells were not through the endosome pathway

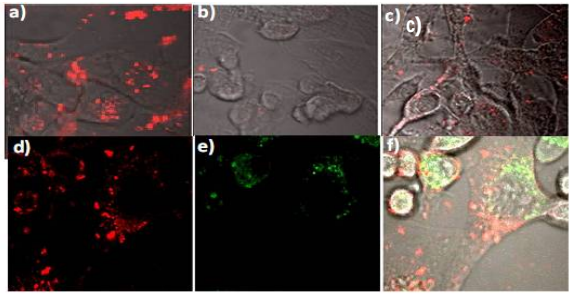


Fig. 4. (a) MPG-mediated delivery of QDs-MPG images into MDA-MB-231 cells after 30 min incubation at 37 ^{o}C; (b) merge image of QDs-COOH with MDA-MB-231 cells; (c) MPG-mediated delivery of QDs images into MDA-MB-231 cells after 10 min incubation at 4 ^{o}C; (d) two-color in different location studies of QDs-MPG (red) and (e) organelle tracking dye (green) that delivered into MDA-MB-231 cells independently the endosome pathway; (f) merge image of QDs-MPG, organelle tracking dye and MDA-MB-231 cells.

Quantum dots tailed for siRNA delivery and intracellular imaging. In vitro delivery experiments were conducted on SKBR3 cells which has the target of HER2 gene. QDs based on the use of Poly (maleic anhydride-alt-1-decene) modified with Dimethylamino propylamine for surface coatings (QDs- modified) which are able to penetrate cell membrane and disrupt endosomal organelles because of the positive charges multivalent amine groups. The essential steps in the use of QDs for siRNA are (1) the formation of QDs-siRNA complexes, (2) the cellular internalization of the QDs-siRNA and (3) the subsequent release of the siRNA to cell cytoplasm. The uptake of HER2 siRNA-conjugated QDs is monitored with laser confocal microscopy. QDs was excited by 405 nm laser, FITC was excited by 488 nm laser, RFP was excited by 543 nm laser, and at the same time, QDs signals was collected on 570 nm, FITC signals was obtained on 520 nm, RFP was collected on 590 nm. QDs-siRNA exhibit high delivery efficiency because the complexes facilitate their diffusion and entry as their small size and after they are endocytosed into cells, the tertiary amines and carboxylic groups are protonated which will rupture the endosomes and release siRNA and QDs. Figure 5 shows the QDs-siRNA complexes attach to SKBR3 cells immediately, the enter cells which suggesting efficiency transport into cells. When mixing QDs-siRNA into SKBR3 cells within 30 min, only QDs fluorescence are visible and nearly no siRNA-FITC signals can be visible, indicating that FRET between QDs and siRNA-FITS have occurred. When observed at 2.5 hours, the siRNA-FITC started to separate from QDs and distributed in the cytoplasm, indicating the efficient endosome escape has occurred. More direct evidence has come from intracellular colocalization studies, in which QDs-siRNA-FITC and RFP organelle tracking dyes are co-delivered into living cells. This colocalization can be readily detected because the QDs, siRNA-FITC and RFP organelles have different fluorescence emission peak. If the QDs are able to escape from the organelles and are released into the cytoplasm, their fluorescence signal will be separated with the RFP dye. The three color colocalization results obtained from QDs, siRNA-FITC and RFP organelles dye. Significantly, QDs and siRNA-FITC are diffuse intracellular distribution is observed. The RFP organelles dye signals decrease at 2.5 h also demonstrated the disruption and QDs and siRNA release have occurred.

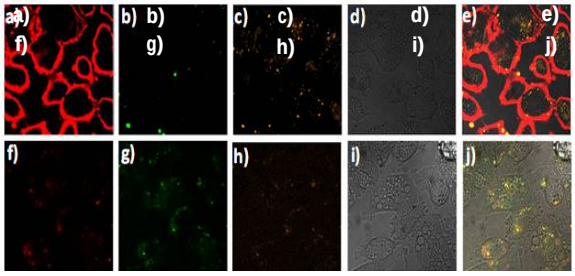


Fig.5. Fluorescence imaging of QDs (a) and siRNA-FITC (b) complexes as well as RFP Organelle tracking dye (c) and SKBR3 cells (d) within 30 min incubation with SKBR3 cells; (e) is merge of QDs, siRNA-FITC, RFP Organelle tracking dye and SKBR3 cells; Fluorescence imaging of QDs (f) and siRNA-FITC (g) complexes as well as RFP Organelle tracking dye (h) and SKBR3 cells (i) within 2.5 h incubation with SKBR3 cells; (j) is merge of QDs, siRNA-FITC, RFP Organelle tracking dye and SKBR3 cells. Cytotoxicity. In order to test whether internalization of QDs complex led to cytoxicity, HeLa cells were grown for 24 hours in the presence of QDs-COOH, QDs-FA, QDs-MPG and QDs-modified. Results showed no toxicity was found based on cell proliferation count when compared to control cells which were not incubated with QDs-COOH, QDs-FA, QDs-MPG and QDs-modified at a concentration up to 0.5 mg/ml by MTT assay (Figure 6), and there are no morphological alterations observed when compared with the control cells.

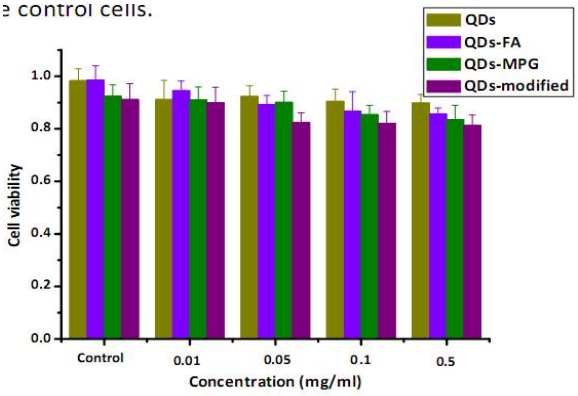


Fig. 6. a) cytotoxicities of QDs-COOH (Dark Yellow), QDs-FA (Violet), QDs-MPG (Olive) and

QDs-modified (Purple) at different concentrations after 24 hours incubation with HeLa cells by MTT assay.

CONCLUSION

In this article, we prepared water-soluble particles by entrapping hydrophobic TOPO-coated CdSeS QDs by poly (maleic anhydride-alt- 1-tetradecene) which we refer to as QDs-COOH. The QDs-COOH comprised a large fraction of carboxyl on the surface of QDs. QDs-COOH were functionalized with folic acid conjugation by using of EDC/NHS. QDs were also linked with MPG by electrostatic interactions. QDs were firstly modified with Dimethylamino propylamine surface coatings and then using for delivering siRNA into cancer cells. As a result, the obtained QDs-FA performed significant affinity and specificity to cancer cells when the QDs-COOH had no such efficacy. Moreover, the competitive experiment also verified QDs-FA enter cancer cells through folate-receptor mediated endocytosis pathway. The QDs-MPG particles exhibit high penetration and stable, rapid enter into living cells. QDs-siRNA were able to rapidly penetrate cell membranes and slowly disrupt endosomal organelles because of multivalent amine groups coated on them. In summary, we have demonstrated that QDs can be effectively used as a fluorescent vector for cancer cells targeting and anti-cancer drug delivery.

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