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RESEARCH ARTICLE

Comparative Studies of High Contrast Fluorescence Imaging Efficiency of Silica-coated CdSe Quantum Dots with Green and Red Emission

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ABSTRACT

Herein we report the possibility of using green and red emitting Cadmium selenide (CdSe)- Silica core-shell quantum dots (abbreviated as CS QDs) for remarkable stem and cancer cellular imaging, efficient cellular uptake and fluorescence imaging of semi and ultra-thin sections of tumor for in vivo tumor targeted imaging applications. The comparative studies of high contrast cellular imaging behaviours of the CS QDs with green and red emission have been exploited to visualize rabbit adipose tissue-derived mesenchymal stem cells (RADMSCs) and human cervical cancerous (HeLa) cells in vitro. The in vitro cellular uptake features of QDs were performed in cultured HeLa cells using Confocal Laser Scanning Microscopy (cLSM) after staining with 4,6-diamidino-2-phenylindole (DAPI). The in vitro cellular imaging and uptake results showed that the both green and red emitting CS QDs were efficiently taken up by the cells and exhibits excellent emission from the cytoplasm. Successively, the in vivo tumor targeting was conducted using both QDs, of Dalton's Lymphoma Ascites (DLA) cells bearing solid tumor mice. Fluorescence imaging and effective tumor targeting characteristics of QDs at tumor site were confirmed by the semithin (~15 µm thickness) and ultrathin sections of tumor (~100 nm thickness) observed under cLSM. Generally, these in vitro and in vivo results are presented for showing the efficient cellular localization of the green and red emitting CS QDs in tumor. Found that the red emitting QDs is exhibit stronger fluorescence than green emitting one. These observations shed light in their potential applications for cellular imaging of tumor cells.

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INTRODUCTION

Nanotechnology is playing a significant role in cancer diagnosis and treatment. Nanomedicine is an emerging method for treating cancer. The potential of nanomedicine is extended to early cancer detection as well as combination therapies that can initiate treating cancers earlier and more effectively (1,2). The marvellous properties shown by nanoparticles and fluorescent nanocrystals unwrap wide opportunities in developing advanced tools in imaging and therapy applications. The conventional organic fluorescent dyes lack emission stability and brightness when taken to buffered media for biological studies and difficulty in signal detection due to autofluorescence. An excellent alternative is semiconductor nanoparticles or quantum dots (QDs), as they are photostable and highly bright in emission. Besides, emission

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tunability as a function of size and room for surface functionalisation makes them novel fluorescence markers in biomedical filed. QDs have variety applications as fluorescent probes and anticancer drug carriers, due to their potential optical properties and structure (3-6).

Nanomaterials can easily circulate in the blood vessels and interact with cancer cells during cancer therapy. Nanoparticle size is considered as an effective factor in cancer therapy as it enters into the tumor tissue via the EPR effect. The size regime of nanoparticles is small compared to cells and cellular organelles facilitate the tumor cell localization through active targeting and also the small size is appropriate for passive targeting to tumor tissue. Nanoparticle sizes less than 30 nm are prone to renal filtration while approximately 250 nm are ideal candidates for phagocytosis (7-9). For passive targeting of QDs, the size and surface properties must be engineered. That is to enhance the targeting time, the optimum size of QDs should be limited to below 100 nm (10).

Many studies show that QDs are promising labelling agents for long-term imaging of cells. An example is fluorescent labelling to track the transport processes in cells as well as the path of single membrane-bound molecules (11,12). Another added property of QDs is in live cell imaging. Bioimaging of fixed cells with QDs is useful and sufficient for many applications while live cell microscopy is essential for visualizing cellular processes, which is greatly more complicated. Many studies proved that QDs are passed into the cell *via* a non-specific uptake cellular mechanism. The same mechanism was used to follow the migration of QDs into breast tumor cells on a substrate coated with QDs; the emission from cell was increased due to the uptake of QDs with and without delivering vehicles, leaving a dark path (13-16).

Numerous studies focus the use of QDs as fluorescent labelling agent to visualize the *in vitro* and *in vivo* biological processes (17-19). QDs exhibits specificity in tumor targeting in living subjects when surface is properly functionalised with peptides, proteins or antibodies. The stable and long term emission of QDs enable easy capturing of images without much complication (20-22). These observations prove that QDs have opened up a new opportunity for visualising biomolecular processes inside, even in the living cells.

Usual synthesis methods yields QDs with hydrophobic ligands. The hydrophobicity of QDs can be override by various approaches like replacing alky ligands with siloxane shell (23-25). The siloxane shell ensures cytocompatibility of CdSe QDs which has been well established by many groups (26-28). This is because in this core-shell structure, the core constituents viz., Cd^{2+} and Se^{2-} ions are well covered by the silica shell preventing leaching and surface oxidation, even in the biological media. So, silica cover offers multi advantages; it makes QDs dispersible in water and eliminates the toxicity. A detailed study of the cytocompatibility of silanised QDs was reported from our group (29). Time and concentration dependent studies confirmed that the internalized CS QDs were non-toxic even at longer incubation periods with higher QD concentrations. Besides, the non-specific cellular uptake and subcellular localization of Silica-QDs was also investigated (30). The application of QDs for sensitive bioassays and *in vitro* and *in vivo* cellular imaging has been investigated by many research groups, including ours (31-36). Still, many points are yet to be addressed when aiming particularly for *in vivo* applications. Our previous findings proved the efficient emission properties of green and red emitting CS QDs under different biological environments (30, 36). In this study, for the first time we report a comparative study on the cellular distribution and imaging as well as tumor targeted imaging of green emitting and red emitting CS QDs in cancer cells, stem cells and semi-thin and ultra-thin sections of tumor tissues under identical biological milieu.

The main objective of this comparative study is to illustrate that the green and red emitting CS QDs can be used as high contrast labelling agent for *in vitro* imaging applications of semi-thin and ultra-thin tumor sections. The bright emission characteristics of the QDs have been exploited in *in vitro* imaging of rabbit adipose tissue-derived mesenchymal stem cells (RADMSCs) and human cervical (HeLa) cancerous cells. Confocal laser scanning microscopy (cLSM) was used for high contrast fluorescence imaging in both stem and cancer cell models. 4,6-diamidino-2-phenylindole (DAPI) was used to stain nucleus for cellular uptake study using confocal microscopy. The *in vivo* fluorescence imaging efficiency of CS QDs has been studied in the semi-thin and ultra-thin section of tumour tissues of Swiss albino tumor bearing mice.

EXPERIMENTAL

Synthesis and characterisation of QDs was done by following in house developed protocols, as reported earlier from our group (29, 30). The New Zealand rabbit adipose tissue-derived mesenchymal stem cells (RADMSCs) and Human cervical cancer cell (HeLa) were maintained as per standard protocol; at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Stem Cellular Imaging

Stem cells were cultured on coverslips, and maintained till 85% cell confluence was achieved. 100 nM dispersion of prepared core-shell QDs in PBS buffer were added to the cells and incubated at 37 °C with 5% CO_2 for 4 hours. Then the coverslips was rinsed with pre-heated PBS at 37 °C three times. After fixing with 3.7% PFA, fluorescence images were recorded on a cLSM.

Cancer Cellular Imaging

HeLa cells was used for cancer cell imaging studies. Cells were cultured on coverslips, and maintained until >85% cell confluence was observed. 100 nM dispersion of prepared core-shell QDs in PBS buffer were added to the cells and incubated at 37 °C with 5% CO_2 for 4 hours. Then the coverslips were rinsed three times with pre-heated PBS at 37 °C. After fixing with 3.7% PFA, fluorescence images were recorded on a cLSM.

Cellular uptake and localization

HeLa cells were used to follow the *in vitro* cellular uptake and localization of the CS QDs. Cells were cultured on coverslips and maintained until >85% cell confluence was observed. 100 nM dispersion of prepared core-shell QDs in PBS buffer were added to the cells and incubated at 37 °C with 5% CO_2 for 4 hours. Then the coverslips were rinsed three times with pre-heated PBS at 37 °C. After fixing with 3.7% PFA and staining with DAPI stain, fluorescence images were recorded on a cLSM.

Fluorescence imaging of semi-thin and ultra-thin sections of tumor

Animal model

Normal Swiss Albino Mice (Male, 6 weeks old, weight 20–25 g) were used for the *in vivo* study. The animal house facility of Biochemistry department, University of Kerala has been utilized. The institutional ethical guidelines were completely followed as per Committee for the Purpose of CPCSEA rules [Sanction No: IAEC-KU-9/06-07/BC-AA(8)(ii)], Government of India for maintaining the experimental animals. All *in vivo* experiments were performed in triplicate.

Solid tumor development and In Vivo tumor-targeting

The solid tumor model was built up by subcutaneous administration of Daltons Lymphoma Ascites cells (1×10^6 cells/animal) into the mice through the back right hind limb (33). The solid tumor development and *in vivo* tumor-targeting studies of QDs was done systematically by following in house developed protocols, as reported earlier from our group (29, 30,36).

Fluorescence imaging of semi-thin sections (~15 µm) of tumor

For this experiment, tumor area of tumor bearing mice were frozen and sectioned with a cryomicrotome (CM 3050S, Leica, Germany) to get ~15 µm semi-thin sections. After that, the sections were stained by utilizing DAPI stain and examined under a cLSM.

Fluorescence imaging of ultra-thin sections (~100 nm) of tumor

Tumor tissues was fixed and sectioned by ultramicrotome (LKB; Bromma-2088-Ultratome®V, Sweden), to get 100 nm ultra-thin sections. After sectioning, it was examined under a cLSM. Subsequently, few sections were subjected for toludine blue staining and examined under a light microscope (Leica-DMIL, Germany).

RESULTS AND DISCUSSION

For biological applications, the primary attention is for biocompatibility of cadmium chalcogenides based QDs. As well known, heavy metals like cadmium are toxic and may leach out from QD surface to the cell culture medium resulting in cytotoxicity (39, 40).

To ensure cytocompatibility, we have used silica coated cadmium selenide QDs (Silica-QDs), where the siloxane shell prevents the leakage of core elements. Also, the biocompatible silica shell helps ready dispersion of QDs in aqueous media, which is critical for biological studies and applications (29). The main objective of this comparative study was

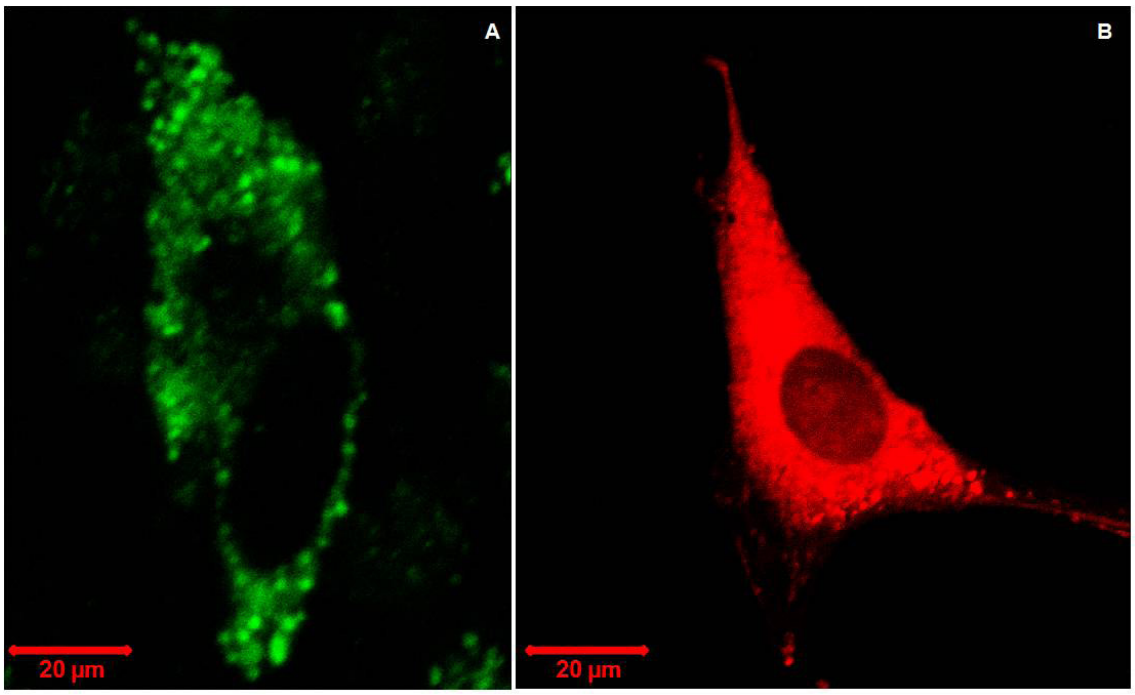

Fig. 1. Stem cellular imaging using silica-coated CdSe QDs. RADMSCs stained with green emitting (A); red emitting (B)QDs

to demonstrate that green and red emitting CS QDs can be used as remarkable fluorescent label for high contrast tumor-targeted imaging and diagnosis application using cLSM. For this, silica-coated water soluble CdSe QDs were prepared first and using it, *in vitro* and *in vivo* fluorescence imaging of semi and ultra-thin sections of tumor were systematically recorded. CdSe QDs covered with trioctylphosphine oxide (TOPO) were synthesised and rendered water dispersible by replacing TOPO with silica shell. QD preparation, silica over coating and characterisation was done as reported earlier from our group itself (29, 30, 36). The synthesized QDs showed the green (at 560 nm) and red emission at 680 nm in PBS buffer (pH 7.3). The size and shape distribution and homogeneity was confirmed using HRTEM. The overall size of QDs after silica coating were estimated as ~5.5 nm and ~6.5 nm for green and red emitting QDs, respectively and details have been published elsewhere (30, 36). The QDs were found to be dispersible, stable and luminescing in biological conditions.

Out of many potential applications, the most important one belongs to biomedical applications - cellular labelling and imaging. The work described through this article, we have used two different cell models- cancer cells and stem cells, to monitor the *in vitro* cellular imaging effectiveness of CS QDs as a function of time. The images obtained from the cLSM studies of stem and cancer cells, undoubtfully prove the role of CS QDs as fluorescent labels (Figs.1 and 2). The high contrast stem cellular imaging behaviours of the green and red emitting CS QDs have been exploited to visualize stem cells. CS QDs exhibited strong emission in the stem cells, which is known to *in vitro* florescence imaging in the cytoplasm. Fig. 1 A. shows the remarkable stem cellular imaging efficiency of green emitting QDs. Fig. 1 B. shows the outstanding stem cellular imaging efficiency of red emitting QDs. Morphological damage to the cells upon treatment with the QDs was not observed during the incubation period (*data not shown*). No morphological changes of the cells during incubation with QDs is an indication of biocompatibility QDs because of silica over coating. Also, the strong emission without change in λ_{max} proved that the electronic structure of QDs is intact in the biological environment. These observations make sure that the CS QDs might be a choice of material as fluorescent labelling agent for *in vitro* stem cellular imaging applications. Fluorescence images obtained from

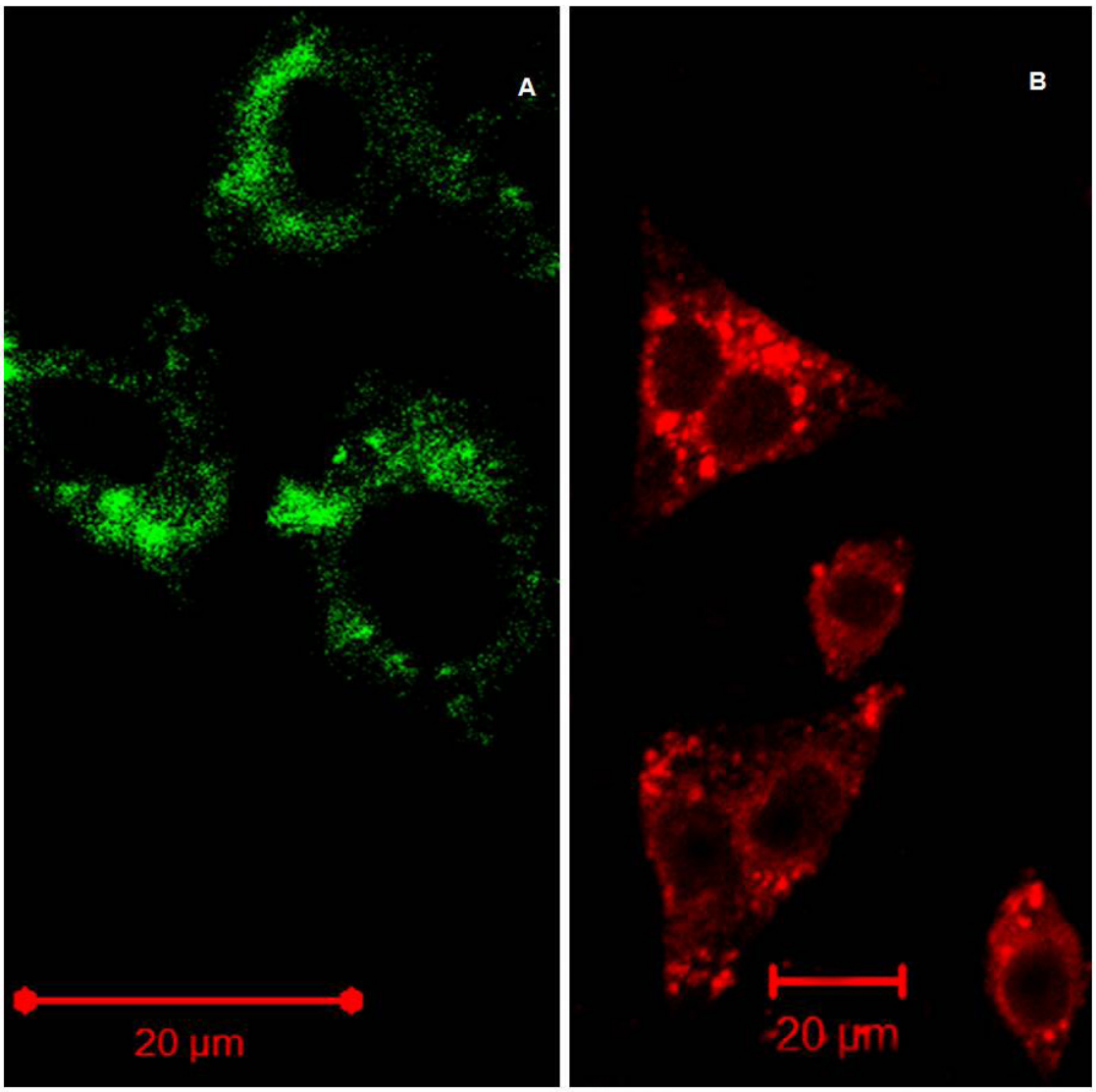

Fig. 2. Cancer cellular imaging using Silica-coated CdSe QDs. HeLa cells stained with green emitting (A); red emitting (B) QDs

cancer cells (HeLa) using green emitting CS QDs are shown in Fig.2A. Strong emission form the cell clearly confirms the internalization of QDs by cell *via* endocytosis. Fig.2B represents the same result under identical conditions, but with cells treated with red emitting CS QDs. As per these observations, it could be established that the CS QDs are apt for *in vitro* cancer cellular imaging, as QD emission is stable and bright under biological experimental conditions.

In contrast to organic fluorophores, QDs have high two-photon absorption cross section which can be made useful for three dimensional *in vivo* two-photon microscopy. This is an emerging biological and medical application of QDs, which necessitates water soluble of QDs, designed for specific uptake by cells. Direct synthesis in water or synthesis in organic solvents followed by ligand exchange are the two options to make water soluble quantum dots. In this case, silica over coating enhances the stability, solubility as well as high contrast fluorescence property. To evaluate the uptake specificity of CS QDs, we treated the cells with both green and red emitting QDs separately. HeLa cells were used to monitor the *in vitro* cellular uptake of CS QDs as a function of time. Cells were

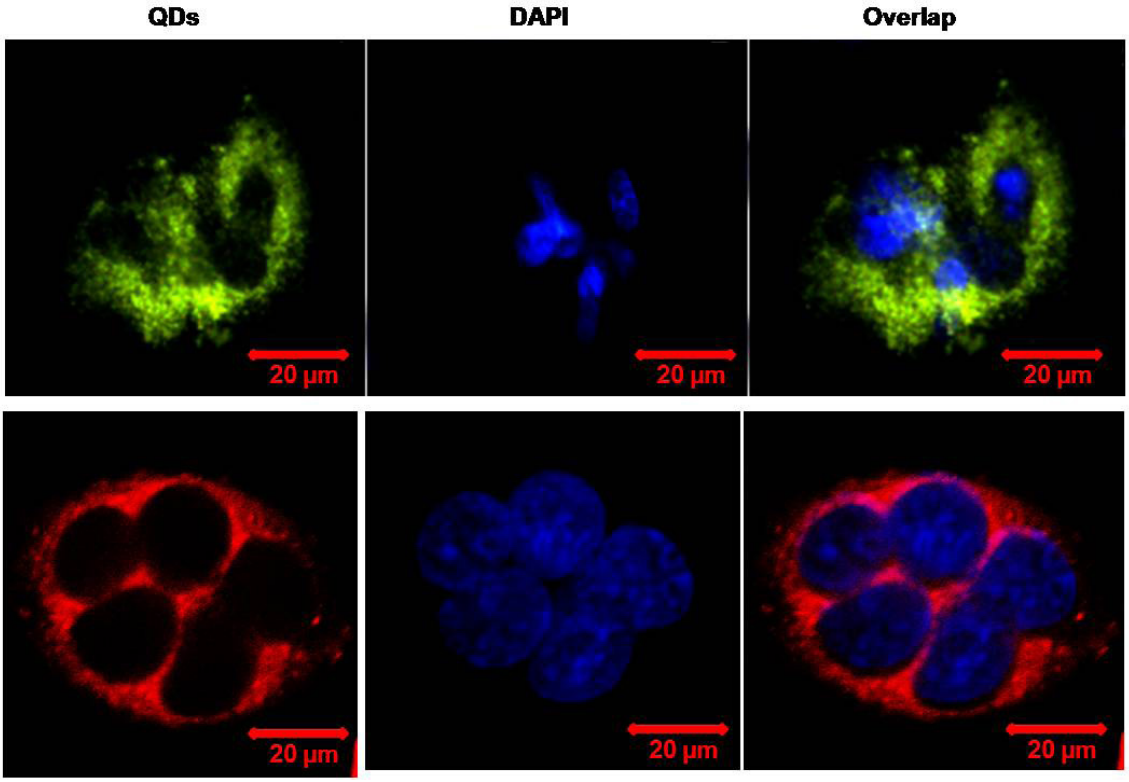

Fig. 3. Efficient non-specific cellular uptake of silica-coated CdSe QDs by Hela cells. Top row: green emitting QDs. Bottom row: Red emitting QDs. Nuclei were counter stained blue with DAPI.

first incubated with QDs for four hours followed by washing to remove unbound QDs. We were able to see a uniform emission pattern in majority of the cells, when observed with cLSM (Fig.3). The emission was observed from cytoplasm as well as in the vicinity of nucleus, in case of both cancer and stem cells under study. These results confirmed the substantial uptake of QDs into cytoplasmic vesicles. The fluorescence seen within the cells is attributed to the trapping of QDs in the endocytic intracellular vesicles. Jaiswal *et al.,* have reported an approach for cellular internalization of QD conjugates, which was postulated by endocytosis (28). Thus internalized CS QDs were found to be non-toxic during the study period. Biocompatibility of these core-shell QDs were proved in our previous studies (29). It indicates that the toxic core constitutes, cadmium and selenium ions are well hindered by the siloxane shell from leaching out. The cytocompatibility of synthesized green and red emitting CS QDs before cellular imaging experiments were performed, and results highlights their biocompatible nature under biological conditions (*data not shown*). To our belief, this is the first report of comparing the efficiency of green and red emitting CS QDs for the cellular uptake and imaging studies in cancer cells. The vital part for developing bio-friendly QDs for biomedical applications is the easiness of selective targeting through surface functionalisation. Targeting moieties can be attached to QD surface *via* covalent attaching functional groups to the silica shell (41) or using high-affinity streptavidin-biotin bioconjugation (42).

In this report, we used CS QDs without further modification and in view of focusing non-specific tumor targeting application. Therefore, both green and red emitting CS QDs were used for *in vivo* tumor-targeted imaging experiments. QDs were applied to tumor mice by intravenous injection. After the predetermined time interval (4 h) mice were euthanized, tumor region was sectioned using cryo-microtome and ultra-microtome followed by cLSM imaging. Fig.4 represents the *in vivo* fluorescence imaging of semi and ultra-thin

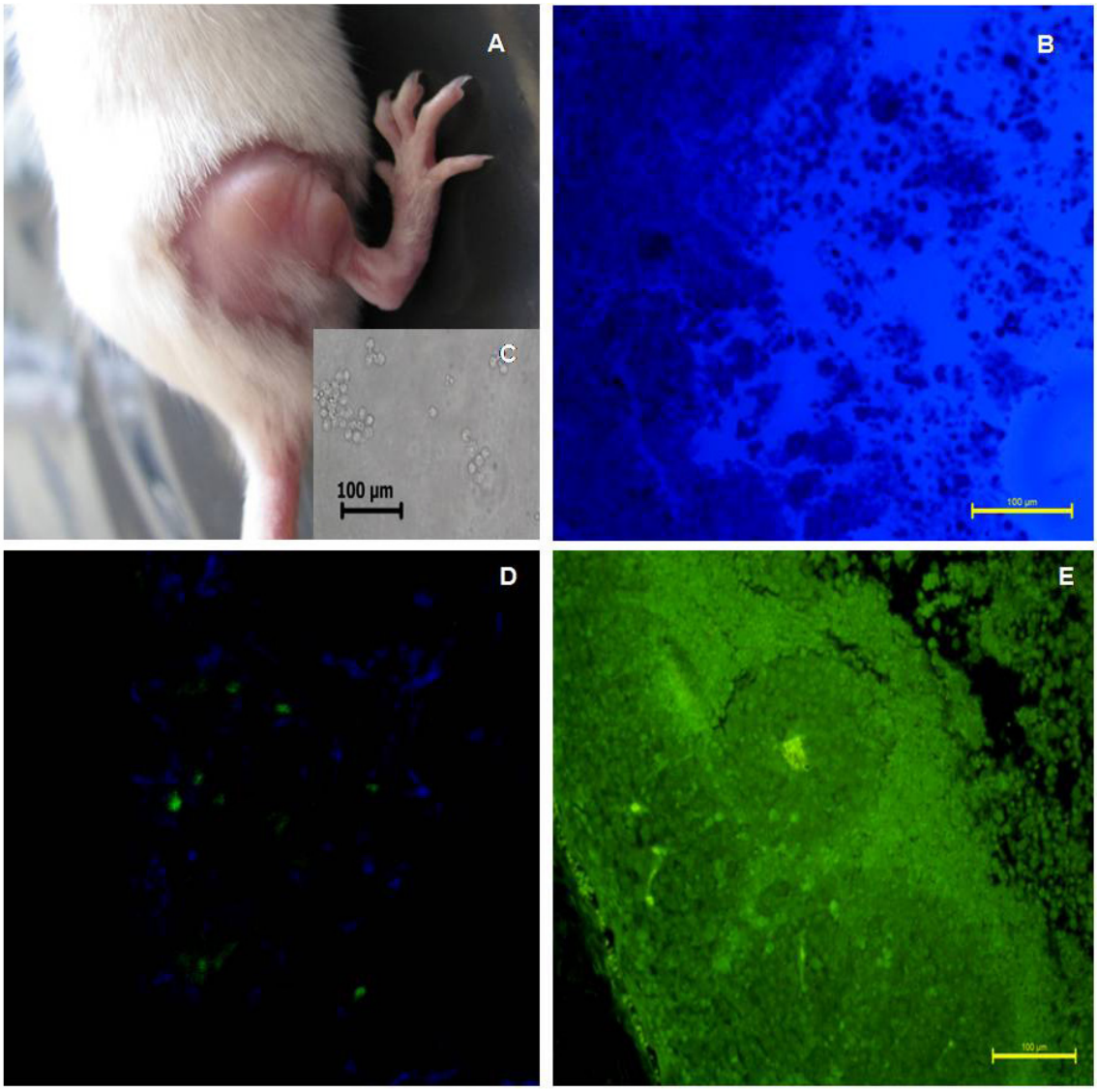

Fig.4. In vivo tumor targeting and fluorescence Imaging of semi and ultra-thin sections of tumor with green emitting silica-coated CdSe QDs. (A) photograph of tumor mice;(B) Phase contrast micrograph of DLA cells;(C) light microscopic image of ultrathin section; (D) cLSM image of the cryosection;(E) cLSM image of the ultrathin of tumor

sections of tumor with green emitting CS QDs. Interestingly, after 4 h time interval, the injected QDs was targeted at tumor site, and it was further confirmed by fluorescence imaging using clSM studies. Quantification of injected QDs in the tumor region was made by using ICP-OES and the results were again confirmed tumor targeting efficiency of CS QDs (*data not given*). Fig.4A represents photograph of tumor mice after 4 h injection; Fig.4B represents thus obtained phase contrast micrograph of DLA cells. Image of toluidine blue stained ultrathin tumor sections under light microscopy (Fig.4C) showed the presence of growing DLA cells in the tumor. The strong emission from the cryosections (~8 µm thickness) of the tumor region was observable under cLSM. Blue colour indicates DAPI stained nucleus of DLA cells (Fig. 4D). Ultrathin sections of tumor tissue also exhibited strong emission which highlights the tumor-targeted imaging efficiency of green emitting CS QDs in living mice (Fig.4E). Overall, these results suggest that confining CS QDs to

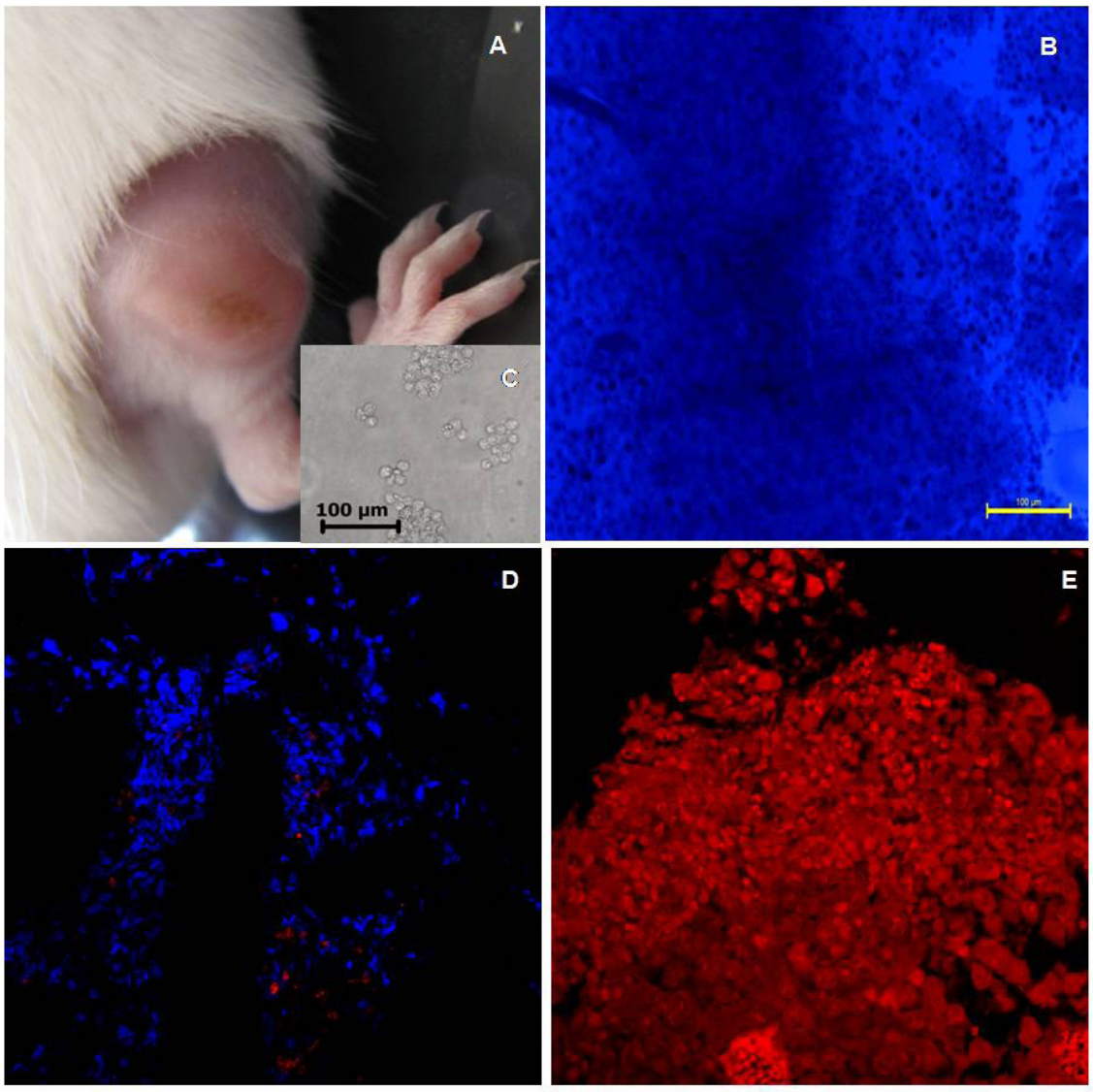

Fig.5. In vivo tumor targeting and fluorescence Imaging of semi and ultra-thin sections of tumor with red emitting silica-coated CdSe QDs. (A) photograph of tumor mice;(B) Phase contrast micrograph of DLA cells;(C) light microscopic image of ultrathin section; (D) cLSM image of the cryosection;(E) cLSM image of the ultrathin of tumor

the tumor compartment increased their systemic dispersion and deposition on tumor tissues.

Interestingly, *in vivo* fluorescence imaging of tumor cells with red emitting CS QDs showed excellent localisation of in the tumor site (Fig.5), when compared to green emitting CS QDs as shown in the previous section. Evident from the results shown in Figs.5D and E, the excellent red fluorescence signal appeared in the semithin and ultrathin sections of tumor after 4 h injection. Nucleus were counterstained blue with DAPI. In addition, Fig.5A represents photograph of tumor mice after 4 h injection; Fig.5B represents thus obtained phase contrast micrograph of injected DLA cells for tumor development. Image of toluidine blue stained ultrathin tumor sections under light microscopy (Fig.5C) showed the existence of growing DLA cells in the tumor region. These results confirmed the outstanding of fluorescence imaging of semi and ultra-thin sections of tumor using red emitting CS QDs. Overall, we found that the red emitting CS QDs shows strong contrast imaging, uptake efficiency and outstanding fluorescence imaging of *in vivo* semi and ultra-thin

sections of tumor using cLSM, possibly because of silica shell encapsulation on CdSe QDs. With these excellent properties and strong uptake and high contrast imaging characteristics, CS QDs might become an extremely promising probe for *in vivo* cancerous cell imaging applications.

CONCLUSIONS

The comparative fluorescence imaging efficiency studies of stem and cancer cells, semi and ultra-thin sections of tumor, cellular uptake and *in vivo* tumor targeting using green and red emitting silica coated cadmium selenide (CdSe) quantum dots (QDs) are systematically followed in this report. The fluorescence imaging using cLSM in both stem cells (RADMSC) and in cancer cells (HeLa) confirmed that CS QDs both green and red emitting has excellent cellular imaging efficiency. In addition, cellular uptake studies in cultured HeLa cells using cLSM after staining with DAPI showed that both QDs were efficiently taken up by the cells and exhibits excellent fluorescence from the cytoplasm. Targeting of QDs at the tumor site were confirmed by the Strong and stable fluorescence from the semithin and ultrathin cryosections of tumor when observed under cLSM. Moreover, the tumor targeting results confirmed that red emitting CS QDs exhibits stronger fluorescence than green emitting one. Overall, this study infers that green and red emitting CS QDs could be used as high contrast fluorescence imaging probe for cellular and cancer imaging applications.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest.

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