Cytotoxicity studies of II-VI semiconductor quantum dots on various cancer cell lines

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Abstract

Enhanced understanding of diseases at the molecular level has made a paradigm shift towards identifying new biological indicators especially in nanomaterials. It is important to make Quantum Dots (QDs) more than just passive bio-probes/labels for biological imaging and cellular studies as they offers "smart" multifunctional nano-platforms. For any biomedical, optoelectronic device application, evaluation of cytotoxicity coupled with cellular uptake and internalization of QDs are imperative. This paper describes the cytotoxic studies of hydrophilic and hydrophobic QDs, capped with polyvinyl pyrrolidone (PVP) and oleic acid in human breast adenocarcinoma MCF-7, Human Embryonic Kidney (HEK-293) and Ehrlich Ascites Carcinoma (EAC) cancer cells that indicated a concentration and time dependent response in a 48 hr assay. The enhanced fluorescence emitted from the cytoplasm confirmed that the QDs were efficiently internalized by the cells. 35% cytotoxicity was observed by core-shell ZnSe/CdSe QDs in HEK-293 cells, while the hydrophobic CdSe exhibited less cytotoxicity in both MCF-7 and EAC cell lines in 48 hrs. Increased LDH leakage and decreased MTT reduction was observed in a time dependent manner. The decrease rate of LDH was found in PVP-CdSe relative to the value obtained from untreated/control cells post 24 hr. The oleic acid coating renders the core-shell CdSe QDs to be more hydrophobic thus making them less toxic due to possibly weak interaction with the cells, and low ionization of cadmium. Based on our experimental observation the sequence of cytotoxicity of tested QDs was hydrophilic greater than hydrophobic in all three cell lines.

Keywords: Quantum dots, cytotoxicity, hydrophilic, cellular uptake, internalization, cells.

Introduction

Inorganic nanocrystals made of III-V(GaN [**1**], GaP [**2**], GaAs [**3**], InP [**4,5**], or InAs [**6,7**] or II-VI (ZnS [**8**], CdS **[9**], CdSe [**10**], CdTe [**11**], core/shell ZnSe/CdS [**12**], CdSe/ZnS, CdSe/CdS [**13**] CdTe/ZnS, and CdTe/CdS [**14**] semiconductor with size variation of 1-10 nm are achieving the desired efficiency in diagnostics, therapeutics and targeted delivery. Due to electronic confinement and size dependent property, band-gap of quantum dots (QDs) can be tuned between 1.5-3.0 eV, which results in tailored fluorescence. Quantum confinement effect leads to photo-stability and light emission with a large extinction coefficient over a wide range of wavelengths, which finds numerous applications in biology [**15**], biotechnology [**16**] and biodiagnostics [**17**]. Usually, organic dyes are widely used as common fluorophores for bio-imaging but organic dyes are susceptible to changes in the physiological environment and are photo-bleached under imaging circumstances limiting their use in multi-colored imaging. Such drawbacks in organic dyes may arise because of two inherent properties. Firstly, dyes have a relatively broad emission spectrum; therefore, overlap the generated signals from other dyes. Secondly, dyes require a suitably excited light within a specific narrow wavelength range and often desire nearly equal number of excitation light resource as the dyes used. Colloidal QDs have potential benefits over dyes in their property such as continuous absorption, narrow emission spectra, and their environmental stability due to robust nature (being inorganics). QDs therefore have created a sort of revolution in nanotechnology for application in biological sciences [**18-20**, **21-23**]. In biology QDs are used for *in vivo* animal imaging, fluorescence energy transfer, gene technology, fluorescent labeling of cellular proteins, cell tracking, pathogen, and toxin detection. The cytotoxicity of QDs has been observed in large number of *in vitro* studies affecting cell growth and viability. Jamieson *et al.* [**21**] described briefly, that the extent of cytotoxicity depends on number of factors such as dose, capping materials, size of QDs, surface chemistry, coating material bioactivity and processing parameters [**24-26**]. QDs are usually made of heavy metals such as Cd and Pb that are known to have toxic effects in its free form [**27**] still the challenges are enormous as the mechanisms by

which QDs whose sizes are above the renal excretion threshold (5.5 nm hydrodynamic diameter) are degraded *in vivo,* are still not well understood, leading to the concern that the QDs may accumulate in certain organs for extended periods of time that may potentially be harmful [**28**]. Some reports have indicated that when appropriately coated, QDs may be retained in the body for over two years' post injection while still preserving their fluorescent properties [**26**].

Cellular uptake in many cell types occurs by a nonspecific uptake mechanism by phagocytosis or indiscriminate engulfing of the QDs. Tracking the migration of metastatic tumor cells on a substrate coated with red emitting QDs may be exploited to measure the fluorescence within the cells, intensity may be increased due to the uptake of QDs, leaving behind a dark path [**29-32**]. Overexposure to QDs can cause the absorption at portal entry and distribution in the body. Degradation to more toxic metabolites can cause interactions with macro molecules like DNA, RNA, proteins and phospholipids, and that may adversely reflect in terms of genetics, carcinogenic, immune toxicity as well as repro-toxicity. Therefore, unintended adverse effects of QDs exposure is a growing concern both academically and socially. Malam *et al.* [**33**] reported cytotoxicity of fullerenes, bucky balls, carbon tubes and liposomes. Iga *et al.* [**34**] and Jamison *et al.* [**35**] reported toxicity of nanoshells, dendrimers and quantum dots, Chaloupka *et al.* [**36**] have reported on silver and gold nanoparticles, Park *et al.* [**37**] too have reported on cytotoxicity of Ag nano particles size less than <3 nm on RAW264.7 cells. Zang *et al.* [**38**] discusses the effect on human umbilical vein endothelial cells *in-vitro*, when treated with mercapto-succinic acid capped CdTe QDs. Similarly, Luong *et al.* [**39**] have reported QDs on fibroblastic V79 cells, Yao-bo *et al.* [**40**] reported DNA damage with CdTe capped with thioglycolic acid QDson HL-7702 cells. Su *et al.* [**40**] observed cell death on K562 and HEK 293T cell line with CdTe, CdTe/CdS, CdTe/CdS/ZnS QDs. The cytotoxicity on MCF-7 breast cancer cells when incubated with cysteine, mercaptopropionic acid and N-acetyl cysteine capped CdSe/ZnS was reported by Cho *et al.* [**42**]. Similarly, Krishner *et al.* [**43**] highlighted the adverse effects of mercaptopropionic acid capped CdSe/ZnS on MDA-MB-435S breast cancer cells. Wang *et al.* [**44**] reported studies on CaCo-2 (human colon carcinoma) cell culture with CdSe/ZnS capped with poly ethylene glycol. Khalil *et al.* [**45**] reported toxicity of mercapto-acetic acid capped CdSe *per se* in mice. In view of above, it is earnestly desired that the toxicity issues be addressed and once the toxicity of QDs is reduced or eliminated, it has the potential to revolutionize the field of biomedicine that includes cancer detection, drug delivery, imaging and real time of monitoring of cellular processes in diseases. Further, the suitability of a lactate dehydrogenase (LDH) assay for evaluation of nanoparticle toxicity was performed. The level of extracellular LDH released from damaged cells was measured as an indicator of cytotoxicity, as the assay relies on measuring the activity of LDH in catalyzing the reaction: where NADH is reduced to β-nicotinamide adenine dinucleotide. Since NADH has a peak absorbance at 340 nm, the rate of decrease in NADH level maybe measured to determine LDH activity if the reaction starts with known levels of NADH and pyruvate and an unknown level of LDH. The absolute values of the slopes were taken as substitutes for LDH activity of the samples (in a unit of μM/min/ml) [**46**]. Therefore, investigation of toxicity and safe clinical use of QDs is imperative to realize the potential of QDs. We have focused on synthesis of various types of II-VI semiconductor QDs by applying different strategies and characterization [**47-50**]. In this paper, hydrophobic CdSe and Core-shell ZnSe/CdSe synthesized by using cyclohexeno-1,2,3-selenadiazole precursor as well as hydrophilic CdSe QDs were synthesized using a coating of polyvinyl pyrrolidone (PVP), oleic acid. Subsequently their cytotoxicity studies were performed on MCF-7, HEK-293, and EAC cell lines. QDs prepared by our group may find effective application in various fields such as optoelectronics, biomedical and therefore its application in detection of deceased cells. In order to study such applications, it is important to understand the toxicity of such tiny particle. Thus, we needed to understand internalization, cellular uptake induced cytotoxicity of QD sand for that we assigned MTT and LDH assay on three cell lines using hydrophilic, hydrophobic QDs are presented hereunder.

Experimental

Materials and methods

Cadmium chloride (99%), Zinc acetate (99%), Selenium (99%), Polyvinyl pyrrolidone (PVP) and Sodium sulphite (99%) were purchased from Aldrich Co. Methanol was purchased from Alfa Aesar. Oleic acid and diphenyl ether were obtained commercially and were used without further purification. Selenium precursor called sodium selenosulfate (Na_2SeSO_3) was prepared as per reported procedure [**49**] and stored in the dark. Similarly, 1,2,3-selenadiazole, a Se-precursor for hydrophobic QDs was prepared and used as described earlier [**47-48**]. The absorption spectra were recorded using JASCO V-570 UV-visible spectrophotometer on Analytik Jena Specord-210 (Germany) at room temperature in toluene. X-ray diffractions patterns were measured by using Cu-Kα (λ=1.5406Aº) radiation having tube voltage with 40 mA current on Mini Flex Rigaku X-ray diffractrometer between 2θ 10-90º. Particle size distribution was measured using Sympatech (France) particle size analyzer at a Laser wavelength of 632 nm.

Preparation of hydrophilic CdSe-PVP QDs

0.5 g of $CdCl_2$ was taken in 50 ml of distilled water containing 0.25 gm of PVP in a clean 250 ml beaker. It was stirred for 5 min. at room temperature followed by drop-wise addition of Na_2SeSO_3 (1:1 ratio). The reaction mixture thus generated was stirred for additional 4-6 hrs; the suspension was centrifuged and washed with methanol thrice *via* centrifugation method. An orange precipitate was collected and air-dried. UV-Visible

spectrum of so-prepared PVP-CdSe quantum dots was recorded in methanol after re-dispersion of freshly isolated product before the final air-drying and was henceforth called as hydrophilic CdSe QDs for all our present studies.

Preparation of hydrophobic core-shell ZnSe/CdSe and CdSe QDs

Typically, a two-step *in-situ* reaction was performed as given below;

Step-one-1.54 g of zinc acetate was taken in 20 ml of oleic acid in a 250 ml two-neck round bottom flask and the mixture was heated at about 100°C. To this hot solution mixture, was added 1.0 g of Cyclohexeno-1,2,3-selenadiazole, pre-dissolved in diphenyl ether (10ml) and the resultant reaction mixture was heated to about 190°C for 2 hrs. The off white to pale colored suspension was formed.

In step-two, this suspension further treated with 1.54 g of cadmium acetate and 1.0 g of same selenadiazole. The final core-shell QDs were isolated as per protocol reported previously by our group [**47**]. CdSe QDs were prepared similarly following reaction of step-one employing cadmium salt instead of zinc metal salt.

Cell Culture and Cytotoxicity

The *in vitro* evaluation was done on Human Embryonic Kidney cell line, HEK-293 and Human breast adenocarcinoma MCF-7 cell line (American Type Culture Collection, Rockville, MD) [**51**]. Confluent flasks were sub-cultured and maintained at 37°C in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Hi media), and antibiotic containing 50 U/mL of penicillin (Sigma) and 50 mg/mL of streptomycin (Sigma, USA) under a humidified atmosphere (5% CO_2). Briefly, $5x10^3$ cells/ well of HEK-293, MCF-7 and EAC cells were plated in 96-well microtiter plates. QDs were then added to the cells at defined concentrations (10 µg/ml, 5 µg/ml and 2.5 µg/mL) and incubated for 24 hrs and 48 hrs. After incubation, 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (5 mg/mL) was then added per well and the plate was incubated for four hours in an incubator. After incubation, the media was discarded carefully without disturbing the formazan crystals in the wells and 150 µl of dimethyl sulfoxide was added to solubilize the formazan crystals formed and read in a 96-well microtiter plate (Synergy HT, Biotek, USA) using a 540 nm filter [**52**]. All measurements were done in triplicates. Percentage viability of the cells was calculated as the ratio of mean absorbance of triplicate readings with respect to mean absorbance of control wells.

$$\text{Cell viability} = (I_{control} - I_{sample}/I_{control}) \times 100$$

Cellular uptake

For understanding the internalization of nanoparticles cellular uptake was assessed in a fluorescence microscope as per published protocols [**53**]. Briefly, MCF-7, HEK-293 and EAC cells were cultured on coverslips till 85% confluence was attained. The cells were further incubated with 10 µg/ml of QDs. The cells were fixed with 4% paraformaldehyde and visualized in a fluorescence microscope (Eclips 90i, Nikon at Mag.20x).

LDH assay

To prepare samples for the LDH assay, cells at a density of 1×10^5 cells/ml (DMEM containing 10% FBS) were seeded in each well of 24-well plates and grown overnight. The cells were washed with HBSS three times and dosed with different concentrations of QDs in DMEM medium containing 1% FBS. After 24 hr exposure, the 24-well plates were shaken briefly to homogenize the released LDH in the cell culture medium and the medium was transferred to micro-centrifuge tubes and centrifuged at 12,000 x g at 4°C for 15 min to remove any cell debris and QDs. 100 µl of each sample was added to the substrate solution and the absorbance was measured at 340 nm using a spectrophotometer (Biotek, USA). The LDH activity of the samples was obtained by measuring the reducing rate of NADH absorbance over time (slope) and therefore, all the slopes thus obtained were negative. Results were normalized with respect to the negative control (expressed as 100%). The positive control consisted in treatment of the cells with 0.9% Triton X-100 and gave leakage values in the range of 700–800% (data not reported). Data were expressed as mean ± SD.

Statistical analysis

The results were expressed as mean ± SD. Comparison among groups were analyzed by One way ANOVA followed by Tukey's test using Prism (5.0) software (Prism software Inc. CA). A p-value of <0.05 was considered significant. Asterisks indicated levels of significance ***$p<0.001$, **$p<0.01$, *$p<0.05$. The statistical analysis was performed by one way anova followed by Tukey's test using Prism Graph pad 5.0

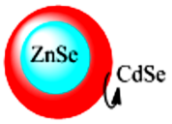
Results and discussion

QDs are a prototypical system in which the mass, charge, monodispersity and exterior chemistry can be effortlessly influenced to expedite the internalization of nanoparticles. Surface coating too has a profound impact on the cellular uptake of QDs. But for any bio-medical applications, the cellular uptake of QDs and mechanism of cell death need to be estimated. Synthesis of mono-dispersed, hydrophilic quantum dots with active bio-conjugation has been highly challenging task as it offers a wide scope for studies. For present study, hydrophobic CdSe and hydrophobic core-shell ZnSe/CdSe were prepared using oleic acid as a surfactant. However; hydrophilic CdSe QDs (PVP-CdSe) were synthesized using a coating of polyvinyl pyrrolidone (PVP). The coloured suspensions/solutions were centrifuged to isolate dry powders. Synthesis of QDs was performed as per schemes I and II as shown below.

$$CdCl_2 + Na_2SeSO_3 + PVP \longrightarrow CdSe\text{-}PVP \quad (I)$$

$C_6H_8N_2Se + Zn(acetate)_2 \longrightarrow ZnSe$ (II)

$+Cd(Ac)_2$



The absorption spectra at room temperature were recorded in methanol after re-dispersing the dried PVP-CdSe QDs. Due to the quantum confinement effect, the hydrophilic PVP-CdSe QDs showed the absorption maximum at 533 nm (2.32 eV) however the hydrophobic CdSe, and core shell ZnSe/CdSe showed the bands at 543 nm (2.26 eV) and 560 nm (2.21 eV) respectively as shown in **Fig. 1**.

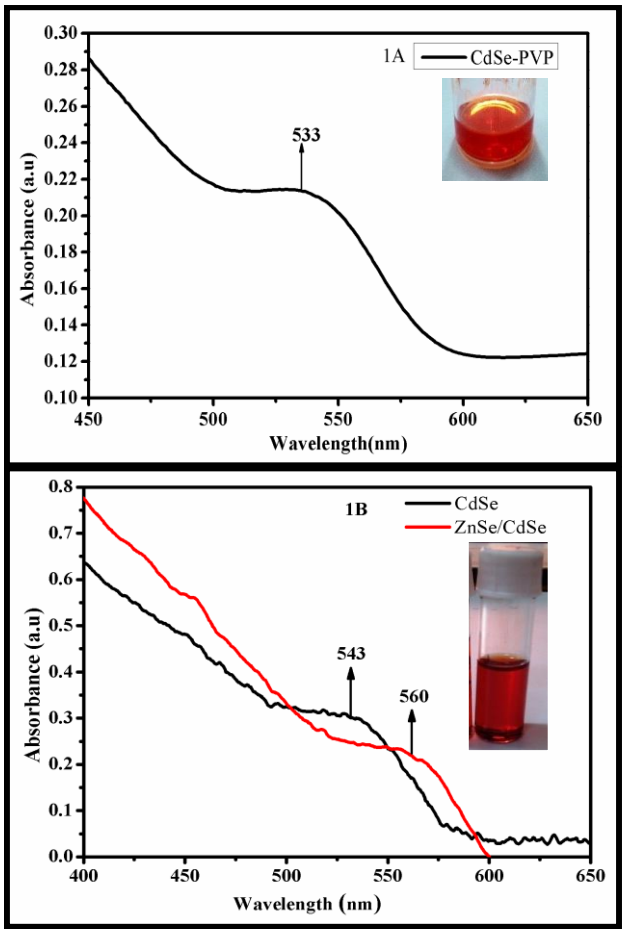


Fig. 1. UV-visible spectrum of (A) PVP-CdSe (inset: dispersed in water). (B) CdSe (black) and core shell ZnSe/CdSe (red) (inset: dispersed in toluene).

In the present case, enhanced band-gap energies of QDs were obtained as against the bulk band-gap of 1.72 eV, that clearly indicated an excellent blue shift in the range of about 150-180 nm with respect to the bulk value of 712 nm. The blue shift is due to the increase in the energy gap from HOMO to LUMO (Highest occupied molecular orbital to lowest unoccupied molecular orbital) because of size quantization effect. The understanding of the band-gap tuning is an essential property for efficient utility of QDs in biological systems. Another important parameter is the particle size distribution which was measured for each type of the QDs employed in the present work as given in SI 1. The size distribution of hydrophilic QDs was between 6.5 to 13 nm. The hydrophilic dispersion may have caused substantial clustering thereby leading to a wider distribution. But, in case of hydrophobic QDs the size distribution was narrower and ranged from 1-8 nm.

TEM (**Fig. 2A**, **2B**, **2C**) of oleic acid capped hydrophobic ZnSe/CdSe and CdSe as well as PVP capped hydrophilic CdSe QDs indicated a spherical morphology but appeared agglomerated in case of PVP-CdSe probably due to the presence of polymer network i.e. polypyrrolidone.

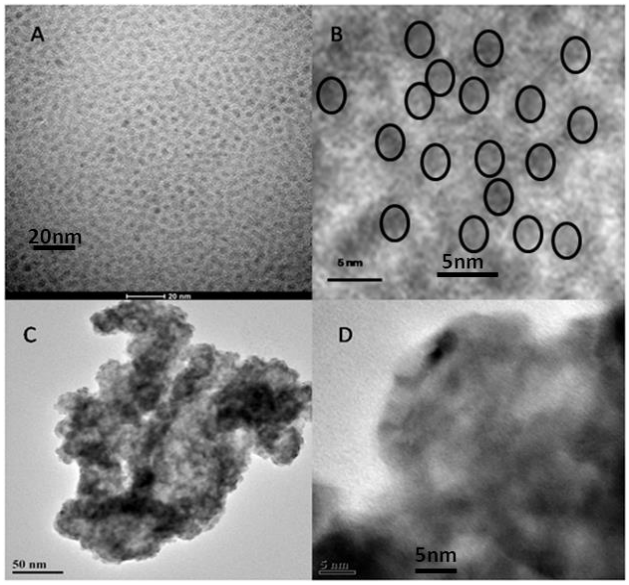


Fig. 2. TEM image of the A) hydrophobic core shell ZnSe/CdSe at resolution of 20 nm, B) hydrophobic CdSe at resolution of 5 nm and C) hydrophilic PVP-CdSeQDs at resolution of 50 nm with (D) signature of lattice fringes at resolution of 5nm.

The size of QDs was in the range of ~ 4-10 nm. Powder X-ray diffraction patterns indicated a characteristic cubic crystal structure of CdSe and the same was observed for ZnSe/CdSe as shown in **Fig 3A**, **3B**). The reflections at 111, 220 and 311 crystal planes matched well with the reported data [**47**] for cubic crystal planes and broad peaks indicated the small particle size of the product. From Scherrer equation, the cluster diameter was estimated to be in between ~ 4-10 nm. A small hump between 2θ values of 31°-33° relating to the slight impurities of elemental selenium was observed in the hydrophilic QDs possibly due to presence of free Se released from the precursor during synthesis. Evidence from the UV-Visible, XRD and EDX spectra shows single phase formation relevant to core-shell QDs. Gradual shift in 2θ from 25.78 for CdSe-PVP to 25.27 for ZnSe/CdSe indicates formation. Additionally, the d-spacing evidence also confirms the formation of ZnSe/CdSe QDs. The calculations are given in **Table 1** below.

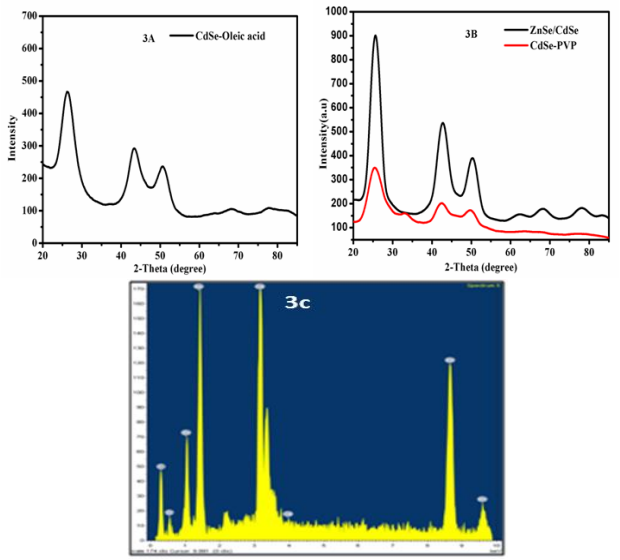


Fig. 3. PXRD spectra of A) CdSe. B) PVP-CdSe (red) and core-shell ZnSe/CdSe (black). C) EDX of the ZnSe/CdSe QDs.

Table 1. d-spacing and 2θ value from XRD.

QDs	Crystal Planes		
	111 d-spacing(nm) / 2Θ (deg.)	121 d-spacing(nm)/ 2Θ(deg.)	311 d-spacing(nm)/ 2Θ(deg.)
ZnSe/ CdSe	3.48/25.27	2.15/41.92	1.84/49.33
CdSe-oleic acid	3.32/25.72	2.09/43.02	1.78/51.18
CdSe-PVP	3.45/25.78	2.09/43.07	1.77/51.42

Cellular uptake induced cytotoxicity of HEK-293, MCF-7 and EAC

QDs have potential applications in nano-medicine as drug delivery vehicles, diagnostic agents and are emerging as an ideal system amongst the inorganic materials where the size, surface chemistry, charge and mono-dispersity can be easily controlled. The cellular uptake studies indicated that the PVP capped QDs were easily internalized in HEK-293, EAC and MCF-7 cells lines as shown in **Fig. 4-6** but the hydrophobic CdSe and hydrophobic core-shell ZnSe/CdSe QDs were not internalized as much. Since the PVP coated QDs are hydrophilic in nature, thereby improving the solubility of CdSe in water and therefore its uptake was probably by receptor mediated endocytosis that maybe correlated with the enhanced cytotoxicity. While CdSe quantum dots are hydrophobic in nature, they are internalized by the lipid bilayer indiscriminately as evident by the fluorescent intensity, but decreased cytotoxicity. The enhanced fluorescence visualized in the cells may be attributed to the trapping of hydrophilic CdSe-PVP, hydrophobic CdSe and hydrophobic ZnSe/CdSe QDs in the endocytic intracellular vesicles [**52**, **53**] as shown in **Fig. 8**. Jaiswal *et al.* also reported a similar observation whereby the fluorescence observed from cells was attributed to the internalization of QDs by endocytosis and the overall mechanism of QDs uptake can be explained as shown in **Fig. 7**. Hence, these observations are in unison with earlier reports indicating the uptake of QDs. Around 85%, 70% and 50% cytotoxicity was observed in case of hydrophilic (CdSe) and hydrophobic (CdSe, ZnSe/CdSe) QDs at a concentration of 10µg/ml in 48 hrs. Hydrophobic core-shell (ZnSe/CdSe) QDs showed ~35% less cytotoxicity in 48 hrs in HEK-293 cell lines compared to other QDs. But only 16% cytotoxicity was indicated in 24 hrs in HEK-293 in case of ZnSe/CdSe as shown in **Fig. 4**. Shielding of cationic groups by functionalizing with oleic acid decreased both cytotoxicity and efficacy of internalization of QDs where both the efficacy and cytotoxicity were presumably linked to the cationic charge.

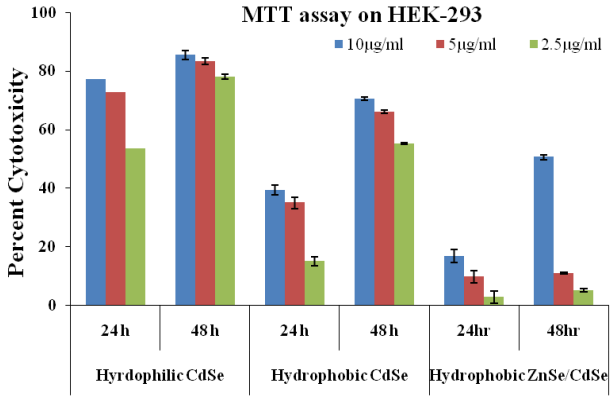


Fig. 4. Dose and Time dependant cytotoxicity of hydrophilic PVP-CdSe, hydrophobic CdSe and hydrophobic core shell ZnSe/CdSe quantum dots on HEK-293 cell lines.

Similarly, in MCF-7 cells around 88%, 76% and 79% cytotoxicity was observed in case of hydrophilic (CdSe) and hydrophobic (CdSe, ZnSe/CdSe) QDs at a concentration of 10µg/ml in 48 hrs. Hydrophobic core-shell (CdSe) QDs showed ~10% less cytotoxicity in 48 hrs in MCF-7 cell lines compared to other QDs. But similar cytotoxicity was indicated in 24 hrs in MCF-7 cytotoxicity for hydrophobic CdSe and ZnSe/CdSe as shown in **Fig. 5**. In cancerous cells hydrophobic ZnSe/CdSe is only 9% less cytotoxic than hydrophilic CdSe and hydrophobic CdSe (12% less).

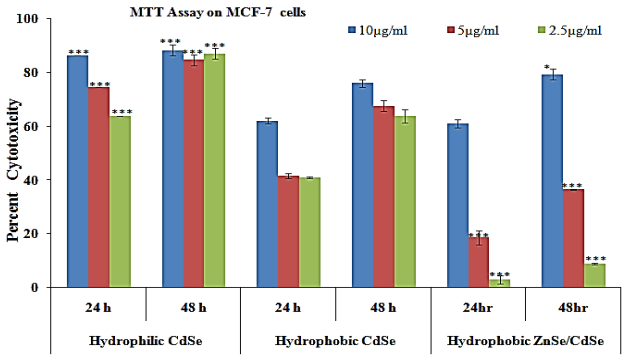


Fig. 5. Dose and Time dependant cytotoxicity of hydrphilic PVP-CdSe, hydrphobic CdSe and hydrophobic core shell ZnSe/CdSe quantum dots on MCF-7 cell lines.

Hence, the hydrophobic ZnSe/CdSe was also showing an enhanced anti-cancer efficacy. But, negligible cytotoxicity was observed at a concentration of 2.5 µg/ml of hydrophobic CdSe and ZnSe/CdSe in EAC cell line.The biocompatibility of QD cannot be limited to only solubility of QDs but biocompatibility relates to reduced toxicity to biological systems, like alteration in cell's regular events. Clift and Brandenberger [**54**] reported that the QDs confine within endosomes and lysosomes and are therebyexposedto an acidic micro-milieu. Mancini and Kairdolf [**55**] observed that hypochlorous acid, present in phagocytic cells, oxidized the polymer-encapsulated CdS/ZnS-capped CdSe QDs with solubility of cadmium, zinc, sulfur, and selenium species.

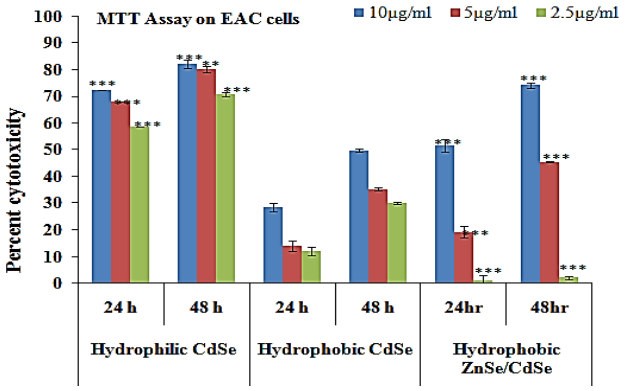


Fig. 6. Dose and Time dependant cytotoxicity of hydrphilic PVP-CdSe, hydrphobic CdSe and hydrophobic core shell ZnSe/CdSe quantum dots on EAC cell lines.

While the release of free cadmium and ROS production are often discussed in isolation, it is more likely that these mechanisms act in concert to produce QD toxicity as shown in **Fig. 7**. Around 82%, 49% and 74% cytotoxicity was observed subsequently in case of hydrophilic (CdSe) and hydrophobic (CdSe) QDs at a concentration of 10µg/ml in 48 hrs. Hydrophobic core-shell (CdSe) QDs showed ~33 % less cytotoxicity in 48 hrs in EAC cell lines compared to other QDs. But only 28% cytotoxicity was indicated in 24 hrs in EAC, cytotoxicity of hydrophobic CdSe as shown in **Fig. 6**. Here the overall observation in hydrophilic QDs is more cytotoxic than hydrophobic QDs.

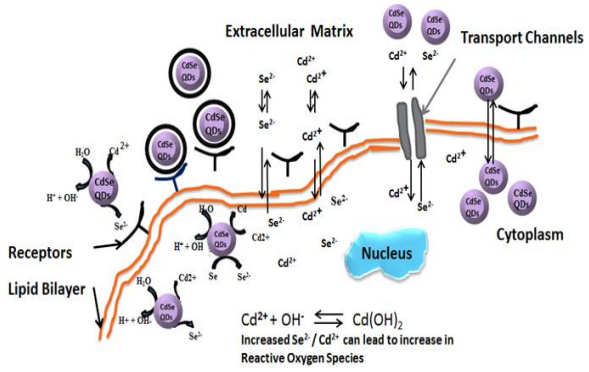


Fig. 7. Proposed mechanism of cytotoxicity of quantum dots at extracellular matrix and transport channels.

King-Heiden *et al.* [**56**] have demonstrated that free cadmium release could not explain the toxicity of CdSe QDs or ZnS-capped CdSe QDs to the zebrafish embryo *Daniorerio*. Internalization based on shape and size facilitates QDs to reach organelles that are otherwise unreachable by metal ions. This may possibly be the cause of variations in the levels of cytotoxicity when compared to the basic metals. Based on the pathway used for uptake by the cell, QDs are wrapped into small intracellular vesicles, transferred from the margins of the cell membrane to the perinuclear region as demonstrated by Parak and Boudreau [**31**]. On the other hand, the ionization of QDs may occur in the cytoplasm resulting in excess of cadmium ions in the cytoplasm, where they are appropriated by metallothioneins. QDs can cause DNA mutations without causing cell death, and the effect is evident in the future generations of cells in progressive carcinoma. In our studies, it is evident that the hydrophobic CdSe and core-shell ZnSe/CdSe QDs do not release free cadmium or Cd^{2+} and Se^{2-} ions, resulting in low toxicities while the hydrophilic PVP-CdSe QDs release Cd^{2+} and Se^{2-} ions thereby resulting in 90% cytotoxicity. A surface coating perceptibly plays an important part in cell death, but is limited primarily to cellular uptake. To assess whether xenobiotic changes are occurring in the cell membrane, it was imperative to do the LDH leakage assay, as the activity of the cytoplasmic enzyme LDH is directly proportional to the cellular damage. It has been observed that the LDH assay could detect cytotoxicity with high sensitivity, whereas the MTT assay could not detect differences at high QD concentrations (>8.3 µg/mL CdSe/ZnS QDs) Young Joo Choi *et al.* [**57**]. Quantitative assessment of the activity of cytoplasmic LDH was measured by the oxidation of NADH or by reduction of the MTT. The release of the cytosol marker enzyme lactate dehydrogenase to the culture medium was determined after 24 hours of QDs treatment on EAC, HEK and MCF-7 cancer cell lines. The cell membrane damage was directly proportional to the lactate dehydrogenase released after QDs exposure.

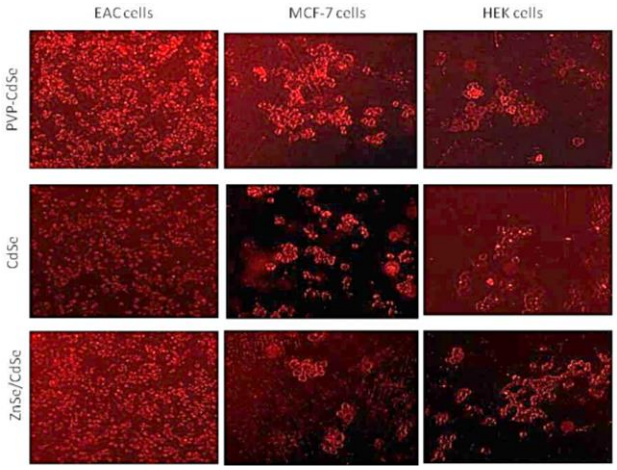


Fig. 8. EAC, MCF-7 and HEK-293 cell lines were treated with 10µg/ml of hydrophilic PVP-CdSe, hydrophobic CdSe and hydrophobic core shell ZnSe/CdSe quantum dots, incubated for 3 hrs, then fixed with 4% para-formaldehyde, and viewed under microscope [Nikon 90i] at Magnification 20x.

The results indicated that the treatment with ZnSe/CdSe QDs induced significant membrane damage in MCF-7 and EAC cell lines at concentrations 10 µg/ml. After exposure to QDs the leakage of enzymes was different in different cell lines. LDH was slightly inhibited by CdSe followed by ZnSe/CdSe in HEK-293 cells and slightly increased in EAC but the highest leakage rate was observed by PVP-CdSe treated cells. The LDH assay, which is a sensitive detection method for necrotic cell death, revealed significant and consistent increases in LDH release. The results observed for cytotoxicity assays such as MTT and LDH using cancer cell line revealed that QDs (CdSe, PVP-CdSe, ZnSe/CdSe) increased LDH leakage and decreased MTT reduction as shown in **Fig. 9**.Though the high toxicity at high QD concentration range can lead to high LDH release, the release is limited by the limited number of cells available. When all cells are dead and no further LDH can be released, further increase of QD concentration will only decrease the released LDH.

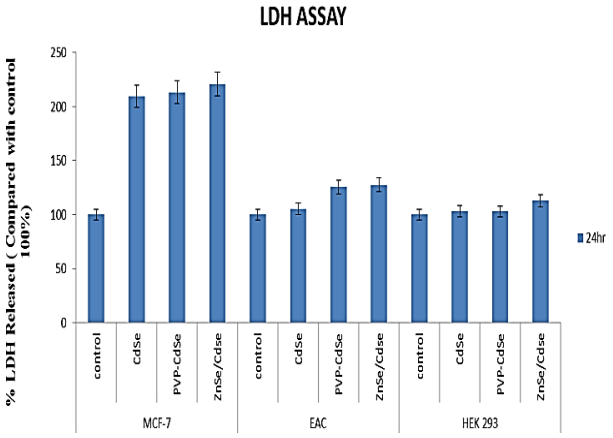


Fig. 9. LDH assay of hydrophobic CdSe, hydrophilic CdSe and hydrophobic ZnSe/CdSe inMCF-7, HEK-293 and EAC cell lines, post 24 hr exposure at dose of each 10µg/ml.

The cytotoxicity of QDs may be related to their physicochemical states as observed by Bruchez *et al.* [**58**]. We observed that the major QDs toxicity was directly proportional to the opined release of metal ions (e.g., Cd^{2+}) which is in unison with the earlier reports [**59**, **60**]. Generally, all the cells are susceptible to cellular modifications culminating in DNA damage.

Conclusion

New chemical strategies have constantly been evolving to overcome the low aqueous solubility that severely hampered the biological applications of QDs. The present work described the synthesis of core/shell quantum dots by use of organoselenium compound essentially by the known strategy but for a different combination of core and shell. The so-obtained quantum dots were thoroughly characterized by various moder tools to establish the size range of less than 5 nm. Differences in particle size distribution can be useful in cytotoxic studies which indeed have been observed in the present studies where hydrophilic QDs have shown more toxicity in comparison to hydrophobic QDs. The cellular uptake and cytotoxicity of QDs are associated with their physicochemical properties. The observed cytotoxicity was both concentration and time dependent which varies with the cell line used. It was observed that the cytotoxicity of QDs can be altered by meaningful coatings around the surface e.g. while QDs with PVP coating showed 85% cytotoxicity at a concentration of 10µg/ml in 48 hrs, the ZnSe/CdSe oleic acid coated QDs showed ~35% less cytotoxicity during the same time in both HEK-293 and MCF-7 cell lines but in EAC, CdSe has shown reduced cytotoxicity. The enhanced fluorescence intensity evident within the cells confirmed internalization of QDs into cytoplasmic vesicles. The PVP-CdSe QDs were easily dispersible in aqueous media and easily generates $Cd^{2+}$and Se^{2-} ions that are extremely toxic [**60**]. PVP-CdSe exhibited enhanced *in vitro* cytotoxicity probably due to faster ionization in endocytic compartments. Hydrophobic core-shell QDs showed reduced cytotoxicity due to the hydrophobic nature coupled with the electronic confinement effect of QDs that resulted in reduced bioavailability of toxic metal to the cells. Also, low toxicity was achieved by replacing Cd with Zn as these QDs were less sensitive to environmental changes like thermal, biochemical and photochemical. The sequence of cytotoxicity due to its bioavailability may be depicted as hydrophilic being more toxic than hydrophobic in order of EAC, MCF-7 and HEK-293 cell lines. LDH widely exists in cell membranes and cytoplasm, and is released from cells into culture supernatants immediately after cell damage. Therefore, photo-spectrometric assessment for cell viability through the extracellular leakage of LDH can be applied for the evaluation of QDs Mori *et al.* [**61**]. For validating the LDH assay, we focused on NADH oxidation by NPs in the presence of pyruvate, because it is the mixture of NADH and pyruvate that is used as the substrate solution for performing the LDH assay. Even if NPs could have some effect on NADH oxidation on a time scale of hours, this effect could still be negligible after a few minutes, which leaves enough time to complete a normal measurement session of the assay. The results observed for cytotoxicity assays such as MTT and LDH using cancer cell line revealed that QDs (CdSe, PVP-CdSe, ZnSe/CdSe) increased LDH leakage and decreased MTT reduction post 24 hrs exposure. Our results establish that hydrophobic CdSe QDs as exceptionally valuable tools for cell tracking, molecular imaging and labeling to evaluate the events in cell division and also in in-vivo tumors studies especially metastasis of cancer, inflammation and other diseases. We, hereby propose oleic acid coated QDs as biocompatible and ideal system for quantifying cellular and *in vivo* metastatic cancer imaging with respect to toxicity. Further studies are imperative to investigate the clearance mechanism of QDs from living systems i.e. the bio safety aspect for *in vivo* applications, toxicological and pharmacokinetic investigations concerning oxidation, degradation, excretion, tenacity and immune response of QDs need to be methodically assessed.

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Author's contributions

Authors have no competing financial interests.

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Supplementary information

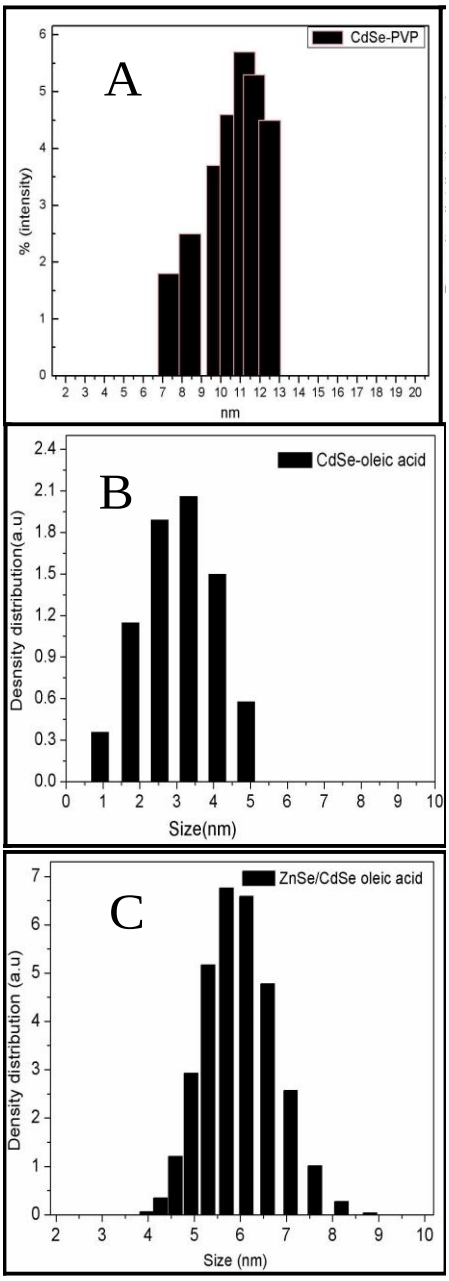

Fig. SI. Particle size distribution profile of (A) ZnSe/CdSe (B) CdSe and (C) PVP-CdSe QDs.