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Synthesis and characterization of Gadolinium doped ZnSe quantum dots for fluorescence imaging of cancer cell

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Gadolinium-doped ZnSe (Gd:ZnSe) Quantum dots (Qds) were synthesized using ethylene glycol as a stabilizing agent. The fluorescence spectrum exhibit an emission peak at 435nm for ZnSe, whereas the Gd doped ZnSe exhibit green emission at 547nm. In vitro imaging studies of MCF-7 cancer cells were carried out using fluorescent probes constructed using Gd-ZnSe Folate.

Breast Cancer is a serious health issue and major cause of death among the women around the globe [1]. Semiconductor nanomaterials also known as Qds possess great physical and chemical properties making them suitable candidate as an alternative fluoroprobe in the imaging and diagnosis of cancer disease [2,3]. Cadmium based semiconductors such as CdSe,CdS etc show high quantum yield and good photostability. They are color – tunable, highly stable and exhibit zero scattering [4]. But these cadmium based Qds are highly toxic and cannot be used for biological and medical applications[5]. Hence, it is essential to prepare less cytotoxic Qds with more biocompatibility,higher luminescence and photo stability.Recently, non-cadmium based low-toxic ZnSe based nanostructures have been reported as distinctive material for fluorescent labels, biological assays, imaging of cells and tissues, and in vivo investigations [6].

ZnSe possesses a wide band gap with bulk band gap of 2.7 eV at room temperature [7] as it is used for and hence is used in the fabrication of optoelectronic devices, solar cells [8], LED [9] and biological labelling [10]. Though ZnSe possesses interesting physical and chemical properties, it has very low fluorescence properties.For biological application like cancer cell imaging, the fluorescent emission properties would be preferably above 500nm. Hence, it is essential to enhance ZnSe fluorescence emission for biological cell imaging application.

Metal ion doping in Qds were usually carried out ~~by~~ using highly poisonous chemicals and reagents and also Qds were prepared by organometallic routes. Recent results showed that the metal ion doped ZnSe quantum dotswere carried out with high temperature, toxic chemicals and reagents. The Qds prepared by organometallic routes are more stable butcannot be directly dispersed in the water phase due to their hydrophobic nature. In order to convert it to hydrophilic surface and obtain water dispersible compound, surface modification is required. The recent studies showed that the higher luminescence water-dispersible ZnSe nanocrystalscan be developed in aqueous solution [12, 13].

The rare earth metal ion Gd has been proved to be excellent whendoped in to the ZnO matrix resulting in enhanced fluorescence properties as reported by Yanlan Liu et.al., [11].Moreover, The Qds optical properties can be tuned from visible – NIR by change of their particle size (~<30nm). Qds exhibit different color of emission with change in size [14]. The PL energy of the Gd^{3+} doped CdSe observed red shift compare with undoped CdSe, this was due to the diameter of the Gd^{3+} doped CdSe increased compare with undoped CdSe [15]. Inorder to tune the emission property, ZnSe may be doped with a suitable element, to increase the emission characteristics of ZnSe. So we have chosen Gd as the dopant andpreparedGadolinum doped ZnSe quantum dots modified with (3-Aminopropyl)triethoxysilane (APTES) and conjugated with folic acid as a fluorescent probe to image the cancer cells in-vitro.This method is cost effective and simple when compared to organometallicsynthesis; it hasvery lowtoxicity, highstabilityand can be prepared in bulk quantities. Experimental studies indicated that these fluorescent nanoprobes possess great potential in breast cancer cell imaging.

All chemicals and solvents used in this experiment were of analytical grade reagent and were used without further purification. Ethylene glycol (99%), selenium powder (200Mesh, 99.9%), $Zn(OAc)_2.2H_2O$(99.9%), $Gd(NO_3)_3$. $6H_2O$ (99.9%), and $NaBH_4$ (99%), were purchased from Aldrich Chemical Co., and APTES (99.9%) purchased from Alfa aesar. Minimum Essential Medium(MEM) was purchased from Hi Media Laboratories, Fetal Bovine Serum (FBS) was purchased fromCistron laboratories Trypsin, methylthiazolyldiphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO)were purchased from SRL. All other chemicals and reagents were obtained from Sigma Aldrich.Millipore water was used in all experiments.

MCF-7cell line were obtained from NCCS, Pune. The cells were maintained in MEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Sodium hydrogen selenium (NaHSe) was prepared according to the synthesis method reported by Daniel L. Klaymanet.al.,[16].

$$4NaBH_4 + 2Se + 7H_2O \longrightarrow 2NaHSe + Na_2B_4O_7 + 14H_2$$

10 mL of ethylene glycol was added slowly to 100 mL of 0.1 M $Zn(OAc)_2$ under constant stirring, in to which freshly preparedNaHSe solution was injected immediately. The reaction was carried out under N_2atmospherefor about 8h and the temperatureof the reaction mass was maintained at 100°C. Finally, the obtained precipitate wasisolated by centrifugation (5000 rpm). Gd:ZnSe Qds were synthesized by the same procedure with slight modification, i.e., 10 mL of 0.01 M $Gd(NO)_3.6H_2O$ was taken along with 90 mL of 0.1 M $Zn(OAc)_2$.

Gd:ZnSe Qds were dispersed in 10 mL of toulene, to which 1 mL of APTES in ethanol was added and stirred at room temperature for 30 minutes, and then the reaction mixture was kept refluxed at 80°C for 30 minutes. The obtained APTES modified Gd:ZnSe APTES-Gd:ZnSe Qds was purified by centrifugation followed by washing with ethanol. Finally, the purified Gd:ZnSe Qds wasredispersed in water in the dark environment for further experiments.

0.05mM of Folic acid (FA) in DMSO was added to the 10mg of APTES-Gd:ZnSe Qds in CH_2Cl_2(10ml) in presence of N,N'-dicyclohexylcarbodiimide (DCC) (0.05 mM). This mixture was stirred for 30min at room temperature. The obtained product (FA-APTES-Gd: ZnSe) was separated by centrifugation and then washed with CH_2Cl_2.

MTT Assay was carried out to evaluate the cell viability of MCF-7 upon FA-APTES-Gd: ZnSe Qds treatment.The concentration of NP was determined based on previous literature [17]. A wide range of concentration was taken to assess the viability.Cells (1×10^5/well) were plated in 24-well plates and incubated at 37˚C with 5% CO_2. At confluence, various concentrations of the nanoprobe were added and incubated for 24hrs. After incubation, the medium was removed from the well andthe cells were washed with phosphate buffered saline. MTT was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells to dissolve the formazan crystal formed and the absorbance was measured at 570nm with a UV- Spectrophotometer. Measurements were performed and

the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula: % cell viability = mean of absorbance of treated group / mean of absorbance of control group × 100%.

Cells were cultured on sterile cover glass in DMEM containing62.5 µg/mlof FA-APTES-Gd: ZnSe for 1h and 6 h at 37°C in a humidified atmosphere containing 5% CO_2. Before imagingthe medium was discardedand washed with phosphate buffered saline (PBS) to remove excess probes.

X-ray diffraction pattern of the prepared QDs were charecterized with Bruker D-8 Advance X-ray diffractometer (Cu K_α (1.5418Å)). UV–Vis absorption spectra were obtained using Shimadzu RF-5301. Fluorescence measurements were performed on an Agilent Varian Cary Eclipse spectrophotometer (FL1201M002). In both experiments, a 1cm path –length quartz cuvette was used. Surface morphology and size were examined by HRTEM (FEI-TECHNAI, G2-Model (T-30 S TWIN)) with an accelerating voltage 250kV and elemental analysis were done by EDAX. FTIR spectrophotometer (Perkin Elmer spectrum -II) was used to characterize the folic acid conjugated Gd:ZnSeQDs. The QDs labelled MCF-7 cells were imaged using confocal microscope (Zeiss LSM 510 META). The ZnSe and Gd:ZnSe were prepared in aqueous solution by nucleation doping strategy [18,19]. Zinc acetate, Gadolinium nitrate and Se powder were taken as precursors to prepare the QDs. Gadolinium doping of ZnSe QDs was achieved by replacing partially zinc acetate with gadolinium nitrate. Fig 1. Shows the XRD pattern of the ZnSe and Gd-doped ZnSe QDs. ZnSe shows the diffraction peaks which correspond to Zinc blende structure with JCPDCS card No. 37-1463. Gd-ZnSe also exhibited similar phase structure and lattice parameters which are consistent with that of ZnSe. From the results we observed the diffraction pattern does not show any peak of secondary phases of gadolinium like Gd_2O_3. It was also indicated that the diffraction peak intensities are affected by doping Gdon to the ZnSe matrix.The diffraction intensitymight decrease by doping with small atom. However doping with a larger Gd^{3+} results in more scattering centres and hence, results in increased peak intensity [20]. This confirms dopant Gd present in the ZnSe matrix. The crystallite size of ZnSe and Gd:ZnSe Qds was calculated using Scherrer equation and found to be 3.5 and 8 nm.

$$D = \frac{0.9\lambda}{\beta_{1/2} cos\theta}$$

Where λ is the wavelength of x-ray radiation, β is the full width at half maximum (FWHM) of the peaks at the diffracting angle θ.

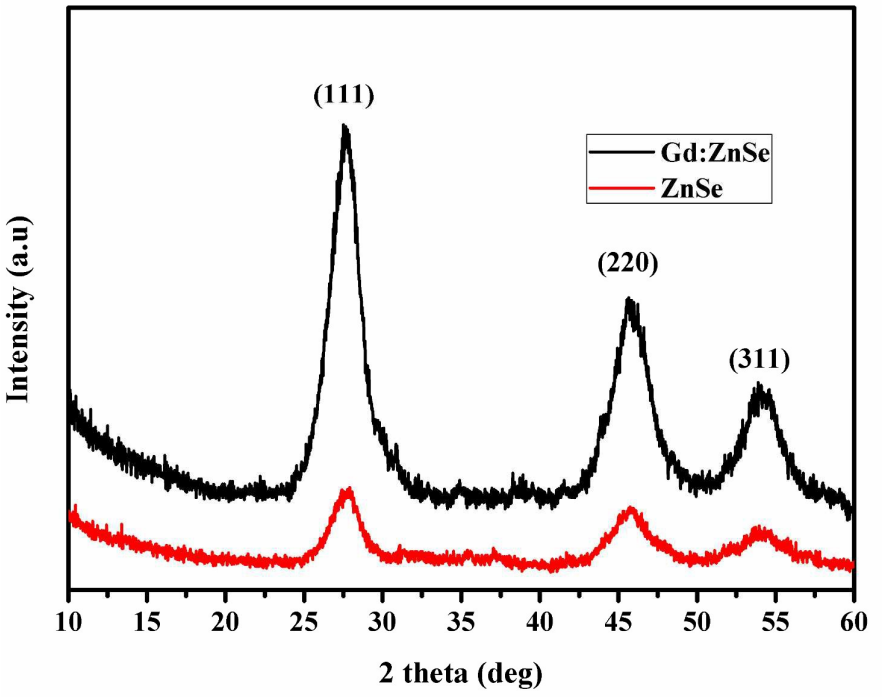

Fig 1: XRD patterns of the ZnSe and Gd:ZnSe Qds.

Fig 2 shows the optical absorption feature of ZnSe and Gd:ZnSe Qds. A sharp absorption peak appears at 362 nm for ZnSe whereas Gd doped ZnSe shows 365nm indicating that the injected metal ions are isomorphic to Zn^{2+} ions and decrease the band gap.

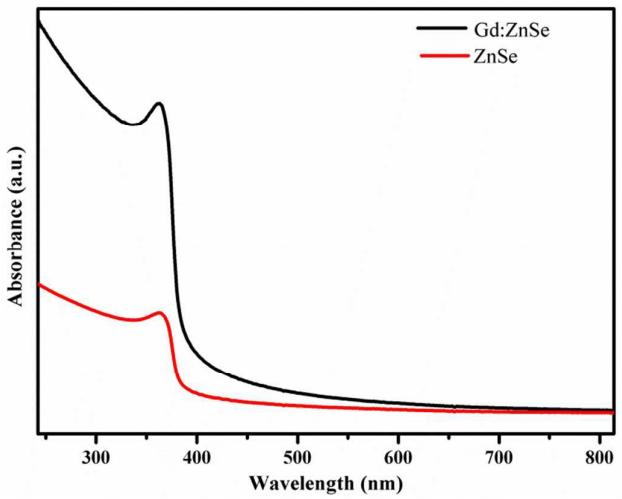

Fig 2: UV-Vis absorption spectra of ZnSe and Gd:ZnSe Qds

Fig 3 shows the corresponding Fluorescence emission spectra of ZnSe and Gd:ZnSe excited at 350nm. The emission spectrum of ZnSe shows bands at 435nm. The Gd:ZnSe shows emission at 547nm. This is attributed due to increased in particle diameter compare with undoped ZnSe. These enhanced emission band indicated that the Gd^{3+} trapped on ZnSe matrix.

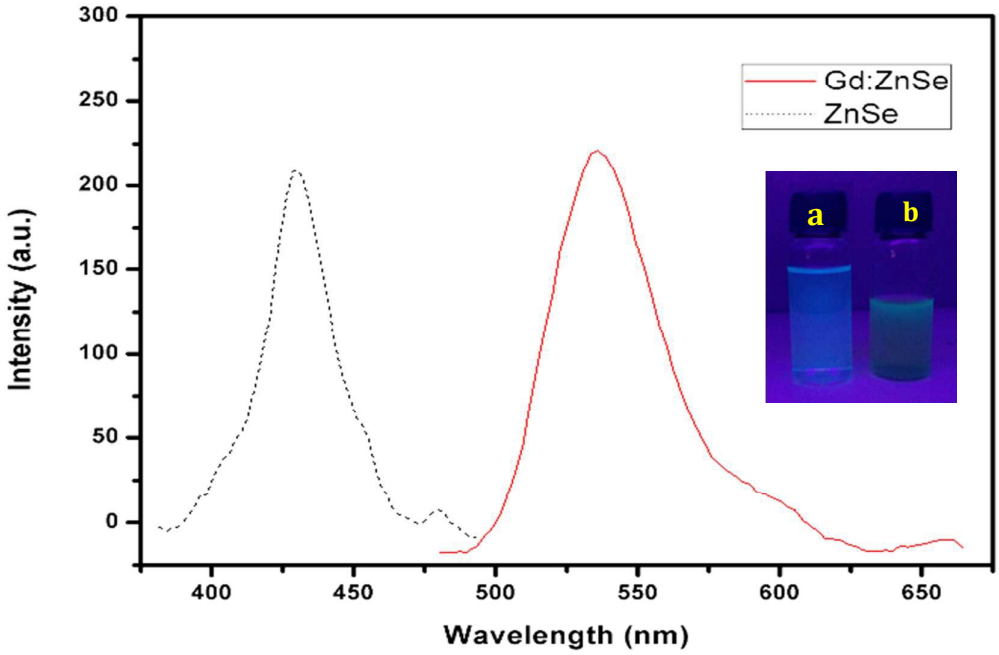

Fig 3: Fluorescent emission spectra of (a)ZnSe (b) Gd:ZnSe Qds (Inset: Photograph of (a)ZnSe (b) Gd:ZnSe Qds irradiated under UV; λ – 360nm)

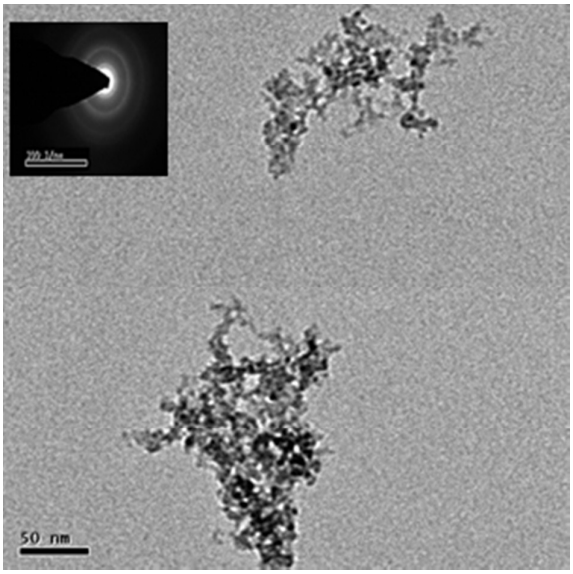

Fig 4:(a) HRTEM imagesof the Gd:ZnSe Qds. Inset:SAED of the Gd:ZnSeQds.

The presence of ethylene glycol as a stabilizing agent provides the stability and size control of the Gd:ZnSe Qds. Fig 4(a) shows the high-resolution TEM image of the Gd doped ZnSe Qds, confirming the size of the Qds as 8nm. The Fig 4(b) shows the corresponding selected area diffraction (SAED) patternof the Gd:ZnSe Qds. From these studies, it was found that the prepared Gd:ZnSe Qds crystal are grown withwell definedcubic structure, SAED pattern, consistingof discrete and bright rings. This indicatesthat the prepared Qds are amorphous in nature which might be due to the surface of the Qds comprising organic moiety.The SAED pattern also confirms zinc blende-structured Gd:ZnSe Qds grow along the (200) direction.

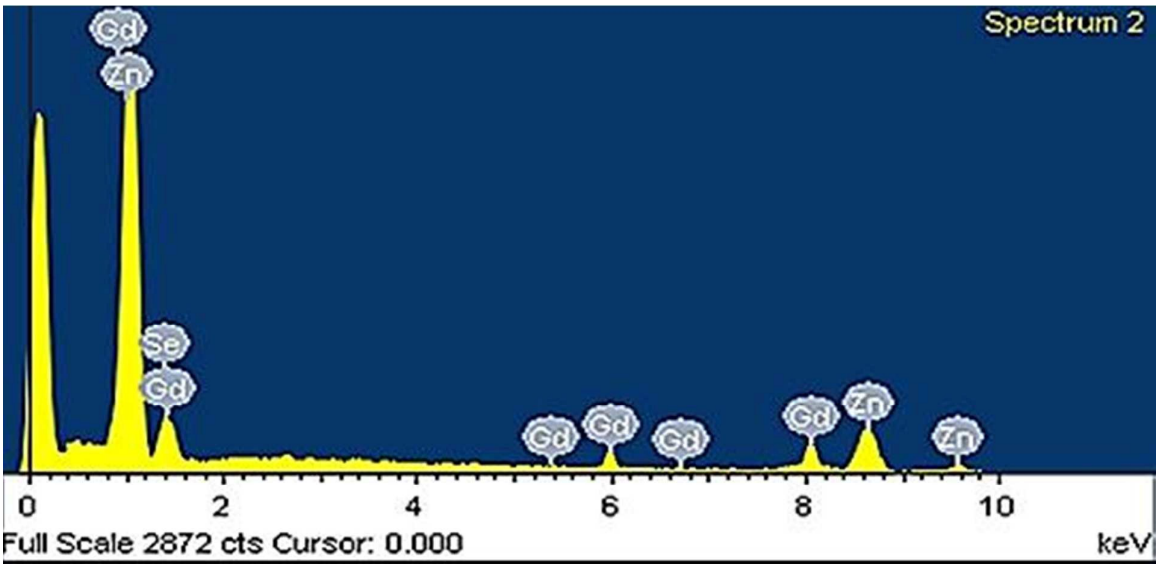

Fig 5. EDAX spectrum of the Gd:ZnSe Qds

Compositional analysis of the Gd:ZnSe Qds was done by EDAX. Figure 5 shows a typical EDAX spectrum of Gd:ZnSe Qds, the presence of zinc and selenium along with Gadolinium can be observed, which is in agreement with the results obtained with x ray difraction analysis..The quantitative analysis Gd present on Gd:ZnSe Qds was obtained from Inductive Coupled Plasma Emission Spectrometer (ICP-OES). which indicates the presence of Gadolinum (6.64mg/l), Zinc(626mg/l) and Selenium(173mg/l) quantitatively.

For fluorescence imaging application Gd^{3+} doped ZnSe Qds was modified with APTES and then it was conjugated with folic acid using DCC cross linking reagent. Fig 6. Shows the FT-IR spectra of Gd:ZnSe Qds, APTES- Gd:ZnSe Qds, and FA-APTES-Gd:ZnSe. In

Fig 6.,peaks at 3441cm^{-1}is OH stretching, 2878cm^{-1} and 1464cm^{-1} indicate CH bending which is attributed by ethylene glycol present over the Gd:ZnSe surface.

The APTES-Gd:ZnSe Qds showedweak band at 2923 cm^{-1}, which can be assigned to the alkylgroups [$(CH_2)_n$] present in APTES. The absorption bands at1561cm^{-1}is attributable to an NH_2 scissor vibration, suggesting thepresence of the amino groups of APTES molecules in the terminalposition of the propyl chain.In addition,the broad band at 1116 cm^{-1}is probably attributable to the stretch vibration of Si-O-Si. All thesefacts suggest that APTES has been successfully linked to Gd^{3+} doped ZnSe QDs surface. FA-APTES-Gd:ZnSe showed the characteristic IR absorption peaks of FA at 1605 cm^{-1} (benzene, conjugated double absorption), 1697 cm^{-1} (ester bond), and 1481cm^{-1} (hetero-ring,conjugated double bond),which confirms the folic acid conjugated with FA-APTES-Gd:ZnSe Qds.

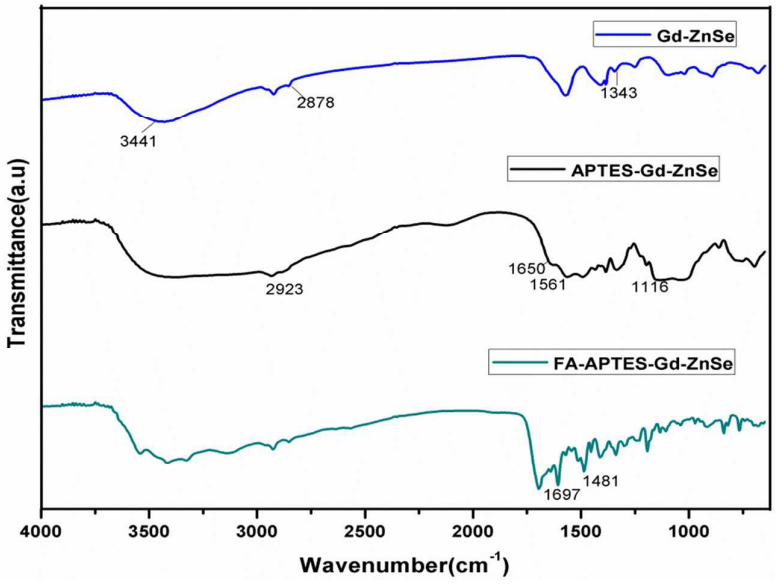

Fig 6: FTIR spectroscopy of Folate conjugated Gd:ZnSe Qds

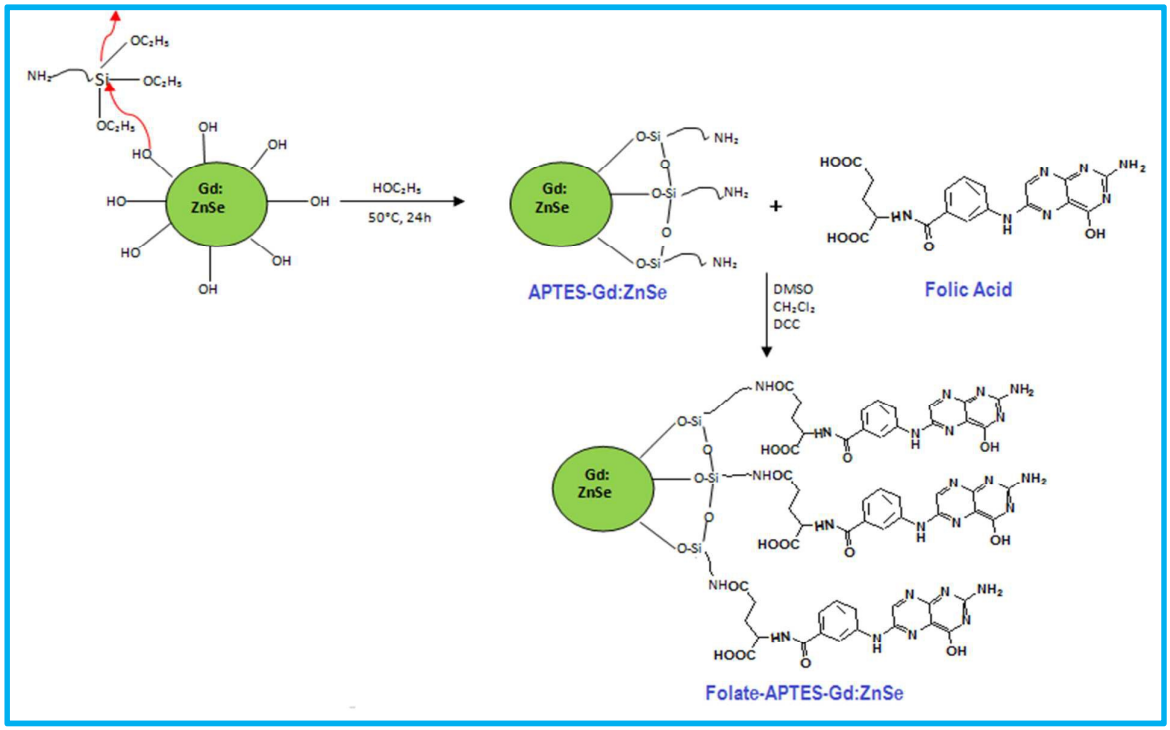

Fig 7: Schematic illustration for the synthesis of folate conjugated with APTES modified Gd^{3+} doped ZnSe quantum dots fluorescence probe.

The folic acid conjugated on gadolinium doped ZnSe quantum was schematically illustrated in Fig 7. Figure 8 shows the toxicity effects of varying concentrations from 0 to 250 µg/mL of FA-APTES-Gd:ZnSe nano fluorescence probes on MCF-7 cell viability using an MTT assay.

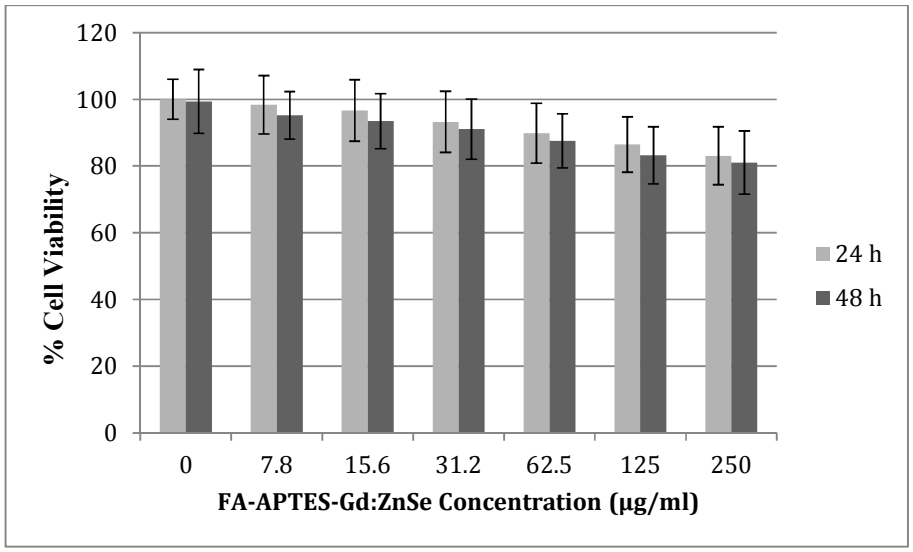

Fig 8: MTT assay of FA-APTES-Gd:ZnSe Qds on MCF – 7 Cell line

The concentration (0 to 250 µg/ml) dependent effect of FA-APTES-Gd:ZnSe probes on MCF-7 cell viability was measured using MTT assay and the results are shown in Fig 8. MCF-7 cells were cultured in FA-APTES-Gd:ZnSe probe containing medium for 24h and 48h. The results suggest that the cell viability has decreased in a dose dependent manner. Even at higher concentration of (31.2µg/ml) FA-APTES-Gd:ZnSe probes the viability of MCF-7 cells were above 92%, thus making it suitable for use in biological system due to its low cytotoxicity.From these results we can conclude that the obtained Qds fluorescence probe can be a better alternative to organic fluorescent probes for imaging in vitro due toits low cytotoxicity nature.

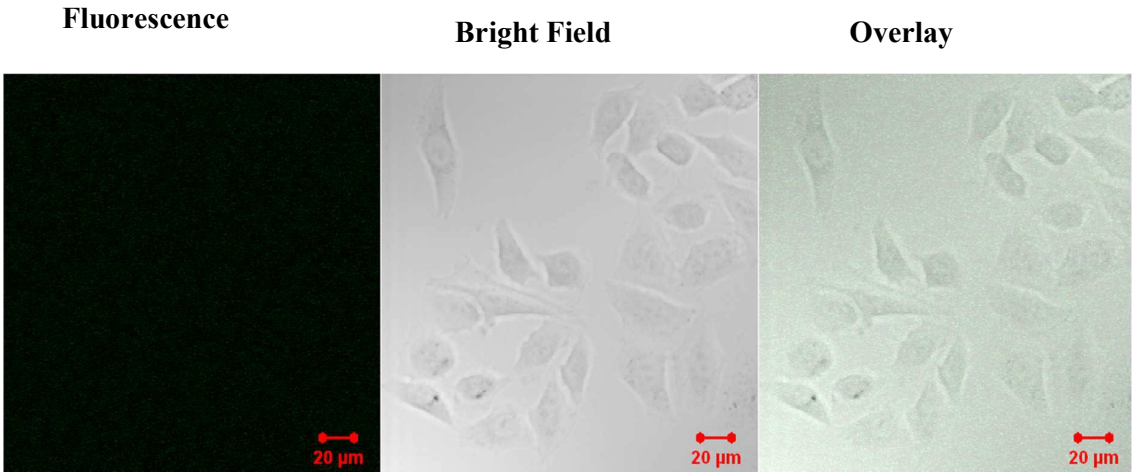

Fig 9. Confocal microscopic images of MCF 7 - 1 hour incubation (Without adding FA-APTES-Gd:ZnSeQds)

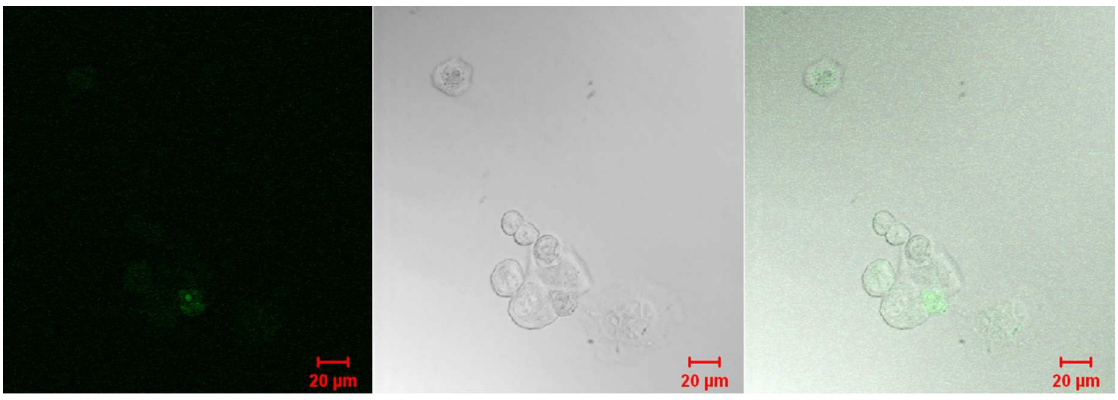

Fig 10. Confocal microscopic images of MCF 7 - 1 hour incubation Cocultured with FA-APTES-Gd:ZnSeQds

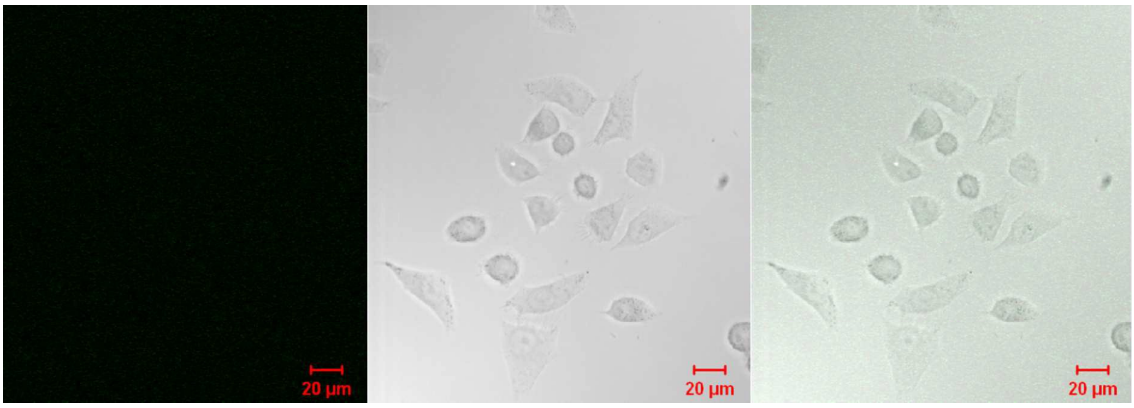

Fig 11. Confocal microscopic images of MCF 7 - 6 hour incubation (Without adding FA-APTES-Gd:ZnSeQds)

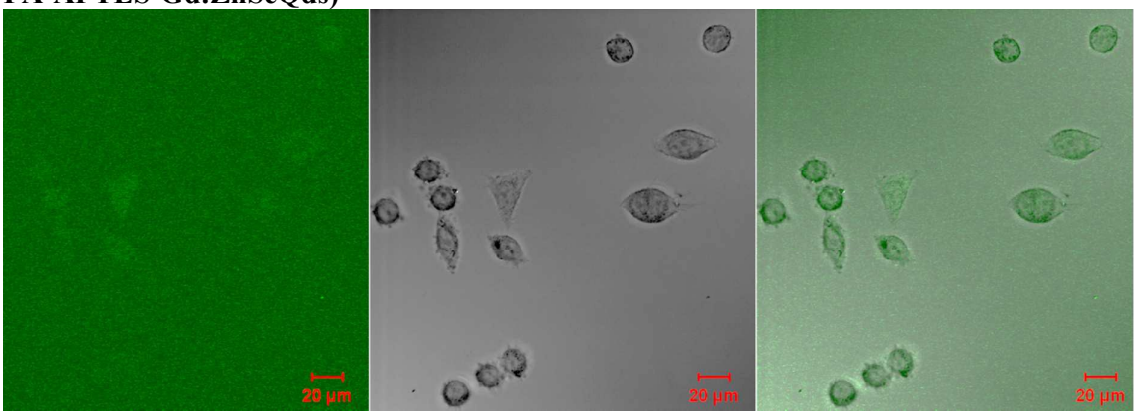

Fig 12. Confocal microscopic images of MCF 7 Cocultured with FA-APTES-Gd:ZnSeQds - 6 hour incubation

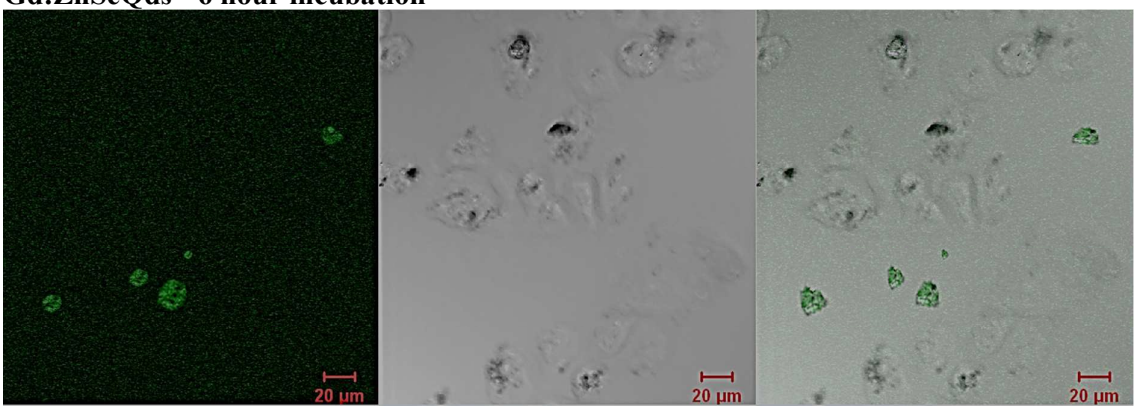

Fig 13. Confocal microscopic images of MCF 7 - Cocultured with APTES-Gd:ZnSeQds-6 hour incubation

The internalization of the FA-APTES-Gd:ZnSe Qds by MCF-7 was further established by confocal microscopy from Fig 9-13. Fig 9. Shows Confocal microscopic images of MCF 7 - 1 hour incubation without adding FA-APTES-Gd:ZnSeQds, indicates no fluorescence was observed. Fig 10 shows MCF 7 - 1 hour incubation Cocultured with FA-APTES-Gd:ZnSe Qds. It can be seen that slightly green fluorescence emission was observed. Fig 12. Showed Confocal microscopic images of MCF 7 Cocultured with FA-APTES-Gd:ZnSeQds - 6 hour incubation. This indicate the ability of the FA-APTES-Gd:ZnSe Qds (62.5µg/L) to penetrate cancer cells and serve as an internal fluorescence label and showed green fluorescence. It is described by, FA was conjugated on APTES-Gd:ZnSe surface of nano-particulate carriers to deliver therapeutic agents within cells via receptor mediated endocytosis. MCF-7 cancer cells are overexpress folic acid receptors and have high requirement for folic acid [21]. Qds can enter into the intracellular cytoplasm via endocytosisand selectively deliver the Qds to MCF-7 cells and to label them.

MTT assay and fluorescence studies revealed that the obtained Qds fluorescence probe can be a better alternative to organic fluorescent probes for imaging in vitro due to its low cytotoxicity nature.

Conclusion

In summary, Gd:ZnSe Qds were synthesized by a simple wet chemical route using ethylene glycol as stabilizer. The prepared Gd:ZnSe Qds exhibited emission at 547 nm due to increased in of Gd:ZnSe compared with ZnSe. It was a convenient, efficient method to obtain the rare earth metal ion-doped ZnSe nanomaterial which can be directly dispersed in the water phase. The average size of Qds was about 3.5-8 nm confirmed by HRTEM analysis. Gd-doped ZnSe QDs were indexed to cubic zinc blende ZnSe crystals. EDAX analysis further confirms Gd^{3+} ions present in the synthesized Gd:ZnSeQDs. Very low cytotoxicity effects of FA-APTES-Gd-ZnSe QDs on MCF – 7 cell lines were observed by MTT Assay. The obtained QDs fluorescence probe can be a better alternative to organic fluorescent probes for imaging of MCF-7 cancer cells by in vitro due to their low cytotoxicity and ease in preparation.

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