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One pot synthesis of luminescent Mn doped ZnSe nanoparticles and their silica based water dispersible formulation for targeted delivery of doxorubicin

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The manganese doped zinc selenide nanoparticles (ZnSe:Mn NPs) have been synthesized by thermolysis method using oleic acid and oleylamine as capping agents, and 1-octadecene as solvent. Coating of mesoporous silica is done on ZnSe:Mn (ZnSe:Mn@mSilica) which is further functionalized with amine functional groups by treating with (3-aminopropyl)trimethoxysilane. Further pegylation is done to achieve water dispersibility by conjugating carboxyl groups of poly(ethylene glycol) diacid with the amine groups. These pegylated NPs are subsequently treated with ethylenediamine followed by acrylic acid. Conjugation of tris-(hydroxymethyl-aminomethane) is performed by Michael-type addition reaction to afford ZnSe:Mn@mSilica-PEG-Tris-OH. These tris functionalized NPs have exhibited broad emission ranging from 590-620 nm that is an indicative for their suitability in diagnosis and monitoring progress of cancer treatment. To explore the usefulness of increased surface area because of mesoporosity, doxorubicin is loaded on ZnSe:Mn@mSilica-PEG-Tris-OH NPs through silyl ether linkage and evaluated for cytotoxicity against WEHI-164 mouse fibrosarcoma and RAJI human hematopoietic origin cancer cell lines. A decrease in 12% of cell viability of WEHI-164 cells while 30% decrease in RAJI cell lines ($IC_{50} \approx 45$ nM) are observed. This shows that our formulation has more cytotoxic in RAJI cancer cell lines than that of WEHI-164 cancer cells. These results reveal that the formulation has potential for the application in drug delivery and diagnosis in chemotherapeutics.

Keywords: ZnSe:Mn NPs, Pegylation, Silyl ether linkage, Doxorubicin, Luminescent nanoparticles

The quest of luminescent nanoparticles (NPs) for using clinically as optical bio-imaging agents as well as drug delivery vehicles in diagnosis and therapy of many diseases especially cancer, has been recognized as major frontier research area[1]. Initially fluorescent organic dyes[2] have been conjugated with drug delivery vehicles like liposomes, micelle, and silica based NPs[3]. But unlike conventional organic fluorophore, the luminescent NPs possess distinct photophysical properties, like broad excitation, size-tunable emission and high photostability rendering them promising fluorescent imaging agents. The surface functionalization of such NPs with specific biological moieties enables loading of drug, drift to cancer site and deliver drugs at cancer site. It was observed that the use of biological moieties that can sense specific physiological conditions like acidic pH of tumor site reduces the side effect with the increased therapeutic index. In addition to these, the luminescence property enables them a suitable modality for theranostic use, an emerging therapeutic paradigm[4].

In this perspective, various types of NPs based formulations were developed[5]. Among them, NPs with inorganic origin such as carbon nanotubes, gold nanoparticles and especially quantum dots of some semiconductor materials are main focus. Semiconducting inorganic nanocarriers have exhibited potential to be used for many biomedical applications, like drug delivery, cellular imaging[6] and in photodynamic therapy for cancer treatment[7]. Mainly semiconductor based NPs mostly in the form of quantum dots (QDs) that are made up from groups II-IV, III-V or IV-VI like CdSe, CdS, CdTe, PbSe, GaAs, InP, InAs, etc. were employed because of their exclusive electronic and optical properties[8]. However, toxicity originated from heavy metals like cadmium,

lead, or arsenic, which are deteriorated from the lattice into biological systems if the surfaces are not properly protected by the shells or ligands, has limited their practical biomedical applications[9]. Additionally, the major concern for the use of QDs in biological applications is inability to cross the cell membrane owing to their size and chemical nature and nonspecific cellular binding of quantum dots[10,11].

In view of these drawbacks, the researchers have developed NPs based on heavy metals other than Cd[12]. Among them, ZnSe based materials like zinc chalcogenides doped with Mn^{2+}, Eu^{2+}, Cu^{2+}, or Ga^{2+} exert low intrinsic toxicity and exclusive photophysical properties[13,14]. The cytotoxicity evaluations for these ZnSe NPs have revealed their suitability for biological applications ranging from diagnosis to drug delivery as evidenced for their non-toxicity up to certain concentration[15,16].

Considering these facts, Mn doped ZnSe luminescent NPs were synthesized and coated with mesoporous silica with objective of loading doxorubicin (adriamycin) in maximum amount by increased surface area due to porous nature of mesoporous silica[17,18,19]. The loading of doxorubicin was done after functionalization using synthetic chemistry strategies and evaluated for cytotoxicity against Burkitt's lymphoma cancer cells (RAJI)-human hematopoietic origin cancer cell lines and Walter and Eliza Hall Institute (WEHI-164)-mouse fibrosarcoma cancer cell lines. The drug release kinetics was also evaluated. The results are reported herein.

Materials and Methods

The elemental selenium (Se), 3-bromopropionic acid, sodium borohydride ($NaBH_4$), oleic acid, 1-octadecene, oleylamine, zinc acetate dihydrate [$Zn(OAc)_2.2H_2O$], manganese acetate tetrahydrate [$Mn(OAc)_2.4H_2O$], polyoxyethylene (5) nonylphenylether (IGEPAL-CO 500), cetyl trimethyl ammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), ammonia solution (30%), (3-aminopropyl)trimethoxysilane (APTMS), polyethylene glycol (PEG) diacid, tris-(hydroxymethyl)aminomethane hydrochloride (Tris HCl), dimethyldichlorosilane (DMDCS) and N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) and doxorubicin (Dox) were purchased from commercial sources. All reactions were performed under an inert atmosphere of argon (Ar). The selenium compound, $(SeCH_2CH_2COOH)_2$ was synthesized and purified by recrystallization in hot water. The solvents were purified by standard procedures and were distilled prior to use[20]. The melting point was determined in capillary tube and is uncorrected. Elemental analysis was carried out on Flash EA 1112 Series CHNS analyzer. NMR spectra were recorded on a Bruker Ascend™ 400 MHz spectrometer operating at 400.13 (^1H), 100.61 ($^{13}C\{^1H\}$) and 76.31 MHz ($^{77}Se\{^1H\}$) in DMSO-D_6. ^1H and $^{13}C\{^1H\}$ NMR chemical shifts are relative to internal DMSO peak. The $^{77}Se\{^1H\}$ NMR chemical shifts were relative to external Ph_2Se_2 in $CDCl_3$ (δ 463.0 ppm relative to Me_2Se (0 ppm). The powder X-ray diffraction (XRD) patterns were recorded on a PANalytical X′pert PRO X-ray diffractometer using Cu-Kα radiation in the 2θ range of 10-70° with step size 0.02° and time gap of 1.20 s. The XRD patterns were analyzed by comparing with standard reported data. The transmission electron microscopic (TEM) image was recorded on Libra-120 plus TEM (Carl Zeiss, Germany) operated at 120 kV. The cancer cell lines *viz.*, mouse BALB/c, fibrosarcoma (WEHI-164) and human hematopoietic origin cancer cell lines (RAJI) were obtained from the National Center for Cell Sciences (NCCS), Pune, India. The cytotoxicity of formulation was evaluated using flow cytometer - Guava Flow cytometer (Luminex Corporation, USA).

Synthesis of 3, 3′-diselenodipropionic acid [$(SeCH_2CH_2COOH)_2$][21]

In a three-necked round bottom flask, the elemental selenium (1.5 g, 19.00 mmol) suspended in distilled water and $NaBH_4$ (0.72 g, 19.95 mmol) were reacted by stirring and followed by refluxing for 30 min and then cooling to room temperature. To this, the 3-bromopropionic acid (2.90 g, 19.00 mmol) was added. The reaction mixture was stirred further for 2 h till the formation of a yellow precipitate, worked up with diethyl ether (3 × 50 mL) and then concentrated *in vacuo* to afford the yellow powder which was recrystallized in hot water to afford pale yellow crystalline solid product, [$(SeCH_2CH_2COOH)_2$] (2.4 g, 83 %) in optimum yield. M. p. 142 °C. Analysis Calcd. for $C_6H_{10}O_4Se_2$: C, 23.70, H, 3.30%; Found: C, 23.30, H, 3.20%. ^1H NMR (DMSO-D_6) δ: 2.74 (t, J = 6.9 Hz, 2 H, $SeCH_2$), 3.04 (t, J = 6.9 Hz, 2 H, $SeCH_2CH_2$); $^{13}C\{^1H\}$ NMR (DMSO-D_6) δ: 24.2 (J_{Se-C} = 75.0 Hz, $SeCH_2$), 35.7 ($SeCH_2CH_2$), 173.4 (C=O); $^{77}Se\{^1H\}$ NMR (DMSO-D_6) δ: 316 ppm.

Synthesis of Mn doped ZnSe NPs

In a three-necked round bottom flask, zinc acetate (0.5 g), $(SeCH_2CH_2COOH)_2$ (2.02 g) and manganese acetate (20 mg) were mixed with oleic acid (4 mL), oleylamine (15 mL) and 1-octadecene

(30 mL). In the inert atmosphere of argon, the reaction mixture was allowed to dissolve and heated slowly with the heating rate of 4 °C per minute till 320 °C. During the heating process, changes in the appearance of the reaction mixture to a yellow coloured solution at 150 °C and then to dark red at 250 °C were observed. After heating at 320 °C for 2 h and cooling to room temperature, the NPs were precipitated out by addition of acetone (~100 mL) in excess, collected by centrifugation and washed repeatedly (3-4 times) with acetone. After drying under an IR (Infrared) lamp, the NPs were characterized with powder XRD and TEM analyses.

Coating of mesoporous silica on Mn doped ZnSe NPs: ZnSe:Mn@mSilica[22]

To oleic acid coated ZnSe:Mn NPs (200 mg) dispersed in cyclohexane (4 mL), cetyl trimethyl ammonium bromide (CTAB, 800 mg) dissolved in water (20 mL) was added and stirred for overnight till the clearly transparent solution was observed. After adjusting the pH of the solution to 11 by controlled addition of ammonia solution (500 μL), TEOS (1.5 mL) was added slowly over a period 1 h. After stirring the reaction mixture at 60 °C for 1 h and stirring at room temperature for 5 h, the NPs were collected. For getting mesoporosity in the NPs, removal of CTAB was done by solvent extraction method using ethanolic ammonium nitrate solution. The obtained ZnSe:Mn@mSilica NPs were collected by centrifugation and characterized by TEM analysis.

Functionalization of mesoporous silica coated over Mn doped ZnSe nanoparticles: ZnSe:Mn@mSilica-NH_2

In a round bottom flask containing nanoparticles (ZnSe:Mn@mSilica) (300 mg) dispersed in dried toluene (5 mL), APTMS (2 mL) and triethylamine (1 mL) were added and the reaction mixture was stirred for overnight (~ 10 h). The functionalized NPs, ZnSe:Mn@mSilica-NH_2 were collected by centrifugation and washed with ethanol and finally with acetone.

Synthesis ZnSe:Mn@mSilica-PEG

In a round bottom flask, PEG-diacid (200 mg) was treated with DCC (900 mg) at 0 °C in DMF (dimethylformamide) (5 mL). After stirring for 6 h at room temperature, ZnSe:Mn@mSilica-NH_2 NPs (200 mg) and catalytic amount of DMAP were added, stirred for another 6 h and followed by addition of excess ethylenediamine (2 mL). After stirring for overnight, the NPs were obtained by centrifugation, washed with acetone and dried under an IR lamp.

Synthesis of ZnSe:Mn@mSilica-PEG-Acrylic

In a round bottom flask, the NPs of ZnSe:Mn@mSilica-PEG (126 mg) were dispersed in DMF (5 mL). In another round bottom flask containing acrylic acid (200 mg, 140 μL), DCC (859 mg) was added at 0 °C, transferred to the round bottom flask containing the dispersed NPs and stirred at room temperature. After stirring the reaction mixture for overnight, the NPs were collected by centrifugation, washed with acetone, dried under an IR lamp and characterized with FT-IR spectroscopy (υ in cm^{-1}): 3700, 1690, 1540, 800.

Tris functionalization: ZnSe:Mn@mSilica-PEG-Tris-OH

To acrylic functionalized NPs, ZnSe:Mn@mSilica-PEG-Acrylic (100 mg) dispersed in methanol (10 mL), tris-(hydroxymethyl-aminomethane) (250 mg) and sodium bicarbonate (500 mg) were added and stirred. After stirring for overnight, NPs were collected by centrifugation, washed with acetone and dried under an IR lamp. The NPs were characterized with FT-IR spectroscopy (υ in cm^{-1}): 3277, 2890, 1655, 1556, 1054, 795, 455.

Preparation of formulation: Loading of Dox over surface functionalized ZnSe:Mn@mSilica-PEG-Tris-O-Si(Me_2)-O-Dox

To the ZnSe:Mn@Silica-PEG-Tris-OH NPs (25 mg) dispersed in dry DMF (2 mL), a large excess of DMDCS (1 mL) and imidazole (5 mg) were added at room temperature. The reaction mixture was stirred for overnight (≈ 10 h). Then this reaction mixture was centrifuged followed by washing with DMF (2 × 5 mL) and collected the NPs. These NPs were further dispersed in dry DMF (2 mL) followed by addition of doxorubicin (2 mg) and imidazole (4 mg) under subdued light conditions. Then the reaction mixture was covered with aluminium foil to protect from light. After stirring for 5 h, the NPs were collected by centrifugation, and then washed with ethanol (5 mL) till there was no colour of Dox persisted in supernatant. The NPs (20 mg) were dried *in vacuo* and then characterized by FT-IR and UV-visible absorption spectroscopy. TEM images were taken by placing a drop of well dispersed NPs in hexane onto a carbon coated copper grid and after drying under vacuum.

Cytotoxicity evaluation of ZnSe:Mn@mSilica-PEG-Tris-O-Si(Me_2)-O-Dox: flow cytometer studies[23]

Cell culture

WEHI-164 mouse fibrosarcoma cell lines and RAJI human hematopoietic origin cancer cell lines

were obtained from the National Center for Cell Sciences (NCCS) Pune, India and cultured in DMEM (Dulbecco's Modified Eagle Medium) and RPMI (Roswell Park Memorial Institute) medium, respectively, that were supplemented with 10% serum (Invitrogen Corporation, CA, USA) and antibiotic solution. Cells were grown in humidified 5% CO_2 atmosphere in incubator at 37 °C and were passaged for every alternate day.

Cytotoxicity studies

The cytotoxicity of ZnSe:Mn@mSilica-PEG-Tris-O-Si(Me_2)-O-Dox and control NPs i.e., ZnSe:Mn@mSilica-PEG-Tris-OH was evaluated against WEHI-164 mouse fibrosarcoma and RAJI human hematopoietic origin cancer cell lines using flow cytometer. Around 0.1 million cells were seeded in each well of a 96 well plate and incubated for overnight in humidified incubator with 5% CO_2. The test compounds were prepared in PBS. The cells were treated with concentration of 20 nM and 50 nM for 48 h. The cells were harvested, washed with PBS and re-suspended in 1 mL of PBS. Around 20 µL of cell suspension was mixed with 20x of Guava cell viability reagents. Samples were analyzed on flow cytometer. The viability was expressed as absolute percentage of viable and dead cells.

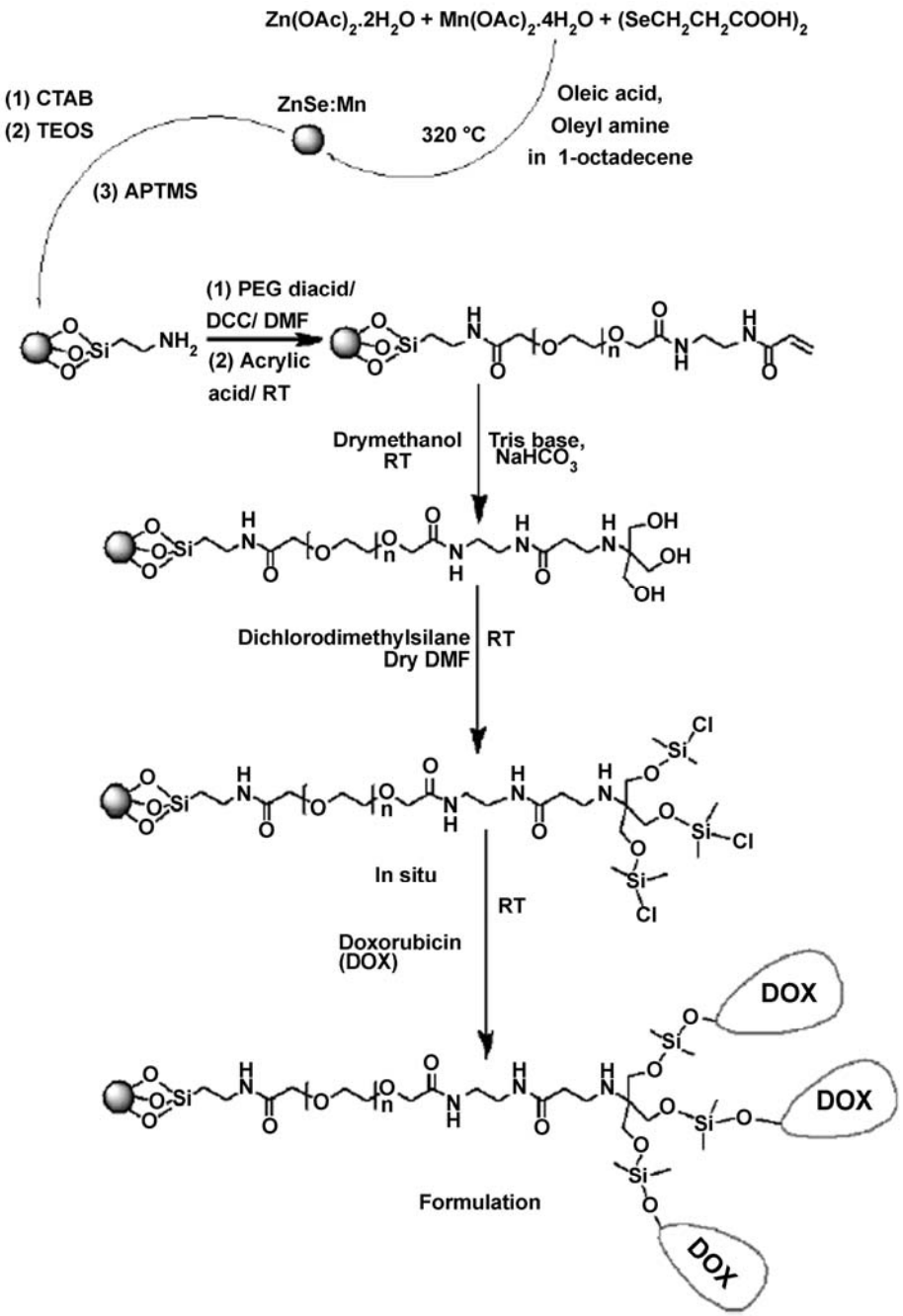
Results and Discussion

Various synthetic methods have been reported for synthesis of Mn doped ZnSe (ZnSe:Mn) based NPs, nanotubes, nanosheets, microflower or colloidal form or its core-shell analogues. Some of prominent methods are microwave-assisted solvothermal method, hydrothermal method[24-27], thermolysis[28] or simple thermal treatment[29] and use of simple chemicals[30]. We have employed thermolysis of organoselenium compound, $(SeCH_2CH_2COOH)_2$ along with Zn and Mn compounds using oleylamine and oleic acid as capping agents and 1-octadecene solvent at 320 °C for synthesis of ZnSe:Mn NPs (Scheme 1).

The obtained NPs were characterized with powder XRD analysis. Three diffraction peaks observed at 2θ: 28.6°, 47.8° and 55.8° matched with corresponding planes *i.e.*, (111), (220) and (311) lattice planes of the standard cubic zinc blende structure (JCPDS file No. 80-0021; Fig. 1). The morphology of well dispersed, sonicated ZnSe:Mn NPs was studied with TEM imaging (Fig. 2a) and found to have the spherical NPs with uniform shape and size of 10 nm. This shows that the NPs are monodisperesed (uniform size) which is the required property for biological applications[31].

The coating of these NPs by oleic acid renders them hydrophobic and unsuitable for biological applications. To make biocompatibility for health related applications; usually transfer to hydrophilic form from hydrophobic can be achieved by surface modifications[32]. Hence, we coated NPs with mesoporous silica by a modified Stöber et al., method producing ZnSe:Mn@SiO_2 NPs. The characterization was done by powder XRD which revealed the formation of cubic phase ZnSe (JCPDS PDF no. 01-088-2345) with SiO_2 (JCPDS PDF no. 821576) (Fig. 1). The confirmation of silica shell above ZnSe NPs was done by TEM image (Fig. 2b) due to the difference in the refractive index of the shell (SiO_2) and the core (ZnSe:Mn). Determination of the thickness of the shell and overall size of the NPs was done statistically by employing hundred NPs. The thickness of the shell was found to be in the range of 60±1 nm and overall size of the NPs was in the range of 70±10 nm. The ZnSe:Mn@SiO_2 NPs were further functionalized with -NH_2 functional groups by treatment of ZnSe:Mn@mSiO_2 with APTMS in basic medium. The amine functional groups present on the ZnSe:Mn@SiO_2-NH_2 were characterized by FT-IR spectroscopy (Fig. 3). The bands at 1035 cm^{-1} are assigned for Si-O-Si symmetrical and asymmetrical stretching[33]. The bands at 780 cm^{-1} (N-H wag), 1650 cm^{-1} (N-H bending) and the broad band centered at 3300 cm^{-1} confirm the presence of amine groups on the surface of ZnSe:Mn@SiO_2-NH_2[34].

Using amine functional groups on the surface of ZnSe:Mn@SiO_2-NH_2 NPs, pegylation was performed by PEG-diacid to afford the water dispersible and PEG functionalized NPs (ZnSe:Mn@mSilica-PEG)[35]. One of the carboxyl groups was attached to the NPs and another carboxyl group was free. The free carboxyl group was allowed to react with ethylenediamine forming amine functionalized NPs. They were further treated with acrylic acid to obtain ZnSe:Mn@mSilica-PEG-Acrylic NPs[36]. Further treatment with tris(hydroxymethylaminomethane) in methanolic solution in presence of sodium bicarbonate yielded the ZnSe:Mn@Silica-PEG-Tris-OH. These NPs were characterized with FT-IR spectroscopy as well as photoluminescence. The presence of broad peaks centered at 1673 cm^{-1} and 3432 cm^{-1} confirm the formation of the functionalization. When photoluminescence studies



Scheme 1 — Schematic representation for the synthesis of nano-formulation loaded with doxorubicin

were conducted, broad emission at the range of 580-615 nm wavelengths was observed (Fig. 4). It is indicative that the said NPs have potential for bio-imaging in diagnosis and treatment in desired area of anticancer applications.

The tris functionalized NPs were treated with DCDMS to render the intermediate with silyl compound which further treated with Dox to load the Dox through silyl ether bond. The formulation, ZnSe:Mn@mSilica-PEG-Tris-O-Si(Me_2)- O-Dox was confirmed by FT-IR spectroscopy. The presence of broad peak centered at 1636 cm^{-1} for C=O of amide of ZnSe:Mn@mSilica-PEG-Tris-O-Si(Me_2)-O-Dox and carbonyl of Dox and 3363 cm^{-1} from O-H stretching vibrations. The presence of peak at 3021 cm^{-1} for C-H stretching vibration of aromatic carbons of Dox also further confirmed the loading. The amount of Dox loaded was quantified by UV-visible absorption

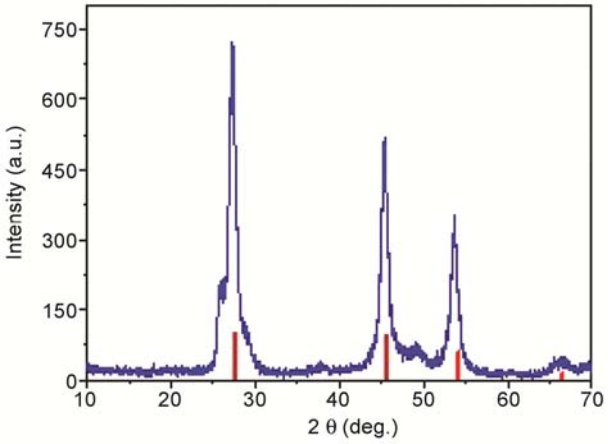


Fig. 1 — Powder XRD pattern for ZnSe:Mn NPs

spectral analysis. It was observed that the amount of Dox loaded was 22% of the total weight of the formulation. This formulation was evaluated for its cytotoxic ability against the RAJI-human hematopoietic origin cancer cell lines and WEHI-164 mouse fibrosarcoma cell lines using flow cytometer. The formulation exhibited potential cytotoxicity as evidenced from flow cytometry (Fig. 5). In case of WEHI-164 cell lines 12% cell killing while in case of RAJI cancer cell lines 35% cell killing ($IC_{50} \approx 45$ nM) when treated with the formulation nanomolar (nM) level of concentration of the formulation. From the results, it is evident that the formulation has the potential for the application in drug delivery and diagnosis in chemotherapeutics.

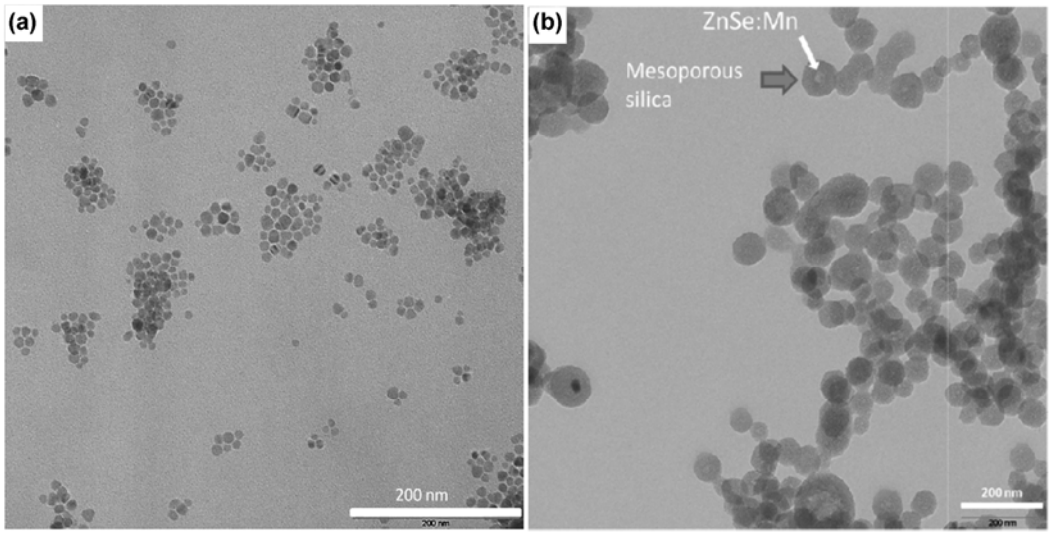


Fig. 2 — TEM images of (a) Mn doped ZnSe and (b) silica coated ZnSe:Mn NPs

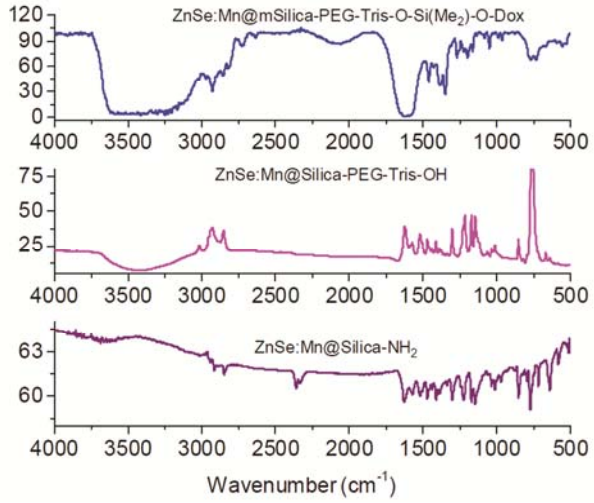


Fig. 3 — FT-IR spectra indicating the progress of surface functionalization of ZnSe:Mn NPs

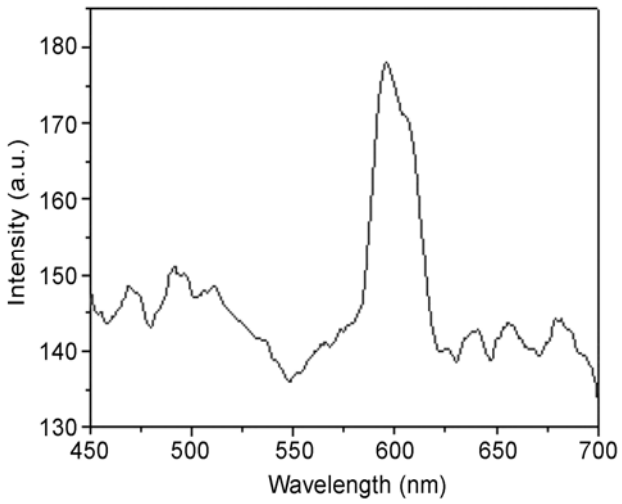


Fig. 4 — Emission spectrum of ZnSe:Mn@mSilica-PEG-Tris-OH with 280 nm excitation

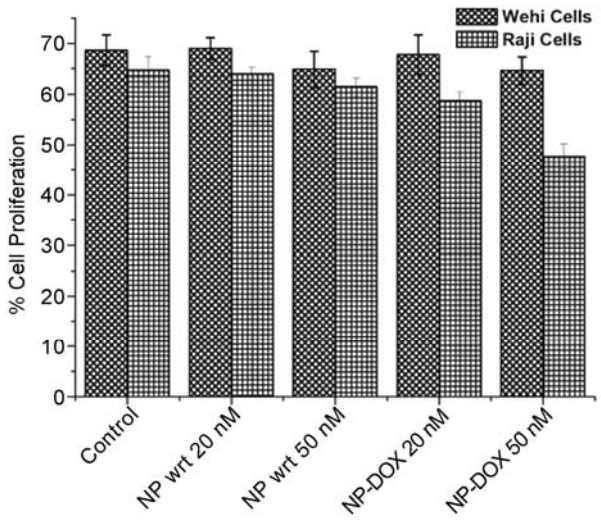


Fig. 5 — Cytotoxicity evaluation of the formulation against WEHI-164 and RAJI cancer cell lines by flow cytometer

Conclusions

We have synthesized ZnSe:Mn NPs coated with mesoporous silica which on further surface modifications to afforded amine functional groups. Further pegylation, followed by sequential treatment with acrylic acid followed by tris(hydroxymethyl-aminomethane) to achieve the Tris fictionalized NPs, ZnSe:Mn@mSilica-PEG-Tris-OH. These NPs exhibited emission at 590 nm. Then the drug doxorubicin was loaded on ZnSe:Mn@mSilica-PEG-Tris-OH through silyl ether linkage. This obtained formulation, MnZnSe@mSilica-PEG-Tris-O-Si(Me_2)-O-Dox was evaluated for the cytotoxicity against RAJI human hematopoietic origin and WEHI-164 mouse fibrosarcoma cell lines which revealed potent cytotoxicity. From these results it is evident that the formulation has the potential for the application in drug delivery and diagnosis in cancer treatment.

Supplementary Data

The NMR [^{1}H, $^{13}C\{^{1}H\}$, $^{77}Se\{^{1}H\}$] and FT-IR spectra for characterization $(SeCH_2CH_2COOH)_2$ (DSePA), FT-IR spectra for authentication of surface chemical modifications on NPs and flow cytometer graphs during cytotoxicity evaluation are given in the supplementary data. The supplementary data associated with this article are available in the electronic form at http://nopr.niscair.res.in/jinfo/ijca/IJCA_60A(03)348-355_SupplData.pdf.

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