

Cytotoxicity and cellular uptake of ZnS: Mn nanocrystals biofunctionalized with chitosan and aminoacids

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A b s t r a c t

Highly luminescent, manganese doped, zinc sulphide (ZnS:Mn) nanocrystals biofunctionalized with chitosan and various aminoacids such as L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine were synthesized by chemical capping co-precipitation method at room temperature, which is a simple and cost effective technique. The synthesized nanocrystals were structurally characterized by TEM, XRD, EDXS and FT-IR spectroscopy techniques. They possess high colloidal stability with strong orange red photoluminescence emission at 598 nm. The intensity of orange red emission has been observed to be maximum in L-citrulline capped ZnS:Mn nanocrystals in which the emission at 420 nm is effectively quenched by surface passivation due to capping. Taking into consideration the prospects of these highly luminescent, bio-compatible ZnS:Mn nanocrystals in bio-imaging applications, cytotoxicity studies were conducted to identify the capping combination which would accomplish minimum toxic effects. ZnS:Mn nanocrystals biofunctionalized with chitosan, L-citrulline, glycine, L-arginine, L-serine and L-histidine showed least toxicity up to 10 nM concentrations in mouse fibroblast L929 cells, which further confirms their cytocompatibility. Also the ZnS:Mn nanocrystals biofunctionalized with L-arginine showed maximum uptake in in vitro studies carried out in human embryonic kidney cells, HEK-293T, which shows the significant role of this particular amino acid in fetoplacental nutrition. The present study highlights the suitability of aminoacid conjugated ZnS:Mn nanocrystals, as promising candidates for biomedical applications.

Introduction

Semiconductor nanomaterials have received much attention in the last two decades as efficient fluorescent probes for live cell imaging of cellular and sub-cellular processes [1–6]. A major requirement for expanding their biological application [7–9] demands the synthesis of monodispersed, water soluble and bio-functionalized nanoparticles, non toxic to animal cells. Several strategies have been adapted previously for biofunctionalization [10–16] of nanoparticles for diverse applications such as cell labeling [17–22], cell tracking, in vivo imaging [23–25] and multiplexing [26–28]. Biofunctionalization of nanomaterials involves the attachment of bio-recognition molecules such as proteins, peptides, anti-bodies, carbohydrates, nucleic acids, aminoacids and polymer materials as capping agents. These bio-compatible capping agents bind to the surfaces of the nanoparticles during synthesis and provide surface passivation, capable of protecting the nanoparticle surfaces from oxidation. Such surface passivation effects often lead to higher luminescence intensity and quantum efficiency in semiconductor nanocrystals. These surface modified nanocrystals can be attached to the target bio-molecules via covalent bonding or self assembly in the same phase. The conjugation of surface modified nanoparticles with specific biomolecules allows researchers to target the desired location with reduction in overall toxicity and enhanced efficiency of the imaging probes [29].

The synthesis and optical characterization of water dispersible, ZnS:Mn nanocrystals capped with L-valine, cysteine, histidine, arginine, methionine and chitosan have already been reported earlier [30–33]. The present work is aimed at synthesizing fluorescent ZnS:Mn nanocrystals suitable for bio-imaging applications, using chitosan and amino acid ligands as capping materials, which is an effective technique for the synthesis of bio-compatible semiconductor nanocrystals. Chitosan is well-known as a capping agent due to its significant chemical and biological properties such as hydrophilicity, biocompatibility, biodegradability, and antibacterial properties [34]. The passivation layer of chitosan on the surface of nanocrystals makes them bio-compatible so that they can be attached to biomolecules such as DNA [35], RNA, proteins [36,37], and peptides [38]. More recently, amino acid ligands have been developed as effective surface capping agents in the synthesis of semiconductor nanocrystals with controllable size distribution. Surface modification of these nanocrystals by suitable aminoacid ligands makes these nanocrystals bio-compatible. Organic ligands that bind to the surfaces of the nanoparticles during synthesis provide the required surface passivation that protects the nanoparticle surfaces from oxidation and minimizes the electronic trapping capabilities of surface defects, both of which lead to a high photoluminescence efficiency in semiconductor nanocrystals. The presence of reactive amine and –OH groups in aminoacids makes them good candidates for biomedical and pharmaceutical applications.

Manganese doped zinc sulphide (ZnS:Mn) is a highly pursued phosphor material which exhibits excellent luminescence characteristics including high luminescence intensity, narrow emission bands, low photobleaching, large Stokes' shift and high photo-chemical stability [39–43]. Its high luminescence efficiency and stability at ambient temperatures are critical properties required for biological applications. Detailed studies on the toxicity of these nanoparticles at cellular and

subcellular levels are mandatory for approving their biological applications. Several groups have investigated the toxicity of nanoparticles based on various factors including shape, size, surface chemistry, chemical composition, surface activity and solubility [44–48].

Recommendations for the use of nanoparticles in various fields have emerged as a result of these initial toxicity studies. A few reports have appeared recently on toxicity studies of quantum dots (QDs) in cells and animals [49,50]. Cell culture experiments have shown that QDs undergo design-dependent intracellular localization which can cause cytotoxicity by releasing free toxic materials into solution and by generating free radical species. In animal experiments, QDs preferentially enter the liver and spleen following intravascular injection which undergo minimal excretion if larger than 6 nm, and appear to be safe to the animal. The present work is focused on the cytotoxicity and cellular uptake studies of ZnS:Mn nanoparticles biofunctionalized with chitosan and various aminoacids. The cytotoxicity of these biofunctionalized nanoparticles was evaluated in mouse fibroblast cells and cellular uptake was investigated in human embryonic kidney cells employing MTT assay and fluorescent microscopic techniques.

From the in vitro toxicological analysis, it has been observed that ZnS:Mn nanocrystals capped with L-citrulline are less toxic and hence highly bio-compatible for biomedical applications.

It is also seen that the viability of cells bio conjugated with L-arginine capped ZnS:Mn nanocrystals is quite close to that of cells conjugated to L-citrulline capped nanocrystals. The in vitro cellular uptake study in HEK293T cells has shown that ZnS:Mn nanoparticles capped with L-arginine has the maximum uptake compared with those capped with L-citrulline, L-histidine and chitosan. These biofunctionalized nanocrystals have been synthesized using simple and cost effective chemical capping co-precipitation technique. The present investigations have proved that aminoacid capped ZnS:Mn nanoparticles, which show strong fluorescent emission are highly bio-compatible and economically viable. In this context, the present work is novel and significant and proposes the suitability of these biofunctionalized nanocrystals as fluorescent probes in biological applications.

Materials and methods

Synthesis of bio-compatible ZnS:Mn nanocrystals

Synthesis of chitosan capped ZnS:Mn nanoparticles

In the present work ZnS nanoparticles doped with manganese (Mn) were prepared by a chemical capping co-precipitation method in which zinc acetate [$\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$] (Merck Specialities Private Limited, Mumbai, >98%), manganese acetate [$\text{Mn}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$] (Merck Specialities Private Limited, Mumbai, >99.5%) and sodium sulphide $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (Merck Specialities Private Limited, Mumbai, 98%) were used as the reactants. These nanoparticles were stabilized by steric hindrances upon using chitosan as a capping agent. The inorganic wet chemical synthesis used to prepare ZnS:Mn nanocrystals was carried out at room temperature in water for all of its inherent advantages of being simple and cost effective. This method is similar to that described by Bhargava et al. [51,52] with a suitable modification. With Mn doping high luminescence occurs at around 597 nm [53]. Chitosan is a bio-compatible capping agent which prohibits the diffusion of ions from the

solution and restricts the growth of nanocrystals. In this method, 0.1 mol L⁻¹ zinc acetate and 0.01 mol L⁻¹ manganese acetate were mixed in 50 ml of water along with 0.001% of chitosan to which 0.1 mol L⁻¹ sodium sulphide was added dropwise to form chitosan capped ZnS:Mn nanoparticles.

Synthesis of aminoacid capped ZnS:Mn nanoparticles

The aminoacid capped manganese doped zinc sulphide nano-particles were synthesized by the same method described as above. The chemicals used were, Zn (CH₃COO)₂·2H₂O (Merck Specialities Private Limited, Mumbai, >98%), Mn(CH₃COO)₂·4H₂O (Merck Specialities Private Limited, Mumbai, >99.5%), Na₂S·9H₂O (Merck Specialities Private Limited, Mumbai, 98%), L-citrulline (C₆H₁₃N₃O₂), L-lysine (C₆H₁₄N₂O₂), 99%), L-arginine (C₆H₁₄N₄O₂), L-serine (C₃H₇NO₃), L-histidine (C₆H₉N₃O₂) and glycine (C₂H₅NO₂) (LOBA CHEMIE PVT, LTD, 99%). Here, 0.1 M zinc acetate, 0.01 M manganese acetate and 0.025 M the aminoacid were mixed in 50 mL of water to which 0.1 M aqueous solution of sodium sulphide was added dropwise to form aminoacid capped ZnS:Mn nanoparticles.

The mixtures thus obtained were kept under constant stirring for one and a half hour and then filtered and dried in an oven at 40 °C. These powder samples were found to be readily dispersible in water and used for further detailed studies. Introduction of different methods for the synthesis of nanoparticles and optimization of various conditions involved in the synthesis have significantly improved the quality of the nanoparticles.

Characterization

The X-ray powder diffraction (XRD) patterns of the as prepared ZnS:Mn nanoparticles were recorded on a Rigaku X-ray diffractometer with Cu K₁ radiation (1.5418 Å) operating at 30 kV and 20 mA. Scanning was carried out in the 2θ range from 20° to 60° at a scan speed of 2° per minute. Transmission electron microscope (TEM) image of the synthesized ZnS:Mn sample was obtained using JEOL 3010 instrument. The energy dispersive X-ray spectrum (EDXS) was obtained with JEOL Model JSM-6390LV scanning microscope. Fourier transform infrared (FT-IR) spectra of the samples were obtained with AVATAR 370 DTGS FTIR spectrophotometer in the wave number range 400–4000 cm⁻¹. The PL emission spectra of the samples were recorded with Fluoromax-3 Spectrofluorimeter using Xe lamp as the excitation source.

In vitro toxicological analysis

Cytotoxicity of ZnS:Mn nanoparticles coated with chitosan and different aminoacids was evaluated using, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium chloride (MTT) assay. Here, 1 × 10⁶ mouse fibroblast L929 cells were inoculated into 96 well tissue culture plates containing Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS] and incubated for 48 h at 37 °C. The cells were copiously washed with phosphate-buffered saline [PBS], and the medium was exchanged with DMEM containing different concentrations of capped ZnS:Mn nanoparticles ranging from 0.1 to 1000 nM. The cells were incubated for 24 h at 37 °C and subjected for MTT assay following standard protocol. Briefly, nanoparticle treated cells were supplemented with 50 μL of MTT solution (5 mg mL⁻¹) prepared in PBS and kept for incubation

under dark at 37 LC for 3 h. Subsequently, the viability of the cells was measured as a function of reduction of MTT to insoluble formazan by mitochondrial dehydrogenase enzyme of healthy cells. Formazan crystals were dissolved in dimethyl sulphoxide and the absorbance was recorded at 570 nm using a microplate reader (Biotech, USA).

In vitro cellular uptake study in HEK293T cells

HEK293T cells were used for the uptake studies. The cells were grown in DMEM with 10% FBS under the standard condition of 37 LC and 5% CO₂. For uptake studies, cells were seeded on cover slips in 24-well plate at a density of 10,000 cells/0.3 cm² and were incubated at 37 LC, 5% CO₂. After reaching ~60% confluency, cells were incubated with 2 IL of each type of capped ZnS:Mn nanoparticles and incubated for another 2 h. Controls were also kept without adding any nanoparticles. The medium was completely removed and 20% drug diluted in DMEM with 10% FBS was added to the corresponding wells excluding control well. The cells were kept in CO₂ incubator for 2 h. After incubation the medium was removed and cells were very gently washed with PBS for three times. After PBS wash, cells were fixed with 4% paraformaldehyde for 15 min at 4 LC. The cells were washed with PBS thrice. Nuclear staining of cells was done by adding 200 IL of the staining dye DAPI, to each well and incubating at room temperature for 20–30 min. DAPI was removed and washed with PBS thrice. The cover slips were very carefully lifted from the wells and mounted on glass slides with fluoromount. The cells were then visualized under UV microscope for the presence of nanoparticles and photographs were taken using Andor 885 camera. In order to check whether the concentration of the nanoparticles used for the uptake study affects the viability of the cells, the MTT assay as previously described was also performed.

Results and discussion

XRD analysis

The structural analysis of the capped ZnS:Mn powder samples is carried using XRD technique and Fig. 1 shows the XRD patterns of the Mn doped ZnS nanoparticles capped with chitosan and the aminoacids, L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine. The samples are having cubic crystal structure and the diffraction data are in agreement with the JCPDS data for ZnS (JCPDS card No.050566). The XRD patterns are quite broad with three main peaks corresponding to reflections from the (111), (220) and (311) planes which suggest zinc blende crystal structure. The particle size is determined from the full width at half maximum (FWHM) of the diffraction peaks, using Debye–Scherrer formula

$$d = \frac{a}{\sqrt{h^2 + k^2 + l^2}}$$

where d, k, b and h are the average particle size, the wavelength of the X-ray, full width at half maximum intensity expressed in radians and diffraction angle respectively. The ZnS:Mn nanoparticles capped with chitosan, L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine are found to have particle size around 10.4 nm, 10 nm, 12.9 nm, 8.4 nm, 8.8 nm, 8.2 nm and 10.7 nm respectively

TEM analysis

The TEM image of a typical capped ZnS:Mn sample (L-citrulline capped ZnS:Mn) was recorded to estimate the crystallite size and the image is shown in Fig. 2(a). The average crystallite size is approximately 10 nm. The average particle size obtained from TEM analysis is found to be approximately equal to that determined from the XRD peaks using the Debye–Scherrer formula. The energy dispersive X-ray spectrum (EDXS) of this typical sample is shown in Fig. 2(b), which provides the elemental composition of the sample. The presence of zinc, sulphur, manganese, carbon and oxygen in the powder sample can be confirmed from the spectrum. The presence of carbon and oxygen in the EDX spectrum is an indication of the effective capping of ZnS:Mn by the aminoacid, L-citrulline.

FTIR analysis

The FTIR spectra of the capped ZnS:Mn nanocrystals are shown in Fig. 3. The major peaks appearing at 662 cm^{-1} , 1014 cm^{-1} and 1400 cm^{-1} in Fig. 3 are attributed to the characteristic vibrations of the ZnS:Mn. The strong peaks appearing around 3400 cm^{-1} and 1550 cm^{-1} are assigned to the vibrational modes of zinc coordinated $-\text{NH}_2$ and $-\text{COO}$ groups for the corresponding aminoacids and chitosan. The IR spectra provide ample confirmation for the attachment of the aminoacid ligands and chitosan onto the surfaces of the ZnS:Mn nanoarticles to provide a water dispersible nature for the originally hydrophobic ZnS:Mn.

Photoluminescence studies

Photoluminescence spectra of Mn doped ZnS nanoparticles capped with chitosan and the aminoacids, L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine, recorded at room temperature under an excitation of 330 nm are shown in Fig. 4. The PL spectra obtained from the capped ZnS:Mn samples show intense orange red emission peaks at 598 nm. The small broad peaks observed around 420 nm can be due to the presence of defect states arising from zinc ion vacancies in the ZnS crystal lattice. The PL peak intensity of ZnS:Mn capped with L-citrulline and L-serine at 598 nm is found to be much higher than the peak intensity in other samples. Though the emission peak intensity at 598 nm of ZnS:Mn, capped with L-citrulline and L-serine are the same, the intensity of the peak at 420 nm is found to be less in the case of L-citrulline capped ZnS:Mn, compared to that in L-serine capped ZnS:Mn [54]. This indicates that L-citrulline is more effective as a fluorescent probe for biomedical applications.

In vitro toxicological analysis

Cytotoxicity of uncapped ZnS:Mn nanoparticles and ZnS:Mn nanoparticles capped with L-citrulline, L-lysine, chitosan, L-arginine, L-serine, L-histidine and glycine was evaluated using MTT assay in mouse fibroblast L929 cells for 24 h and the results are shown as bar diagram in Fig. 5. Studies carried out on the viability of cells (%) at different concentrations of these ZnS:Mn nanocrystals have shown that nanocrystals capped with L-citrulline are less toxic when compared to others up to 10 nM concentration and hence highly bio-compatible at this concentration. L-arginine is also found to be an efficient capping agent to make ZnS:Mn nanocrystals bio-compatible for 1 nM concentration.

In vitro cellular uptake study in HEK293T cells

In vitro cellular uptake study in HEK293T cells was carried out using ZnS:Mn nanocrystals capped with L-arginine, L-citrulline, L-histidine and chitosan and the cells were observed using fluorescence microscope and the images are shown in Fig. 6. All the nano-crystals showed cellular uptake at 1nM concentration. The concentration of all the capped ZnS:Mn nanocrystals, used for the uptake studies was fixed at 1nM, since, at this concentration the cells were found to be viable. Of all the capped nanocrystals, ZnS:Mn capped with L-arginine showed maximum uptake when compared with L-citrulline, L-histidine and chitosan. ZnS:Mn nano-particles capped with L-arginine at the concentration 1nM was found to be taken up by more number of cells. This is due to the fact that L-arginine is considered to be an essential amino acid for young developing mammals, suggesting a significant role for arginine in tissue growth [55]. It has been reported that L-arginine enhances cell proliferation and reduces apoptosis in human endo-metrial RL95-2 cells [56]. It also serves as a precursor for many molecules that are important for cellular physiology, including proline, glutamate, creatine, nitric oxide (NO) and polyamines, making L-arginine one of the most versatile amino acids. Wu et al. [57] have reported an unusual abundance of L-arginine in porcine allantoic fluid, suggesting a role for this particular amino acid in fetoplacental nutrition.

Conclusions

Bio-compatible ZnS:Mn nanocrystals have been synthesized using chitosan and amino acid ligands as capping materials. These capped ZnS:Mn nanocrystals show orange emission at 598 nm and the emission intensity of L-citrulline capped ZnS:Mn is found to be the maximum. The toxicity of these nanocrystals has been assessed in mouse fibroblast L929 cells using the colorimetric MTT in vitro assay and effects on human cells have also been investigated.

Studies on the cytotoxicity of uncapped and capped ZnS:Mn nanocrystals with chitosan, L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine as capping agents, show that nanocrystals capped with L-citrulline are less toxic for 0.1 nM concentration and hence highly bio-compatible. With a multitude of opportunities for nanomaterial use in pharmaceutical and medical applications, a thorough understanding of associated systemic toxicity is critical.

In vitro cellular uptake studies in HEK293T cells using these bio-compatible ZnS:Mn nanocrystals show that ZnS:Mn nanoparticles capped with L-arginine (1 nM concentration) has the maximum uptake suggesting a role for this particular amino acid in fetoplacental nutrition. These results highlight the importance of using appropriate amino acids for the bioconjugation of ZnS:Mn nanocrystals. These nanocrystals have safe physico-chemical properties which will enable them to be utilized as ideal agents for intracellular tracking and biomedical imaging.

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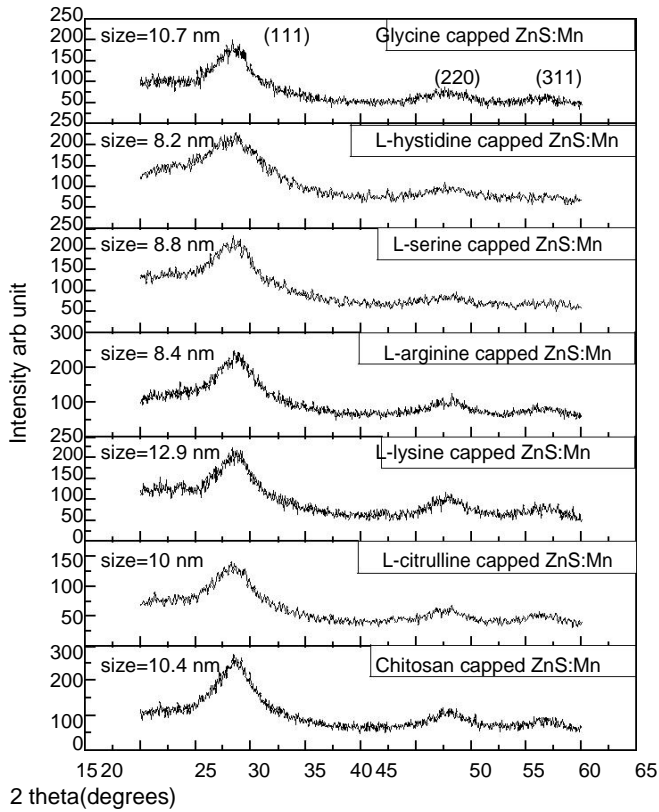


Fig. 1. XRD patterns of ZnS:Mn nanoparticles capped with chitosan, L-lysine, L-arginine, L-serine, L-histidine and glycine.

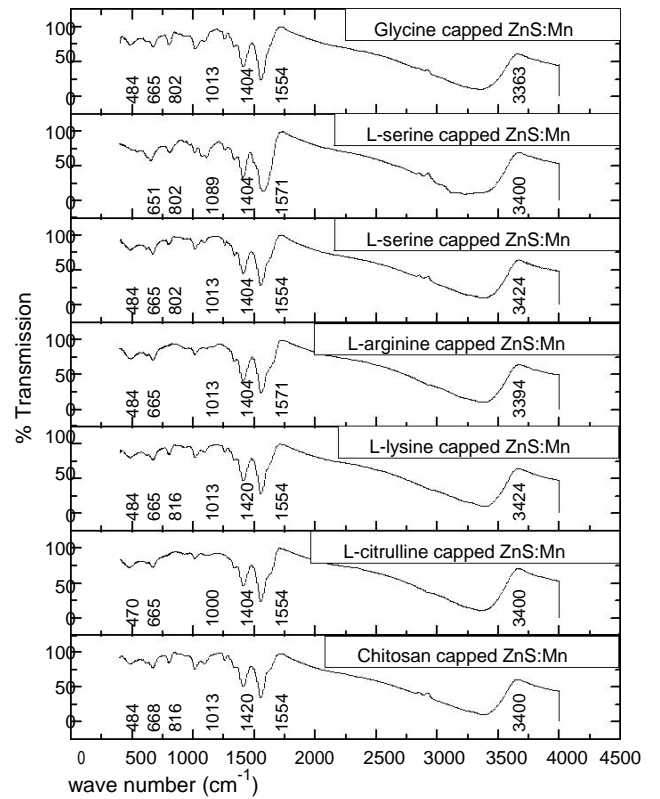
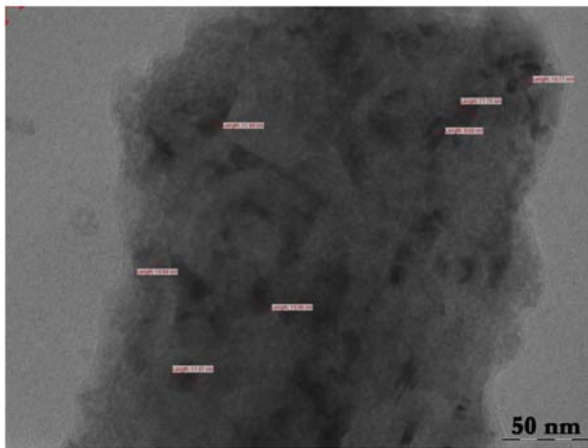
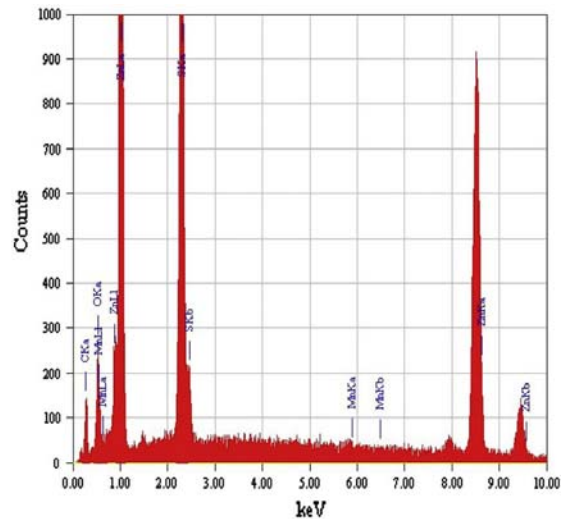


Fig. 3. FT-IR spectra of ZnS:Mn nanoparticles capped with chitosan, L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine.



(a)



(b)

Fig. 2. (a) TEM image (scale bar: 50 nm) and (b) EDX Spectrum of ZnS:Mn nanoparticles capped with L-citrulline.

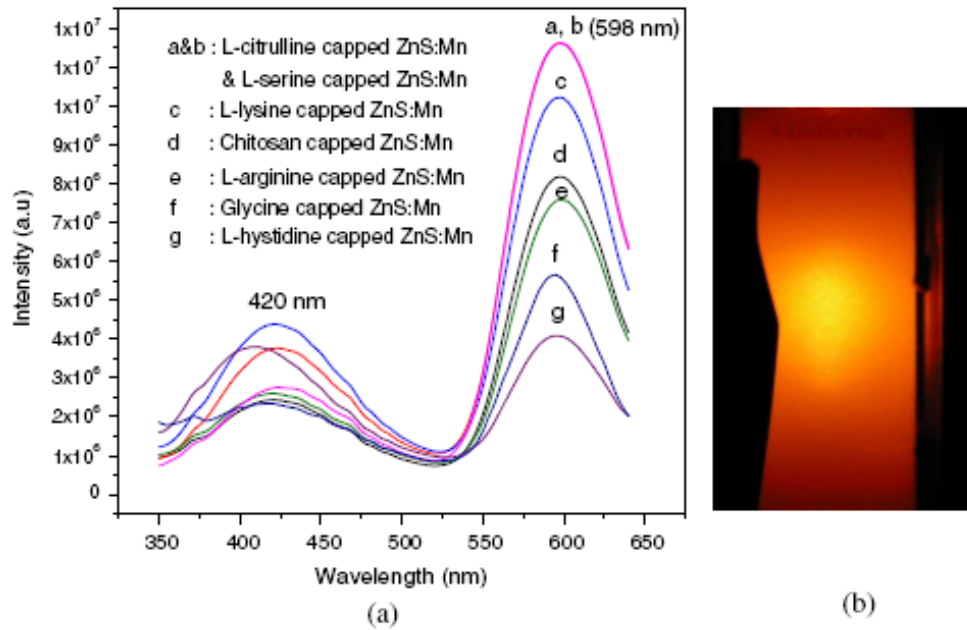


Fig. 4. (a) PL spectra of ZnS:Mn nanoparticles capped with chitosan, L-citrulline, L-lysine, L-arginine, L-serine, L-histidine and glycine and (b) orange red emission observed from capped ZnS:Mn nanoparticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

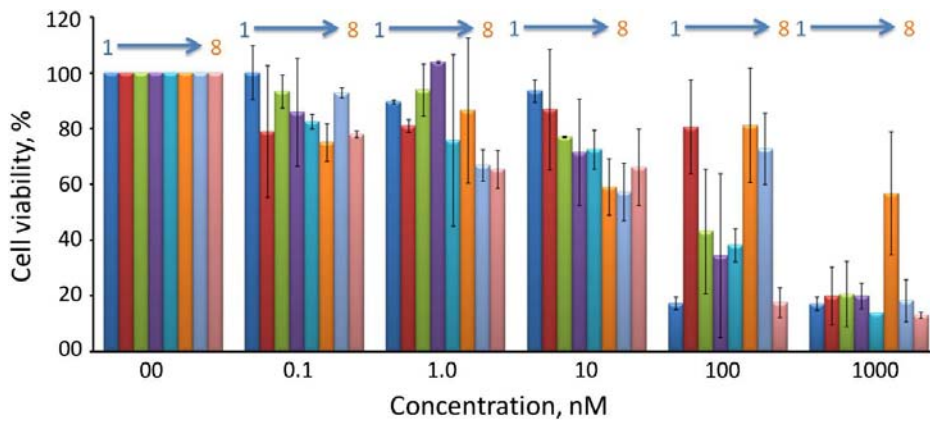


Fig. 5. Bar diagram showing cell viability (%) at different concentrations of ZnS:Mn capped with L-citrulline (1), L-lysine (2), chitosan (3), L-arginine (4), L-serine (5), L-histidine (6) and glycine (7) and uncapped ZnS:Mn (8).

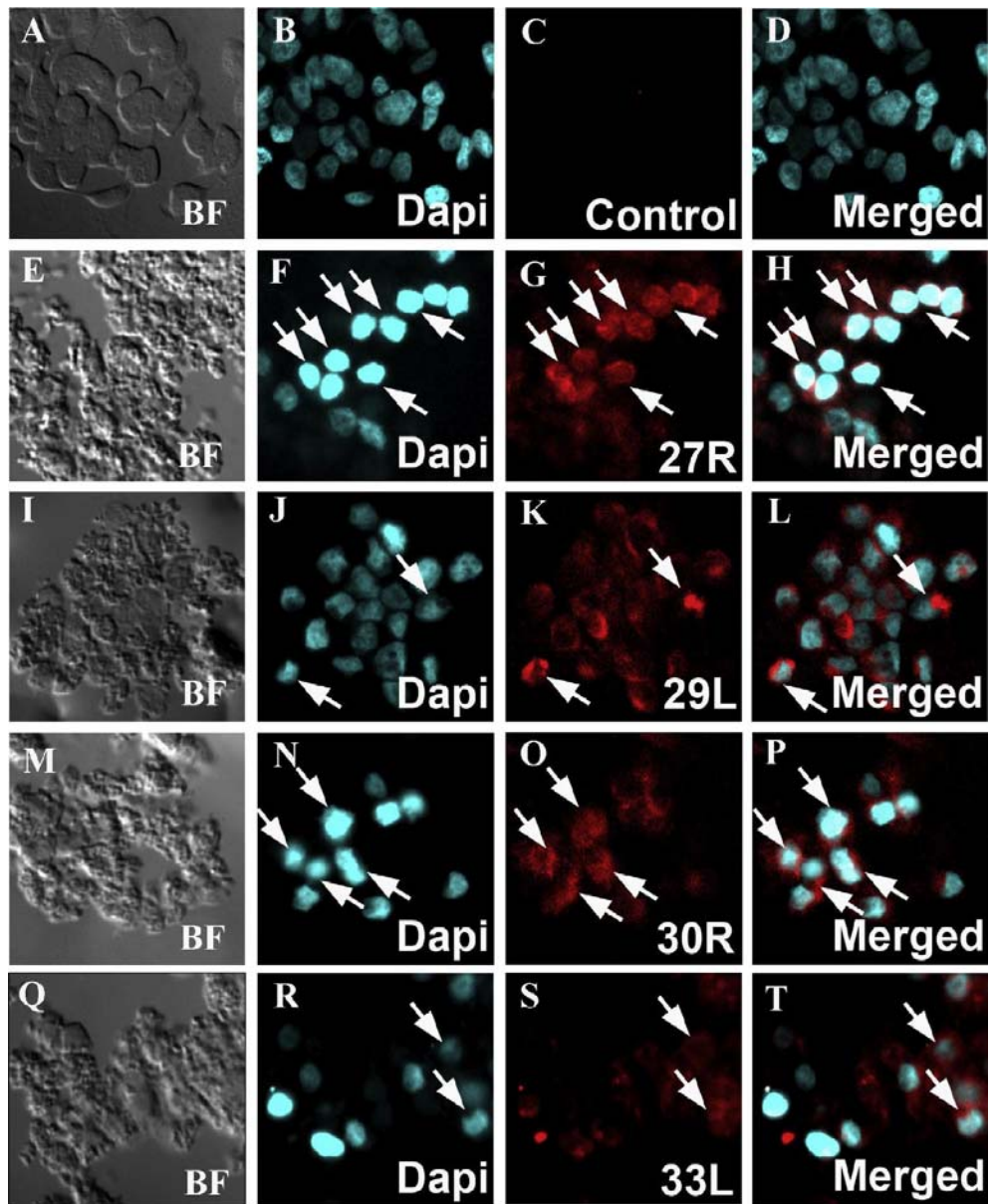


Fig. 6. HEK293T cells were cultured in the presence of nanocrystals capped with L-arginine (27R) (E–H), L-citrulline (29L) (I–L), L-histidine (30R) (M–P) & chitosan (33L) (Q–T) and their uptake was observed under UV microscope. A and B represent the cells cultured without the nanocrystals. BF = bright field. DAPI staining shows nuclei of the cells.