

Hydroxyapatite nanocrystals dually doped with fluorescent and paramagnetic labels for bimodal (luminomagnetic) cell imaging

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Abstract Hydroxyapatite nanocrystals (HAp NCs) were prepared that contain both a fluorescent label (fluorescein) and the paramagnetic label Ho^{3+} ion. Synthesis was performed by a surfactant-free aqueous method using commonly available precursors and dopants. The resulting HAp NCs display a spindle like hexagonal phase morphology. Their average diameter and length are 10 and 60 nm, respectively. The photoluminescence of the HAp NCs displays substantial variation with concentration of the fluorophore. The co-dopant Ho^{3+} endows the nanocrystals with paramagnetism and has little effect on the luminescence of the fluorescent label. The surface of the nanocrystals was coated with polyethyleneimine and the resulting particles were covalently conjugated to folic acid in order to enable targeting of the folate receptor that is over expressed in cancer cells. The HAp NCs are biocompatible as proven by an MTT assay that revealed no apparent toxicity in doses as high as $800 \mu\text{g mL}^{-1}$ after a 48 h incubation period. The HAp NCs were used as a fluorescent bioimaging probe for in vitro imaging of HeLa cells. The characterization of the magnetic properties indicates the paramagnetic nature of the nanocrystals with a magnetization of $7.3118 \text{ emu g}^{-1}$, and this property makes the system potentially suited for application as a Ho^{3+} based contrast agent for T2 magnetic resonance imaging.

Keywords Hydroxyapatite nanocrystals · Luminomagnetic nanoparticles · Holmium · Bimodal imaging · Cell imaging

Introduction

Hydroxyapatite ($\text{HAp}, \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a green natural material with good biocompatibility, biodegradability, bioresorbability, osteogenesis, osteoconductivity and osteoinductivity. HAp is the most important constituent of biological tissues such as bone and teeth. It is favoured in many biological applications and plays an inimitable role in the field of bone tissue engineering [1, 2]. The design and synthesis of multifunctional nano systems with high biocompatibility are very significant for clinical applications. Several bifunctional luminomagnetic nanocrystals based on HAp have been fabricated and adapted for biological applications including theranostics and multimodal imaging. In majority of such systems, lanthanide dopants offer luminescence and magnetism. For instance, Eu or Tb doping offers luminescence and Gd offers magnetism to such systems [3–6].

Ho^{3+} (magnetic moment $10.6 \mu\text{B}$), the paramagnetic lanthanide ion with highest magnetic moment is seldom used to obtain luminomagnetic nanoparticles and ^{166}Ho doped HAp is employed for radiosynovectomy applications [7]. Several Ho-doped nanocrystals were developed and most of them focused on the upconverting luminescence properties and related biological applications [8–12]. In this context, our aim is to develop Ho^{3+} doped luminomagnetic HAp NCs for biomedical applications. Ho^{3+} doping can provide substantial paramagnetic properties to HAp NCs. Innovations revealed that Ho^{3+} as such is luminescent and the near infrared Ho^{3+} luminescence in solution is exceedingly weak with a quantum yield of 10^{-5} . This low quantum yield limits the luminescent bioanalytical applications of Ho^{3+} doped nanocrystals

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[13–15]. Thus an additional fluorescent label is required to develop Ho^{3+} doped luminomagnetic HAp. Lanthanide ions such as Eu, Tb etc. have interesting luminescence properties are generally employed in bioimaging [16] and water dispersible fluoridated HAp: Ln^{3+} ($\text{Ln} = \text{Eu}$ or Tb) luminescent nanoparticles are successfully employed in cell imaging [17]. Surface PEGylated fluoridated hydroxyapatite with Eu or Tb dopants also used in cellular imaging [18]. Tb^{3+} alone can act as a fluorescent label for cellular imaging applications [19]. However, Ho^{3+} act as a quencher of lanthanide luminescence during down conversion and hence another choice is organic fluorophores. Organic dyes however, suffer from lack of photostability and are prone to photobleaching due to solvent effect. The photobleaching can be reduced by the encapsulation of the dye inside a rigid matrix [20, 21]. Here we select the organic fluorescent label fluorescein isothiocyanate (FITC), which is extensively utilised in bioimaging due to its desirable spectral characteristics [22]. HAp nanocrystals functionalised with FITC were synthesized and used in cancer cell imaging [23].

We describe a facile surfactant free wet chemical method to develop luminomagnetic FITC and Ho^{3+} dual-doped HAp NCs as well as the successful application of the obtained system in the *in vitro* cancer cell imaging of HeLa cells through suitable surface conjugation using folic acid (FA). To the best of our knowledge, the present system is the first Ho^{3+} doped HAp based luminomagnetic system for bioimaging.

Materials and methods

Chemical and reagents

Calcium hydroxide ($\text{Ca}(\text{OH})_2$) and Ortho-phosphoric acid (H_3PO_4) were purchased from Spectrum (www.spectrumchemicals.com). Ammonium hydroxide (25 % NH_3) was purchased from Qualigens (www.qualigensfinechemicals.com). Holmium nitrate ($\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$), solid FITC, Poly ethyleneimine (PEI, MW 25 kDa), Folic acid, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxy succinimide (NHS) were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Human cervical cancer (HeLa) cells and MCF-17 cells were procured from NCCS, Pune, India (www.nccs.res.in). The phosphate buffer (pH 7.4) was prepared freshly. All chemicals were used as received without further purification. Deionised water was used in the experiments throughout.

Apparatus

Powder X-ray diffraction (XRD) was obtained on wide angle X-ray scattering, using a Bruker AXS D8 Advance X-ray powder diffractometer (www.bruker.com) and the source of

X-ray used is $\text{CuK}\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$). Energy dispersive X-ray spectrum (EDX) was recorded using JEOL Model JED-2300 spectrometer (www.jeol.com). Scanning electron microscopy (SEM) images were taken using JEOL Model JSM-6390 LV scanning electron microscope. Atomic force microscopy (AFM) images were taken by using a Bruker Dimension Edge atomic force microscope. High resolution transmission electron microscope (HRTEM) images were acquired with a FEI Tecnai T 30 (www.fei.com) transmission electron microscope, operating at 300 kV of acceleration voltage. Room temperature photoluminescence (PL) of all the samples were studied using Jasco FP-750 spectrofluorometer (www.jascoinc.com). The magnetic properties (M-H curve) were measured at room temperature using a Lakeshore VSM 7410 (www.lakeshore.com). Fourier transform infrared (FTIR) spectra were recorded with a Shimadzu IR Prestige 21 spectrometer (www.shimadzu.com) and the scanning range is from 400–4000 cm^{-1} .

Synthesis of FITC and Ho^{3+} dual-doped HAp nanocrystals

FITC- Ho^{3+} -HAp nanocrystals were synthesised by a reported protocol with adequate modifications [6]. In a typical surfactant free aqueous wet chemical procedure, 20 mL of 0.5 M $\text{Ca}(\text{OH})_2$ (10 mmol) is heated to 100 °C in a 250 mL beaker, to which 20 mL of 0.3 M (6 mmol) ortho-phosphoric acid was added and stirred. After 5 min, 3 mL of 0.1 M (0.3 mmol) holmium nitrate, $\text{Ho}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ was added drop-wise at the rate of 500 $\mu\text{L min}^{-1}$, with continuous stirring over a period of 2 h at 80 °C and the pH was maintained at 7.4 using 1 M NH_4OH throughout the reaction. 4 mg (0.01 mmol) of solid FITC is then added to the reaction mixture and stirred for another 3 h without heating, in the dark. The reaction mixture is then incubated at room temperature overnight, centrifuged and washed three times with hot distilled water. It was then dried, powdered using mortar and pestle and stored. Similar procedure repeated with varying amounts of dopants.

Synthesis of surface aminized FITC- Ho^{3+} -HAp nanocrystals

The surface aminization is carried by using poly ethyleneimine (PEI). In a typical procedure, 10 mg of the FITC- Ho^{3+} -HAp nanocrystals were taken in 50 mL RB flask and 10 mL of 0.01 % PEI (MW 25 kDa) were added and stirred for 12 h. After the reaction, the sample was centrifuged and washed with distilled water twice to remove the unconjugated polymer. The resultant surface aminized nanocrystals were suspended in phosphate buffer.

Folic acid (FA) conjugation of surface aminized FITC-Ho³⁺-HAp nanocrystals

The covalent binding of FA to the surface aminized FITC-Ho³⁺-HAp nanocrystals was conducted using a modified N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)/N-hydroxy succinimide (NHS) reaction, in which FA, EDC and NHS are used in the optimized molar ratio 1:1:2.5 [24]. In a typical procedure amine reactive and ester activated FA was prepared by reacting 5 mg (1.1×10^{-5} mmol) FA and 2.2 mg (1.1×10^{-5} mmol) EDC in 7 mL deionised water for 30 min in the dark at room temperature. 3.2 mg (2.75×10^{-5} mmol) NHS was then added to EDC-FA system and stirred well for 6 h under same condition. The obtained FA-NHS ester was reacted with 10 mg of surface aminized FITC-Ho³⁺-HAp nanocrystals in phosphate buffer at pH 7.4 for 3 h at room temperature, which resulted in FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals.

Cytotoxicity of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals

In vitro cytotoxicity was measured by performing methyl thiazolyl tetrazolium (MTT) assays on human cervical cancer HeLa cells. Cells were maintained in culture in DMEM under standard culture conditions. 1×10^4 cells/well were seeded in 96 well plates and allowed to attach. After attachment, medium containing different concentrations (10, 20, 30, 40 and 50 $\mu\text{g mL}^{-1}$) of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals were added and incubated for different time intervals (1, 2 and 4 h). After incubation, the medium was removed and supplemented with fresh medium containing MTT and incubated for 4 h. After removing the medium containing MTT, the formazan crystals formed were solubilised in DMSO and the absorbance was measured at 570 nm. Same procedure repeated with high concentration (800 $\mu\text{g mL}^{-1}$) of the nanocrystals for different incubation time (12, 24 and 48 h).

Fluorescent microscopic studies for folate receptor targeted imaging

HeLa cells were seeded on 96 well plates and maintained in culture for 24 h for attachment. After removing the unattached cells, medium containing 50 $\mu\text{g mL}^{-1}$ of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals were supplemented and incubated for another 4 h. After removing the excess nanocrystals, images were captured using a Live Cell Bioimager (BD pathway TM Bioimage System, BD Biosciences).

Results and discussion

Choice of materials

In order to design and fabricate a new biocompatible nano-system based on HAp, separate luminescent and paramagnetic labels are selected as dopants. The magnetism aims at the potential use of the system as MRI contrast agents. As a T1 contrast agent, Gd³⁺ based nanosystems are widely explored. Hence the paramagnetic lanthanide ion Ho³⁺ with high magnetic moment than Gd³⁺ is selected and Ho³⁺ can offer T2 contrast property as well. The luminescence of the system aims at bioimaging and lanthanide ion labels are widely used to provide luminescence. However Ho³⁺ markedly quenches the luminescence of Eu³⁺ and Tb³⁺ during down conversion. Thus organic dyes are considered and FITC is selected as fluorescent label due its promising fluorescence and wide applicability in bioimaging. The luminescence and magnetism together make the system adaptable for bimodal imaging.

Fabrication of FITC- Ho³⁺- HAp nanocrystals

Several synthetic methods have been developed for the fabrication of monodispersed HAp NCs with and without dopants. The structural diversity, crystallinity, phase purity, Ca/P ratio and size of the HAp NCs depends on the synthetic procedure adopted [25]. Here we employed an already reported surfactant free aqueous wet chemical synthesis with suitable modification [6]. During the synthesis, the first dopant Ho³⁺ is added at a rate of 500 $\mu\text{L min}^{-1}$ and after which the second dopant FITC was added with vigorous stirring at an optimum pH of 7.4 throughout the reaction. This controlled and separate addition of the dopants enhances the doping efficiency and associated functional properties. The addition of the second dopant FITC and subsequent reaction was carried out in the dark at room temperature, which effectively prevents the photobleaching of FITC.

As a result of doping, the free sites in the crystal lattice of HAp are occupied by dopants. This resulted in the slight distortion of lattice structure of HAp. However, the stoichiometry of HAp remains unaltered during doping. The interaction between the organic dye and HAp is probably through the dipolar interaction between polar groups of the dye with that of HAp. There is no covalent bond formation between FITC and HAp, which is inferred from the IR spectra.

Characterisation of FITC- Ho³⁺- HAp nanocrystals

X-Ray powder diffraction studies were carried out with perfectly dried and finely powdered FITC- Ho³⁺- HAp nanocrystals. The XRD pattern obtained is shown in Fig. 1a. The observed diffraction peaks clearly reveals the crystalline nature of the sample. The data obtained are consistent with the

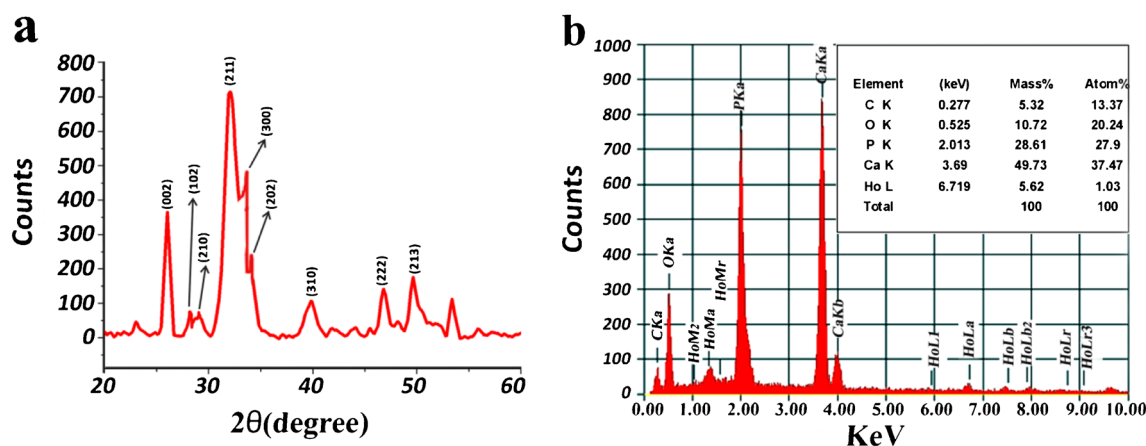


Fig. 1 **a** X-diffraction spectrum and **b** Energy dispersive X-ray analysis spectrum of FITC-Ho³⁺-HAp nanocrystals

values in the JCPDS (09–0432) database for hexagonal Ca₁₀(PO₄)₆(OH)₂ in P63m space group. The lattice constants of FITC-Ho³⁺-HAp nanocrystals were slightly deviated from undoped HAp NCs and it is attributed to the distortion of the HAp lattice structure in the presence of the dopants. Moreover the broad nature of the peaks suggests the formation of nanosized particles, because the peak broadening was normally caused by the finite size effect of the crystallites below a certain limit.

The chemical purity and elemental compositions of FITC-Ho³⁺-HAp nanocrystals were tested by energy dispersive X-ray (EDX) analysis. The EDX spectrum of a typical HAp nanocrystal synthesized by adding 3 % Ho³⁺ and 0.4 % of FITC (with respect to concentration of calcium) is shown in Fig. 1b and the spectrum displays elemental peaks related to Ca, P, O, C and Ho. This result confirms the detectable levels of dopants within HAp matrix and the atomic percent of the elemental dopant Ho³⁺ is found to be 1.03.

AFM images of the FITC-Ho³⁺-HAp nanocrystals are shown in Fig. 2a and b. The images were taken in the tapping mode. From the 3D height sensor image (Fig. 2a), it is clear

that nanocrystals exhibit spindle like morphology with considerable polydispersity.

From the AFM data, the average diameter and length of the nanocrystals were obtained and the values are 26 nm and 65 nm respectively. The phase image (Fig. 2b) confirms that the crystal size is in the nano regime. The surface morphology of the nanocrystals was further investigated by SEM. The SEM images of the finely powdered nanocrystals are shown in electronic supplementary material (Fig. S1, Electronic Supplementary Material, ESM).

The HRTEM images of the FITC-Ho³⁺-HAp nanocrystals are shown in Fig. 3a. From the HRTEM, it is clear that the nanocrystals exhibit spindle like morphology with structural diversity and polydispersity.

The nanocrystals dimensions obtained from HRTEM are more accurate and values are less than that obtained from the AFM data. The average diameter and length are 10 nm and 60 nm respectively. The careful analysis of the image reveals the existence of ordered lattice planes (marked portion of the inset of Fig. 3a). The HRTEM image of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals (Fig. 3b) confirmed

Fig. 2 AFM image (a) 3D height sensor image and (b) phase image of FITC-Ho³⁺-HAp nanocrystals

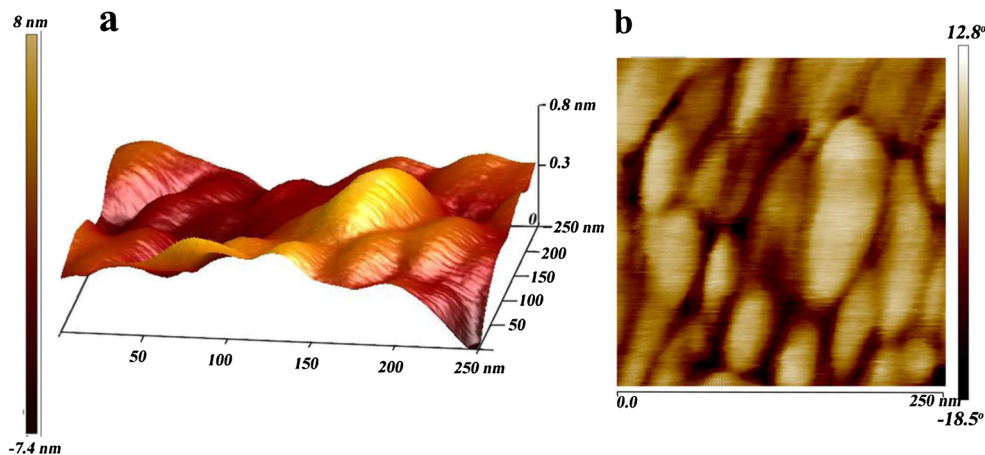
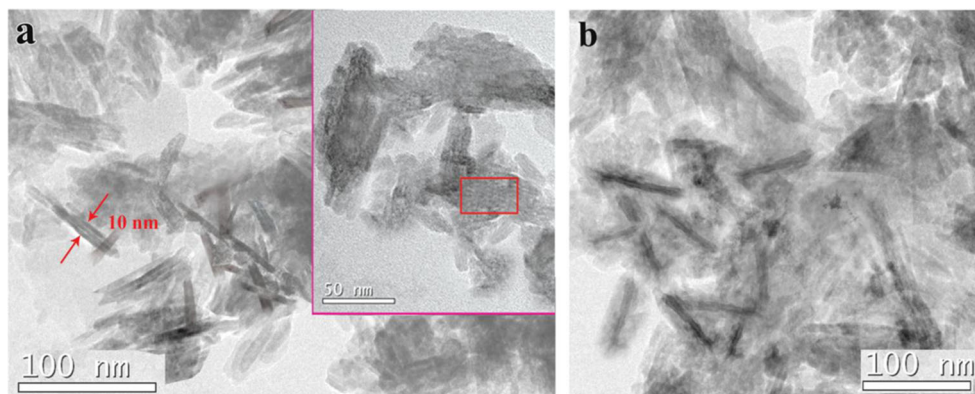


Fig. 3 HRTEM image of (a) FITC- Ho^{3+} -HAp nanocrystals (magnified image is shown in *inset*) and (b) FA conjugated surface aminized FITC- Ho^{3+} -HAp nanocrystals



the existence of the nanosized particles after conjugation with FA.

Photoluminescence properties of FITC- Ho^{3+} - HAp nanocrystals

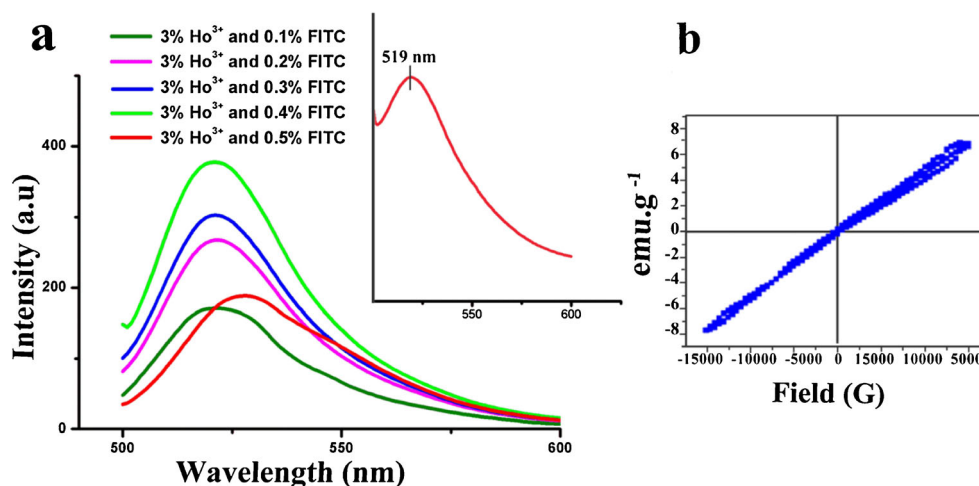
HAp NCs with different concentrations of FITC and fixed concentration of Ho^{3+} were prepared by similar procedure. The concentration of FITC and Ho^{3+} were expressed with respect to the concentration of calcium, which is initially used for the synthesis of HAp NCs. The fluorescence emission properties of all the samples were recorded at room temperature and the spectra obtained is shown in Fig. 4a. The photoluminescence (PL) studies of the present system reveals that the paramagnetic dopant Ho^{3+} has little effect on the emission properties of the fluorophore, FITC. However the incorporation of the Ho^{3+} ions causes a slight red shift, which can be inferred by comparing the PL spectra of HAp NCs doped with FITC alone (*inset* of Fig. 4a) and HAp NCs with both FITC and Ho^{3+} . In the former case the emission maxima is observed at 519 nm and in the latter case it is observed at 528 nm. The slight red shift confirms the incorporation of Ho^{3+} into the lattice structure of HAp NCs. The luminescence

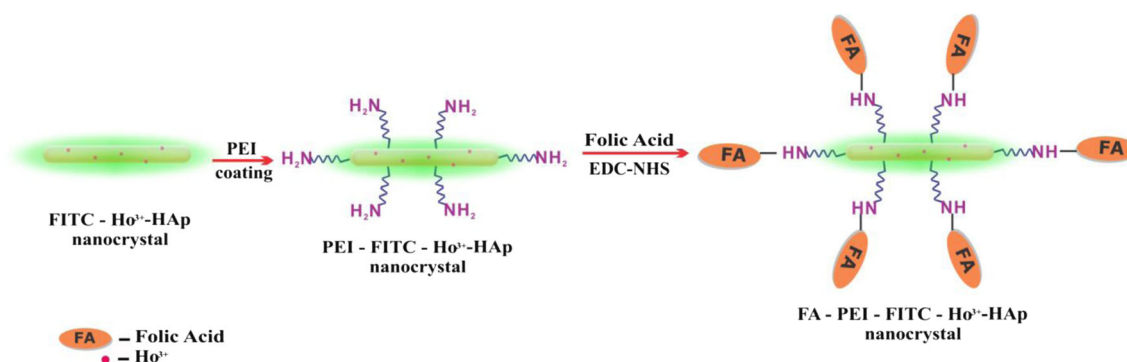
emission intensity increases with increase in concentration of FITC (from 0.1 to 0.4 %) and for a concentration of 0.5 % of FITC, a marked quenching effect is observed with substantial red shift. As the concentration of the dye increases the interaction between the neighbouring molecules also increases. This interaction lowers excited state energy levels of the molecules and causes a red shift. The decrease in intensity observed at high concentration of FITC is due to the self-quenching [22]. Thus the concentration of FITC for maximum luminescence intensity is optimized as 0.4 %.

Magnetic properties of FITC- Ho^{3+} - HAp nanocrystals

The lanthanide ion dopant Ho^{3+} ion imparts magnetism to FITC- Ho^{3+} -HAp nanocrystals. The magnetic properties of the said nanocrystals were studied by using VSM. The room temperature magnetisation (M-H) curve of HAp NCs synthesized by using 3 % of Ho^{3+} and 0.4 % of FITC (with respect to concentration of Ca) is shown in Fig. 4b. The M-H curve clearly indicates the paramagnetic nature of the nanocrystals with a magnetization value of $7.3118 \text{ emu g}^{-1}$. Biocompatible and paramagnetic nanoparticles are extensively used as contrast agents in MRI. Most of such contrast agents are based on

Fig. 4 a Fluorescence emission spectra of FITC- Ho^{3+} - HAp nanocrystals (*inset* shows the spectrum of FITC-HAp nanocrystals without Ho^{3+} dopant) and (b) the room temperature magnetisation Vs field (M-H) curve





Scheme 1 Schematic illustration of surface aminization and FA conjugation of FITC-Ho³⁺-HAp nanocrystals

Gd (T1 contrast agents) and Fe (T2 contrast agents) [26]. Recently, T2 contrast agents based on paramagnetic lanthanide ions such as Dy, Ho, Tb and Er have been developed [27, 28]. The Dy³⁺ and Ho³⁺ have very short electronic relaxation time due their highly anisotropic ground state and hence they are efficient T2 contrast agents in intermediate and high magnetic field strengths. The present system can be explored for their potential application as holmium based T2 MRI contrast agents.

Surface modification and FA conjugation of FITC-Ho³⁺-HAp nanocrystals

In order to make the FITC-Ho³⁺-HAp nanocrystals adaptable for in vitro cancer cell imaging, they are surface aminized and then conjugated with FA. The selection of FA is due to the fact that it is a promising cancer targeting ligand that can targets the folate receptors (FR) over expressed in most of the cancer cells. Scheme 1 illustrates the surface aminization and FA conjugation of FITC-Ho³⁺-HAp nanocrystals.

The surface aminization is carried out by coating with PEI, which is an amine rich dendrigraft polymer. The obtained surface aminized nanocrystals is then subjected to FA conjugation through the well established EDC coupling route using FA, EDC and NHS in a specified ratio(1:1:2.5). In this method, the FA is made amine reactive by connecting with EDC, which reacts with γ -COOH group of FA to form a succinimidyl ester. NHS was then added to get FA-NHS ester, which is finally reacted with surface aminized nanocrystals to get FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals and the FA conjugation is happened through the amide linkage.

The surface aminization and FA conjugation steps were analytically confirmed by using FTIR spectroscopy. Figure 5a shows the FTIR spectrum of bare FITC-Ho³⁺-HAp nanocrystals. The bands at 3570 and 634 cm⁻¹ are respectively attributed to ν (OH) stretching vibration and ν (OH) librational vibration of hydroxyl group of the lattice. The broad band around 3440 cm⁻¹ is assigned to hydrogen bonded -O-H group in absorbed water. The weak bands at 1460 and

877 cm⁻¹ are attributed to vibration modes of ν_2 (CO₃²⁻) and ν_3 (CO₃²⁻) respectively. The carbonates are produced from atmospheric CO₂, during the course of the reaction. The band at 1037 cm⁻¹ corresponds to ν_3 antisymmetric stretching of P-O bond. The bands at 604 and 567 cm⁻¹ is due to ν_4 vibration of O-P-O bond of phosphate group [29]. The incorporation of FITC in HAp is indicated by the formation of a band at 3163 cm⁻¹(due to aromatic -OH group) and bands at 2333 cm⁻¹, 2359 cm⁻¹(characteristic of -N=C=S). FITC contains an aromatic keto group and it can be manifested by a

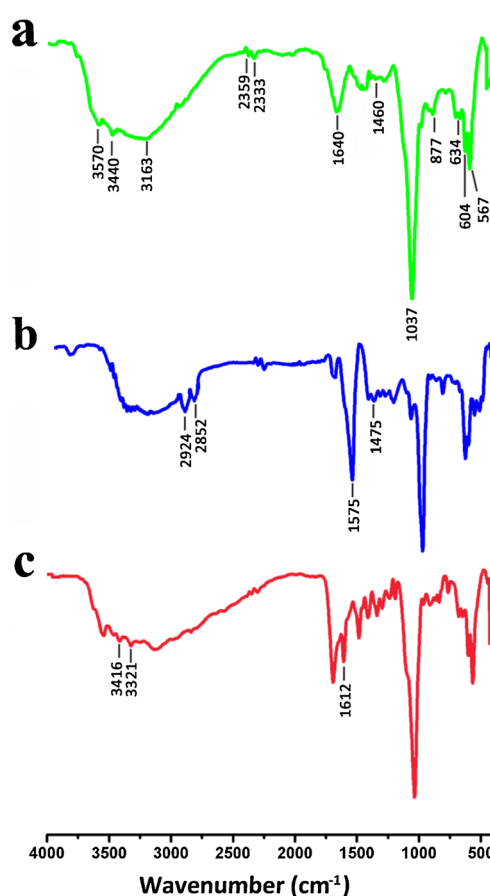
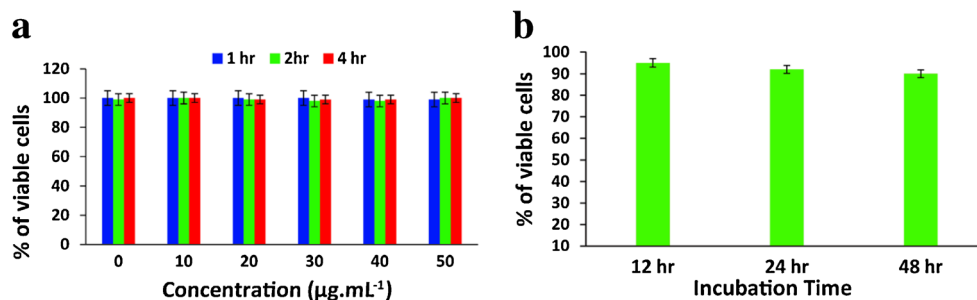


Fig. 5 FTIR spectra of (a) FITC-Ho³⁺-HAp nanocrystals (b) PEI coated FITC-Ho³⁺-HAp nanocrystals and (c) FA conjugated PEI coated FITC-Ho³⁺-HAp nanocrystals

Fig. 6 MTT assay showing the non-toxicity of (a) FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals and (b) FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals at high concentration of 800 $\mu\text{g mL}^{-1}$



peak near 1700 cm^{-1} . In the present spectrum this peak is merged with acidic carbonyl peak at 1697 cm^{-1} and ν_2 bending band of H_2O to form a broad peak at 1640 cm^{-1} . The functional group peaks of HAp as well as FITC remain intact and this is a clear indication of the fact that there is no covalent linkage between FITC and HAp.

The FTIR spectrum of PEI coated FITC-Ho³⁺-HAp nanocrystals (Fig. 5b) contain characteristic bands at 1475 cm^{-1} (bending vibration of C-H bond), 1575 cm^{-1} (bending vibration of N-H bond of primary and secondary amine), 2852 cm^{-1} and 2924 cm^{-1} (both due to C-H stretching). PEI is a dendrigraft polymer with primary (25%), secondary (50%) and tertiary amine (25%) moieties [30]. It forms electrostatic interpenetrating linkages with HAp at the surface. As the peaks for the functional groups of HAp and PEI remain intact in the IR spectrum, there is no evidence of covalent linkage between PEI and HAp. As a result of PEI coating, a thin shell is formed on the surface of HAp [31].

The FTIR spectra of FA conjugated PEI coated FITC-Ho³⁺-HAp nanocrystals (Fig. 5c) contain characteristic bands of FA at 3416 cm^{-1} (N-H stretching of amines), 3321 cm^{-1} (N-H stretching of amides) and 1612 cm^{-1} (stretching vibration of C=O in amide linkage). Thus the FTIR study confirms successful surface aminization and subsequent FA conjugation.

Cytotoxicity of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals

In order to explore the immense application of nano particles in the field biomedical imaging, the bio compatibility of the particles under consideration should be ensured. Before the usage of FA conjugated surface aminized FITC-Ho³⁺-HAp

nanocrystals as bioimaging probes, their toxicity is tested using MTT assay in HeLa cells. The said nanocrystals were found to be nontoxic up to $50\text{ }\mu\text{g mL}^{-1}$ for 4 h incubation and the results are given in Fig. 6a. The toxicity is also tested at high concentration of the nanocrystals ($800\text{ }\mu\text{g mL}^{-1}$) for high incubation time (12, 24 and 48 h) and the results again proved their non toxic nature (Fig. 6b).

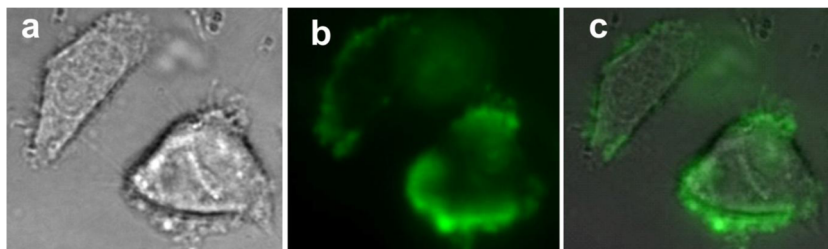
The effect of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals on the morphology of MCF-7 cells were also tested using standard procedures and the results are given in electronic supplementary material (Fig. S2, ESM). The results indicated that up to $800\text{ }\mu\text{g mL}^{-1}$ concentration, the morphology of the cells remains the same. On the basis of the MTT and cell morphological analysis, it can be inferred that the said nanocrystals are biocompatible and nontoxic to live cells and hence promising candidates for bioimaging.

Folate receptor targeted in vitro cancer cell imaging

The receptor targeted delivery of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals on HeLa cells was studied by incubating the nanocrystals for 4 h and the fluorescence is monitored by a fluorescence microscope. The images obtained are shown in Fig. 7a, b and c.

The studies revealed that the said nanocrystals were able to recognise the cell surface folate receptor and appeared as green fluorescence around the plasma membrane. The cell imaging is through the specific interaction between FA and the FR on the cell surface [32]. Thus the said nanocrystals were successfully employed as a bioimaging probe in the in vitro cancer cell imaging.

Fig. 7 Fluorescent microscopic images showing the interaction of FA conjugated surface aminized FITC-Ho³⁺-HAp nanocrystals with HeLa cells (a) phase contrast image (b) fluorescent image and (c) merged image



Conclusion

Luminomagnetic FITC and Ho^{3+} dual-doped HAp nano crystals with spindle like morphology have been synthesised by wet chemical method without the aid of surfactants. The nanocrystals were characterised and their luminescent and magnetic properties were explored. The variation in luminescence observed with the change in concentration of the fluorophore (FITC) was studied and the concentration for which, maximum luminescence intensity is optimised. Except marginal red shift, the Ho^{3+} doping does not influence the luminescence intensity of nanocrystals. The Ho^{3+} doping imparts paramagnetism to the system. The prepared nanocrystals were surface aminized by PEI coating and conjugated with FA. The nanocrystals were analysed for toxicity through MTT assay and the effect of nanocrystals on cell morphology were also tested. The result revealed that the nanocrystals are nontoxic as well as have no effect on morphology of cells even at high concentration of $800 \mu\text{g mL}^{-1}$. The FA conjugation enabled the nanocrystals to target cancer cells through FA-FR interaction. The luminomagnetic nanocrystals were successfully employed in the in vitro cell imaging of HeLa cells. In future, the magnetic properties can be explored for the potential use of the nanocrystals as Ho-based MRI contrasting agents and the present system can also be employed for targeted drug delivery.

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References

- Hui J, Wang X (2014) Hydroxyapatite nanocrystals: colloidal chemistry, assembly and their biological applications. *Inorg Chem Front* 1: 215–225
- Palmer LC, Newcomb CJ, Kaltz SR, Spoerke ED, Stupp SI (2008) Biomimetic systems for hydroxyapatite mineralization inspired by bone and enamel. *Chem Rev* 108:4754–4783
- Liu Z, Wang Q, Yao S, Yang L, Yu S, Feng X, Li F (2014) Synthesis and characterization of $\text{Tb}^{3+}/\text{Gd}^{3+}$ dual-doped multifunctional hydroxyapatite nanoparticles. *Ceram Int* 40:2613–2617
- Chen F, Huang P, Zhu Y-J, Wu J, Zhang C-L, Cui D-X (2011) The photoluminescence, drug delivery and imaging properties of multifunctional $\text{Eu}^{3+}/\text{Gd}^{3+}$ dual-doped hydroxyapatite nanorods. *Biomaterials* 32:9031–9039
- Chen F, Huang P, Zhu Y-J, Wu J, Cui D-X (2012) Multifunctional $\text{Eu}^{3+}/\text{Gd}^{3+}$ dual-doped calcium phosphate vesicle-like nanospheres for sustained drug release and imaging. *Biomaterials* 33:6447–6455
- Ashokan A, Menon D, Nair S, Koyakutty M (2010) A molecular receptor targeted, hydroxyapatite nanocrystals based multi-modal contrast agent. *Biomaterials* 31:2606–2616
- Unni PR, Chaudhari PR, Venkatesh M, Ramamoorthy N, Pillai MRA (2002) Preparation and bioevaluation of ^{166}Ho labelled hydroxyapatite (HA) particles for radiosynovectomy. *Nucl Med Biol* 29:199–209
- Mader HS, Kele P, Saleh SM, Wolfbeis OS (2010) Upconverting luminescent nanoparticles for use in bioconjugation and bioimaging. *Curr Opin Chem Biol* 14:582–596
- DaCosta MV, Doughan S, Han Y, Krull UJ (2014) Lanthanide upconversion nanoparticles and applications in bioassays and bioimaging: a review. *Anal Chim Acta* 832:1–33
- Yu X, Liang S, Sun Z, Duan Y, Qin Y, Duan L, Xia H, Zhao P, Li D (2014) Microstructure and upconversion luminescence in Ho^{3+} and Yb^{3+} co-doped ZnO nanocrystalline powders. *Opt Commun* 313:90–93
- Antic Z, Lojpur V, Nikolic MG, Dordevic V, Ahrenkiel PS, Dramicanin MD (2014) Strong emission via up-conversion of $\text{Gd}^{2+}:\text{Yb}^{3+}$, Ho^{3+} nanopowders co-doped with alkali metals ions. *J Lumin* 145:466–472
- Yang Y (2014) Upconversion nanophosphors for use in bioimaging, therapy, drug delivery and bioassays. *Microchim Acta* 181:263–294
- Werts MHV (2011) Near-infrared luminescent labels and probes based on lanthanide ions and their potential for applications in bioanalytical detection and imaging. In: Haenninen P, Haerma H (eds) *Lanthanide luminescence: photophysical, analytical and biological aspects, springer series on fluorescence, vol 7*. Springer, Heidelberg, pp 142–143
- Moore EG, Szigethy G, Xu J, Palsson LO, Beeby A, Raymond KN (2008) 3-Hydroxypyridin-2-one complexes of near-infrared (NIR) emitting lanthanides: sensitization of holmium(III) and praseodymium(III) in aqueous solution. *Angew Chem Int Ed* 47: 9500–9503
- Zhang J, Badger PD, Geib SJ, Petoud S (2005) Sensitization of near-infrared-emitting lanthanide cations in solution by tropolonate ligands. *Angew Chem Int Ed* 44:2508–2512
- Dosev V, Nichkova M, Kennedy IM (2008) Inorganic lanthanide nanophosphors in biotechnology. *J Nanosci Nanotechnol* 8:1052–1067
- Hui J, Zhang X, Zhang Z, Wang S, Tao L, Wei Y, Wang X (2012) Fluoridated Hap : Ln^{3+} ($\text{Ln} = \text{Eu}$ or Tb) nanoparticles for cell imaging. *Nanoscale* 4:6967–6970
- Zhang X, Hui J, Yang B, Yang Y, Fan D, Liu M, Tao L, Wei Y (2013) PEGylation of fluoridated hydroxyapatite (FAP): Ln^{3+} nanorods for cell imaging. *Polym Chem* 4:4120–4125
- Li X, Zeng H, Teng L, Chen H (2014) Comparative investigation on the crystal structure and cell behaviour of rare-earth doped fluorescent apatite nanocrystals. *Mater Lett* 125:78–81
- Altinoglu EI, Russin TJ, Kaiser JM, Barth BM, Eklund PC, Kester M, Adair JH (2008) Near-infrared emitting fluorophore-doped calcium phosphate nanoparticles for in vivo imaging of human breast cancer. *ACS Nano* 2:2075–2084
- Tabakovic A, Kester M, Adair JH (2012) Calcium phosphate-based composite nanoparticles in bioimaging and therapeutic delivery applications. *WIREs Nanomed Nanobiotechnol* 4:96–112
- Imhof A, Megens M, Engelberts JJ, de Lang DTN, Sprik R, Vos WL (1999) Spectroscopy of Fluorescein (FITC) dyed colloidal silica spheres. *J Phys Chem B* 103:1408–1415
- Liu H, Chen F, Xi P, Chen B, Huang L, Cheng J, Shao C, Wang J, Bai D, Zeng Z (2011) Biocompatible fluorescent hydroxyapatite: synthesis and live cell imaging applications. *J Phys Chem C* 115:18538–18544
- Ma J, Huang P, He M, Pan L, Zhou Z, Feng L, Gao G, Cui D (2012) Folic acid-conjugated $\text{LaF}_3:\text{Yb}$, Tm @ SiO_2 nanoprobe for targeting dual-modality imaging of upconversion luminescence and X-ray computed tomography. *J Phys Chem B* 116:14062–14070

25. Shojai MS, Khorasani M-T, Khoshdargi ED, Jamshidi A (2013) Synthesis methods for nanosized hydroxyapatite with diverse structures. *Acta Biomater* 9:7591–7621
26. Nicolay K, Strijkers G, Grull H (2013) Gd-Containing nanoparticles as MRI contrast agents. In: Merbach A et al (eds) *The chemistry of contrast agents in medical magnetic resonance imaging*, 2nd edn. Wiley, UK, pp 449–483
27. Norek M, Peters JA (2011) MRI contrast agents based on dysprosium or holmium. *Progress NMR* 59:64–82
28. Vuong QL, Doorslaer SV, Bridot JL, Argante C, Alejandro G, Hermann R, Disch S, Mattea C, Stapf S, Gossum Y (2012) Paramagnetic nanoparticles as potential MRI contrast agents: characterization, NMR relaxation, simulations and theory. *Magn Reson Mater Phys* 25:467–478
29. Cao H, Zhang L, Zheng H, Wang Z (2010) Hydroxyapatite nanocrystals for biomedical application. *J Phys Chem C* 114: 18352–18357
30. Murakami Y, Rikimra S, Sugo K, Kawamura K, Ogawa T, Masashiro H, Okuyama T (2008) Preparation of polyethyleneimine-hydroxyapatite and its chromatographic use. *J Liq Chromatogr Relat Technol* 32:407–417
31. Murakami Y, Sugo K, Hirano M, Okuyama T (2011) Surface chemical analysis and chromatographic characterization of polyethyleneimine-coated hydroxyl apatite with various amounts of polyethyleneimine. *Talanta* 85:1298–1303
32. Ai J, Xu Y, Li D, Liu Z, Wang E (2012) Folic acid as delivery vehicles: targeting folate conjugated fluorescent nanoparticles to tumors imaging. *Talanta* 101:32–37