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Upconversion fluorescence imaging of HeLa cells using ROS generating SiO₂-coated lanthanide-doped NaYF₄ nanoconstructs

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Inorganic nanomaterials able to generate reactive oxygen species (ROS) are promising components for modern medical applications. Activated by near-infrared light, up-converting β-NaYF₄ doped with Er³⁺–Yb³⁺ and Tm³⁺–Yb³⁺ pair ions nanoparticles (UCNPs), have a wide range of applications in biological imaging as compared to traditional reagents excited by ultra-violet or visible light. We analysed the green-red and the blue-red luminescence to explain the mechanism of the upconversion depended on the surface condition. The influence of SiO₂ coating on the cytotoxicity of the as-produced UCNPs towards HeLa cancer cells was reported. We demonstrated a possibility of a direct UCNPs application to photodynamic therapy, without need to attach additional molecules to their surface. The presence of Tm³⁺–Yb³⁺ pair ions, thus ROS generation capability, renders the SiO₂ shell coated nanoparticles to become potentially useful theranostic agent.

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1 Introduction

Presently, cancer is one of the most prevalent diseases. In Europe, this pathology is responsible for 20% of deaths, with more than 3 million new cases and 1.7 million deaths every

year. Actually, after cardiovascular diseases, cancer is the most important cause of deaths and morbidity in Europe (WHO). Fluorescent techniques are known as ultra-sensitive diagnostic tools to identify some pathogenic cells at the molecular level. Optical imaging is extremely useful in biomedical research for early detection of pathogenic cells as well as for therapy. Many types of luminophores are currently applied in bioimaging (*e.g.* quantum dots, fluorescent proteins, organic dyes, dye doped silica nanoparticles, metallic nanoparticles). However, application of organic probes for luminescence imaging of living organism has some limitations. For instance, the excitation of the traditional bio-labels usually requires UV or VIS light, leading to: (I) low signal-to-noise ratio due to high biological autofluorescence, (II) small light penetration depth, and (III) possible cell photodamages.^{1,2}

Therefore, it is desirable to use fluorescent biolabels that can be excited by near infrared (NIR) light. In particular, NIR light is safe to the human body and can penetrate into tissues up to several centimetres.^{3,4} Upconversion nanoparticles (UCNPs), which can convert photons of lower energy (*e.g.* NIR light) to higher energy (*e.g.* visible light) via a two-photon or multiphoton upconversion mechanisms,⁵ represent a new class of fluorescent biolabels. UCNPs have a relatively high quantum yield, as compared to a two-photon excitation of organic dyes, or quantum dots used in two-photon microscopy, narrow emission

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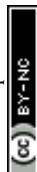
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peak, large anti-Stokes shift, good chemical stability, and relatively low toxicity.^{6,7} Since UCNPs are NIR light active, the signal-to-noise ratio and sensitivity of the optical detection can be improved due to the absence of autofluorescence.^{8–10} Therefore, UCNPs are promising alternatives to traditional fluorescent biolabels for cell imaging and possess prominent potential in biological and clinical applications such as photodynamic therapy (PDT). In this therapy as a lethal factor are using reactive oxygen species (ROS).¹¹ In traditional case of PDT, photosensitizer is activate by visible light to generate ROS, as described above this kind of light has limitation at tissues penetration. Materials with upconverting properties could be used to activation of photosensitizer directly in the environment of tumor. UCNPs excited by NIR light transfer absorbed energy to photosensitizer molecules and cause generation of ROS.^{12,13}

The most efficient host material for the 980 nm upconversion is the hexagonal-phase (β -phase) NaYF_4 . The β - NaYF_4 host is frequently doped with rare-earth (RE) ions, as for instance Yb^{3+} - Er^{3+} or Yb^{3+} - Tm^{3+} ion pair.^{14–17} Monodisperse UCNPs are usually synthesized in organic solvents at high-temperatures. However, the most commonly used hydrophobic capping agent, namely oleic acid, limits their biological applications.^{17–22}

The coating of the nanoparticles (NPs) by thin silica (SiO_2) layer or metal oxides makes them dispersible in water. The use of SiO_2 for external coating of lanthanide-doped UCNPs is an attractive alternative because the surface chemistry of silica spheres is well documented and silica is known to be relatively harmless while using in biological systems.^{23–25} Moreover, the thickness and the surface properties of SiO_2 shell can be easily adjusted to favour secretion of SiO_2 -coated UCNPs through the kidneys.²⁶

Both types of materials, surface unmodified UCNPs and core-shell with solid oxide, have a potential to attach some molecules.²⁷ This fact enhances their application in selected cancer therapy called photodynamic therapy. In this case UCNPs must be connect with photosensitizer molecules.^{28,29} Presented nanoparticles, doped with thulium ions, are an alternative system, avoiding unstable (photobleaching) molecules of photosensitizers.³⁰ Wide emission spectrum, dependent on the ions pair (Yb^{3+} - Tm^{3+}), allows to apply the upconverting system for generation of reactive oxygen species without any additional reagents like photosensitizer molecules present in the traditional photodynamic therapy.³¹ High energy emitted UV light is capable to decompose water molecules to toxic radicals.

Herein, we report on a general method of silica-coating β - NaYF_4 :20% Yb^{3+} ,2% Er^{3+} and β - NaYF_4 :20% Yb^{3+} ,0.2% Tm^{3+} NPs. The NaYF_4 host matrix has a very low phonon energy and therefore minimizes the quenching of the lanthanide ions excited-state, which results in a high quantum yield for upconversion luminescence (UCL).^{32–39} Depending on the RE doping, under NIR excitation UCL emission of different colours is obtained, thus making the obtained UCNPs interesting for multicolour bioimaging, basic material in modern photodynamic therapy.

In brief, we report on the synthesis of functionalized SiO_2 -coated UCNPs with sizes smaller than 20 nm, which form stable

aqueous suspensions and therefore can be applied for biological optical imaging. The SiO_2 -coating improves the overall biocompatibility of the UCNPs and makes them suitable for functionalization with biologically-active molecules.

2 Methods

2.1. Materials

Trifluoroacetic acid (99%, Sigma-Aldrich), sodium trifluoroacetate (>99%, Sigma-Aldrich), yttrium(III) oxide (99.99%, Sigma-Aldrich), ytterbium(III) oxide (99.9%, Sigma-Aldrich), erbium(III) oxide (99.9%, Sigma-Aldrich), thulium(III) oxide (99.9%, Sigma-Aldrich), 1-octadecene (>95%, Sigma-Aldrich), oleic acid (99%, Chempur), cyclohexane (99%, Chempur), ethanol (99.8%, Chempur), polyoxyethylene(5)nonylphenylether IGEPAL CO-520 (Sigma-Aldrich), ammonium hydroxide solution (30%, Sigma-Aldrich) and tetraethyl orthosilicate TEOS (98%, Sigma-Aldrich) were used without further purification.

2.2. Synthesis of β - NaYF_4 :20% Yb^{3+} ,2% Er^{3+} and β - NaYF_4 :20% Yb^{3+} ,0.2% Tm^{3+} nanoparticles

The upconverting NPs were synthesized by homogeneous solution coprecipitation method. The nanoparticles were prepared from precursors (trifluoroacetate salts of yttrium, ytterbium, and erbium or thulium). The trifluoroacetate salts were chemically prepared according to method reported previously.⁴⁰

The main synthesis was carried out at non-oxide waterless reaction environment using solution of oleic acid and octadecene, at 330 °C for 30 minutes.⁴¹

2.3. Synthesis of β - NaYF_4 :20% Yb^{3+} ,2% Er^{3+} @ SiO_2 nanoparticles

The β - NaYF_4 :20% Yb^{3+} ,2% Er^{3+} NPs were surface coated with silica to improve their functionality. Solution of NPs suspended in cyclohexane was mixed with IGEPAL CO-520 and the mixture was sonicated for 10 min. Next ammonia was added dropwise and stirred for 30 min. Then, TEOS was added and the solution was stirred for 24 h at room temperature. The NPS was cleaned by mixture of ethanol/water solution (1 : 1) five times after the reaction.⁴²

2.4. Characterization of NaYF_4 :20% Yb^{3+} ,2% Er^{3+} , NaYF_4 :20% Yb^{3+} ,0.2% Tm^{3+} , and NaYF_4 :20% Yb^{3+} ,2% Er^{3+} @ SiO_2 nanoparticles

The size distribution and morphology of both β - NaYF_4 :20% Yb^{3+} ,2% Er^{3+} and β - NaYF_4 :20% Yb^{3+} ,0.2% Tm^{3+} NPs were assessed by scanning electron microscopy (SEM) using a Zeiss Auriga Neon 40 microscope at an acceleration voltage of 5 kV. For SEM imaging samples were prepared by dropping the NPs' solution onto the surface of a $0.5 \times 0.5 \text{ cm}^2$ silicon wafer. The presence of the SiO_2 shell was confirmed using high-resolution transmission electron microscopy (HRTEM). Elemental analysis of the NPs was performed using the energy-dispersive spectroscopy (EDX) accessory connected to the TEM system (FEI Tecnai Osiris operating at 80 kV acceleration voltage).



The crystal structure of the NPs was determined using TEM and X-ray diffraction (XRD). XRD measurements were conducted in a Philips X'Pert Pro Alpha1 MPD (Panalytical) diffractometer in the 2θ range of $10\text{--}150^\circ$ (15 h pattern scanning at 1.5406 nm wavelength).

The upconversion luminescence of the NPs was measured in an optical system comprising a 980 nm continuous wave (CW) laser (Lumics model LU0980D300-DNA014) as the optical excitation source and a Jobin Yvon-SPEX 270 M monochromator equipped with a CCD camera. Luminescence spectra were measured in the 500 to 700 nm range. A shortpass filter cut-off 750 nm (ThorLabs FESH0750) was employed while recording the luminescence data.

2.5. Incubation of HeLa cells with $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ nanoparticles

The standard HeLa cell line derived from cervical cancer was used in this study. The HeLa cells were routinely cultured with DMEM (Dulbecco's modified eagle medium) containing 10% fetal calf serum (FCS). Cell cultures were kept at 37°C in a humidified atmosphere containing 5% of CO_2 . Cells were cultured in 6-well plate dishes ($6 \times 10\text{ cm}^2$) at a density of 100 000 per plate. The cells were incubated with $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ NPs without oleic acid (procedure describe in Section 2.4.1) and with $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs. 291 μl of the sonicated initial aqueous colloidal suspension of $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ NPs (3.44 mg ml^{-1}) was dissolved in 709 μl miliR water (the initial aqueous colloidal suspension of $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}, 2\%\text{Er@SiO}_2$ NPs contained 3.75 mg ml^{-1} and 267 μl of this sample was sonicated with 733 μl MiliR water to obtain 1 mg ml^{-1} concentration). Next, 2 μl of each diluted suspension was added to a 10 cm^2 dish with HeLa cells and incubated for 24 h. Then, the medium was changed to DMEM.⁴³

2.6. Procedure of ligand-free $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ nanoparticles preparation

Cyclohexane suspension of the NPs was precipitated by ethanol and washed by several cycles of sonication and centrifugation. Removal of oleic acid ligand took place through the using of different solution to dissolve NPs such as: HCl solution (0.1 M), acidic ethanol solution (pH 4), ethanol. Finally, the NPs were re-dispersed in 1 ml distilled water.^{44,45}

2.7. Observation of $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ nanoparticles inside the HeLa cells

The main technique for imaging both $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs was confocal microscopy, using a Zeiss 710 NLO system equipped with an infrared femtosecond laser (Coherent, Chameleon). The three channels were observed: the first, with excitation at 980 nm (femtosecond laser), was used for NP imaging in the 500–730 nm range; the second, with excitation at 705 nm (femtosecond laser), was used for imaging the nucleus marked by the Hoechst marker while detecting in the 425–475 nm range; the third, with excitation at 488 nm (continuous laser), was used to image the actine

filaments build cell's cytoskeleton marked by antibodies labelled with fluorophore AlexaFluor 488 imaging with detection in the 495–572 nm range. This approach allows for imaging the marked structures and the UCNPs inside the selected cells.

Samples for confocal microscopy imaging were prepared according to the following procedure. Cells were inoculated onto $2 \times 2\text{ cm}^2$ slides and placed in the 6-well plate at 100 000 per plate. After 24 h incubation materials were separated by sonication and transfected as previously described (Section 2.4). Then, the medium was removed and the cells were washed twice with 2 ml of phosphate-buffered saline (PBS). Firstly, the cells were fixed by adding 2 ml of 3.7% formaldehyde in PBS and incubated for 20 min. After fixing, the cells were washed twice using 2 ml of PBS. Secondly, the cell's membrane was permeabilized using 2 ml of 1% solution of TritonX-100 in 5% FBS/PBS (5% solution of Fetal Bovine Serum in PBS) and incubated for 10 min. Next, the cells were washed twice with 2 ml of PBS and permeabilization was blocked by adding 2 ml of 5% FBS/PBS and the samples were incubated for 30 min. Samples were washed with PBS and the cytoskeleton was stained with the primary rabbit actine antibody. 25 μl of the antibody in 5% FBS/PBS solution was dropped onto the slide with cells and covered with a capping slide. Samples were incubated for 1 h and then the capping slide was removed. The samples were washed twice using PBS. Using the same protocol the secondary antibody connected with AlexaFluor 488 was attached to the primary antibody. This step of preparation was conducted in dark to protect the fluorescent dye from photobleaching. The nucleus was marked using the Hoechst 33342 (25 μl) added.

2.8. Cytotoxicity of $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ nanoparticles

The cytotoxicity of the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs was evaluated by the MTT viability assay. The cells (10 000 per well) were incubated in each well of a 96-well plate with the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs in the following concentrations: 0.1, 1, 5, 10 and 50 $\mu\text{g ml}^{-1}$ in medium for 24 and 48 h. Following incubation, the cells were washed three times with the culture medium. Then, the MTT solution (5.0 mg ml^{-1}) was added. After incubation for another 4 h the MTT medium was removed from each well, DMSO was added and the mixture was shaken at room temperature to dissolve the reacted dye. The optical density (OD) was measured at 570 nm with a microplate reader. The cytotoxicity was calculated as follows: cytotoxicity = $(B \times 100\%) / A$, where A is the absorbance of the cells incubated with the culture medium (as a control) and B is the absorbance of the cells incubated with or without the NPs.

2.9. Transmission electron microscopy of the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ nanoparticles inside the HeLa cells

The presence of the NPs' inside the HeLa cells was confirmed by TEM imaging. Upon incubation with $1\text{ }\mu\text{g ml}^{-1}$ of $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ or $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs for 24 h HeLa cells pellets were fixed with 2% paraformaldehyde (Sigma



Aldrich, Germany) and 2.5% glutaraldehyde (Merck KGaA, Darmstadt, Germany) in 0.1 M cacodylate buffer, pH = 7.4 at 4 °C for 2 h, post-fixed with 1% osmium tetroxide (Sigma, USA) for 1 h, dehydrated in the graded series of ethanol (from 30% to 99.8%) and propylene oxide (ABCR GmbH, Germany), embedded in Agar 100 resin kit R1031 (Agar, USA) and polymerized at 60 °C for 24 h and sectioned (60 nm) using a RMC ultramicrotome (USA). The ultrathin sections were stained with uranyl acetate and lead citrate. Grids were examined with a JEM 1200EX electron microscope.

2.10. Reactive oxygen species generation by β -NaYF₄:20% Yb³⁺,0.2%Tm³⁺@SiO₂ nanoparticles

The potential application of the presented nanoparticles to generate ROS was proved by electron paramagnetic resonance spectroscopy (EPR) after NIR light excitation. Water solution of NPs at 15 mg ml⁻¹ concentration was mixed with spin trap buffer, as a spin trap used 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), which shows EPR signal after reaction with the radicals.

The experiment was performed using as a near infrared sources: prototype LEDs system with maximum emission by 980 nm developed at University of Brasília, which worked with following parameters: 1 W cm⁻² of power density, 30 minutes of irradiation (working cycle: 30 seconds of irradiation, 1 minute break). EPR signal was measured every ten minutes during 20 minutes of irradiation using Bruker EMX plus EPR spectrometer with X-Band using a 4119HS resonator with 5 G modulation, 200 G sweep width, and 20 mW microwave power. The ROS production was performed by spin trapping using by CMH cyclic hydroxylamine and Krebs Hepes buffer, with controlled time to air exposure to avoid false positives results.

3 Results and discussion

The structural properties and the phase purity of the β -NaYF₄:20%Yb³⁺,2%Er³⁺ and β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ UCNPs were examined by XRD measurements. The XRD patterns of the UCNPs are shown in Fig. 1. As can be observed the diffraction peaks of both NPs are well defined and the peak positions and intensities agree well with the values for the hexagonal NaYF₄ crystal.^{7,46}

The size distribution of the UCNPs was determined by using XRD results and SEM images (Fig. 2a and b). From the line broadening of the (021) XRD peak of the samples β -NaYF₄:20%Yb³⁺,2%Er³⁺ and β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ and using the Debye-Scherrer formula an average crystallite size of 15.0 and 21.6 nm were calculated, respectively. Typical the UCNPs observed by SEM are well dispersed and very much uniform in size, with an average diameter of about 20.5 (±2.5) nm for the Er³⁺-doped and 22.3 (±0.9) nm for the Tm³⁺-doped NPs, as shown in the histograms presented in Fig. 2a and b. The average size values assessed from the SEM micrographs matched the average particle size obtained from the XRD data. Deviation in the average particle size obtained from the SEM and XRD data can be due to inaccuracy of the size estimation while using the

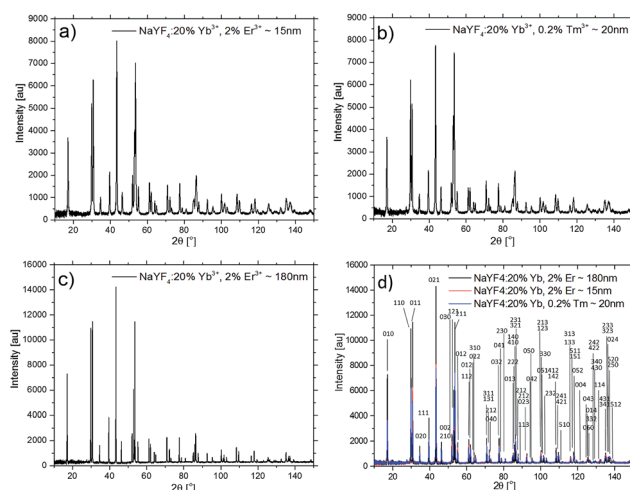


Fig. 1 XRD patterns of: (a) β -NaYF₄:20%Yb³⁺,2%Er³⁺ (~15 nm of diameter); (b) β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ (~20 nm); (c) β -NaYF₄:20%Yb³⁺,2%Er³⁺ (~180 nm) and (d) all diffractograms grouped together. Note that big crystals (c) were taken as reference.

XRD technique. The as-produced NPs have a regular spherical shape (Fig. 2), which is beneficial to their application in the cellular imaging.

The SiO₂-shell thickness and morphology of the β -NaYF₄:20%Yb³⁺,2%Er³⁺@SiO₂ UCNPs were assessed by TEM. Typical TEM images of the NPs are shown in Fig. 2c. After coating with SiO₂, the average size of the Er³⁺-doped NPs increased to about 24.6 (±3.7) nm. Nevertheless, after SiO₂-coating the shape and size monodispersity remained almost unchanged, as it observed on Fig. 2c, where the SiO₂-shell thickness is about 3.0 nm.

The EDX data are shown in Fig. 3. The EDX spectra confirm the presence of all elements (Na, Y, Yb, Er, F) in the β -NaYF₄:20%Yb³⁺,2%Er³⁺ and (Na, Y, Yb, Tm, F) in the β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ NPs (Fig. 3a and b). EDX maps show homogenous distribution of the elements forming the core and the shell of the NPs. Fig. 3c presents core, β -NaYF₄:20%Yb³⁺,2%Er³⁺ NPs and Fig. 3d shows the SiO₂ shell on the NPs.

The EDX data in Fig. 3 revealed homogeneous distribution of all elements in both types of the NPs. Likewise, a homogeneous elemental distribution is observed in the β -NaYF₄:20%Yb³⁺,2%Er³⁺@SiO₂ NPs (see Fig. 3d). Silicon and oxygen are in the same location as other elements within the NPs. Indeed, silica forms a shell around the NPs which is visible in the Si EDX map in Fig. 3d. This finding confirms that the NPs were efficiently coated by SiO₂.

Before the cytotoxicity measurements of β -NaYF₄ NPs, the oleic acid was detached from the surface. Actually, the oleic acid acts as a surfactant capable to cap ligands while introducing the hydrophobic character to the NPs. Therefore, the oleic acid-coated UCNPs are not suitable for biological application, but can be easily removed out from the NP surface by HCl washing while the remaining functional groups associated with oleic acid can be identified by FT-IR spectroscopy (Fig. 4).

The oleic acid exhibits an IR band around 3430 cm⁻¹, corresponding to the hydroxyl group's stretching vibration. Two



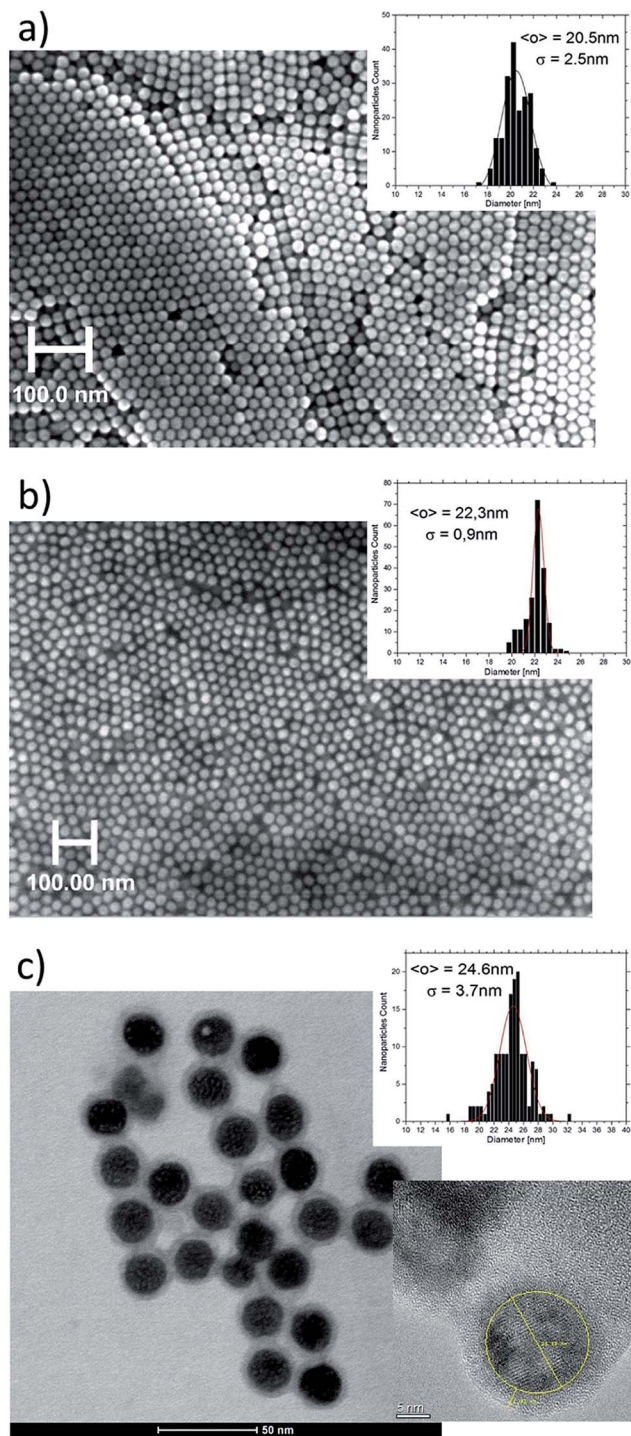


Fig. 2 SEM images of (a) β -NaYF₄:20%Yb³⁺, 2%Er³⁺ NPs; (b) β -NaYF₄:20%Yb³⁺, 0.2%Tm³⁺ NPs (inserts show particle size histograms obtained from 200 counting); (c) TEM image of β -NaYF₄:20%Yb³⁺, 2%Er³⁺@SiO₂ NPs (inserts show particle size histogram and HRTEM micrograph showing).

features peaking at 1632 and 1460 cm⁻¹ are associated with the asymmetric and symmetric stretching vibrations of the carboxylic group (COO⁻), respectively. The two peaks at 2924 and 2854 cm⁻¹ can be assigned to the asymmetric and symmetric stretching vibrations of methylene group,

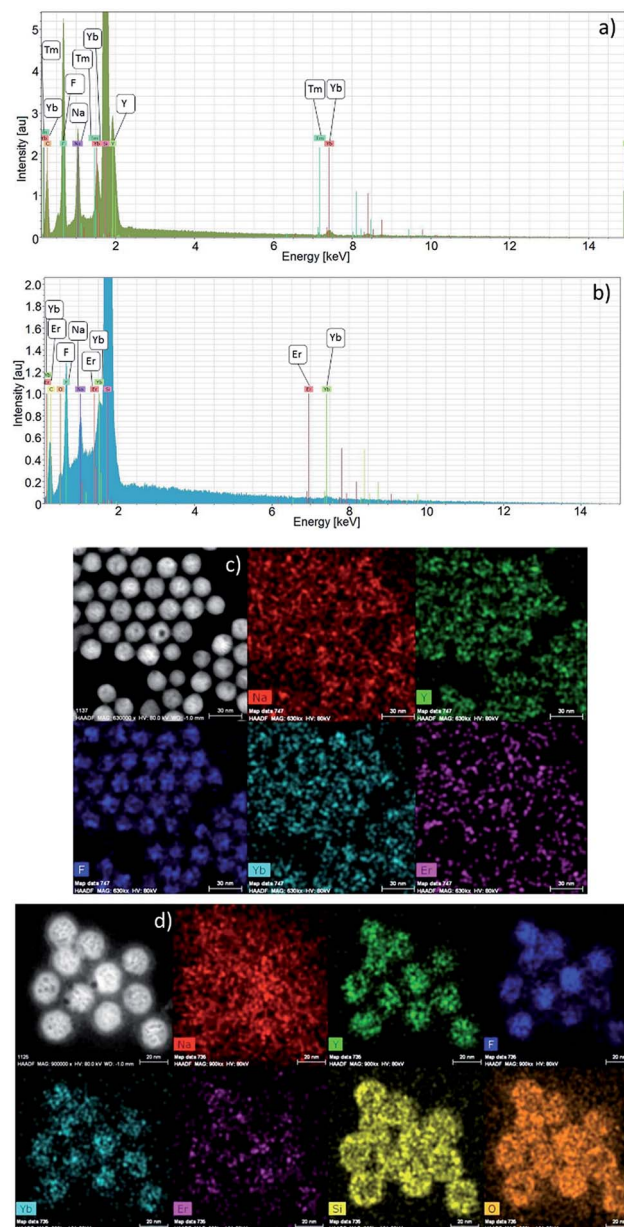


Fig. 3 EDX spectra of the (a) β -NaYF₄:20%Yb³⁺, 0.2%Tm³⁺ and (b) β -NaYF₄:20%Yb³⁺, 2%Er³⁺ NPs. EDX elemental distribution maps of the (c) β -NaYF₄:20%Yb³⁺, 2%Er³⁺ NPs and (d) β -NaYF₄:20%Yb³⁺, 2%Er³⁺@SiO₂ NPs.

respectively. As shown in Fig. 4, after the HCl purification, the quenching of the features in the FT-IR spectrum in the 4000–1000 cm⁻¹ range indicates that the pure (oleic acid free) NaYF₄:20%Yb³⁺, 2%Er³⁺ NPs were obtained.

The upconversion spectra of 1 mg ml⁻¹ suspensions of β -NaYF₄:20%Yb³⁺, 2%Er³⁺ and β -NaYF₄:20%Yb³⁺, 0.2%Tm³⁺ NPs in cyclohexane under 980 nm laser diode excitation (power density 12.14 W cm⁻²) are shown in Fig. 5a and d. The insets of Fig. 5a and d show a digital photograph of the total upconversion luminescence of the same solution under the described excitation condition.



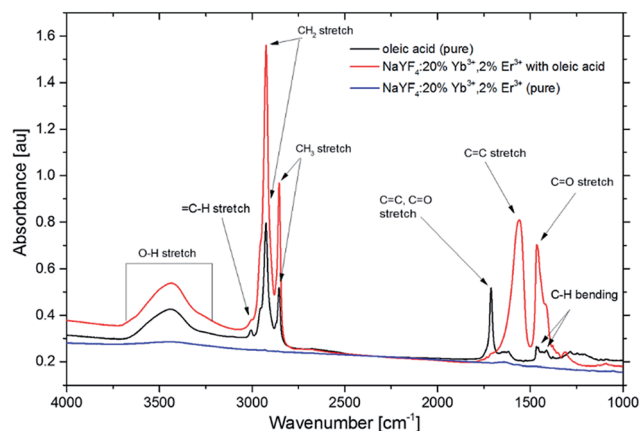


Fig. 4 FT-IR spectra of oleic acid, β -NaYF₄:20%Yb³⁺,2%Er³⁺ NPs with oleic acid and β -NaYF₄:20%Yb³⁺,2%Er³⁺ NPs after HCl washing.

In order to determine the number of photons responsible for the upconversion mechanism the intensity of the upconversion emission (UCL) was recorded as a function of the 980 nm excitation (Fig. 5c and f).

Stable suspension of the NPs in aqueous medium is very important for effective bioimaging applications. Therefore, for the biological applications the silica-coated NPs (β -NaYF₄:20%Yb³⁺,2%Er³⁺@SiO₂) were produced. As a result of surface modification the β -NaYF₄:20%Yb³⁺,2%Er³⁺@SiO₂ NPs are stable in water and emit strong upconverting fluorescence (Fig. 5b and e).

The emission bands of the β -NaYF₄:20%Yb³⁺,2%Er³⁺ and the β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ NPs after NIR light excitation are shown in Fig. 5a and d. They can be assigned to the transitions between the 4f–4f levels of the Er³⁺ and Tm³⁺ ions. The spectrum of the β -NaYF₄:20%Yb³⁺,2%Er³⁺ NPs (see Fig. 5a) exhibits three emission bands. The two strong green emissions at 540 and 550 nm originate from the ²H_{11/2} to ⁴I_{15/2} and from ⁴S_{3/2} to ⁴I_{15/2} transitions, respectively. The red emission at 654 nm can be assigned to the transition from ⁴F_{9/2} to ⁴I_{15/2} levels.^{47,48}

Four Tm³⁺ emission bands were observed in the β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ NPs (Fig. 5d) with maximum position of peaks at 474, 645, 697 and 800 nm.

In order to determine the number of photons responsible for the upconversion mechanism, the intensity of the upconversion luminescence (UCL) was recorded as a function of the power of 980 nm excitation (see Fig. 5c and f). The UCL intensity (I_{up}) in the lanthanide-doped UCNPs depends on the laser power (P) excitation. This dependence can be expressed as: $I_{up} \sim P^n$, where n describes the quantity of absorbed photons in the luminescence process. Fig. 5c shows the green and red Er³⁺ upconversion emission intensities in quadratic power dependence at low excitation, indicating the two-photon upconversion mechanisms. For the Tm³⁺-doped NPs (see Fig. 5f) three- and two-photon power dependencies were observed for the ¹G₄ to ³H₆ and ³H₄ to ³H₆ emissions. Similar observation has been reported previously in the literature.⁴⁷

The upconversion mechanisms for the Er³⁺/Yb³⁺ and Tm³⁺/Yb³⁺ ion couples within the β -NaYF₄ crystal are well-known⁴⁹

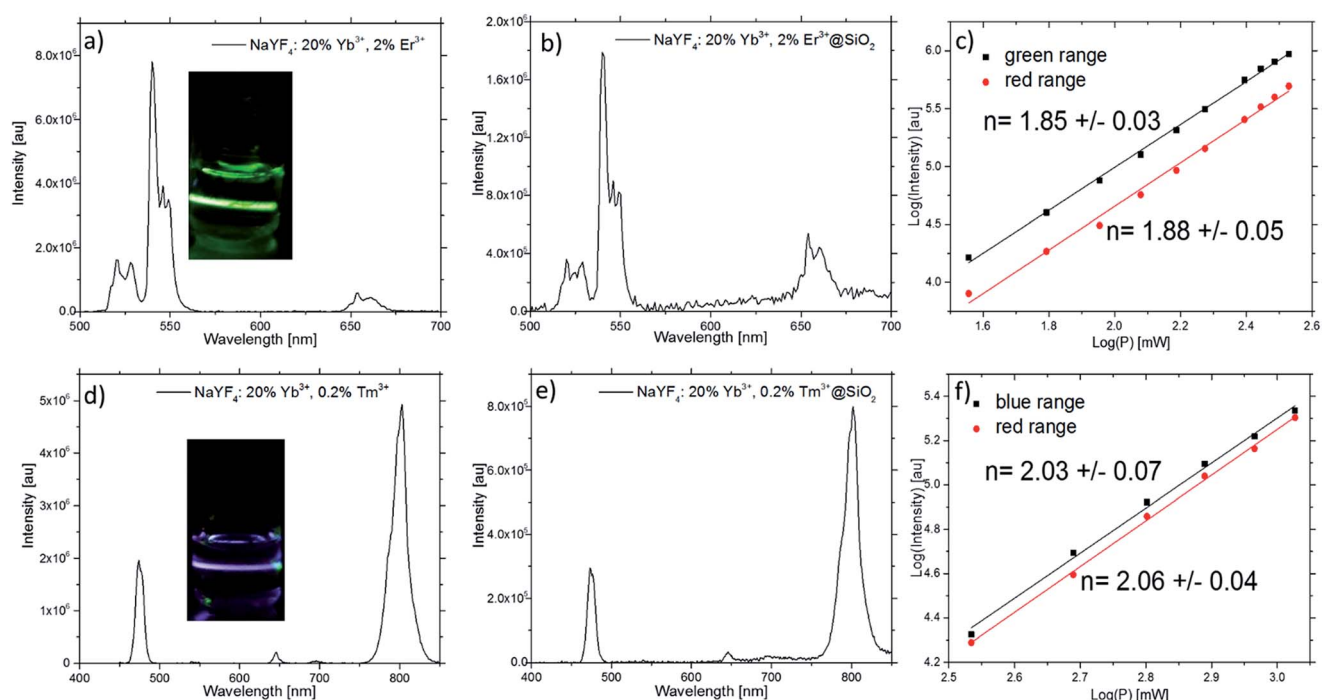


Fig. 5 Luminescence spectra of the NPs suspensions (1 mg ml⁻¹) in cyclohexane excited with a 980 nm laser diode (power density 12.14 W cm⁻²): (a) β -NaYF₄:20%Yb³⁺,2%Er³⁺, (d) β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺ and 1 mg ml⁻¹ in water suspension of (b) β -NaYF₄:20%Yb³⁺,2%Er³⁺@SiO₂ and (e) β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺@SiO₂ NPs excited with a 980 nm laser diode (power density 12.14 W cm⁻²). Double log plot of power dependence of the upconverted emissions of 1 mg ml⁻¹ suspensions of the NPs in cyclohexane excited at 980 nm: (c) β -NaYF₄:20%Yb³⁺,2%Er³⁺ and (f) β -NaYF₄:20%Yb³⁺,0.2%Tm³⁺. Symbols are experimental data while the straight lines are least-squares fits to the data points.



and schematically represented in Fig. 6. In the case of β -NaYF₄:20%Yb³⁺,2%Er³⁺ the excitation energy is transferred from the Yb³⁺ ion in the ²F_{5/2} state to the Er³⁺ ion in the ⁴I_{11/2} state. The second energy transfer from the Yb³⁺ ion can populate the ⁴F_{7/2} state of the Er³⁺ ion. The Er³⁺ ion can relax non-radiative (without emission of photons) to the ²H_{11/2} and ⁴S_{3/2} levels before emitting green light *via* ²H_{11/2} → ⁴I_{15/2} and ⁴S_{3/2} → ⁴I_{15/2} transitions. Alternatively, the Er³⁺ ion can relax and populate the ⁴F_{9/2} level, leading to the red ⁴F_{9/2} → ⁴I_{15/2} emission. The ⁴F_{9/2} level may also be populated from the ⁴I_{13/2} level of the Er³⁺ ion by absorption of a 980 nm photon, or by energy transfer from an Yb³⁺ ion, with the ⁴I_{13/2} state being initially populated *via* the non-radiative ⁴I_{11/2} → ⁴I_{13/2} relaxation.⁴⁷

Nanoparticles doped by Tm³⁺ ions are characterized by four main emission bands (see Fig. 5d) with wavelength 474, 645, 697 and 800 nm. After absorption of 980 nm by Yb³⁺ ions,

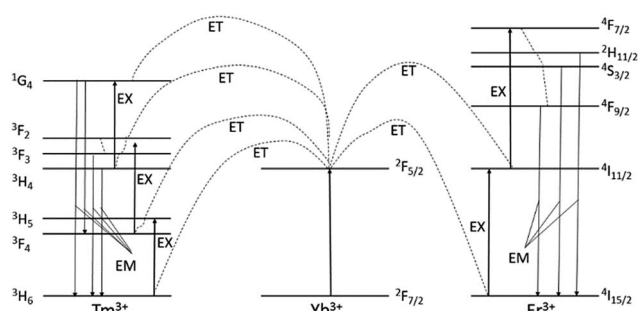


Fig. 6 The energy level diagrams of the Er³⁺, Tm³⁺ and Yb³⁺ dopant ions and the corresponding upconversion mechanisms under 980 nm excitation. The ET interrupted arrows represent energy transfer (non-radiative transition), EX – excitation and EM – emission of visible photons (radiative transition).^{51–53}

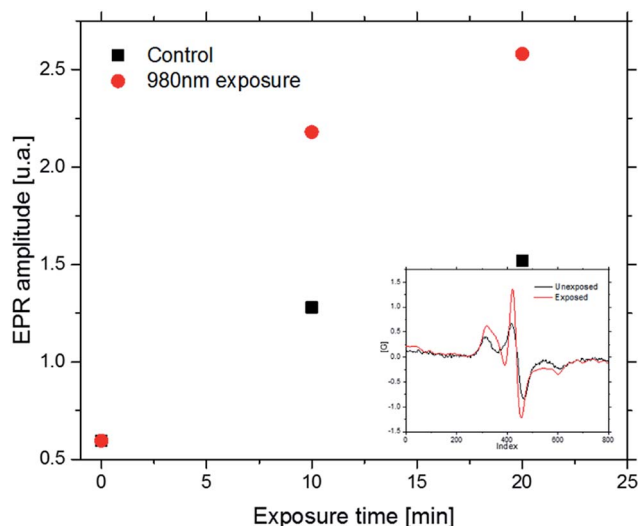


Fig. 7 EPR spectroscopy graph of CMH signal. Spin trap was activated by reactive oxygen species from decomposition of water by NPs emitted high energy light. Control group shows non irradiated solution. Tested group was irradiated during 30 minutes with 980 nm NIR sources at 1 W cm⁻² power density, respectively by LEDs system. Insert shows example spectrum (after 20 minute of irradiation) using to make a graph.

energy is transferred from ²F_{5/2} ytterbium state to ³H₆, ³H₅, ³F₂ and ¹G₄ states, respectively. In consequence of energy transfers and non-radiative transitions between the levels followed the emission from states: ¹G₄ → ³H₆, ¹G₄ → ³F₄, ³F₃ → ³H₆ and ³H₄ → ³H₆ respectively. These emission bands are described above (Fig. 6).^{52,53}

As shown in Fig. 5a and e presence of the SiO₂ shell, has an impact to the physical aspect of the luminescence. Analysis of the core NaYF₄:20%Yb³⁺,2%Er³⁺ NPs emission spectrum points to the higher luminescence intensity of the green area compared to the red area. The ratio of the two luminescence areas has different characteristic for the NPs coated by SiO₂. In this material the intensity of the green luminescence is lower than the red. This difference is due to the modification of the NPs surface which offsets the surface effects and contribute to the energy of phonons relaxation, which takes part in the upconversion, as we reported previously.⁵⁴ The silicon oxide shell enhances the non-radiative decay which causes higher quantity of the radiative emission from ⁴F_{9/2} level than ⁴S_{3/2} level (see Fig. 6).

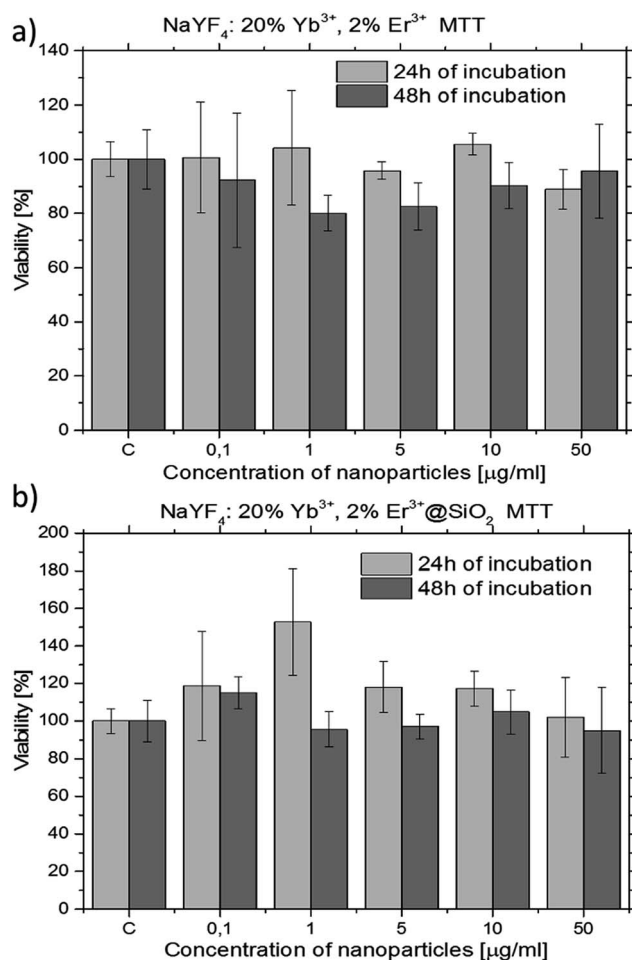


Fig. 8 Cell viability values estimated from the MTT assay versus concentration of the β -NaYF₄:20%Yb³⁺,2%Er³⁺ NPs without oleic acid (a) and β -NaYF₄:20%Yb³⁺,2%Er³⁺@SiO₂ NPs (b). Cells were incubated with 0–50 µg ml⁻¹ of the NPs at 37 °C, for 24 h (a, c) and 48 h (b, d). Each data point is represented as mean (SD from 4 trials).



We have synthesized and characterized $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 0.2\%\text{Tm}^{3+}\text{@SiO}_2$ nanoparticles exhibiting several promising properties useful in medical applications: bio imaging of cells and tissues and modern PDT. A high efficiency NIR upconversion and, due to presence of $\text{Tm}^{3+}\text{-Yb}^{3+}$ pair ions, ROS generation capability, renders the SiO_2 shell coated nanoparticles to become potentially useful theranostic agent.

The experiment, performed under standard conditions, was aimed to test the possibility of $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 0.2\%\text{Tm}^{3+}\text{@SiO}_2$ NPs to generate ROS without any additional organic molecules. Comparison of the control sample-without the LED irradiation and the NPs suspension exposed to the light, shows enhanced level of the CMH spin trap signal. Intensity of the EPR signals for the control and LED illuminated samples were summarized (Fig. 7). We observed the 70% increased in the EPR signal after 10 min of irradiation and 70% after 20 min of irradiation. The ROS generation from Tm-doped nanoparticles is potentially promising for the future PDT therapy.

In order to investigate the cytotoxicity of both $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ (without oleic acid) and $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs a MTT assay, using HeLa cell line, was performed to determine the effect of the UCNPs on the cell proliferation after 24 and 48 h (Fig. 8).

One of the most important aspect in the design and the synthesis of biomarkers is their toxicity. In order to investigate

the cytotoxicity of $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ (without oleic acid) and $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs a MTT assay was used. No significant difference in the cell proliferation was observed in the absence or presence of $0.1\text{--}50\text{ }\mu\text{g ml}^{-1}$ UCNPs (see Fig. 8). The cellular viabilities were estimated to be greater than 90% after 24 h and 80% after 48 h for the $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ NPs (Fig. 8a) and almost 100% after 24 and 48 h for the $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs (Fig. 8b). These data show that the $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ NPs have relatively low cytotoxicity after 24 and 48 h of incubation even at relatively high concentration ($50\text{ }\mu\text{g ml}^{-1}$). The $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs have no apparent cytotoxicity (after 48 h of incubation, the cellular viabilities were 100% even at the NPs concentration of $50\text{ }\mu\text{g ml}^{-1}$).

Cellular uptake of both $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ (without oleic acid) and $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs by HeLa cells was visualized by multiphoton confocal imaging (Fig. 9a and b). Cells stained by the NPs were exposed to 980 nm irradiation using a femtosecond Ti:sapphire pulsed laser while their typical upconversion luminescence was measured in 500–730 nm channel for the green and red emissions. In addition, the cytoskeleton of the HeLa cells was stained by the antibodies conjugated with the AlexaFluor 488 for a better visualization of the cells. The blue color indicates the nucleus stained by Hoechst 33342 channel (excitation: 705 nm – femtosecond

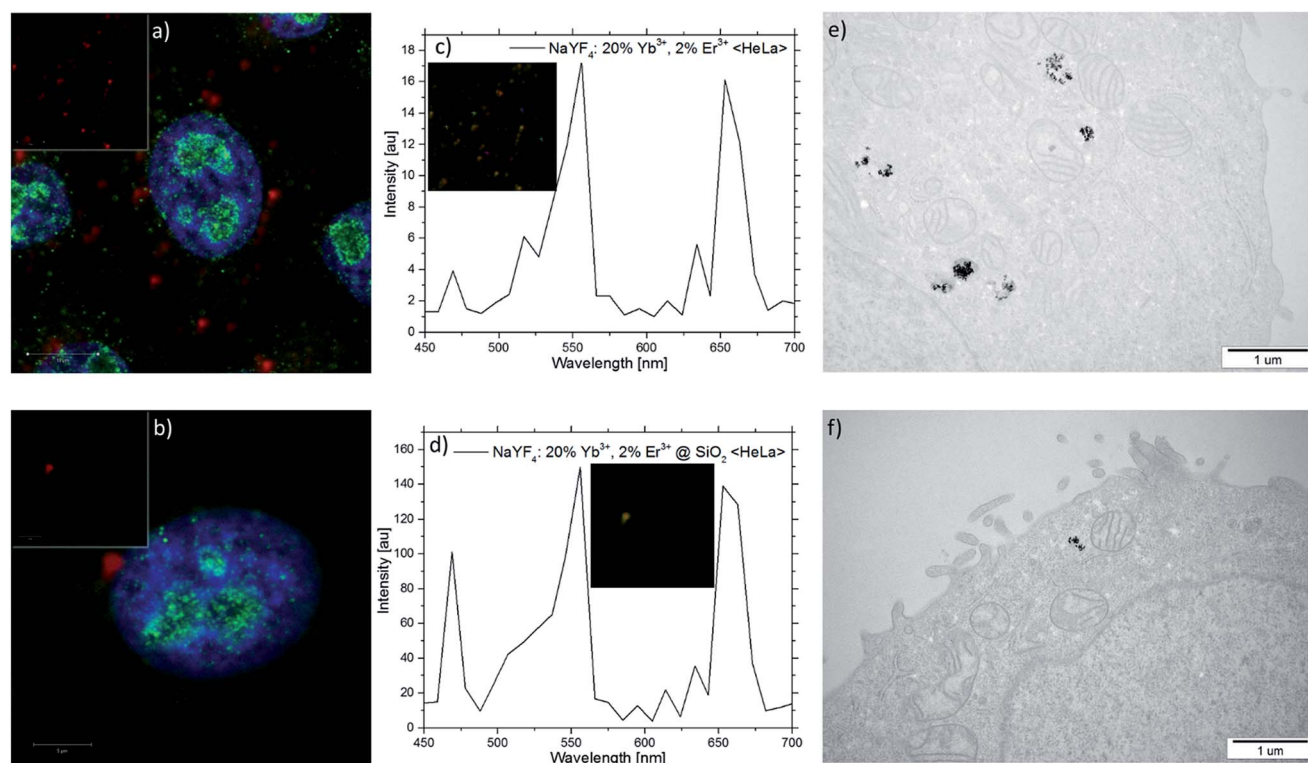


Fig. 9 Confocal images of the HeLa cells incubated with the (a) $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ and (b) $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs after 24 h of incubation with 0.001 mg ml^{-1} of the NPs concentration. The insets in the panels (a) and (b) show the upconversion channels. Luminescence spectra of the (c) $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ and (d) $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs inside the HeLa cells excited with the 980 nm wavelength light at $1.11\text{ W }\mu\text{m}^{-2}$ laser power. Insets in (c) and (d) show images with luminescent spots. TEM images showing the (e) $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}$ and (f) $\beta\text{-NaYF}_4\text{:20%Yb}^{3+}, 2\%\text{Er}^{3+}\text{@SiO}_2$ NPs within HeLa cells (incubation with $1\text{ }\mu\text{g ml}^{-1}$ of NPs at $37\text{ }^\circ\text{C}$, for 24 h).



laser, power density: $0.72 \text{ W } \mu\text{m}^{-2}$, detection: 425–475 nm). Green color indicates the actine cytoskeleton filaments marked by the antibody labeled with AlexaFluor 488 (excitation: 488 nm – CW, power density: $0.0375 \text{ W } \mu\text{m}^{-2}$, detection: 495–572 nm). Red color indicates the UCNPs channel (excitation: 980 nm – femtosecond laser, power density: $1.11 \text{ W } \mu\text{m}^{-2}$; detection: 500–730 nm). A spectrum recorded from the cells showed that the luminescence exhibits two emission bands, with maxima at 556 and 653 nm after 980 nm of excitation (Fig. 9c and d). Fig. 9e and f shows the TEM images of (e) SiO_2 -uncoated and (f) SiO_2 -coated NPs (without oleic acid) inside the HeLa cells.

The overlay of the upconversion channel and cytoskeleton channel indicate that the UCL signal distributions are strongly correlated with the cytoskeleton of HeLa cells, meaning that the NPs spontaneously enter into the cells and locate within the cytoplasm. In contrast, the HeLa cells incubated with the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}@\text{SiO}_2$ NPs (Fig. 9b) show weaker luminescence in the upconverting green channel under the same conditions, suggesting a lower uptake by the HeLa cells. This may be due to differences in the NP surface charge. The spectrum recorded from the HeLa cells shows that the luminescence exhibits two characteristic emission bands, with maxima at 556 and 653 nm (Fig. 9d).

It should be pointed out that no measurable autofluorescence signal was observed after 980 nm excitation in the upconverting channel of the HeLa cells labelled with both $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}@\text{SiO}_2$ NPs (insets of Fig. 9a and b). Moreover, in the upconverting channel a high signal-to-noise ratio, with relatively high upconversion intensity, was observed and no background fluorescence was noted. As far as the biological imaging is concerned, the finding is an interesting and useful feature of the UCNPs,⁵⁰ which has not been obtained in the traditional single-photon or two-photon fluorescent imaging.^{55–66} While excited with the 980 nm laser light the biological samples presented weak absorption with no luminescence in the visible region. Fig. 9e and f shows that SiO_2 -coated and SiO_2 -uncoated NPs (without oleic acid) were trapped within cytosolic vesicular structures after being up-taken by the HeLa cells, suggesting that the NPs' cellular internalization took place by endocytosis in both cases.

4 Conclusions

The present report describes a successful synthesis and characterization of the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 0.2\%\text{Tm}^{3+}$ NPs. Under NIR light irradiation the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ NPs exhibited intense green and red luminescence while the $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 0.2\%\text{Tm}^{3+}$ NPs exhibited intense ultra-violet, blue and red luminescence. UV emission range is capable to generate reactive oxygen species in aqueous environment. Compared to traditional therapies based on ROS generation, we presented materials which could generate ROS without any other components attached to the nanoparticles. This property allows to solve a common problems with traditional systems containing photosensitizers (photobleaching, dose limitation). The synthesized core NPs were surface coated with silica, having the silica shell thickness

about 3 nm. The nanoparticle's core/shell morphology, its structure and surface modification were confirmed by XRD, TEM, SEM, EDX, and FTIR measurements. All synthesized nanomaterials formed stable aqueous colloids exhibiting green or blue luminescence under NIR laser irradiation ($\lambda_{\text{ex}} = 980 \text{ nm}$). The cytotoxicity assays revealed that all the prepared nanomaterials are relatively nontoxic even in relatively high concentration ($50 \mu\text{g ml}^{-1}$) for 48 h incubation. The $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}$ and $\beta\text{-NaYF}_4\text{:}20\%\text{Yb}^{3+}, 2\%\text{Er}^{3+}@\text{SiO}_2$ NPs were visualised inside the cytoplasm of HeLa cells. Moreover, no apparent background fluorescence was observed inside the HeLa cells after the NIR light excitation with the strong up-conversion of the nanoparticles. Our report shows several promising applications of the inorganic, lanthanide-doped nanophosphors, in particularly, as potential biomarkers, contrast agents, and drug-delivery systems.

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