UP-CONVERTING FLUORIDE NANOPARTICLES FOR BIOMEDICAL APPLICATIONS

LIVIA PETRESCU[1], OTILIA CINTEZA[2], TUDOR ROŞU[2], ŞERBAN GEORGESCU[3] and DAN MIHĂILESCU[1]

[1]Faculty of Biology, University of Bucharest, Splaiul Independentei No 91-95, sector 5, Bucharest, Romania.
E-mail: draghicilivia@yahoo.com
[2]Faculty of Chemistry, University of Bucharest
[3]National Institute for Laser, Plasma and Radiation Physics, Laboratory of solid-state quantum electronics

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Labeling with fluorescent markers is a technique widely used in medical practice and biomedical research. Need to develop new types of markers more sensitive, ease to use, less destructive and less expensive is becoming more acute. Lately, phosphate-based biomarkers with up-conversion (infrared excitation and visible emission) have attracted attention, due to advantages it presents in relation to classical markers (excited with ultraviolet radiation): avoid tissue degradation, lack or low autofluorescence, deep tissue penetration of excitation radiation, low toxicity and long stability of markers. In this paper we report methods of synthesis, characterization, coating and biocompatibilization of up-converting fluoride nanoparticles. In order to test biomedical applicability we performed experimental studies of cell application and cell imaging with these up-converting nanoparticles. Our conclusion is that fluoride nanoparticles doped with lanthanide ions have promising application like biolabels in biomedicine.

Key words: luminescence, fluorescence, nanoparticles, up-conversion, $NaYF_4$, biomarkers.

INTRODUCTION

Nanotechnology include a wide range of research, processes, technologies, applications and concepts of the multidisciplinary fields of physics, chemistry, biology, medicine, engineering, electronics, materials science. Evolution of research in this area was stimulated by the concomitant development of technical means and approaches, enabled to investigate the properties of materials at the atomic level and use this information in various branches of science. Fusion between nanotechnology and nanoscience has produced a real revolution with particularly strong impact in biotechnology. Bioimaging is a medical branch that enjoys the attention of nanotechnology research because of the necessity of developing new diagnostic methods and more sensitive, efficient and cheaper studies. Use of optically active nanoparticles such quantum dots[1, 2], the metal particles (gold)[3, 4] or those of oxides, fluorides and metal salts (VO_5, YO_3, $NaYF_4$) is a very promising field in bioimaging due to optical properties of these nanoparticles.

Of these, dielectric crystalline particles (oxides, fluorides) doped with lanthanide ions (Yb^{3+}, Er^{3+} and Tm^{3+}), known as up-converting nanoparticles or up-converting nanophsphors, are intensively studied because of their capacity to fluorescence emission by infrared excitation[5–7]. This is a very useful feature for *in vitro* studies. Lately applications are bioimaging expanding *in vivo*, allowing obtaining images in deep tissue where the ultraviolet radiation does not penetrate[8–10].

Up-conversion was first reported in 1966 and first applications were in public lighting and paint industry. Since 1990 it has also been studied for applications in biomedical research.

Up-conversion mechanism consists either of the sequential absorption of the same ion energy levels (absorption of the ground state followed by absorption of excited states) or by excitation of two different centers of energy followed by non-radiative transfer (Figure 1)[11].

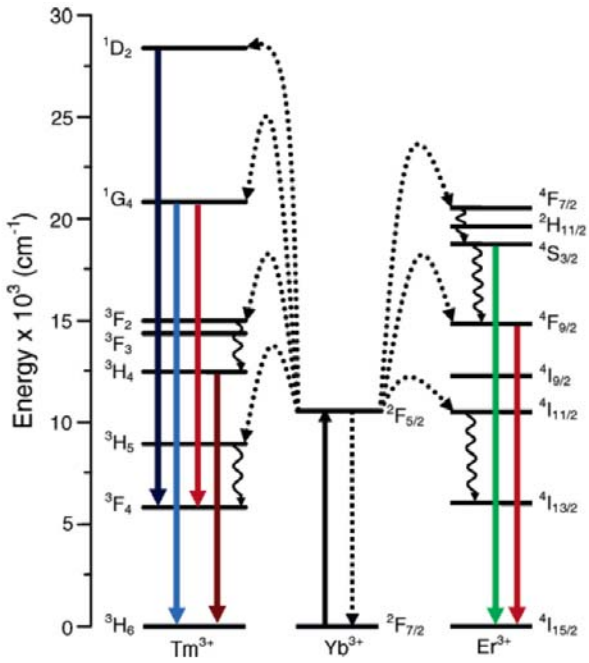

Fig. 1. Schematic illustration of the transition level of energy for Yb^{3+}, Tm^{3+} and Er^{3+}.

Up-converting phosphors are ceramic materials where rare lanthanide ions are embedded in the crystalline network. The material absorbs infrared radiation and emitting visible radiation using real power levels[12].

Markers with up-conversion have several advantages compared with classical fluorescent markers: lack or low tissue autofluorescence (leading to a high contrast), avoidance of tissue degradation due to ultraviolet exposure, avoidance of the photobleaching (optical properties of up-converting nanoparticles are unaffected by their environment because of the host lattice), long term stability of the samples, deep penetration of infrared radiation, low toxicity. The cost of this method is lower, so it is more accessible to potential users.

The particles used in many studies for biological applications have relatively large size (hundreds of nanometers) and are obtained by grinding of commercial phosphors. The main disadvantage is that although larger particles have a stronger fluorescence and is easier to detect, their size and irregular shape may result in a non-specific binding.

The result is lower reproducibility of analytical methods and difficulties in applying powder on biological samples. To get better results is recommended to use particles with lower size and more uniform structure.

Up-conversion phenomenon has been studied in a wide range of compounds and in according to existing data in the literature the maximum efficiency was obtained for Y_2O_3, Y_2O_2S, YVO_4 and $NaYF_4$,[13–15].

Our studies focus on the synthesis, characterization and biocompatibilisation of the fluoride nanoparticles doped with rare lanthanide ions ($NaYF_4:Er^{3+}:Yb^{3+}$) for use as markers to identify various analytes in biological environment or for highlighting the molecules of interest in the target cells.

EXPERIMENTAL PROCEDURES

Synthesis of $NaYF_4$ nanoparticles

There are a variety of physical and chemical methods for $NaYF_4$ synthesis (hydrothermal method[16], co-precipitation, thermolysis, polyol method), and according to working conditions, the properties obtained for the powders are different.

In our studies powders were doped with Er and Yb ions and synthesized by the hydrothermal method. Nanoparticles were prepared using aqueous solution of $Y(NO_3)_3$, aqueous solution of $Yb(NO_3)_3$, aqueous solution of $Er(NO_3)_3$, and aqueous solution of NaF as precursors. We applied the same principle of the method, but we used different ligands: EDTA (ethylenediaminetetraacetic acid), sodium citrate and citric acid.

Depending on the molar ratio, chelating agent, pressure and temperature were obtained particles with different crystallization shapes and sizes. The obtained material was characterized through X-ray diffraction (XRD), fluorescence spectroscopy and electron microscopy.

Nanoparticles characterization

Phase composition and average size of coherence domain were determinate by X-ray diffraction analysis (XRD). Measurements were performed with Bruker-AXS D8-ADVANCE diffractometer, using copper K_α radiation. Study of the fluorescence spectrum and measuring the kinetics powders luminescence were performed at room temperature using an experimental assembly consist of: luminescence excitation source (Xenon lamp with filters to separate a field centered on 395 nm, laser diode emitting at 980 nm), YAG laser: frequency doubled Nd for luminescence

kinetics measurements, a Jarrell-Ash monochromator, photomultiplier with spectral response S-20 and acquisition system (lock-in amplifier and multichannel analyzer). Size and shape of nanoparticles were obtained using an electron microscope Zeiss Evo 50. Electron microscope was used with a LaB_6 cathode in high vacuum mode. In this way the resolution is to 2 nm according to specifications. To achieve high-resolution observations of morphology, samples were coated with a thin layer of gold (thickness approximately 20 nm). Layers of gold were deposited using an installation of the DC sputtering. Hummer installation type 6 is designed specifically for electron microscopy coatings for obtaining a grain diameter of 2 nm.

Nanoparticles coating for biofunctialisation

Coating of nanoparticles is one of the essential conditions of their application to cells, thereby eliminating or at least diminishing some of the major issues: the aggregation of particles, low stability and degree of toxicity. In order to encapsulation of nanocrystals in biocompatible materials, samples synthesized using the chelating agent EDTA was dispersed in different organic solvents, in polyelectrolyte solutions and in biocompatible polymer solutions (chitosan with amino functional groups - Figure 2). Dispersion was performed by sonication continued for 3 hours, using an ultrasound bath with 700 W average power.

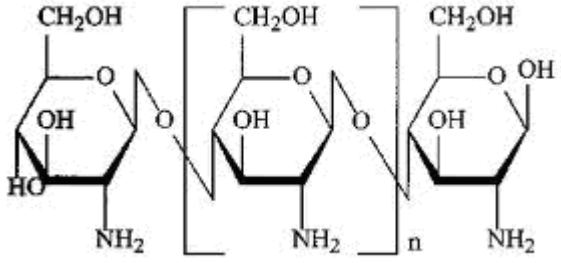

Fig. 2. Chitosan: β-1,4-poli-D-glucozamine.

We focused on Chitosan encapsulation of $NaYF_4$ nanocrystals with average size of 61 nm after sonication. After encapsulation, size distribution was measured using dynamic light scattering (DLS) with the NanoZetasizer Malvern to determine the presence of aggregates.

To confirm the results of coating and biofunctionalisation, the samples thus obtained were analyzed by electron microscopy. Suspensions containing nanoparticles were deposited (volume 10 microliters) on glass plates and dried at a temperature of 50 degrees in the air.

After drying, the layer containing both nanoparticles and the polymer was coated by DC sputtering with a thin layer of gold. Coverage was achieved in pulsed deposition to minimize the effect of the process on the integrity of the polymer / nanoparticles layer. Gold thickness was about 20 nm.

Culture of HEK 293 cells

We used for our experiments HEK 293 line cells (Human Embryonic Kidney), widely used in laboratory studies, being easily grown and transfected with various genes (or combinations of genes). From a cell line can be done about 30 passages.

Terms of culture

Medium: DMEM supplemented with 100 units / ml penicillin G sodium, 100 mg / ml streptomycin, 4 mM L-glutamine, and 10% fetal bovine serum.

Conditions of incubation: 37°C, 5% CO_2, humid atmosphere. Incubation of less than 37°C did not cause the cells to adhere (remain in suspension), giving the impression that they are death. Cells are kept frozen in liquid nitrogen, where they put out for cultivation protocol.

Thawing cells

Quick defrost cells by immersion in water bath at 37°C (2–3 min by constant stirring). Transfer cells in a culture flask. Add of 4 ml of culture medium followed by agitation for easy distribution cell on the surface growth. Put the culture in the incubator.

The next day is examined under a microscope. Healthy cells are bright, expose a flat surface and adhere well to the plate. Expand the culture through passages made at 2–4 days, when confluence reached 70–80%. Cells should not be very confluent, but not too rare (indicated 100 cells/mm^2)

Resuspended cells using 1X tripsin-EDTA.

Cell application of coated $NaYF_4:Er^{3+}:Yb^{3+}$

HEK 293 cells were plated 24 h before exposure to chitosan/$NaYF_4$ nanocrystals. The cells were next incubated with the nanocrystals at different concentrations up to100 mg/ml for 24 h. The cells were washed thoroughly thrice with 1X PBS before viewing them under the microscope.

Observation of cells was done on inverted microscope Olympus CKX41, and the infrared excitation was generated by a laser diode JOLD-200-CABN-4A. Radiation was conducted by an optical fiber and infrared output flux was focused through a collimation system. Radiation wavelength emitted by the diode was 935.6 nm under optimal excitation of the phosphor (980 nm).

RESULTS AND DISCUSSIONS

X-ray diffraction

The X-ray diffraction indicated two phases of $NaYF_4:Er^{3+}:Yb^{3+}$ depending on chelating agent and on the synthesis conditions: cubic phase α-$NaYF_4$ (according to standard JCPDS No. 77-2042) and hexagonal phase β-$NaYF_4$ (according to standard JCPDS No. 28-1192 or standard No. 16-0344). For samples chelated with citrate or EDTA the hexagonal phase is dominant, while in the case of using citric acid as a chelating agent dominant phase is the cubic one (Figure 3).

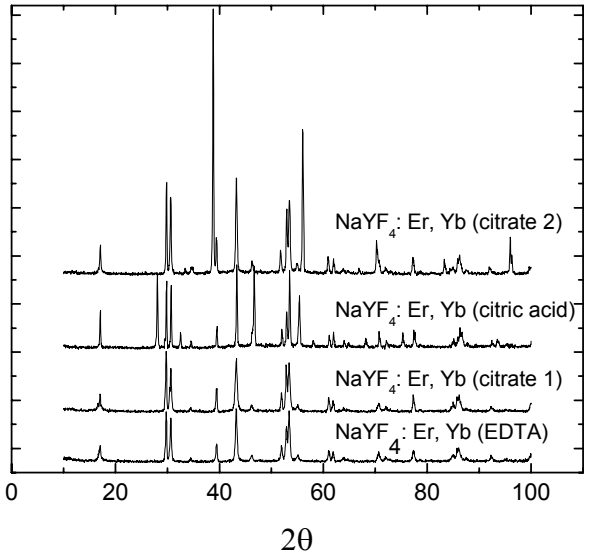

Fig. 3. XRD patterns obtained on four samples $NaYF_4$ using different chelating agents.

Fluorescence spectroscopy

Two samples obtained with different chelating agents were tested: one sample was obtained with citrate and the other sample was obtained using citric acid as chelating agent. Excitation was made by up-conversion to Yb^{3+} ($2F_{7/2} \rightarrow 2F_{5/2}$) and transferred to Er^{3+} (Figures 4 and 5).

The comparative analysis of the two spectra obtained reveal a changed ratio between band corresponding to the transition $4F_{9/2} \rightarrow 4I_{15/2}$ (red), depending on the chelating agent. For chelating with citrate, green band is dominant (Figure 4) while for the chelating of citric acid is more intense red band (Figure 5).

It shows a narrowing of the luminescence lines with increasing crystallite size.

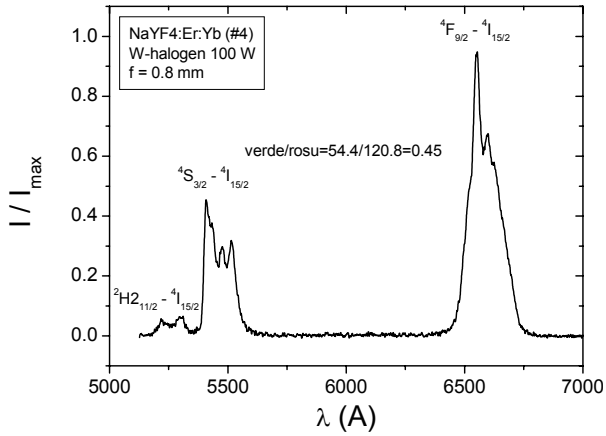

Fig. 4. Fluorescence spectrum by up-conversion excitation for $NaYF_4$ (chelating agent citric acid).

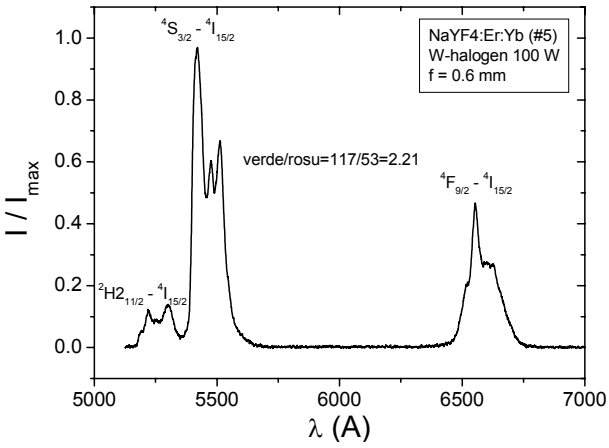

Fig. 5. Fluorescence spectrum by up-conversion excitation for $NaYF_4$ (chelating agent citrate).

Electronic microscopy

Following the analysis of samples by scanning electron microscopy we observe both the hexagonal and cubic phase, hence particle morphology are strongly dependent on synthesis conditions. Note the long, hexagonal prismatic crystals and presence of two microcrystal dimensional groups (Figure 6).

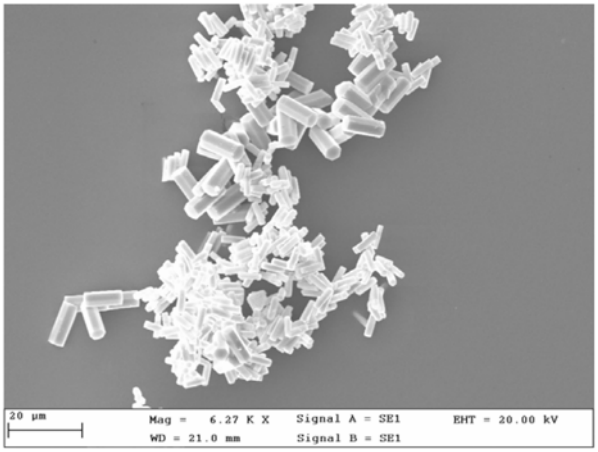

Fig. 6. Scanning image for $NaYF_4$ powder obtained by chelation with EDTA.

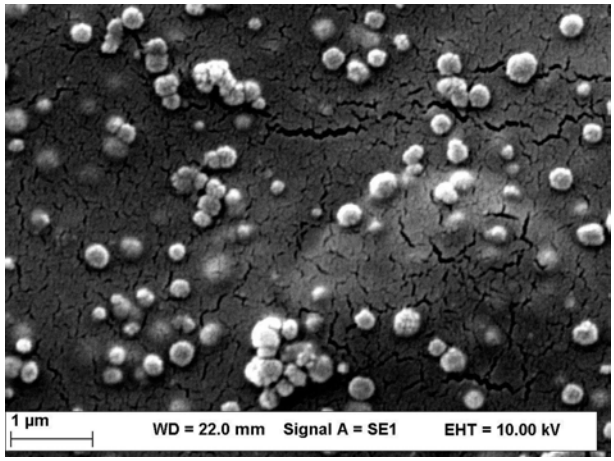

Fig. 8. Spherical particles of 100 nm order. After interaction with the electron beam cracks appear in the composite layer.

Characterization of coated nanocrystals

We found a very good stability of samples dispersed in polymer, independent of the electric charge of medium. Chitosan did not show a variation of stability depending on concentration (in the 0.1–5% concentrations domain). That reason does not justify the choice of optimal concentrations of Chitosan for relatively dilute dispersions: 1–2 mg / ml nanophosphores.

In Figure 7 is represented the plot which describe the variation in size of nanocrystals stabilized with different concentrations of Chitosan.

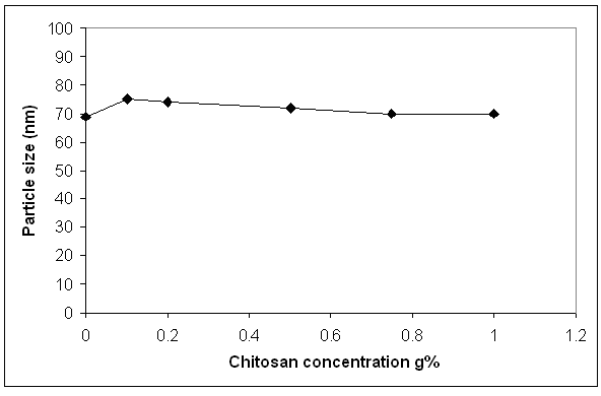

Fig.7. Variation of the size of nanocrystals depending on the concentration of Chitosan (dispersion 1mg/ml $NaYF_4$).

After analysis by electron microscopy we observed spherical particles with diameters of order 100 nm (Figure 8).

Cell application of coated $NaYF_4$

$NaYF_4$ nanoparticles coated with Chitosan were incubated in physiological conditions (at 37^0C, 5% CO_2 and humid atmosphere) of HEK 293 cell monolayer for 24 hours. Different concentrations of nanoparticles have been chosen: 1,5,10,15,25,50,100 µg/ml. After incubation, the samples were washed three times with 1XPBS to remove unbound particles. As a control sample was used a HEK 293 cell monolayer culture without addition of nanoparticles. Remaining cells were counted after incubation using a hemacytometer. The percentage of survival cells incubated with nanoparticles was obtained by reference to the control culture (Figure 9).

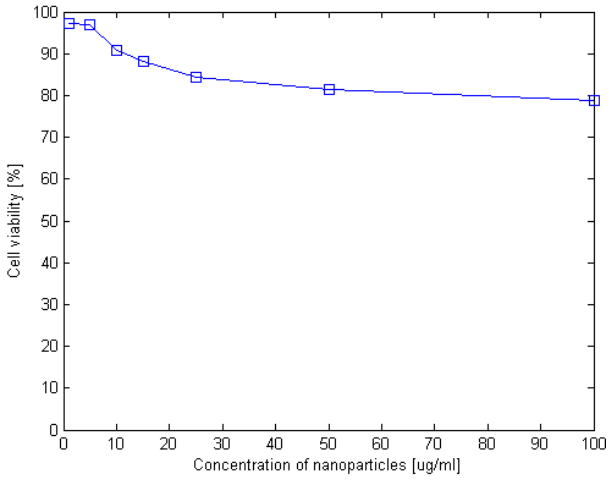

Fig. 9. The viability of HEK 293 cells depending on the dose of nanoparticles applied.

Nanocrystals show a good fluorescence (Figure 10), signal to background contrast is increased, due of low autofluorescence of background. Increasing the laser power gradually increases the efficiency of phosphorus without increasing noise simultaneously. Cells with coated nanoparticles showed a good survival rate after 24 hours of incubation, even at high concentrations of nanoparticles.

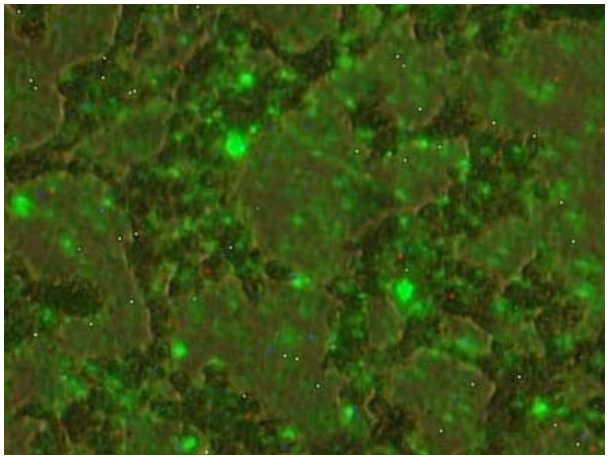

Fig. 10. HEK 293 cells after 24 hours of incubation with $NaYF_4$ 15 μg / ml.

CONCLUSIONS

Synthesis, characterization, coating and biocompatibilisation of fluoride up-conversion nanoparticles have been reported in this paper. Synthesis was performed by hydrothermal method. Characterization was performed by X-ray diffraction, fluorescence spectroscopy and electron microscopy. Coating and biofunctionalisation were made by dispersion in biopolymers like Chitosan. Experimental cell application and cell imaging of these up-converting nanoparticles have been investigated.

Due low cytotoxicity and good signal to background contrast, fluoride nanoparticles doped with lanthanide ions have promising application like biolabels in biomedicine.

Our future work focus on: better control of crystals size, biocompatibilization by biopolymers coating of particles (layer-by-layer method) and specific antibody binding for immunofluorescence and other applications.

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REFERENCES

1. Ho, Y.P. and K.W. Leong, *Quantum dot-based theranostics.* Nanoscale. **2**(1): p. 60–8.
2. Alcor, D., G. Gouzer, and A. Triller, *Single-particle tracking methods for the study of membrane receptors dynamics.* Eur J Neurosci, 2009. **30**(6): p. 987-97.
3. Stone, J., S. Jackson, and D. Wright, *Biological applications of gold nanorods.* Wiley Interdiscip Rev Nanomed Nanobiotechnol.
4. Arvizo, R., R. Bhattacharya, and P. Mukherjee, *Gold nanoparticles: opportunities and challenges in nanomedicine.* Expert Opin Drug Deliv. **7**(6): p. 753–63.
5. Rantanen, T., *et al.*, *Tandem dye acceptor used to enhance upconversion fluorescence resonance energy transfer in homogeneous assays.* Anal Chem, 2007. **79**(16): p. 6312–8.
6. Boyer, J.C., L.A. Cuccia, and J.A. Capobianco, *Synthesis of colloidal upconverting NaYF4: Er3+/Yb3+ and Tm3+/Yb3+ monodisperse nanocrystals.* Nano Lett, 2007. **7**(3): p. 847–52.
7. Xiong, L.Q., *et al.*, *Synthesis, characterization, and in vivo targeted imaging of amine-functionalized rare-earth up-converting nanophosphors.* Biomaterials, 2009. **30**(29): p. 5592–600.
8. Chatterjee, D.K. and Z. Yong, *Upconverting nanoparticles as nanotransducers for photodynamic therapy in cancer cells.* Nanomedicine (Lond), 2008. **3**(1): p. 73–82.
9. Couleaud, P., *et al.*, *Silica-based nanoparticles for photodynamic therapy applications.* Nanoscale. **2**(7): p. 1083–95.
10. Ungun, B., *et al.*, *Nanofabricated upconversion nanoparticles for photodynamic therapy.* Opt Express, 2009. **17**(1): p. 80–6.
11. Wang, F. and X. Liu, *Recent advances in the chemistry of lanthanide-doped upconversion nanocrystals.* Chem Soc Rev, 2009. **38**(4): p. 976–89.
12. Xue, X.J., *et al.*, *Synthesis and upconversion luminescence of NaYF4:Yb3+,Tm3+/TiO2 nanocrystal colloidal solution.* J Nanosci Nanotechnol. **10**(3): p. 2028–31.
13. Chatterjee, D.K., A.J. Rufaihah, and Y. Zhang, *Upconversion fluorescence imaging of cells and small animals using lanthanide doped nanocrystals.* Biomaterials, 2008. **29**(7): p. 937–43.
14. Jiang, S., *et al.*, *NIR-to-visible upconversion nanoparticles for fluorescent labeling and targeted delivery of siRNA.* Nanotechnology, 2009. **20**(15): p. 155101.
15. Zheng, K., *et al.*, *Upconversion luminescence properties of Yb3+, Gd3+, and Tm3+ co-doped NaYF4 microcrystals synthesized by the hydrothermal method.* J Nanosci Nanotechnol, 2010. **10**(3): p. 1920–3.
16. Sun, Y., K.E. Addison, and M.N. Ashfold, *Growth of arrays of Al-doped ZnO nanocones by pulsed laser deposition.* Nanotechnology, 2007. **18**(49): p. 495601.