VISUALIZATION OF Nd$^{3+}$-DOPED LaF$_3$ NANOPARTICLES FOR NEAR INFRARED BIOIMAGING VIA UPCONVERSION LUMINESCENCE AT MULTIPHOTON EXCITATION MICROSCOPY

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Abstract

Recent developments in the field of biophotonics facilitate the raise of interest to inorganic nanoparticles (NPs) doped with Nd$^{3+}$ ions, because of their near-infrared (NIR) absorption. These NPs are interesting bioimaging probes for deep tissue visualization, while they can also act as local thermometers in biological tissues. Despite the good possibilities for visualization of NPs with Nd$^{3+}$ ions in NIR spectral range, difficulties arise when studying the cellular uptake of these NPs using commercially available fluorescence microscopy systems, since the selection of suitable luminescence detectors is limited. However, Nd$^{3+}$ ions are able to convert NIR radiation into visible light, showing upconversion properties. In this paper we found optimal parameters to excite upconversion luminescence of Nd$^{3+}$:LaF$_3$ NPs in living cells and to compare the distribution of the NPs inside the cell culture of human macrophages THP-1 obtained by two methods. Firstly, by detecting the upconversion luminescence of the NPs in VIS under NIR multiphoton excitation using laser scanning confocal microscopy and secondly, using transmission electron microscopy.

Keywords: Nd$^{3+}$-doped nanoparticles, near-infrared, upconversion luminescence, multiphoton excitation, laser scanning confocal microscopy.


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Introduction

Optical imaging plays an important role in biomedical research and clinical diagnosis. Obtaining optical images from the depth of biological tissue is a serious scientific task, since biotissue is heterogeneous and has a strong scattering and absorption by various components. NIR spectral region (700-950 nm) is most suitable for excitation during in vivo visualization due to minimal absorption by biotissue.

In the last decade a lot of attention has been paid to the inorganic NPs containing rare-earth ions, as a promising class of nanomaterials for biophotonics. The advantages of rare-earth ions as luminescent labels include narrow-band radiation, a large spectral shift between the excitation and emission wavelengths, which is characteristic for the up- and down-conversion, long luminescence lifetime, high photostability of materials and low toxicity, minimization of autofluorescence of biological tissues by time resolved fluorescence spectroscopy and the greatest penetration depth when NPs are excited in the NIR spectral range [1]. Rare-earth ions can be excited through multiple electronic states, and, due to internal conversion, can show luminescence bands in a wide range of UV, VIS, and IR including the second biological window of optical transparency in short-wavelength infrared (SWIR) [2].

NPs doped with Nd3+ ions, are increasingly considered as an improvement for the upconversion system of ion pair, one of which is the sensitizer Yb3+, with the possibility of excitation by 800 nm [3-6]. The absorption cross section of Nd3+ at 808 nm is 1.2×10^-19cm^2, about ten times larger than that of Yb3+ at 980 nm [7], which is conducive to improve the efficiency of upconversion process through Nd-sensitizing [8]. Also, the Nd3+-containing NPs are perspective as bioimaging probes [9] and non-invasive contactless fluorescence temperature sensors [10]. The water colloids of Nd3+:LaF3 NPs synthesized by hydrothermal microwave treatment have already shown themselves to be excellent fluorescent agents for bioimaging in the NIR spectral range [11].

Despite the good possibilities for visualization of NPs with Nd3+ ions in NIR spectral range, difficulties arise when studying the cell uptake of these NPs using the methods of common VIS fluorescent microscopy that are associated with the selection of suitable detectors of luminescence [12-14]. Fortunately, the materials doped with Nd3+ ions can convert NIR radiation into VIS, while Nd3+ ions can simultaneously act as both sensitizers and activators of upconversion. In this case, the probability of emission in VIS is higher at high pump densities, when high-energy levels of most of the Nd3+ ions in one NP are populated [15].

The present work demonstrates the visualization of the intracellular distribution of Nd3+:LaF3 NPs by laser scanning confocal microscopy with multiphoton excitation in the NIR spectral range by pumping into the F^2^2^/2^ 4H_{9/2} (795 nm) and F^3^3^/2^ 4S_{3/2} (738 nm) levels of Nd3+ and detection of two-photon and three-photon upconversion luminescence in the VIS range.

Materials and methods

**Synthesis of Nd3+-doped lanthanum trifluoride NPs**

We use water based hydrothermal microwave treatment (HTMW) synthetic approaches to crystalline 4% Nd3+:LaF3 NPs. NPs with such Nd3+ doping concentration
is selected as having the highest NIR luminescence brightness. For the synthesis of LaF₃ NPs doped with 4% Nd³⁺ ions, 0.48 mM La(NO₃)₃, 6H₂O and 0.02 mM Nd(NO₃)₃·5H₂O were dissolved in 15 ml deionized water (dH₂O). The solution of rare-earth salts was added dropwise to the 5 mM NH₄F solution in 25 ml dH₂O under vigorous stirring. To improve the dispersibility of the obtained NPs, 1 g of biocompatible surfactant polyvinylpyrrolidone (PVP, average Mₚ ~55000, Aldrich) was added to the solutions. The surfactant was added to the rare-earth nitrates solutions before precipitation. The freshly precipitated gel was diluted with 10 ml dH₂O and left stirring for 15 min. The solution was transferred into a 100 ml Teflon autoclave and placed under microwave irradiation for 2 hours at 200°C using a microwave digestion laboratory device Speedwave Four (2.45 GHz, 1 kW maximum output power, Berghof, Germany). The resulting solution was cooled, centrifuged using a Heraeus Multifuge X1 (Thermo Fisher Scientific, USA) and washed several times with dH₂O. The resulting powder was redispersed in dH₂O.

Characterization of Nd³⁺:LaF₃ NPs

The X-Ray Diffraction (XRD) analysis of the Nd³⁺:LaF₃ NPs synthesized using HTMW treatment was performed as earlier (16). The NPs demonstrate pure and highly crystalline LaF₃ phase.

The morphology of the Nd³⁺:LaF₃ NPs was studied by means of high-resolution transmission electron microscopy (HR TEM) using the Titan 200 instrument (FEI, USA) with a field emission gun operating at 200 kV. The sample was prepared by dropping NPs colloidal solution onto a formvar or holey carbon coated copper (grid 3 mm in diameter) followed by the evaporation of the solvent.

Hydrodynamic sizes of Nd³⁺:LaF₃ NPs in dH₂O were determined by multiscatter spectrometer of dynamic light scattering Photocor Complex (Photocor, Russia). ζ-potential measurements were determined using a Zetasizer Nano ZS (Malvern Instruments, UK) analyzer in dH₂O at 25°C. All measurements were performed in triplicate.

The absorption spectra of Nd³⁺:LaF₃ NPs were recorded on a spectrophotometer U-3400 (Hitachi, Japan).

Confocal microscopy

Intracellular Nd³⁺:LaF₃ NPs distribution was studied using human monocytic cell line derived from an acute monocytic leukaemia patient (THP-1). THP-1 cells were cultured in Roswell Park Memorial Institute Medium (RPMI-1640) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. Cells were sub-cultured every seventh day. For confocal microscopy experiments, monocytic cells were differentiated into macrophage-like cells using Concanavalin A (ConA). Cells were seeded at a density of 100 000 cells/cm² on glass bottom dishes with cell culture medium containing 30 μg/ml ConA for three days. During this time, cells attach to the glass bottom and develop macrophage-like morphology. Next, macrophages were incubated with Nd³⁺:LaF₃ NPs (100 μg for 50 000 cells) for 2±72 hours.

For microscopy the cells were finally washed twice with pre-warmed phosphate buffered saline (PBS). For visualization of lysosomes the washed cells were incubated in PBS with 50 nM LysoTracker Green DND-26 (Molecular Probes) during 20 min at 37°C in 5% CO₂. The nuclei were stained in PBS with 2 nM Hoechst 33342 (Molecular Probes) during 10 min at 37°C in 5% CO₂. To acquire images a laser scanning microscope LSM-710-NLO (Zeiss, Germany) was used. The 63x oil Plan-Apochromat objective with numerical aperture (NA) of 1.4 was used.

The upconversion luminescence of Nd³⁺:LaF₃ NPs were excited with a pulse femtosecond Chameleon Ultra II laser system (Coherent, USA), tunable in the 690÷1060 nm range, 80 MHz pulse laser, 140 fs pulse width.

The power density produced by the scanning laser beam emerging from the objective lens in the object plane was calculated as follows. The size of this focusing laser spot, assuming uniform illumination, is a function of the excitation wavelength (λₑx) and the parameter NA of the objective:

\[ S_{\text{spotsize}} = \frac{1.22 \lambda_{\text{exc}}}{NA} \]

Thus, for a wavelength of 738 nm and a 63xOil objective with aperture NA = 1.4, the spot size was ~640 nm, for a wavelength of 795 nm ~690 nm. Accordingly, for the 1% laser power or 1 mW measured at the output of the objective with the LabMax-T0 laser power meter (Coherent, USA) the power densities are 0.313 MW/cm² (for 738 nm laser) and 0.263 MW/cm² (for 795 nm laser).

The dose of laser radiation with a single scan at speed 2.55 μs/pix was 0.80 J/cm² for 738 nm laser and 0.67 J/cm² for 795 nm laser, respectively.

The luminescence emission was detected by the 32 channel GaAsP detector in VIS spectral range 400÷750 nm. To discriminate between Nd³⁺ upconversion luminescence and fluorescence of LysoTrackerGreen DND-26 or Hoechst 33342, “Online Fingerprint” mode was used. For this purpose, the upconversion luminescence spectra of the Nd³⁺:LaF₃ NPs and fluorescence of LysoTrackerGreen DND-26 or Hoechst 33342 at the same two photon excitation were detected beforehand. As fluorescence of LysoTrackerGreen DND-26 or Hoechst 33342 has gently sloping broad line in the range 400–600 nm, and upconversion luminescence Nd³⁺ ions has characteristic comb of narrow peaks, then the total fluorescence corresponding to each pixel can be decomposed into the components (17).

The Nd³⁺:LaF₃ NPs upconversion luminescence intensity dependence from pump power

The Nd³⁺:LaF₃ NPs upconversion luminescence intensity dependence in VIS spectral range 400÷750 nm on 140 fs pulse width 80 MHz pulse laser pump power.
varying in 0.1÷5.5 MW/cm² at 738 and 795 nm wavelengths was measured using 32 channel GaAsP photomultiplier detector in LSM-710-NLO. The obtained spectral images were used to plot the intensity dependences of the upconversion luminescence for individual Nd³⁺ electronic transitions.

**Transmission electron microscopy (TEM)**

For TEM experiments, THP-1 cells were differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA). The cells were seeded at a density of 50 000 cells/cm² in cell culture medium in the presence of PMA at final concentration of 200 nM for differentiation for three days. After differentiation, macrophages were incubated with Nd³⁺:LaF₃ NPs (100 μg for 100 000 cells) for 48 hours.

Cells were then gently washed with pre-warmed PBS, trypsinized for 5 min at 37°C in 5% CO₂ and fixed with 4% methanol-free paraformaldehyde (PFA) overnight in the fridge to produce pellets. The pellets were then washed three times with double distilled water (ddH₂O) and 0.1 M cadoxylate buffer. For TEM contrast, the pellets were stained with 2% osmium tetroxide (OsO₄) and 1.5% potassium ferricyanide for 1 hour. Next, the pellets were washed with ddH₂O and gradually dehydrated using an ethanol gradient (20%, 40%, 60%, 70%, 80%, 90%, 95%, 100% (3x)) for 5 min. The pellets were then incubated with ddH₂O and gradually dehydrated using an ethanol gradient (20%, 40%, 60%, 70%, 80%, 90%, 95%, 100% (3x)) for 5 min. The pellets were then embedded to epoxy resin (EPON 812) according to procedures described in the manufacturer’s protocol. Resin blocks were cured in the oven for 72 hours, trimmed with a razor blade and sectioned in 100 nm sections using an ultramicrotome. Acquired thin sections were imaged using JEOL 2000FX at 80 kV.

**Results and discussion**

Nd³⁺-doped LaF₃ nanoparticles are synthesized via microwave assisted hydrothermal reaction. HR TEM results show that synthesized Nd³⁺:LaF₃ NPs are crystalline with elongated or hexagonal form and a size around 15 to 20 nm (Inset on the Fig. 2). The hydrodynamic size of the particles in the colloid is about 70 nm. The colloid remains stable more than 6 months without noticeable precipitation, because of PVP envelope for each NP. The ζ-potential of these NPs was 13.7±0.9 mV, which is similar to the results obtained for other PVP-functionalized nanoparticles.

The energy level scheme for the trivalent neodymium ions in a Nd³⁺ LaF₃ crystal is plotted on the base of literature data (Fig. 1) [18, 19].

The absorption spectra, NIR luminescence spectra of the Nd³⁺:LaF₃ NPs aqueous colloidal solution, obtained by excitation with 800 nm wavelength at 1 W/cm² of continuous wave (CW) laser, and upconversion luminescence obtained with 738 nm and 795 nm wavelengths of femtosecond laser, at 1 MW/cm² average power density are presented in Fig. 2.

At both types of excitation, 738 nm and 795 nm, the upconversion luminescence has almost equal intensity and shows different spectral bands of Nd³⁺ ions, followed by nonradiative relaxation to the 4F₃/2 metastable state. The laser excitation at 738 nm induces the 4I⁹/₂ → 4F⁷/₂, 4I⁷/₂ transition of Nd³⁺ ions, followed by nonradiative relaxation to the 4F₃/2 metastable state. At a sufficient power density, the next photon induced absorption transition from the excited 4F₃/2 state (excited state absorption process, ESA). In addition, there is a resonance cross-relaxation (4F₃/2 → 4I⁹/₂, 4I⁷/₂ transitions from this state, but it is not involved in the upconversion. Thus, the ²P₃/₂ level is excited, followed by nonradiative relaxation to the ²G₅/₂, ²K₃/₂ metastable state and then to the ²G₇/₂ state. When the third photon is absorbed, the levels higher than ²P₃/₂ are populated (Fig. 1).

The upconversion luminescence intensity Ivis in the VIS spectral range depends on the pumping power I_p as Ivis ∝ I_p^n where n is the number of NIR photons which are absorbed for emission of one photon in the VIS range [20].

However, in practice, deviations from this dependence can be observed. The slope of the dependence
of the upconversion luminescence intensity on the pump power is determined by the competition between relaxation processes and upconversion during the population of excited states of the acceptor. The slope depends on energy transfer to impurity ions, energy migration among donor ions, the inhomogeneous distribution of doping ions in the matrix, and temperature [21]. In our experiments, the dependence of the intensity of the transition 2P3/2 → 4I13/2 and 4D3/2 → 4I15/2 (420÷460 nm), 2P1/2 → 4I13/2 and 4G7/2, 2K₁₃/₂ → 4I₉/₂ (500÷550 nm), 4G5/2 → 4I₉/₂ and 4G7/2, 2K₁₃/₂ (560÷600 nm), 4D3/2 → 4F₃/2 and 4G7/2, 2K₁₃/₂ (620÷660 nm) on the incident pump power (in the range 0.5÷2 MW/cm²) gives values of 1.5, 1.5, 1.3 and 1.5 for n, respectively, which indicate two-photon upconversion processes (Fig. 3).

The exponent n, if smaller than unity, is related to deactivation processes. The upconversion efficiency for studied NPs is far from ideal, but using a pulsed laser for excitation makes it possible to produce images without visible cell structures damage. Similar orders of power density (35 kW/cm² ÷ 3.6 MW/cm²) of CW 730 nm laser was used to obtain two-photon, three-photon image and four-photon image of Nd³⁺-doped NPs [15]. Fast scanning speeds can cause artifacts in fluorescence imaging because of long lifetimes from Nd³⁺ NPs. The authors of this article noted the streaking artifacts at the scan speed more than 100 μs/pixel. We use scan speed 2.55 μs/pixel to reduce the laser heating effects on cells, and at such scan speed the streaking effect was not observed.

The images in the Fig. 4 are obtained at the simultaneous excitation of the LysotrackerGreen DND-26 or Hoechst 33342 and Nd³⁺:LaF₃ NPs by laser 795 nm with a power density 1 MW/cm². Confocal microscopy indicates cellular uptake of Nd³⁺:LaF₃ NPs. After incubation of live cells with NPs, it
Visualization of Nd³⁺:doped LaF₃ nanoparticles for near infrared bioimaging via upconversion luminescence at multiphoton excitation microscopy

is confirmed that NPs are located inside the cells. The fluorescence obtained from NPs coincides quite well with the signal from the stained lysosomes. However, it is also seen that some NPs are attached to the inner and outer membranes. Also, some of the bigger aggregates, not associated with the uptake, are visible. From the confocal microscopy images it is obvious that the Nd³⁺:LaF₃ NPs are located in macrophage-like THP-1 cells closer to the cytoplasm membrane, but also occur throughout the cytoplasm. In the cytoplasm, NPs formed clusters, feasibly, are occur in small spherical endosome-like organelles, but not all organelles containing NPs are stained as lysosomes. Some of the NPs are attached to the surface of the cells from the outside, and in addition, the NPs aggregates at the bottom of the Petri dish can be seen. Perhaps such a distribution of NPs is associated with their positive surface charge.

A more detailed Nd³⁺:LaF₃ NPs distribution within the THP-1 cell can be seen on the TEM image (Fig. 5). The images clearly indicate uptake of NPs into vesicles and their aggregation in the live cells. On the right image on the Fig. 5 protrusion of the plasma membrane for phagocytosis is observed. TEM indicate the presence of NPs in the endosomes and on the plasma membrane outside the cell.
Cellular uptake of NPs is strongly dependent on their surface charge. It has been shown that positively charged NPs are more permeable for cells [22]. Studied PVP envel-
oped Nd³⁺:LaF₃ NPs show a small positive charge. In general, hydrophilic sodium fluoride-based NPs doped with lanthanide ions have very low toxicity to granulocytes from the phagocytosis point of view [23]. Their uptake by macrophage-like cells THP-1 was observed from two hours to three days.

**Conclusion**

Accumulation of Nd³⁺:LaF₃ NPs in living cells was demonstrated by detecting upconversion luminescence with laser scanning confocal microscopy. Despite the low quantum yields of the VIS upconversion luminescence for the Nd³⁺:LaF₃ NPs in aqueous colloidal solutions, when pulse excitation is used, it is possible to reliably record the VIS signal of Nd³⁺:LaF₃ NPs upconversion luminescence at 1 MW/cm² power density, still not destroying the cells. The excitation at 738 nm, induces the 4I₉/₂→F₄/₂, 2S₃/₂ transition of Nd³⁺ ions and 795 nm, induces the 4I₉/₂→H₉/₂ gives almost equal the upconversion luminescence intensity from the Nd³⁺ bands.

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