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ABSTRACT

We report on the high-resolution deep-tissue imaging using novel water-dispersible upconversion nanoparticles (UCNPs) \( \beta \)-NaYF\(_4\) :Yb\(^{3+}\):Tm\(^{3+}\). Luminescence from the UCNP embedded into tissue-mimicking phantoms at the depth of 4 mm epi-illuminated with 975-nm laser radiation was detected. Fiber-optic detection shows 2-times better resolution compared with that obtained using CCD-based imaging modality. The conversion efficiency of upconversion particles and their cytotoxicity to HeLa cells were also investigated and reported.

Keywords: optical imaging, upconversion nanoparticles, luminescent nanoparticles, biophotonics, tissue-mimicking phantoms

1. INTRODUCTION

Imaging of biological tissue is a challenging task. Methods developed for this purpose can be divided into optical and others. Drawbacks of non-optical technique are either invasiveness (X-rays), very high costs (magnetic resonance imaging (MRI)) or insufficient resolution (acoustics). Optical methods offer such advantages as non-invasiveness, high resolution but relatively low (in comparison to the methods above) penetration depths. Optical modalities showing impressive (sub-micron) resolution such as femtosecond-laser-based optical coherence tomography (OCT) or nonlinear optical tomography (NLOT) operate predominantly in the superficial tissue area (up to 1 mm) and are quite expensive (several hundreds of thousands dollars) because of use of a femtosecond laser.

Optical diffuse tomography, an optical tissue imaging method, finds important practical applications in medicine [1]. Among the latest achievements worth mentioning are monitoring of physiological activity of tissue by measuring level of blood oxygenation [2], measurement of bilirubin levels in newborns [3], and many others. This method has such advantages as physiological functional informativity, instrumental simplicity, noninvasiveness, cost-effectiveness and is well accepted by patients. Imaging of tissue is achievable at a depth of several centimetres. However, spatial resolution at the millimeter level and non-specificity are the weaknesses of the technology, and additional techniques are required to overcome these limitations (an example of this approach is the photo-acoustic tomography), adding substantially more complexity to the instrumental part of the technology.

An obvious way to improve the localization of tissue to be diagnosed is labeling it by luminescent materials, known as exogenous luminophores. Examples of exogenous luminophores are fluorescent dyes such as fluorescein, widely used for visualization of blood flow e.g. in the eye fundus [4]. Spectrally selective registration reduces the background autofluorescence of tissue and improves the localization of the labeled tissue. However, strong absorption and scattering of the probing light as well as fluorescence in the visible spectral range and incomplete suppression of the autofluorescence by spectral methods make this approach a non-ideal solution.
A promising solution of labeling could be the use of anti-Stokes (upconversion) luminophores (phosphors) also known as upconversion nanoparticles (UCNPs). A serious hurdle for the use of these materials for deep optical sensing has long been their micron size, making the targeted delivery of these markers in vivo very difficult. However, technological breakthroughs of the recent years [5] have resulted in production of nano-sized water-dispersible UCNPs, making the above-mentioned approach exceptionally attractive. In UCNPs, a NaYF₄ matrix is doped with lanthanide ions e.g. ytterbium and erbium (or thulium) and determine its key (nonlinear) optical properties.

There are two outstanding properties of UCNPs in the context of deep optical tissue imaging applications in medicine: a) a spectrally well-positioned narrow absorption line (975-980 nm) in the so-called biological tissue transparency window, which provides deeper penetration of light with minimal absorption and scattering in living tissue and b) a long luminescence decay time, measured in milliseconds, allowing to implement simple optical schemes of delayed (time-gated) registration, which can almost completely eliminate the background autofluorescence from the surrounding tissue. Thus, one can expect unprecedentedly high sensitivity, localization and penetration depth of optical imaging. Absence of UCNPs photobleaching adds attractiveness to these materials and the optical modality.

Here we report on the deep-tissue (from 4 mm) imaging of UCNPs located under a layer of a tissue-mimicking phantom upon irradiation by 975-nm light. Toxicity of the commercial upconversion particles and their conversion efficiency are determined and reported.

2. SYNTHESIS OF UPCONVERSION NANOPARTICLES

Nanoparticles of programmable size and crystal phase were synthesized from a solution of sodium metal salts and oleic acid in an oxygen-free atmosphere at elevated temperatures. The mixture of YO₃ (0.78 mmol), Yb₂O₃ (0.2 mmol) and Tm₂O₃ (0.02 mmol) was refluxed in 70% trifluoroacetic acid (20 ml) for ca. 6 h. The resulting clear solution was cooled down to room temperature and the solvent was evaporated. The obtained residue was dried under vacuum at 0.1 torr for 3 h and thoroughly ground in an agate mortar until a fine homogeneous powder was produced. This powder was mixed with sodium trifluoroacetate (2 mmol), oleic acid (6 ml), and 1-octadecene (6 ml) in a three-neck flask equipped with a thermometer and a magnetic stirrer, and stirred at 100 °C under vacuum for 30 min. The degased and water-free mixture was gradually heated to 290 °C at a rate of 6 °C/min and kept at this temperature for 45 min under an argon atmosphere. The temperature was then raised to 310 °C for 70 min. Next, the solution was cooled, suspended in propanol-2 (130 ml), and centrifuged at 6000 rpm for 30 min (Z206A centrifuge, Hermle, Germany). The as-synthesized particles were washed with absolute ethanol four times and dried. The particles were then dissolved in chloroform (10 ml), precipitated with propanol-2 (50 ml), and centrifuged at 4000 rpm for 10 min twice. The final product was dried at room temperature [6]. All chemicals were purchased from Sigma-Aldrich (Germany).

Fig. 1. TEM image (a) and size distribution histogram (b) of the as-synthesized upconversion particles.
3. PHANTOM FABRICATION

For testing of the synthesized UCNPs, homogeneous tissue-mimicking phantoms were fabricated in-house from polyvinyl chloride plastisol, PVCP (M-F Manufacturing Co., USA) according to the procedure recently developed by the authors [7, 8]. PVCP is a mixture of an oily liquid and microscopic plastic particles. TiO₂ nanoparticles with a mean size of 510 nm (Fig. 2) (Sigma-Aldrich, Germany) were added to simulate phantom scattering properties: concentration of 3 mg/dl results in a scattering coefficient \( \mu_s = 5 \text{ mm}^{-1} \) and anisotropy factor \( g = 0.57 \) at 975-nm wavelength. After baking for 40 min in the oven heated to 200 °C and subsequent cooling, the phantoms become solid. They are stored between glass slides at room temperature and humidity. The phantom properties were retrieved from measurements using a spectrophotometric system (Optronic laboratories, USA) equipped with integrating spheres: total transmittance, total reflectance, and collimated transmittance for 1-mm-thick samples were used as an input for a program implementing an inverse adding-doubling method [9].

![Fig. 2. SEM image (a) and size distribution (b) of the used TiO₂ powders.](image)

4. IMAGING: FIBER-OPTIC DETECTION VS. CCD

Two imaging modalities were compared in terms of their spatial resolution performance: a fiber-optic-based setup and an option with a CCD camera (Fig. 3). A continuous-wave diode laser emitting at 975 nm excited a luminescent label consisted of UCNPs located under a 4-mm-thick tissue-mimicking phantom. The 100-μm-thick label with a diameter of 2 mm was fabricated by mixing UCNPs with a transparent (within 400-1000 nm spectral range) monomer polymerized after UV treatment.

![Fig. 3. Two schematic diagrams of the imaging modalities: a) using a fiber-optic probe, b) CCD camera based full field imaging. Adapted from [10].](image)
The home-made fiber bundle comprised one illuminating (central) fiber and eight (surrounding) detecting fibers; fiber diameters were 600 μm with numerical apertures NA = 0.22. Emitted luminescence (in the vicinity of 800 nm) was detected by a spectrophotometer (Ocean Optics HR 4000), with the excitation radiation cut. The disadvantage of the fiber modality is that it requires scanning over a sample, while CCD camera captures an entire pixelated image (within its field of view) in parallel during a single exposure. Figure 4 shows the experimental results: accuracy of fiber detection is twice better than that of CCD camera (0.45 cm vs. 1 cm).

![Normalized light intensity detected by either the CCD or the fiber bundle on the surface of the phantom. UCNP label is located 4 mm under the tissue-mimicking phantom. The horizontal axis indicates the shift of the detector position relative to the location of the UCNP label. Adapted from [10].](image)

**5. CHARACTERISATION OF COMMERCIAL PHOSPHORS**

**5.1 Optical study**

Due to very small amount of above-mentioned home-made UCNPS, we performed necessary characterisation of UCNP key properties using commercially available luminophores PTIR660-UF (Phosphor Technology, UK), instead. Their morphology and size distribution are depicted in Fig. 5. As it is seen, these particle size range exceeds the nanoparticle size range.

![SEM image (a) and size distribution (b) of commercial luminescent upconversion particles.](image)
Luminescence spectra were recorded using a home-made setup (Fig. 6(a)). It comprised a CW diode laser PL980P330J (Thorlabs, USA) with maximum emission power of 330 mW at 975 nm (bandwidth 1.5 nm), coupled into a single-mode fiber (SMF: HI1060) with 1.5-mm Teflon loose tube jacket and FC/AC connector. A long-pass dichroic mirror DMLP900 (Thorlabs, USA) transmitting laser light, a set of objectives (40×, NA = 0.30; 40×, NA = 0.65) and a band-pass interference filter FES0550 (470-750 nm) transmitting luminescence but cutting off pump light and a PixeLINK PL-B741F CMOS camera (1280x1024) with a Kenko 12-mm F1.4 objective were assembled in a cage cube. The camera will later be used for deep-tissue imaging. Luminescence spectra (Fig. 6(b)) were detected by a spectrophotometer Wave Star V (Ophir Optronics, Israel) operating in 570-1070-nm spectral range fiber-coupled to an integrating sphere. The acquired spectrum of the investigated luminophores was primarily located in the spectral range of 640-700 nm which marginally fell into the biological tissue transparency window hence beneficial for tissue imaging.

An important characteristic of the luminescent particles is conversion efficacy defined as a ratio of the emitted and absorbed power. For such measurements, commercial powders were sandwiched between two glass slides and sealed with an adhesive tape. The sample was placed on the top flange of an integrating sphere for collecting luminescence and was irradiated through a 40× objective lens. The diameter of the focal spot was estimated from sizes of holes burnt in a polymethyl methacrylate (PMMA) and was measured to be 10 μm. A spectrophotometer AVANTES was calibrated using 975-nm and 680-nm diode lasers to directly calculate power from spectrophotometric measurements. It was assumed that a half of the luminescence was collected by the integrating sphere, since the sample was located on top of it. The power of the luminescence collected by the integrating sphere exhibited saturation at the higher pump (excitation) intensity (Fig. 7(a)). The conversion efficacy varied within 0.12%-0.16% depending on the pump intensity, with decreasing trend (Fig. 7(b)).
5.2 Toxicity study

Potential utility of the luminophores for biomedical applications critically depends on whether they are not hazardous to biological organisms, which is conventionally tested by running viability of cells at various concentrations of the luminophores. In particular, we report on the cytotoxicity test using the upconversion luminophores using HeLa cells.

HeLa cells (Biocenter Oulu, Finland) were kept at 37 °C and 5% CO₂ in complete DMEM medium supplemented with 10% bovine serum, 1% penicillin-streptomycin (all from Sigma, USA).

For determination of the limits of the investigated luminophore concentrations, the cells were kept in 6-well plates and incubated overnight with the luminophores at concentrations of 30 μg/ml, 3 μg/ml, 0.3 μg/ml, 0.03 μg/ml and 0.003 μg/ml. After incubation, the samples were investigated in phase-contrast regime by a microscope (Fig. 8). The concentration of 30 μg/ml was found causing cell apoptosis, probably due to induced stress not related to the intrinsic cytotoxicity of our sample. In contrast, the concentrations of up to 0.03 μg/ml did not affect cell viability. Thus, more thorough analysis of particles toxicity was carried out at the luminophore concentrations of 0.3-3 μg/ml.

Fig. 8. Microscopy pictures of the cells samples, overnight incubated with luminophores at various concentrations. Small light dots indicate live cells.
For the MTT assay, the cells were kept in 96-well plates and incubated with the luminophores at concentrations of 0.37 μg/ml, 0.75 μg/ml, 1.5 μg/ml and 3 μg/ml overnight, four-fold. After incubation, the MTT solution (Sigma, USA) in final concentration of 0.5 μg/ml was added, and the cells were incubated for 4 h in the dark at 37 °C. Then, the supernatant was removed; 200 μl DMSO (ACS grade DMSO, Amresco, USA) was added to dissolve the formed formazan crystals. The samples were centrifuged at 12000g for 5 min in 1.5-ml Eppendorf flasks (Eppendorf Minispin, Germany), and supernatant was transferred into 96-well plates. Absorbance values at 492 nm were registered by a microplate reader Power Wave (Bio-Tek Instruments, USA).

As it can be seen from Fig. 9, the viability of the cells (respiratory activity) after treatment with the luminophores at the concentrations ranging from 0.37 to 3 μg/ml decreased with the increase of particle concentration, from 60% to 26% (with the control corresponding to 100%).

![Fig. 9. Cell viability revealed by an MTT assay for HeLa cells at different concentrations of luminophores.](image)

For the fluorescent test with propidium iodide (PI), the cells were maintained in 6-well plates and incubated with luminophores at concentrations of 0.37 μg/ml, 0.75 μg/ml, 1.5 μg/ml and 3 μg/ml overnight. After incubation, PI solution (Sigma, USA) in final concentration of 3 μg/ml was added, and cells were incubated for 20 min in the dark at 37 °C. Fluorescence of the samples was registered at 600-650 nm by Olympus IX81 epi-fluorescent inverted microscope equipped with a filter cube TRITC 41002c (Chroma).

![Fig. 10. A histogram of the viability of HeLa cells treated with luminophores estimated by the PI-test.](image)
Figure 10 also indicates decrease of live cells with increase of particle concentration: the luminophores in concentration of 0.37 μg/ml decrease the number of live cells from 91% (control) to 67%, while for 3 μg/ml concentration it results in 34%.

Summarizing, the luminophores are cytotoxic at μg/ml concentrations and decrease cells viability was detected versus their concentration and found to be proportional. Both modalities, the MTT assay and propidium-iodide-based test showed similar results.

6. CONCLUSION

We reported on the investigations of the upconversion particle properties, and optical system imaging resolution properties in luminophore-assisted imaging. We demonstrated that the fiber-optic scanning modality exhibited the improved spatial resolution in comparison with that of the CCD-based full field modality (0.45 cm vs. 1 cm for 4-mm imbedding depth of the luminescent target). Micro-sized luminophores were also characterized by measuring their conversion efficacy (0.12%-0.16%) and their cytotoxicity properties, with the concentration of 0.37 μg/ml found to cause cell apoptosis.

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Introduction

The European Conference on Biomedical Optics (ECBO), part of World of Photonics Congress in Europe, took place in Munich, Germany on 12-16 May 2013. Its ~500 attendees were treated to five days of talks, posters, technical exhibits and special events, with ample opportunities for discussing biophotonics science, making personal and scientific connections, and finding out the latest trends in our rapidly evolving interdisciplinary field. The ECBO program was organized into nine thematic sub-conferences, and the chairs of each solicited manuscript summaries from their respective oral and poster presenters. These were compiled into the 2013 ECBO proceedings volumes, with the one you are currently holding in your hands containing selected manuscripts from the Novel Biophotonic Techniques and Applications (NBTA) sub-conference.

The NBTA presentations were thematically diverse, as indicated by the session titles – Novel Tissue Assessments I and II, Polarization and Coherence, Shedding Light on Cells, and Photons, Phonons, and Mechanics. As you read this volume, you will encounter interesting biophotonics studies using speckle, SERS, FRET and other novel methods for advanced tissue assessment. Work on nanoparticle constructs and surface-plasmon resonances were also well represented at NBTA. Such breadth and variety nicely illustrates the diversity of research in this exciting field. We hope that as you read this and the other proceedings volumes of the EBCO 2013, you will be inspired to (continue to) actively contribute to biophotonics research!

Alex Vitkin
Arjen Amelink