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NIR photo-driven upconversion in $NaYF_4$:Yb,Er/PLGA particles for *in vitro* bioimaging of cancer cells

Lidija Mancic[a,*], Aleksandra Djukic-Vukovic[b], Ivana Dinic[c], Marko G. Nikolic[d], Mihailo D. Rabasovic[d], Aleksandar J. Krmpot[d], Antonio M.L.M. Costa[e], Dijana Trisic[f], Milos Lazarevic[g], Ljiljana Mojovic[b], Olivera Milosevic[a]

[a] Institute of Technical Sciences of the Serbian Academy of Sciences and Arts, Belgrade, Serbia
[b] Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Serbia
[c] Innovation Center of the Faculty of Chemistry, University of Belgrade, Serbia
[d] Photonic Center, Institute of Physics Belgrade, University of Belgrade, Zemun, Belgrade, Serbia
[e] Department of Chemical and Materials Engineering, Pontifical Catholic University of Rio de Janeiro, Rio de Janeiro, Brazil
[f] Clinic for Pediatric and Preventive Dentistry, School of Dental Medicine, University of Belgrade, Serbia
[g] Institute of Human Genetics, School of Dental Medicine, University of Belgrade, Serbia

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ABSTRACT

Lanthanide-doped fluoride up-converting nanoparticles (UCNPs) represent the new class of imaging contrast agents which hold great potential for overcoming existing problems associated with traditionally used dyes, proteins and quantum dots. In this study, a new kind of hybrid $NaYF_4$:Yb,Er/PLGA nanoparticles for efficient biolabeling were prepared through one-pot solvothermal synthesis route. Morphological and structural characteristics of the as-designed particles were obtained using X-ray powder diffraction (XRPD), scanning and transmission electron microscopy (SEM/TEM), energy dispersive spectroscopy (EDS), Fourier transform infrared (FTIR) and photoluminescence (PL) spectroscopy, while their cytotoxicity as well as up-conversion (UC) labeling capability were tested *in vitro* toward human gingival cells (HGC) and oral squamous cell carcinoma (OSCC). The results revealed coexistence of the cubic (*Fm*-3*m*) and hexagonal ($P6_3/m$) phase in spherical and irregularly shaped nanoparticles, respectively. PLGA [Poly(lactic-*co*-glycolic acid)] ligands attached at the surface of UCNPs particles provide their enhanced cellular uptake and enable high-quality cells imaging through a near-infrared (NIR) laser scanning microscopy (λ_{ex} = 980 nm). Moreover, the fact that NaYF4:Yb,Er/PLGA UCNPs show low cytotoxicity against HGC over the whole concentration range (10–50 μg/mL) while a dose dependent viability of OSCC is obtained indicates that these might be a promising candidates for targeted cancer cell therapy.

1. Introduction

Head and neck cancers represent the sixth most common cancer worldwide, with the highest incidence rates in Melanesia, South-Central Asia and Central and Eastern Europe [1]. Among them, oral squamous cell carcinoma (OSCC) is the most common malignant epithelial neoplasm affecting the oral cavity. The incidence of oral cavity cancer appearance is higher in males and as the major risk factors are considered smoking, alcohol use, smokeless tobacco use and human papillomavirus (HPV). Early stages of disease are asymptomatic and very similar to other mucosal diseases and if diagnosed at advanced stages result with a 5-year survival rate of around 50%. Fatal outcomes are mostly caused by local recurrence and neck lymph node metastasis [2]. Therefore, advancements in both, early diagnosis and therapy, are necessary and most likely will come from innovative non-invasive selective optical techniques [3]. Among the various optical diagnostic methods, fluorescence imaging is of immense importance since that provides accurate visualization of the molecular and functional processes in the human body [4].

Moreover, with the current progress in designing of the new hybrid multifunctional lanthanide-doped up-conversion nanoparticles (UCNPs) whose excitation/emission falls into the biological tissue transparency window, superior optical diagnostic and targeted drug delivery is expectable in the near future [5, 6]. Due to efficient two-phonon excitation and the large anti-Stocks shift UCNPs are able to emit visible or UV photons under excitation by near-infrared (NIR) light, to achieve deeper tissue penetration, and to exhibit higher photochemical stability in comparison with a traditionally used fluorophores [7]. The

* Corresponding author.
E-mail address: lidija.mancic@itn.sanu.ac.rs (L. Mancic).

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effectiveness of these materials is principally dependent on the crystal structure and phonon energy of a host matrix, as well as, on the choice and concentration of lanthanide dopants. Efficient lanthanide pairs which can easily upconvert low energy photons into higher ones comprise ytterbium (Yb^{3+}) as a sensitizer and erbium (Er^{3+}), thulium (Tm^{3+}) or holmium (Ho^{3+}) as activator. The rich energy levels and long-lived intermediate excited states of activators provide various energy transfer pathways for UC emissions in visible spectra. Following initial Yb^{3+} excitation by 980 nm laser, energy transfer upconversion (ETU), excited state absorption (ESA), photon avalanche (PA), cooperative energy transfer (CET) and cross-relaxation (CR) processes take place determining resulting fluorescence efficiency of UCNPs [8]. Among many compounds, fluoride based cubic or hexagonal crystal lattices of $NaYF_4$ phase have been extensively studied as the most efficient hosts because of their low phonon energy (*i.e.*, 350 cm^{-1}), which minimize harmful non-radiative relaxations, and high optical transparence necessary for migration of NIR photons. In particular, hexagonal phase exhibits approximately one order of magnitude higher UC emission in comparison to cubic counterpart since it possess a higher degree of asymmetry, multisite occupation with lanthanide dopant ions and shorter distance among them. Since that particle size also affects the luminescence efficiency (higher energy transfer loss is attributed to the higher density of the surface defects) different synthesis strategies were developed to highlight tailored performances of specially designed core-shell, hybrid and composite UCNPs for cell imaging and tracking, drug delivery, photodynamic therapy and target recognition of biospecies [9, 10]. Although showing remarkable characteristics during both *in vitro* and *in vivo* investigations studied structures were usually obtain through complex multi-steps procedure which involves decomposition of organometallic compounds in oleic acid and subsequent ligand exchange (or oxidation, coating, intercalation, *etc.*). Usage of toxic and hazardous substances during synthesis raised deep concerns regarding potential toxicity of synthesized UCNPs [11–13]. Recently, it was shown that such obtained UCNPs could regain their cytotoxicity upon interaction with cells if weakly coordinated surface groups are used to render them biocompatible after synthesis [14]. Thus, the safe biological application of UCNPs implies establishing of facile and reliable procedure which would minimize the usage of toxic solvents and provide hydrophilic reactive surface *in situ* toward enhanced conjugation of proteins and drugs. One-pot polymer assisted hydrothermal approach, originally reported by Wang et al. [15] is a simple procedure which enables direct synthesis of water-dispersible UCNPs. To date, it was used for *in situ* functionalization of UCNPs surface with a wide range of biocompatible capping ligands, including carboxylic and amine/imine groups of polymers, such as: polyethylenimine (PEI), polyacrylic acid (PAA), polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) [16–18]. Among others, we have also shown that PVP-, PEG- and EDTA-assisted hydro/solvothermal route, performed in a controlled manner, led to the generation of hydrophilic/biocompatible upconverting particles with a different shape (spherical, rod, prisms, octahedron and desert-rose) [19, 20]. Here, we reported for the first time usages of poly(lactic-*co*-glycolic acid) (PLGA) *during* solvothermal synthesis of $NaYF_4$:Yb/Er nanoparticles. PLGA, approved by the United States Food and Drug Administration and European Medicine Agency for pharmaceutical application and human use, is one of the most widely utilized biodegradable polymer with a minimal toxicity associated with its use as scaffolds for tissue engineering or for delivery of macromolecular therapeutics [21–23]. For example, PLGA nanoparticles have been proposed as a delivery medium of an amphiphilic Gd^{3+} complex developed for the need of high sensitive magnetic resonance imaging (MRI) and imaging guided drug delivery applications [24]. Similarly, it was shown that pH-responsive PLGA(UCNPs/doxorubicin hydrochloride) nanocapsules obtained through self-assembly strategy could act as T1-weighted contrast agents MRI, cell imaging label and effective chemotherapy drug delivery system [25]. Due to fact that PLGA comprises both, hydrophilic and hydrophobic moiety, it was usually used in combination with PEG in the form of amphiphilic block-copolymer for posterior UCNPs coating *via* flash nanoprecipitation. PEG-PLGA layer formed in such way provides excellent UCNPs colloidal stability in deionized water, buffers and serum media, but in the case of a thicker layer formation decrease of the upconversion luminescence was observed [26]. Recently, fabrication of a multifunctional nanovector for simultaneous gene delivery and real-time intracellular tracking based on UCNPs modified by positively charged amphiphilic polymer and PEG–PLGA copolymer was reported [27]. In accordance to the literature, PLGA solely was used, up to date, only for coating of the bare UCNPs through intercalation process, but obtained biocompatible particles sized around 250 nm exhibited negligible cellular uptake and mild cytotoxicity (cellular viability of about 80%) when applied at concentration of 62.5 μg/mL to the human keratinocyte and fibroblast cells [28].

In this study, a new kind of hybrid NaYF4:Yb,Er/PLGA nanoparticles for efficient cell labeling was prepared through one-pot solvothermal synthesis using non-toxic reagents. Hence, PLGA functional groups attached *in situ* to the UCNPs surface ensure excellent biocompatibility without compromising upconverting process. Furthermore, NaYF4:Yb,Er/PLGA UCNPs demonstrated dose-dependent cytotoxicity against oral squamous cell carcinoma (OSCC) without damaging healthy non-cancerous human gingival cells (HGC), so could be considered as promising and reasonably safe candidate for theranostic.

2. Materials and methods

2.1. Synthesis and characterization of $NaYF_4$:Yb,Er/PLGA UCNPs

All of the chemicals used for PLGA mediated solvothermal synthesis of $NaY_{0.8}Yb_{0.17}Er_{0.03}F_4$ were purchased from Sigma-Aldrich. Deionized water was used throughout. Defined stoichiometric amounts of rare earth nitrates (5 mmol in total) were dissolved in 15 ml of deionized water and then mixed with 5 ml of NaF solution (1.75-fold excess) and 0.1 g of PLGA (lactide:glycolide, 75:25; Mr. 66,000–107,000) dissolved in 40 ml of acetone. Obtained mixture was stirred for 15 min, transferred to 100 ml Teflon lined autoclave and sealed. Since that PLGA is thermally stable until 250 °C under atmospheric pressure [29] synthesis was carried out at twice lower temperature of 120 °C with a continual stirring (100 rpm) for 24 h. After cooling, the as-prepared $NaYF_4$:Yb,Er/PLGA UCNPs were washed with acetone by centrifuging (8000 rpm, 10 min) and dried at 80 °C for 3 h.

The $NaYF_4$:Yb,Er/PLGA UCNPs were characterized by the X-ray powder diffraction (XRPD) using Bruker D8 Discovery equipped with a Cu-Kα source (λ = 1.5406 Å). The pattern was recorded with a step scan of 0.02° and accounting time of 5 s per step. Powders microstructural data were acquired through combined La Bail and Rietveld refinement in Topas 4.2 software. For cubic α- and hexagonal β-$NaYF_4$ phase refinements were carried out in *Fm*-3*m* (No. 225) and *P*63/*m* (No. 176) space groups, respectively. The morphological features of the as-prepared particles were investigated by means of both, scanning and transmission electron microscopy (JEOL JSM-6701F SEM and JEOL JEM 2010 TEM operating at 200 kV at phase contrast and selected area electron diffraction (SAED) modes) coupled with energy dispersive spectroscopy (EDS). For the aforementioned analyses the particles were suspended in isopropyl alcohol and dropped directly on the stub (for SEM) or on lacey carbon film supported on a Cu grid after 20 min sonication (for TEM). The SemAfore 5.21 JEOL software was used to construct particle size distribution diagrams, while Fourier processing of high resolution TEM images was performed with Digital Micrograph 3.7.4 Gatan Inc. software. Detection of the PLGA ligands on the particles surface was done by Fourier transform infrared spectroscopy (FTIR) using Thermo Scientific Nicolet 6700 spectrophotometer (Thermo Fisher Scientific) with a Smart iTR Diamond Attenuated Total Reflectance accessory. Spectra were recorded using typically 128 scans at the resolution of 4 cm^{-1}. Dynamic light scattering measurements of