Comparison of Two Strategies for the Synthesis of Upconverting Nanoparticles as Biological labels

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Abstracts
Oleic acid-coated upconverting nanoparticles were prepared by hydrothermal and solvothermal synthesis. The properties of as-prepared NaYF₄:Yb,Er nanoparticles were characterized, including transmission electron microscope, X-ray diffraction pattern and photoluminescence spectrum. The effects of reaction conditions (reaction time and temperature) and the concentration of rare earth dopants have been investigated in each strategy. Optical properties of upconverting nanoparticles synthesized by two strategies were compared as well. For biological application, the surface ligands were exchanged from oleic acid to hexanedioic acid with carboxyl-functionalized groups. The results indicated upconverting nanospheres (45 ± 2 nm) and nanoplates (150 ± 5 nm) prepared by solvothermal synthesis had higher photoluminescence intensity with uniform size distribution, whereas hydrothermal synthesis produced small upconverting nanocubes (8-20 nm). Upconverting nanoparticles in hydrothermal synthesis showed better optical properties while increasing the reaction time and decreasing the molar ratio of water. The ratio of green and red emissions of NaYF₄:Yb,Er nanoparticles could be adjusted through changing the concentration of rare earth dopants. Furthermore, hexanedioic acid mediated ligand-exchange process transferred NaYF₄ nanoparticles from hydrophobic to hydrophilic with great potential as biological labels.

Key Words: upconverting nanoparticles; hydrothermal synthesis; solvothermal synthesis; ligand-exchange; comparison

1. Introduction
Fluorescence-based detection is widely used in biological labeling and imaging. Conventional fluorescent labeling materials such as organic dyes and semiconductor quantum dots (QDs) show visible and near-infrared (NIR) fluorescence under excitation of high-energy light. Organic dyes are easily accessible and versatile to use but they do have considerable disadvantages. Commonly, organic dyes are susceptible to photobleaching or degradation in certain conditions. In recent years nanoparticle technology has become a research focus as its fundamental and practical importance is well known, especially in the case of fluorescent materials. For example, QDs with small diameter show brighter and more stable
fluorescence than common organic dyes. Additionally, their emission wavelength can be tuned from visible to NIR light by variation of their diameter [1,2]. However, the application of QDs still has many limitations, especially the toxicity of their heavy metal components while \textit{in vivo} applications. On the other hand, UV excitation of down-conversion materials will cause severe damage to biological tissues and strong background fluorescence as \textit{in vivo} biolabels. Compared to the above down-conversion fluorescent materials, upconverting nanoparticles (UCNs), which emit higher-energy photos after absorbing lower-energy NIR photos, have many advantages including narrow emission, deep tissue penetration, high chemical and optical stability, less photodamage to living organisms, weak autofluorescence from cells and tissues, low background fluorescence and high sensitivity for detection.

In the past decade, upconversion in lanthanide-doped materials have been proposed to be more suitable for ideal biolabels. The upconversion process is mainly located in the lattice of the host materials in which the lanthanide ion is embedded in. To the best of our knowledge, among the reported upconversion materials, NaYF\textsubscript{4} is one of the most efficient host materials for \textit{in vivo} upconversion fluorescence. There are two phases of the crystal structure of NaYF\textsubscript{4} cubic phase and hexagonal phase. Upconversion efficiency of the pure hexagonal phase NaYF\textsubscript{4} is much higher than cubic phase NaYF\textsubscript{4}. The emission wavelength of the UCNs is not size-dependent as it is for QDs and multicolor emission can easily be accomplished by varying host crystal and rare earth dopants. Many studies have been performed to obtain NaYF\textsubscript{4} nanoparticles (nanocubics, nanospheres, nanoplates, nanorods, etc) via hydrothermal synthesis [3,4], solvothermal synthesis[5], thermal decomposition[6,7], sol-gel and homogenous precipitation. Among these routes, sol-gel and homogenous precipitation are low temperature methods. However, the UCNs synthesized using these two methods always have low crystallinity and require post-treatment or annealing at high temperature up to 1000 \textdegree{C}. The powder products with large size cannot be easily redispersed in the solvent which make them unsuitable for biological application. Thermal decomposition of mixed trifluoroacetates is an effective method to synthesize high quality cubic phase and hexagonal phase NaYF\textsubscript{4}, but pyrolysis of trifloroacetates produces very toxic fluorinated and oxyfluorinated carbon species. Thus, the experimental procedure must be carried out carefully in the ventilation. Moreover, a liquid-solid solvothermal reaction has been reported to produce oil-dispersed hexagonal phase NaYF\textsubscript{4} whereas the excessive NaF reactants are difficult to be removed. Very recently, Zhang et al. [5] proposed a efficient and user-friendly solothermal method for the synthesis of oleic acid (OA)-coated UCNs with controllable shape and upconversion fluorescence. Researchers also produced hydrophobic and hydrophilic UCNs through hydrothermal synthesis with proper photoluminescence intensity (PL) for biological application. Both solvothermal and hydrothermal synthesis can obtain UCNs with small size (<50 nm), high lumiscence and good redispersibility.

Although recent advances in the synthetic methods for UCNs, the UCNs with hydrophobic ligands on their surface still cannot be used directly in biological applications due to low solubility in water and unfavorable surface properties. Therefore, it is prerequisite to prepare water-soluble UCNs bearing functional groups (such as –COOH, –NH\textsubscript{2} or –SH) on their surface to conjugate with biomolecules, which will promote the development of UCN-based biolables. Three strategies have been developed for transferring hydrophobic OA-coated UCNs to hydrophilic UCNs with functionalized groups on the surface for bio-conjugation. One strategy is ligand-exchange processes which replace the original organic ligands with hydrophilic ones, such as hexanedioic acid (HDA) [8], citrate [9], thioglycollic acid (TGA) [10] and so on. The procedure has usually been performed at high reaction temperature, but it has less adverse effect on the quality of the UCNs. The second strategy is based on the encapsulation of hydrophobic UCNs with amphiphilic copolymer (such as polyvinylpyrrolidone, poly(acrylic
acid), polyethylene glycol and polyethylenimine) or silica [11-13]. However, the processes are complicated and not easy to control the reaction precisely. The third strategy involves converting surface OA ligands through certain chemical reactions like Lemieux-von Rudloff oxidation which selectively oxidize a carbon-carbon double bond to give two carboxylic acids [14]. Hydrophilic UCNs can be stable in aqueous solution for more than one month.

Hydrophilic UCNs with surface functionalized groups have great potential for biological applications, including bioimaging for cancer cells in vitro and real-time targeted imaging of tumor bearing mouse in vivo, photodynamic therapy, immunoassay for antibody, bioassay for RNA and DNA based on fluorescence resonant energy transfer (FRET), etc [14-16]. UCNs absorb NIR light with deep tissues penetration and less autofluorescence and convert it to visible light or even NIR light (about 800 nm). Meanwhile, UCNs have outstanding luminescent stability under long-term excitation in comparison with down-conversion fluorescence materials.

In this study, UCNs with OA ligands were prepared by hydrothermal and solvothermal synthesis. As-prepared hydrophobic OA-coated UCNs were converted into hydrophilic via a ligand-exchange process with HDA. The transmission electron microscope (TEM), X-ray diffraction (XRD) pattern and upconverting emission spectrum of OA-coated UCNs were characterized in each strategy. The effects of reaction time and temperature for the synthesis of UCNs were investigated. Moreover, the ratio of green and red emission was adjusted by changing the concentration of rare earth dopants. Optical property of UCNs before and after ligand-exchange process was measured as well. The differences of as-prepared UCNs prepared by hydrothermal and solvothermal synthesis were compared for further biological applications.

2. Experimental section

2.1 Materials

Y(NO₃)₃·6H₂O, Yb(NO₃)₃·5H₂O and Er(NO₃)₃·5H₂O (purity >99.9%) were purchased from Aladdin. Rare earth chlorides (LnCl₃, Ln: Y, Yb and Er) were prepared by dissolving the corresponding oxides (Y₂O₃, Yb₂O₃ and Er₂O₃ from Aladdin and China National Medicine Corporation Ltd.) in 10% hydrochloric solution and then evaporating the water completely. Diethylene glycol (DEG; 98%) were purchased from Aladdin. Hexanedioic acid, NaOH, NH₄F and NaF were purchased from China National Medicine Corporation Ltd. 1-octadecene (90%) and oleic acid (90%) were all purchased from Sigma-Aldrich and used as starting materials without further purification.

2.2 Synthesis of oleic acid-coated upconverting nanoparticles

2.2.1 Hydrothermal synthesis of NaYF₄:Yb,Er nanoparticles

NaYF₄:Yb,Er nanoparticles were prepared by a modified hydrothermal process [4]. In a typical synthesis, NaOH (1.2 g, 30 mmol), ethanol (10 mL), and oleic acid (20 mL) and different amount of water were mixed under agitation to form a homogeneous solution. Subsequently, 0.6 mmol (total amounts) of rare-earth nitrate (1.2 mL, 0.5 mol/L LnNO₃) aqueous solution was added under magnetic stirring. Then 1.0 M aqueous NaF (4 mL) solution was added dropwise to the above solution. After agitating for another 10 min, the mixture was transferred to a 50 mL autoclave, sealed, and hydrothermally treated at 160 °C for 12 h. The autoclave was cooled to room-temperature naturally, and the products were collected at the bottom of the container by cyclohexane, washed with cyclohexane/ethanol (1:6 v/v) for three times and centrifuged to obtained power products.
2.2.2 Solvothermal synthesis of NaYF₄:Yb,Er nanoparticles

NaYF₄:Yb,Er nanoparticles were synthesized following this protocol: Rare earth chlorides solution (0.5 mol/L) were prepared by dissolving the corresponding oxides in 10% hydrochloric solution. Different molar ratio of RECl₃ (Y: Yb: Er) were added to a 100 ml flask containing 6 ml oleic acid (OA) and 15 ml 1-octadecene (ODE). The solution was heated to 160 °C for 30 min and then cooled down to room temperature. Thereafter, 10 mL methanol solution of NH₄F (0.148 g) and NaOH (0.100 g) was added dropwise under stirring for 30 min. After methanol evaporated, the solution was heated to 300 °C under nitrogen for 1 h and cooled down to room temperature. The resulting nanoparticles were precipitated by the addition of ethanol, collected by centrifugation. The products were washed with cyclohexane/ethanol (1:6 v/v) for three times.

2.3 Ligand-exchange of oleic acid-coated NaYF₄:Yb,Er nanoparticles with HDA

Ligand-exchange of UCNs was prepared following Zhang’s report [8]. Ligand exchange was carried out as follows: The DEG solution (10 mL) containing HDA (500 mg) was heated up to 110 °C for 30 min with vigorous stirring under nitrogen. A chloroform solution of UCNs (20 mg) was injected into the above solution, then heated to 240 °C and kept at this temperature for about 1.5 h until the solution became clear. After the solution was cooled to room temperature, excess aqueous solution was added. The UCNs were isolated by centrifugation and decantation. Finally the sample was washed three times with pure water and redispersed in deionized water for analysis.

2.4 Characterization

Powder XRD measurement was carried out using a Philips X’Pert PRO X-ray diffractometer. Sizes and morphologies of UCNs were determined at 200 kV using a JEOL JEM-2100 high-resolution transmission electron microscope (HR-TEM). Up-conversion luminescence spectra were measured with S2000 eight-channel optical fiber spectrographotometer (Ocean Optics corporation, America) and a 0-2000 mW adjustable laser (980 nm, Shaanxi Scitower Photoelectricity Equipment co., LTD., China) was used as the external excitation source. All optical measurements were performed at room temperature.

3. Results and discussion

3.1 Size and morphology of NaYF₄ nanoparticles

TEM images of UCNs with different synthesis routes and conditions were shown in figure 1. Nanocubes were generated by hydrothermal synthesis with sizes of 8-12 nm, as the volume ratio of OA, ethanol and water is 20:10:9 (Figure 1A). While reducing the volume of water to 4 mL, the size distribution enlarged to 15 ± 5 nm (Figure 1B). A few nanorods with the length of 900 nm and the width of 20 nm could be observed in this sample (data were not shown). The results indicate the amount of water influence the growth of UCNs, since OA and produced UCNs are not dissolved in water. OA coated UCNs can easily grow with less molar ratio of water and become larger in size.

For UCNs prepared by solvothermal synthesis, nanospheres and nanoplates were obtained. The size distribution was 45 ± 2 nm in the reaction system of 6 mL OA and 15 mL ODE. While increasing the reaction time and temperature without changing the ratio of reagents, the shape of as-prepared UCNs was almost changed to plate-like crystals with larger size (150 ± 5 nm). A few cubic impurities could also be observed in figure 1D.
Figure 1 TEM images of NaYF₄:Yb,Er nanoparticles with different synthesis methods and conditions: hydrothermal synthesis (A) OA/ethanol/water=20:10:9 (v/v/v) (ruler=20 nm) and (B) OA/ethanol/water=10:5:2 (v/v/v) (ruler=50 nm); solvothermal synthesis at different reaction temperatures (C) 300 °C for 1 h (ruler=50 nm) and (D) 310 °C for 1 h (ruler=200 nm).

In comparison of these two synthesis strategies, hydrothermal synthesis with more amount of water can generate small upconverting nanocubes (less than 20 nm). However, solvothermal synthesis always produced nanoplates and nanospheres with more uniform size distribution than hydrothermal synthesis, the shape of which can be adjusted through adding different amount of OA and ODE. According to Li’s report [3], hydrothermal synthesis also produced nanorods with large size when increasing hydrothermal reaction time and temperature. All the as-prepared UCNs were monodisperse suggesting that the long-chain OA ligands on their surface prevent aggregation.

3.2 Structure of NaYF₄ nanoparticles

The X-ray diffraction (XRD) pattern result of the NaYF₄ nanoparticles prepared by solvothermal synthesis was shown in figure 2. The diffraction peaks positions and intensities confirmed the existence of hexagonal (JCPDS card 16-0334) and cubic (JCPDs card 77-2042) phase NaYF₄ nanoparticles (standard cards were not shown). Most of OA-coated UCNs
synthesizing via solvothermal route were hexagonal phase. The concentration of OA ligands affected the growth rates of hexagonal-phase NaYF₄ nanoparticles in different direction.

3.3 Optical characterization of NaYF₄ nanoparticles

Photoluminescence spectrum of prepared NaYF₄ nanoparticles powder were measured at room temperature and given in figure 3. The typical emission peaks of the NaYF₄:Yb,Er nanoparticles were located at 407, 521, 539 and 651 nm, which due to the transitions between energy levels ⁴H⁹₂, ⁴H₁₁₂, ⁴S₃₂ and ⁴F₉₂ to ⁴I₁₅₂ of Er³⁺.

Figure 3A presented upconverting emission of NaYF₄:Yb,Er nanoparticles synthesizing through hydrothermal method. PL intensities in different reacting condition demonstrated the amount of water affected the emission intensity of UCNs. UCNs with larger size had higher PL intensity. For hydrothermal synthesis, decreasing the amount of water and increasing the reaction time and temperature can both enhance upconverting emission of UCNs (Figure 4). Moreover, for solvothermal synthesis, reducing the amount of OA and increasing reaction temperature (below the boiling point of ODE) and time will increase the PL emission.

Figure 3 Upconversion spectrum of NaYF₄:Yb,Er powders with different synthesis strategies and rare earth ion doped ratio: (A) NaYF₄:20 mol%Yb,2 mol%Er nanoparticles prepared by hydrothermal synthesis; (B) NaYF₄:18 mol%Yb,2 mol%Er nanoparticles prepared by solvothermal synthesis.
Photostability of OA-coated UCNs were investigated in 90 minutes (Figure 5). UCNs have higher optical and chemical stability than conventional organic dyes and QDs, which is significant for bioimaging in cells or in animal models. Nanoplates prepared by solvothermal route showed stronger upconverting emission compared with nanospheres (solvothermal synthesis) and nanocubes (hydrothermal synthesis). Previous reports revealed that UCNs with hexagonal phase showed 4.4 times stronger PL intensity than cubic phase powders [5], and the different is even bigger when the size of UCN powder is of nanoscale order. NaYF₄:Yb,Er nanoparticles prepared by hydrothermal synthesis with 4 mL water showed approximate upconverting emission comparing with solvothermal synthesized NaYF₄:Yb,Er nanospheres (6 mL OA, 300 °C for 1 h).

3.3 Ligand-exchange of NaYF₄ nanoparticles
NaYF₄ nanoparticles were synthesized by hydrothermal and solvothermal route in the presence of OA molecules. In this reaction process, OA molecules were coated onto the outer face of the UCNs through the interaction between their carboxyl
groups and rare earth ions. Since the alkyl chains of OA were left outside, the as-prepared UCNs were hydrophobic and easily dispersed in nonpolar solvents, such as hexane, cyclohexane, chloroform, etc. After the addition of polar solvents like ethanol and acetone, OA coated UCNs were deposited and centrifuged to obtain power products.

To convert hydrophobic OA coated UCNs into carboxyl functionalized derivatives, an efficient ligand-exchange process was carried out to obtain hydrophilic UCNs. HDA molecules were used to replace the original OA ligands in DEG solvent at high reaction temperature without obvious adverse effects on the optical property of the UCNs (Figure 6). The HDA molecules with six carbons have proper length to chelate to the surface of UCNs. High reaction temperature can enhance the efficiency of the ligand-exchange process. The HDA coated UCNs showed strong upconverting emission and high water solubility. Most importantly, HDA-coated UCNs with carboxyl-functionalized groups become a promising candidate for biological labeling.

![Figure 6 Photoluminescence spectrum of UCNs before (A) and after (B) HDA ligand-exchange.](image)

### 3.4 The effect of rare earth doped concentration on the PL intensity ratio of green and red emissions

Upconverting emission peaks of NaYF₄:Yb,Er nanoparticles were located at 407, 521, 539 and 651 nm, which could show green and yellow color observed by naked eyes. Red color can be observed through suitable optical filters. The intensity of green (540 nm) and red (650 nm) emission peaks can be adjusted by changing the ratio of rare earth dopants. Table 1 presented the effects of rare earth doped concentration on the PL intensity ratio of green and red emissions. When the concentration of Er³⁺ is constant, increasing the concentration of doped Yb³⁺ ions could obviously enhance red emission, but had little effects on the green emission of NaYF₄:Yb,Er nanoparticles (Figure 7). Adjustable green and red emissions are useful for multicolor biological labeling.
Figure 7 PL intensity ratio of green and red light with different doped Yb$^{3+}$ ions

| Y (molar%) | Yb (molar%) | Er (molar%) | PL intensity ratio of green and red light |
|------------|-------------|-------------|------------------------------------------|
| 80         | 18          | 2           | 2.414                                    |
| 75         | 23          | 2           | 2.054                                    |
| 70         | 28          | 2           | 1.644                                    |
| 65         | 33          | 2           | 1.499                                    |
| 60         | 38          | 2           | 1.188                                    |

Table 1 PL intensity ratio of green and red light with different molar ratio of rare earth dopants

4. Conclusion

In this study, we have synthesized NaYF$_4$:Yb,Er nanoparticles via hydrothermal and solvothermal routes and transferred hydrophobic OA-coated UCNs to hydrophilic HDA-coated UCNs with higher water solubility. TEM results indicated the as-prepared UCNs were monodisperse with uniform size distribution. Nanocubes, nanospheres and nanoplates were obtained by changing the molar ratio of reagents. UCNs prepared with less amount of water in hydrothermal synthesis and synthesized by solvothermal route showed strong upconversion fluorescence. Moreover, HDA ligand-exchange hardly had any adverse effects on luminescence properties of the UCNs. The carboxyl-functionalized groups of HDA can further conjugate with tumor targeting ligands, antibodies, DNA, proteins, etc. High luminescent UCNs have great potential for biological labeling, bioimaging in vitro and in vivo. Future work will involve targeted localization of these UCNs tumors in vivo in small animals for targeted bioimaging and photodynamic therapy.

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