Biomaterials 34 (2013) 1722–1731

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Sandwich-structured upconversion nanoparticles with tunable color for multiplexed cell labeling

Qingqing Dou [a], Niagara Muhammad Idris [a], Yong Zhang [a,b,c,*]

[a] Department of Bioengineering, National University of Singapore, Singapore 117574, Singapore
[b] NUSNNI-NanoCore, National University of Singapore, Singapore 117576, , Singapore
[c] NUS Graduate School for Integrative Sciences & Engineering, National University of Singapore, Singapore 117456, Singapore

ARTICLE INFO

*Article history:*
Received 15 September 2012
Accepted 10 November 2012
Available online 30 November 2012

*Keywords:*
Nanoparticle
Fluorescence
Cell culture
Confocal microscopy

ABSTRACT

The need for a more efficient biological label to meet their burgeoning utility in rapidly developing multiplexing applications may be realized through the recent advent of upconversion nanoparticles (UCNs). UCNs fabricated to-date, however, are either not displaying strong fluorescence or have limited available colors. Here, we report on fabricating sandwich-structured UCNs with a $NaYbF_4$ matrix sandwiched between two $NaYF_4$ layers. Such sandwich design allows for efficient absorption of the excitation energy by the absorber ion-rich $NaYbF_4$ layer that then transfers it to the adjacent $NaYF_4$ layers on either side for an improved fluorescence efficiency. By doping different emitters into each of the shells and adjusting their thickness, different color output tunable based on the RGB color model were obtained. In this study, multicolor UCNs with strong emission intensity have been facilely synthesized and used for multiplex detection of three subcellular targets with a single near-infrared excitation wavelength.



## 1. Introduction

The arduous task of piecing together complex cellular events can now be performed with ease by multiplexing. By throwing a rainbow of fluorescent colors to a cell sample, more than one target can be visualized simultaneously in the same cell, thus allowing several events to be captured in a single snapshot, altogether reducing the amount of reagents, consumables and sample required, besides minimizing sampling errors and eases the inclusion of internal controls [1]. Indeed, such multiplexing capability is made possible with the advent of multicolor fluorescence. Recently, upconversion nanoparticles (UCNs) with highly unusual optical properties and emission wavelength in the ultraviolet (UV), visible (VIS) and near-infrared (NIR) range upon excitation by a single wavelength of NIR light, has come into vogue as a novel group of fluorescent label that is foreseen to overcome current limitations of conventional labels [2,3]. The use of NIR as an excitation light source gives it a competitive advantage as practically near-zero background visible fluorescence of the sample is generated due to lack of efficient endogenous absorbers in the NIR spectral range besides the fact that most biomolecules do not possess the upconverting property [4]. Due to this 'transparency' in the NIR window, its use is anticipated to allow deep access [5] to tissue specimens being investigated besides offering astounding detection sensitivity for tracing minute amount of target molecules due to the aforementioned nil autofluorescence background and reduced light scattering. When used in a multiplex detection set-up, cross-talking between excitation and emission lights can also be greatly reduced. Moreover, UCNs' capability of multicolor emissions at a single NIR excitation wavelength allows simultaneous excitation of the different colors with ease. In addition, their inherent exceptional photostability feature coupled with low photo-damage (NIR excitation light being generally harmless to biomolecules at low dose) to cells and delicate proteins makes them an attractive tool for long-term live cell imaging. The ability to manipulate the color output of these UCNs is therefore of particular importance in harnessing their unique optical property to generate novel, superior fluorescent tags for multiplexing applications.

In a study done by Nann and coworkers [6], four color-UCNs were successfully fabricated by doping Tm, Ho, Er and Yb lanthanide ions into $NaYbF_4$ and $NaYF_4$ lattices, albeit at low quality as displayed by their non-uniformity in size, irregularity in shape and having a much lower intensity compared to their bulk counterpart. In the same year, Liu et al. [7] also reported a method of fine-tuning upconversion emission color by adjusting the Er/Tm ratio co-doped

* Corresponding author. Department of Bioengineering, Faculty of Engineering, Block EA #03-12, National University of Singapore, 9 Engineering Drive 1, Singapore 117576, Singapore. Tel.: +65 65164871; fax: +65 68723069.
*E-mail address:* biezy@nus.edu.sg (Y. Zhang).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.11.011

into the nanocrystals. Similarly, Yan et al. [8] documented synthesizing $NaYF_4$:Yb,Tm nanocrystals with an overall output color of blue, purple and red by altering the particles' size and their Yb/Tm doping concentrations. In yet another study to synthesize multicolor UCNs, Li et al. [12] reported on the method of co-doping Er and Tm ions into $NaYF_4$ matrix to achieve green, red, and NIR upconversion luminescence. Undeniably, however, the above methods are easily succumbed to fluorescence quenching due to cross-relaxation that occurs when Er or Tm ions are doped in the same crystal matrix as other rare earth dopants [13,14] such that the concentration of Er and Tm ions can only be tuned within a certain range, thus making color tuning by this method very much limited. As such, it is difficult to obtain multicolor emission UCNs with strong fluorescence by solely adjusting their Er/Tm ratio. In yet another piece of work by Yan et al. [15], it was reported that the upconversion emission of $NaYF_4$ UCNs was size dependent and that their green/red emission ratio ($f_{g/r}$) was affected by coating an undoped $\alpha$-$NaYF_4$ shell. Although multicolor UCNs were obtained by manipulating these parameters, nanocrystals emitting different colors were of different sizes, thus hindering their potential for downstream applications. Li et al. [16] also fabricated multicolor emission upconversion nanospheres based on fluorescence resonance energy transfer (FRET) occurring between UCNs and organic dyes (ODs) or quantum dots (QDs) that have been encapsulated in the silica shell of the UCNs. However, the multicolor emission was largely dependent on and limited by the FRET efficiency from UCNs to the encapsulated ODs or QDs. Hence, the abovementioned efforts in deriving multicolor UCNs were made at the expense of the particle's upconversion fluorescence intensity. In view of this, Capobianco [17] and Chow [18] developed a strategy to enhance the fluorescence of UCNs by coating an undoped matrix shell on the nanocrystals (Capobianco: $NaGdF_4$:Yb,Er@$NaGdF_4$ and Chow:$NaYF_4$:Yb,Er/Tm@$NaYF_4$, respectively) to minimize quenching of fluorescence due to surface defects and surface-associated ligands.

To obtain a high efficiency of upconversion fluorescence, the sensitizer and activator should be close enough for dipole–dipole interactions to occur. Otherwise, it would result in a much weaker dipole–quadrupole and quadrupole–quadrupole interactions that would give rise to a weak upconversion had the sensitizer and activator been positioned further apart [5]. On the other hand, the concentration of lanthanide ions should also be kept low to avoid the quenching effect from deleterious cross-relaxation and inefficient cooperative upconversion. Therefore, sandwich-structured nanoparticle with Yb sensitizer-rich content packed in its middle layer could provide a solution to this problem. In this work, we report on the synthesis of such sandwich-structured nanoparticle comprising of a middle $NaYbF_4$ matrix layer sandwiched between two $NaYF_4$ matrix layers for an enhanced energy absorption effect by the middle sensitizer-rich layer to produce highly fluorescent UCNs with tunable emission based on the RGB color model. Here, the middle $NaYbF_4$ matrix layer is expected to perform three functions: (i) its rich content of Yb absorber ions allows for maximum absorption of the excitation energy that is then transferred to the adjacent $NaYF_4$ layers lying on either side; (ii) it repairs the surface defects on the nanocrystal core and thus minimizes fluorescence quenching; (iii) its own upconversion emission serves as a color source that can be used to adjust the overall output emission color. By altering the dopant components in each layer and adjusting the layer thickness, any desired upconversion emission color can be obtained based on the RGB model. This new approach to tune emission colors of UCNs with strong emission by the sandwich design of an energy-accumulating matrix between layers of a sandwich construct is foreseen to generate a superior fluorescent tool for a wide range of multiplexing applications. In this study, their feasibility for use in multiplex detection was also assessed by further surface functionalization of these multicolor UCNs with different antibodies to target multiple cellular markers on one cell simultaneously.

## 2. Materials and methods

### 2.1. A ($NaYF_4$) core UCNs synthesis

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. $NaYF_4$: 20% Yb, 2% Er (or 0.3% Tm) nanocrystals were synthesized following protocols reported previously with modification [16,19]. 0.8 mmol $YCl_3$, 0.20 mmol $YbCl_3$ and 0.02 mmol $ErCl_3$ (or 0.003 mmol $TmCl_3$) were mixed with 6 ml oleic acid and 15 ml octadecene in a 50 ml flask. The solution was heated to 150 °C to form a homogenous solution, and then cooled down to room temperature (RT). A solution of 4 mmol $NH_4F$ and 2.5 mmol NaOH in 10 ml of methanol was next added into the flask and stirred for 30 min. Subsequently, the solution was slowly heated to remove the methanol followed by degassing at 100 °C for 10 min. It was then heated to 300 °C and maintained at that temperature for 1.5 h under an argon atmosphere. The solution was allowed to cool to RT before the nanocrystals were precipitated out from the solution with acetone. They were then washed thrice with ethanol/water (1:1 *v*/*v*) and finally dispersed in cyclohexane for subsequent use.

### 2.2. AB ($NaYF_4$@$NaYbF_4$) core–shell UCNs synthesis

1 mmol $YbCl_3$, and 0.02 mmol $ErCl_3$ (or 0.003 $TmCl_3$) were mixed with 6 ml oleic acid and 15 ml octadecene in a 50 ml flask. The solution was heated to 150 °C to form a homogenous solution, and then allowed to cool down. Solution of the core nanocrystals dispersed in cyclohexane that was earlier obtained from previous step was next added to the flask. The solution was maintained at 70 °C so as to remove the cyclohexane solvent and then subsequently cooled down to RT. Following this, a solution of 4 mmol $NH_4F$ and 2.5 mmol NaOH in 10 ml of methanol was added into the flask and stirred for 30 min. Then, the solution was slowly heated to remove the methanol followed by degassing at 100 °C for 10 min. Subsequently, the solution was heated to 300 °C for 1.5 h under an argon atmosphere. The solution was once again cooled down before the nanocrystals were precipitated out from the solution with acetone. This was washed thrice with ethanol/water (1:1 *v*/*v*) and the resultant AB nanocrystals were dispersed in cyclohexane for the next layer of coating.

### 2.3. ABA sandwich UCNs synthesis

0.8 mmol $YCl_3$, 0.20 mmol $YbCl_3$ and 0.02 mmol $ErCl_3$ (or 0.003 mmol $TmCl_3$) were mixed with 6 ml oleic acid and 15 ml octadecene in a 50 ml flask. The solution was heated to 150 °C to form a homogenous solution, and then allowed to cool down. Core–shell AB nanocrystals dispersed in cyclohexane solution as obtained earlier from previous step were next added into the flask. The solution was maintained at 70 °C to evaporate off the cyclohexane and then cooled down to RT. A solution of 4 mmol $NH_4F$ and 2.5 mmol NaOH in 10 ml of methanol was added into the flask and stirred for 30 min. Subsequently, the solution was slowly heated to remove the methanol, degassed at 100 °C for 10 min, and then heated to 300 °C for 1.5 h under an argon atmosphere. The solution was once again cooled down before the nanocrystals were precipitated out from the solution with acetone. This was washed thrice with ethanol/water (1:1 *v*/*v*). The resultant nanocrystals obtained are the sandwich ABA UCNs.

### 2.4. Conjugation of antibodies to UCNs

Antibodies were covalently conjugated to UCNs using the EDC–NHS chemistry. UCNs were first carboxylized with carboxyethyl silane triol sodium salt. 0.25 ml of CO-520, 4 ml of cyclohexane and 1 ml of 0.02 M ABA UCNs dispersed in cyclohexane were mixed in a bottle followed by sonication. 0.04 ml of ammonia (33 wt%) was then added into the bottle and this was sealed before it was shaken fiercely to form a transparent emulsion. 5 μl of TEOS and 5 μl of carboxyethyl silane triol sodium salt was next added into the solution followed by vigorous stirring of the solution at 60 rpm for two days. The product was precipitated out by ethanol, washed twice with ethanol/water (1:1 *v*/*v*) and then stored in DI water. 1 ml of 2 mM UCNs was activated with 1 μl of 0.2 mg $\mu l^{-1}$ N-hydroxysuccinimide and 1 μl of 0.3 mg $\mu l^{-1}$ 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride with vigorous shaking for 15 min. After which, the activation buffer was removed and the mixture topped up with fresh DI water. 20 μl of 4 μg $\mu l^{-1}$ antibody solution was added to the activated particles and incubated at 4 °C for 3 h. Details of the antibodies are as follows: anti-HER2 (AbD Serotec, Kidlington, Oxford, UK); anti-BMPR2 (N-term) (Abgent, San Diego, CA, USA); anti-PDGFRα (Cell Signaling Technology, Beverly, MA, USA); anti-α-tubulin (Cell Signaling Technology, Beverly, MA, USA). This was followed by washing of the particles twice with water, with centrifugation step at 5000 rpm for 5 min in between the washings. Finally, the UCNs–antibody conjugates were re-suspended and stored in 1 ml of DI water.