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Multiphoton Imaging of Upconverting Lanthanide Nanoparticles in Three Dimensional Models of Cancer

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Abstract

While upconverting lanthanide nanoparticles have numerous advantages over other exogenous contrast agents used in scanned multiphoton imaging, their long luminescence lifetimes cause images collected with non-descanned detection to be greatly blurred. We demonstrate herein the use of Richardson-Lucy deconvolution to deblur luminescence images obtained via multiphoton scanning microscopy. Images were taken of three dimensional models of colon and ovarian cancer following incubation with NaYF₄:Yb,Er nanoparticles functionalized with an antibody for EGFR and folic acid respectively. Following deconvolution, images had a lateral resolution on par with the optimal performance of the imaging system used, ~1.2 μm , and an axial resolution of ~5 μm . Due to the relatively high multiphoton excitation efficiency of these nanoparticles, it is possible to follow binding of individual particles in tissue. In addition, their extreme photostability allows for prolonged imaging without significant loss in luminescence signal. With these advantageous properties in mind, we also discuss the potential application of upconverting lanthanide nanoparticles for tracking of specific, cancer relevant receptors in tissue.

Keywords

upconversion; lanthanide; nanoparticle; 2p; microscopy; erbium

1. INTRODUCTION

Upconverting lanthanide nanoparticles (UNPs) are a family of luminescent contrast agents with numerous advantages over more traditionally used molecular fluorophores.^{1,2} Excitation of these nanoparticles typically utilizes the $^2F_{7/2} \rightarrow ^2F_{5/2}$ electronic transition in trivalent ytterbium ions at 980 nm, and subsequent nonradiative energy transfer to neighboring erbium and thulium ions, to produce anti-Stokes shifted luminescence. This multiphoton excitation process is fundamentally different from multiphoton absorption because it makes use of real, metastable energy levels within the lanthanide ions to build up energy greater than that of a single exciting photon. For this reason, multiphoton excitation of UNPs is several orders of magnitude more efficient than multiphoton excitation of most molecular fluorophores, such that femtosecond laser sources and tightly focused excitation light are no longer strict requirements for multiphoton imaging. In addition, UNPs are

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extremely photostable, and can be imaged for long periods of time with no loss of luminescence, except under extreme illumination intensities.^{3,4} Of particular interest to our group are upconverting nanoparticles consisting of a NaYF₄ host matrix codoped with Yb³⁺ and either Er³⁺ or Tm³⁺.

While there are many advantages to using UNPs as exogenous contrast agents for multiphoton imaging, there remain several hurdles impeding their practical application. UNPs are most commonly synthesized via a thermal decomposition method that uses oleic acid as a capping ligand, rendering the nanoparticles hydrophobic. For biological applications, hydrophilic contrast agents are often preferable, especially when working with antibodies and cell surface receptors like epidermal growth factor receptor (EGFR) and folate receptor alpha (FR α), two proteins often overexpressed in colon and ovarian cancers. In order to render UNPs hydrophilic and provide surface groups for functionalization, we coat UNPs with a lipid monolayer.^{5,6}

In addition, upconverting lanthanide materials have long luminescence lifetimes. While these lifetimes can be advantageous in some circumstances, they are a hindrance when imaging using scanning microscopy techniques. When detecting photons by descanning through a confocal pinhole, the majority of luminescence photons are emitted after the microscope has scanned past the particles, resulting in a loss of signal. As a result, higher excitation powers are necessary to image UNPs using confocal microscopy. If the UNPs are instead imaged with non-descanned detection, as is the case in typical multiphoton microscopy, luminescence appears streaked along the fast scan axis, obscuring nanoparticle location. We have recently demonstrated one possible solution to the problem of UNP streaking, Richardson-Lucy (R-L) deconvolution using luminescence lifetimes obtained through single photon counting.⁵ This method allows for the use of a standard multiphoton microscopy setup when imaging with UNPs, with only a minor impact on acquisition time.

Finally, the axial resolution of UNPs is dependent on excitation power.⁵ Because luminescence lifetimes are long, and multiphoton excitation occurs via real metastable excited states, saturation of initial excited states occurs at lower excitation powers than are typically used in multiphoton microscopy. This saturation results in the nanoparticles behaving as though they were being excited through a single photon absorption process, resulting in a loss of optical sectioning.

Herein, we demonstrate the use of R-L deconvolution to enable multiphoton microscopy of targeted UNPs in three dimensional models of colon and ovarian cancer. NaYF₄:Yb,Er nanoparticles were synthesized, coated with lipids, and conjugated to either an antibody for EGFR or folic acid. Anti-EGFR-PEG-lipid-UNPs were introduced to *ex vivo* colon taken from Vil-Cre mice treated with azoxymethane (AOM), and folate-PEG-lipid-UNPs were introduced to collagen constructs seeded with CAOV3 ovarian cancer cells. In addition, we explore two different methods for maintaining good axial resolution while imaging UNPs, a requirement for optical sectioning techniques.

2. MATERIALS AND METHODS

2.1 Synthesis of small upconverting lanthanide nanoparticles

Small UNPs, ~2 nm in diameter, were synthesized by a thermal decomposition previously described by Mai et al.⁷ All reagents were obtained from Sigma Aldrich unless otherwise specified. In a typical synthesis, 5 mmol of lanthanide oxides at a ratio of 78:20:2 Y:Yb:Er were added to a solution made up of 25 mL deionized water and 25 mL trifluoroacetic acid, forming a cloudy, white suspension. This solution was then heated to 80 °C and magnetically stirred overnight to form lanthanide trifluoroacetate precursors. Precursors were separated from solution by rotor evaporation, suspended in 20 mL of tertiary butanol, and lyophilized. Once dry, 2 mmol of lanthanide trifluoroacetate precursors and 2 mmol of Na(C₂F₃O₂) were added to a 100 mL 3-neck flask containing 6.58 mL oleylamine (Acros Organics, New Jersey, USA), 6.32 mL oleic acid (Acros Organics, New Jersey, USA), and 12.8 mL 1-octadecene. The resulting solution was then heated to 100 °C and magnetically stirring while alternating between Ar gas flow and vacuum to remove oxygen and water from the reaction vessel. Following 1 hour at 100 °C, the solution was rapidly heated to 280 °C and maintained at this temperature for 1 hour. Once cooled to room temperature, nanoparticles were precipitated with ~80 mL of ethanol and centrifuged at 6000 ×g for 10 minutes. The supernatant was discarded and the pellet was washed several times with ethanol. UNPs were then dried overnight under vacuum and redispersed in chloroform.

2.2 Synthesis of large upconverting lanthanide nanoparticles

Synthesis of large UNPs, ~40 nm in diameter, was performed using a method described in several publications.^{8–10} In a typical synthesis, 2 mmol of lanthanide acetates at a ratio of 78:20:2 Y:Yb:Er were added to 12 mL of oleic acid and 34 mL 1-octadecene in a 100 mL 3-neck flask. The resulting cloudy mixture was then heated to 125 °C for 1 hour. The resulting solution was clear and either colorless or pale yellow. Once cooled to room temperature, 10 mmol of NaOH and 5 mmol of NH₄F were dissolved in 20 mL of methanol and added to the reaction vessel. Methanol, oxygen, and water were removed from the reaction vessel by alternating between Ar flow and vacuum pressure while heating to 100 °C. The solution was maintained at 100 °C under vacuum until bubbles stopped forming. The solution was then heated to 310 °C for 1 hour. Once cooled to room temperature, ~80 mL of ethanol was added to precipitate UNPs, which were separated from solution by centrifugation at 6000 ×g for 10 minutes. Following several washes with ethanol, particles were dried overnight under vacuum and redispersed in chloroform.

2.3 Lipid coating of UNPs

NaYF₄:Yb,Er nanoparticles were coated with a lipid monolayer using a method previously described by our group. Lipids were added to chloroform at a concentration sufficient to coat a batch of nanoparticles once. To functionalize 80 mg of nanoparticles with carboxylic acid surface groups for use in anti-EGFR conjugation, DPPC, DPPE-[methoxy(PEG)2000], and DSPE-[carboxy(PEG)2000] (Avanti Polar Lipids, Alabama, USA) were used at a molar ratio of 95:4.5:0.5 DPPC:DPPE:DSPE. For functionalization with folic acid, 10 mg of UNPs were coated with DSPC, DSPE-[methoxy(PEG)2000], and DSPE-[folate(PEG)5000] (Avanti Polar Lipids, Alabama, USA) at a molar ratio of 79.9:20:0.1. The lipid/UNP

suspension was then dried using N₂ flow to evaporate most of the chloroform followed by vacuum pressure to remove any remaining chloroform. Once dry, 5 mL of 10 mM MES buffered saline and 2 mL of 1X PBS were added to the lipid/UNP mix for carboxy-PEG-lipid-UNPs and folate-PEG-lipid-UNPs respectively. The resulting solutions were sonicated for several hours at temperatures above the transition temperatures of the majority lipid constituents. Lipid-UNPs were separated from other lipid structures by ultracentrifugation for 30 minutes. The supernatant was discarded, and the lipid-UNP pellet was redispersed in buffer and stored at 4 °C until used.

2.4 Conjugation of anti-EGFR

6.5 mg N-hydroxysulfosuccinimide, 5.31 µL N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, and 235 µL of deionized water were added to 300 µL of the coated particles in 10 mM MES. After 10 minutes of gentle stirring at room temperature, 30.6 µg of anti-EGFR rabbit polyclonal IgG (Santa Cruz Biotechnology, California, USA) were added. The solution was stirred overnight at 4 °C. Purification was by dialysis against DI water with a 300 kDa membrane cutoff. Following dialysis, the presence of bound anti-EGFR was confirmed by the presence of a UV absorption peak at ~280 nm. Particles were used for tissue staining and subsequent imaging immediately following purification.

2.5 UNP Characterization

NaYF₄:Yb³⁺,Er³⁺ nanoparticles were sized by quasi-elastic light scattering using a ZS-90 Zetasizer (Malvern, Worcestershire, United Kingdom). Emission spectra were measured by a QE65000 back-thinned CCD spectrometer (Ocean Optics, Florida, USA) using a 980 nm laser diode for excitation. Luminescence decays were measured by a single photon counting avalanche photodiode (PerkinElmer, Vaudreuil, Québec, Canada) and multichannel scaler (Picoquant, Berlin, Germany) using a tunable optical parametric oscillator (OPO) pumped by a Nd:YAG laser Q-switched at 20 Hz with a 3 ns pulse width for excitation. Luminescence rise and decay constants were determined by fitting lifetime data to $-Ae^{-t/\tau_1} + Be^{-t/\tau_2} + BKG$, where τ_1 is the rise constant and τ_2 is the decay constant.

2.6 Staining *ex vivo* mouse colon

AOM treated Vil-Cre mice were euthanized with CO₂ and proximal colon tissue was removed. Colon tissue samples were washed twice with 1X PBS, then incubated with 100 µL of either anti-EGFR conjugated UNPs or unconjugated UNPs at ~4 mg/mL for 1 hour at room temperature. Following incubation, colons were washed twice in 1X PBS, then splayed open on a glass slide, submerged in 1X PBS, covered with a glass coverslip, and imaged.

2.7 Staining ovarian cancer 3D models

CAOV3 cells were seeded onto a collagen matrix and grown for 3 days in Dulbecco's modified eagle medium with low glucose and 10% fetal bovine serum. For staining, CAOV3/collagen constructs were washed twice with 1X PBS, then incubated with 500 µL at 1 mg/mL of either folate-PEG-lipid-UNPs or carboxy-PEG-lipid-UNPs in folate free RPMI

at 37 °C for 1 hour. Following incubation, constructs were washed twice with 1X PBS, placed in a well containing 1X PBS, and imaged.

2.8 Multiphoton microscopy

Multiphoton scanning microscopy was carried out using a Ti:Sapphire laser with ~100 fs pulse width and a repetition rate of 80 MHz for excitation and a commercial laser scanning microscope (LaVision Biotec, Bielefeld, Germany). All images were collected using a 20X water immersion objective lens with a NA of 0.95. Emission from the nanoparticles was collected at approximately 665 and 377 nm following 980 nm excitation. Tissue autofluorescence was collected at 460 nm following 780 nm excitation. SHG images of collagen were collected at ~377 nm following 780 nm excitation. Signal was detected using H7442A-40 PMTs (Hamamatsu Photonics, Hamamatsu City, Japan). Deconvolution was performed by a custom script developed in MATLAB (MathWorks, Massachusetts, USA) using the Richardson-Lucy function.⁵ Colon images were collected to a depth of 100 μm , with a step size of 1 μm between images. Ovarian cancer construct images were collected to a depth of 150 μm , with a step size of 2 μm between images.

3. RESULTS

Nanoparticles synthesized using the method described in section 2.1 had a diameter of 2.76 nm before coating and 31.9 nm after lipid coating. These nanoparticles were used in preparation of FR α targeted UNPs. Nanoparticles synthesized using the method described in section 2.2 had diameters of 62.22 and 38 nm before coating, 43.73 and 42 nm after lipid coating, and were used for EGFR targeting and as a control for non specific binding by CAOV3 cells respectively. No peak was observed at 280 nm for anti-EGFR conjugated nanoparticles, however, scattering from the sample may have been obscuring the absorption peak. As can be seen in figure 1, binding of UNPs to colon tissue did not correlate well with cellular structures, and instead bound preferentially to larger structures that was very likely feces that was not removed during wash steps. R-L deconvolution resolved bound particles well, removing the majority of streaking effects. Optical sectioning was maintained through the use of low excitation power, ~167 μW . Autofluorescence shown in gray in figure 1 was collected following excitation at ~20 mW.

Figures 2 and 3 demonstrate binding of COO-PEG-lipid-UNPs and folate-PEG-lipid-UNPs to tissue mimics made up of collagen gels seeded with CAOV3 cells. Binding of COO-PEG-lipid-UNPs was highly correlated to cellular structures, though it is unclear whether UNPs were taken up by the cells, or simply bound to the cell membrane. Folate-PEG-lipid-UNPs preferentially bound to the collagen gel, and little correlation between UNP luminescence and cellular structures was observed. UNP luminescence was detected through a 377 nm emission filter with 50 nm bandpass. This luminescence is the result of a three photon excitation process, and is completely free of endogenous fluorescence background. In addition, optical sectioning was maintained at an excitation power of ~40 mW. Collagen SHG and NAD/NADH autofluorescence images were also excited at an average power of 40 mW.

4. DISCUSSION

Both synthesis techniques used resulted in UNPs of expected sizes, with the method described in section 2.2 above yielding particles an order of magnitude larger than those synthesized with the method described in section 2.1. Lipid coating of 2.76 nm particles resulted in UNPs 31.9 nm in diameter. It is likely that lipid coating is not a viable option for very small nanoparticles due to the extreme radius of curvature demanded of any monolayer created on that scale. I suspect that nanoparticles cluster together until they reach a size conducive to lipid coating, and then cease to aggregate. TEM will be used to confirm this at a future date. Of the two sets of UNPs used in the experiments presented in this paper, one decreased in diameter following coating, from 62.22 nm to 43.73 nm. Immediately following coating, this sample had an average diameter of ~100 nm. When left to sit over night, some of the particles precipitated out of solution. Subsequent removal of precipitated particles likely resulted in a reduction in the average size of suspended particles, as larger particles fall out of solution more easily.

Conjugation of COO-PEG-lipid-UNPs to anti-EGFR did not result in a strong absorption peak at ~280 nm. This may be due to low concentration of the bound protein, or due to a complete absence of anti-EGFR attached to UNPs. Samples conjugated using the described method scattered UV light significantly, making determination of successful conjugation difficult. Due to the coating method used, introduction of DSPE-[folate(PEG)5000] during lipid coating is nearly guaranteed to result in some degree of UNP functionalization. However, we have not yet quantified the degree of functionalization to determine how much of the DSPE-[folate(PEG)5000] associates with other lipid structures.

Binding of UNPs was largely nonspecific, and may have been the result of a number of factors. In the case of anti-EGFR-UNPs, there may not have been sufficient anti-EGFR bound to UNPs to result in specificity capable of overcoming nonspecific binding. Even if anti-EGFR was properly bound to the UNPs, there may not be sufficient cellular expression of EGFR to overcome the nonspecific uptake of UNPs by feces and other structures in the colon. Binding to CAOV3 and collagen in the ovarian cancer mimics was also not specific to FR α . There may have been charge interaction between folate and collagen, or FR α expression may have been low as the cells were not kept in low folate or folate free medium for a substantial length of time.

R-L deconvolution of images obtained resulted in good lateral and axial resolution, ~1.2 μ m along the fast scan axis and ~5 nm axial. Both low excitation power and imaging three photon excited luminescence at high excitation power result in optical sectioning with similar axial resolutions. While low excitation powers are generally preferable when avoiding changes in tissue morphology and cell death, greater excitation power may allow for simultaneous acquisition of endogenous fluorescence and UNP luminescence via 980 nm excitation, which is vital for long term tracking of individual cell receptors.

5. CONCLUSIONS

In conclusion, we have demonstrated that R-L deconvolution may be used in conjunction with stack acquisition of scanning multiphoton microscope images to generate high resolution, three dimensional images of upconverting lanthanide nanoparticles in *ex vivo* colon tissue and a rudimentary model of ovarian cancer. UNP binding was loosely correlated with tissue structures, but specific binding to membrane bound receptors commonly overexpressed in cancer cells was absent. Future work will focus on improvements in anti-EGFR conjugation to upconverting nanoparticles, cell preparation, and nanoparticle synthesis.

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REFERENCES

- [1]. Zhou J, Liu Z, Li F. Upconversion nanophosphors for small-animal imaging. *Chem. Soc. Rev.* 2012; 41:1323–1349. [PubMed: 22008740]
- [2]. Wang F, Banerjee D, Liu Y, Chen X, Liu X. Upconversion nanoparticles in biological labeling, imaging, and therapy. *Analyst.* 2010; 135:1839–1854. [PubMed: 20485777]
- [3]. Chatterjee DK, Rufaihah AJ, Zhang Y. Upconversion fluorescence imaging of cells and small animals using lanthanide doped nanocrystals. *Biomaterials.* 2008; 29:937–943. [PubMed: 18061257]
- [4]. Wang Y, Tu L, Zhao J, Sun Y, Kong X, Zhang H. Upconversion luminescence of β -NaYF₄: Yb³⁺, Er³⁺@ β -NaYF₄ core/shell nanoparticles: excitation power density and surface dependence. *J. Phys. Chem. C.* 2009; 113:7164–7169.
- [5]. Gainer CF, Utzinger U, Romanowski M. Scanning two-photon microscopy with upconverting lanthanide nanoparticles via Richardson-Lucy deconvolution. *J. Biomed. Opt.* 2012; 17(7): 076003. [PubMed: 22894486]
- [6]. Li LL, Zhang R, Yin L, Zheng K, Qin W, Selvin PR, Lu Y. Biomimetic surface engineering of lanthanide-doped upconversion nanoparticles as versatile bioprobes. *Angew. Chem. Int. Ed.* 2012; 51:1–6.
- [7]. Mai HX, Zhang YW, Sun LD, Yan CH. Size- and phase-controlled synthesis of monodisperse NaYF₄:Yb,Er nanocrystals from a unique delayed nucleation pathway monitored with upconversion spectroscopy. *J. Phys. Chem. C.* 2007; 111:13730–13739.
- [8]. Li Z, Zhang Y. An efficient and user-friendly method for the synthesis of hexagonal-phase NaYF₄:Yb, Er/Tm nanocrystals with controllable shape and upconversion fluorescence. *Nanotechnology.* 2008; 19:345606. [PubMed: 21730655]
- [9]. Qian HS, Zhang Y. Synthesis of hexagonal-phase core-shell NaYF₄ nanocrystals with tunable upconversion fluorescence. *Langmuir.* 2008; 24:12123–12125. [PubMed: 18839973]
- [10]. Abel KA, Boyer JC, van Veggel FCJM. Hard proof of the NaYF₄/NaGdF₄ nanocrystal core/shell structure. *J. Am. Chem. Soc.* 2009; 131:14644–14645. [PubMed: 19824725]

Figure 1.

Proximal colon from a AOM treated Vil-Cre mouse stained with anti-EGFR-PEG-lipid-UNPs, false colored red here. UNP emission was collected through a filter with a 665 nm center wavelength and 45 nm bandpass. Autofluorescence was measured using a filter with a 460 nm center wavelength and 80 nm bandpass and is shown in grayscale. UNPs were excited with 980 nm light and tissue autofluorescence was obtained using 780 nm excitation. Images are 510×510 pixels and cover a 400 μm by 400 μm area. Dwell time was 9.2 μs/pixel. The bottom image is from a point of view orthogonal to the other images in the figure.

Figure 2.

CAOV3 cells seeded on a collagen construct and stained with COO-PEG-lipid-UNPs, false colored red here. UNP emission and collagen SHG were collected through a filter with a 377 nm center wavelength and 50 nm bandpass. Autofluorescence was also measured using a filter with a 460 nm center wavelength and 80 nm bandpass and is shown in grayscale. UNPs were excited with 980 nm light and tissue autofluorescence was obtained using 780 nm excitation. Images are 510×510 pixels and cover a 400 μm by 400 μm area. Dwell time was 9.2 μs/pixel. The bottom image is from a point of view orthogonal to the other images in the figure.

Figure 3.

CAOV3 cells seeded on a collagen construct and stained with folate-PEG-lipid-UNPs, false colored red here. UNP emission and collagen SHG were collected through a filter with a 377 nm center wavelength and 50 nm bandpass. Autofluorescence was also measured using a filter with a 460 nm center wavelength and 80 nm bandpass and is shown in grayscale. UNPs were excited with 980 nm light and tissue autofluorescence was obtained using 780 nm excitation. Images are 510×510 pixels and cover a 400 μm by 400 μm area. Dwell time was 9.2 μs/pixel. The bottom image is from a point of view orthogonal to the other images in the figure.