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Synthesis, Characterization and Biosensing Application of Photon Upconverting Nanoparticles

Manoj Kumar and Peng Zhang

Department of Chemistry, New Mexico Institute of Mining and Technology, Socorro, NM 87801, USA

Abstract

Phosphor/fluorescent molecules/particles have been widely used in various applications for quite some time. Typically, light with longer wavelength(s) is emitted when excited by shorter wavelength light. The opposite effect also exists, where a phosphor particle is excited with an infrared or red light and emits color(s) of shorter wavelengths, a process called up-conversion. Materials with upconverting properties have narrower absorption and line emission spectra than their down-converting counterparts. Because most non-target materials in a complex mixture do not possess such photon up-conversion properties, a dramatically improved S/N ratio is expected in sensing and luminescence reporting applications. This makes photon upconverting materials ideal for identification of trace amounts of target molecules. Here we report the synthesis, characterization and DNA detection application based on $\text{NaYF}_4:\text{Yb}^{3+}, \text{Er}^{3+}$ photon upconverting nanoparticles. The design of a nucleotide sensor for the detection of point mutation associated with sickle cell disease is described. The underlying principle for the detection is luminescence resonance energy transfer (LRET), with the photon upconverting nanoparticle as the donor and a dye, N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), as the acceptor. The detection scheme is based on a sandwich-type hybridization format. The presence of the target DNA is indicated by the increase of the normalized acceptor's emission. Based on photon upconverting nanoparticles, which display high S/N ratio and no photobleaching, the DNA sensor demonstrates high sensitivity and specificity. The results demonstrate great potential of such nanomaterials as oligonucleotide sensors.

Keyword list

Photon upconversion; DNA detection; Energy transfer; Nanoparticles

Introduction

Advances in the field of optics has lead to wide acceptance of optical based biosensors, due to their ability to do remote sensing, highly non-invasive for *in vivo* studies and to provide high selectivity and sensitivity.[1] Biosensors relying on photonic interaction with analytes for detection require some kind of fluorescent/luminescent labeling. For many years organic fluorophores have been used as conventional fluorescent tags. These dyes follow stokes law and absorb one high-energy photon and emit one low-energy photon. These dyes in solution exhibit broad emission and excitation bands and tend to show compromised photo stability at prolonged exposure to intense excitation sources. Use of such dyes in the design of

biosensors has played very crucial role in the development of optical biosensors. Nevertheless, there has been constant desire in the research community to seek for higher selectivity and sensitivity for the designed sensors. In an effort to achieve higher selectivity and sensitivity, use of intense excitation source, low noise from background, strong signals from the dyes becomes highly desirable feature for biosensing.

In contrast to organic dyes there exists a type of materials, which follow anti-stokes law and absorb two or more low-energy photons and emit one high energy photon.[2] These materials are known as photon upconverting materials. These materials absorb in near infra red (NIR) region, are highly photostable, exhibit narrow emission and excitation bands. Thus photon upconverting materials demonstrate outstanding prospects for use in biosensing as well as in imaging-based application as luminescent tags. There have been many materials exhibiting upconversion properties, although photon upconverting materials based on $\text{NaYF}_4: \text{Yb}^{3+}, \text{Er}^{3+}$ or $\text{NaYF}_4: \text{Yb}^{3+}, \text{Tm}^{3+}$ demonstrate higher luminescent property due to low phonon- photon coupling. The emission bands of the upconverting materials depend upon the choice of the acceptor ions. These upconverting materials find extensive use in display technologies, probable use in sensing applications, imaging and many others.

In recent years many researchers have demonstrated the efficient use of photon upconverting particles in biosensing.[3–6] Application of photon upconverting particles in imaging and sensing has drawn considerable interest of researcher towards the synthesis and characterization of such nanoparticles. Synthesis of nanoparticles with or without scaffolding of 5 – 50 nm range without compromising the quantum yield and their subsequent use in bio-related application is highly desirable. In this proceeding, we present our ongoing efforts in the direction of synthesis and characterization of photon upconverting particles such as $\text{NaYF}_4: \text{Yb}^{3+}, \text{Er}^{3+}$, along with their subsequent use in DNA sensing applications.

Experimental

Chemicals

All chemicals were used as bought without further purification. NaF , $\text{YbCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{YCl}_3 \cdot 6\text{H}_2\text{O}$, potassium phosphate mono basic (K_2HPO_4) and potassium phosphate dibasic (KH_2PO_4), ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), tetraethyl orthosilicate (TEOS) were purchased from Sigma Aldrich, where as cyanogens bromide (CNBr) was purchased from Fisher Scientific. Phosphate buffer of require pH and concentrations were prepared by mixing KH_2PO_4 and K_2HPO_4 in calculated amounts. All DNA sequences used were purchased from Integrated DNA Technologies (Coralville, IA).

Synthesis of photon upconverting $\text{NaYF}_4: \text{Yb}^{3+}, \text{Er}^{3+}$ nanoparticles

Photon upconverting particles were synthesized in laboratory using wet chemistry route. The synthesis route was a modification over previous method described elsewhere.[7] Here we tend to briefly present the synthesis procedure followed. 3.2 ml of YCl_3 , 0.68 ml of YbCl_3 , 0.12 ml ErCl_3 , mixed together with 4ml of EGTA of 0.2 M concentration in a clean glass vial. This mixture was stirred overnight and then 12 ml of 0.83M NaF was added to the

stated mixture, allowed to react for 2 hours at 130°C in autoclave, and then nanoparticles were separated from the solution by centrifuging at 14000 rpm. The synthesized nanoparticles were washed with acetone and then with DI water several times to wash out organic matter. After several washing synthesized nanoparticles were annealed at 325°C for 3 hours.

TEM characterization of NaYF₄: Yb³⁺, Er³⁺ nanoparticles

Synthesized nanoparticles were dispersed in methanol and sonicated thoroughly using BRANSON Sonifier. A drop of the suspension was deposited on a copper-grid covered with carbon-coated Formavar film (Electron Microscopy Sciences, PA). Samples were analyzed using JOEL TEM.

Luminescence characterization of NaYF₄: Yb³⁺, Er³⁺ nanoparticles

Synthesized nanoparticles after annealing at 325 °C were suspended in 100 mM phosphate buffer (pH 7.0) by rigorous stirring. The suspension was excited at 975nm, using a diode laser (CrystaLaser, NV) at ~ 300 mW. Emission slit was fixed at 2nm and PMT was operated at 1kV.

Silica coating

The synthesized nanoparticles (NaYF₄: Yb³⁺, Er³⁺) were coated with a thin silica layer to allow further attachment of recognition elements. The procedures for coating were a variation of the well-known Stöber reaction.[8] In brief, the synthesized nanoparticles were suspended in 80 ml of 2-propanol by sonicating thoroughly. To the suspension 8.94 ml of 28% ammonium hydroxide, 7.5 ml of DI water and 0.1 ml of TEOS were added. To ensure uniform coating, mixture was allowed to stir continuously for 24 hours and then, coated nanoparticles were separated by centrifuging at 14000 rpm. The separated nanoparticles were thoroughly washed with methanol and DI water and collected as thick slurry for later use.

Oligonucleotide sequences

Four Oligonucleotide sequences based on desired detection scheme were ordered. These sequences were 13, 11, 26 and 26 bases long, respectively, as listed in Table 1. Various targets ordered were based on the segments of the human hemoglobin beta chain (HBB). The 13-base long oligo target, labeled as DNA_probe, was modified at its fifth prime end with amine, for attachment of DNA with silica-coated photon upconverting nanoparticles. DNA_Mis is a mutant form of HBB, available at NIH's NCBI gene bank as (AY356351).[9] The acceptor dye, N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), is attached to the 11-base long oligonucleotide (DNA_FI).

Attachment of DNA to silica coating

The amine modified DNA was attached to the silica modified nanoparticles. The following protocol was based on 100 mg of silica coated nanoparticles. Silica coated particles were weighed and washed twice in 10 ml phosphate buffer of pH 8.0, with 1 mM EDTA. After washing the nanoparticles were separated and re-suspended in the 9.5 ml of sodium

carbonate buffer of 2 M concentration. To the suspension 1 mg of cyanogen bromide dissolved in 0.5 ml of acetonitrile was added and allowed to stir for 2 minutes. Activated nanoparticles were separated out by centrifuging at 14000 rpm and then washed several times with ice cold phosphate buffer (pH 8.0) followed by DI water. Then these particles were re-suspended in 5 ml of phosphate buffer (pH 8.0) to which ~420 μ L of 50 μ M DNA_probe was added. Conjugation reaction was allowed to carry out at 4 °C for 24 hours. After 24 hours reaction mixture was quenched with large amount of phosphate buffer of pH 7.0. DNA-conjugated nanoparticles were separated and washed several times with the phosphate buffer of pH 7.0 and then re-suspended in ~ 600 μ L phosphate buffer of pH 7.0 for later use.

Assay measurements

Assay measurements were carried out using spectrofluorometer (Photon Technologies International) equipped with R928 photon multiplier tube from Hamamatsu. To one of the side ports, diode laser of ~ 975 nm and ~1W power was externally attached. Spectrofluorometer was also attached with a water bath (NESLAB, RTE-10) to maintain the sample temperature. PMT was operated at 1.1KV, with laser output of 400 mW, slit width of 2nm and cuvette temperature at 22 °C, for all the measurements.

70 μ L of DNA-conjugated nanoparticles suspension and 60 μ L of 1 μ M DNA_FI were mixed together as blank, and three spectra were acquired. To the blank, 1 μ M of DNA_Tar or 4 μ M of DNA_Mis was added with increment of 2.5 μ L. Data were acquired 25 minutes after each addition to allow for hybridization. Hybridization reaction was carried out in phosphate buffer of pH 7 and 100 mM concentration.

Results and discussion

TEM characterization of photon upconverting nanoparticles

Synthesized nanoparticles after annealing were characterized for the size distribution. The micrograph acquired (Figure 1) for the nanoparticles show the size distribution of nanoparticles ranging from 50 nm to 100 nm. The size of the synthesized nanoparticles was determined to be 72 nm on randomly taking average of 50 nanoparticles.

Luminescent characterization of photon upconverting nanoparticles

The synthesized photon upconverting nanoparticles exhibit little luminescence before annealing. After annealing at 325 °C for four hours, they display intense luminescence upon excitation at ~975 nm, shown in Figure 2. These bands in visible region corresponds corresponding to $^4S_{3/2} \rightarrow ^4I_{13/2}$, $^4F_{9/2} \rightarrow ^4I_{15/2}$, $^2S_{3/2} \rightarrow ^4I_{15/2}$, and $^2H_{11/2} \rightarrow ^4I_{15/2}$ electronic transitions. The energies associated with these electronic transitions are approximately 11800, 15000, 18200 and 18800 cm^{-1} respectively, as reported in the literature.[10]

Scheme of the detection

The detection scheme was based on Luminescent Resonance Transfer (LRET) in a sandwich format, where two short oligonucleotides capture one long target (Figure 3). One of the short

probes was attached to the nanoparticles; whereas the other short oligonucleotide was labeled with TAMRA. Photon upconverting particles served as energy donor whereas TAMRA acted as energy acceptor. The donor-acceptor pair was chosen based on the spectral overlap from the emission of nanoparticles (~ 525 nm to 550 nm) with the excitation band of TAMRA (~550 nm). The dye absorbed emission at 550 nm and emitted at 575 nm. When DNA-conjugated nanoparticles and DNA_FI were added as a blank, there was little energy transfer between the two. Upon addition of perfectly matched target, DNA duplexes between DNA_Probe, DNA_FI and DNA_Tar were formed, leading to LRET between the donor and the acceptor. Thus increase in the emission from the TAMRA accompanied with subsequent increase in concentration of target DNA concentration would indicate any hybridization occurred.

Sensitivity of the detection

The sensitivity of the detection was calculated based on the detection of the matched target. Upon hybridization of probe and target lead to increase in the emission of dye, normalized with respect to the emission from the donor nanoparticles. Such ratiometric detection schemes not only account for any disturbance inherent to the measuring and surrounding environment but also increase the dynamic range of the detection.[3,11] Signal f was calculated as the percentage increase in the emission of the dye, mathematically given as

$$f = [(I_{575}/I_{537}) - (I_{575}/I_{537})_{\text{blank}}] / (I_{575}/I_{537})_{\text{blank}}$$

This calculated f value was plotted against the increase in concentration of the target in the blank due to subsequent additions. Based on S/N = 3 ratio, first five points of the perfectly matched targets were used to determine the detection limit, as 0.65 nM. The detection limit reported was found comparable to or better than many other reported schemes.

Selectivity of the detection scheme

In order to critically assess the selectivity capability of the sensor, DNA_Mis, a single nucleotide variant (sickle cell anemia, point mutation gene portion), was chosen, presented in the Table 1. The point mutation is flanked by two guanines, which provides higher stability to annealing or to destabilizing duplex and hence offering challenge to any kind of superior sensors. In bioanalytical analysis success of sensor critically depends on its ability to distinguish desired targets from matrix element present in very high concentration compared to desired target. Thus in order to test the superiority of the sensor, DNA_Mis was taken in 4× greater concentration to the perfectly matched target. The signal f plotted for DNA_Tar and DNA_Mis against concentration yields a linear response of slope which is 7.5 times more sensitive for the perfectly matched target than for the mismatch target.

Conclusion

DNA probe of our design has demonstrated the ability to clearly distinguish perfectly matched target from the single nucleotide variant at four time higher concentration as compared to matched targets. The proposed design of DNA probe based on photon

upconverting nanoparticles is full of promise for high selective and sensitive oligonucleotide detection. These nanoparticles probably can be used for detecting genes responsible for cancer, without further amplification and transcription of RNA into DNA.

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Figure 1.
TEM micrograph of photon upconverting particles after annealing

Figure 2.
Luminescent spectra of photon upconverting nanoparticles after annealing, suspended in phosphate buffer of pH 7 of 100 mM concentration and 1mM EDTA.

Figure 3.
Sandwich format for detecting DNA Targets

(a)

(b)

Figure 4.

Plot of f value vs.: (a) $[DNA_Tar]$ and (b) $[DNA_Mis]$, upon excitation of photon upconverting particles at 975 nm.

Table 1

Oligonucleotide	Sequence	No. of bases
DNA_Probe	5'-/AmineC ₆ /CAGTAACGGCAGA-3'	13
DNA_FI	5'-/TAMRA/TCTCCACAGGA-3'	11
DNA_Tar	5'-TCCTG <u>T</u> GGAGAAGTCTGCCGTTACTG-3'	26
DNA_Mis	5'-TCCTG <u>A</u> GGAGAAGTCTGCCGTTACTG-3'	26