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Highly sensitive and selective oligonucleotide sensor for sickle cell disease gene using photon upconverting nanoparticles

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Abstract

We report the design of an oligonucleotide sensor for the detection of point mutation associated with sickle cell disease. The sensor was based on luminescence resonance energy transfer between a donor and an acceptor. Photon upconverting nanoparticles (NaYF₄ doped with Yb³⁺ and Er³⁺) were used as the donor and a conventional fluorophore, N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), as the acceptor. The sensor could detect the perfectly matched target, in the background of the mismatched target or other oligonucleotides of random sequences. The detection limit of this sensor towards perfectly matched target was calculated to be 120 femtomoles, with no photobleaching. Oligonucleotide sensors of such design demonstrate high sensitivity and specificity.

Keywords

Photon upconversion; sickle cell disease; oligonucleotide detection; point mutation; locked nucleic acid

1. Introduction

Extensive efforts have been invested in the identification of specific variations and mutations in human genome. DNA detection techniques have been an active field of study for years due to their widespread use in various applications such as, forensic analysis, gene therapy, and diagnosis and treatment of genetic diseases. These applications demand high sensitivity and specificity. Many techniques such as polymerase chain reaction (PCR), DNA microarray, and surface plasmon resonance have laid a foundation for DNA detection. All of these techniques utilize optical, electrical, electrochemical or gravimetric detection schemes (Foder et al., 1991; He et al., 2000; Fritzsche et al., 2003; Wang et al., 2003).

DNA hybridization-based detection is a major technique for the diagnosis of genetic disease where clinical symptoms are linked to DNA sequence alterations. DNA hybridization is highly specific and becomes very sensitive when coupled with an optical detection scheme. Optical detection schemes usually require some kinds of labeling such as fluorophore tagging (Zhao et al., 2003), or the use of molecular beacons (Tyagi et al., 1996; Zhang et al., 2001). Typically, fluorophores and quantum dots are used for signaling the detection of the hybridization of DNA (Edgar et al., 2006). These photoluminescent species are usually excited at shorter wavelengths and emit at longer wavelengths. Yet, organic fluorophores tend to suffer from photobleaching, while the toxicity and cytotoxicity of quantum dots somewhat limit their use. Thus, there is an ongoing effort to search for better labeling reagents.

Photon upconversion is a phenomenon where, upon the excitation of low energy photons, high energy photons are emitted. This is achieved through multi-photon processes (Auzel 1973 and 2004). Materials with upconverting properties are much less common than their downconverting counterparts (Suyver et al., 2005), usually including some kinds of lanthanide ions. There have been reports of using photon upconverting materials in such applications as display and bioassay, among others (Niedbala et al., 2001; Corstjens et al., 2003 and 2005; Kuningas et al., 2005).

Photon upconversion process is generally less efficient than the downconversion process. However, because most nontarget materials in a complex matrix do not possess such photon upconversion properties, a good signal-to-noise ratio can be expected regardless of the weak signal. This makes photon upconverting materials ideal for identification of trace amounts of target molecules in sensing and luminescence reporting applications. In addition, photon upconverting materials display narrow emission and lack of autofluorescence and have no photobleaching. All of these are desired features for luminescence-based sensors.

$\text{NaYF}_4:\text{Yb}^{3+}$ (doped with Er^{3+} or Tm^{3+}) have been recognized as one of the most efficient photon upconverting phosphors. When they are excited by an Infrared (~ 975 nm) source, various strong visible bands appear, depending on the type of the dopant. In a previous report, we demonstrated a scheme for detecting oligonucleotide targets, based on such photon upconverting nanoparticles of 60-120 nm in size, with high specificity and selectivity (Zhang et al., 2006).

Sickle cell disease is a medical condition where the red blood cells become sickle-shaped. These sickle-shaped blood cells are stiff and tend to form clumps, which block blood flow in the blood vessels leading to the limbs and organs. Blocked blood vessels may cause pain, infections, and organ damage. It is known that the gene defect associated with sickle cell disease is the mutation of a single nucleotide (from A to T) of the β -globin gene, resulting in a valine instead of a glutamate to be expressed. Sickle cell disease occurs when a person inherits two sickle cell genes from the parents. People inheriting a single sickle cell gene from a parent will not develop the disease, but will have sickle cell trait, which means they can pass the sickle cell gene on to their own children. There are more than 70,000 Americans with sickle cell disease, with even more, about 2 million, Americans having sickle cell trait. It is thus of practical interest to diagnose sickle cell disease, and furthermore, to detect sickle cell trait.

The underlying principle for the detection is luminescence resonance energy transfer (LRET). There have been recent reports regarding the use of LRET with photon upconverting materials (Zhang et al., 2006; Kuningas et al., 2006 and 2007) to detect targets such as DNA and small molecule. In this paper we report the design of a nucleotide sensor based on photon upconverting nanoparticles, following the scheme first described in the previous report (Zhang et al., 2006). We use 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 two capturing probes are labeled with the donor and the acceptor, respectively. The presence of the target DNA is indicated by the increase of the normalized acceptor's emission. The fact that photon upconverting particles do not photobleach allows the sensor to be excited by a highly intense laser source, which helps improve the sensor's performance.

2. Materials and methods

2.1 Chemicals

All chemicals were used as received without further purification. NaF, $\text{YCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{YbCl}_3 \cdot 6\text{H}_2\text{O}$, ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) and

ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. Cyanogen bromide (CNBr) was purchased from Fisher Scientific. Phosphate buffer solution was prepared from KH_2PO_4 and K_2HPO_4 . All DNA strands were purchased from Integrated DNA Technologies Inc (Coralville, IA).

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

The photon upconverting $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles were home-made, based on the procedures in the literature (Yi et al., 2004) with some modifications of our own. In brief, 3.2 ml of YCl_3 , 0.68 ml of YbCl_3 , 0.12 ml of ErCl_3 , and 4 ml of EGTA (all of 0.2 M aqueous solutions) were mixed in a glass vial under stir overnight. Then 12 ml of 0.83 M NaF solution was added to the mixture under stir for two hours. The vial was then placed in an autoclave for two hours at 130 °C. Particles were separated from the solution by centrifuging at 13400 rpm. These particles were then thoroughly washed, first with methanol and then by acetone. They were then annealed at 350 °C for four hours.

2.3 TEM characterization

The $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles were first suspended in methanol through sonication to ensure the extraction of representative dispersion volumes. Then a drop of suspension was deposited on a Formvar-covered carbon-coated copper grid (Electron Microscopy Sciences, PA) and let dried at room temperature. The nanoparticles were analyzed using a JEOL TEM at an accelerating voltage of 80 kV.

2.4 Oligonucleotides sequences

Several single-stranded DNA were designed, with sequences listed in Table 1. The DNA probes were based on the target sequence of Homo sapiens mutant hemoglobin beta chain (HBB) gene (AY356351), available at NIH's NCBI GenBank (NCBI). The sequence of DNA_Tar was a segment of the HBB gene. The sequence of DNA_Mis was a segment of the variation of HBB, responsible for the sickle cell disease. There was only a single base difference between DNA_Tar and DNA_Mis, at the 8th-base position from the 3'-end as indicated by the underline. Two of the short oligonucleotides, DNA_Np and DNA_Fluo, were complementary to different portions of DNA_Tar. The DNA_Np strand was amine-modified at its 5'-end, to facilitate conjugation to the $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles. This strand also included a locked nucleic acid (LNA)-modified oligonucleotide (+A), as indicated by the '+' sign in the front. DNA_Fluo strand was labeled at its 5'-end with a fluorophore, TAMRA, acting as an energy acceptor.

2.5 Silica Coating on Nanoparticles

The $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles were coated with a thin layer of SiO_2 through a variation of the well-known Stöber process (Santra et al., 2000). In brief, 30 mg of nanoparticles was dispersed in 80 ml of n-propanol under sonication using a Branson Sonifier 450. Then, 8.94 ml of 28% ammonia, 7.5 ml of de-ionized water, and 10 μl of tetraethyl orthosilicate (TEOS) were added. The mixture was under vigorous stirring at room temperature for 24 hours. The resulting nanoparticles were then centrifuged and collected. They were thoroughly washed in methanol and de-ionized water, respectively. A thin SiO_2 layer was thus deposited on the nanoparticles surface. Silica coating thickness can be controlled by varying the amount of TEOS. After final washing, coated $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles were left in the slurry form.

2.6 Attachment of DNA probe to the photon upconverting nanoparticles

DNA probe were ready to attach to the photon upconverting nanoparticles after the $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles were coated with the silica layer. The following protocols were used to attach the amine-modified DNA (DNA_Np) to the nanoparticles (Bangs Lab): The coated $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles were weighed and washed twice with coupling

buffer (0.1 M phosphate buffer of pH = 8.0, 1mM EDTA). For every 100 mg of nanoparticles, 10 ml of phosphate buffer was used. After a second wash the nanoparticles were suspended in 9.5 ml of activation buffer (2 M Na₂CO₃). One gram of CNBr was dissolved in 0.5 ml of acetonitrile per 100 mg of nanoparticles. This solution was added to the suspended nanoparticles, and the reaction was allowed to proceed for two minutes. The reaction mixture was quenched by adding a large volume of ice-cold de-ionized water. The nanoparticles were then separated and suspended into 5 ml of coupling buffer, and DNA_Np was added. The concentration of DNA_Np was set at approximately five times in excess of the calculated monolayer. The reaction was carried out for 24 hours at 4 °C under constant stirring. These conjugated NaYF₄:Yb³⁺,Er³⁺ nanoparticles were then separated, and washed several times with the storage buffer (0.1 M phosphate buffer of pH = 7.0, 1 mM EDTA) before being stored at 4 °C.

2.7 Photoluminescence Measurements

The photoluminescence measurements were carried out at room temperature using a spectrofluorometer (Photon Technologies International) equipped with a Hamamatsu R928 photomultiplier tube (PMT) operating at 1.1 kV. Instead of using the built-in Xenon lamp as the illumination source, a 975 nm diode laser (CrystaLaser, NV) with a maximum power of 1 W was attached to the side port of the spectrofluorometer, and used to excite the sample. The emission slit was set at 2 nm for all measurements.

The various concentrations used during the experiment were as follows: 1 μM for DNA_Fluo; 0.25 μM for DNA_Tar and DNA_Mis. For every series of measurements, the mixture of 80 μL DNA_Fluo, 6.4 mg of photon upconverting nanoparticles conjugated with DNA_Np, and 120 μL of phosphate buffer (0.1 M, pH = 7.0, 1mM EDTA) was first treated as blank, and photoluminescence spectrum was taken. Various volumes of the targets (DNA_Tar or DNA_Mis) were then added to the blank, and spectra were taken. There was a 10-minute waiting period between every addition of the targets and the measurement, to allow for complete hybridization. All measurements were carried out under a constant temperature of 27 °C, with the output of the diode laser set at 400 mW.

3. Results and discussions

3.1 Characterization of the photon upconverting nanoparticles

The home-made photon upconverting nanoparticles were characterized both spectroscopically and microscopically. Figure 1 shows a typical photoluminescence spectrum of the NaYF₄:Yb³⁺,Er³⁺ nanoparticles, after annealing, upon the excitation by a diode laser of around 975 nm. Figure 2 is a TEM image of the nanoparticles, which shows the particle size ranging from 60 nm to 100 nm, with an average of 72 nm (calculated by averaging 50 nanoparticles randomly selected in the TEM image). We noticed that, before annealing, the NaYF₄:Yb³⁺,Er³⁺ nanoparticles displayed weak photon upconversion, consistent with previous report in the literature (Yi et al., 2004). Note that the photon upconverting nanoparticles are not susceptible to photobleaching, even under intense laser irradiation (~500 mW) for extended period of time (Figure 3).

3.2 Design of DNA probes

The principle of this design is shown in Figure 4. A sandwich-type hybridization format was adopted by using two shorter oligonucleotides with designed sequence to capture the longer target oligonucleotide. While designing oligonucleotide strands of DNA, proper care was taken to avoid any loops and secondary structure in both short oligonucleotides. One of the short oligonucleotides was covalently bound to the photon upconverting nanoparticles, while the other was labeled with a fluorophore. TAMRA was chosen as the fluorophore in this study,

because its excitation spectrum overlaps with the emission spectrum of the upconverting nanoparticles. Upon excitation by an infrared laser, the photon upconverting nanoparticles would emit visible light. Without the target oligonucleotide, the two shorter oligonucleotides were separate. Energy transfer between the photon upconverting nanoparticles and TAMRA was negligible. The addition of the target oligonucleotide brought the fluorophore close to the nanoparticle. Thus energy transfer took place between the photon upconverting nanoparticles and TAMRA as shown in Figure 5, where the NaYF₄:Yb³⁺,Er³⁺ nanoparticle emission (537 nm) decreased while TAMRA emission (575 nm) increased as the amount of the target oligonucleotide increased. By monitoring the TAMRA emission upon an infrared excitation, one can detect the presence of the target oligonucleotide.

In this study, the difference between the two oligonucleotide targets is rather small (from A to T), a challenge for the sensor design. To improve the specificity, LNA-modified oligonucleotide was included in one of the probes (DNA_Np). A locked nucleic acid is a modified RNA nucleotide, modified with an extra bridge connecting the 2' and 4' carbons of the ribose moiety. This modified conformation enhances the base stacking, significantly increasing the thermal stability of oligonucleotides. LNA-modified oligonucleotides have been shown to possess very highly hybridization affinity toward their complementary DNA/RNA strands. (Vester et al., 2004) The inclusion of LNA-modified oligonucleotide helps to improve the capability of differentiating the single-base (A-T) mismatch between the normal gene and the mutant, as shown in later section.

3.3 Sensitivity of the detection

We have determined the sensitivity of this method to detect the perfectly-matched target, DNA_Tar. Taking advantage of the ratiometric nature of this detection scheme, the intensity of TAMRA emission ($\lambda \sim 575$ nm) was normalized with respect to the emission of the upconverting particle ($\lambda \sim 537$ nm), so as to have less influence from environmental factors affecting optical measurements, and a larger dynamic range (Zhang et al., 2001 and 2006). The results are shown in Figure 6, where an f value is plotted against the concentration of the target DNA. The parameter f is defined as, $f = (I_{575}/I_{537})/(I_{575,0}/I_{537,0}) - 1$, where I_{575}/I_{537} is the normalized TAMRA emission at a given target DNA concentration (taking into consideration the slight volume change) and $I_{575,0}/I_{537,0}$ is the corresponding value for the blank solution. This f parameter indicates the percentage increase of the normalized TAMRA emission over the blank. The slope of the first 4 data points was used to calculate the detection limit. Based on the standard deviation and the $S/N = 3$ criteria, the detection limit for DNA_Tar is calculated to be 0.6 nM, equivalent to 120 femtomoles.

Note that, because the NaYF₄:Yb³⁺, Er³⁺ nanoparticles do not photobleach, higher power excitation source can be used to increase the signal level. Besides, the assay volume (~ 0.2 mL in this study) can also be reduced by, for example, using a microscopic system. We thus believe that the sensitivity of the sensor can be further improved.

3.4 Selectivity of the detection

The nucleotide sensor of this design displays high specificity. To demonstrate, a DNA sequence with a single-nucleotide mismatch, DNA_Mis, was used as the target instead of the perfectly matched DNA_Tar. The sequence of DNA_Mis corresponds to the gene variation that is responsible for the sickle cells disease. The difference in the two sequences, as highlighted in Table 1, leads to a single-base mismatch between DNA_Np and part of DNA_Mis, while DNA_Fluo and DNA_Mis remain perfectly matched. The plot of f value against the DNA_Mis concentration is shown in Figure 6. The results show a clear discrimination of the single-nucleotide (A-T) mismatched targets. Notice that the A-T mismatch in the DNA_Tar/DNA_Mis is flanked by two guanine (G) nucleotides, which makes it even harder to resolve

the mismatch. We thus expect that oligonucleotide sensors of such design could achieve better sensitivity and specificity for other single-nucleotide mismatches.

To further study the discrimination capability of the designed probes, we carried out a separate control experiment, where an oligonucleotide of random sequence (DNA_Random) totally non-complementary to the two probes, was used as the target. The results, also shown in Figure 6, indicate that oligonucleotides of random sequence barely interfere with the assay. This is an important feature that has great implication in clinical applications. It would allow the detection of sickle cell trait in people not developing the disease.

4. Conclusions

In conclusion, we report the detection of point mutation of the gene associated with sickle cell disease, using a nucleotide sensor based on photon upconverting nanoparticles. The method displays high sensitivity and selectivity that differentiates single base mismatch. It can also detect the perfectly matched target in a matrix of DNA mixtures, which allows the detection of sickle cell trait. The use of photon upconverting particles as LRET donor allows the excitation power to be greatly increased, as the photon upconverting particle is extremely stable against photobleaching, thus improving the sensor's sensitivity. The inclusion of a locked nucleic acid-modified oligonucleotide helps improve the sensor's discrimination capability. All of these features are very appealing for such type of DNA/RNA sensor to be used in practical applications.

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Figure 1. Photoluminescence spectrum of the NaYF₄:Yb³⁺,Er³⁺ nanoparticles used in this study, excited by a 975 nm diode laser.

Figure 2.
TEM image of the $\text{NaYF}_4:\text{Yb}^{3+},\text{Er}^{3+}$ nanoparticles used in this study.

Figure 3.
Time course measurement at 537 nm emission of NaYF₄:Yb³⁺,Er³⁺ nanoparticles excited by a 975 nm diode laser at ~500 mW.

Figure 4.
Schematic of the oligonucleotide sensor design.

Figure 5. Normalized intensities of 537 nm and 575 nm emissions (excited at 975 nm), as DNA_Tar was introduced into the mixture of DNA_Np and DNA_Fluo.

Figure 6.

Change of f -value as target DNA concentration was increased in respective runs. f value is defined in text. Both DNA_Np and DNA_Fluo are in excess compared to the targets in all runs. All excited by a 975 nm diode laser at 400 mW.

Table 1

Probes and targets DNA sequences.

Oligonucleotide	Sequence	No. of bases
DNA_Np	5'-/AmineC ₆ /TTC TTC +ACA GGA-3'	12
DNA_Fluo	5'-/TAMRA/CAG GTG CAC C-3'	10
DNA_Tar	5'- GGT GCA CCT GAC TCC TGT GGA GAA G -3'	25
DNA_Mis	5'- GGT GCA CCT GAC TCC TGA GGA GAA G -3'	25
DNA_Random	5'- GTT GCG TCG ATC CTT CCT GGG CAT GG -3'	26