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Design of a Highly Sensitive and Specific Nucleotide Sensor Based on Photon Upconverting Particles

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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, a process called downconversion. 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 upconversion. Photon upconverting materials convert lower-energy light to higher-energy light, which is realized through excitation with multiple photons. Materials with upconverting properties are much less common than their downconverting counterparts.[1] They typically have narrower absorption and line emission spectra, as compared to the downconverting materials. Because most nontarget materials in a complex mixture do not possess such photon upconversion properties, a dramatically enhanced signal-to-noise ratio is expected in sensing and luminescence reporting applications. This makes photon upconverting materials ideal for identification of trace amounts of target molecules.

To the other front, DNA/RNA analysis is of great importance in molecular biology, genetics, and molecular medicine. Extensive efforts have been invested in identification of specific variations and mutations in human genome. DNA hybridization-based detection is a major technique for the diagnosis of genetic disease where clinical symptoms are linked to DNA sequence alterations.[2,3] One would naturally think of applying materials with photon upconverting properties for the sensitive detection of oligonucleotides. Indeed, there have been reports of upconverting phosphor particles as reporters to detect single-stranded nucleic acids[4,5] where these particles were used as direct labeling reagents. In this report, we propose and demonstrate a novel scheme of developing a highly sensitive and specific single-stranded nucleotide sensor based on the photon upconverting particles. The design is versatile and easy to implement.

The principle of this design is shown in Figure 1. Here, a sandwich-type hybridization format is adopted by using two shorter oligonucleotides with designed sequence to capture the longer target oligonucleotide. One of the short oligonucleotides is covalently bound to the photon upconverting particle, while the other is labeled with a fluorophore. The fluorophore is so chosen that its excitation spectrum overlaps with the emission spectrum of the upconverting particle. Upon illumination by an infrared laser, the photon upconverting particles would emit visible light. In the presence of the target oligonucleotide, the fluorophore is brought close to the particle, and energy transfer takes place, leading to the light emission from the fluorophore. Essentially, by monitoring the fluorophore emission upon an infrared excitation, one can detect the target oligonucleotide.

We have applied this strategy by synthesizing several single-stranded DNAs with nucleotide sequences listed in Table 1. DNA1 (16mer) was amine-modified at the 5′-end, while DNA2 (10mer) was labeled at its 5′-end with a fluorophore, carboxytetramethylrhodamine (TAMRA), whose excitation and emission peaks are 559 and 583 nm, respectively. The photon upconverting particles used in this study emit green color (peak at ~537 nm) when excited by a diode laser of 975 nm. After the particles are coated with a thin layer of SiO_2 through the well-known Stöber process, the amine-modified DNA1 is covalently attached to this SiO_2 layer following procedures described in a previous study.[6] The sequences of DNA1 and DNA2 are so chosen that they are each complementary to a portion of the target oligonucleotide, DNA_tar. Solutions containing DNA1-photon upconverting particles and DNA2-TAMRA, respectively, are then mixed. In the absence of target DNA_tar, the particle attached to DNA1 and the TAMRA-labeled DNA2 are far apart, and energy transfer from the particles to TAMRA is trivial upon 975 nm illumination. With the addition of the 26-base target DNA_tar into the mixture, the signal intensity corresponding to the TAMRA emission increases significantly. This indicates that TAMRA and the photon upconverting particle are brought close and stabilized by formation of double-stranded DNA and energy transfer takes place. Figure 2 shows the photoluminescence spectra of the mixture in 50 mM PBS buffer solution (pH = 7.4 and 1 mM EDTA), under 975 nm excitation, before (line A) and after (line B) the addition of DNA_tar at room temperature. These results demonstrate that a nucleotide sensor can be developed based on the unique feature of photon upconverting particles.

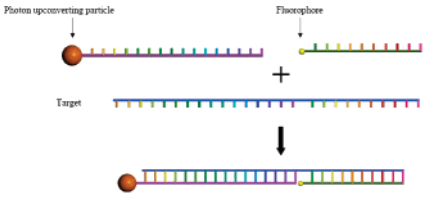

Figure 1. The schematic of the nucleotide sensor design.

Table 1. Probes and Targets DNA Sequences

DNA_1	5′-(C6NH)-CCATGCCCAGGAAGGA-3′
DNA_2	5′-(56-TAMRA)-TCGTCGCTAC-3′
DNA_tar	5′-GTA**G**CGACGATCCTTCCTGGGCATGG-3′
DNA_mis	5′-GTA**C**CGACGATCCTTCCTGGGCATGG-3′

The most significant feature of this type of nucleotide sensor is its high sensitivity, mainly owing to the lack of autofluorescence upon 975 nm excitation. We have determined the sensitivity of this method to detect the target DNA_tar. The results are shown in Figure 3. Line A in Figure 3 plots I_{575nm} against a range of target DNA concentrations and shows a reasonably good linear relationship. Note that nucleotide sensors of this type have the characteristics typical for a ratiometric dye and that the ratio of $I_{TAMRA}/I_{upconverting}$ can be used as the signal indicator instead of I_{TAMRA} only. Since $I_{upconverting}$ decreases when energy transfer takes place, the

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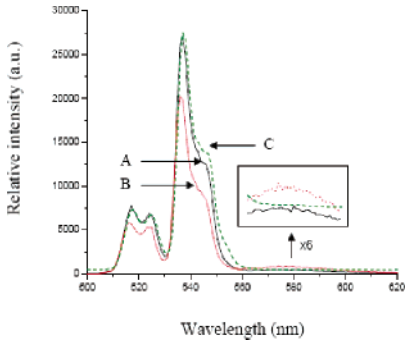

Figure 2. Photoluminescence spectra of the mixture of DNA1-photon upconverting particles and DNA2-TAMRA at 975 nm excitation: (A) before addition of DNA_tar; (B) after addition of DNA_tar; (C) after addition of DNA_mis. The mismatched [DNA_mis] is 4 times that of [DNA_tar]. Particle-bound DNA1 and free TAMRA-labeled DNA2 concentrations are present in excess during the annealing reaction.

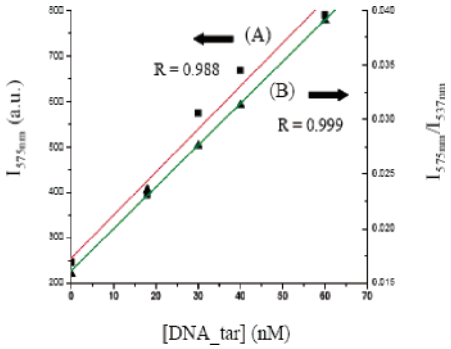

Figure 3. (a) I_{575nm} vs [DNA_tar]; (b) I_{575nm}/I_{537nm} ratio vs [DNA_tar], excited at 975 nm. Both [DNA1] and [DNA2] are in excess compared to the [DNA_tar].

$I_{TAMRA}/I_{upconverting}$ ratio will have better linearity, less influence from environmental factors affecting optical measurements, and a larger dynamic range, as compared to I_{TAMRA}.[7] This further provides a built-in calibrating feature.

We therefore plot I_{575nm}/I_{537nm} ratio against [DNA_tar] in Figure 3, line B. The excellent linear relationship permits for quantitative detection of the target concentration. Based on the standard deviation of the linear fit and the $S/N = 3$ criteria, the detection limit for DNA_tar is calculated to be 1.3 nM, which is comparable to that for many other DNA probes.[8,9] We should point out that this detection limit is achieved with the low excitation power of around 50 mW. Since these lanthanide ions-based photon upconverting particles are extremely stable against photobleaching, more powerful laser sources could be used, thus improving the detection sensitivity further. This is a significant advantage over most of the fluorescence detection schemes based on downconversion where photobleaching is a major limitation in increasing the excitation power.

The nucleotide sensor of this design also 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 difference in the two sequences, as highlighted in Table 1, leads to a single-base mismatch between DNA2 and part of DNA_mis, while the DNA_mis and DNA1 remain perfectly matched. Line C in Figure 2 shows the photoluminescence spectra at room temperature of the DNA1/DNA_mis/DNA2 mixture, where [DNA_mis] is 4 times as concentrated, while the [DNA_tar], [DNA1], and [DNA2] are the same as those in line B. The results show a clear discrimination of the single-nucleotide mismatched target.

We further obtained a thermal denaturation profile of the DNA1/DNA_tar/DNA2 complex, where TAMRA emission at 575 nm was monitored under 975 nm excitation, while the hybridization temperature was increased. The results, as in Figure 4 (line A), clearly showed that there were two annealing transitions for the complex at the temperatures corresponding to the melting points of DNA1 and DNA2, respectively. A similar profile of the DNA1/DNA_mis/DNA2 mixture was also obtained and shown in Figure 4 (line B). To illustrate the DNA sequence-dependent specificity of the signal, [DNA_mis] is 10 times that of [DNA_tar] as in the DNA1/DNA_tar/DNA2 mixture. Based on the two profiles in Figure 4, we see that the specificity of detecting the perfectly matched target can be further improved if the temperature of the measurement is increased from room temperature to about 50 °C. This result also suggests that one can adjust the respective length of the two capturing probes to achieve the best specificity at a certain operation temperature. In this regard, a nucleotide sensor of this type is advantageous over some other types of nucleotide sensor, such as molecular beacons, as it offers the flexibility in designing nucleic acid probes for single-nucleotide polymorphisms studies.

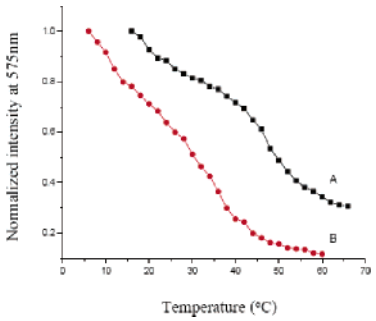

Figure 4. Thermal denaturation profile (normalized I_{575nm} vs temp) of the sandwich complexes excited at 975 nm: (A) DNA1/DNA_tar/DNA2 complex; (B) DNA1/DNA_mis/DNA2.

In conclusion, we have demonstrated a new design of a nucleotide sensor, which uses photon upconverting particles as energy donor and an appropriate fluorophore as an energy acceptor in a sandwich assay format. Such design displays high sensitivity, high specificity, and self-calibrating capability. By using Er^{3+}-doped $NaYF_4$ particles as donor and TAMRA as acceptor, we can quantitatively detect the perfectly matched target DNA with a detection limit of 1.3 nM, and distinguish targets with single-nucleotide variation, using a 50 mW excitation source. We expect nucleotide sensors of this type to be effective for applications in both DNA/RNA detection and protein-DNA/RNA interaction studies, on the basis of high sensitivity, low background, and lesser dependence on optical geometry and photobleaching.

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