Biosensors

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Fluorescence Resonant Energy Transfer Biosensor Based on Upconversion-Luminescent Nanoparticles**

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The rapid development of pharmacogenomic research and drug discovery, diagnostics of infectious and genetic diseases, and methodologies for forensic and genetic identification is now challenging chemists to find more efficient biological labels than traditional organic dyes which are resistant to photobleaching, nontoxic, biocompatible, monochromatic,

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highly luminescent, and, more importantly, ultrasensitive in both in vitro and in vivo bioassays. To find fluorophores that can meet these requirements, various semiconductor quantum dots (QDs) with tunable size-dependent emission, high quantum yields of photoluminescence (PL), broad excitation spectra, and narrow emission bandwidths have been developed and successfully applied in biological analyses.[1–5] However, they also have intrinsic limitations. On the one hand, their inherent toxicity and chemical instability limits their application in biological detection and medical diagnosis.[6] On the other hand, although these QDs work well under laboratory conditions, an increase in their background signal may be noted in the presence of interfering biomolecules (such as green-fluorescent proteins) and other fluorescent organic molecules that are usually present in biological and environmental samples and which can also be excited by UV radiation. The sensitivity of detection would hence be lowered. To improve the sensitivity, fluorescence resonant energy transfer (FRET) has been introduced into QD-based bioanalysis of molecular structure and protein–protein, protein–nucleic acid, and other interactions.[7,8] Owing to the specificity and the intrinsic sensitivity of FRET to small changes in donor–acceptor distances,[9] these FRET-QD systems have been demonstrated to show higher sensitivity. However, the inherent limitations of the fluorescence of QDs, including high background noise, potential toxicity, and instability, as mentioned above, cannot be eliminated by incorporating FRET technology and still restrict their application in the analysis of biological samples.

Hence, to find a more appropriate luminescent label remains a challenging task and is crucial to the development of modern gene technology and medical sciences. Upconversion (UC) nanophosphors, which are excited in the infrared region instead of the UV region to give emission in the visible domain, may be such an alternative. These UC nanophosphors show a high chemical stability, high quantum yields, and low toxicity, and their optical properties can be tuned by variation of lanthanide dopants and the host matrix.[10] In particular, the fluorescence from biological samples (background) upon excitation with IR radiation is extremely low as the interfering biomolecules in question absorb in the UV (not the IR) region. All these favorable properties have indicated the great potential of UC nanoparticles in the analysis of biological and environmental samples, and especially for fluorescence imaging in vivo.[11] Furthermore, if FRET technology can be coupled with bioassays based on UC nanoparticles, a further enhancement in the selectivity and sensitivity of detection can be expected. However, to the best of our knowledge, no such system has yet been reported. Herein, we demonstrate a novel biosensor for the detection of trace amounts of avidin which is based on FRET between bioconjugated UC nanoparticles and gold nanoparticles.

The UC nanoparticles we chose are $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Er^{3+}$. As can be seen in the spectrum shown in Figure 1 a, the nanoparticles show three emission bands at $\lambda =$ 525, 540, and 655 nm, which correspond to energy-transfer processes from the excited states $^2H_{11/2}$, $^4S_{3/2}$, and $^4F_{9/2}$, respectively, to the ground state $^4I_{15/2}$.[12] However, the peak at 540 nm is the main peak, while the other peaks are very

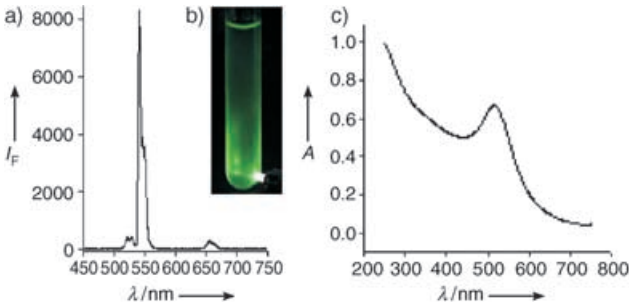

Figure 1. a) Upconversion luminescence spectrum of hexagonal-phase upconversion nanoparticles in solution upon excitation with a 980-nm laser; b) green luminescence observed from sample. c) UV/Vis absorption spectrum of 7-nm gold nanoparticles in aqueous solution.

weak in comparison, which makes it an excellent single-emission biological label. As typical organic fluorescent dyes display broad emission spectra with a long tail, an "impurity" in the form of the emission from the nanoparticles can introduce spectral cross-talk between different detection channels which thus makes them unsuitable for biological applications and creates difficulties in the quantification of the relative amounts of different probes. An ideal probe for multicolor experiments should emit at spectrally resolvable energies with a narrow, symmetric emission spectrum, and the complete set of probes should be excitable at a single wavelength.[2] Here, the monodispersed nanoparticles emit strong and pure green fluorescence with a symmetric and narrow emission spectrum upon excitation at a single wavelength (980-nm laser), which makes them more suitable as a probe for multicolor biological detection. The strong green UC luminescence from suspensions of the nanoparticles in water upon excitation with a 980-nm laser is easily visible to the naked eye.

It is also well-known that gold nanoparticles have good absorption properties in the UV region.[13] The 7-nm gold nanoparticles used in our experiments show a strong absorption at $\lambda \approx 520$ nm, which matches well with the UC emission of $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Er^{3+}$. According to the theory of FRET, when the absorption of the energy acceptor is close to the emission of the phosphor and when the donor and the acceptor are close enough, the emission of the energy donor (upconversion phosphor nanoparticles) will be quenched by the energy acceptor (gold nanoparticles). Thus it is intrinsically possible to couple the UC $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Er^{3+}$ nanoparticles (energy donors) with 7-nm gold nanoparticles (energy acceptors) to give a FRET biosensor.

To test the UC phosphor-based FRET biosensor, first, we functionalized the UC nanoparticles by the layer-by-layer (LbL) method[14] to introduce an NH_2 group that can be attached to biotin. The LbL approach is based on the electrostatic attraction between the oppositely charged species deposited, and its major advantage is that it permits the preparation of coated colloids of different shapes and sizes, with uniform layers of diverse composition as well as controllable thickness.[14] To enable the application of the green upconversion phosphors as fluorescent biological probes, the 50-nm upconversion hexagonal-phase nanoparticles were

functionalized by the LbL method (Figure 2; see also Supporting Information). Zeta potential experiments indicated that the UC nanoparticles were negatively charged under weakly basic conditions (Figure 3a), so the fabrication of the polyelectrolyte was performed at pH 8.5. The fabrication process slightly affected the luminescence of the phosphors. The functionalized upconversion-luminescent nanoparticles were then conjugated with biotin according to a reported procedure[15] (see Supporting Information). The phosphor/PAH/PSS/PAH nanocomposites were characterized by analysis of the zeta potential (Figure 3b) and FTIR spectroscopy (Figure 4). The zeta potential of the nanocomposites alternated from negative to positive values with the alternating absorption of the polyelectrolyte, which indicated that PAH and PSS were absorbed alternately onto the surface of the phosphor nanoparticles. Figure 4 presents a series of peaks corresponding to the stretching vibrations of organic functional groups in the polyelectrolyte absorbed onto the phosphor nanoparticles. Strong bands at $\tilde{\nu}=3433\ cm^{-1}$ indicate the N–H stretch for the amine group in PAH. The weak bands at $\tilde{\nu}=1647$ and $1414\ cm^{-1}$ result from the vibrations of the C–C skeleton of the benzene rings in PSS. The band at $\tilde{\nu}=739\ cm^{-1}$ results from the bending vibration of the N–H bond in the primary amine in PAH which further indicates the existence of amine groups on the surface of the functionalized phosphor nanoparticles. Thus, the data from zeta potential and FTIR experiments indicated that the fabrication of phosphor/PAH/PSS/PAH nanocomposites was successful. Similarly, we prepared biotin-conjugated gold nanoparticles (Au-biotin; see Supporting Information). Thus, on the basis of Au-biotin nanoparticles and biotinylated UC phosphor nanoparticles, the FRET system was developed as shown in Figure 5.

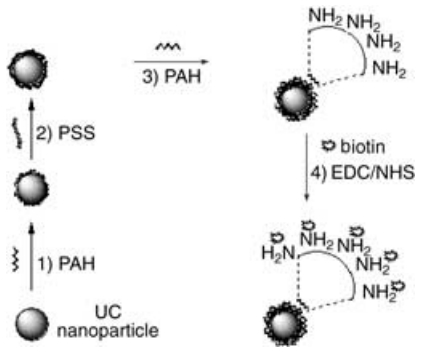

Figure 2. Schematic illustration of the functionalization of the upconversion nanoparticles. PAH = poly(allylamine hydrochloride); PSS = poly(styrene sulfonate), EDC = 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NHS = *N*-hydroxysuccinimide.

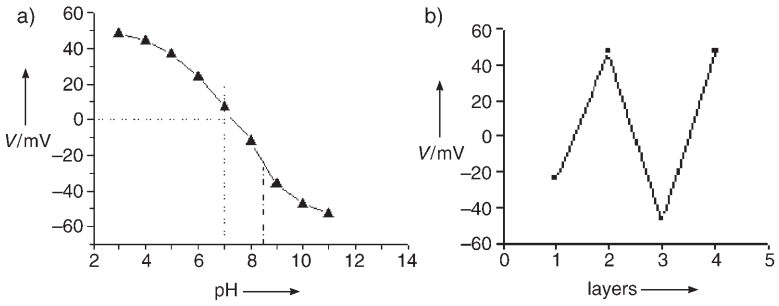

Figure 3. Zeta potentials a) of the upconversion-luminescent nanoparticles at different pH values and b) of negatively charged (under weakly basic conditions) phosphor nanoparticles as a function of the number of layers of the polyelectrolyte: 1) naked phosphor nanoparticle; 2) phosphor/PAH; 3) phosphor/PAH/PSS; 4) phosphor/PAH/PSS/PAH.

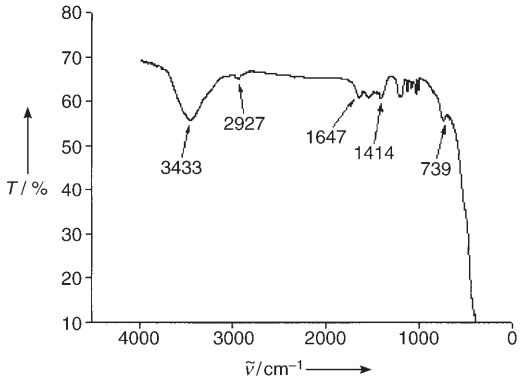

Figure 4. IR spectrum of the as-prepared phosphor/PAH/PSS/PAH nanocomposite.

To demonstrate the practicality of the UC nanoparticle-based FRET method, the probe was applied to the detection of trace amounts of avidin. Different concentrations of avidin were added to the mixture of the biotin-phosphor nanoparticles (80 nM) and Au-biotin nanoparticles (144 nM), and PL spectra were measured as a function of the concentration of avidin. When avidin was added, Au-biotin nanoparticles were conjugated to the surface of the UC phosphor-biotin nanoparticles through the sensitive and selective interaction between avidin and biotin. As shown in Figure 6a, the luminescence was gradually quenched with increasing amounts of avidin added to the system. Figure 6b shows the linear relationship between the relative intensity of PL (F_0/F) of the system and the concentration of avidin, wherein F_0 and F represent the intensity of luminescence in the absence and presence of different amounts of avidin, respectively. The

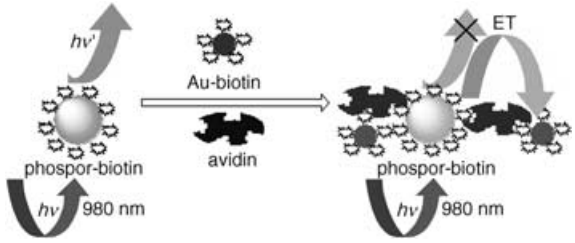

Figure 5. Scheme of the FRET system, with phosphor-biotin nanoparticles as energy donors and Au-biotin nanoparticles as energy acceptors, in the analysis of avidin. ET = energy transfer.

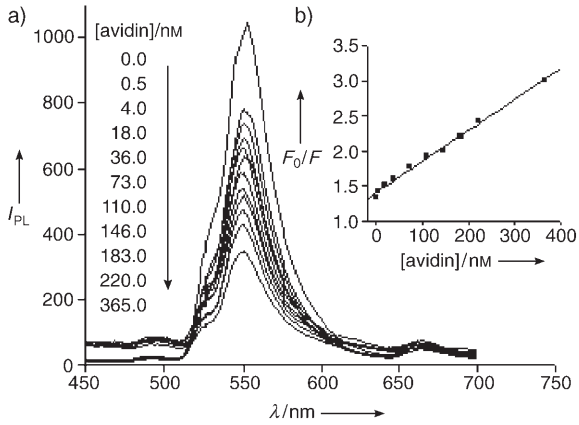

Figure 6. FRET-based assay of avidin: a) PL spectra of the FRET system at different concentrations of avidin as indicated (nM); b) linear relationship between the relative intensity of PL (F_0/F) and the concentration of avidin in the system.

intensity of upconversion luminescence varied linearly with the concentration of avidin in the solution from 0.5 nM to 370 nM. The results indicated that a FRET system based on upconversion-luminescent nanoparticles was reasonable and feasible.

To further test the FRET system based on the upconversion-luminescent nanoparticles, we also prepared $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Tm^{3+}$ nanoparticles using solvothermal technology. The as-prepared upconversion-luminescent $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Tm^{3+}$ nanoparticles emit violet fluorescence with single excitation at $\lambda = 980$ nm. These nanoparticles were also bioconjugated with biotin and applied to a FRET study using Au-biotin nanoparticles to quench the violet emission (see Supporting Information). The results showed that the FRET system based upon the single-emission upconversion nanoparticles was very efficient and versatile. Applications of the FRET system in fluorescence immunoassays, DNA detection, and fluorescence imaging are in progress.

In conclusion, hexagonal-phase $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Er^{3+}$ and $Na(Y_{1.5}Na_{0.5})F_6:Yb^{3+},Tm^{3+}$ nanoparticles with good crystallinity, strong single-emission upconversion fluorescence, good monodispersibility, and controllable size have been successfully prepared. The synthetic methodology also can be used to prepare other single-emission upconversion-luminescent nanoparticles. A FRET system with upconversion-luminescent nanoparticles as energy donors and gold nanoparticles as energy acceptors has been developed and applied to detect trace amounts of avidin. The results indicated that such a FRET system is sensitive and simple for use in biological analyses. Further studies may pave the way to wider applications of these upconversion phosphors in ultrasensitive multicolor detection of nucleic acids and proteins, fluorescence immunoassays, and fluorescence imaging performed in vitro and in vivo.

Experimental Section

Bioconjugation of nanoparticles: Biotin (0.1 mmol) was added to morpholine ethanesulfonic acid (MES) buffer solution (pH 6.0, 2.5 mL) containing functionalized luminescent nanoparticles (20 mg; ≈50 nm), and the mixture was ultrasonicated for 10 min. Then, EDC (0.1 mmol) and NHS (0.2 mmol) were added, and the mixture was mixed slowly with a vortex stirrer for 3 h. Excess biotin, EDC, and NHS were removed by three repeated separation/wash/redispersion centrifugation cycles. The biotin-conjugated phosphors were dispersed in water (10 mL) and stored at 4 °C.

Determination of avidin: Different concentrations of avidin were added to the mixture of the biotin-phosphor nanoparticles (concentration fixed at 80 nM) and biotin-Au nanoparticles (concentration fixed at 144 nM), and the mixtures were mixed thoroughly. After stirring the mixtures with a vortex stirrer at room temperature for 30 min and ultrasonication for a further 5 min, PL spectra were measured of the samples.

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