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Aptamer optical biosensor without bio-breakage using upconversion nanoparticles as donors†

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LRET-based optical biosensor of an aptamer-upconversion conjugate was constructed. It is demonstrated that photosensitized breakage and damage of aptamers are eliminated by employing UCNPs as donors, and the as-designed biosensor is specific and sensitive in the detection of ATP.

Aptamers are a special class of nucleic acids (DNA or RNA), that can bind with high affinity and specificity to a wide range of target molecules, such as organic dyes, amino acids, antibiotics, peptides, vitamins, proteins and even whole cells or microorganisms. As recognition elements in biosensors, aptamers are superior to traditional antibodies, in terms of their small size, simple production, long storage, high reproducibility and easy modification.[1,2] More importantly, aptamers can be folded into diversely defined tertiary structures, resulting in a shape-complementary recognition between aptamers and targets *via* hydrogen bonds, van der Waals interactions or base-stacking interactions. The significant conformational change upon target binding offers remarkable flexibility and convenience in designing novel biosensors.

An often adopted approach is to use homogeneous luminescence resonance transfer (LRET) assay, which is specific, sensitive, fast, and simple in detection of ligand–receptor binding. This strategy explores covalent attachment of a pair of donor/acceptor molecules onto aptamers. Taking advantage of the target induced conformational change of the aptamer, the change of distance between donor/acceptor molecules will alter the efficiency of LRET, leading to a corresponding change in luminescence intensities. Although organic molecules have been widely used as energy donors and acceptors in a variety of LRET-based aptamers biosensor studies, many suffer from intrinsic limitations such as pH dependence, susceptibility to photobleaching, and narrow absorption bands coupled with broad emission bands, all of which can compromise signal transduction performance. To circumvent these problems, recently quantum dots (QDs) were used as energy donors instead of conventional organic dye molecules.[3–8] However, QDs are still problematic due to their high background noise, potential toxicity, intrinsic blinking and chemical instability.[9] Furthermore, QDs are usually excited by UV or short wavelength radiation, which can lead to nucleic acid component damage.[10–12] In particular, it is worth mentioning that photoactivation of QDs is not favourable for stability of conjugated nucleic acids. Recent work has demonstrated that chemically induced free radicals and photo-induced reactive oxygen intermediates (ROI) in the process of light radiation on QDs can result in nucleic acid strand breakage and nucleobase damage.[13] The inevitable disturbances would significantly impair the performance of aptamer biosensors utilizing QDs as donors, such as recognition capability, reusability, and long-term stability.

To solve the problem of photo-induced ROI on the breakage and damage of aptamers, we propose and validate a novel and simple optical biosensor based on aptamer-upconversion nanoparticles (UCNPs) bioconjugates. Although there are several reports on the use of UCNPs (such as $NaYF_4 : Yb^{3+}$, Er^{3+}) as QDs substitutes due to the unique characteristics of these new class of photoluminescent labels,[14–16] there has been little study on the breakage and damage of photoactivated UCNPs upon biological samples. In this work, it is proved for the first time that, in contrast to photoactivated QDs, the upconversion phosphors in themselves do not directly produce ROI under near-infrared (NIR) illumination, illustrating that photosensitized breakage and damage upon aptamers are eliminated by employing UCNPs as donors. It is also demonstrated that the as-designed biosensor is specific and sensitive in detection of adenosine triphosphate (ATP).

The principle of this design is shown in Scheme 1. Two synthetic DNA oligonucleotides are employed: the first one is modified with biotin at the 5′-end so as to bind to avidin-functionalized $NaYF_4 : Yb^{3+}$, Er^{3+} UCNPs through biotin–avidin interaction (denoted UCNPs_DNA), which consists of an aptamer domain and a 'nonsense' sequence. The second one is labeled with a conventional fluorophore, *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′-end

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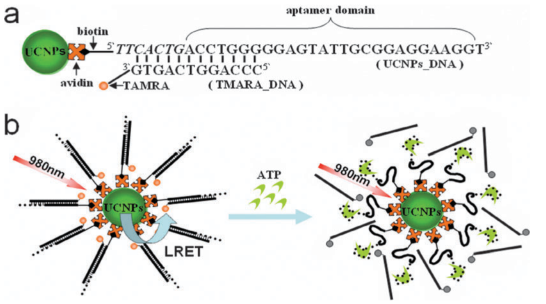

Scheme 1 Schematic of aptamer-UCNPs biosensor for ATP detection based on LRET: (a) design of aptamer-UCNPs bioconjugates and (b) the LRET process of aptamer-UCNPs bioconjugates for ATP detection.

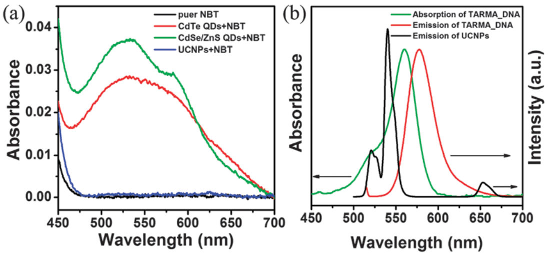

Fig. 1 (a) NBT assay of ROI production by QDs and UCNPs. (b) Absorption (green) and emission spectra (red) of TAMRA_DNA aqueous solution. For comparison, the upconversion emission spectra (black) of the $NaYF_4 : Yb^{3+}, Er^{3+}$ UCNPs aqueous solution under excitation of 980 nm are also shown.

(denoted TAMRA_DNA). The TAMRA_DNA is designed to be complementary to a partial segment sequence of the UCNPs_DNA near the UCNPs. With this arrangement, the two oligonucleotides naturally assemble to form the duplex state, keeping the UCNPs and TAMRA in close proximity with each other in the absence of the target. Upon excitation with a 980 nm laser, the luminescence signals of UCNPs and TAMRA are observed simultaneously as a consequence of LRET. Conversely, in the presence of targets, aptamer–target complex structure should be preferentially stabilized because the aptamer is selected on the basis of target binding. Conformational change of the aptamer induces the complete or partial dissociation of TAMRA_DNA from the hybridization duplex, leading to increased separation between UCNPs and TAMRA, which is accompanied by a change in the optical readout.

The energy donor UCNPs were prepared in the organic phase, followed by phase transfer to obtain water-soluble, small amido-functionalized UCNPs using 2-aminoethyl dihydrogenphosphate (AEP) molecules as surface coating agents to replace the original ligands (see ESI† for details). Fig. S1 shows the TEM images of $NaYF_4 : Yb^{3+}, Er^{3+}$ UCNPs before and after phase transfer. The average diameter of the nanoparticles is about 20 nm. The phosphate groups of the AEP molecule could serve as an anchoring moiety *via* its strong interaction with rare-earth ions (Ln^{3+}) on the UCNPs surface while the amino group induces aqueous solubility. Moreover, the free amino groups lead to biological functionality (see Fig. S2, ESI†).

To testify that the energy donor UCNPs do not generate ROI in process of light radiation, nitroblue tetrazolium (NBT), which is reduced by ROI leading to an increase of the absorption band, was used as a ROI detector.[17] By measuring the intensity increase of the NBT absorption band in the 450–700 nm range, the generation of ROI can be monitored.[13] Fig. 1a illustrates the absorbance change for CdTe bare core QDs, CdSe/ZnS core/shell QDs and the ligand exchanged UCNPs, respectively. Without light illumination, the NBT absorbance intensity in all three solutions shows negligible change over time. When the solutions were illuminated with 488 or 980 nm light, the absorbance intensity of NBT in QDs (CdTe and CdSe/ZnS QDs) solutions increased dramatically. By contrast, the absorption band of NBT with photoactivated UCNPs was unchanged.

To further estimate the effect of photoactivated QDs and UCNPs on photo-damage and breakage of aptamer molecules, agarose gel electrophoresis was carried out. Fig. S3 (ESI†) shows the agarose gel images of aptamer with and without QDs (UCNPs) before and after photoactivation, as compared with that of untreated aptamer (see ESI† for details), indicating that UCNPs, which possess excellent optical properties and lead to no photosensitized breakage and damage of aptamer molecules, are good candidates for donors in LRET-based aptamer biosensors.

The UV-Vis absorption spectrum of TAMRA (acceptor) overlaps well with the emission band centered at 540 nm of the $NaYF_4 : Yb^{3+}, Er^{3+}$ UCNPs (donor), as shown in Fig. 1b. Through the well-established specific interaction between avidin and biotin, biotinylated duplex strands of DNA with designed aptamer sequence were attached to avidin-functionalized UCNPs. Under illumination at 980 nm, the hybridization format induced close proximity between UCNPs and TAMRA labeled at 3′-termini of the TAMRA_DNA, leading to the occurrence of LRET from UCNPs to TAMRA, which was observed in a titration experiment as shown in Fig. 2a, where a broad and featureless TAMRA emission band (~577 nm) gradually appears, and simultaneously the green upconversion emissions bands between 514 and 560 nm of UCNPs decreases significantly, proportional to the amount of biotinylated duplex strands of DNA. This observation can be understood in such that increasing the amount of DNA would in fact bring more and more TAMRA close to UCNPs and hence enhance LRET efficiency. It is noted that red emission between 635 and 680 nm of UCNPs was also quenched although to a small extent, which is related with the upconversion mechanism: among other feeding channels, the population of the red emission level ($^4F_{9/2}$) is partially derived from green emission level ($^4S_{3/2}$, $^4F_{7/2}$) *via* a nonradiative relaxation process.[18]

To demonstrate the feasibility of the approach using UCNPs in sensor design, ATP was tested as an example. ATP is an important substrate of numerous biochemical reactions in living cells. As a 27-nt single-strand oligodeoxynucleotide, the ATP DNA aptamer forms a tertiary structure containing two nonequivalent ATP binding sites flanked by reversed G–G and sheared G–A mismatches upon ligand binding, which has stem and stem-loop arms.[19,20] In this work, titration experiments were carried out by increasingly adding ATP to the aforementioned LRET system to examine whether the upconversion luminescent intensity change could be used to label ATP quantitative assay. As expected, the luminescence intensity corresponding to the TAMRA emission gradually decreases following the increase of the upconversion green emission of

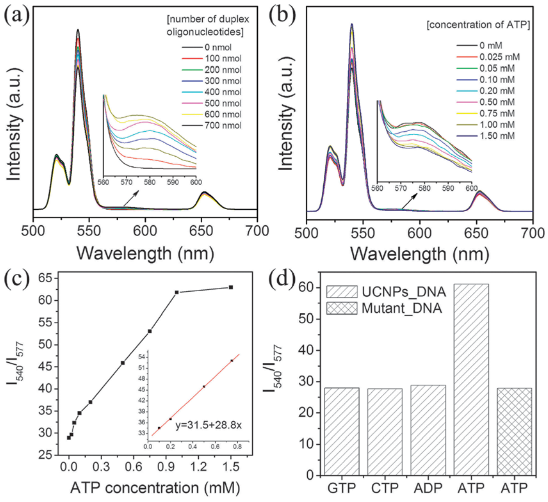

Fig. 2 (a) Luminescence spectra of a mixture of avidin-functionalized $NaYF_4:Yb^{3+}, Er^{3+}$ UCNPs in the presence of different concentrations of duplex DNA under excitation at 980 nm. (b) Luminescence spectra of the aptamer-UCNPs bioconjugates in the presence of various ATP concentrations. (c) Plot of I_{540}/I_{577} value *vs.* ATP concentration. Inset: the linear relationship of the I_{540}/I_{577} value and ATP concentration ranging from 0.1 to 0.75 mM. (d) Specificity of the aptamer-UCNPs bioconjugates toward potential nucleotide interferences and the response of ATP to mutant aptamer.

UCNPs until a plateau is reached (Fig. 2b), in which the I_{540}/I_{577} ratio at 1.2 mM ATP presents no great difference from that at 1.5 mM ATP (Fig. 2c). This observation can be understood as that, in the absence of the target ATP, the UCNPs_DNA naturally binds to the TAMRA_DNA, this duplex should have enough strength to bring the TAMRA and the UCNPs into close proximity so that efficient LRET can occur. Upon introducing the target, the aptamer prefers to form the aptamer–target complex rather than the DNA duplex, triggering the release of the TAMRA_DNA from the UCNPs_DNA. The dissociation of the TAMRA_DNA is accompanied by the reverse changes in emission intensities of the donor and acceptor. Saturation of the I_{540}/I_{577} was observed with increase of the concentration of target ATP which indicates that the potential steric hindering effects resulting from contacting reactive aptamer with the UCNPs rigid surface will limit the amount of aptamers undergoing conformation change. There is a good linear relationship *vs.* the concentration of target ATP in the measured range of 0.1–0.75 mM (inset of Fig. 2c). The limit of detection (LOD) is determined to be 20 μM, which is defined following the 3SD/*m* principle (SD and *m* represent standard deviation and slope rate, respectively).

Specificity was studied by comparing the emission signal at the presence of ATP and three other ATP analogues, *i.e.* adenosine diphosphate (ADP), cytidine triphosphate (CTP) and guanosine triphosphate (GTP). Without exception, the results in Fig. 2d show a clear discrimination between ATP and the analogues. The I_{540}/I_{577} value does not show much change after the addition of ADP, CTP and GTP, completely different from the scenario when ATP was introduced, where an apparent change was easily detected—clear evidence that the ATP aptamer was able to recognize ATP specifically. To further determine the specificity of this luminescence-signaling aptamer, one oligonucleotide analogue that contained two point mutations in the aptamer domain at positions 6 (G to C) and 14 (T to A) from the 3′-end as highlighted in Table S1 (ESI†) was synthesized in a control experiment. These mutations have been proven previously to cause loss of the ATP-binding ability of the DNA aptamer.[21] The observed specificity pattern in Fig. 2d clearly demonstrated that differences of the two bases in oligonucleotide sequence lead to aptamer malfunction due to the high specificity of the aptamer toward its target molecule.

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