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A paper-based multiplexed resonance energy transfer nucleic acid hybridization assay using a single form of upconversion nanoparticle as donor and three quantum dots as acceptors

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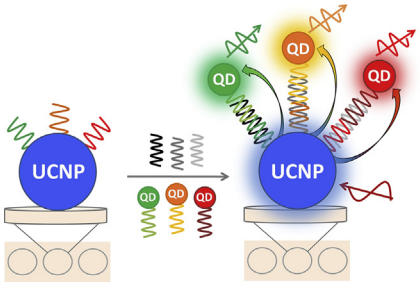
HIGHLIGHTS

- Use of a single form of UCNP as LRET donor and three QDs as acceptors.
- NP emission peaks resolved using only optical band-pass filters.
- Triplexed LRET-based nucleic acid hybridization assay.
- Improved sensitivity and selectivity compared to analogous assays.

GRAPHICAL ABSTRACT



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ABSTRACT

Monodisperse aqueous upconverting nanoparticles (UCNPs) were covalently immobilized on aldehyde modified cellulose paper via reductive amination to evaluate the multiplexing capacity of luminescence resonance energy transfer (LRET) between UCNPs and quantum dots (QDs). This is the first account of a multiplexed bioassay strategy that demonstrates the principle of use of a single form of UCNP as donor and three different color emitting QDs as acceptors to concurrently determine three analytes. Broad absorbance profiles of green, orange and red QDs that spanned from the first exciton absorption peak to the UV region were in overlap with a blue emission band from UCNPs composed of $NaYF_4$ that was doped with 30% Yb^{3+}, 0.5% Tm^{3+}, allowing for LRET that was stimulated using 980 nm near-infrared radiation. The characteristic narrow and well-defined emission peaks of UCNPs and QDs allowed for the collection of luminescence from each nanoparticle using a band-pass optical filter and an epi-fluorescence microscope. The LRET system was used for the concurrent detection of uidA, Stx1A and tetA gene fragments with selectivity even in serum samples, and reached limits of detection of 26 fmol, 56 fmol and 76 fmol, respectively.

1. Introduction

Upconversion nanoparticles (UCNPs) are lanthanide doped inorganic crystals with multiple narrow and well-defined emission peaks. Upconversion is based on the sequential absorption of two or more photons in the NIR or IR region of the electromagnetic

Abbreviations: BPM, Base Pair Mismatch; Indocarbocyanine, Cy3; FRET, Fluorescence Resonance Energy Transfer; FC, Fully complementary; GSH, reduced L-glutathione; LRET, Luminescence Resonance Energy Transfer; NP, Nanoparticle; OA, Oleic Acid; PEA, o-phosphorylethanolamine; PMT, photomultiplier tube; TMAH, tetramethylammonium hydroxide; UCNP, Upconverting Nanoparticle.

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spectrum followed by emissions spanning the UV to NIR region. Excitation using NIR radiation minimizes autofluorescence from biological samples and reduces optical background associated with scatter from UV and visible excitation sources [1]. These properties have made UCNPs attractive for use in bioassays for the detection of nucleic acids and proteins [1–4].

The tuneable narrow emission profiles governed by the electronic structure and concentration of the lanthanide dopants allow for UCNPs to be used as multiplexing agents in bioassays. The use of UCNPs as passive labels for multiplexing has been widely explored [5–10], and has primarily been of interest owing to the opportunity for NIR excitation of optical processes. However, their use as LRET donors has been limited to the concurrent detection of two biomolecules [11–13]. A further attribute suitable for analytical applications is that LRET methods offer access to ratiometric methodology that provides for good precision [14]. Rantanen et al. reported a dual parameter sandwich-based nucleic acid hybridization assay using two colors from one UCNP as LRET donors and two molecular dyes as acceptors with a limit of detection of 28 fmol [12]. He et al. demonstrated the first UCNP-based LRET assay on paper for the detection of matrix metalloproteinase-2 (MMP-2) [15]. Recently, He et al. reported a portable UCNP-based paper device for the detection of cocaine based on the quenching of UCNP luminescence by gold NPs [16]. Zhou et al. demonstrated a two-plex assay on paper using dye-labelled nucleic acids [13]. A similar study for a sandwich-based single-plex hybridization assay demonstrated limited sensitivity and selectivity and had a limit of detection of 146 fmol [17]. Our research group has reported a sandwich-based hybridization assay on paper using UCNPs as donors and QDs as acceptors with a limit of detection of 13 fmol for the HPRT1 housekeeping gene fragment [18].

While QDs are used as LRET accepters herein, they are more typically used as donors in fluorescence resonance energy transfer (FRET)-based bioassays. Multiplexed FRET-based hybridization assays have been reported with a maximum of one QD donor and two molecular dye acceptors. Three-plex detection was achieved only when two FRET channels between green emitting QD donor and Cy3 and Rhodamine Red-X acceptors were used, while the third channel was based on the direct excitation of Pacific Blue [19]. The work was extended for the simultaneous detection of four different targets on optical fibres with the addition of red emitting QD donors and Alexa Fluor 647 acceptor. Detection limits of 1–7 nM were achieved for dual labelled nucleic acid reporters [20].

The FRET multiplexing capacity for one donor was limited by the small Stoke's shift and broad emission profiles of molecular dyes. Herein, QDs are used as LRET acceptors. In addition to their characteristic photostability and high extinction coefficient compared to molecular dyes, QDs have broad absorption profiles in the UV and blue region of the spectrum. This allows the fluorescence of any color emitting QD to be sensitized by blue emitting UCNPs. Both UCNPs and QDs have narrow and well defined emission bands, allowing for QD emission peaks to be resolved in the visible region of the spectrum using only optical band pass filters. LRET has been well characterized between UCNPs and QDs [21,22], and in particular between lanthanides and QDs [23,24]. Forster distance between UCNP and QDs was characterized to be 15 Å [22].

Herein, we use a paper-based assay to evaluate the capacity for multiplexing using LRET between UCNPs and QDs. The immobilization of UCNPs on paper offers a facile layer by layer assembly of the assay without the need for tedious purification steps and avoids problems associated with aggregation of nanoparticles in solution. The simultaneous detection of three nucleic acid targets using one UCNP as donor and three different color emitting QDs as acceptors allows for the controlled placement of QDs in close proximity to the UCNPs for LRET. The large surface area associated with the three dimensional matrix of paper, in addition to the contraction of wet paper upon drying that brings donors and acceptors in closer proximity, can give up to a 10 fold enhancement in ratiometric signal by LRET [25].

This work presents the first account of LRET between a single form of UCNP and three acceptors. The photoluminescence from three different color emitting QDs was concurrently sensitized by blue emission of a single form of UCNP. Three independent optical channels for green, orange and red emitting QDs were isolated using optical band pass filters. Examination of the potential of the LRET system for higher-order multiplexing was done using a sandwich-based hybridization assay for the concurrent detection of uidA, Stx1A and tetA gene fragments. The uidA sequence is diagnostic of *Escherichia coli* [26], the Stx1A gene encodes the production of Shiga toxins [27], and the tetA gene indicates resistance to tetracycline [28]. These markers offer potential for the detection of *E. coli*, its pathogenicity and its resistance to an antibiotic.

2. Experimental

A full list of Materials and Instrumentation can be found in the Supporting Information.

2.1. Synthesis of $NaYF_4$: 0.5% Tm^{3+}, 30% Yb^{3+}/$NaYF_4$ core/shell UCNPs

Oleic acid (OA) capped $NaYF_4$: 0.5% Tm^{3+}, 30% Yb^{3+}/$NaYF_4$ core/shell UCNPs were synthesized according to previous reports [29]. Core $NaYF_4$: 0.5% Tm^{3+}, 30% Yb^{3+} were synthesized by first stirring 456.2 mg, 253.4 mg and 4.2 mg of $Y(CH_3CO_2)_3.xH_2O$, $Yb(CH_3CO_2)_3.4H_2O$, $Tm(CH_3CO_2)_3.xH_2O$, respectively, in 30 mL of octadecene and 12 mL of OA at 115 °C under vacuum for 30 min. The clear solution was then cooled to 50 °C under argon and a 20 mL solution of methanol containing 0.20 g of NaOH and 0.30 g of NH_4F was added. The cloudy solution was stirred for 30 min before the temperature was raised to 75 °C to evaporate the methanol. Then the solution was rapidly heated to 300 °C and maintained at this temperature for 1 h while stirring. The solution was allowed to cool to room temperature and the core UCNPs were collected in ethanol and were separated by centrifugation. The core UCNPs were re-suspended in hexanes and recaptured with ethanol and centrifugation. The core UCNPs were stored in hexanes overnight at 4 °C.

Core UCNPs were capped with a $NaYF_4$ shell. 573.8 mg of $Y(CH_3CO_2)_3.xH_2O$ was stirred in 30 mL of octadecene and 12 mL of OA at 115 °C under vacuum for 30 min. The clear solution was allowed to cool to 80 °C under argon before the core UCNPs in hexanes were added. After the hexanes evaporated, the temperature was lowered to 50 °C and a 20 mL solution of methanol containing 0.14 g of NaOH and 0.26 g of NH_4F was added. The reaction temperature was increased to 75 °C to evaporate the methanol before it was rapidly increased to 300 °C and maintained for an hour while stirring. The reaction was allowed to cool to room temperature and the core/shell UCNPs were captured with ethanol and centrifugation. The core/shell UCNPs were re-suspended in hexanes and recaptured with ethanol and centrifugation three times. The OA capped core/shell UCNPs were stored in hexanes at 4 °C for subsequent modification.

2.2. Preparation of water soluble UCNPs

OA capped UCNPs were made water soluble via ligand exchange with o-phosphorylethanolamine (PEA) according to previous reports [30]. 100 mg of OA capped UCNPs in 2 mL of