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Expanding the scope of biomolecule monitoring with ratiometric signaling from rare-earth upconverting phosphors

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Abstract: Upconversion (UC) is a powerful multi-photon mechanism that converts low energy photons into higher energy emission. One of the most investigated UC systems is upconverting phosphors (UCPs). Here, a new, one-pot synthetic procedure was used to prepare water dispersible, visibly emissive, rare-earth doped UCPs that were capped with the functional groups oleic acid (OA), 6-aminohexanoic acid (AHA), and 6-maleimidohexanoic acid (MHA). These synthesised UCPs were characterised by UC luminescence, dynamic light scattering (DLS), transmission electron microscopy (TEM), and powder X-ray diffraction (pXRD). This study expands upon our previous proof-of-principle work in demonstrating the use of UCPs (both synthesised and commercial) to detect on the level and function of biological analytes, from enzymes to key disease biomarkers (PETNR, glucose oxidase, vitamin B12, and cytochrome c). By tailoring the absorption profile of the biomolecule cofactors to the UCP emission, a wide-range of analytes can be utilised. We also demonstrate the ability of our system to reversibly monitor the addition of enzyme substrates via repeat oxidation and reduction of pentaerythritol tetranitrate reductase (PETNR).

Introduction

In the last decade, rare-earth upconverting phosphors (UCPs) have emerged as a unique class of nanoparticles (NPs) with a wide range of potential applications, including optical memory,[1] security,[2] and theranostics.[3-5] Upconversion (UC) luminescence is a phenomenon by which the emitted photons are of a higher energy than those absorbed, a reverse of the case in classical downconverted rare-earth luminescence (and by extension sensitised lanthanide(III), or Ln(III), emission).[6] UCPs generally display photoluminescence in the visible region of the electromagnetic spectrum upon excitation with near-infrared (NIR) light.[7] The non-linear multiphoton process(es) involved in UC is similar to multi-photon absorption (MPA).[8] Unlike the virtual excited state that makes MPA possible however, in UC, the sequential absorption of NIR photons leads to the population of real, metastable excited states (of ~ µs lifetime in the systems investigated here). These long-lived, ladder-like energy levels are a prerequisite for achieving UC. Such a structure is a key characteristic of many lanthanide ions, making them one of the most studied ions for UC to date. The form and efficiency of the UC process can be tuned by altering the individual Ln(III) ion doping levels in these systems.[9] In order to achieve efficient UC, a donor-acceptor pair of ions is often employed. Of these pairs, Yb(III) → Er(III) and Yb(III) → Tm(III) are the most commonly used and are the donor-acceptor system used in this study. There are a number of excellent reviews for a more comprehensive outlook of UC mechanism and UCP development.[7,10-12]

Scheme 1. Simplified schematic showing biosensor design of our “on/off” reversible energy transfer between rare-earth upconverting phosphors (UCPs) and biomolecules, in this case the interaction between a Tm(III)-doped UCP and flavoprotein (PETNR).[7,8]

Supporting information for this article is given via a link at the end of the document.
UCPs present a number of potential advantages over traditional fluorophores, including far higher photostability compared to organic dyes. Due to the nature of the NIR excitation, there is almost no auto-fluorescence generated when using biological samples/media. This lack of noise, in combination with generally low associated toxicities[14] and increased penetration depth with NIR light, has led to a particular focus on bioimaging applications. [4,15,16]

Our main curiosity with respect to UCPs is in the development of potential biosensing applications, a growing area of interest in the field of these UCPs. [17-19] We previously reported the first example of utilising UCPs to directly monitor the redox state of an enzyme. [13] For this prototypical system, we used pentaerythritol tetraneitrato reductase (PETNR), a flavoprotein belonging to the old yellow enzyme family of NAD(P)H- (reduced nicotinamide adenine dinucleotide (phosphate)) dependant enzymes. [20] In this study, we demonstrated that the presence of the enzyme could be detected by variation in energy transfer from Tm(III)-based UCPs to the flavin cofactor, flavin mononucleotide (FMN), of PETNR in its oxidised and 2-electron reduced state. Energy transfer could then effectively be turned “on” or “off” by varying the oxidation state of the enzyme, as the spectral overlap is much weaker when FMN is in its reduced state. As only one of the two main emission bands of the Yb(III)-Tm(III) doped UCP exhibits appreciable spectral overlap with the oxidised FMN cofactor, only the 475 nm emission band (1G4 → 3H6 transition) is quenched upon enzyme addition. By normalising the emission intensity of this band to that of the 800 nm emission band (3H6 → 3H4 transition), ratiometric analysis can be used to improve accuracy of measurements and negate issues with variation in dispersability of the UCPs from scan to scan.

Here, we wished to explore the scope of our proposed system and to investigate the compatibility of this energy transfer mechanism towards different UCP systems and a variety of biomolecules; glucose oxidase, cytochrome c, and vitamin B12 (Scheme S-1). In addition, we wished to expand upon our initial study to investigate whether enzyme substrates could be detected. If achievable, substrate detection could allow a much broader avenue for the development of sensors for an array of small-molecule analytes. The concept for our cofactor-responsive biosensor system is summarised in Scheme 1.

Results and Discussion

Synthesis of nanoparticles

In order to ensure the ability to translate our approach to differently derived upconversion systems, the UCPs used in this work were either microscale commercial phosphors or synthesised nanoscale particles. The commercial phosphors were donated by Phosphor Technology Ltd. (PTIR475 and PTIR545) and were the same systems used in our previous report.[13]

With a view to enabling future covalent conjugation strategies, in studies beyond those presented here, we decided to synthesise particles with bifunctional, ligand coatings. We adapted procedures by Cao et al.[21] and He et al.[22] to synthesise UCPs coated with oleic acid (OA), 6-aminohexanoic acid (AHA), and 6-maleimidohexanoic acid (MHA). The OA should control UCP growth,[8] the AHA should allow high dispersibility in aqueous solutions,[21] and the MHA should enable covalent attachment if required for future applications. This new, one-pot solvothermal synthetic approach is shown in Scheme 2.

MHA (in EOH) and OA were added to a basic solution of AHA and mixed briefly to form an homogenous solution (OA:AHA:MHA = 6:4:1). To this mixture was added the appropriate mixture of lanthanide chloride salts, followed by slow addition of NaF. The mix was heated in a Teflon™-lined Parr reaction vessel (120-200 °C). After cooling, the particles were obtained by centrifugation before being washed and dried. Particles were then resuspended at the appropriate concentration for subsequent analysis and UC applications. For further synthetic details, please refer to the experimental section. The physical properties of the particles were analysed by infrared (IR) spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM), powder X-ray diffraction (pXRD), and UC emission.

In order to attempt to visualise the ligand coating, infrared (IR) spectroscopy was performed on the NP samples. NP1-Tm and NP2-Er display similar IR spectra (Figure 1b), with NP1-Tm giving a more intense signal with multiple peaks. In comparison, UCPs without capping ligands have very few IR bands in their spectra (Figure S-2a). However, from the data it is difficult to determine the proportion of ligands bound to the UCP surface, as all ligands used have similar functional groups. AHA appears to be present as the N-H amine stretch can be seen at 3330 cm⁻¹ in both spectra. The ligands all contain alkyl chains, which are responsible for the CH₂-CH₃ and CH₃-CH₃ stretching seen at...
2940 cm\(^{-1}\) and 2870 cm\(^{-1}\), the CH out of plane bending at 723 cm\(^{-1}\), and CH\(_2\) scissor vibration and CH\(_2\) deformation at 1478 cm\(^{-1}\) and 1389 cm\(^{-1}\), respectively. The peaks at 1640 cm\(^{-1}\) and 1510 cm\(^{-1}\) indicate the presence of carboxyl groups (present in all three ligands) and the peaks at 1268 cm\(^{-1}\) and 1199 cm\(^{-1}\) suggest C-N stretching and bending, a bond that is present in both AHA and MHA. Whilst IR spectroscopy is not the ideal tool to study the ligand surface of the particles and does not provide any information on the proportion of ligands bound to the UCPs, it does however indicate which functional groups are/are not present. \(^1\)H NMR spectroscopy would be a superior technique to study the small molecule ligands, but here it is not feasible due to limited solubility at the concentrations required for NMR spectroscopy and the presence of paramagnetic lanthanide(III) ions in all particles that may lead to spectral shifting and broadening.

The TEM data (Figure 1c) show NP1-Tm to be around 100 nm in size, correlating with the DLS data suggesting an average size of 110 (±7.4) nm (Figure S-3a). However, the TEM micrographs show aggregation of these UCPs, which is supported by the DLS bimodal size distribution and the biexponential decay of the correlogram (Figure S-3). DLS data of NP2-Er suggest a larger size (ca. 200 nm), with a possibly greater extent of aggregation resulting in large, micron scale particles in solution also. From the TEM data collected, it appears as though the drying stage during sample preparation causes significant aggregation that is not fully seen in solution, where the particles appear to be well suspended over time. Such artefacts are well known to occur in TEM[20] and we have not been able to find a sample preparation technique that does not result in significant aggregation to date.

The pXRD data of NP1-Tm and NP2-Er (Figure 1d) indicate predominantly hexagonal phase UCPs are present in the sample (compared to ICSD data No. 51916). However, the presence of an additional peak at 28 degrees indicates that cubic phase UCPs are also in the sample, implying the synthesis of mixed phase UCPs.

The capping ligands do appear to have successfully improved the NP stability in solution. The NP1-Tm and NP2-Er particles remain dispersed in aqueous solution for at least a few weeks. This situation is unlike the commercial PTIR systems, where a white sediment is observed after only a few hours. However, aggregation is still seen in the TEM and suggested by the DLS data, so the procedure does not seem to produce monodisperse UCPs according to these techniques.

Upon excitation of Yb(III) with nIR excitation source (450 mW CW 980 nm diode laser), UC emission can be observed in both NP1-Tm and NP2-Er (Figure 1a). As expected, NP1-Tm displays the typical Tm(III) emission bands at 451 nm (\(\text{I}_2 \rightarrow \text{F}_4\) transition), 475 nm (\(\text{I}_4 \rightarrow \text{H}_6\) transition), 650 nm (\(\text{I}_4 \rightarrow \text{F}_4\) transition) and 800 nm (\(\text{H}_4 \rightarrow \text{H}_6\) transition), while NP2-Er shows characteristic Er(III) emission bands at 410 nm (\(\text{I}_2 \rightarrow \text{I}_{15/2}\) transition), 525 nm (\(\text{H}_{11/2} \rightarrow \text{I}_{15/2}\) transition), 545 nm (\(\text{S}_{3/2} \rightarrow \text{I}_{15/2}\) transition) and 660 nm (\(\text{F}_{5/2} \rightarrow \text{I}_{15/2}\) transition). To note, the 410 nm emission band in the Er(III) doped NP2-Er (albeit comparatively weak compared to the green and red bands) is clearly visible, indicating that vibrational decay processes are minimised in these UCPs.

While further synthetic methods could be performed to refine the NP synthesis to produce smaller and better-defined particles, for the screening work we performed here, the UCPs synthesised were determined sufficient for the purpose. Our main desire was highly emissive particles that could be easily dispersed in aqueous solution, which was successfully achieved with the method chosen. For an overview of potential UCP syntheses, please refer to recent reviews covering this subject[4,5,24-26].

**Biomolecule detection**

In order to expand on our previous reports[13] and investigate the scope of our UC biosensor systems, it was necessary to expand to other biomolecules and UCPs. If our initial aims would also work with a variety of biomolecules then, ultimately, it would allow the development of a wide range of biosensors. Our initial studies focused on a flavoenzyme, PETNR. This enzyme was chosen as the absorption spectrum of its oxidised FMN cofactor overlaps strongly with the 475 nm emission band of Tm(III)-doped UCPs (Figure 2a). In theory, however, any biomolecule containing a chromophore with an appropriate spectral overlap should be able to be incorporated in a similar manner.
As such, in addition to PETNR we also chose to incorporate glucose oxidase (GOx), vitamin B₁₂ (vitB₁₂), and cytochrome c (cyt c) as model systems for our approach. These systems were chosen as they represent a wide variety of biologically important molecules that each contain a chromophore capable of overlapping with one or more emissive bands of Tm(III)- or Er(III)-doped UCPs (Figure 2). If spectral overlap is much stronger with one of the UC emissions bands over the others, then ratiometric analysis can be performed by taking advantage of the distinct and highly separated emission signals. In all of our studies, this difference in spectral overlap is apparent. Hence, in all data presented here, Tm(III)-doped UCPs have been plotted normalised to the 800 nm emission band and Er(III)-doped UCPs are normalised to the 660 nm emission band, as these bands have negligible spectral overlap with our chosen chromophores and so are generally unaffected by biomolecule addition. Ratiometric analysis of the UCP emission bands was performed in all cases as it reduces errors arising from variance in dispersibility, particle size, and laser power during titration experiments.²⁷

Before studying new biomolecule systems, however, we also wanted to expand the system to look at different UCP systems. Therefore, it was as necessary to repeat our initial PETNR studies with the synthesised UCPs described above.

Note, there is some discussion in the literature as to the exact nature of the mechanism behind the energy transfer process occurring here and elsewhere. In our previous study we labelled the mechanism as Förster resonance energy transfer (FRET), as others had previously done before us.²²⁻²⁶ However, there is currently insufficiant evidence that the energy transfer process can be classified as FRET (including exhibiting changes in the lifetime kinetics of the donor moiety), or to warrant classing the phenomenon as an inner filter or ‘nanolamp’ effect.²¹ or other similar processes. Therefore, throughout the rest of this work we simply refer to the process as ‘energy transfer’ and acknowledge that further work is required to investigate the exact mechanisms involved.

**Tm(III)-based UCP sensors**

**PETNR**

In order to assess whether our synthesised UCPs are as effective for our biosensor system as the commercial PTIR475 phosphors we had previously utilised, we first measured the response to PETNR to ensure we could repeat our previously published results.¹³ While an order of magnitude less emissive than the PTIR samples, NP1-Tm/NP2-Er are substantially smaller and more easily dispersible in aqueous buffers, making solution state studies much simpler and more reproducible. As the Tm(III)-based system NP1-Tm is the most comparable to PTIR475 and shows the greatest overlap with the absorption of PETNR, this UCP was chosen for this study.

In order to compare the potential of NP1-Tm to act in the same manner as PTIR475, a titration with NP1-Tm and PETNR was performed using the same concentrations and conditions reported previously.¹⁸ PETNR (1.1 mM stock solution, 10 μM increments from 0 – 60 μM, 20 μM increments from 60 – 200 μM) was titrated against a 0.1 mg/mL dispersion of NP1-Tm in aqueous buffer (100 mM TRIS, 10 mM NaCl, pH 7.0). A decrease in emission intensity of the 451 and 475 nm bands of NP1-Tm was observed with increasing concentrations of PETNR (Figure S-4). The ratio of the 451 and 475 nm bands against the normalised 800 nm band was plotted and compared to the ratio observed when using the commercial PTIR475 UCPs (Figure S-4c). For both UCPs a decrease in the 475/800 nm ratio is observed upon addition of PETNR. However, a sharper decline in the ratio is observed for NP1-Tm in comparison to...
PTIR475 from 0 – 60 µM PETNR. This trend is likely due to the higher initial ratio of the 475 and 800 nm bands for NP1-Tm.

The limit of detection (LOD) of 31 µM calculated using NP1-Tm (see Table S-1) was determined to be a factor of two higher than with PTIR475 (LOD = 17 µM), although both are within the same order of magnitude as achieved by related UCP biomolecule systems.\(^{31}\) It should be noted that some aggregation of PETNR was observed during the titration as a white precipitate, resulting in some variation in emission peak ratios between scans. All measurements were performed in triplicate and averaged to minimise such errors.

The luminescence lifetimes of the 475 and 800 nm emission bands of NP1-Tm and PTIR 475 were measured before and after the addition of 100 µM and 200 µM PETNR (Table S-2). Note, due to the loss of emission intensity of the 475 nm band after addition of 200 µM of PETNR, for titrations with NP1-Tm the signal was too weak to record accurate lifetime values. It should also be noted that the luminescence lifetimes are an order of magnitude longer for NP1-Tm in comparison to PTIR475. This extended lifetime may suggest the ligand coating is likely reducing non-radiative quenching processes occurring via interaction with solvent molecules.\(^{32}\) However, different lattice environments can also have a dramatic effect on luminescence lifetimes of the ions contained inside them, making it difficult to compare the two, very different, sets of particles.

The luminescence lifetime values for both the 475 and 800 nm bands remain unchanged upon PETNR addition despite substantial quenching of the 475 nm signal, suggesting static quenching occurring with both UCP systems. In both cases, Stern-Volmer analysis results in an almost identical linear decrease in emission intensity being observed in the 0-60 µM range of added PETNR (\(k_{sv} \text{NP1-Tm} = 0.012 \text{ µM}^{-1}\), PTIR475 \(k_{sv} = 0.014 \text{ µM}^{-1}\)). Beyond 60 µM, the gradient of the \(k_{sv}\) curve increases with both systems. Such an increase could indicate a mixing of dynamic and static quenching processes, which could conceivably be due to the different environments present in a NP suspension.

As the UCP luminescence lifetimes remain unchanged throughout the addition, it is difficult to assess the dominant energy transfer mechanism. Both static quenching, which is possible through the protein binding to the particle surface, or emission-reabsorption are potential mechanisms that would cause quenching without a lifetime change. There may also be additional quenching mechanism present, such as inner filter effects, though these have been minimised through maintaining constant volume and analysing the low concentration ranges of analytes. For this study, LOD analysis was only applied to analyte concentrations where inner filter effects and other quenching processes appear to be minimal (i.e. < 50 µM).

Despite the lack of observable evidence, it would also be premature to rule out FRET as the mechanism. While it is often assumed that FRET processes are accompanied by large changes in donor emissive lifetimes, these changes can in fact be rather subtle, particularly in the case of UCPS. A recent report on FRET from UCPS to quantum dots indicated only a 7% change in the shorter component of the donor lifetime, which could only be observed immediately following the excitation pulse.\(^{33}\) It is possible that such small changes in the decay may be obscured by the envelope of the rise of the emission signal following excitation along with the tail fitting procedure employed (Figure S-5c). Further analyses are ongoing to fully determine the processes involved.

**Glucose oxidase, GOx**

We next wanted to determine if the observed interaction between Tm(III)-based UCPS could be extended to other biomolecules beyond PETNR. Due to its ability to diagnose diabetes, glucose detection has long been a model for demonstrating biosensor ability.\(^{34,35}\) and so we turned our attention towards glucose oxidase, GOx.\(^{36}\) GOx is highly specific for \(\beta\)-o-glucose, and catalyses the oxidation of this substrate into glucono-\(\delta\)-lactone, with a relative activity of 100%. Glucono-\(\delta\)-lactone then spontaneously hydrolyses to o-gluconic acid. The enzyme is also able to catalyse the oxidation of a number of monosaccharides, hydroxyl compounds and nitroalkenes, although with much lower reaction rates (< 30% in comparison to \(\beta\)-o-glucose).\(^{36}\)

UCP-based glucose sensors have the potential to overcome some of the drawbacks with current systems, particularly with potential contamination and/or autofluorescence from other species in solution, and have the added benefit of long emissive lifetimes and high stability to photobleaching in comparison to other fluorescence based systems.\(^{6,12}\) These potential advantages have led to the development of UCP-based glucose sensors\(^{32,37}\) mainly utilising the \(\mathrm{H}_2\mathrm{O}_2\) produced during the reaction between glucose and GOx to determine glucose concentration. However, GOx also contains a flavin adenine dinucleotide (FAD) cofactor, which contains the same core chromophore (isoalloxazine) as the FMN in PETNR and in its oxidised form overlaps with the 451 and 475 nm emission bands of Tm(III)-doped UCPS (e.g. NP1-Tm). As with PETNR, there is negligible overlap with the 650 and 800 nm UCP emission bands, so ratiometric analysis can be used to monitor energy transfer from UCPS to GOx.

A similar procedure to that carried out with PETNR was performed with GOx from *Aspergillus niger*. Incremental amounts of GOx (10 µM increments from 0-60 µM, 20 µM increments from 60 – 100 µM and 50 µM increments from 100 – 200 µM, 200 µM stock solution) were added to a 0.1 mg/mL dispersion of NP1-Tm in aqueous buffer (10 mM TRIS, 100 mM NaCl, pH 7.0). A decrease in the emission intensity of the 451 and 475 nm bands of NP1-Tm is observed upon increasing concentration of GOx (Figure 3). The intrinsic errors observed for this titration are much lower in comparison to those observed for the PETNR titration with NP1-Tm reported above. Unlike with
PETNR, where protein aggregation was observed during similar experiments, the GOx appeared more stable in solution. This increased stability is likely the cause for the improved reliability and less error prone reading from scan to scan with GOx and led to a slightly lower LOD being calculated (10 µM, Table S-1). Due to solubility issues, a more dilute stock solution of GOx had to be used in comparison to PETNR. The ratiometric nature of the analysis negates issues with dilution effects but here, 0.1 mg/mL NP1-Tm was also added to the stock solution of GOx as an added precaution to maintain the UCP at a constant concentration.

It should be noted that beyond 150 µM GOx, the decrease in emission intensity appears to plateau and full quenching of the 451 and 475 nm bands was not observed. If the energy transfer process was due to the UCPs acting as ‘nanolamps’ as proposed by Hirsch et al.\textsuperscript{[31]} then it may be expected that increasing quantities of GOx should lead to complete quenching of these bands with this mechanism. It seems more feasible that the energy transfer may only be occurring from emissive ions closer to the surface of the particles and it is not possible to quench emitters closer to the centre. While the distances involved when considering an entire UCP are far beyond those involved in classical energy transfer processes, it has previously been reported that these distances can be greatly exceeded in the unique environment apparent within a NP lattice structure.\textsuperscript{[38]}

There have been additional reports labelling the effect as luminescence resonance energy transfer (LRET).\textsuperscript{[29]} which has been shown to work more efficiently over greater distances than traditional FRET.\textsuperscript{[40]} It is also not unreasonable to imagine that the majority of emitter ions are excited closer to the surface of the particle, as this has the largest surface area and lowest required penetration depth. In this case, energy transfer from near-surface ions to overlapping chromophores would seem more likely. We acknowledge further work needs to be performed to understand the underlying photophysical processes that allow these biosensor systems to work.

**Er(III)-based UCP sensors**

**Vitamin B\textsubscript{12} (vitB12) and cytochrome c (cyt c)**

Up to now, our efforts had been focused on Tm(III)-based UCPs and their interactions with flavins. While Er(III)-based UCPs do not show the same effect with flavin chromophores due to poor spectral overlap (Figure S-6), they should be able to undergo similar energy transfer with suitable chromophores absorbing around one of the main emissive Er(III) peaks. We chose to focus on the interactions between vitamin B\textsubscript{12} (vitB12) and PTIR545 and between cytochrome c (cyt c) and NP2-Er.

**Figure 3.** (a) Normalised emission spectrum of NP1-Tm UCPs during addition of GOx (200 µM aqueous stock, 10 µM increments from 0-60 µM, 20 µM increments from 60-100 µM and 50 µM increments from 100-200 µM). A decrease in the emission intensity is observed in the 451 and 475 nm emission bands of NP1-Tm, but not in the 800 nm, allowing normalisation of the spectra and subsequent ratiometric analysis. (b) Expansion of the 475 nm emission band. (c) Ratio of the 475:800 nm emission intensities of NP1-Tm as a function of added GOx. Standard error bars are shown in the graph.

**Figure 4.** (a) Normalised emission spectrum of NP2-Er UCPs during addition of cyt c (1 mM aqueous stock, 10-200 µM range). A decrease in the emission intensity is observed in the 525 and 545 nm emission bands of NP2-Er, but not in the 660 nm, allowing normalisation of the spectra and subsequent ratiometric analysis. (b) Expansion of the 500-580 nm emission range. (c) Ratio of the 545:660 nm emission intensities of NP2-Er as a function of added cyt c. Standard error bars are shown.

VitB12 is known to have a key role in central nervous system function, DNA synthesis, red blood cell formation, and metabolism.\textsuperscript{[41]} Deficiency of VitB12 can cause severe and...
irreversible physiological harm (e.g., pernicious anaemia), while increased levels can be a biomarker for a number of diseases.\textsuperscript{[42]} Cyt c is known to be released from cells during apoptosis, resulting in it being a key biomarker in monitoring and understanding a number of diseases on the cellular scale.\textsuperscript{[43]} These features have led to vitB12\textsuperscript{[44,45]} and cyt c\textsuperscript{[43,46]} being sought as targets for a number of biosensor systems. Both vitB12 and cyt c have an absorbance band that overlaps with the 525 and 545 nm emission bands of NP2-Er, indicating that the presence of both analytes should be detectable by monitoring the change in the ratio between the 525 and 545 nm bands vs the 660 nm band, which displays negligible spectral overlap in both cases (Figure 2).

Analogously to the NP1-Tm studies, titration experiments were carried out by adding incremental additions of vitB12 and oxidised cyt c to PTIR545 and NP2-Er respectively. As expected, a decrease in emission intensity of the 525 and 545 nm bands was observed in both cases (Figure 4). Lack of significant spectral overlap with the 660 nm Er(III) emission band enabled ratiometric analysis of the resulting spectra, by plotting the ratio of 525/545:660 nm emission intensity as a function of added analyte. As with the previous PETNR/GOx experiments, no notable change in lifetime was observed upon addition of either analyte over the concentrations tested, indicating static quenching processes are likely the dominant effect (the similarity in Stern-Volmer analyses between each system is illustrated in Figure 5). The calculated LODs were again on the \( \mu \text{M} \) scale, with cyt c and vitB12 providing the lowest detection limits (4 and 1 \( \mu \text{M} \)), respectively, Table S-1 to date with our solution-based system. As there is some overlap with Tm(III)-doped UCP emission bands, they can also be used to detect these biomolecules. The drastically lower spectral overlap, however, leads to a much smaller reduction in emission intensity and significant increases in experimental error (Figure S-9).

Figure 5. Stern-Volmer analyses of UCP emission bands upon the addition of (a) PETNR, (b) GOx, (c) vitB12, (d) cyt c. In each case, an initial linear increase in \( \frac{I_0}{I} \) is observed while \( \frac{I}{I_0} \) remains unchanged, suggesting static quenching processes for all systems tested. Beyond 60-100 \( \mu \text{M} \) of added analyte, the gradient of the intensity plot increase in all systems, suggesting the presence of additional quenching mechanisms.

**Analyte detection using PETNR**

Through the studies presented here, we are confident that the energy transfer from UCPs to biomolecules we have proposed as a model for biosensor development is transnational to any biomolecule with a suitable chromophore. In order to develop a more universal sensor system, however, it is also necessary to demonstrate that our technique can be advanced towards enzyme substrate detection. If this detection method can be accomplished, then it is possible to imagine the development of synthetic, chromophore-containing proteins that be designed for the detection of an array of desired analytes. By utilising the enzyme/protein as the part of the sensor with intrinsically high selectivity and specificity and the UCP as the luminescence readout, a broad range of biosensors could be readily achieved.

In order to test the feasibility of such a system, we returned to our initial PETNR/PTIR475 system to investigate if it could be used to detect substrates of the enzyme.

The absorption spectra of reduced and oxidised PETNR indicate that energy transfer from Tm(III)-doped UCPs can only occur when the flavin cofactor is in its oxidised form as the 2-electron reduced flavin is essentially colourless (Figure S-11). The change of spectral overlap during redox of the enzyme allows the energy transfer process to effectively be switched “on” and “off”. Utilising these “on/off” states offers the prospect of using energy transfer to monitor redox reactions of flavin cofactors involved in enzyme catalytic processes. Reduced PETNR reduces its substrates via a two electron reduction, becoming oxidised itself in the process (see Figure S-10). Monitoring the ratio between the emission intensity of the 475 and 800 nm emission peaks of PTIR475 allows the change in the redox state of PETNR to be determined, providing an indirect method of monitoring enzyme turnover.

Figure 6. (a) Natural substrates of PETNR used for monitoring the redox behaviour of PETNR during enzyme turnover, 2-cyclohexen-1-one (CH) and ketoisophorone (KI). (b) Normalised ratio of the 475:800 nm emission band of a 0.1 mg/mL dispersion of reduced PTIR475 in aqueous buffer (150 \( \mu \text{M} \)).
enzyme, 100 mM TRIS, 10 mM NaCl, pH 7.0) upon increasing addition of the two substrates (CH: 0 – 300 µM, 50 µM aliquots, 10 mM stock; KI: 0 – 120 µM, 20 µM aliquots, 10 mM stock solution). A decrease in the emission ratio is observed upon addition of both CH and KI, indicating the presence of oxidised PETNR undergoing energy transfer from the UCPs.

In order to test the potential of PTIR475 UCPs to monitor enzyme turnover, two known substrates of PETNR were investigated; 2-cyclohexen-1-one (CH) and ketoisophorone (2,6,6-trimethyl-2-cyclohexen-1,4-dione, KI). In both cases, anaerobic conditions were maintained throughout, as molecular oxygen is known to compete with the substrates for the oxidation of the reduced FMN cofactor of PETNR.\(^{[47]}\)

In an anaerobic glove box, 150 µM of oxidised PETNR was added to a dispersion of PTIR475 (0.1 mg/mL) in degassed aqueous buffer (100 mM TRIS, 10 mM NaCl, pH 7.0). NAD(P)H was then added to reduce the PETNR (400 µM for CH, 300 µM for KI) and the mixture visibly turned from yellow to colourless. Following the reduction, incremental additions of the desired substrate (CH: 0 – 300 µM, 50 µM aliquots, 10 mM stock solution; KI: 0 – 120 µM, 20 µM aliquots, 10 mM stock solution) were added until the 475:800 nm ratio of the PTIR475 emission bands no longer decreased (Figure 6). The return of the yellow colour of oxidised PETNR could also be visibly observed in the solution upon addition of CH or KI. These observations indicate the enzyme substrates are being reduced by PETNR, with the enzyme itself becoming oxidised and turning “on” the energy transfer mechanism between PTIR475 and PETNR. As energy transfer from the UCPs to PETNR can only occur when the FMN cofactor is in its oxidised form, the concentration of oxidised flavin can be determined by the change in UCP emission.

A much lower concentration of KI (100 µM) was required to fully quench the PTIR475 475 nm emission peak in comparison to CH (300 µM). This reduced concentration coincides with the increased catalytic efficiency (\(K_{cat}/K_m\)) of PETNR for transforming the KI (404 s\(^{-1}\)mM\(^{-1}\)) in comparison to CH (5 s\(^{-1}\)mM\(^{-1}\)).\(^{[47]}\) Therefore, the conversion of CH into its product, cyclohexen-1-one, is a much slower process. A delay of 20 min was maintained between each substrate addition, but it is possible that not all CH was converted during this time.

Ideally, the system should also show reversibility to allow repeated use. In order to test whether such a system could be used more than once, PETNR was repeatedly reduced and oxidised three times using NAD(P)H as the reducing agent and CH/KI as the oxidising agent. As with the substrate titrations, this repeated redox was performed in anaerobic conditions to limit the effect of molecular oxygen. Upon repeated reduction and oxidation of PETNR, a clear “on-off” reversibility in the UCP 475:800 nm emission intensity ratio is observed for both CH and KI (Figure 7). There is significant variation in the ratio intensity, which is likely due to sedimentation of the UCPs and some aggregation of the enzyme (visible as the formation of white agglomerates). Increasing amounts of NAD(P)H and CH/KI were also required to restore or reduce the 475 nm UCP emission band each subsequent cycle. Despite these limitations, the pattern observed shows that the system is reversible and could theoretically be developed into a multi-use biosensor, if the stability and reproducibility could be improved. The observations also indicate that a substantial amount of the enzyme is retaining its activity upon multiple redox cycles in the presence of UCPs.

Figure 7. (a) Normalised 475:800 nm emission intensity ratio of PTIR475 upon repeated reduction and oxidation of PETNR. Reduction was performed by NAPDH and oxidation by (a) CH or (b) KI. The ratio is high (0.75–1.2) when PETNR is in its reduced form (energy transfer “off”) and low (0.2–0.4) when PETNR is in its oxidised form (energy transfer “on”). Standard error bars are shown.

Conclusions

This study expands upon our previous proof-of-principle work\(^{[13]}\) in demonstrating the use of UCPs to detect on the level and function of analytes that span enzymes to key disease biomarkers; here, PETNR, glucose oxidase, vitamin B\(_12\) and cytochrome c. Depending on the absorption spectrum of the analyte of interest, Yb(III):Tm(III) or Yb(III):Er(III) doped commercial or ligand capped NaYF\(_4\) UCPs are able to spectroscopically report on the presence of and redox state(s) of important biomolecule cofactors in a ratiometric manner via energy transfer from the nanoparticle emission to the visible chromophore of the analyte in question with calculated limits of detection in the low micromolar range. In this regard, tailoring the absorption profile of the UCP emission to the biomolecule/cofactor of interest means a wide-range of analytes can be utilised. In addition to the examples shown here, due to the nature of the tunable UCP emission, in principle, it would be possible to incorporate any biomolecule with a chromophore that absorbs in the visible-NIR region of the electromagnetic spectrum (here, 400–820 nm). Not every potential analyte contains such a chromophore however, and so the demonstration of using PETNR to sense substrate turnover is a key finding. It should be trivial to utilise this system for detection of PETNR substrates of greater practical importance, such as 2,4,6-trinitrotoluene (TNT).\(^{[48]}\) More importantly, however, this example paves the way for the design of synthetic flavin-containing (or other suitable chromophore) proteins for a wide array of small-molecule analytes of interest.
One area for improvement is the micromolar LOD concentrations with these systems at present. The work presented here however, highlights the broad scope of our approach and increases confidence in potential translation to real-world applications. Strategies to reduce current limitations and increase applicability, such as covalent conjugation of biomolecules and/or incorporation into devices, are currently on going.

**Experimental Section**

**General details**

CH, ErCl₃·6H₂O, FAD, GOx, KI, NAD(P)H, NH₄F, TmCl₃·6H₂O, YCl₃·6H₂O and YbCl₃·6H₂O were purchased from Sigma Aldrich. Ethylene glycol and NaOH were purchased from Fisher Scientific. AHA, MHA and NaF were purchased from Alfa Aesar. Cyt c was purchased from CalbioChem, FMN was purchased from AppliChem and OA was purchased from VWR. All chemicals were used as received. Deionised water was obtained from a Millipore Synergy water purification system. The commercial UCPs, PTIR475 and PTIR545, were kindly donated by Phosphor Technology Ltd. PETNR was prepared, purified, and its activity tested according to a literature procedure.[49]

Steady state emission spectra were recorded in quartz cuvettes on an Edinburgh Instrument FP920. Phosphorescence lifetime spectrometers were equipped with a 450 W xenon lamp (with single 300 mm focal length excitation and emission monochromators in Czerny Trunier configuration) and a red sensitive photomultiplier in Peltier (air cooled) housing (Hamamatsu R928P) and a custom built (Edinburgh Instruments) 980 nm diode laser operating in either CW mode or pulsed mode, with variable repetition rate. The average power and repetition rate of the diode laser was kept constant for all experiments. All scans were performed in triplicate and an average taken to minimise errors arising from variable aggregation/solubility from scan to scan. In addition, emission band ratios could be affected by temperature changes, which could arise through the heating effect of the irradiation source. Provisional experiments using a Digi-sense type K thermocouple thermometer indicated small, but significant changes in certain bands and slight heating effects upon prolonged irradiation (Figure S-12, Table S-5). Therefore, irradiation times (130 s) and volumes of solutions (3 mL) were kept constant throughout to avoid possible misinterpretation arising from these variables, as temperature changes were negligible under these conditions.

Lifetime data of 0.1 mg/mL UCP samples in DI H₂O or TRIS buffer (pH 7.0) were recorded following 980 nm excitation in pulsed mode using time correlated single photon counting (PC9900 plug-in PC card for fast photon counting). Lifetime values were obtained by tail fit on the data obtained, and the quality of fit judged by minimization of reduced chi-squared and residuals squared.

Fourier transform-infrared (FTIR) analysis was performed on a Thermo Scientific Nicolet 5700 FT-IR Spectrometer using with the attenuated total reflection (ATR) method on dry solid UCP samples. The data were analysed with OMNIC software.

A PANalytical XPert powder X-ray diffractometer was used to perform pXRD analysis. Dry UCP samples were placed on a silicon crystal sample holder and measured at a scanning speed of 0.047 °/s in the 26 range from 10° to 90° using graphite-monochromated Cu-Kα radiation (λ = 1.54 Å). The crystal phases of the UCPs were determined by comparison with data from the Inorganic Crystal Structure Database (ICSD).

A Malvern Zetasizer Nano ZS was used to perform DLS measurements. 1 mg/mL UCP samples in DI H₂O were sonicated for 10 minutes and then analysed at 20 °C in a DTS1070 capillary cell. A 633 nm laser was used as the excitation source and a backscattering NiB.J detector at 173 ° to the laser was used to collect the scattered light. The data were analysed using the general method with the Mark-Houwink parameters α = 0.428, k = 7.67 x 10⁵ cm²/s. The results quoted are the average of three measurements.

TEM samples were prepared by placing a droplet of a dilute, aqueous solution of a UCP sample onto a copper mesh TEM grid, flash coated with palladium, and left to air dry.

UV-vis spectra were recorded in quartz cuvettes on a Cary 60 spectrometer (Agilent), with a path length of 1. Sample concentrations were determined using known extinction coefficient values and compared to the literature.

**Synthesis of NP1-Tm (NaYF₄: 20% Yb³⁺, 0.2% Tm³⁺) and NP2-Er (NaYF₄: 20% Yb³⁺, 2% Er³⁺)**

This procedure was adapted from previously published methods.[21,22] A solution of NaOH (24.4 mmol) and AHA (5g, 38 mmol) were dissolved in H₂O (5 mL). MHA (2 g, 9.5 mmol) was dissolved in ethanol (8.5 mL). H₂O and ethanol solutions mixed to form a homogenous solution. OA was added (17 mL, 54 mmol) and stirred for 10 min. 1.2 mL LnCl₃ (0.5 M. Tm UCPs; 20% Yb³⁺, 0.2% Tm³⁺. Er UCPs; 20% Yb³⁺, 2% Er³⁺) and stirred for a further 10 min. 4 mL aqueous NaF (1 M) added dropwise. Stirred for 10 min then placed in a Teflonlined reaction vessel (Parr Instrument, 302AC T304) and heated at 120 °C for 30 min before increasing to 200 °C for 5 h. The solution was centrifuged (2665 g, 10 min), washed with ethanol (3 x 10 mL) and dried overnight in a vacuum desiccator to give NP1-Tm (Yield: 214.8 mg) and NP2-Er (Yield: 234.8 mg) of Tm³⁺ and Er³⁺ doped UCPs respectively. Both samples were obtained as white powders.

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**Keywords:** lanthanide • upconversion • energy transfer • biosensor • enzyme

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A one pot synthesis of thulium and erbium lanthanide doped upconverting phosphors capped with three different functional groups ratiometrically sense the presence of cofactors in key biomolecules through energy transfer and report on enzyme activity of flavoproteins with substrates.

*Upconversion biosensors

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