Evaluating spectral overlap with the degree of quenching in UCP luminescence energy transfer systems

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Abstract
The use of organic based fluorophores has been firmly established as a key tool in the biological sciences, with many biological-sensing methods taking advantage of Förster Resonance Energy Transfer (FRET) between different fluorescent organic based dyes following one photon excitation. Nevertheless, the employment of UV-visible absorbing dyes as fluorescent tags and markers typically suffer from several drawbacks including relatively high energy of excitation wavelength, photobleaching and competitive autofluorescence, which often limits their effectiveness and longevity both in vitro and in vivo. As an alternative, lanthanide doped upconverting phosphors (UCP) have emerged as a new class of materials for use in optical imaging and RET sensing; they exhibit high photo- and chemical stability and utilise near infrared excitation. Approaches to sensing a given analyte target employing upconverting phosphors can be achieved by engineering the UCP to operate analogously to fluorescent dyes via Luminescence Resonance Energy Transfer (LRET) and such systems are now becoming central to optically sensing low concentrations of biologically important species and performing distance measurements. Similarly to FRET, the LRET process is distance dependent and requires spectral overlap between the absorption of the acceptor luminophore and the emission of the donor moiety, yet essential measures of the relationship between spectral overlap and the degree of quenching have not yet been established. To address this, we have investigated the Stern-Volmer relationship for a set of six commonly functionalised organic dyes and seven biomolecules that contain key chromophoric co-factors with Gd₂SO₄:Yb:Er (PTIR545) and Gd₂SO₄:Yb:Tm (PTIR475) UCPs under low power nIR excitation, and found that for the organic dyes a linear relationship between spectral overlap and degree of quenching is observed. However, this linear relationship is observed to break down for all the biomolecules investigated.

Introduction
The development of biosensors has received great interest over the last decade as elevated or changed levels of biomolecules such as proteins, DNA and carbohydrates amongst others within the body can provide an important indication of disease [1]. The ability to detect these changes in biomolecules enables more effective treatment, as early detection improves prognosis and ultimately reduces cost [2]. Optical biosensors are the most common type of biosensor [3]. They have many advantages over conventional analytical techniques; they are highly sensitive, rapid and reproducible allowing real time analysis in complex mixtures. They are simple to operate, do not require extensive sample preparation and are small in size and cost effective compared to some other methods [3, 4]. These optical biosensors commonly work on the principal of Luminescent Resonance Energy Transfer (LRET), the non-radiative transfer of energy from a
interaction is distance dependent (range 1–10 nm) and requires a spectral overlap between the absorption of the acceptor and the emission of the donor [5, 6].

Unfortunately, many optical biosensors that use organic dyes or quantum dots (QD) as the probe molecule suffer from several limitations such as overlap of excitation and emission bands, autofluorescence of the biological media, photobleaching and photoblinking [7, 8]. Lanthane doped upconverting phosphors (UCP) are a unique class of probes for use as LRET or apparent energy transfer quenching biosensors (depending on the exact mechanism of quenching involved) as they do not suffer from these same limitations. Upconversion is a powerful non-linear process involving the sequential absorption of two photons of longer wavelength (lower energy) resulting in emission of light of shorter wavelength (higher energy) [8, 9]. For lanthanide (YbIII and ErIII/TmIII) co-doped UCPs this is characterised by intra 4f-4f excitation in (1)

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...involved (as they do not suffer from these same limitations. Upconversion is a powerful non-linear process involving the sequential absorption of two photons of longer wavelength (lower energy) resulting in emission of light of shorter wavelength (higher energy) [8, 9]. For lanthanide (YbIII and ErIII/TmIII) co-doped UCPs this is characterised by intra 4f-4f excitation in the near infra red (nIR, 980 nm) and the emission of visible light—for YbIII:TmIII doped nanoparticles at 475 nm (\( ^1G_4 \rightarrow ^3H_6 \)) and 800 nm (\( ^1D_2 \rightarrow ^2F_3 \)) and for YbIII:ErIII at 410 nm (\( ^3H_2 \rightarrow ^4I_{15/2} \)), 520 nm (\( ^3H_{15/2} \rightarrow ^4I_{15/2} \)), 545 nm (\( ^5S_{5/2} \rightarrow ^4I_{15/2} \)) and 660 nm (\( ^5P_{9/2} \rightarrow ^4I_{15/2} \)) [9, 10]. This large anti-Stokes shift leads to facile separation of UCP excitation and emission [11]. The use of nIR excitation also has two added benefits of deeper tissue penetration and no autofluorescence of omnipresent chromophoric containing biomolecules in samples, as they exhibit negligible absorption in this wavelength range [7, 12]. The presence of multiple emission bands for UCPs additionally enables ratiometric sensing since the acceptor fluorophore can be chosen so that only one of the emission bands that overlaps with the acceptor absorption is affected. In such cases, all other bands remain unchanged. This helps to negate effects such as laser power and probe concentrations, thus improving the sensitivity and precision of measurements [13].

UCPs are also chemically stable, therefore they are less toxic, and photostable [7, 12]. A final advantage of using UCPs is their long emission lifetimes enable time gating spectroscopic methods to be used [11]. All these advantages result in an improved signal to noise ratio for biosensors containing UCPs [7].

Although many examples exist in the literature of UCP based LRET biosensors for the detection of biomolecules such as; DNA [14–18], proteins [19–22], antioxidants [23], glutathione [24, 25] and enzymes [26–30], the relationship between the spectral overlap of the biomolecule absorption with the UCP emission bands has not been explored, which would be of great benefit in the design of new LRET systems. Here we set out to investigate this relationship, six organic dyes and seven biomolecules whose absorption profile overlapped with either the 475 nm band of Gd₂SO₄:Yb:Tm (PTIR475) or the 545 nm band of Gd₂SO₄:Yb:Er (PTIR545) UCPs were chosen as representative systems for the studies.

**Experimental**

The approximate micron sized Gd₂SO₄:Yb,Tm (PTIR475) and Gd₂SO₄:Yb,Er (PTIR545) upconverting phosphors were donated by Phosphor Technology Ltd; spectroscopic analysis on these commercial UCPs indicate that, to the best of our knowledge, these nanoparticles are not functionalised [26]. The size distribution of the UCPs was determined by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM), see Supplementary Information is available online at stacks.iop.org/MAF/8/045003/mmmedia. DLS data (as solutions of 1 mgUCP/ml in TRIS buffer at pH 7.0) indicates a size of 1432 nm for PTIR475 with a PDI of 0.358, and a size of 2964 nm with a PDI of 0.823 for PTIR545. These sizes do not correlate particularly well with the sizes obtained from the TEM (values obtained from TEM are an average of 10 imaged UCPs per sample), which gives a size estimation of 765 nm for PTIR475 and 320 nm for PTIR545 respectively. This discrepancy is more than likely due to aggregation of the UCPs in the TEM images (as commonly seen in these systems) and partial sedimentation in the DLS. The enzymes, PETNR and GFP were donated by the Hay group (The University of Manchester) and the enzyme BM3Heme475 was donated by the Munro group (The University of Manchester). Vitamin B12 (hydrocobalamin, VitB12) was purchased from Generon Ltd, SAMSa fluorescein (SF) was purchased from Life Technologies Ltd and Fluorescein maleimide (FM) was purchased from Vector Laboratories Ltd. All other solvents, reagents and biomolecules were purchased from Sigma-Aldrich. All reagents and solvents were used as received. Molar absorption extinction coefficients of the organic dyes and the biomolecules (glutathione reductase) were determined experimentally at three wavelengths; the absorption maximum of the dye or biomolecule co-factor, and at 475 nm and 545 nm corresponding to the emission maxima of the Gd₂SO₄:Yb:Tm and Gd₂SO₄:Yb:Er UCPs respectively. For glutathione reductase (GR), difficulties in reproducibility of the molar extinction coefficients were encountered, therefore the literature extinction coefficient of 11 300 M⁻¹ cm⁻¹ at the absorption maxima of 460 nm reported by Massey was used alongside an experimentally collected spectrum that matched the literature values (see V. Massey, C.H. Williams Jr J. Biol. Chem., 1965, 240, 4470–4480).

Emission spectra of the upconversion phosphors were recorded on an Edinburgh Instrument FP920 spectrofluorometer using a 45 mW continuous wave (CW) 980 nm diode laser and a red sensitive photomultiplier tube (PMT). Lifetime data of these UCPs was recorded using a 45 mW pulsed 980 nm diode...
laser using time correlated single photon counting (PCS900 plug-in PC card). The lifetime data was fitted using the tail fit function in the Edinburgh Instrument Software Package and the goodness of fits judged on chi [2] and residuals squared. All spectra were corrected for the excitation source and the detector response using the correction files provided in the software, but in the case of the UCPs, the emission spectra were not corrected for the detector response in order to observe the blue emission bands of the upconverting phosphors with respect to the nIR bands more clearly. The data for the Yb:Er doped UCPs was normalised to the 660 nm band and the Yb:Tm doped UCPs to the 800 nm band as these bands lie outside of the wavelength range of interest for the organic dyes and biomolecules and are therefore unaffected by their presence, this allows for ratiometric detection methods and negates the probe concentration dependency.

All dye and biomolecule titrations were carried out in a similar manner: incremental additions of the dye/ biomolecule were added to a 1 mg ml⁻¹ dispersion of PTIR-UCP in buffer. Preparation of the biomolecules is described in reference [31]. For all the dye titrations, PETNR and vitamin B12 titrations a 100 mM TRIS pH 7 buffer was used. For the GFP, BM3Heme, Cytc and GR titrations a 100 mM PBS pH 7.4 buffer was used. For the GO titrations, a 100 mM sodium acetate pH 5.1 buffer was used.

In order to ensure that the degree of quenching observed in the dye/biomolecule titrations is due to the presence of the dye/biomolecule only, the experimental conditions were kept constant for all of the titrations:

1. The concentration of the UCP was 1 mg ml⁻¹.
2. A buffer was used to maintain a constant pH (either 100 mM TRIS pH 7 or 100 mM PBS pH 7.4 or 100 mM sodium acetate pH 5.1, see supplementary information for buffer control experiment).
3. The titrations were carried out at room temperature (298 K, see supplementary information for temperature control).
4. The total volume of the dye/biomolecule added was kept to a minimum to avoid dilution effects (≤5% dilution, see supplementary information for the dilution control experiment).
5. The concentration of the dye at the wavelengths of interest was kept below 0.2 absorption units in order to minimise any inner filter effects (this was calculated from the extinction coefficient of the dye at the wavelength of interest, see tables 1 and 2).
6. The data presented below is the averaged data set of three repeats (to ensure reproducibility) and has been normalised to the other band in the UCP emission spectrum, i.e. the 660 nm band for PTIR454 and the 800 nm band of PTIR475. This band lies outside the spectral range of the acceptor dye and therefore its intensity is independent of the dye concentration, normalisation to this band allows ratiometric analysis of the system (which negates probe concentration dependency - important for these systems as they are dispersions with variable concentration per scan) [26].

**Results and discussion**

In this study, the fluorescent dyes; fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RBITC), rhodamine 6G ethylene diamine (R6GNNH₂), SAMS fluoescin (SF), fluoescin maleimide (FM) and methyl red (MR) were chosen to represent commonly used dye molecules in FRET studies, whereas the biomolecules; enhanced green fluorescent protein (GFP), pentaerythritol tetranitrate reductase (PETNR), heme domain (residues 1–472) of *Bacillus megaterium* cytochrome P450 BM3 (BM3Heme), Cytochrome C (cytC), vitamin B12, glucose oxidase (GO) and glutathione reductase (GR) were chosen to represent key co-factor containing biomolecules often implemented in disease or used for sensing and for their respective absorption spectral features which overlap significantly with the upconversion emission bands of Er³⁺ and Tm³⁺ (Chart 1 and figure 1). The enzyme PETNR was chosen to supplement our previous studies [26, 30]. In principle, the degree of quenching of the UCP emission brought about by each organic dye or co-factor, here represented by the Stern-Volmer constant, $K_{SV}$, will be linearly correlated to the magnitude of the spectral overlap integral (I) of the dye/biomolecule absorption band with the UCP emission band.

In order to experimentally determine the relationship between spectral overlap and degree of quenching, the spectral overlap must first be calculated. An absorption spectrum of the acceptor (FITC) and an emission spectrum of the donor (PTIR475 and PTIR454) was recorded (figures 2(a) and (b)) and the spectral overlap integral (I) with either the 475 nm emission band of PTIR475 or the 545 nm band of PTIR454 was calculated using the following equation [32]:

$$I = \int F_\lambda(\lambda)\varepsilon_\lambda(\lambda)\lambda d\lambda$$

Where $F_\lambda$ is the normalized fluorescence intensity of the donor emission band (either the 475 nm band or the 545 nm band of PTIR475 and PTIR454 respectively), and $\varepsilon_\lambda$ is the extinction coefficient of the acceptor (dye/molecule) over that band [32]. For FITC, $I$ was calculated to be $2.79 \times 10^{15}$ nm⁻¹ M⁻¹ cm⁻¹ for the 475 nm band and $1.63 \times 10^{14}$ nm⁻¹ M⁻¹ cm⁻¹ for the 545 nm band, validating the observation
Table 1. Summary of the extinction coefficient (ε), spectral overlap integral (f), Stern-Volmer constant (Ksv), limit of detection (LoD) and limit of quantification (LoQ) for each dye with each UCP; PTIR475 (475 nm emission band) and PTIR545 (545 emission band).

| Dye     | UCP Band (nm) | ε (mol⁻¹ d m⁻³ cm⁻¹) | Spectral Overlap (nm² M⁻¹ cm⁻¹) | Ksv (M⁻¹) | LoD (µM) | LoQ (µM) |
|---------|---------------|------------------------|---------------------------------|-----------|----------|----------|
| FITC    | 475           | 46500                  | 2.79 × 10⁻¹⁵                    | 97000 ± 3400 | 0.10 ± 0.03 | 0.35 ± 0.04 |
|         | 545           | 612                    | 1.63 × 10⁻¹⁴                    | 6400 ± 290  | 0.14 ± 0.06 | 0.46 ± 0.05 |
| RBITC   | 475           | 7460                   | 6.00 × 10⁻¹⁴                    | 22000 ± 890 | 0.12 ± 0.04 | 0.40 ± 0.04 |
|         | 545           | 98000                  | 8.26 × 10⁻¹⁵                    | 240000 ± 6400 | 0.08 ± 0.03 | 0.26 ± 0.03 |
| R6GNH2  | 475           | 446                    | 3.13 × 10⁻¹³                    | 390 ± 53   | 0.41 ± 0.1  | 1.4 ± 0.1  |
|         | 545           | 448                    | 4.83 × 10⁻¹³                    | 150 ± 32   | 0.64 ± 0.2  | 2.1 ± 0.2  |
| MR      | 475           | 12800                  | 5.93 × 10⁻¹⁴                    | 260000 ± 950 | 0.11 ± 0.04 | 0.36 ± 0.04 |
|         | 545           | 622                    | 6.21 × 10⁻¹³                    | 1600 ± 72  | 0.14 ± 0.05 | 0.46 ± 0.05 |
| SF      | 475           | 66700                  | 3.76 × 10⁻¹⁵                    | 101000 ± 1600 | 0.05 ± 0.02 | 0.15 ± 0.02 |
|         | 545           | 757                    | 1.22 × 10⁻¹⁴                    | 5300 ± 67  | 0.04 ± 0.01 | 0.13 ± 0.02 |
| FM      | 475           | 43000                  | 2.79 × 10⁻¹⁵                    | 85000 ± 6700 | 0.23 ± 0.08 | 0.78 ± 0.08 |
|         | 545           | 0                      | 5.86 × 10⁻¹³                    | 1100 ± 290 | 0.78 ± 0.3  | 2.6 ± 0.3  |

Table 2. Summary of the extinction coefficient (ε), spectral overlap (f), Stern-Volmer constant (Ksv), limit of detection (LoD) and limit of quantification (LoQ) for each biomolecule with each UCP; PTIR475 (475 nm emission band) and PTIR545 (545 emission band).

| Enzyme | Wavelength (nm) | Extinction coefficient (M⁻³ cm⁻¹) | Spectral Overlap (nm² M⁻¹ cm⁻¹) | Stern-Volmer Constant (Ksv) (M⁻¹) | Limit of detection (LoD) (µM) | Limit of quantification (LoQ) (µM) |
|--------|-----------------|----------------------------------|---------------------------------|-----------------------------------|------------------------------|-----------------------------------|
| PETNR  | 475             | 10584                            | 5.19 × 10⁻¹⁴                    | 82700 ± 890                       | 0.32 ± 0.11                   | 1.07 ± 0.11                       |
|        | 545             | 2.35                             | 1.24 × 10⁻¹⁵                    | 2700 ± 760                        | 0.84 ± 0.29                   | 2.82 ± 0.28                       |
| GFP    | 475             | 53838                            | 2.94 × 10⁻¹⁵                    | 239000 ± 18000                    | 0.23 ± 0.08                   | 0.77 ± 0.08                       |
|        | 545             | 140                              | 3.22 × 10⁻¹⁵                    | 422000 ± 12000                    | 0.83 ± 0.27                   | 2.74 ± 0.27                       |
| BM3Heme| 475             | 8186                            | 4.46 × 10⁻¹⁴                    | 13000 ± 1500                      | 0.42 ± 0.14                   | 1.40 ± 0.14                       |
|        | 545             | 10913                           | 9.36 × 10⁻¹⁴                    | 210000 ± 860                      | 0.16 ± 0.05                   | 0.52 ± 0.05                       |
| Oxidised CytC | 475       | 5672                            | 3.11 × 10⁻¹⁴                    | 560 ± 970                         | 0.51 ± 0.17                   | 1.71 ± 0.17                       |
|        | 545             | 8571                            | 7.44 × 10⁻¹⁴                    | 8720 ± 970                        | 0.26 ± 0.08                   | 0.85 ± 0.09                       |
| Reduced CytC | 475    | 3119                            | 1.84 × 10⁻¹⁴                    | 0 ± 2600                         | 6.2 ± 2.04                    | 20.7 ± 2.1                        |
|        | 545             | 13215                           | 1.46 × 10⁻¹⁵                    | 230000 ± 16000                    | 0.21 ± 0.07                   | 0.70 ± 0.70                       |
| VitB12  | 475             | 7563                            | 4.38 × 10⁻¹⁴                    | 6590 ± 290                        | 0.51 ± 0.17                   | 1.68 ± 0.17                       |
|        | 545             | 12415                           | 9.73 × 10⁻¹⁴                    | 11900 ± 1100                      | 0.072 ± 0.02                  | 0.24 ± 0.02                       |
| GR     | 475             | 9970                            | 5.07 × 10⁻¹⁴                    | 92900 ± 19000                     | 0.61 ± 0.20                   | 2.30 ± 0.23                       |
| GO     | 475             | 9670                            | 4.49 × 10⁻¹⁴                    | 37800 ± 23000                     | 1.83 ± 0.60                   | 6.11 ± 0.61                       |

from figures 2(a) and (b) that this dye has a better spectral overlap with the 475 nm band. The spectral overlap of all other dyes/biomolecules (data located in the Supplementary Information) has been calculated and the data summarized in table 1 (dye data) and table 2 (biomolecule data).

The second step is to calculate the degree of quenching; this is given by the Stern-Volmer constant (Ksv), the gradient of the fit of I0/I. The dye/biomolecule was sequentially titrated into a solution of the UCPs and an emission spectrum (figures 2(c) and (d) show the sequential titration of FITC into a solution of PTIR475 and PTIR545 respectively) and lifetime recorded.

From the Stern-Volmer plots; the limit of detection (LOD), the lowest analyte concentration that can be determined from the blank, see equation (2) [33, 34] and limit of quantification (LoQ, the lowest analyte concentration that can be determined with precision under the conditions of the test, see equation (3)) [33, 34] can also be calculated using the linear regression model to fit the entire I0/I data for the band of interest:

\[
\text{LoD} = \frac{3S_a}{b} \quad (2)
\]

\[
\text{LoQ} = \frac{10S_a}{b} \quad (3)
\]

Where S_a is the error of the fit and b is the gradient of the fit.

The Stern-Volmer plots (I0/I and τ0/τ) for the titration of FITC into PTIR475 (figure 2(d)) and PTIR545 (figure 2(f)) are shown below. As sequential amounts of FITC are titrated into a solution of PTIR475 and PTIR545, a decrease in both the 475 nm (31.0%) and 545 nm (29.7%) bands is observed. A Ksv of 97000 ± 3400 M⁻¹ was obtained for PTIR475 and a Ksv of 6400 ± 290 M⁻¹ for PTIR545 was obtained. These Stern-Volmer constants show that there is a higher degree of quenching for PTIR475 than PTIR545 by FITC, validating the hypothesis that this is due to the greater spectral overlap of the dye with the 475 nm band.
It is expected that the LoD and LoQ would be lower for the system with the better overlap, as it should be the most sensitive. For the FITC titrations, a lower LOD of 0.10 μM and LoQ of 0.35 μM was obtained for the PTIR475 titrations than PTIR545 (0.14 μM and 0.46 μM respectively) as expected. The KSV, LoD and LoQ values were calculated for all dye/biomolecule titrations and is summarised below in tables 1 and 2.

The lifetime data was also recorded at each increment during the titrations; the lifetime data for PTIR475 is monoexponential, and an initial lifetime of 99.1 ± 0.1 μS was obtained. During the course of the titration there is minimal (within error) change in the lifetime value and after 4.9 μM addition of FITC the lifetime remains 97.5 ± 1.3 μS. The lifetime fit for PTIR545 is biexponential, containing two species (ratio 40:60) with initial lifetime values of 65.1 ± 1.7 μS and 164.3 ± 2.4 μS respectively. Again the lifetime changes very little during the course of the titration and after addition of 67.6 μM FITC, lifetime values of 63.9 ± 0.8 μS and 163.1 ± 1.1 μS are obtained. As the lifetime of both species is unaffected by increasing FITC concentration, for clarity in figure 2 only the lifetime data for the predominant species has been plotted. This lack of change in the lifetime value for the UCPs would suggest that the method of quenching observed is static quenching, however this might not be the case. This lack of change in the lifetime data has previously been observed in UCP systems and has been suggested as being due to the fact that only the surface lanthanide ions are quenched [26]. As these UCPs are very large (on the μm scale, see TEM data in Supplementary Information), the core lanthanide ions in the body of the UCP should remain unaffected, seemingly masking any small changes in the lifetime of the surface emitters. We also considered the possibility of inner filter effects being the dominant quenching pathway, which would also explain the non-statistically relevant lifetime changes of the UCPs in the presence of the dye molecules. However, analysis of log(I0/I) (Supplementary Information, SI20) data did not show significantly more linear trends that the I0/I presented. Since we designed the experiment to minimise inner filter effects, we believe the unusual lifetime data is mainly due to other factors.

Footnote: Due to the small spectral overlap of flavoenzymes with the 520/545 nm band of PTIR545, and due to the small KSV obtained for the titration of PETNR into a solution of PTIR545, the titrations of GO and Glutathione Reductase (GR) were carried out only with the PTIR475 UCPs.

It is expected that the system that possesses the greatest degree of spectral overlap of enzyme absorption with UCP emission, will experience the most efficient LRET/apparent energy transfer and therefore will have the highest KSV and lowest LoD and LoQ. The expected trend is therefore: SF > FM > FITC > RBITC > MR > R6GNH2 and GFP > PETNR > GO > VitB12 > BM3Heme > Oxidised CytC > Reduced CytC for the Yb:Tm and RBITC > FITC > R6GNH2 > FM > SF > MR and reduced CytC > VitB12 > BM3Heme > oxidised CytC > GO > GFP > PETNR for the Yb:Er PTIR-545 UCPs.
As can be seen from tables 1 and 2, in almost all cases (the only exception is R6GNH₂ for the PTIR545 titrations), the UCP that has a better spectral overlap with the dye/biomolecule will be quenched to a greater extent. The organic dyes; FITC, SF, FM and MR, and the biomolecules; GFP, PETNR, GO and GR, have a greater spectral overlap with the 475 nm band of PTIR475 than with the 545 nm band of PTIR545 and the data show a greater KSV for these dyes/biomolecules with PTIR475 than PTIR545 as expected.

Figure 1. (a) Spectral overlap of the absorption spectra of FITC, RBITC, R6GNH₂, MR, SF and FM with the emission spectra of PTIR475 and PTIR545. All spectra recorded in 100 mM TRIS pH 7. Emission spectra recorded after CW excitation at 980 nm and reported uncorrected for detector response. PTIR545 has been normalised to the 545 nm band and PTIR475 has been normalised to the 475 nm band. Concentrations varied between dyes (from 3–35 μM) and all spectra have been normalised to their maximum absorption between 400–600 nm in order to clearly indicate spectral overlap. In all titrations, concentrations were chosen such that the maximum absorption was below 0.2, to avoid the inner filter effect TRIS pH 7. (b) Spectral overlap of the absorption spectra of GFP, PETNR, BM3Heme, CytC, vitamin B12, GO and GR with the emission spectra of PTIR475 and PTIR545. The emission spectra, PETNR absorption and VitB12 absorption were recorded in 100 mM TRIS pH 7. GFP, BM3Heme, CytC and GR absorption were recorded in 100 mM PBS pH 7.4 and the GO absorption was recorded in 100 mM sodium acetate buffer pH 5.1. Emission spectra recorded after CW excitation at 980 nm and reported uncorrected for detector response. PTIR545 has been normalised to the 545 nm band and PTIR475 has been normalised to the 475 nm band. Concentrations varied between biomolecules (from 6–60 μM) and all spectra have been normalised to their maximum absorption between 400–600 nm in order to clearly indicate spectral overlap.
RBITC, BM3Heme, CytC and Vitamin B12 on the other hand have a greater spectral overlap with the 545 nm band of PTIR545 than the 475 nm band of PTIR475 and again the data shows a greater $K_{SV}$ for these dyes/biomolecules with PTIR545 than PTIR475.

For the dye titrations a linear relationship is observed between spectral overlap and degree of quenching for both the PTIR475 and PTIR545 systems. There are small deviations from the expected ordering, such as FITC and FM for PTIR475, due to the systems not being sensitive enough to distinguish between dye molecules that have similar spectral overlaps (both $2.79 \times 10^{15}$ nm$^4$ M$^{-1}$ cm$^{-1}$), however these small deviations are within error ($K_{SV} = 97000 \pm 3400$ and $85000 \pm 6700$ M$^{-1}$ respectively) and therefore are negligible. They do not detract from the linear relationship. Also it should be noted that for the PTIR545 titrations, R6GNH2 has the smallest $K_{SV}$ despite having the third largest spectral overlap, this deviation appears to be a result of the poorer solubility of this dye under the conditions employed, but again, this does not detract significantly from the observed linear relationship.

Figure 2. (a) figure showing the overlap of the extinction coefficient of the dye acceptor (FITC) with the emission of the 475 nm band of PTIR475, (b) figure showing the overlap of the extinction coefficient of the dye acceptor (FITC) with the emission of the 520/545 nm band of PTIR545, (c) Normalised emission spectrum showing only the 475 nm band of PTIR475 UCPs (1 mg ml$^{-1}$ in TRIS pH 7) during the addition of FITC. Excitation at 980 nm. The graph is normalised to the emission intensity at 800 nm in order to negate the probe (UCP) concentration dependency and allow for ratiometric analysis, (d) The Stern-Volmer plot for the titration of sequential amounts of FITC into a solution of PTIR475, (e) Normalised emission spectrum showing only the 545 nm band of PTIR545 UCPs (1 mg ml$^{-1}$ in TRIS pH 7) during the addition of FITC. Excitation at 980 nm. The graph is normalised to the emission intensity at 660 nm and (f) The Stern-Volmer plot for the titration of sequential amounts of FITC into a solution of PTIR545.
However for the biomolecule titrations, it can be seen from figure 3 below that this linear relationship is not observed. This is most likely due to the size of the biomolecules varying more widely than the organic dyes (the organic dyes have a size range of 266.78 $\text{g mol}^{-1}$, whereas the biomolecules have a size range of 158.6 kDa) resulting in significantly different diffusion rates of the biomolecules to the UCPs in order for this quenching mechanism to occur. Also conformational changes of the biomolecules resulting from favourable electrostatic interactions with the surface of the UCPs that may alter the average distance of the co-factor to the emissive metal cations cannot be ruled out.

It is interesting to note from tables 1 and 2 above, that the LoD and LoQ data neither match the predicted trend from the spectral overlap nor match the trend observed from the ordering of the experimental $K_{SV}$ values obtained. It is expected that the LoD and LoQ will be smallest for the dye/biomolecule that has the largest spectral overlap, or the dye/biomolecule that has the largest $K_{SV}$ as the system should be most sensitive to the dye/biomolecule. However there does not appear to be any consistent trend in the LoD and LoQ ordering, the reason for this most likely lies in the large errors obtained for the titrations. These large experimental errors are a result of the size and composition of the system. Analytical DLS and TEM data (Supplementary Information) show that the phosphors used are in the 0.5–1.5 $\mu\text{m}$ size range. This causes errors for a number of reasons; first being that the non uniform size distribution will lead to discrepancies with the concentrations of emitter Ln$^{III}$ ions, with potentially differences in the concentrations (or cation cluster sizes) of emitter ions at the surface of the nanoparticles due to different degrees of Ln$^{III}$ ion migration. Since LRET is distance dependent (usually occurs $<10$ nm), it is likely that only the surface emitters will be affected by the presence of the dye and the main core of the phosphors will be unaffected. However, differences in protein size within the selection studied herein alongside possible fluctuations in protein conformation upon electrostatic interaction with the UCPs (and therefore the distance between co-factor of the protein and the UCP surface emitter ions) coupled with possible additional collisional deactivation mechanisms and possible inner filter effects [27], are most likely to be the main reasons for the unanticipated trend between $K_{SV}$ and $J$. Such effects therefore need be taken into consideration.

Figure 3. (a) Plot of Stern-Volmer constant versus spectral overlap for all six dyes with the PTIR475 UCPs, (b) Plot of Stern-Volmer constant versus Spectral overlap for all six dyes with the PTIR545 UCPs, (c) Plot of Stern Volmer constant versus spectral overlap for the biomolecules with PTIR475 and (d) Plot of Stern Volmer constant versus spectral overlap for the biomolecules with PTIR545.
Conclusion

In conclusion, it has been shown that if there is an overlap of the absorption of a chromophoric molecule, such as an organic dye or biomolecule, with the emission band of the UCP then energy transfer can occur from the UCP to the dye molecule. Due to this LRET or apparent energy transfer process, during the sequential titration of a dye/biomolecule into a solution containing UCPs a ratiometric quenching of the emission band of the UCP is observed. Six organic dyes (FITC, RBITC, R6G/NH2, MR, SF and FM) and seven biomolecules (GFP, PETNR, BM3Heme, CytC, VitB12, GO and GR) were investigated with different absorption maxima, and therefore different extinction coefficients at 475 and 545 nm and different spectral overlaps with the UCP bands. The organic dyes; FITC, SF, FM and MR, and the biomolecules; GFP, PETNR, GO and GR, have a greater spectral overlap with the 475 nm band of PTIR475 and were therefore expected to quench this band to a greater extent than the 545 nm band of PTIR545 in which the spectral overlap integral is much smaller. The molecules RBITC, BM3Heme, CytC and Vitamin B12 on the other hand have a greater spectral overlap with the 545 nm band of PTIR545 than the 475 nm band of PTIR475 and as such were expected to quench this band to a greater extent, and indeed this was found to be the case. For the organic dyes a linear relationship was determined between spectral overlap and $K_{SV}$ for both the PTIR475 and PTIR545 UCPs. However a linear relationship between spectral overlap and $K_{SV}$ for the biomolecules with both these UCPs was not observed. It is postulated that this is due to the relative sizes of the biomolecules affecting the diffusion rates of the biomolecule to within the necessary distance of the UCPs in order for energy transfer to occur. However, here, the occurrence of inner filter effects cannot be ruled out completely [27]. Interestingly, the prediction that the limit of detection (LoD) and limit of quantification (LoQ) would be directly related to the spectral overlap of the dye/biomolecule at the band of interest; with the larger the spectral overlap the more efficient the energy transfer process and the lower the LoD and LoQ, did not hold true for these systems and no conclusive trend could be drawn from these data, suggesting that improved quenching may well occur in systems where the reporter chromophore is covalently attached to the UCP at a known distance; such studies are currently in progress.

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References

[1] Zhang F 2015 Photon Upconversion Nanomaterials (Berlin, Heidelberg: Springer Berlin Heidelberg) 978-3-662-45597-5 (https://doi.org/10.1007/978-3-662-45597-5)
[2] 2014 Saving lives, averting costs. An analysis of the financial implications of achieving earlier diagnosis of colorectal, lung and ovarian cancer A report prepared for Cancer Research UK, September 2014 by Incisive Health: https://www.incisivehealth.com/wp-content/uploads/2018/08/Saving-lives-averting-costs.pdf
[3] Damborsky P, Vitel J and Katrilik J 2016 Essays Biochem. 60 91–100
[4] Dey D and Goswami T 2011 J. Biomed. Biotechnol. 2011
[5] Aoki K, Komatsu N, Hirata E, Komiya Y and Matsuda M 2012 Cancer Sci. 103 614–9
[6] Hussain S A, Dey D, Chakraborty S, Saha J, Roy A D, Chakraborty S, Debnath P and Bhattacharjee D 2015 Sci. Letts. J. 4 119–47 arXiv:108.6559
[7] Tan G R, Wang M, Hsu C Y, Chen N and Zhang Y 2016 Adv. Opt. Mater. 4 984–97
[8] Xing H, Wei T, Lin X and Dai Z 2018 Anal. Chem. Acta 1042 71–8
[9] Sedimeier A and Gorris H H 2015 Chem. Soc. Rev. 44 1526–60
[10] Zhu X J, Su Q Q, Feng W and Li F Y 2017 Chem. Soc. Rev. 46 1025–39
[11] DaCosta M V, Doughan S, Han Y and Krull U J 2014 Anal. Chim. Acta 832 1–33
[12] Haase M and Schäfer H 2011 Angew. Chemie - Int. Ed. 50 5808–29
[13] Wang X, Chang H, Xie J, Zhao B, Liu B, Xu S, Pei W, Ren N, Huang L and Huang W 2014 Coord. Chem. Rev. 273–274 201–12
[14] Saleh S M, Ali R, Hirsch T and Wollbeis O S 2011 J. Nanoparticle Res. 13 4603–11
[15] Cai W and Chen F 2017 Hybrid Nanomaterials: Design, Synthesis, and Biomedical Applications (Boca Raton, FL: CRC Press)
[16] Kumar M, Guo Y and Zhang P 2009 Biosens. Bioelectron. 24 1522–6
[17] Kumar M and Zhang P 2009 Langmuir 25 6602–4
[18] Zhang P, Rogelj S, Nguyen K and Wheeler D 2006 J. Am. Chem. Soc. 128 12410–1
[19] Millan K M and Mikkelson S R 1993 Anal. Chem. 65 2317–23
[20] Chen Z, Chen H, Hu H, Yu M, Li F, Zhang Q, Zhou Z, Yi T and Huang C 2008 J. Am. Chem. Soc. 130 3023–9
[21] Wang M, Hou W, Mi C, Wang W and Xu Z 2009 Anal. Chem. 81 1–4
[22] Cen Y, Wu Y M, Kong X J, Wu S, Yu R Q and Chu X 2014 Anal. Chem. 86 7119–27
[23] Chen H, Yuan F, Wang S, Xu J, Zhang Y Y and Wang L 2013 Biosens. Bioelectron. 48 19–25
[22] Feng Zhao D C, Noll J-F, Nielsen and Fessler J A 2012 IEEE Trans. Med. Imaging 31 1713–23
[23] Zhai Y, Zhu C, Ren J, Wang E and Dong S 2013 Chem. Commun. 49 2400
[24] Deng R, Xie X, Vendrell M, Chang Y T and Liu X 2011 J. Am. Chem. Soc. 133 20168–71
[25] Zhang Y, Tang Y, Liu X, Zhang L and Lu Y 2013 Sensors Actuators, B Chem. 185 363–9
[26] Harvey P, Oakland C, Driscoll M D, Hay S and Natrajan L S 2014 Dalton Trans. 43 5265–8
[27] Wilhelm S, Del Barrio M, Heiland J, Himmelstoß S F, Galbán J, Wolfbeis O S and Hirsch T 2014 ACS Appl. Mater. Interfaces 6 15427–33
[28] Arppe R, Mattsson L, Korpi K, Blom S, Wang Q, Riuttamäki T and Soukka T 2015 Anal. Chem. 87 1782–8
[29] Lu J, Chen Y, Liu D, Ren W, Lu Y, Shi Y, Piper J, Paulsen I and Jin D 2015 Anal. Chem. 87 10406–13
[30] Oakland C, Andrews M B, Burgess L, Jones A, Hay S, Harvey P and Natrajan L S 2017 Eur. J. Inorg. Chem. 2017 5176–85
[31] Burgess L 2018 PhD Thesis The University of Manchester
[32] Goryashchenko A S, Khrenova M G and Savitsky A P 2018 Methods. Appl. Fluoresc. 6 022001
[33] Armbruster D A and Pry T 2008 Clin. Biochem. Rev. 29 Suppl 1 S49–52
[34] Sanagi M M, Ling S L, Nasir Z, Hermawan D, Wan Ibrahim W A and Naim A A 2009 J. AOAC Int. 92 1833–8