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Supporting Information

A Continuous Luminescence Assay for Monitoring Kinase Activity: Signalling the ADP/ATP Ratio using a Discrete Europium Complex

Sarah H. Hewitt, Jonathan Parris, Romain Mailhot and Stephen J. Butler*

Department of Chemistry, Loughborough University, Loughborough, LE11 3TU.

1. General Considerations

All chemicals used were purchased from standard chemical suppliers and used without further purification. PKAc was obtained from New England Biolabs inc. as a 2.5×10^6 units mL^{-1} (13 μM) solution, and used without further purification. [**Eu.1**]$^+$ was synthesised using a synthetic protocol described previously.[1]

Measurements of pH were carried out using a Jenway 3510 pH/mV meter with a Jenway combination electrode or a Jenway 3020 pH meter with an Aldrich glass combination pH electrode, both calibrated using buffer solutions of pH 4.00 ± 0.01, 7.00 ± 0.01 and 10.00 ± 0.01.

1.1 Optical Spectroscopy

UV/Vis absorbance spectra were measured using a GENESYS 10S UV/Vis spectrophotometer (Thermo Scientific) and VISIONlite version 5.2 software.

Emission spectra were recorded on an SPEX Fluoromax luminescence spectrometer using dM300 version 3.12 software. Emission spectra were obtained using a 40 μL Hellma Analytics quartz cuvette (Art no. 111-10-K-40), unless stated otherwise. With excitation at 330 nm, reading emission in the range 550 – 720 nm (for titrations) or 570 – 635 nm (for enzyme assays), using an integration time of 0.5 seconds, increment of 0.5 nm and excitation of emission slits of 0.5 nm.

Lifetime measurements were performed using a Perkin Elmer LS55 luminescence spectrometer with FL Winlab Molecular Spectroscopy version 4.00.02 software. Lifetime measurements were typically obtained by indirect excitation of the Eu(III) ion *via* the quinoline chromophore using a short pulse of light (at 330 nm) followed by monitoring the integrated intensity of the light emitted at 615 nm during a fixed gate time, tg, after a delay time, td. Measurements were made for a minimum of 20 delay times, covering 3 or more lifetimes. A gate time of 0.1 ms was used, and the excitation and emission slits were set to 10 and 5 nm respectively. The obtained decay curves were plotted in Microsoft Excel and fitted to the equation:

$$I = A_0 + A_1 e^{-kt}$$

I: intensity at time t following excitation; A_0: intensity when decay has ceased

A_1: pre-exponential factor; k: rate constant for the depopulation of the excited state

1.2 Biophysical measurement procedures

All biophysical analyses were carried out in degassed 10 mM HEPES, pH 7.0 buffer. Concentrated [**Eu.1**]$^+$ stocks were made at ~2.5 mg mL^{-1} in methanol and the accurate concentration determined using the UV/Vis absorbance at 332 nm (ε = 12 500 M^{-1} cm^{-1})[1] in 10 mM HEPES, pH 7.0. In all biophysical experiments, the [**Eu.1**]$^+$ concentration was kept at 8 μM concentration throughout. Stocks of ATP, ADP, GTP and GDP were made up at 20 or 50 mM and adjusted to pH 7.0 by addition of minimal volumes of 1 M NaOH. Stocks of $MgCl_2$ and DTT were made up at 50 mM in 10 mM HEPES, pH 7.0.

General procedure for titrating [Eu.1]$^+$ with anions

A stock solution of [**Eu.1**]$^+$ was prepared in 10 mM HEPES, pH 7.0. Stock solutions of the anion (sodium salt, usually 10 mM, 20 mM, 50 mM) were also prepared in 10 mM HEPES buffer solution at pH 7.0. To a 1 cm cuvette was added a solution of the probe so that the concentration of probe reached 8 μM and the Eu emission spectrum was recorded. Aliquots of the anion solution were then added, ensuring that the total added volume did not exceed 50 μL. After each addition, the solution was mixed with a pipette and the emission spectrum was recorded. Typically, 20 equivalents of the anion were added to the solution for titrations in water and up to 200 equivalents in a medium containing $MgCl_2$. Each titration was repeated twice. To determine association constants (reported as log K_a values) for the bound anion, the ratio of two wavelength bands (605–630/580–600 nm) was plotted as a function of anion concentration. The titration data was analysed using a nonlinear least-squares curve fitting procedure, based on a 1:1 binding model described by the equation below:

$$FB = \frac{\frac{1}{K_a}+[X]+[Eu]-\sqrt{(\frac{1}{K_a}+[X]+[Eu])^2+4[X][Eu]}}{2[Eu]}$$

FB: fraction bound, calculated by $(I-I_0)/(I_1-I_0)$, where I = intensity at [X], I_0 = initial intensity and I_1 = final intensity

[X]: total concentration of anion in solution

[Eu]: total concentration of Eu(III) complex

K_a: association constant

Stability of Complex [Eu.1]$^+$

Complex **[Eu.1]$^+$** was found to be kinetically stable in aqueous solution over extended time periods, as determined by mass spectrometric data and by comparing the emission spectral form, intensity and lifetime at various stages over a six month time period. Furthermore, the ATP and and ADP adducts of [**Eu.1**]$^+$ are kinetically inert under the conditions used in our enzymatic studies (e.g. 10 mM HEPES buffer containing 3 mM Mg^{2+} ions). The control experiment shown in Figure 4 (main article) in the absence of enzyme shows less than 10% change in luminescence of [**Eu.1**]$^+$ during the time course of the reaction (75 minutes), consistent with the presence of the stable ternary complex [**Eu.1**]$^+$-ATP**.** Similarly, in the presence of 0.4 uM PKAc, the emission intensity of [**Eu.1**]$^+$ reaches a plateau after approximately 25 minutes, signalling complete conversion of ATP to ADP and the formation of a stable [**Eu.1**]$^+$-ADP adduct. Any loss of Eu(III) from the complex would result in a significant decrease in emission intensity over time – this was not observed during the time frame of the enzyme experiment. For examples of analogous stable europium complexes, bearing trans-related azaxanthone units, see reference 2.

Anion titrations in an aqueous medium containing $MgCl_2$

Variable volumes of ATP, ADP or AMP in 3 mM $MgCl_2$ and 8 μM [**Eu.1**]$^+$ in 10 mM HEPES (pH 7.0) was added to 50 μL of 3mM $MgCl_2$ and 8 μM [**Eu.1**]$^+$ in 10 mM HEPES (pH 7.0). The emission spectra (λ_{exc} = 330 nm, λ_{em} = 550 – 720 nm) were recorded after each addition. The ratio of the intensity of the bands at 605–630/580–600 nm was taken and used to determine association constants.

pH titration

The emission spectrum of 2 mL of 1 mM ADP, 8 μM [Eu.1]$^+$ in 10 mM HEPES buffer at pH 6.40 was taken (λ_{exc} = 330 nm, λ_{em} = 550 – 720 nm). 1 M or 100 mM NaOH was added 2 μL at a time to the solution, to increase the pH by ~0.2 units until pH 8.5 and the emission spectra (λ_{exc} = 330 nm, λ_{em} = 550 – 720 nm) taken each time. The ratio of intensity at 616.5/599

ATP to ADP and GTP to GDP titration

Variable amounts of a solution of 1 mM ADP or GDP, 3 mM $MgCl_2$ and 8 μM [**Eu.1**]$^+$ in 10 mM HEPES (pH 7.0) was added to 50 μL of 1 mM ATP or GTP, 3 mM $MgCl_2$ and 8 μM [**Eu.1**]$^+$ in 10 mM HEPES (pH 7.0), with stated concentrations of other additives (DMSO, DTT, staurosporine) in both the ATP and ADP solutions and the emission spectra (λ_{exc} = 330 nm,

λ_{em} = 550 – 720 nm) recorded after each addition. The ratio of the intensity at 616.5 nm to 599.5 nm was taken.

PKAc enzyme assay

In all PKAc reactions the PKAc was added to the kemptide, ATP, DTT, $MgCl_2$ and [**Eu.1**]$^+$ in 10 mM HEPES, pH 7.0 to a final volume of 48 µL and concentration of 1 mM Kemptide, 1 mM ATP, 2 mM DTT, 3 mM $MgCl_2$ and 8 µM [**Eu.1**]$^+$. Emission spectra (λ_{exc} = 330 nm) were obtained over the range 570–635 nm, with 96 s between each recording.

Staurosporine inhibition

The PKAc was added to the kemptide, ATP, DTT, $MgCl_2$, DMSO, staurosporine and [**Eu.1**]$^+$ in 10 mM HEPES, pH 7.0 to a final volume of 48 µL and concentration of 0.4 µM PKAc, 1 mM Kemptide, 1 mM ATP, 2 mM DTT, 3 mM $MgCl_2$, 0, 0.2 or 0.4 µM staurosporine, 1% DMSO and 8 µM [**Eu.1**]$^+$. Emission spectra (λ_{exc} = 330 nm) were obtained over the range 570–635 nm, with 96 s between each recording.

Mass spectrometry

High resolution mass spectra were obtained on a Thermofisher Q-Exactive orbitrap mass spectrometer. Solutions of ~60 µM [**Eu.1**]$^+$ with ~500 µM ATP or ADP in a mixture of methanol/water (1:1 v/v) were injected and the spectrum recorded.

2. Spectroscopic studies of [Eu.1]$^{+}$

Anion titration data measured in 10 mM HEPES, pH 7.0

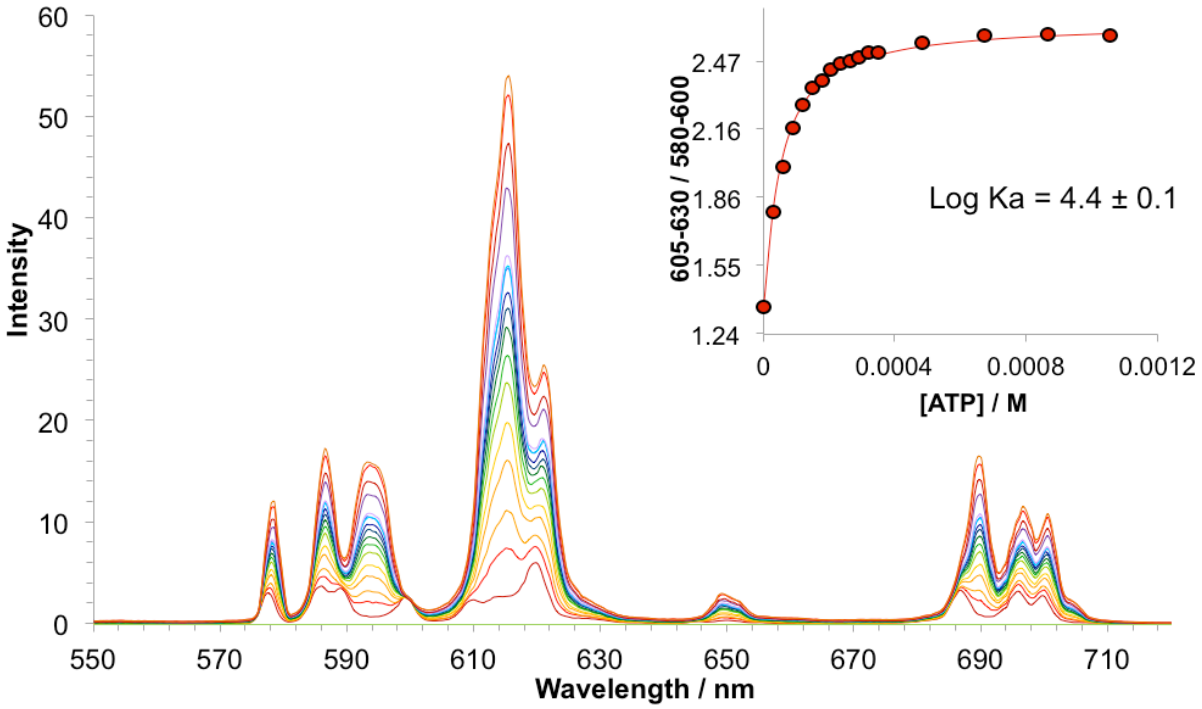

Figure S1: Changes in emission spectra of **[Eu.1]**$^{+}$ (5 μM) as a function of added ATP at pH 7.0. The inset shows the fit to the experimental data, for log K_a = 4.4 ± 0.1. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

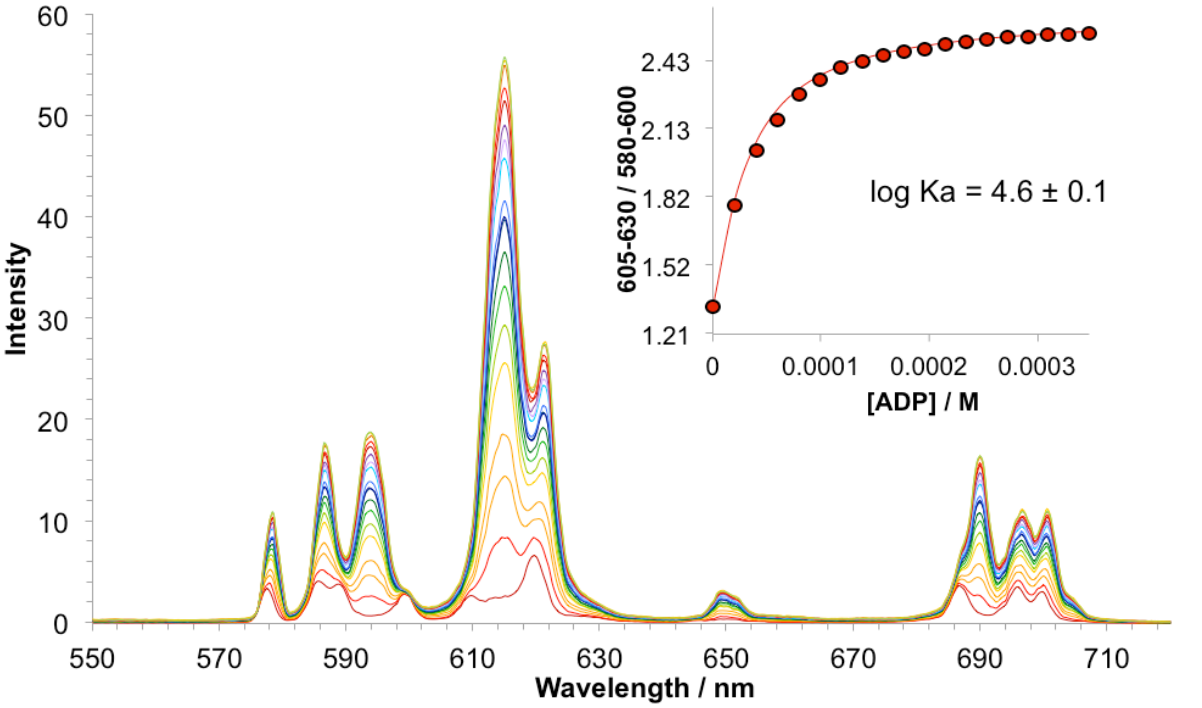

Figure S2: Changes in emission spectra of **[Eu.1]**$^{+}$ (5 μM) as a function of added ADP at pH 7.0. The inset shows the fit to the experimental data, for log K_a = 4.6 ± 0.1. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

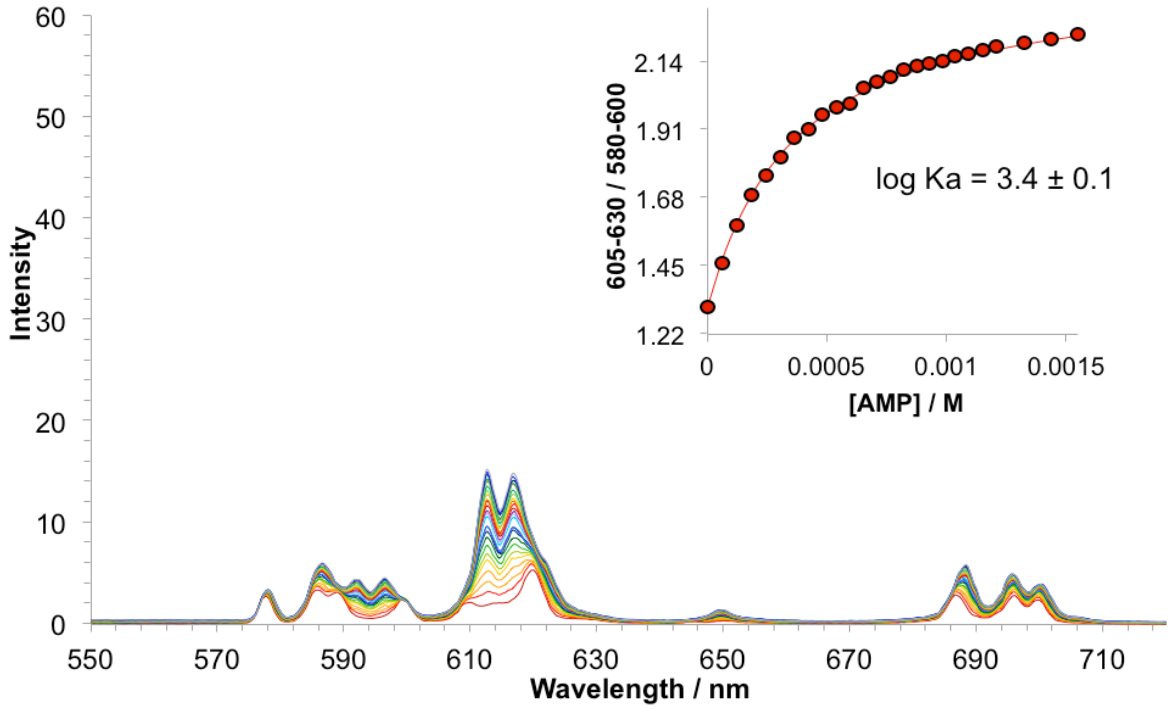

Figure S3: Changes in emission spectra of **[Eu.1]**$^{+}$ (5 μM) as a function of added AMP at pH 7.0. The inset shows the fit to the experimental data, for log K_a = 3.4 ± 0.1. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

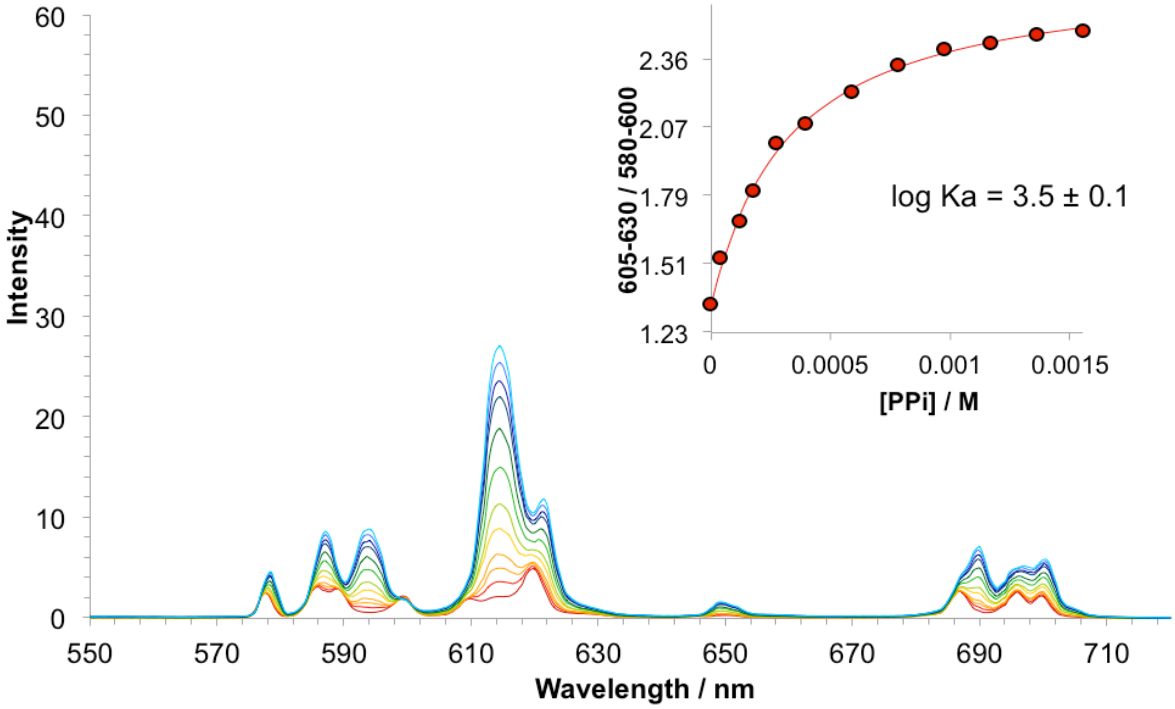

Figure S4: Changes in emission spectra of **[Eu.1]**$^{+}$ (5 μM) as a function of added pyrophosphate (Ppi) at pH 7.0. The inset shows the fit to the experimental data, for log K_a = 3.5 ± 0.1. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

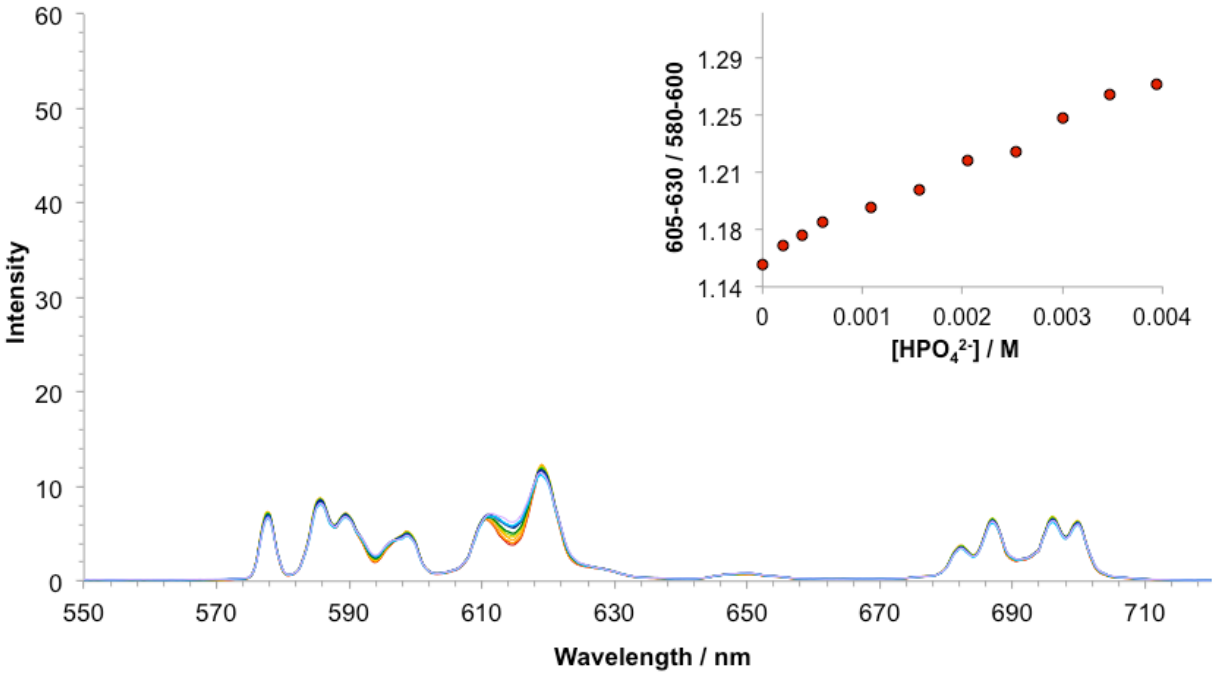

Figure S5: Minor changes in emission spectra of **[Eu.1]**$^{+}$ (5 μM) as a function of added hydrogen phosphate (0–4 mM) at pH 7.0. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

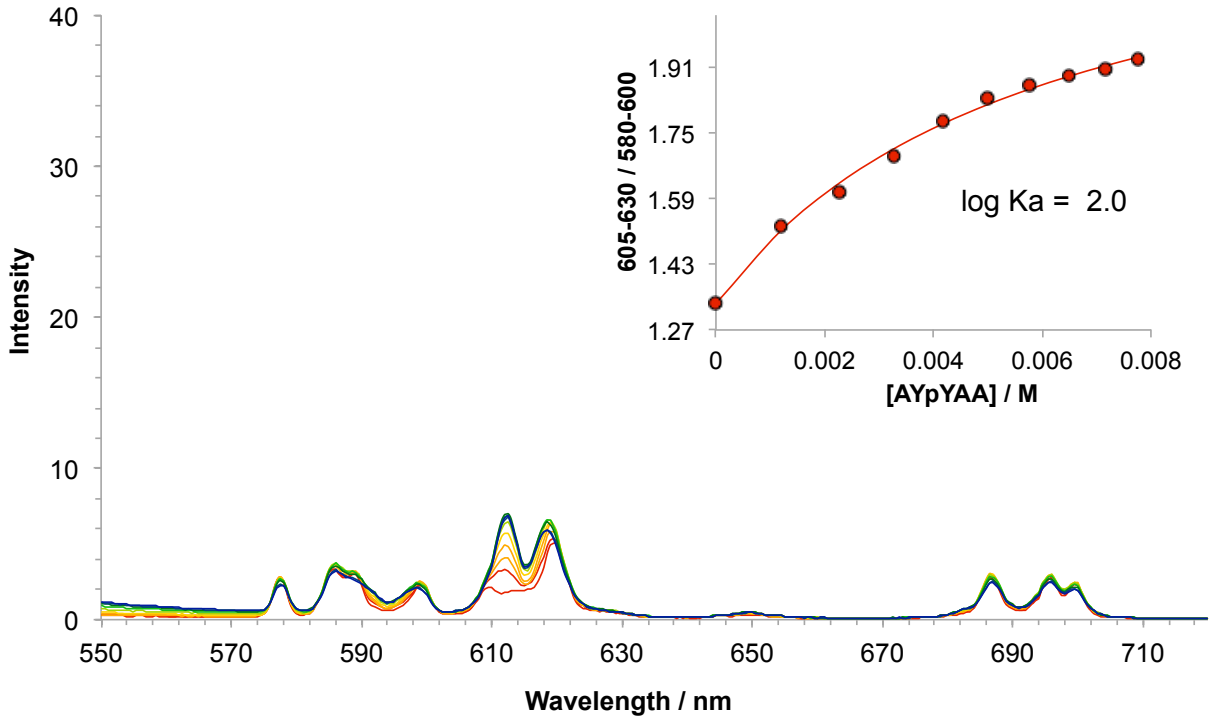

Figure S6: Changes in emission spectra of **[Eu.1]**$^{+}$ (5 μM) as a function of added monophosphorylated peptide AYpYAA (0–8 mM) at pH 7.0. The inset shows the fit to the experimental data, for log K_a = 2.0 ± 0.1. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

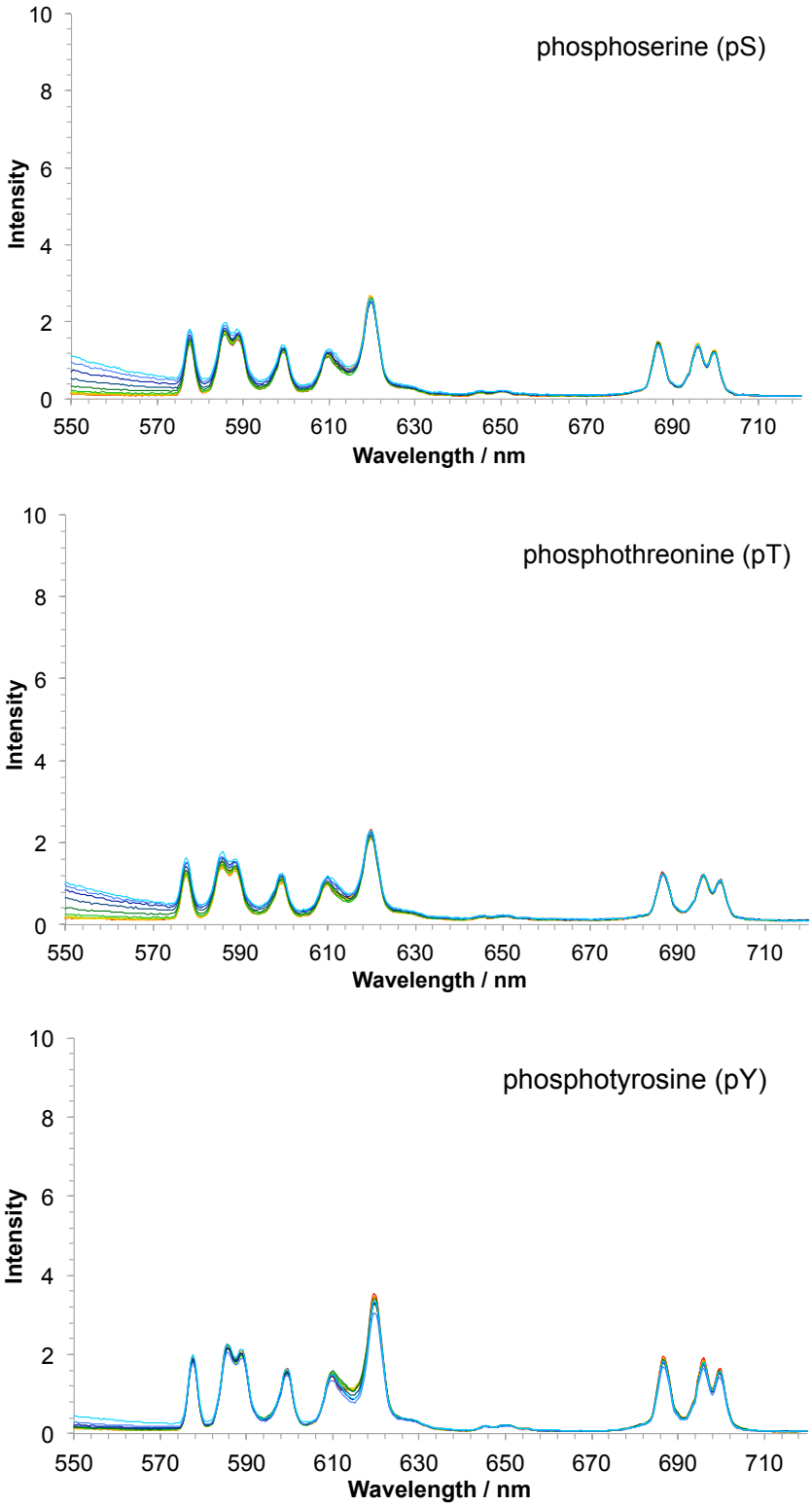

Figure S7: Minor changes in emission spectra of [**Eu.1**]$^{+}$ (8 μM) as a function of added phosphorylated amino acids pS, pT, or pY (0–4 mM each) at pH 7.0. Conditions: 10 mM HEPES buffer, λ_{exc} 330 nm, 25 °C.

Mass spectral data of [Eu.1]$^{+}$ bound to ATP and ADP

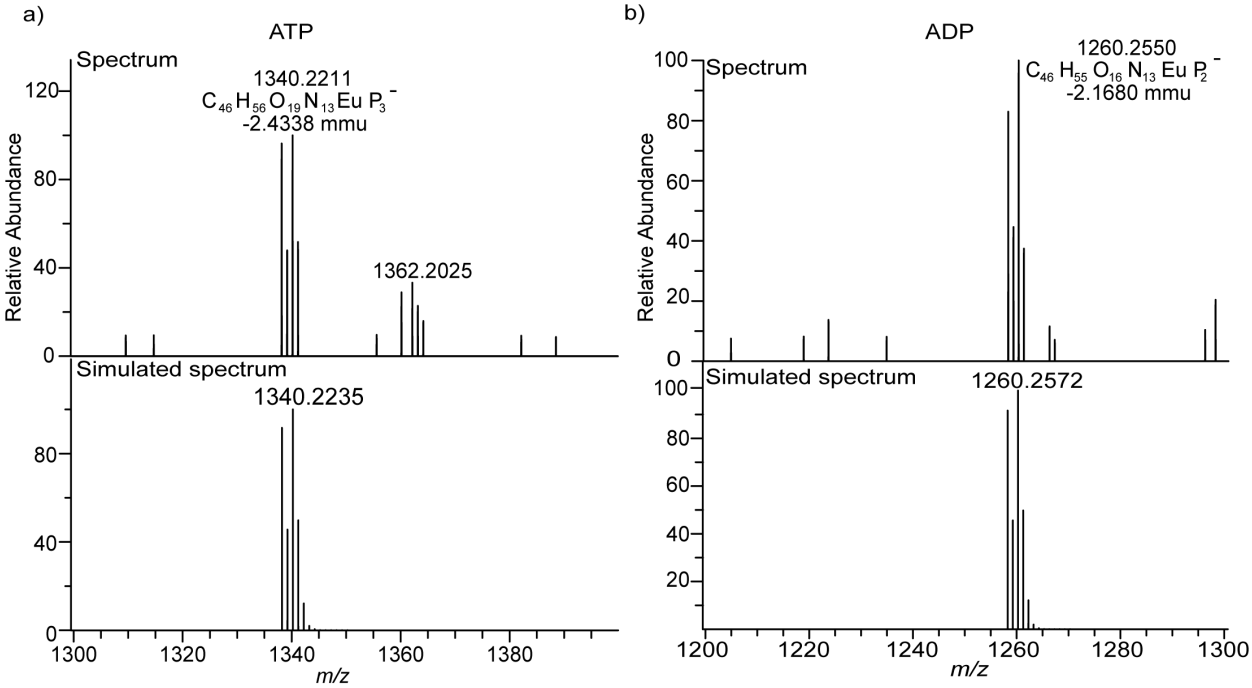

Figure S8: High resolution mass spectra of [**Eu.1**]$^{+}$ complexed to (a) ATP and (b) ADP, showing the formation of 1:1 adducts, in both cases.

Effect of pH

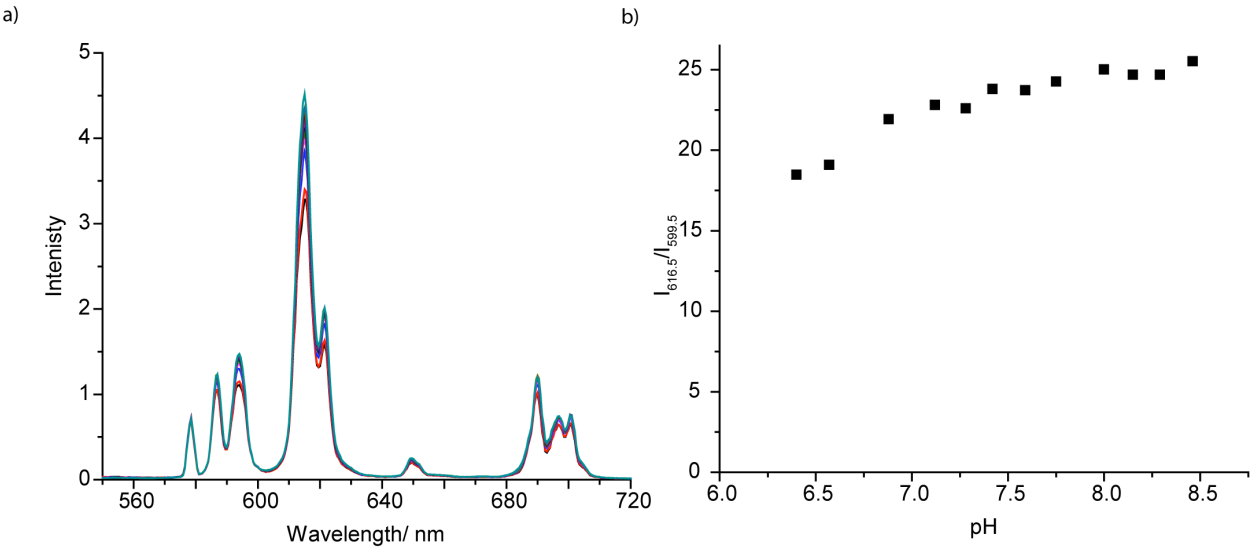

Figure S9: Effect of changing pH. a) Change in emission spectrum of [**Eu.1**]$^{+}$ in the presence of ADP over the pH range 6.4 – 8.5, b) change in ratio of emission intensity at 616.5/599.5 nm as a function of pH. Conditions: 10 mM HEPEs, pH 7.0, 1 mM ADP, 8 μM [**Eu.1**]$^{+}$

$MgCl_2$ and NaCl titrations

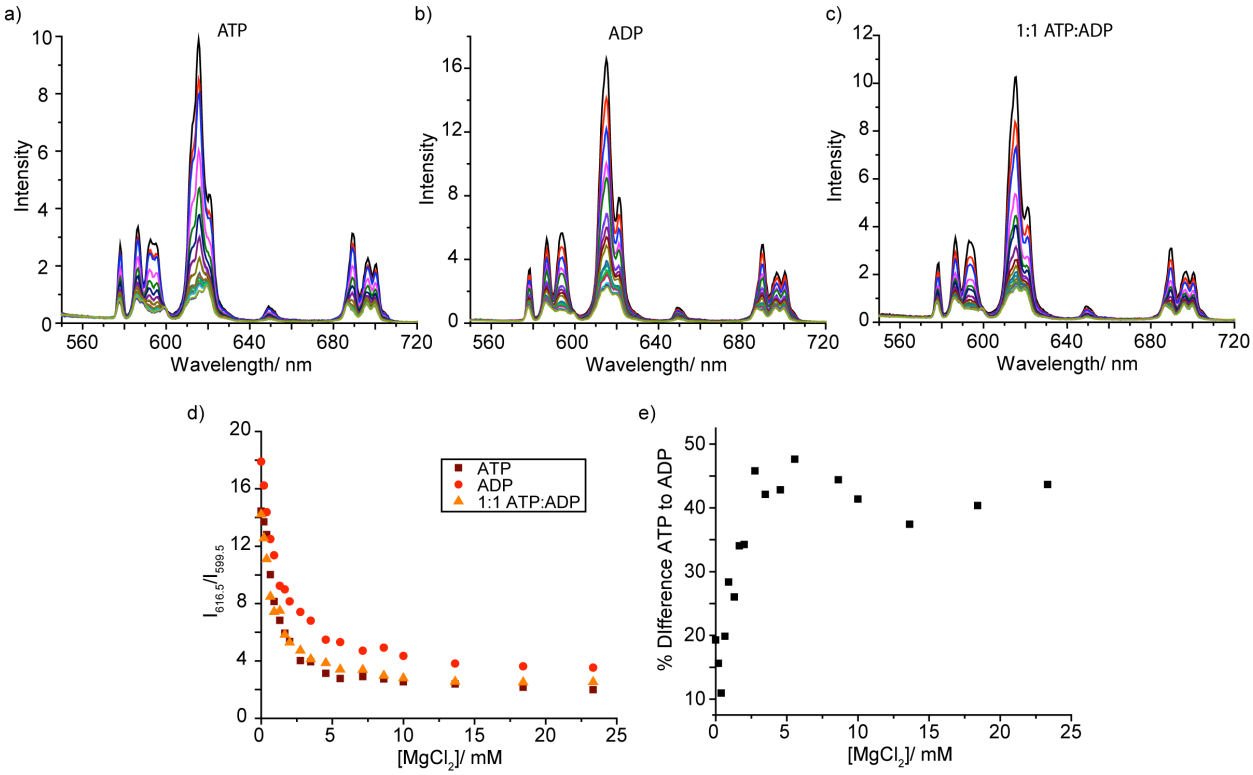

Figure S10: Effect of Mg^{2+} concentration. Changes in emission spectra of [**Eu.1**]$^+$ (8 μM) in the presence of ATP (1 mM) (a), ADP (1 mM) (b) and a mixture of ATP (0.5 mM) and ADP (0.5 mM) (c) as a function of added $MgCl_2$. c) Change in the ratio of emission intensity at 616.5 nm to 599.5 nm as a function of $MgCl_2$ concentration. d) Discrimination of ATP/ADP as a function of $MgCl_2$ concentration. Conditions: 10 mM HEPES, pH 7.0, λ_{exc} 330 nm, 25 °C.

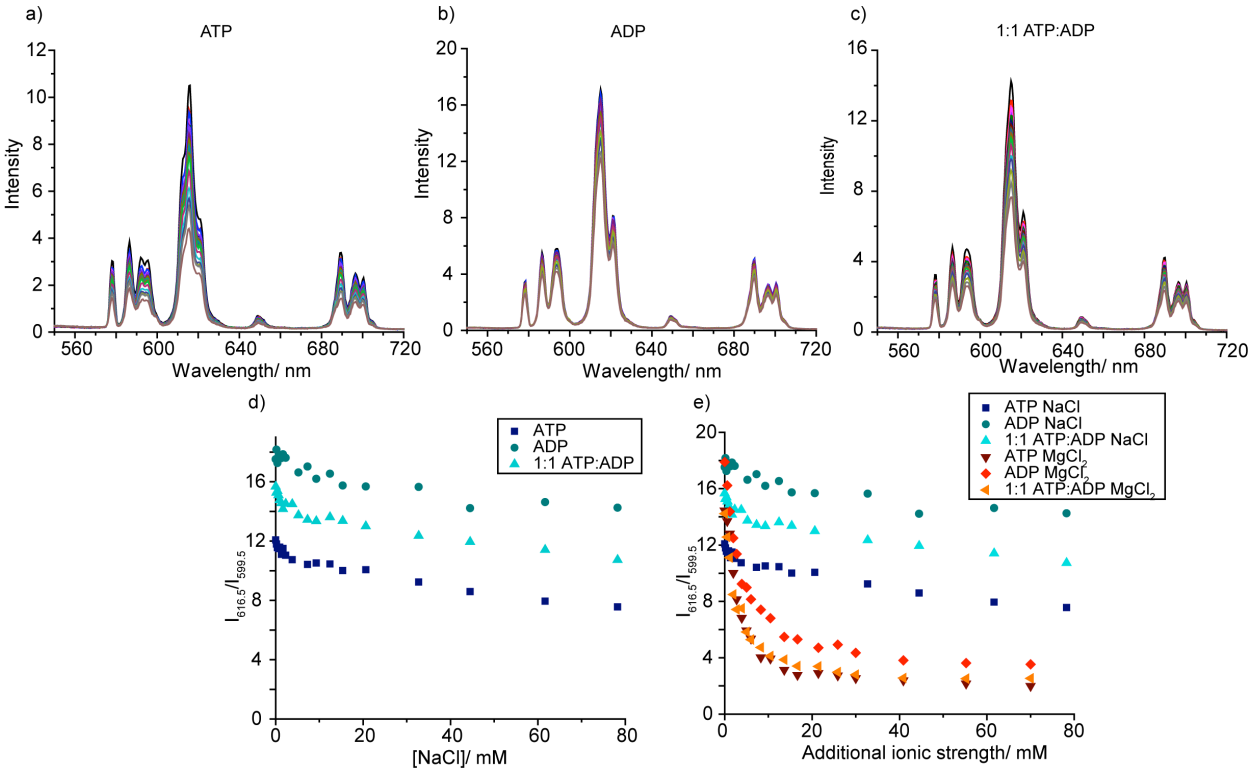

Figure S11: Effect of ionic strength. Changes in emission spectra of [**Eu.1**]$^{+}$ (8 μM) in the presence of ATP (1 mM) (a), ADP (1 mM) (b) and a mixture of ATP (0.5 mM) and ADP (0.5 mM) (c) as a function of added NaCl. c) Change in the ratio of emission intensity at 616.5/599.5 nm as a function of NaCl concentration. d) Comparison of the effect of the additional ionic strength between addition of $MgCl_2$ and NaCl. Conditions: 10 mM HEPES, pH 7.0, λ_{exc} 330 nm, 25 °C.

Anion titrations in 10 mM HEPES (pH 7.0), 3 mM $MgCl_2$

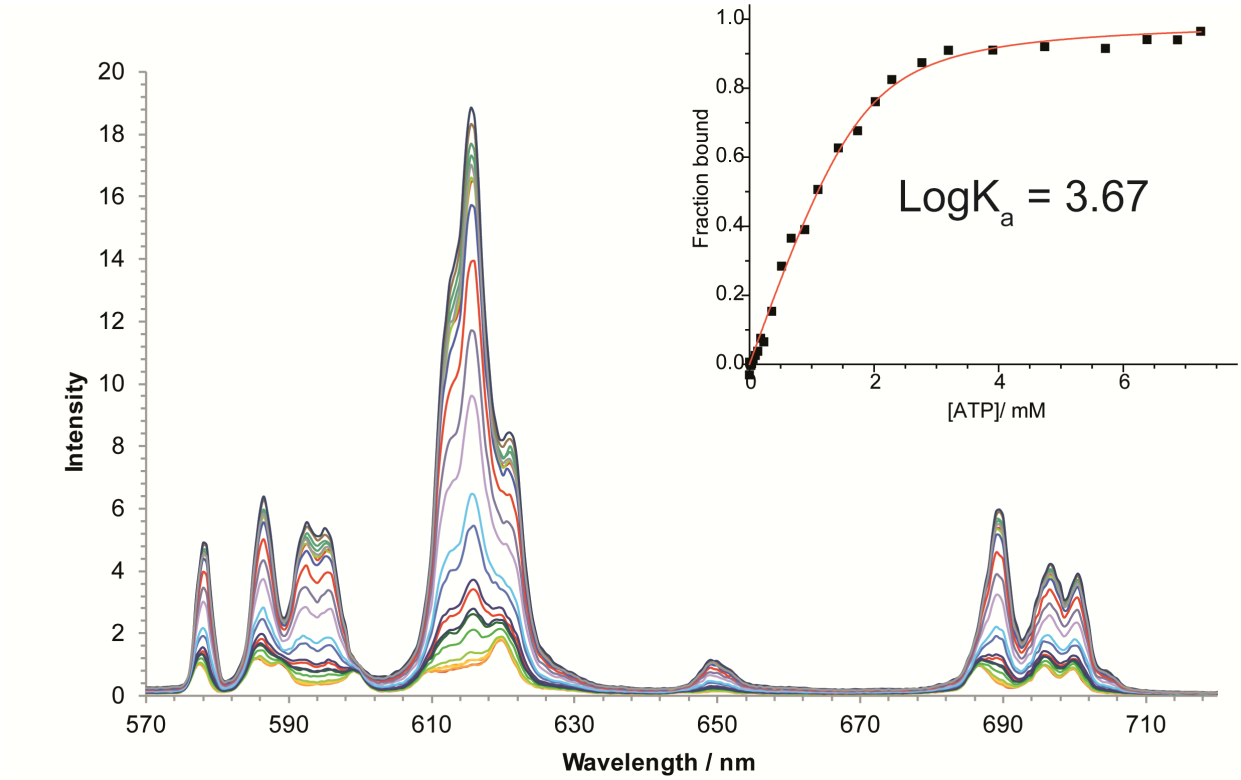

Figure S12: Changes in emission spectra of [**Eu.1**]$^+$ (8 μM) as a function of added ATP in the presence of 3 mM $MgCl_2$ at pH 7.0. The inset shows the fit to the experimental data, for log K_a = 3.7 ± 0.1. Conditions: 10 mM HEPES buffer, pH 7.0, λ_{exc} 330 nm, 25 °C.

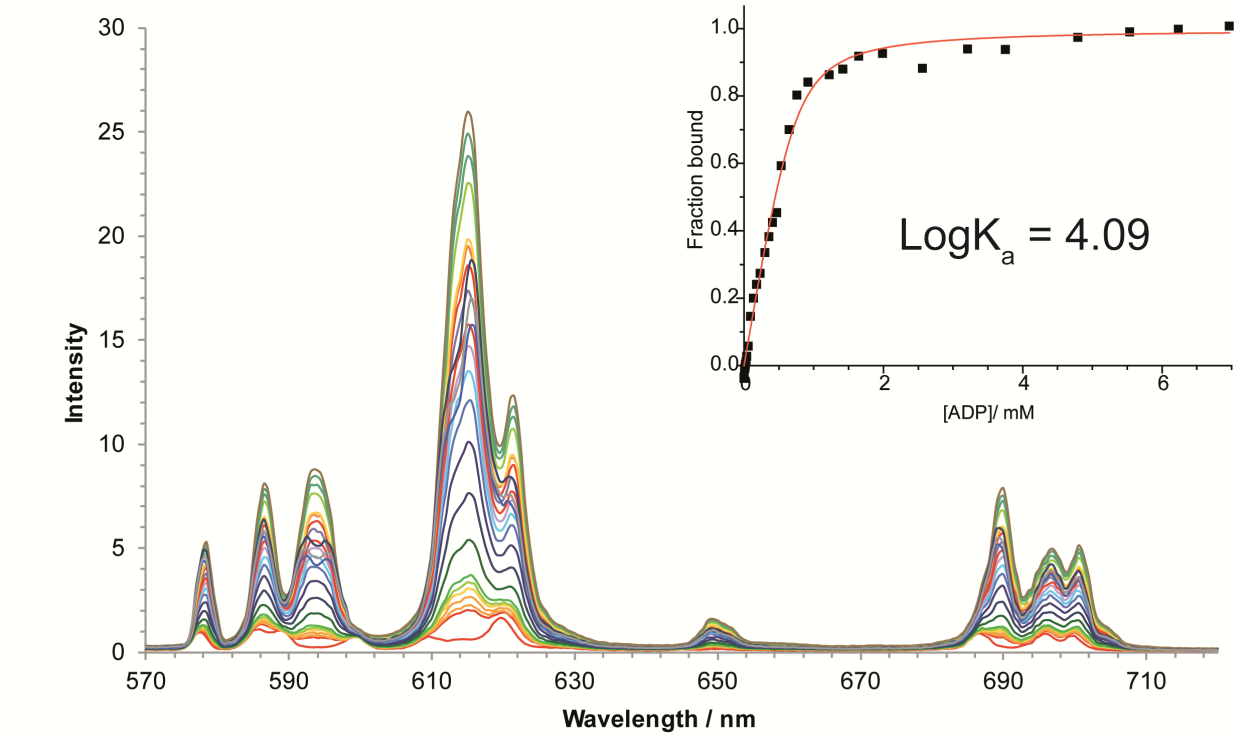

Figure S13: Changes in emission spectra of [**Eu.1**]$^+$ (8 μM) as a function of added ADP in the presence of 3 mM $MgCl_2$. The inset shows the fit to the experimental data, for log K_a = 4.1 ± 0.1. Conditions: 10 mM HEPES buffer, pH 7.0, λ_{exc} 330 nm, 25 °C.

GTP to GDP titration

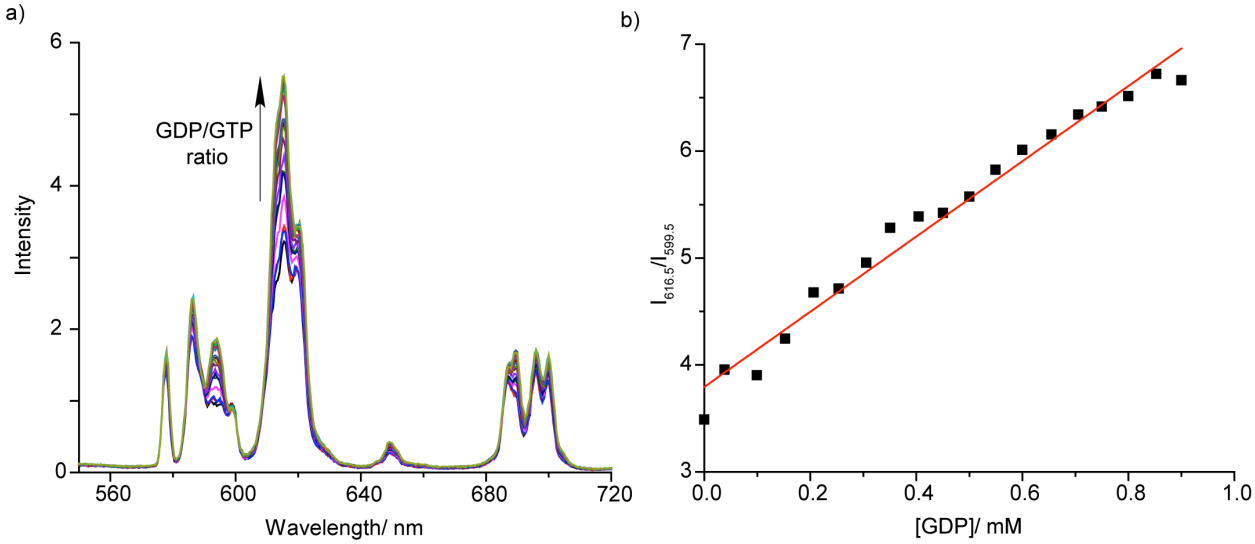

Figure S14: a) Ratiometric change in emission spectra of [**Eu.1**]$^+$ (8 μM) in the presence of varying ratio of GTP/GDP; b) plot of the emission intensity ratio, 616.5/599.5 nm, versus mole fraction of GDP. Conditions: buffered aqueous solution (10 mM HEPES, pH 7.0) containing $MgCl_2$ (3 mM). Concentration of GDP: 0–0.9 mM, total nucleoside concentration, [GTP] + [GDP] = 1 mM. λ_{exc}= 330 nm, 298 K.

Kemptide (peptide substrate) titration

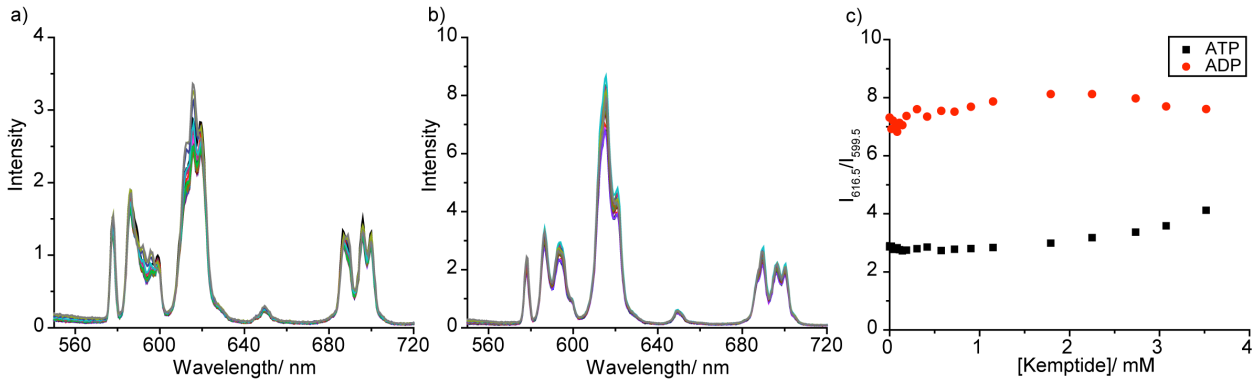

Figure S15: Effect of kemptide (peptide substrate) on the emission spectra of [**Eu.1**]$^+$ in the presence of ATP and ADP. a) and b) Emission spectra of [**Eu.1**]$^+$ in response to increasing kemptide concentration in the presence of ATP (a) and ADP (b). c) Plot of emission intensity ratio 616.5/599.5 nm as a function of kemptide concentration. Conditions: buffered aqueous solution (10 mM HEPES, pH 7.0) containing $MgCl_2$ (3 mM), and 8 μM [**Eu.1**]$^+$, λ_{exc} = 330 nm, 298 K.

Effect of adding DTT

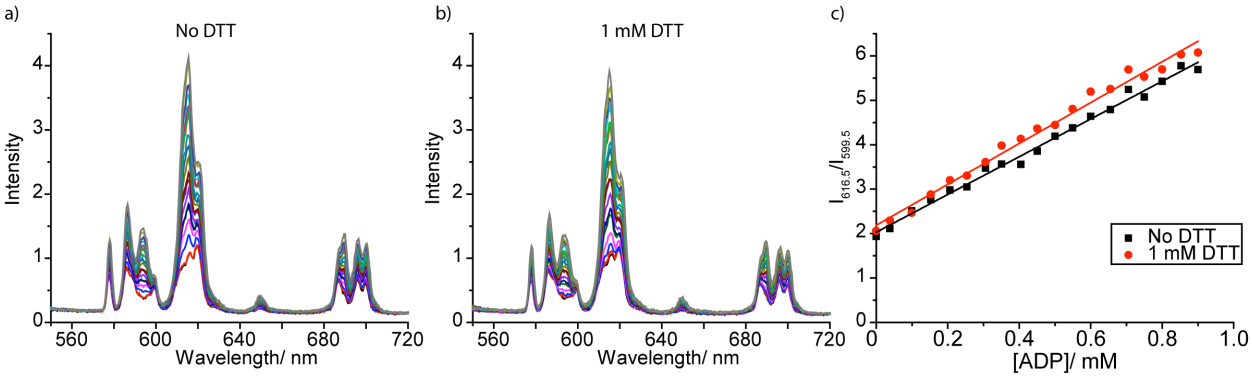

Figure S16: a) Emission spectra of [**Eu.1**]$^{+}$ (8 μM) in the presence of varying ratio of ATP/ADP in the absence (a) and presence (b) of DTT (1 mM); c) plot of the emission intensity ratio, 616.5/599.5 nm, versus mole fraction of ADP. Conditions: buffered aqueous solution (10 mM HEPES, pH 7.0) containing $MgCl_2$ (3 mM). Concentration of ADP: 0–0.9 mM, total nucleoside concentration, [ATP] + [ADP] = 1 mM. λ_{exc}= 330 nm, 298 K.

Effect of adding PKAc

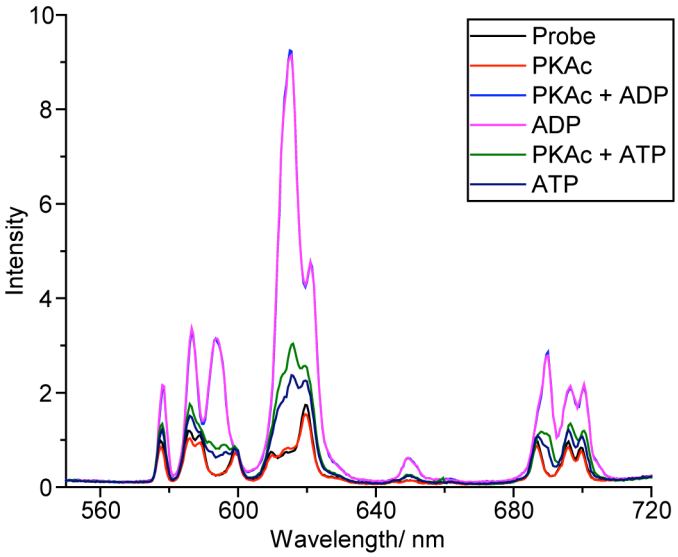

Figure S17: Effect of addition of PKAc (0.8 μM) on the emission spectrum of [**Eu.1**]$^{+}$ (8 μM), alone and in the presence of ATP (1 mM) and ADP (1 mM). Conditions: 10 mM HEPES, pH 7.0, 3 mM $MgCl_2$, λ_{exc}= 330 nm, 298 K

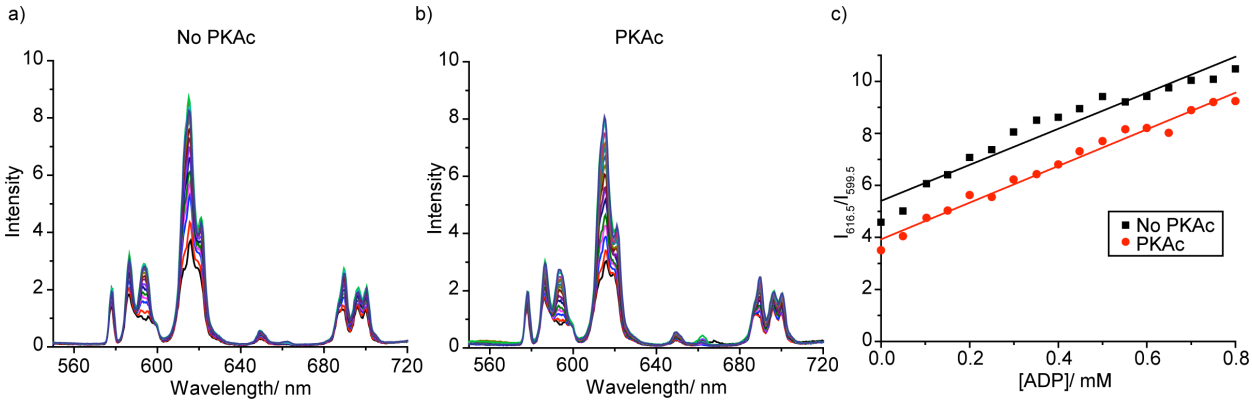

Figure S18: a) Emission spectra of [**Eu.1**]$^{+}$ (8 μM) in the presence of varying ratio of ATP/ADP in the absence (a) and presence (b) of PKAc (0.8 μM); c) plot of the emission intensity ratio, 616.5/599.5 nm, versus mole fraction of ADP. Conditions: 10 mM HEPES, pH 7.0, containing $MgCl_2$ (3 mM). Concentration of ADP: 0–0.9 mM, total nucleoside concentration, [ATP] + [ADP] = 1 mM. λ_{exc}= 330 nm, 298 K.

3. PKAc catalysed phosphorylation of Kemptide

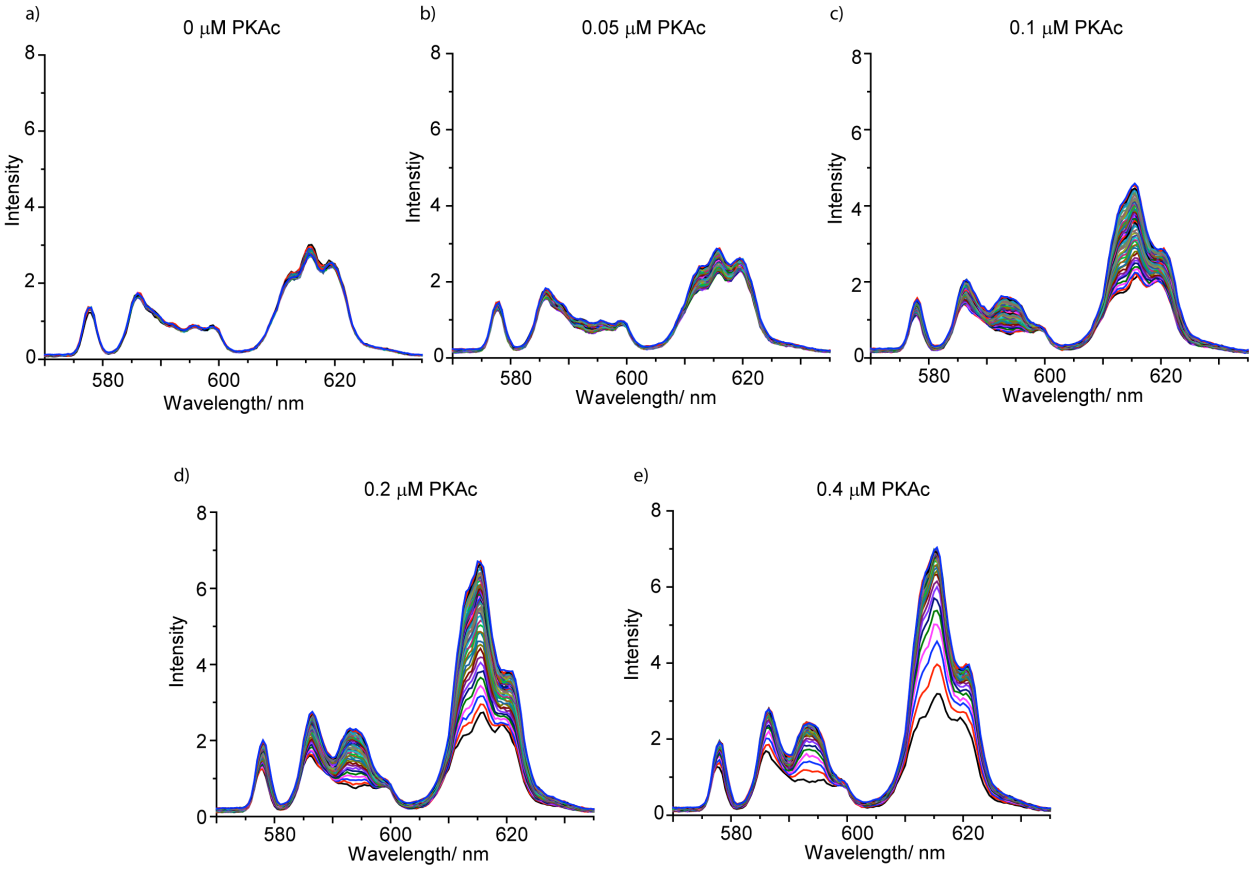

Figure S19: Emission responses over time during the PKAc (variable concentrations) catalysed phosphorylation of kemptide (1 mM) in the presence of 8 μM [**Eu.1**]$^{+}$, ATP (1 mM), DTT (2 mM), $MgCl_2$ (3 mM) in 10 mM HEPES, pH 7.0, (λ_{exc} = 330 nm). a) 0 μM PKAc, b) 0.05 μM PKAc, c) 0.1 μM PKAc, d) 0.2 μM PKAc and e) 0.4 μM PKAc

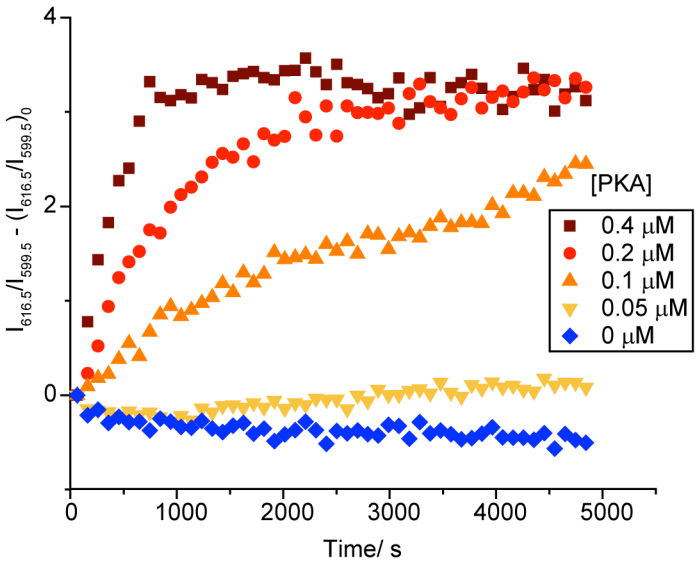

Figure S20: Ratiometric analysis of the enzyme reaction. Plot of the difference in the ratio of intensity at 616.5/599.5 nm between time, t and time 0. Conditions: 8 μM [**Eu.1**]$^+$, 1 mM ATP, 3 mM $MgCl_2$ 2 mM DTT, 1 mM Kemptide in 10 mM HEPES, pH 7.0, λ_{exc} = 330 nm

Effect of adding DMSO

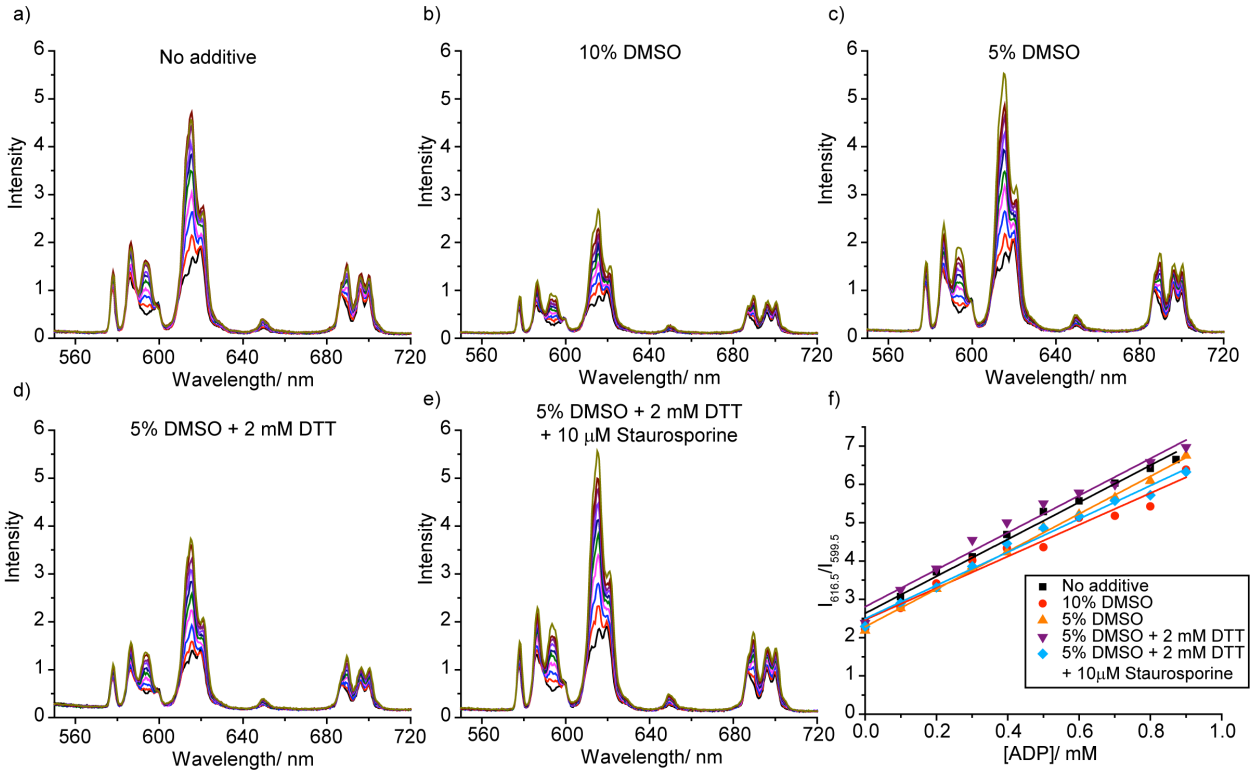

Figure S21: Emission spectra of [**Eu.1**]$^+$ (8 μM) in the presence of varying ratio of ATP/ADP with various additives (none (a), 10 % DMSO (b), 5 % DMSO (c), 5%DMSO + 2 mM DTT (d), 5 % DMSO + 2 mM DTT + 10 μM staurosporine (e)).; f) plot of the emission intensity ratio, 616.5/599.5 nm, versus mole fraction of ADP. Conditions: buffered aqueous solution (10 mM HEPES, pH 7.0) containing $MgCl_2$ (3 mM). Concentration of ADP: 0 – 0.9 mM, total nucleoside concentration, [ATP] + [ADP] = 1 mM. λ_{exc}= 330 nm, 298 K.

Staurosporine inhibition

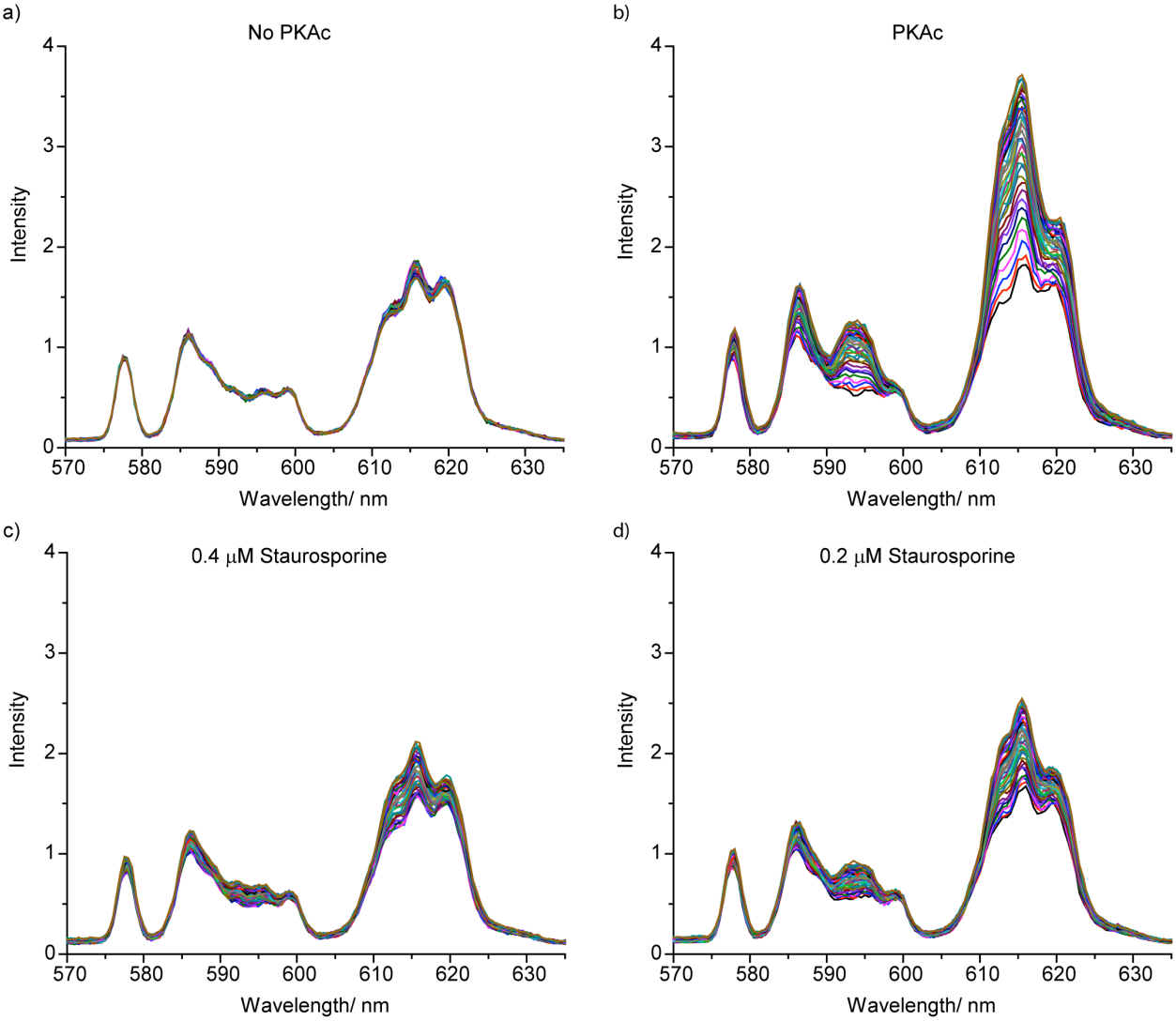

Figure S22: Emission responses over time during the PKAc (0.4 μM) catalysed phosphorylation of kemptide (1 mM) in the presence of staurosporine (variable concentrations), ATP (1 mM), DTT (2 mM), $MgCl_2$ (3 mM), 1 % DMSO in 10 mM HEPES, pH 7.0, (λ_{exc} = 330 nm). a) No PKAc, b) 0 μM staurosporine, c) 0.4 μM staurosporine, d) 0.2 μM staurosporine

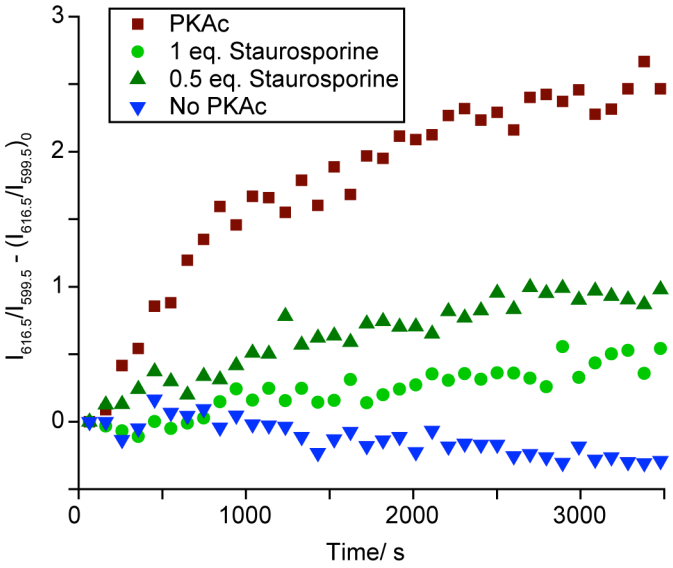

Figure S23: Ratiometric plot of PKAc catalysed phosphorylation of kemptide in the presence of staurosporine. Plot of the difference in the ratio of intensity at 616.5/599.5 nm between time, t and time 0. Conditions: 8 μM [**Eu.1**]$^+$, 1 mM ATP, 3 mM $MgCl_2$ 2 mM DTT, 1 mM Kemptide in 10 mM HEPEs, pH 7.0, λ_{exc} = 330 nm

References

1. S. J. Butler, *Chem. Commun.*, 2015, **51**, 10879.

2. R. Carr, L. Di Bari, S. L. Piano, D. Parker, R. D. Peacock and J. M. Sanderson, *Dalton Trans.,* **2012**, 41, 13154.