SUPPLEMENTARY INFORMATION

A generic approach based on long-lifetime fluorophores for the assessment of protein binding to polymer nanoparticles by fluorescence anisotropy

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I. Synthesis, purification and characterization of the polymer nanoparticles

For the synthesis of the PNPs, 3.45 mmol N-isopropylacrylamide (53 mol%), 2.6 mmol N-*tert*-butylacrylamide (40 mol%), 0.32 mmol acrylic acid (5 mol%), 0.13 mmol N,N'-methylene bisacrylamide (2 mol%) and 20 mg sodium dodecyl sulfate were dissolved in 50 mL ultrapure water. N-isopropylacrylamide was recrystallized from hexane and acrylic acid was passed through an inhibitor remover column before use. The reaction mixture was purged with argon for 50 minutes. The polymerization was carried out at 60 ^{0}C for 3 h with magnetic stirring and argon bubbling after the addition of 300 µL from 100 mg/mL aqueous ammonium persulfate solution. The obtained nanoparticles were purified by dialysis (Spectra/Por 4 RC membrane, 12-14 kDa MWCO, Spectrum Laboratories, Inc.) for 6 days in ultrapure water, changing the water twice a day. The monomer conversion and w/v concentration of the PNPs were calculated by drying 1 ml aliquots of the suspension at 60 °C. The particle size and "molar" concentration were determined by nanoparticle tracking analysis (LM10HS, Nanosight Ltd., Salisbury, UK).

II. Preparation of RuL-Su labelled lysozme (RuL-Lys)

3.8 mg lysozyme was dissolved in 1 ml 100 mM phosphate buffer, pH 7.4, then mixed in the dark with 2.5 mg bis(2,2'-bipyridine)-4'-methyl-4-carboxypyridine-ruthenium N-succinimidyl ester bis (hexafluorophosphate) (RuL-Su) dissolved in 114 µl N,N-dimethylformamide. The molar ratio of RuL-Su was approx. 9-fold compared to the protein. The solution was left for 3 hours at 4 °C and was shaken every 15 minutes. The unreacted dye was removed by dialysis using a 3.5-5 kDa MWCO dialysis device (Spectra/Por Float-A-Lyzer® G2, Spectrum Laboratories, Inc), against 10 mM phosphate buffer, pH 7.4 that was changed after 15, 18, 20 and 48 hours. The concentration of the dye in the labelled protein solution was obtained by measuring the absorbance at its absorption maximum (457 nm), assuming that the protein-conjugated and the free dye have the same molar absorptivity. The molar absorption coefficient of RuL-Su was measured in 10 mM phosphate buffer, pH 7.4. The protein concentration in the labelled protein solution was determined by the BCA total protein assay (Thermo Scientific). From these values the number of dye molecules conjugated to one protein was calculated.

III. Lysozyme activity assay

Lysozyme activity assay was used to quantitate the binding of lysozyme to PNPs after separation by ultracentrifugation. 5 µg/mL lysozyme was dissolved in phosphate buffer (10 mM, pH 7.4). Freeze-dried Micrococcus lysodeikticus cells were resuspended at 150 µg/ml concentration in phosphate buffer (50 mM, pH 6.2). 100 µL lysozyme containing sample solution was added to 2500 µL cell suspension, and the cell lysis was followed at 25 °C by measuring the decrease in absorbance at 450 nm. The slope of absorbance decrease in the first 5 minutes was used as a measure of lysozyme activity.

IV. Interpretation of the absorption spectra of RuL-Su and RuL-Lys

The absorption spectra of the unconjugated ruthenium probe (RuL-Su), the probe-lysozyme conjugate (RuL-Lys) and that of the unlabeled lysozyme are shown in Figure S1, normalized to unity for better comparison.

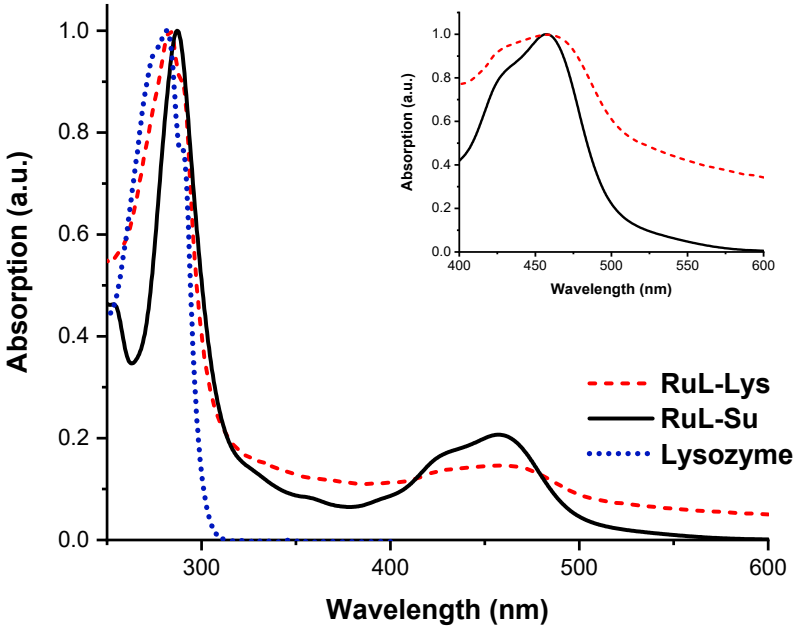

Figure S1: Normalized absorption spectra of RuL-Su, RuL-Lys and lysozyme (the inset shows the normalized spectra in the 400-600 nm range)

The absorption spectrum of RuL-Su is closely similar to the spectrum of the parent ion, $Ru[(bpy)_3]^{2+}$, the photophysical properties of which have been studied extensively [1]. On, the

basis of this similarity, the longest wavelength band appearing at 457 nm is due to an MLCT (metal-to ligand charge transfer) transition, the weak feature at ~350 nm can be assigned to a MC (metal centered) transition and the strong band at 286 nm belongs to an LC (ligand centered) transition (π-π* transition of bipyridine). The absorption spectrum of RuL-Lys is similar to that of the RuL-Su. The absorption maximum at 286 nm is shifted slightly to 283 nm, due to overlap with the absorption maximum of lysozyme (281 nm). The wavelength of the 457 nm absorption peak does not change, but the peak broadens to some extent (Figure S1, inset). Similar phenomenon has been observed by Terpetschnig et al. [2] upon labelling albumin with a similar, unsymmetrical Ru complex.

V. Interpretation of the steady-state anisotropy of RuL-Su, RuL-Lys and RuL-Lys upon adsorption to the PNPs

Observed and calculated steady-state anisotropy data of the unconjugated ruthenium probe, RuL-Su and the probe-lysozyme conjugate, RuL-Lys in the absence and in the presence of polymer nanoparticles are listed in Table S1.

Table S1 Steady-state anisotropy data of RuL-Su, the RuL-Lys conjugate in the absence of PNPs and the RuL-Lys conjugate in the presence of high excess of PNPs.

	τ^* (ns)	r_{st} (-, measured)	r_{st} (-, calculated)
RuL-Su	340	unobservable	0.000061
RuL-Lys	420	0.0045	0.0013
RuL-Lys adsorbed onto PNPs	1100	0.12 (λ_{ex}=457 nm) 0.164 (λ_{ex}=490 nm)	0.119

We used the simplest model for the interpretation of the anisotropy data, in which the fluorescent species is taken as a spherical rotor. Following an excitation with a light pulse, the anisotropy of such fluorophore decays exponentially

$$r(t) = r_0 exp(-t/\theta), \quad \text{(S1)}$$

where r_0 is the limiting anisotropy in the absence of rotational diffusion and θ is the rotational correlation time. θ is related to the hydrodynamic volume of the fluorophore, V, the local viscosity, η and the temperature, T as

$$\theta = \frac{\eta V}{k_B T} \quad \text{(S2)}$$

where k_B denotes the Boltzmann constant.

In case the fluorescence decay of the spherical fluorophore is monoexponential with a time constant τ, the steady-state anisotropy is given by the Perrin equation

$$r_{st} = r_0 \frac{\theta}{\tau + \theta} \quad \text{(S3)}$$

We have estimated the r_{st} values using the previous equations for the different species and compared them to the measured values. The correlation times were calculated, taking estimated or measured values for the hydrodynamic volumes. The τ fluorescence time constants were determined experimentally (Table S1). The limiting anisotropy of the RuL fluorophore, r_0, was taken to be 0.12, equal to the measured steady-state anisotropy of the PNP-bound labelled protein, based upon the following consideration. The average volume of the PNPs determined by nanoparticle tracking analysis was V = $1.08 \cdot 10^6$ nm^3, and the contribution of the adsorbed labelled protein to the volume was neglected. The rotational correlation time of RuL-Lys adsorbed onto PNPs, predicted by the spherical rotor model (Eq. S2) is 206 μs (η=0.798 cP, T=303 °C), much longer than its fluorescence lifetime, τ = 1.1 μs. Thus, its measured r_{st}, 0.12, must be very close to the limiting anisotropy. This assumption was supported by the fact that the limiting anisotropy of RuL conjugated human serum albumin was measured to be 0.17 in a frozen glycerol-water mixture, using 490 nm excitation wavelength [3], and we have found very similar steady-state anisotropy value (0.164) for the PNP-bound labelled protein using the 490 nm excitation wavelength. It is interesting to note, that the r_0 of such unsymmetrical Ru-bipyridyl complexes is strongly dependent on the excitation wavelength [3].

The size of the RuL-Su cation was approximated by the size of the parent cation, $Ru(bpy)_3$ the diameter of which is ≈1.2 nm [4], corresponding to V = 0.905 nm^3, θ = 0.17 ns and r_{st} = 0.000061. Such a low r_{st} value is undetectable by fluorescence spectrometers. The fast rotational diffusion with such a short correlation time could not be followed with our TCSPC system.

The volume of the labelled lysozyme molecule was approximated by the hydrated volume of lysozyme, V = 24.2 nm^3 [5]. Using this value, θ = 4.62 ns was calculated for the rotational correlation time and r_{st} = 0.0013 for the steady state anisotropy. The experimental

value of r_{st} is 0.0045, from which a θ = 16.4 ns rotational correlation time is obtained, approximately 3.5 times larger than the approximated value. This might be explained by the elongated shape of the protein and/or protein association.

VI. References

[1] S. Campagna, F. Puntoriero, F. Nastasi, G. Bergamini, V. Balzani, In *Photochemistry and Photophysics of Coordination Compounds I*, Springer Berlin Heidelberg, Berlin, Heidelberg, **2007**, pp. 117–214.

[2] E. Terpetschnig, H. Szmacinski, J. Lakowicz, *Anal Biochem* **1995**, *227*, 140.

[3] H. Szmacinski, E. Terpetschnig, J. R. Lakowicz, *Biophys Chem* **1996**, *62*, 109.

[4] L. Yang, H. J. Liang, T. E. Angelini, J. Butler, R. Coridan, J. X. Tang, G. C. L. Wong, *Nat Mater* **2004**, *3*, 615.

[5] H. Pessen, T. F. Kumosinski, S. N. Timasheff, *J Agric Food Chem* **1971**, *19*, 698.