Influence of an anti-metastatic ruthenium(III) prodrug on extracellular protein-protein interactions: studies by bio-layer interferometry

Aviva Levina and Peter A. Lay*

School of Chemistry, The University of Sydney, Sydney NSW 2006, Australia
E-mail: peter.lay@sydney.edu.au

Supplementary Material

Experimental Section

The following sources of proteins were used: (i) recombinant human transferrin receptor 1 (CD71), extracellular domain (Cys89 – Phe760), $(His)_6$-tagged at the *N*-terminus, from Sino Biological, China (Cat. No. 11020-H07H); (ii) human apo-transferrin from Sigma (>98% Tf; ≤0.005% Fe; Cat. No. T1147); (iii) human serum albumin from Sigma (>97%; Cat. No. A9511); (iv) purified human IgG (10 mg mL^{-1} in PBS) from Invitrogen (Cat. No. 02-7102). Other reagents of highest available purity grade were from Sigma-Aldrich or Merck. Water was purified by the Milli-Q technique. Synthesis and characterisation of NAMI-A were performed according to the literature methods,[S1] as described previously.[4]

In order to minimise the influence of residual Fe(III) on Tf-TfR1 binding, buffer solutions were treated with Chelex 100 chelating resin (Bio-Rad) for three days, while adjusting the pH values with solutions of ultra-pure HCl (0.10 M, Merck) or NaOH (0.10 M, Aldrich). The purified buffers were filtered through sterile 0.2 μM Minisart RC membrane filters (Sartorius) prior to use. Transferrin was initially dissolved (~0.15 mM; 100 μL) in metal removal buffer (0.10 M MES, 4.0 mM Na_2edta, 0.30 M KCl, pH = 5.60)[14] for ~10 min at 295 K, then the solution was passed through a BioGel P30 gel filtration column (Bio-Rad, 30 kDa cut-off), which was pre-saturated with Chelex-treated metal binding buffer (20 mM HEPES, 25 mM $NaHCO_3$, 140 mM NaCl, pH = 7.40).[17] Protein concentration in the resulting solution was determined spectrophotometrically (ε(278 nm) = 9.3×10^4 M^{-1} cm^{-1}),[S2] and adjusted to 100 μM Tf using the latter buffer. Stock solutions of NAMI-A (~20 mM in H_2O) were prepared within 1 h prior to the experiments (to avoid hydrolysis),[16] their purity and concentration were checked spectrophotometrically (ε(390 nm) = 3.64×10^3 $M^{-1}cm^{-1}$).[16,S3] Stock solutions of Fe(III)-NTA were prepared by mixing aqueous solutions of $Fe(NO_3)_3\cdot 9H_2O$ (50 mM) and Na_3NTA (55 mM), pH_{end} ~ 4.[S4] Solutions of Tf complexes for the binding studies were prepared by incubation of Tf (100 μM) with either Fe(III)-NTA or NAMI-A (200 μM) for 4 h at 310 K in the metal binding buffer (see above).[16,S2] Solutions of HSA and IgG for the binding studies were prepared in Chelex-treated and sterilly filtered PBS (20 mM $H_2PO_4^-/HPO_4^{2-}$, 150 mM NaCl, pH = 7.40). A Ru(III)-HSA adduct was generated by incubation of HSA (0.50 mM) with NAMI-A (0.50 mM) in PBS for 4 h at 310 K.[4,19]

Protein-protein interaction studies were performed on a BLItz instrument (ForteBio, CA, USA)[5] in Advanced Kinetics mode at 295 K, using a drop holder (4 μL solution) for the protein association steps and a tube (250 μL solution) for the background and dissociation steps. The Ni(II)-NTA and protein G coated optical probes

were supplied by ForteBio. Processing of the data was performed using BLItz[5] and Origin[S5] software, and kinetic analyses were performed using Pro-Kineticist[S6] software. Electronic spectra were collected at 295 K on a Hewlett Packard HP8452A diode-array spectrophotometer (300-800 nm, 2 nm resolution). The pH values were measured at 295 K using an Activon 210 pH-meter with an AEP 312 glass-Ag-AgCl electrode that was calibrated daily using Sigma pH standards.

Additional references:

S1 G. Mestroni, E. Alessio and G. Sava, *New salts of anionic complexes of Ru(III), as antimetastatic and antineoplastic agents*. WO9800431, 1998. *Chem. Abstr.* 1998, **128**, ref. 123148; E. Alessio, G. Balducci, M. Calligaris, G. Costa, W. M. Attia and G. Mestroni, *Inorg. Chem.* 1991, **30**, 609; M. Bouma, B. Nuijen, G. Sava, A. Perbellini, A. Flaibani, M. J. van Steenbergen, H. Talsma, J. J. Kettene-van den Bosch, A. Bult and J. H. Beijnen, *Int. J. Pharmaceut.* 2002, **248**, 247.

S2 J. M. El Hage Chanine and D. Fain, *J. Chem. Soc., Dalton Trans.* 1993, 3137.

S3 E. Alessio, G. Balducci, A. Lutman, G. Mestroni, M. Calligaris and W. M. Attia, *Inorg. Chim. Acta* 1993, **203**, 205.

S4 R. J. Bergeron and W. R. Weimar, *J. Bacteriol.* **1990**, *172*, 2650.

S5 *Microcal Origin*, Version 6.0, Microcal Software Inc., Northampton, MA, USA, 1999.

S6 *ProKineticist*, Version 1.06, Applied Photophysics Ltd., Leatherhead, UK, 2001.

Table S1. Calculated first-order rate constants for the association and dissociation steps in BLI experiments.

	Association			Dissociation		
Binding Species	k_1, s^{-1}	k_2, s^{-1}	k_3, s^{-1}	k_{-1}, s^{-1}	k_{-2}, s^{-1}	k_{-3}, s^{-1}
Fe(III)-Tf [a]	0.47(2)	0.093(3)	0.015(2)	0.87(8)	0.092(5)	0.006(1)
apo-Tf [a]	1.00(5)	0.055(2)	0.014(1)	0.88(5)	0.097(2)	0.012(1)
Ru(III)-Tf [a]	0.96(7)	0.061(2)	0.009(1)	0.5(1)	0.075(5)	0.015(1)
Fe(III)-Tf-TfR [b]	0.20(1)	0.012(1)	--	1.0(1)	0.17(3)	0.028(5)
apo-Tf-TfR [b]	0.08(2)	0.007(2)	--	1.2(2)	0.20(3)	0.022(3)
Ru(III)-Tf-TfR [b]	0.07(1)	$1.0(3)\times10^{-4}$	--	1.32(4)	0.19(1)	0.015(2)
HSA [c]	0.065(5)	$6.4(4)\times10^{-3}$	--	0.042(7)	$5.4(4)\times10^{-3}$	--
Ru(III)-HSA [c]	0.042(5)	$5.0(5)\times10^{-3}$	--	0.045(5)	$4.4(2)\times10^{-3}$	--

Notes. Fitting of normalised kinetic curves (Figures 2, 3 and 5 in the main text and Figure S6) was performed with ProKineticist software,[S6] using the models that consisted of two or three sequential first-order reactions. Typical experimental and calculated kinetic curves are shown in Figures S3, S5 and S7. Shown in the table are the mean values and standard deviations (errors in the last significant figures are shown in parentheses) of two kinetic measurements, performed on separate probes. All the measurement were performed at 295 ± 1 K. [a] Binding of Tf (1.0 μM) to TfR (immobilized on a Ni(II)-NTA probe) in the presence or absence of metal ions (2.0 μM) in the metal binding buffer (20 mM HEPES, 25 mM $NaHCO_3$; 140 mM NaCl, pH = 7.40); association and dissociation steps correspond to B and C, respectively, in Figure 1 (main text). [b] Incubation of the Tf-TfR1 complexes (immobilized on a Ni(II)-NTA probe) under simulated endosomal conditions, and dissociation of the resultant apo-Tf-TfR1 complex at pH = 7.40; association and dissociation steps correspond to segments D and E, respectively, in Figure 1 (main text). [c] Binding of HSA (0.50 mM) or its NAMI-A adduct (0.50 HSA + 0.50 mM Ru) to human IgG (loaded on a protein G probe) in PBS (20 mM phosphate, 150 mM NaCl, pH = 7.40); association and dissociation steps correspond to the segments D and E, respectively, in Figure 4 (main text).

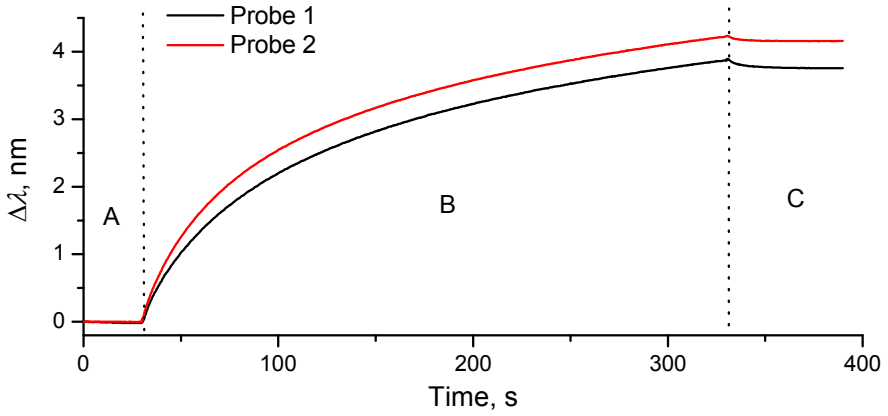

Figure S1. BLI response curves for the binding of $(His)_6$-tagged TfR1 (0.25 mg mL^{-1} in PBS) to Ni(II)-NTA-coated probes[5] at 295 K. Designations of segments: A and C, PBS alone; B, PBS with the protein.

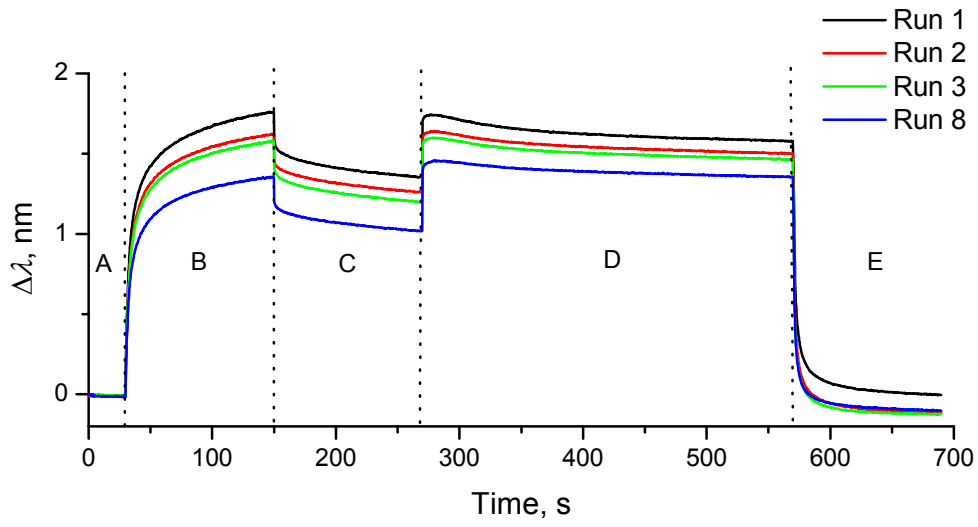

Figure S2. Repeated stripping and re-loading of TfR1 immobilised on a Ni(II)-NTA probe (see Figure S1) with Fe(III)-Tf (1.0 μM Tf, 2.0 μM Fe(III)). Reaction conditions and designations of the segments correspond to those in Figure 1, main text. Although the amounts of Fe(III)-Tf bound to the probe decreased slightly with each run, the kinetic parameters (see Table S1) were not significantly affected.

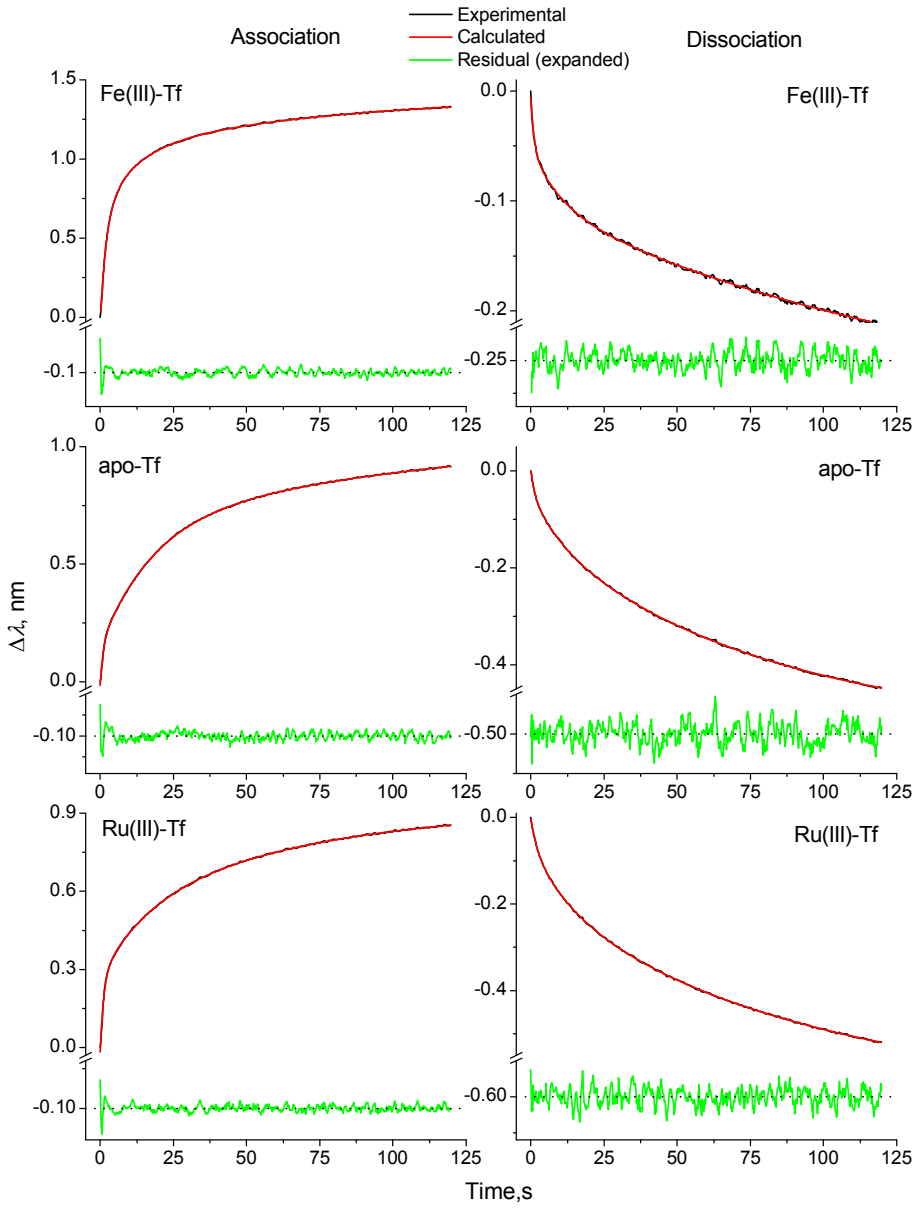

Figure S3. Typical experimental and calculated kinetic curves for the association and dissociation steps (corresponding to the segments B and C in Figure 1, main text) for Tf (1.0 μM) binding to TfR1 in the presence or absence of metal ions (2.0 μM). Kinetic analyses were performed using ProKineticist[S6] software, and the calculated rate constants are listed in Table S1.

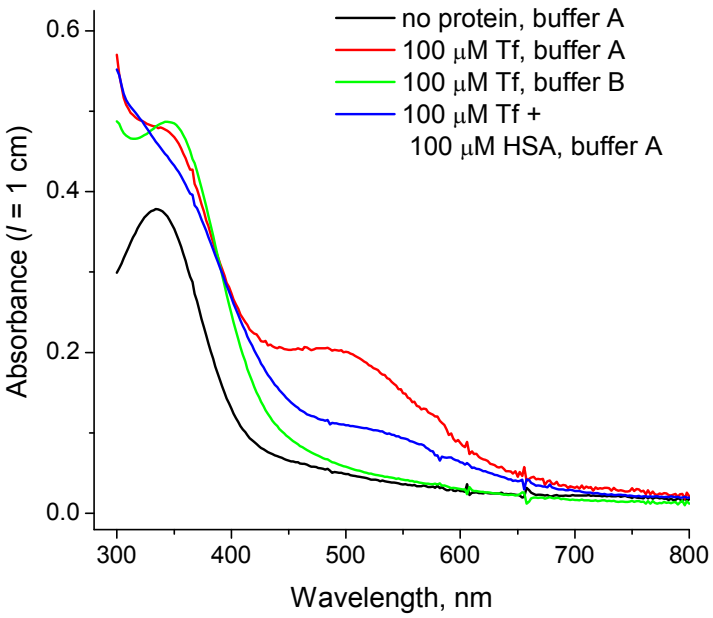

Figure S4. Typical electronic spectra of the reaction products of NAMI-A (200 μM) in aqueous buffer solutions in the presence or absence of proteins (4 h at 310 K). Buffer A: 20 mM HEPES, 25 mM $NaHCO_3$, 140 mM NaCl, pH = 7.40. Buffer B: 20 mM HEPES, 140 mM NaCl, pH = 7.40. Blank solutions for each measurement contained all the components (including the proteins), except for Ru(III).

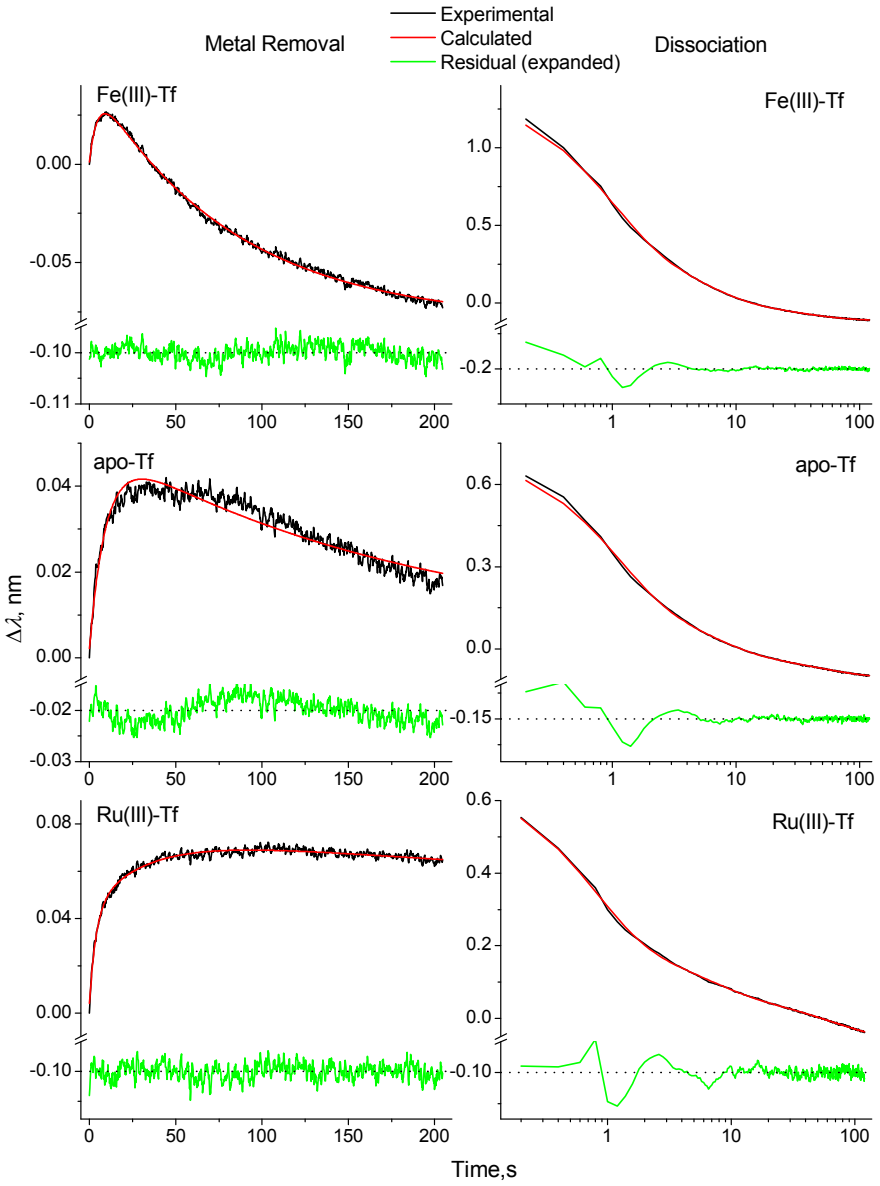

Figure S5. Typical experimental and calculated kinetic curves for the removal of metal ions and dissociation of Tf-TfR1 complexes (corresponding to the segments D and E in Figure 1, main text). Kinetic analyses were performed using ProKineticist[S6] software, and the calculated rate constants are listed in Table S1.

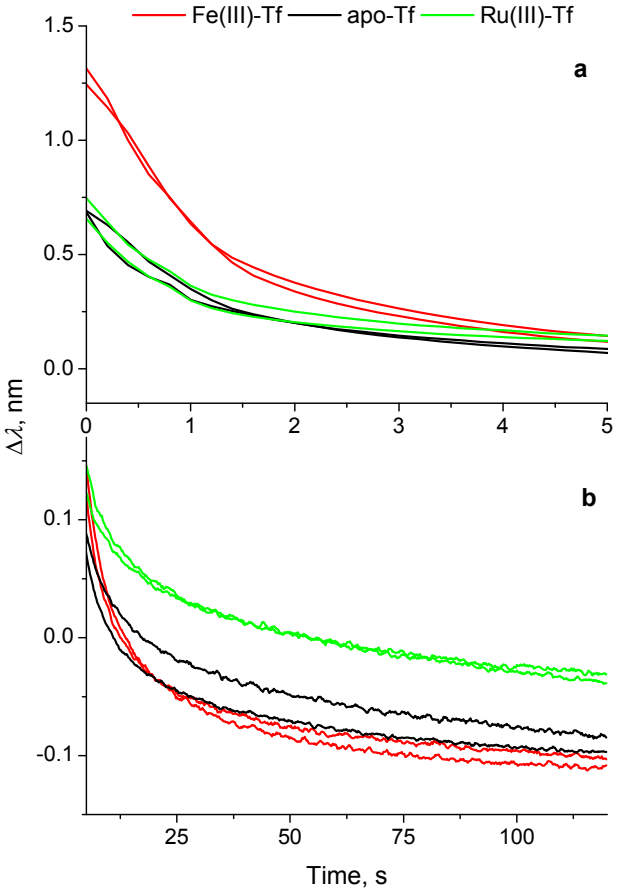

Figure S6. Kinetic curves for the dissociation of Tf-TfR1 complexes (**a:** 0-5 s timescale; **b:** 5-120 s timescale) after the removal of metal ions (segment E in Figure 1, main text). Each condition is represented by two measurements, using different probes. Kinetic parameters are shown in Table S1 and Figure S5.

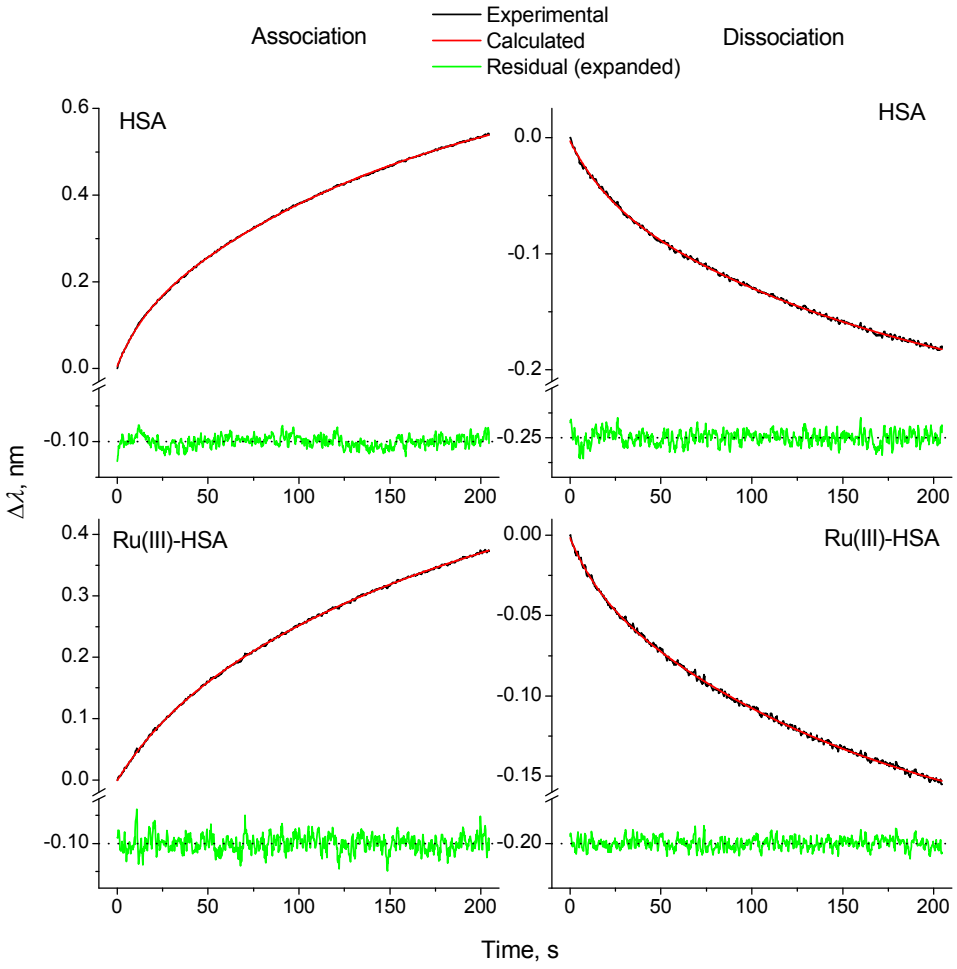

Figure S7. Typical experimental and calculated kinetic curves for the association and dissociation steps (corresponding to the segments D and E in Figure 4, main text) of HSA or Ru(III)-HSA (0.50 mM in PBS) binding to IgG (loaded on a protein G probe). Kinetic analyses were performed using ProKineticist[S6] software, and the calculated rate constants are listed in Table S1.