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

Aviva Levina and Peter A. Lay*

These studies have been greatly assisted by the availability of soluble recombinant proteins that represent the substrate-binding (extracellular) portions of common receptors. In addition to a multi-channel instrument (Octet®) that can be used for high-throughput screening of PPIs, a portable single-channel instrument (BLItz™), which is suitable for directed small-scale studies, has now become available. To our knowledge, no applications of BLI to studies of the biochemicality of protein adducts of metal-based prodrugs have been reported as yet.

One of the outstanding questions in medicinal inorganic chemistry is the role of transferrin (Tf, the main Fe transport protein in blood plasma) in the cellular uptake of “alternative” metal ions, including Ru that is derived from Ru anti-cancer prodrugs. Many biological and abiological metal ions bind to Tf with affinities that are comparable to those for Fe(m), but the resulting adducts are not necessarily recognized and taken up by the cell. Transport of essential Fe into cells involves the binding of Fe(m) ions by the two specific sites of Tf, which changes the conformation of the Tf molecule to allow its high-affinity binding to transferrin receptor 1 (TfR1). The complex is then returned to the cell surface (pH = 7.4), where it dissociates and releases Tf into extracellular space for uptake by the cell inside an endosome, where Fe(m) is released by a combination of changes in acidity (pH < 6) and yet unknown chelators, but the Tf–TfR1 complex remains intact. The complex is then returned to the cell surface (pH = 7.4), where it dissociates and releases Tf into extracellular space for more Fe(m) binding. Various stages of Fe(m)–Tf–TfR1 interactions have been studied in detail, using radiolabelling techniques, immunostaining assays, rapid kinetic measurements by electronic or fluorescent spectroscopy, and surface plasmon resonance (SPR) measurements. The latter technique is related to, but not identical with, BLI.

Here we present the first application of BLI to studies of Tf–TfR1 interactions, using a BLItz instrument† (see ESI† for experimental details). A (His)₆-tagged recombinant human protein that represents the extracellular portion of TfR1 (Cys89-Phe760; Sino Biological, China) was loaded on a Ni(u)–NTA-coated optical probe (Fig. S1 in ESI; † NTA =...
nitrilotriacetate(3−)). The reaction of Fe(m)-loaded human Tf (1.0 μM Tf, 2.0 μM Fe(m)) with this probe at pH = 7.40 (20 mM HEPES,‡ 25 mM NaHCO3, 140 mM NaCl, 295 K) led to rapid Tf binding to TfR1, followed by slow dissociation when the probe was incubated with the buffer alone (red line in Fig. 1, segments B and C). Application of simulated endosomal conditions (100 mM MES,‡ 4.0 mM Na2edta,‡ 300 mM KCl, pH = 5.60)14 caused little change in the probe response within 5 min (segment D in Fig. 1), which is consistent with the Tf-TfR1 complex remaining intact while Fe(m) is released.11,14

A subsequent change to pH = 7.40 caused a rapid dissociation of the TF-TfR1 complex (segment E in Fig. 1), in agreement with the literature data.11,14 Functional TfR1 remained attached to the probe, and could be re-loaded with Fe(m)-Tf several times without significant loss of performance (Fig. S2 in ESI†). The use of apo-Tf (1.0 μM) instead of Fe(m)-Tf resulted in a much lower response of the probe (black line in Fig. 1), although some binding was still observed due to the traces of Fe(III) in the buffer.12 To our knowledge, this is the first observation of the full Fe(m)-Tf-TfR1 receptor binding and dissociation cycle in a single experiment in cell-free systems. A comparison of normalised association and dissociation kinetic curves for Fe(m)-Tf and apo-Tf (segments B and C in Fig. 1) is shown in Fig. 2.

The dissociation constant ($K_{D2}$; a standard measure of the protein–protein binding affinity)11−15 for the Fe(m)-Tf–TfR1 interaction was calculated from segments B and C of the binding curve (red line in Fig. 1), using the 1 : 1 binding model (the only one currently available in the BLItz software), which led to $K_{D2} = 12 ± 2$ nM (mean and SD of four measurements using two different probes). The literature values of $K_D$ depend on the experimental technique, but are generally in the low nM or sub-nM range.8,11−15 Our data were most consistent with the SPR results,15 which used the 2 : 2 binding model (dimeric TfR1 sequentially binds two Fe(m)-Tf molecules) and led to $K_{D1} = 1.9$ nM and $K_{D2} = 29$ nM ($K_{D} = 12$ nM approximates the average of these two constants). However, our preliminary kinetic studies suggested that the Fe(m)-Tf–TfR1 binding mechanism may be more complicated than this, since both association and dissociation kinetic curves were best fitted with sequences of three first-order reactions (Table S1 and Fig. S3, ESI†). Detailed kinetic studies of these reactions are in progress since they may have unraveled previously unrecognised steps in the binding and release that were not obvious in data obtained from other methodologies.

An anti-metastatic Ru(III) complex, NAMI-A (Chart 1), is currently in Phase II human clinical trials.1 In agreement with literature data,16 a reaction of two molar equivalents of NAMI-A with human apo-Tf ([Ru(III)] = 200 μM, [Tf] = 100 μM, 20 mM HEPES, 25 mM NaHCO3, 140 mM NaCl, pH = 7.40, 4 h at 310 K) caused the formation of an absorbance band at ~500 nm in electronic spectra (Fig. S4 in ESI†), similar to that for Fe(m)-Tf.17 As shown in Fig. S4,† this absorbance was only observed in the presence of HCO3− (a synergistic ion for Fe(m)–Tf binding).17 These features are indicative of specific Ru(m) binding to the Fe(m) binding sites of Tf,16 although this has not yet been unambiguously demonstrated (non-specific Ru(m)
binding to surface His residues of Tf is also likely to occur \(^2,10\)

As shown in Fig. 1 and 2 (green lines), the binding of Ru(III)–Tf (1.0 μM apo–Tf, 2.0 μM Ru(III)) to TfR1 was weaker (slower association and faster dissociation) compared not only with that of Fe(III)–Tf, but also with that of apo–Tf. Like Fe(III)–Tf and apo–Tf, both the association and dissociation kinetic curves for Ru(III)–Tf were best fitted with three sequential first-order reactions (Table S1 and Fig. S3†). These results indicated that the binding of Ru(III) to the Fe(III) binding sites of Tf inhibited its interaction with TfR1, so that Tf is unlikely to serve as a specific carrier of NAMI-A into cancer cells.\(^2\) Similar results were recently reported for the binding of various Ru anti-cancer prodrugs to Tf, and for the interactions of the resultant Ru-Tf adducts with TfR1, although no experimental details for NAMI-A were provided.\(^10\) Compared with the previous work that used immunosorptions assays to study the Tf–TfR1 binding,\(^10\) the use of BLI provides a simpler and faster option for assessing the TfR1 binding of various metal–Tf complexes.

In contrast to the binding of Fe(III)–Tf to TfR1 that has been studied by a variety of experimental techniques,\(^11\) the removal of Fe(III) from the Tf–TfR1 complex under simulated endosomal conditions has only been studied by stopped-flow fluorescence spectroscopy in solutions.\(^8,14\) Although the changes in BLI response during the metal ion removal were small (probably due to changes in protein conformation; see segment D in Fig. 1 and 3), they were sufficient for the kinetic analyses (Table S1 and Fig. S5 in ESI†), which offers a valuable alternative technique for the studies of this process. The data displayed in Fig. 3 indicated that the strong binding of apo–Tf to TfR1 at pH < 6 \(^11,12\) was further enhanced in the presence of Ru(III), probably due to the cross-linking of the two proteins by this kinetically inert metal ion.\(^2\) The presence of Ru(III) was also manifested by a slight decrease in the dissociation rate of apo–Tf–TfR1 complex at pH = 7.40, and by partial retention of Tf on the probe after this process (segment E in Fig. 1; see Table S1 and Fig. S5 and S6 in ESI† for details). These data suggested that blocking of Fe(III) delivery to fast-metabolising cancer cells through Ru(III)–Tf binding may be one of the mechanisms of anti-cancer activity of Ru(III),\(^2,9\) but the possible extent of this process in vivo is not yet clear.

Notably, the formation of a Ru(III)–Tf complex (measured from its absorbance at ~300 nm) could be partially suppressed by the addition of an equimolar amount of human serum albumin (HSA), as shown in Fig. S4.† This observation is consistent with those of human clinical trials, which showed that NAMI-A and other Ru-based anti-cancer prodrugs were bound predominantly (>90%) to albumin in the blood of patients (the HSA : Tf molar ratio in human blood is ~15 : 1).\(^18\) Previously, we have shown that Ru(III)–albumin adducts are likely to be responsible for the anti-metastatic action of NAMI-A,\(^4\) despite the fact that the coordination environment of Ru(III) in such adducts is completely different from that in the original prodrug.\(^5,19\) In the current study, we tested the influence of Ru(III)–HSA binding on non-specific interactions \(^20\) of HSA with another major group of blood proteins, immunoglobulins G (IgG; typical total blood plasma concentrations in humans, 7.5–22 mg mL\(^{-1}\), compared with ~40 mg mL\(^{-1}\) for HSA).\(^21\)

Loading of IgG was performed by incubation of a probe coated with protein G (a bacterial protein that strongly binds to human IgG)\(^5\) with a dilute solution of human IgG (50 μg mL\(^{-1}\)) for 2 min at 295 K in phosphate-buffered saline (PBS; 20 mM H\(_2\)PO\(_4^−/\)HPO\(_4^{2−}\), 150 mM NaCl, pH = 7.40), followed by a brief wash with PBS (segments B and C in Fig. 4). This was followed by the association step with concentrated solutions of HSA (0.50 mM in PBS), or of Ru(III)–HSA adduct (pre-formed by the incubation of 0.50 mM HSA with 0.50 mM NAMI-A in

![Fig. 3](image-url) Normalised BLI response curves for the metal ion removal from the Tf–TfR1 complex under endosomal–mimetic conditions \(^18\) (segment D) in Fig. 1. Each condition is represented by two measurements, performed on different probes. Kinetic parameters are given in Table S1 and Fig. S5, ESI†

![Fig. 4](image-url) Typical BLI response curves (295 K) for the binding of HSA or the Ru(III)–HSA adduct (0.50 mM) to human IgG loaded on a protein G probe (see ESI† for experimental details). Designations of the segments: (A) initial buffer background; (B) loading of IgG (50 μg mL\(^{-1}\)); (C) washing of the probe with the buffer; (D) association of HSA; and (E) dissociation of HSA (buffer alone). Buffer used in all the segments: 20 mM H\(_2\)PO\(_4^−/\)HPO\(_4^{2−}\), 150 mM NaCl, pH = 7.40. Jumps due the changing solution viscosity are designated with asterisks.
PBS for 4 h at 310 K4,21 and by the dissociation step in PBS alone (segments D and E in Fig. 4). A comparison of normalised kinetic curves for the association and dissociation steps (Fig. 5) showed that Ru(III) binding significantly inhibited both processes compared with those for native HSA. The kinetics of both processes (either in the absence or presence of Ru(III)) were best described with two sequential first-order reactions (Table S1 and Fig. S7 in ESI†), which means that the HSA–IgG interactions could not be adequately described by simple $K_D$ values.20 Inhibition of protein–protein dissociation in the presence of Ru(III) (Fig. 5 and S6†) can be particularly important for the enhancement of Ru(III)–HSA interactions with collagen, which is a likely crucial step in the anti-metastatic action of NAMI-A.2,4,19

Conclusions

This proof-of-concept work has shown the considerable potential of BLI in the studies of the influences of medicinal metal ions on both strong (TF–TfR1) and weak (IgG–HSA) protein–protein interactions, using an anti-metastatic Ru(III) prodrug, NAMI-A, as an example. Prior to these studies, the method has been validated using the well-understood binding of Fe(III)–Tf to TFR1. Unlike for other experimental techniques, BLI can be used to study the full cycle of TF–TfR1 binding and dissociation in a single experiment. Specific binding of NAMI-A to the Fe(III) binding sites of TF can occur in the absence of excess Fe(III) and albumin, but it is unlikely to have major effects on the cellular uptake of NAMI-A in vivo. Results of IgG–HSA binding studies support the hypothesis that cross-linking of extracellular proteins by Ru(III) is involved in its anti-metastatic activity.2,4 The BLI technique can be expanded to the studies of the roles of metal ions in protein–lipid and protein–nucleic acid interactions.22,23

Acknowledgements

Financial support of this work has been provided by the Australian Research Council (ARC) Discovery grant, including an ARC Professorial Fellowship, to P. A. L. (DP0984722) and funding for an ARC Senior Research Associate for A. L. (DP130103566). We thank Dr Donna Lai (Bosch Institute, The University of Sydney) for assistance in using the BLItz instrument, and Ms. Hannah O’Riley (School of Chemistry, The University of Sydney) for the synthesis of NAMI-A.

Notes and references

†HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MES = 2(N-morpholino)ethanesulfonic acid; edta = $N,N',N''$-ethane-1,2-diaminetetra-acetate(4–).


