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Spectroscopic Analysis of Three Anticancer Ruthenium (II) Complexes and Transferrin: No evidence for Specific or Non-Specific Binding

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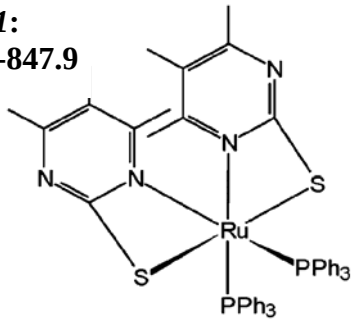
Introduction

Research on ruthenium-complexes has grown over the years as scientists become aware of potential applications in a number of innovative fields. Chief among the attractive qualities of several categories of Ru-complexes are their promising photochemical properties and their usefulness as fluorescent probes (see Appendix). No less intriguing are the anticancer properties shown by many ruthenium(III) and a number of ruthenium(II) complexes synthesized over the past two decades[1,2,3]. Representative Ru(III) complexes studied *in vivo* exhibit inhibition of DNA replication, reduction of RNA synthesis, mutagenic activity, and induction of the SOS repair mechanism[3]. In the case of many Ru(III) complexes, the "activation by reduction" mechanism is invoked in which the inert complexes are reduced *in situ* to ruthenium(II), the species thought to be responsible for direct attack on DNA nucleobases[3].

Renzo Cini at the University of Siena has synthesized three novel ruthenium(II) complexes (Figure 1) and sent them to our lab for spectroscopic analysis. In addition to the ruthenium oxidation state less encountered in literature on anticancer Ru-complexes,

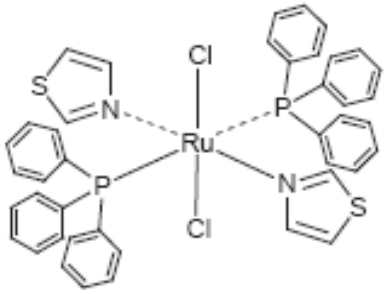
***Fig. 1*:**

Ru-847.9

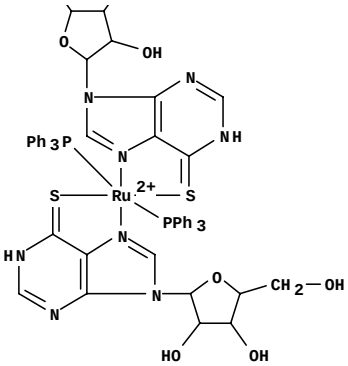


Cis-[Ru(PPh$_3$)$_2$(TPYM)$_2$]
PPh$_3$= triphenylphosphine,
TPYM= 2-thio-1,3,pyrimidine.
Molecular Weight= 847.9 [1]
("Ru-847.9")

Ru-866.8



Trans, trans, trans-[RuCl$_2$(PPh$_3$)$_2$(THZ)$_2$]
THZ= thiazole-1,3.
MW= 866.8 [4]
("Ru-866.8")



- 2 Cl$^-$
- 2.75 H$_2$O

Cis-Ru(PPh$_3$)$_2$(HTPR)$_2$]Cl$_2$x2.75H$_2$O: ***same ligands as the above diagram but with the cis octaherdral arrangement rather than the depicted trans configuration*
HTPR=6-thiopurine-riboside
MW=1314.7 [5]
("Ru-1314.7")

these complexes are especially interesting because the ligands themselves have medicinal properties. Phosphines have shown selective cytotoxic and anticancer properties[1]. Along with antiviral properties, thiopyrimidines possess photochemical properties that may prove useful in the design of new photodynamic cancer therapies[1]. Thiopurines are currently used as antileukemic and antiviral agents[1]. Likewise, thiazoles are present in a number of active drugs[4].

Complexation of anticancer drugs to a metal atom is beneficial for a number of reasons. The inertness of certain metal-ligand linkages protects the ligand drugs against enzymatic degradation[1]. Secondly, the metal complex may have a better hydrophobicity or hydrophilicity than the free ligands[1]. Thirdly, the ligands' activity can be synergistically reinforced by the metal's own anticancer properties[1,4]. Lastly, the metal complex has the potential to release the active drug ligands in a target organ through several biological processes[1].

I. UV/Vis Absorption of the Ruthenium(II) Complexes

All three Ru(II) compounds display signature visible and/or ultraviolet absorption peaks. Ru-847.9 dissolved in chloroform or a buffer solution with 15% DMSO shows a well-defined peak in the visible range at 333 nm or 327 nm respectively. In a buffer solution with less DMSO (4%), a corresponding peak becomes somewhat obscured, yet is nonetheless discernable (Figure 2). This peak at approximately 330 nm lies in the typical range of ligand to metal charge transfers (LMCT) found in other octahedral and non-octahedral ruthenium complexes[2,6,7,8,9], and therefore can be attributed to such.

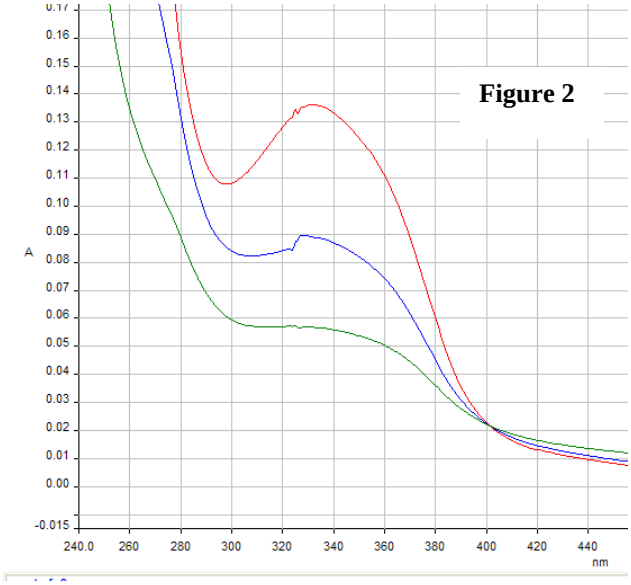


Figure 2

Ru-847.9 in various solvents displays an absorption maximum at ~330 nm.
--- 56.4 μM in chloroform. Blank: chloroform.
--- 20.0 μM in buffer solution and DMSO (15%). Blank: buffer solution and DMSO (15%).
---20.0 μM in buffer solution and DMSO (4%). Blank: buffer solution and DMSO (4%).
0.2 cm cuvettes
buffer solution: (25 mM Tris-base and 10 mM $NaHCO_3$ in deionized-distilled H_2O, pH 7.4)

Similarly, the peak at approximately 378 nm for Ru-1314.7 (Figure 3) is likely a ligand to metal charge transfer band. Ru-866.8's absorption peak is at a significantly

shorter wavelength (at approximately 280 nm, Figure 3), and is probably instead due to ligand absorption, or some combination of metal and ligand absorption that cannot be determined without additional spectroscopic study.

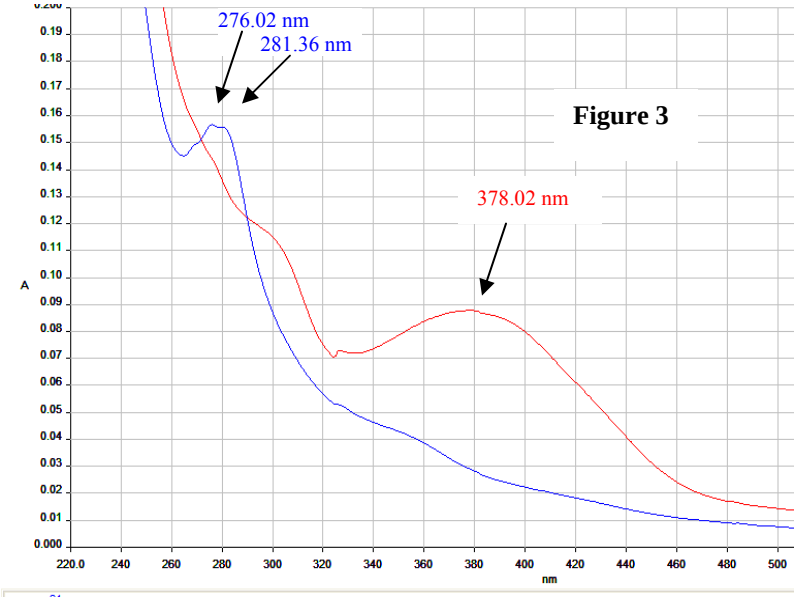


Figure 3

Ru-866.8 and Ru-1314.7

--- 20 µM **Ru-866.8** in buffer solution and DMSO (4%)

--- 20 µM **Ru-1314.7** in buffer solution and DMS0 (4%)

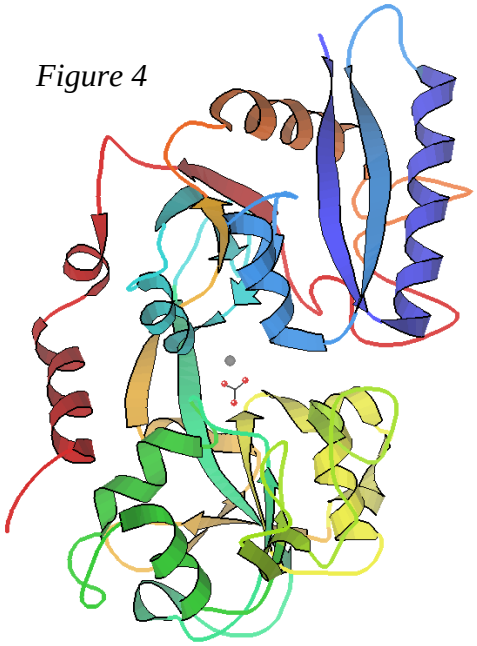
Blank: buffer solution and DMSO (4%)
0.2 cm cuvettes
buffer solution: same as noted in Fig. 2

II. Transferrin

Increased selectivity of a drug reduces systemic toxicity by keeping a high, localized concentration of the drug at target tissue but a low concentration in other regions[10]. The linkage of the drug to biological macromolecules that possess high selectivity for well-defined biological targets is a means to appropriate drug-targeting.

The iron(III)-transport protein transferrin has been looked to as a possible transport vehicle for ruthenium-based drugs because of its abundance in the plasma and because of ruthenium's location just below iron on the periodic table. Human serum transferrin, a glycoprotein of 80 kilodaltons, binds ferric ions and transports them to the hemoglobin-synthesizing immature red blood cells[11] or to the liver for storage as ferritin and hemosiderin[12]. A bicarbonate or carbonate ion is necessary to promote the hinge-like closing of the protein domains around the metal ions[13]. Preliminary X-ray crystallographic studies[12] and amino acid sequencing[14] indicate that the protein is a single polypeptide chain composed of two homologous domains each containing a metal-

Figure 4



Monoferric transferrin with ferric and carbonate ions[16]

binding site. Each metal site contains a distorted octahedron with two tyrosines, one histidine, one aspartate, and the synergistic, bidentate bicarbonate anion as ligands[13]. Despite similarity in overall structure and function, the two sites have been shown to differ in kinetics and pH dependence of binding, thermal stability, iron-donating properties and other characteristics[12,11]. Most trivalent metals, Ru^{3+} included[15], and a few divalent metals bind specifically to the iron-binding sites of the apoprotein[12].

Transferrin enters endosomic compartments within the cell via the transferrin cycle, and could thus theoretically usher the ruthenium-based drugs across the cell membrane into an acidic compartment where the low pH would favor disassociation from the protein[10]. The elevated nutrient requirements, higher membrane permeability, and increased angiogenesis of tumor cells[17], coupled with the disproportionately large number of transferrin receptors on the surface of quickly proliferating tumor cells[2,10,18], could result in selective, elevated uptake of the drug-protein adducts by these target tissues.

Apo-transferrin's distinguishing feature in the ultraviolet and visible light range is an absorption peak at 280nm with a shoulder at 290 nm. Holo-transferrin (diferric transferrin) presents a similar curve over this ultraviolet range, yet is unique in its visible light absorption peak centered on ~465 nm (Figure 5). This latter peak of holo-Tf is assigned to a phenolate to iron(III) charge transfer[19].

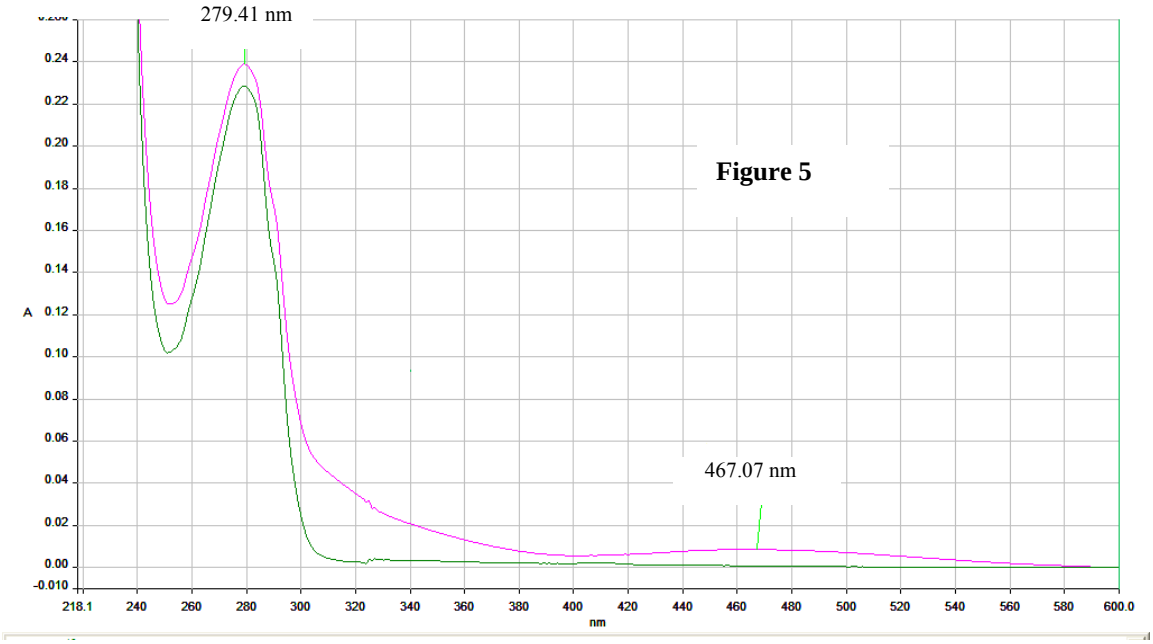


Figure 5

Holo-Transferrin vs. Apo-Transferrin

Blanks: buffer solution
--- Holo-transferrin (diferric transferrin) purchased from Sigma: 10 µM in buffer
--- Apo-Transferrin purchased from Sigma: 10 µM in buffer
0.2 cm cuvettes
buffer solution: same as noted in Fig. 2

Two spectroscopic signals indicate the occupancy of the two metal-binding sites of transferrin by ferric ions. The first is the increase in absorption at 280 nm in going from apotransferrin [A_{280} (1%) = 10.9][11] to holo-transferrin [A_{280} (1%) = 14.0][11] (Figure

5). When iron binds to transferrin, a conformational change occurs in which amino acid ligands from the three different regions of the protein become coordinated to the metal[20].

The second piece of evidence indicating specific metal binding is the LMCT absorption peak at ~465 nm seen in the holo-transferrin spectrum. A corresponding absorption peak can be induced by adding an appropriate source of ferric ions to an apotransferrin solution. For example, a 1.5 mM solution of $Fe(NTA)_2$ of pH 4.0 was made in line with previously published results[11]. The addition of two molar equivalents of this $Fe(NTA)_2$ solution to apo-transferrin in the presence of bicarbonate ion produces an absorption peak analogous to that of holo-transferrin (Figure 6).

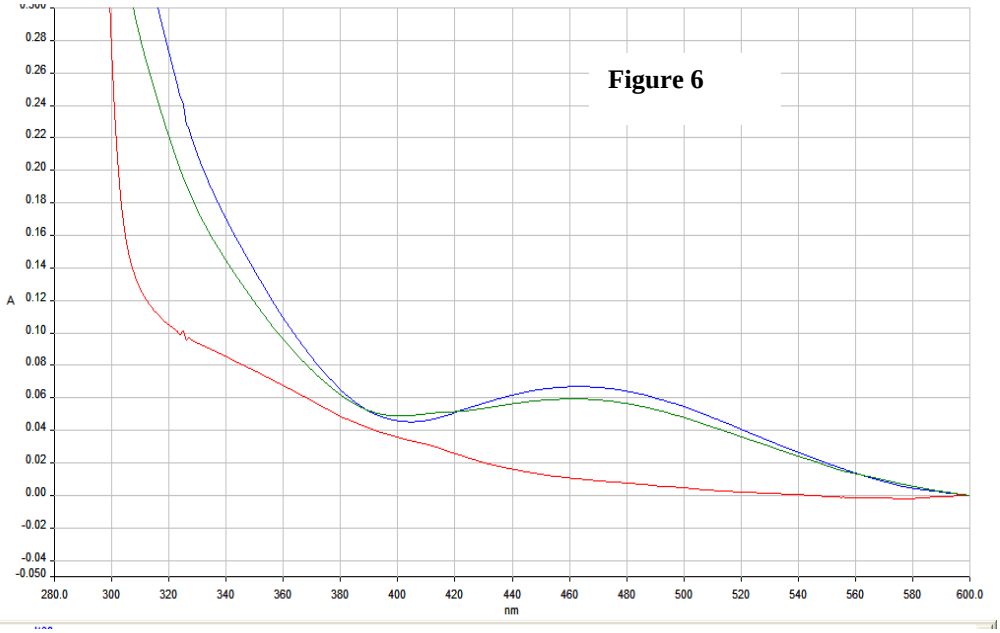


Figure 6

Diferric Transferrin vs. Apo-Transferrin

Blanks: Buffer solution as in Figure 2
--- Holo-Tf purchased from Sigma: 20 µM protein in buffer solution
---Diferric Transferrin: 40 µM $Fe(NTA)_2$ plus 20 µM apo-Tf in buffer solution
---Apo-Tf: 40 µM protein in buffer solution
1 cm masked cuvettes

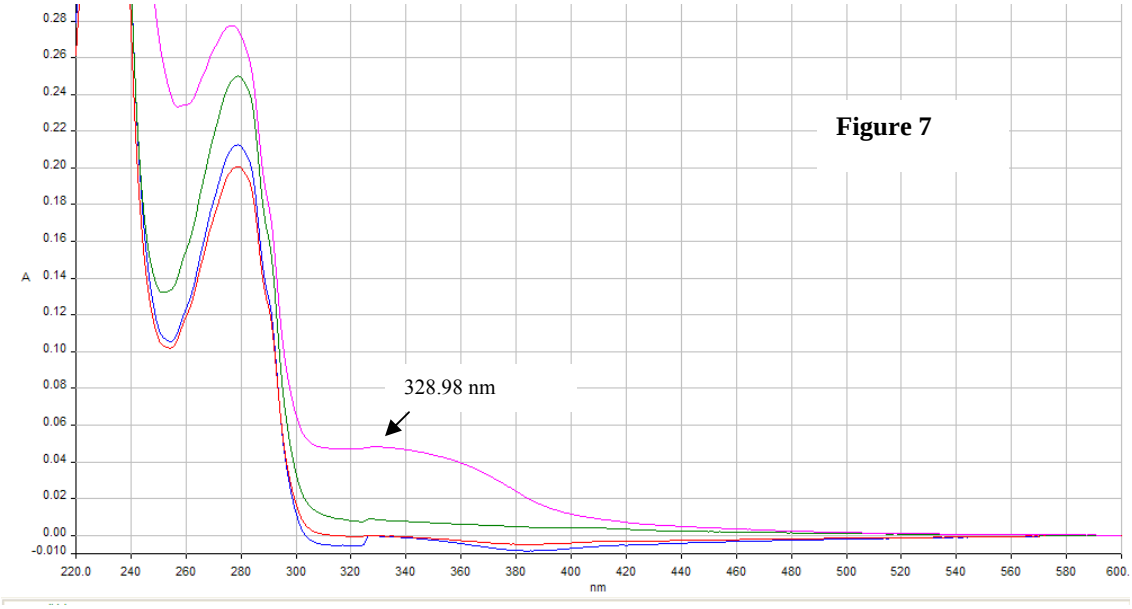
1

[1] $Fe(NTA)_2$ was prepared by dissolving dissolving 15.0 µmol iron filings in 5 mL of 6.0 M HCl over night. A small volume of this iron solution (0.77 mL) was added to 3.0 mL nitrilotriacetate solution (30 µmol NTA in 5 mL deionized-distilled H_2O). The pH of this $Fe(NTA)_2$ solution was adjusted to 4.0 with KOH.

III. Do the three Ruthenium Complexes Bind to Transferrin?

When two or even three molar equivalents of Ru-847.9 are introduced to apo-Tf under the identical conditions used for introduction of $Fe(NTA)_2$ to apo-Tf, spectroscopic signs of specific binding are not observed (Figure 7). The peak at ~329 nm for the sample of apo-Tf, buffer solution, and two equivalents of Ru-847.9 versus a blank of buffer solution (pink curve) can be attributed to Ru-847.9 unbound to transferrin (see Fig. 2).

Furthermore, when Ru-847.9 is placed in the blank cuvette, the resulting transferrin spectrum (Figure 7, red curve) fails to show the characteristic features that distinguish a diferric transferrin spectrum from that of apo-Tf. First and foremost, a change in the protein's absorption in the visible spectrum corresponding to that seen with the addition of ferric ions (i.e., holo-Tf's absorption peak at ~465 nm) is not observed. Secondly, the transferrin absorbance values at 280 nm for apo-Tf in the presence of Ru-847.9 are lower than that for apo-Tf alone. Diferric transferrin has a higher absorbance at 280 nm than apo-Tf, and one would expect the same pattern if ruthenium were binding to transferrin. Together, these two observations seem to indicate that the ruthenium complex does not occupy the iron-binding sites of transferrin.

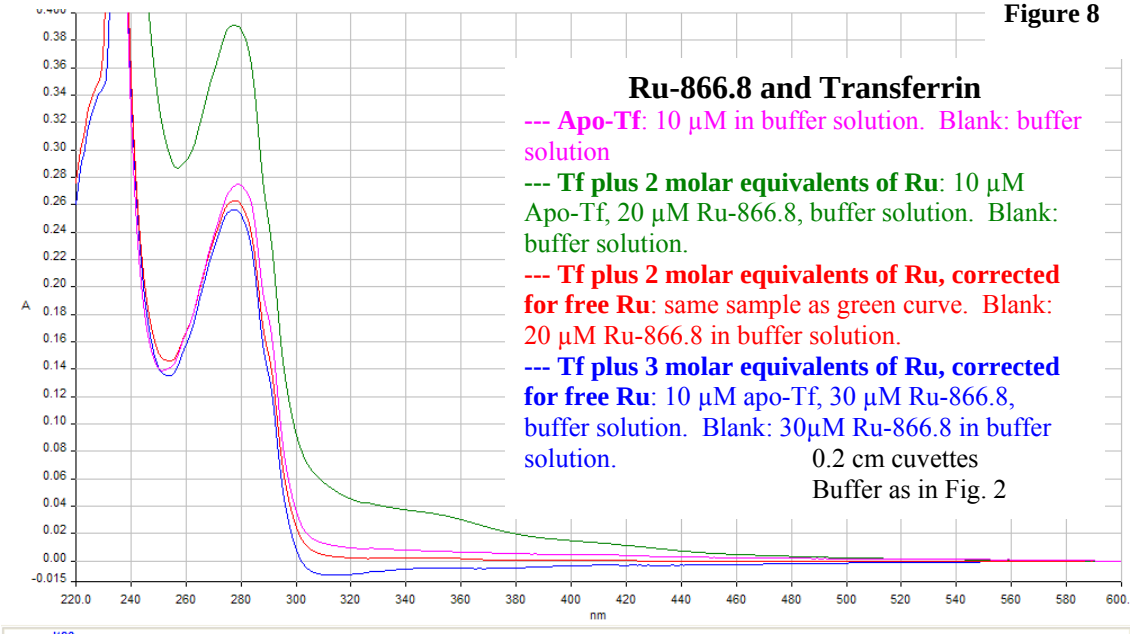


Ru-847.9 and Transferrin

--- **Apo-Tf**: 10 µM in buffer solution. Blank: buffer solution
--- **Tf plus 2 molar equivalents of Ru**: 10 µM Apo-Tf, 20 µM Ru-847.9, buffer solution. Blank: buffer solution.
--- **Tf plus 2 molar equivalents of Ru, corrected for free Ru**: same sample as pink curve. Blank: 20 µM Ru-847.9 in buffer solution.
--- **Tf plus 3 molar equivalents of Ru, corrected for free Ru**: 10 µM apo-Tf, 30 µM Ru-847.9, buffer solution. Blank: 30µM Ru-847.9 in buffer solution.
0.2 cm cuvettes
Buffer solution as in Figure 2

Similarly, the spectra of Ru-866.8 and Ru-1314.7 in solution with apo-Tf fail to conclusively indicate specific binding (Figure 8 and 9).

Figure 8



Ru-866.8 and Transferrin

--- Apo-Tf: 10 µM in buffer solution. Blank: buffer solution

--- Tf plus 2 molar equivalents of Ru: 10 µM Apo-Tf, 20 µM Ru-866.8, buffer solution. Blank: buffer solution.

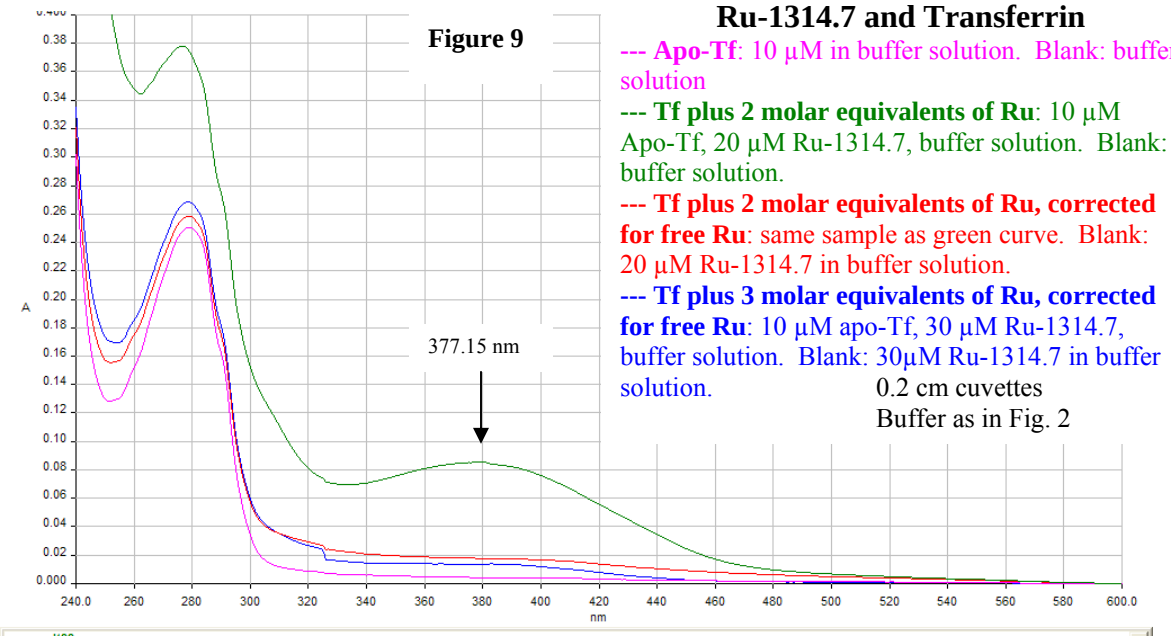
--- Tf plus 2 molar equivalents of Ru, corrected for free Ru: same sample as green curve. Blank: 20 µM Ru-866.8 in buffer solution.

--- Tf plus 3 molar equivalents of Ru, corrected for free Ru: 10 µM apo-Tf, 30 µM Ru-866.8, buffer solution. Blank: 30µM Ru-866.8 in buffer solution.

0.2 cm cuvettes

Buffer as in Fig. 2

Figure 9



Ru-1314.7 and Transferrin

--- Apo-Tf: 10 µM in buffer solution. Blank: buffer solution

--- Tf plus 2 molar equivalents of Ru: 10 µM Apo-Tf, 20 µM Ru-1314.7, buffer solution. Blank: buffer solution.

--- Tf plus 2 molar equivalents of Ru, corrected for free Ru: same sample as green curve. Blank: 20 µM Ru-1314.7 in buffer solution.

--- Tf plus 3 molar equivalents of Ru, corrected for free Ru: 10 µM apo-Tf, 30 µM Ru-1314.7, buffer solution. Blank: 30µM Ru-1314.7 in buffer solution.

0.2 cm cuvettes

Buffer as in Fig. 2

IV. Do the Ru-complexes display fluorescent properties that could be employed to investigate possible binding to transferrin?

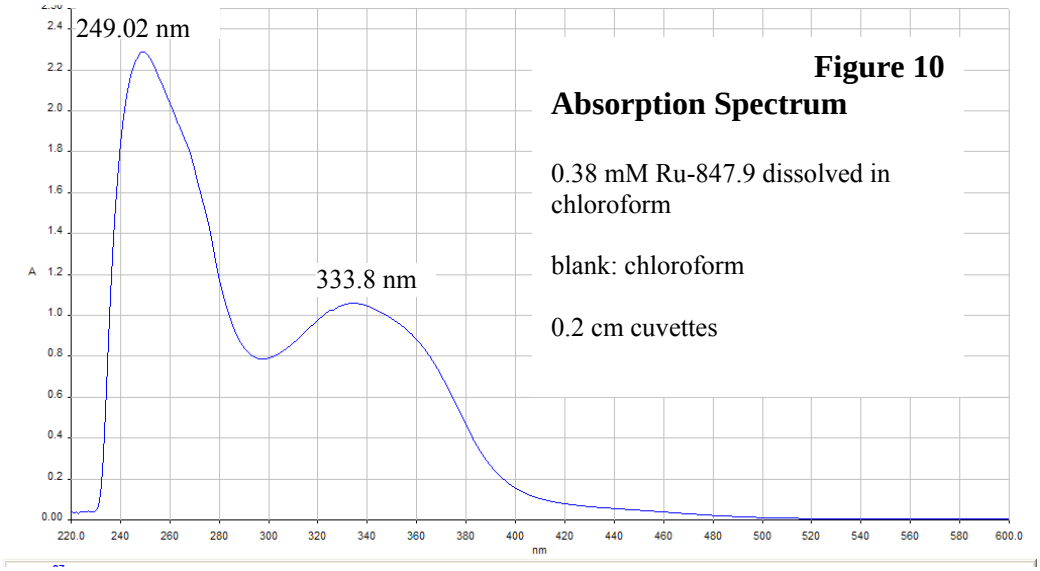
Although the above absorption spectra appear to indicate that the three ruthenium(II)-complexes do not bind specifically to transferrin, several previous studies by other labs have confirmed the ability of various Ru(III)-complexes to bind tightly to several plasma proteins including transferrin[2,3,9,10,17,18,20]. All but one[20] of these referenced ruthenium(III) complexes are coordinated to chloride ions; and, many of the authors of these studies assert that the loss of at least one[3,9] or two[21] coordinated chlorides is a prerequisite for further reactions with proteins. Perhaps our three Ru(II) molecules, two of which are not coordinated to halogens, are unable to access the appropriate binding sites of transferrin as a result of their retention of ligands. In addition, the fact that the protein-bound ruthenium complexes remain in the Ru(III) oxidation state upon binding to transferrin[3] may indicate that our Ru(II)-complexes need to be oxidized prior to binding.

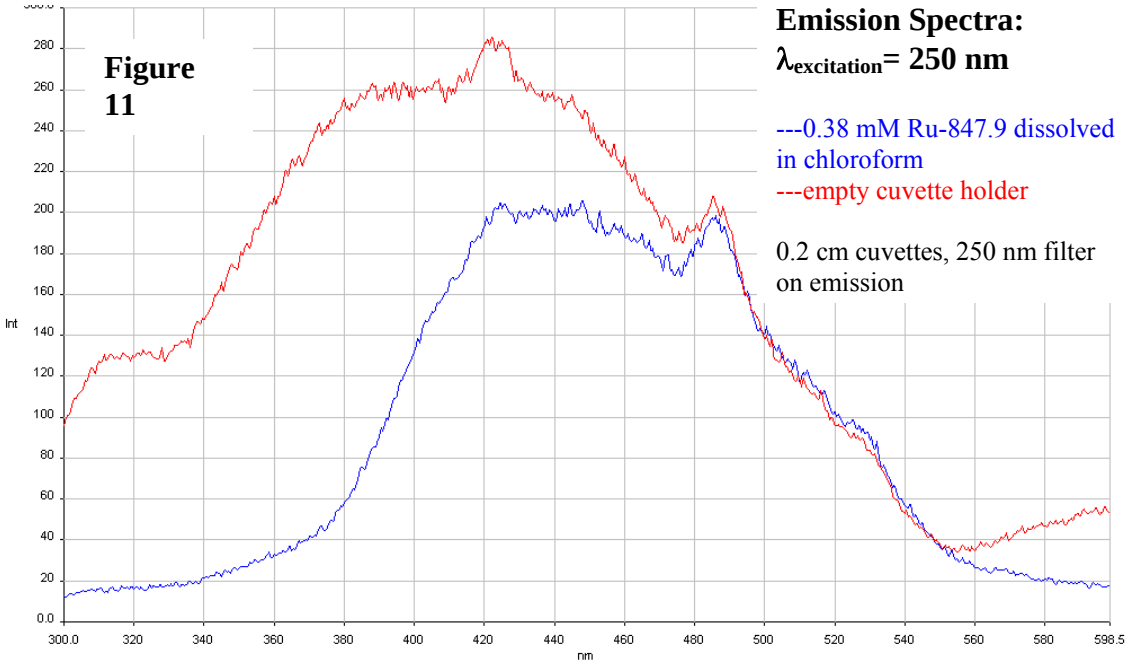
Ru(III) ions bound to NTA ligands have been shown to bind specifically at the iron-binding sites of transferrin[15]. As with iron, ruthenium(III) administered to apotransferrin as Ru(III)-NTA binds in a two to one ratio if bicarbonate ion is available. The resulting UV difference spectrum of $Ru(III)_2Tf$ versus apo-Tf reflects that usually seen for a number of metallotranferrins[15]. However, Ru(III)-complexes have also been shown to bind to transferrin at exposed imidazole rings of histidines[3,9,17,20]. Unlike iron which has a maximum binding ratio of two iron atoms to one transferrin molecule, ruthenium(III)-complexes can bind with up to five[3] or six[20] equivalents per transferrin. That specific binding at the iron-binding sites proper is not occurring between the ruthenium-complexes and transferrin is further demonstrated by the fact that ruthenium modified transferrin retains its ability to bind up to two iron(III) equivalents when $Fe(NTA)_2$ is administered[20].

Documentation of the binding of Ru(III)-complexes to the histidines of transferrin offered hope that our ruthenium(II)-complexes might possess the ability to likewise bind to transferrin. Perhaps fluorescence spectra could elucidate these histidine interactions and show evidence of binding that our absorption spectra failed to indicate. We were optimistic that our three ruthenium(II)-complexes would display useful fluorescent properties because other ruthenium complexes cited in literature have intriguing and valuable fluorescent properties. For example, the emission spectra of ruthenium(III) dendrimers, ruthenium(III) polypyridyl complexes, and cyano-bridged trinuclear polypyridyl complexes of Ru(II) employed as artificial, luminescent chromophores are useful tools for understanding the excited state dynamics of these molecules[6,7,22,23]. Likewise, the emission spectra of several oligonuclear polypyridyl Ru(II) complexes helps elucidate the structure and charge transfer behaviors of these molecules[24]. Furthermore, unsymmetrical Ru-ligand complexes such as $Ru(dcby)(bpy)_2$ are used as anisotropy probes because of their emission properties and long lifetimes[25]. Lastly, $Ru(bpy)_3^{2+}$-pendant dendrimers can serve as sensors for the explosive trinitrotoluene (TNT) based on the quenching of their fluorescence by TNT[26].

Ru-847.9 dissolved in chloroform has an absorption peak at ~333 nm and a larger peak at ~250 nm (Figure 10). However, an emission spectrum of 0.38 mM Ru-847.9

dissolved in chloroform and excited at 250 nm failed to result in emission intensity greater than the background intensity of light scattered by the cuvette holder (Figure 11).



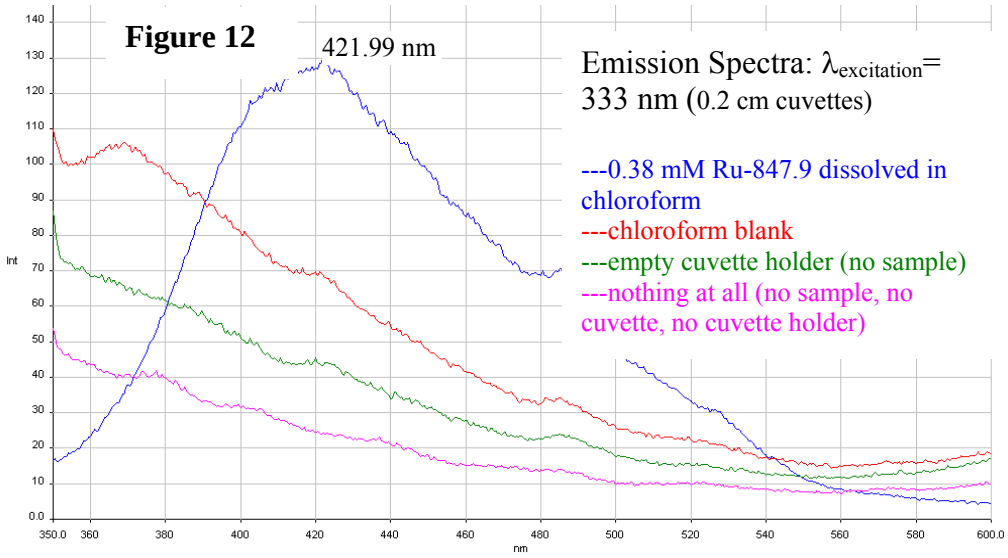


*For all fluorescence spectra, both the excitation and emission slits are 10.0 nm. Scan speed is 200 nm/sec.

An emission spectrum of the same sample excited at 333 nm results in an intensity maximum at 421.99 nm (Figure 12). However, the intensity of this signal is not sufficiently higher than that caused by the solvent alone and other background noise.

In an effort to determine how to maximize the emission signal of the ruthenium complex, an excitation spectrum was analyzed at an emission wavelength of 420 nm. This excitation spectrum indicated that the intensity at 420 nm is stronger with excitation

at 290 nm (Figure 13). Therefore, an emission spectrum of Ru-849.7 in chloroform with an excitation wavelength of 290 nm was collected (Figure 14). However, while the intensity of the signal does increase as hoped, it remains insufficiently greater than the emission intensity of the solvent alone. The fluorescence signal of the ruthenium(II) molecule, even at this relatively high concentration, is inadequate in intensity and would not serve as a conclusive probe in our attempts to elucidate the interactions between transferrin and the ruthenium complex.



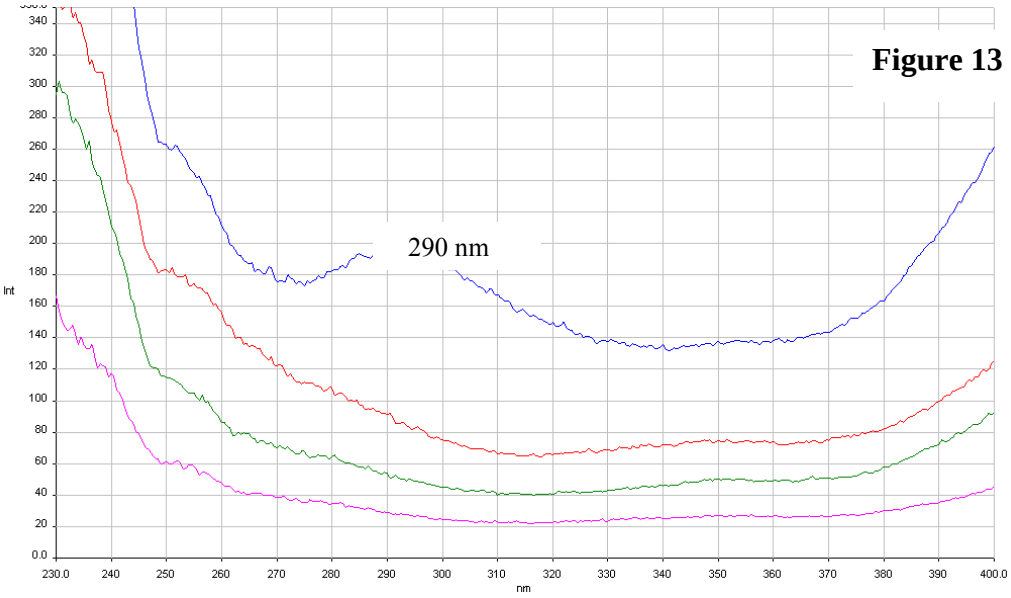


Fig. 11 Excitation Spectra: $\lambda_{emission}$= 420 nm (0.2 cm cuvettes)
---0.38 mM Ru-847.9 dissolved in chloroform
---chloroform blank
---empty cuvette holder (no sample)
---nothing at all (no sample, no cuvette, no cuvette holder)

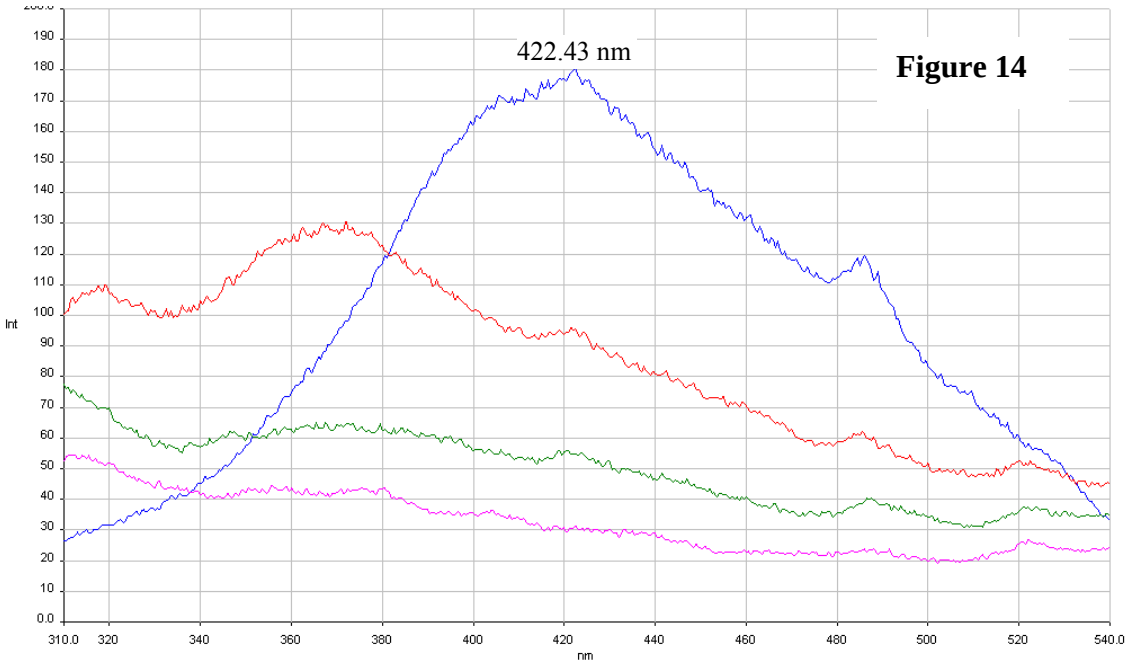


Fig. 12 Emission Spectra: $\lambda_{excitation}$= 290 nm (0.2 cm cuvettes)
---0.38 mM Ru-847.9 dissolved in chloroform
---chloroform blank
---empty cuvette holder (no sample)
---nothing at all (no sample, no cuvette, no cuvette holder)

Conclusion

Absorption spectra of the three ruthenium(II) complexes in solution with apo-transferrin and bicarbonate ion do not show the spectroscopic evidence of specific Tf-metal binding that Fe_2-transferrin displays. It is our belief that these ruthenium complexes are not binding specifically at the metal-binding sites of transferrin.

The fluorescence emission of these three ruthenium(II) complexes at 420 nm is quite weak, presumably due to ligand quenching. It is impossible therefore, to use fluorescence as a means of binding to these secondary sites.

Appendix

A Brief Survey of the Literature: Additional Applications of Ruthenium Complexes

Artificial Photosynthesis

- Ruthenium containing dendrimers (perfectly branched synthetic macromolecules with several side chains radiating from a single core) serve as light-harvesting chromophores that channel absorbed energy to a single reaction center in a fashion analogous to that of natural photosynthetic systems. Ruthenium-polypyridine complexes have ideal luminescence and redox properties for such systems and therefore have the potential to be used in light-emitting diodes, signal amplifiers, fluorescent sensors, frequency converters, and other photonic devices[22].
- Ruthenium polypyridyl complexes take the place of chlorophylls in artificial photosynthesis. The absorption of light by $[Ru\text{-}(bpy)_3]^{2+}$ results from metal to ligand charge transfer (MLCT)[9,10]. The excited states that result are reached with high efficiency, are quite stable, and have sufficiently long lifetimes that facilitate the desired chemical reactions. The appeal of these complexes is due to multiple MLCT transitions, vibronic progressions, and solvent broadening which allow for the absorption of light over a wide range of wavelengths. Alternatively, chemical changes can systematically tune the range at which light is absorbed[7].
- Cyano-bridged trinuclear polypyridyl complexes of Ru(II) are of interest as light-harvesting devices and sensitizers in photovoltaic cells[23].

Understanding Other Biological Processes

- A ruthenium-labeled cytochrome *c* derivative allows for the study of electron transfer from cytochrome bc_1 to cytochrome *c* (Cc), a step in the electron transport chains of mitochondria and many respiratory and photosynthetic prokaryotes. Labeling the ruthenium-Cc derivative, [Ru_z-39-Cc], with $Ru(bpz)_2(dmb)$, a complex able to photooxidize the ferrous heme in Cc, permits the study of electron transfer in the forward, physiological direction[24].
- Oligonuclear polypyridyl Ru(II) complexes model long-range electron and energy transfer processes known to be important steps in respiration, photosynthesis, and DNA oxidative cleavage[25].
- Ruthenium(II) myoglobins are employed in the study of metal ion substitution in heme proteins and the binding of carbon monoxide and dioxygen to myoglobins[26].

Probes

- Because of their long-lived lifetimes, unsymmetrical Ru-ligand complexes such as $[Ru(dcby)(bpy)_2]$ can be used as anisotropy probes for protein hydrodynamics and immunoassays of high-molecular-weight antigens[27].
- $[Ru(bpy)_3]^{2+}$-pendant dendrimers can serve as TNT sensors based on the quenching of their fluorescence by TNT. The high quantum yield, photostability, and redox power of $[Ru(bpy)_3]^{2+}$ make it a very promising fluorescence probe[28].

- The photoreactive reagent AzRu, containing ruthenium and a photoactivable azido group, interacts specifically with Ca^{2+} binding proteins regardless of the proteins' catalytic mechanisms. AzRu thus provides a method of identification of these proteins and characterization of their binding sites[29].

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