Binding of Luminescent Ruthenium(II) Molecular Probes to Vesicles

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A systematic study of the binding of luminescent Ru(II) complexes with α-diimine ligands (2,2′-bipyridine, 1,-10-phenanthroline, and substituted analogues) to neutral (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and anionic (phospholipid was L-α-phosphatidylserine (Brain; sodium salt)) unilamellar vesicles is presented. Excited state lifetime measurements coupled with differential oxygen quenching of the bound and unbound complex provide a quantitative measure of binding. The roles of charge and complex hydrophobicity are examined in regard to binding strength. Binding is strongly dependent on electrostatic attraction, but hydrophobic interactions are equally important and can enhance binding by orders of magnitude. These results provide criteria and guidelines for the design of luminescent inorganic molecular probes for use in biological and, especially, membrane systems.

Introduction

Luminescent transition metal complexes (TMC's) with long-lived excited state lifetimes are currently affecting profoundly many areas of modern photochemistry and photophysics.[1,2] TMC's can have many potential advantages as luminescence probes, including long excited-state lifetimes (τ's) and high luminescence quantum yields. The longer lifetimes of many TMC's make measurements of τ's much easier than with the typical nanosecond organic probes and permit efficient time discrimination from the ubiquitous fluorescences of short-lived organics. In particular, platinum metal complexes with α-diimine ligands have formed the basis for new classes of sensitizers, molecular probes, and sensors.

Among the most successful metal complexes used to date as molecular probes are Ru(II) α-diimine complexes. Such materials have shown exquisite environmental sensitivity, which results in variations in emission maxima, quantum yields, and lifetimes with changes in local environments. All of these quantities have been used to obtain information on local environments and binding modes. This study focuses on the binding of selected ruthenium-based luminescent molecular probes to vesicles. We have systematically examined the variations in binding properties with both the type of vesicle and the structure of the molecular probe. Such knowledge is essential in selecting or designing probes with specific binding properties for different types of environments. Vesicles are model systems for biological membranes,[3] and this work can be applied directly to the design and application of bioprobes.

While there has been a reasonable amount of work on metal complex monolayers for use in sensors[4] and solar energy conversion,[5,6] there is little available work on metal complex binding to vesicles. Using synthetic dihexadecyl phosphate vesicles, quenching and electron transfer from $[Ru(bpy)_3]^{2+}$ (bpy = 2,2′-bipyridine) to viologen have been examined.[7] In the same system, quenching studies have detected different binding sites and stopped-flow measurements have found inside−outside $[Ru(bpy)_3]^{2+}$ flip-flopping.[8] Neither of these papers addressed the effect of metal complex structure on binding affinity.

The practical applications of such research are diverse. Since the cell membranes of all living organisms are composed of phospholipid bilayers and such systems are critical in photosynthesis and vision, information about phospholipid binding interactions has tremendous potential for applications in biochemical and energy conversion research.[9,10] The information obtained about the role of hydrophobic effects vs electrostatic interactions in plasma membrane binding can be used to interpret the cellular binding mechanisms of antibodies, proteins, or essential metabolites. In addition, it may be possible to develop specialized ruthenium-based luminescent tags for use in biological assays of different cellular systems.

Experimental Section

Complexes. The probes consisted of cationic ruthenium complexes with different α-diimine ligands of varying hydrophobicities. The complexes examined were $[Ru(Phphen)_2(phen)](ClO_4)_2$, $[Ru(Ph_2phen)_3](ClO_4)_2$, $[Ru(Ph_2phen)(phen)_2](ClO_4)_2$, $[Ru(Phphen)(phen)_2](ClO_4)_2$, $[Ru(Me_2phen)_3](ClO_4)_2$, $[Ru(phen)_3](ClO_4)_2$, and $[Ru(bpy)_3]Cl_2$ where bpy = 2,2′-bipyridine, phen = 1,10-phenanthroline, Phphen = 5-phenyl-1,10-phenanthroline, Me_2phen = 4,7-dimethyl-1,10-phenanthroline, and Ph_2phen = 4,7-diphenyl-1,10-phenanthroline.

Complexes were available from earlier studies or were synthesized by analogous procedures.[11] All complexes exhibited a single-exponential decay time in solution and had the intense orange luminescence of this class of complexes.

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Synthesis of Vesicles. Phospholipids were from Avanti-Polar-Lipids, Inc. The anionic phospholipid was L-α-phosphatidylserine (Brain; sodium salt) and the neutral phospholipid was 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. The 800 Millipore filters were from the Poretics Corp. All measurements were made with a 3.1 mM MOPS (3-morpholinopropanesulfonic acid) (Fluka) buffer with 12.5 mM NaCl to control pH and ionic strength. Procedures were similar to those in the literature.[12] A Lipex extruder was used for vesicle preparation. All other chemicals were reagent quality, and doubly distilled water was used throughout.

Typically, 2.5 mg of the anionic phospholipid was suspended in 2 mL of the vesicle buffer in a small vial. The vial was flushed with nitrogen to minimize phospholipid oxidation. The phospholipid solutions were the expected opaque, milky color. The solution was then freeze-thawed five times in liquid nitrogen in order to disrupt phospholipid aggregates.

The lipid solutions were extruded 10 times in order to induce vesicle formation. After approximately three extrusions, a distinct bluish tint, indicative of vesicle formation, was noted. This tint was very pronounced by the 10th extrusion, and the solution itself had become more transparent.

The solutions were then centrifuged at 10 000 rpm for 15 min (0−10 °C). The supernate was removed, sealed in a vial under argon, and refrigerated until use. This procedure produces vesicles that are about 800 Å in diameter. Even with care to exclude oxygen, samples did not keep well. All solutions were used within 2 days of preparation. Samples which showed the characteristic yellow color of oxidation products were promptly discarded.

Luminescence Measurements. Intensity titrations were done on a SPEX Fluorolog 2+2 described earlier.[13] Lifetime measurements were performed on the nitrogen laser based system described earlier.[13] All decays could be fit as single or double exponentials:

$$D(t) = A_S \exp(-t/\tau_S) + A_L \exp(-t/\tau_L) \quad (1)$$

where S and L refer to the short- and long-lived components, respectively. Fitting was done by a Marquardt nonlinear least-squares procedure.[14] For the double-exponential decays, the fractional contribution to the preexponential from the short-lived component was taken as

$$F_S = A_S/(A_S + A_L) \quad (2)$$

The quality of the fits is excellent. The residuals are randomly distributed around zero, and the deviations are too small to discern on the decay plots.

All fluorescent titrations were done at 10 μM ruthenium except for one study with $[Ru(Phphen)(phen)_2]^{2+}$ where the concentration was varied from 5 to 20 μM.

Hydrophobic Surface Area Estimations. To estimate the relative hydrophobic areas of the complexes, we calculated the surface areas of the free ligands. This was done by building the ligands in Spartan 5.0 and using minimized energy with PM3. This area includes the entire van der Waals surface. The bonding regions of the nitrogens obviously should not be included, but since the nitrogens are common to all ligands, this area is a constant offset.

Results

In the absence of vesicles, all decays were pure monoexponentials. In deoxygenated vesicles over a wide concentration range, it was found that the decays were only slightly nonexponential, which established a binding interaction, but the nonexponentialities were too small to allow reliable fitting to multicomponent models. Thus, in the absence of quenchers, binding has little effect on the luminescence lifetimes. This problem of reliable data fitting when lifetimes differ by less than a factor of 2 has been observed earlier.[15] We suspected that the small nonexponentiality arose from very similar τ's for the bound and unbound forms of the complex. However, oxygen saturating the solutions greatly accentuated the lifetime differences. Subsequently, all titrations were performed under oxygen-saturated conditions for which the unbound form had a much shorter lifetime than the bound form (factor of >2).

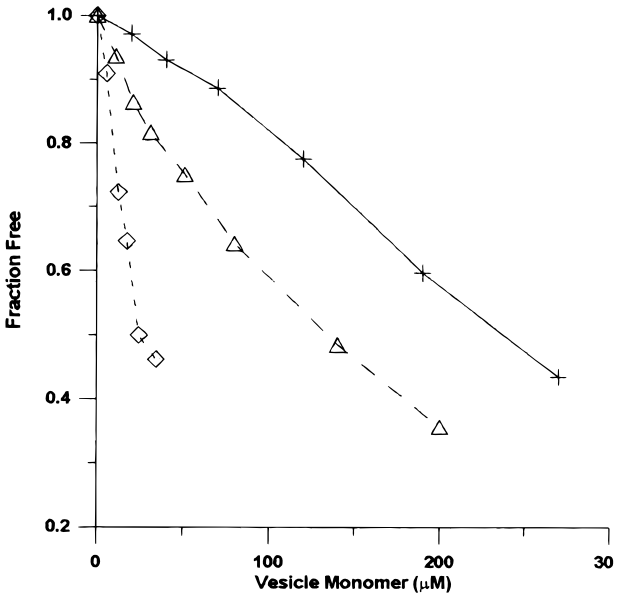

Figure 1. Typical lifetime titrations with the anionic phospholipid: $[Ru(phen)_3]^{2+}$ (+); $[Ru(Me_2phen)_3]^{2+}$ (△); $[Ru(Ph_2phen)phen_2]^{2+}$ (◇). The lines are to connect the points and have no physical significance.

The presence of only two concentration-independent lifetimes indicates that there is no aggregation of the complexes. This is consistent with the failure of all previous work to detect any aggregation in this class of complexes.

For all of the anionic phospholipid titration curves, the data could be fit using two fixed lifetimes. In the absence of vesicles, the lifetime matched the short-lived component in the vesicle solutions. The short-lived component's fractional contribution decreased with increasing vesicle concentration, and at high vesicle concentrations, the fractional contribution of the long-lived component could approach 100%. Thus, we identify the short- and long-lived components with the free and bound forms, respectively. The presence of only two fixed lifetimes indicates that the complex existed in only two discrete forms. Further, the exchange rate between the bound and unbound forms of the complex has to be slow compared to the excited state lifetimes.

Figure 1 shows typical luminescence decay time titrations for three different ruthenium complexes exhibiting a range of hydrophobic substituents. Since the aggregation number of the vesicle is unknown, vesicle concentrations are given as the phospholipid concentration. Figure 2 shows titration curves for $[Ru(Phphen)(phen)_2]^{2+}$ at two different metal complex concentrations.

For the neutral phospholipid, Figure 3 shows intensity titrations for $[Ru(4,7\text{-}Me_2phen)_3]^{2+}$. The complex showed no discernible changes in excited state lifetime with variations in

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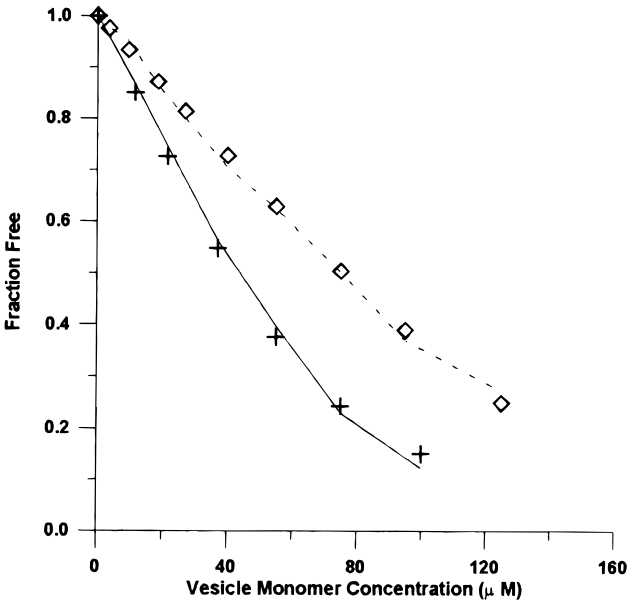

Figure 2. Concentration dependence of the fraction of [Ru(Phphen)-(phen)$_2$]$^{2+}$ bound to the anionic vesicles at 5 (+) and 10 (◇) μM phospholipid concentrations. The lines are global fit of both data sets to a DNA-binding model with $K = 1.8$ and $S = 14$.

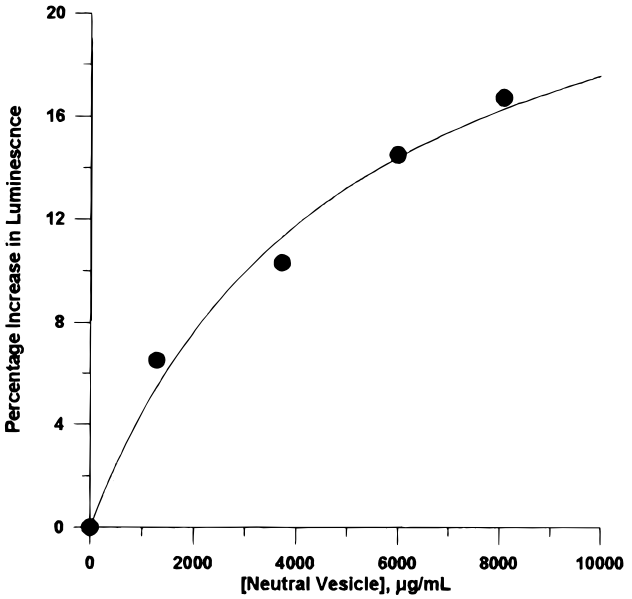

Figure 3. Titration for [Ru(Me$_2$phen)$_3$]$^{2+}$ with the neutral vesicle. The solid line is the best fit for a 1:1 binding model with $K = 2.1 \times 10^{-4}$ μM^{-1} and a limiting intensity of 26.

phospholipid concentration under nitrogen-purged, air-saturated, or pure-oxygen-saturated conditions. It is clear from the changes in intensity data that [Ru(4,7-Me$_2$phen)$_3$]$^{2+}$ does bind. The absence of a concomitant lifetime change must be due to a small simultaneous environmental effect on the radiative and radiationless rate constants. The fit to a simple binding model further supports vesicle binding. Similar results were obtained with [Ru(bpy)$_3$]$^{2+}$ except that the changes were smaller and the curve showed no leveling at the higher vesicle concentrations, which indicates an even weaker binding.

Table 1 shows the binding strengths of all of the complexes. These are given as the concentration of phospholipid required to bind 50% of the metal complex as indicated by an F_S of 0.50. For the [Ru(bpy)$_3$]$^{2+}$ lifetime data in anionic vesicles and the [Ru(4,7-Me$_2$phen)$_3$]$^{2+}$ intensity data with neutral vesicles,

Table 1. Strengths of Binding of Ruthenium Complexes to Anionic Phospholipid Vesicles in Oxygen-Saturated Solutions

complex	vesicle monomer concn for 50% binding (μM)[a,b]	unbound lifetime (ns)	bound lifetime (ns)
[Ru(Ph$_2$phen)$_3$]$^{2+}$	10	165	572
[Ru(Phphen)$_2$(phen)]$^{2+}$	17	180	469
[Ru(Ph$_2$phen)(phen)$_2$]$^{2+}$	25	189	426
[Ru(Phphen)(phen)$_2$]$^{2+}$	75	172	458
[Ru(4,7-Me$_2$phen)$_3$]$^{2+}$	150	151	431
	5000[c]		
[Ru(phen)$_3$]$^{2+}$	225	166	405
[Ru(bpy)$_3$]$^{2+}$	1900	164	260

[a] All measurements were performed at 10 μM ruthenium. [b] Anionic vesicles unless otherwise indicated. [c] Neutral vesicle.

the phospholipid concentration was not high enough to achieve 50% binding, so the data were fit to a

$$D + V \leftrightarrows DV \tag{3}$$

binding model where D is the complex and V is the vesicle. The concentration of phospholipid required to reach 50% of the computed limiting intensity was then evaluated from the binding constant.

If one assumes a DNA-style binding model[16,17] where S vesicle monomers are assumed to bind one probe molecule with an equilibrium constant K associated with the S monomer binding site, then the fraction bound, f_{bound}, is given by

$$b = 1 + KD_0 + (K[V]/S) \tag{4a}$$

$$f_{bound} = [b - (b^2 - (4K^2D_0[V])/S)^{1/2}]/(2KD_0) \tag{4b}$$

where D_0 is the ruthenium probe concentration and [V] is the concentration of vesicle monomer.

Discussion

Electrostatic attraction is clearly an essential element for strong binding in that it brings the complexes to the vesicles where other factors can come into play. A comparison of [Ru-(Me$_2$phen)$_3$]$^{2+}$ with both neutral and anionic vesicles shows that binding is about 30-fold stronger for the oppositely charged versus the uncharged system.

However, when the electrostatic interactions are fixed, there is a huge hydrophobic binding contribution. As shown in Table 1, the variation in binding of the divalent cationic complexes to the anionic vesicles is controlled largely by the hydrophobicity of the metal complexes. The binding strength varies by over 2 orders of magnitude on going from the rather polar [Ru(bpy)$_3$]$^{2+}$ to the extremely hydrophobic [Ru(Ph$_2$phen)$_3$]$^{2+}$. Binding also increases as the number of hydrophobic ligands on a complex increases.

The extent of the hydrophobic versus electrostatic binding can be seen by examining the expected binding strength based on electrostatics. [Ru(bpy)$_3$]$^{2+}$ is the smallest complex with the highest charge-to-volume ratio and should have the strongest electrostatic binding, but in fact it is the poorest binder (200 times weaker than [Ru(Ph$_2$phen)$_3$]$^{2+}$). Even the similarly sized [Ru(phen)$_3$]$^{2+}$ binds 10 times better than [Ru(bpy)$_3$]$^{2+}$. We attribute the differences in binding to the relative hydrophobicity of the organic ligands.

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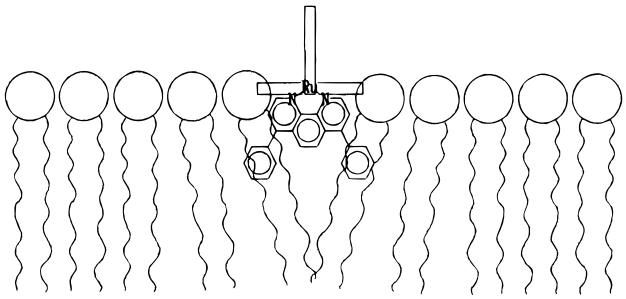

Figure 4. Schematic representation of the binding of metal complexes to vesicles. The rectangles represent the other two additional ligands.

Binding correlates well not only with the hydrophobicity of different ligands but also with the number of hydrophobic groups of the same type. For example, $[Ru(Phphen)_2(phen)]^{2+}$ with two phenyl groups binds about 3−4 times better than $[Ru(Phphen)(phen)_2]^{2+}$ with one phenyl group and is comparable to $[Ru(Ph_2phen)(phen)_2]^{2+}$ with two phenyls. $[Ru(Ph_2phen)_3]^{2+}$ with six phenyls binds about 2−3 times more strongly than $[Ru(Ph_2phen)(phen)_2]^{2+}$. However, in this last case not all six phenyls can be situated to aid in binding. The most likely orientation would have no more than four phenyls suitably oriented for hydrophobic binding. From the standpoint of aiding binding, phenyls are better than methyls and phen's are much better than bpy.

We turn now to the specific location of the complexes in the vesicles. Figure 4 is a schematic representation. Since the complexes are charged cations, they cannot fully embed themselves in the uncharged vesicle interior. The most hydrophobic groups (methyls, phenyls) will be oriented to achieve maximum penetration into the hydrophobic regions of the vesicles. The strong shielding from oxygen quenching of the vesicle-bound complexes establishes that the complexes must be rather deeply buried in order to be shielded from solution oxygen.

We have also fit our data with the simple 1:1 binding model derived from eq 3. For a single metal complex concentration, the simplest form of a 1:1 model gives reasonable fits. However, to account for the metal concentration dependence of the binding, a more complex model is required. We choose a model analogous to that used for binding of dyes to DNA, based on eqs 4. Figure 2 shows the global fit for the 5 and 10 μM concentrations of $[Ru(Phphen)(phen)_2]^{2+}$ with $S = 13$ and $K = 1.8\ \mu M^{-1}$. While the fit for these two concentrations is excellent, inclusion of the 20 μM data set substantially degrades the quality of the fit, but the general features are all preserved and the best fit parameters are similar. The S is certainly reasonable with a substantial number of vesicle monomer units necessary to bind each Ru molecule. The deviations from the model at higher Ru concentrations may be a consequence of the very high loading of the vesicles at the beginning of the titration with a concomitant breakdown of the assumptions of this simple model. Similar S values (5−15) were obtained for the other complexes, but the fitting parameters are so poorly poised that detailed conclusions concerning the fit parameters are unjustified.

We turn now to the issue of binding strength versus complex structure. The trends here with hydrophobicity are very similar to those observed earlier for binding of Ru complexes to micelles in the absence of electrostatic effects. Electrostatic interactions were eliminated by using either an uncharged micelle or complex. A neutral phen complex was found to bind roughly an order of magnitude more strongly than an analogous bpy complex to anionic SDS micelles.[18] Similarly, methyl-substituted phen versus unsubstituted phen increased binding to uncharged Triton micelles by an amount similar to the effect observed on the vesicles. In Triton micelles, $[Ru(Ph_2phen)_3]^{2+}$ binds about an order of magnitude more strongly than $[Ru(Me_2phen)_3]^{2+}$, which is similar to the enhancement on vesicles observed here.[19]

As support of this binding picture, we have plotted the reciprocal 50% binding vesicle concentration versus the hydrophobic ligand area. We assumed in one calculation that the hydrophobic area presented for vesicle binding was the single most hydrophobic ligand. In the second case, we used the area of the two most hydrophobic ligands. The correlation was poor for the single ligand ($r^2 = 0.6$), but reasonable using two ligands ($r^2 = 0.91$). This result supports our view that the binding probably involves two ligands.

In addition, electrostatic enhancement of binding is similar in vesicles to that observed earlier for micelles. Binding of a charged metal complex to an oppositely charged micelle increased binding strength by about an order of magnitude, which is similar to that observed for the charged versus uncharged vesicles with $[Ru(Me_2phen)_3]^{2+}$.

There are still a number of points to be investigated. We do not know whether, in the few-hour time scale of our measurements, the complex can enter the interior solvent region of the vesicles, although Almgren's results, with similar synthetic vesicles,[8] indicate that our systems probably have complexes on both interior and exterior surfaces. We have no information on the structural perturbation of the vesicles, especially at high occupancies. However, it seems unlikely that the vesicles are destroyed since there are no discontinuities in the titrations, and the data are reasonably fit by simple binding models. Given the instabilities of the vesicles to oxidation under our measurement conditions, these will not be easy questions to address. Nevertheless, they do not affect our general conclusions about the role of hydrophobicity and electrostatics in vesicle binding of metal complex probe molecules.

Conclusions

Our results provide models for the structures necessary to design inorganic molecular probes for attaching to membrane-type systems. Similar results should hold for other biological binding sites where both electrostatic and hydrophobic effects are important contributors to binding. Electrostatics are clearly important for enhancing initial interactions, but a high degree of complex hydrophobicity is essential for overall tight binding.

Addition of long-chained aliphatic groups to bpy or phen ligands would significantly enhance binding with minimal structural perturbations to the vesicles. Further work on development of probe molecules with different hydrophobic binding regions is in progress.

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