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Lipid bilayers decorated with photosensitive ruthenium complexes

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Summary, Conclusions and Outlook

7.1. Summary

7.1.1. General Introduction (Ch 1)

Polypyridyl ruthenium complexes are classical tools in photochemistry.^[1-2] Their photophysical properties can be tuned in order to get the desired behavior under light irradiation. In particular, ruthenium complexes with distorted octahedral geometry are capable of photosubstituting one ligand by a solvent molecule upon visible light irradiation (400-600 nm).^[3]

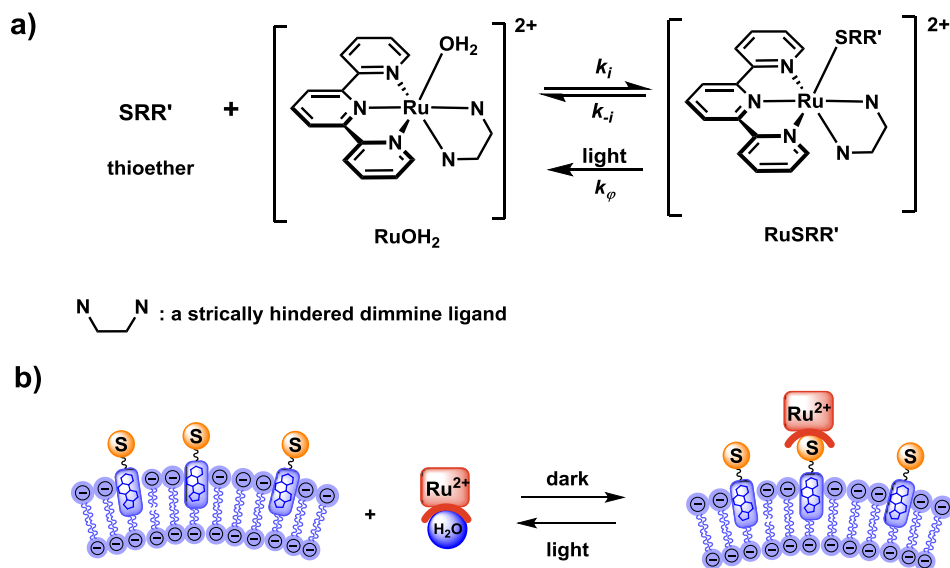
Two potential applications of this kind of complexes are discussed, being the design of light-controlled molecular machines, and light-activatable anticancer prodrugs. In this thesis, a link between these two applications using lipid bilayers was made. Photosubstitution reactions are first studied at the surface of lipid bilayers in order to mimic natural molecular machines. By anchoring monodentate ligands at the membrane, the ruthenium complex can bind thermally to the membrane, and be cleaved by visible light irradiation to realize a model of molecular carrier controlled by light. In the second part the same liposomes functionalized with photosensitive ruthenium complexes are considered for photochemotherapy as the ruthenium aqua complex liberated by light irradiation may be cytotoxic. The ruthenium-functionalized liposomes, which act as pro-drugs, may be delivered to cancer cells. Once taken up they can be activated by light irradiation, resulting in a photosubstitution reaction that releases the active ruthenium aqua complex from the membrane into the cell. Thus, by combining the photochemistry of ruthenium complexes and the biological properties of liposomes we moved from a very fundamental, biomimetic topic dealing with molecular motion, to the second, more applied field of drug delivery.

7.1.2. Ruthenium polypyridyl complexes hopping at anionic lipid bilayer surface through a supramolecular bond sensitive to visible light (Ch 2)

In Chapter 2 the new ruthenium complex $[\text{Ru}(\text{terpy})(\text{dcbpy})(\text{Hmte})]^{2+}$ (RuHmte) is introduced, where terpy is 2,2',6',2''-terpyridine, dcbpy is 6,6'-dichloro-2,2'-bipyridine, and Hmte is 2-methylthioethan-1-ol. Based on kinetics and thermodynamic data it is shown that steric hindrance of the dcbpy ligand induces destabilization of both the ruthenium thioether complex RuHmte and the aqua analogue $[\text{Ru}(\text{terpy})(\text{dcbpy})(\text{H}_2\text{O})]^{2+}$ (RuOH₂). These two species are in fact in thermal

equilibrium at room temperature and in the dark. However, shining blue light allows for selective substitution of the thioether ligand by an aqua ligand, thus shifting the equilibrium towards the formation of the RuOH_2 complex (see Scheme 7.1a). Such light-induced equilibrium shift is shown to be repeatable at least four times in homogenous aqueous solution.

Thermal binding and light-induced unbinding of a thioether ligand to the ruthenium center was also achieved at the surface of negatively charged liposomes (see Figure 7.1b). UV-vis measurements show that the ruthenium aqua complex efficiently coordinates to a membrane-embedded thioether ligand in the dark, and that upon exposure to visible light the Ru-S coordination bond is selectively cleaved to release the ruthenium aqua complex into the solution. This cycle is shown to be repeatable four times by switching on and off the source of visible light. Thus, light-triggered hopping of a ruthenium complex is achieved at a lipid bilayer membrane surface.



Scheme 7.1. a) Thermal equilibrium between $[\text{Ru}(\text{terpy})(\text{N-N})(\text{H}_2\text{O})]^{2+}$ and $[\text{Ru}(\text{terpy})(\text{N-N})(\text{SRR}')]^{2+}$ and the photosubstitution of SRR' ligand by H_2O . SRR' is a thioether ligand such as 2-methylthioethan-1-ol and N-N is a diimine ligand such as dcbpy. b) Light-induced ruthenium binding and un-binding at a negatively charged bilayer membrane.

The light-controlled hopping of a ruthenium complex at the membrane has two requirements: first, the steric hindrance of the ruthenium complex should be high enough to allow for fast thermal binding and photo-induced unbinding. Secondly, the liposomes should be negatively charged so that the ruthenium aqua complexes actually bind to the membrane-embedded sulfur ligands. These two issues are discussed in Chapters 3 and 4, respectively.

7.1.3. Spontaneous formation in the dark, and visible light-induced cleavage, of a Ru-S bond in water: a thermodynamic and kinetic study (Ch 3)

In Chapter 3 the thermal and photochemical reactivity in water of four related ruthenium polypyridyl complexes with the general formula $[\text{Ru}(\text{terpy})(\text{N-N})(\text{Hmte})]^{2+}$ is described, where N-N are the four diimine ligands bpy, biq, dcbpy, or dmbpy (see Scheme 3.1a). For each of these complexes photo cleavage of the Ru-S bond occurs, resulting in the formation of the aqua complex $[\text{Ru}(\text{terpy})(\text{N-N})(\text{H}_2\text{O})]^{2+}$ (RuOH_2). In this chapter it is described how the steric hindrance of the N-N ligand influences both the thermodynamic stability and kinetic lability of the RuHmte and RuOH_2 complexes in the dark. The kinetics of the photosubstitution reactions are reported as well.

Upon increasing the steric hindrance of the N-N ligand, the rates of thermal binding to and thermal cleavage of the Hmte ligand from the ruthenium center increase. A shift was observed along the series bpy, biq, dcbpy, and dmbpy, from a very slow thermal equilibrium between RuOH_2 and RuHmte with $\text{N-N}=\text{bpy}$, to a very fast one with $\text{N-N}=\text{dmbpy}$. The increased lability of the hindered complexes in water is not due to the change of the enthalpy of activation of the substitution reaction (ΔH^\ddagger). Instead, it is due to the variation of the entropy of activation ΔS^\ddagger , which from being negative for bpy and biq, becomes positive for dcbpy and dmbpy. Such change in activation entropy indicates a change in the mechanism of the substitution reaction, from an interchange associative mechanism with bpy and biq ($\Delta S^\ddagger < 0$) to an interchange dissociative mechanism for dcbpy and dmbpy ($\Delta S^\ddagger > 0$).

On the other hand, the quantum efficiency of the photocleavage of the Ru-S bond upon light irradiation also increases along the series $\text{N-N}=\text{bpy}$, biq, dcbpy, and dmbpy. Overall, two requirements were found for shifting with light the equilibrium between the RuHmte and RuOH_2 species in water. First, the thermodynamic stability of the RuHmte complex in water and in the dark must be higher than that of the RuOH_2

complex ($k_{-i} < k_i$) to lead to the spontaneous formation of the thioether complex. If the establishment of thermal equilibrium is too slow however, such as for the least hindered complex with N-N=bpy, RuHmte formation does not occur at room temperature because there is not enough thermal energy to cross the activation barrier of the coordination reaction. Secondly, the rate of the photosubstitution of the Hmte ligand by water must be higher than that of its thermal dissociation ($k_{-i} < k_{\theta}$, see Figure 7.1a). For the most hindered ruthenium complex with N-N=dmbpy this condition is not met, and the thermal lability of RuHmte is so high that light cannot induce a significant shift of the thermal equilibrium between RuHmte and RuOH₂. To conclude, only the moderately hindered complexes, *i.e.*, those with N-N=biq and dcbpy, are suitable for shifting with light the equilibrium between RuHmte and RuOH₂.

7.1.4. Binding of a ruthenium complex to a thioether ligand embedded in a negatively charged lipid bilayer: a two-step mechanism (Ch 4)

As mentioned in section 7.1.2., negatively charged membranes are required for the binding of ruthenium aqua complexes to membrane-embedded thioether ligands. In Chapter 4, the role of the negative charge of the membranes on the coordination reaction occurring at the water-membrane interface is reported. The interaction of the complex [Ru(terpy)(dcbpy)(H₂O)]²⁺ with phospholipid membranes containing either neutral thioether ligands or cholesterol was studied using three different techniques: UV-visible spectroscopy, Langmuir-Blodgett monolayer surface pressure measurements, and Isothermal Titration Calorimetry (ITC). The first technique proved that ruthenium binding to the thioether ligands becomes slower when the electrostatic interaction between the ruthenium cations and the negative liposomes is shielded by higher ionic strengths. Thus, adsorption of the dicationic ruthenium complex at the surface of the negative membranes plays a prominent role in the formation of the Ru-S coordination bond.

Information about the time scale of such adsorption phenomenon and about its thermodynamics was obtained from lipid monolayer surface pressure and ITC measurements. It was shown that the adsorption of the ruthenium aqua complex to the surface of negatively charged monolayers and bilayers is much faster (minutes) than coordination, *i.e.*, ligand exchange (hours). In addition, the adsorption phenomenon was found to be endothermic, *i.e.*, entropy driven. Based on these results a two-step model is proposed for the binding of the dicationic metal complex to the thioether

ligands embedded in negative liposomes. In the first step, the outer leaflet of a negatively charged lipid bilayer quickly adsorbs the positively charged metal complexes, whereas in the second step the Ru-S bond formation occurs *via* two-dimensional diffusion of both reagents at the membrane (see Scheme 4.2). Such two-step reaction at negative membranes is faster, all other conditions being the same, than the corresponding Ru-S bond formation in homogenous solutions.

7.1.5. Liposomes functionalized with ruthenium: towards a tumor-targeted, light-controlled anticancer prodrugs (Ch 5)

In Chapter 5, the potential application of liposomes decorated with photosensitive polypyridyl ruthenium complexes in drug delivery is discussed. Four non-labile ruthenium complexes with the general formula $[\text{Ru}(\text{terpy})(\text{N-N})(\text{SRR}')]^{2+}$ (N-N = bpy (2,2'-bipyridine) or pyimi (phenylpyridin-2-ylmethylene-imine), and $\text{SRR}' =$ thioether ligands with a cholesterol tail, were prepared and were supported on neutral and negatively charged liposomes. All ruthenium-functionalized liposomes are photoreactive; shining blue light on them results in the photocleavage of the ruthenium complex from the liposome surface. The photosubstitution reactions are shown to be faster at human body temperature (37 °C) than at room temperature, and slightly faster at neutral bilayer surfaces than at negatively charged ones.

Cellular uptake experiments on human carcinoma cell lines showed that in the absence of PEGylation, ruthenium-functionalized liposomes built from neutral lipids are better taken up by HepG₂, A2780, and A2780R cancer cells than their analogues built from negatively charged lipids. When PEGylated lipids are introduced in the liposome formulation, the charge of the resulting ruthenium-functionalized stealth liposomes is shielded, which results in a decreased cellular uptake compared to PEG-free liposomes. Moreover, almost equal cellular uptakes were obtained when neutral and negatively charged lipids are used for PEGylated liposomes containing Ru. Overall, the structure of the ruthenium complexes did not affect significantly these uptake results.

Dark cytotoxicity tests with DOPC and DOPG stealth liposomes functionalized with any of the four ruthenium complexes showed that these liposomes are poorly toxic against A2780 and A2780R cell lines, with no significant variation between the different ruthenium complexes. Light cytotoxicity results were obtained on HepG₂ cells for one of the ruthenium complexes supported on non-PEGylated liposomes with different surface charges. The results showed up to five times higher cytotoxicity after light

irradiation than in the dark. Thus, liposomes decorated with ruthenium complexes are promising in drug delivery.

7.1.6. Yellow-light sensitization of a ligand photosubstitution reaction in a ruthenium polypyridyl complex covalently bound to a rhodamine dye (Ch 6)

In Chapter 6 the possibility of extending the photoactivation of polypyridyl ruthenium complexes towards longer wavelengths by photosensitization, is discussed. As mentioned in section 7.1.1. some of these metal complexes have been proposed as light-activatable drugs in phototherapy. However, their potential application *in vivo* is limited since they mostly show high molar absorptivities near 450 nm, *i.e.*, for blue light, which is known to poorly penetrate human tissues.^[4-5]

The photosubstitution of a thioether ligand by a water molecule was studied with 570 nm photons (*i.e.*, yellow light). A rhodamine B dye, which has a high molar absorptivity for yellow light, was covalently bound *via* a short saturated linker to the terpyridine ligand Rtpy in the complex $[\text{Ru}(\text{Rterpy})(\text{bpy})(\text{Hmte})]^{2+}$. The excellent antenna effect of the rhodamine B dye, coupled to efficient energy transfer to the ruthenium center, resulted in faster photosubstitution of the Hmte ligand with yellow photons, than with blue photons.

In this chapter also the rate of photosubstitution reactions is discussed when photons of insufficient energy, compared to that of the ¹MLCT state, are used. Both for the rhodamine B-functionalized ruthenium complex and for its antenna-free analogue $[\text{Ru}(\text{terpy})(\text{bpy})(\text{Hmte})]^{2+}$ the quantum yields upon yellow light or blue light irradiation were found to be comparable. In fact, at constant photon flux it is the extinction coefficient that mostly influences the photosubstitution rate for these complexes, whereas the photosubstitution quantum yield hardly depends on the irradiation wavelength.

7.2. Conclusions and Outlook

7.2.1. General conclusions

In this thesis the thermal- and photo-substitution behavior of polypyridyl ruthenium complexes with the general formula of $[\text{Ru}(\text{terpy})(\text{N-N})(\text{SRR}')]^{2+}$ is described, either at the surface of lipid bilayers, or in homogeneous solutions. It is shown that the successive thermal binding and light-induced unbinding of the cationic ruthenium complex at the surface of the lipid bilayer requires negatively charged liposomes and ruthenium complexes containing moderately hindered N-N bidentate ligands such as

biq or dcby. Our results in homogeneous solution show that changing the steric hindrance of the bidentate ligand influences both the photo- and thermal reactivities of these complexes, by altering the mechanism of the Ru-S bond formation. It is also shown that the Ru-S bond formation at the surface of negative lipid bilayers is faster than the same reaction in homogenous aqueous solutions, and a two-steps mechanism is proposed for the thermal coordination of ruthenium aqua complexes at membrane-embedded ligands.

The application of ruthenium-functionalized liposomes in drug delivery is discussed in Chapter 5. *In vitro* tests on cancer cell lines show that neutral liposomes functionalized with ruthenium compounds are more readily taken up by cancer cells than ruthenium-free liposomes. The liposome samples with ruthenium compounds are shown to be poorly cytotoxic in the dark. After light irradiation, the cytotoxicity increased at least up to five times for ruthenium complexes supported on non-PEGylated liposomes.

Finally, the photoactivation of polypyridyl complexes with low-energy photons was studied using a photosensitization approach. A photosubstitution reaction was made faster upon yellow light irradiation than upon blue light irradiation by covalently linking a rhodamine B dye to the ruthenium complex.

7.2.2. Outlook

7.2.2.1. Molecular motion at the surface of a lipid bilayer

In this research it was shown that a ruthenium complex can hop at the surface of a lipid bilayer in a light-controlled manner. The ultimate goal of this research is to achieve unidirectional motion at the lipid bilayer surface, such as reported linear organic molecular machines.^[6-7] For this aim the thioether ligands at the membrane should be organized in a way to produce a dissymmetric track. The first issue to solve for extending this research is to modify the ruthenium complex in order to detect its lateral position, and possibly to probe binding and unbinding events by single-molecule techniques. One approach is to use fluorescent imaging techniques, which have shown their potential at the single-molecule level. Since ruthenium complexes of the type $[\text{Ru}(\text{terpy})(\text{N-N})(\text{SRR}')]^{2+}$ photosubstitute a ligand under light irradiation their luminescence is very poor. Thus, a fluorophore would need to be covalently linked to the ruthenium complex. As discussed in Chapter 6, however, the emission spectrum of the fluorophore overlapped with the ¹MLCT absorption band of the ruthenium

complex, and the fluorescence of the dye was quenched by Förster energy transfer. To avoid such quenching a fluorophore absorbing in the red region of the spectrum, *i.e.*, at wavelengths higher than 630 nm, should be used. It is expected that in this case the photoreactivity of the metal center and the emission of the fluorophore may be decoupled, which would allow for probing the position of the ruthenium complex *via* excitation of the fluorophore.

7.2.2.2. Liposomes functionalized with ruthenium in photoactivated chemotherapy

Liposomal drug delivery for ruthenium-based anticancer compounds has not been investigated extensively, except for two recent studies in 2012.^[8-9] In Chapter 5, it was shown that liposomes functionalized with polypyridyl complexes are potential candidates for drug delivery. However, our results are only preliminary, and more investigations need to be done in this area. The first important point is to modify the uptake detection method. Our uptake results are currently based on the fluorescence of NBD-PC lipids included in the formulation of the liposomes. However, the excitation wavelength of NBD-PC overlaps with the ¹MLCT absorption band of the ruthenium complex, as a result of which the fluorescence of NBD is partially quenched by the ruthenium complex. As explained in Chapter 5, the extent of fluorescence quenching in the cell culture can be estimated based on the data obtained in absence of cells. However, this estimation remains rather qualitative since the cell environment is different from that of an aqueous buffer. Ideally the amount of ruthenium in cells should be quantified by metal trace analysis methods after uptake experiments. Unfortunately valid ruthenium concentrations in cell lysis solutions could not be obtained using ICP-OES. A more sensitive detection method, such as ICP-MS, should be used in the future.

The dark and light cytotoxicity investigations need to be extended in the future in an optimal condition for different drug exposure times using stealth liposomes. Irradiation of the cells after drug exposure should be performed in at least 5% CO₂ atmosphere and 37 °C. Light intensity and photon flux also should be measured precisely and correctly. Finally, after finding the optimal conditions, all of the *in vitro* tests, *i.e.*, uptake, dark toxicity and light cytotoxicity, should be performed on healthy cells as well to determine the toxicity of such liposomes to these healthy cells and conclude on the selectivity of such prodrugs towards cancer cells.

7.3. References

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