Imaging of alkyne-functionalized ruthenium complexes for photoactivated chemotherapy

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6

SUMMARY, DISCUSSION, AND CONCLUSION

6.1 Summary

6.1.1 PACT as selective treatment against cancer cells

Currently used chemotherapy agents go along with undesirable side effects due to poor selectivity of the drug: healthy cells are as much affected as cancerous cells. To direct the cytotoxicity towards tumor tissue only, cancer cells can be targeted in different ways: either by targeting a protein that is abundant in cancer cells (biological selectivity), or by the selective release of the cytotoxic species only at the tumor site (physical selectivity). The latter can for example be achieved by light. A non-toxic prodrug is injected in the patient and distributes through the whole body. However, only light-activation can trigger the release of the cytotoxic species that causes cell death. Depending on the cytotoxic species, two types of phototherapy are distinguished: photodynamic therapy (PDT), in which the chemotherapy agent and light lead to the production of toxic reactive oxygen species, or oxygen-independent photoactivated chemotherapy (PACT) in which the cleavage of a protecting moiety of the complex leads to the release of the cytotoxic species. The direct interaction of the released cytotoxic moiety with intracellular targets such as proteins or DNA, leads to cell death. Depending on the nature of the released cytotoxic species, different targets and modes of action of the chemotherapy agent are possible. In this thesis, different ruthenium-based photoactivatable complexes are reported and their properties were investigated for their suitability as PACT agents.

6.1.2 Investigation of intracellular behavior of metal complexes

A better understanding of the biological activity of the metal-based anticancer compound allows for improving drug design and tuning drug interaction with its cellular targets. To gather knowledge about the interaction of the complex with biomolecules, many different techniques can be utilized. The interaction of the complex with isolated biomolecules can be studied by *e.g.* mass spectrometry, UV-vis spectroscopy, or X-ray diffraction, and proteomic studies help to understand the effect of a drug on the protein expression within cells. To obtain a complete picture of the effect of the drug on a cell, results of different techniques need to be combined and new methods need to be developed to study the compound under physiologically relevant conditions. In this thesis, the intracellular distribution of a ruthenium-based PACT agent was investigated by fluorophore labeling of the complex in cells *via* copper-catalyzed azide-alkyne cycloaddition (CuAAC).

6.1.3 Alkyne functionalization of photoactivatable ruthenium complex

To attach a reporter tag such as a fluorophore *via* CuAAC to the ruthenium-based PACT agent, it is necessary to functionalize the complex with a click handle. Here, we decided to choose for a minimal handle, *i.e.* a simple alkyne group (Figure 6.1). To date, reaction procedures for the synthesis of alkyne-functionalized ruthenium complexes proceed with low yield due to side reactions in presence of the ruthenium center, or they require silver ions. However, even small traces of the heavy metal are toxic to living cells. Thus, the use of silver ions should be avoided when synthesizing complexes that are intended for therapeutic applications. We developed a new synthetic route to synthesize such alkyne-functionalized complexes as described in **Chapter 2**. Thereby, we optimized the alkyne-protecting group, found that *tert*-butyldimethylsilyl (TBDMS) is strong enough to cope with the metalation and ligand exchange conditions, and on the other hand is easy to remove using fluoride, *i.e.* without involving silver ions.

6.1.4 Improved ruthenium-based PACT agents

PACT is an oxygen-independent way of releasing a toxic species in a light-triggered manner, in order to fight cancer. Ruthenium-based anticancer compounds suitable as PACT agents have to be stable in the dark, activated by light, and show cytotoxic behavior after light activation. $[Ru(tpy)(bpy)(Hmte)](PF_6)_2$ is known to be photoactivatable, but this complex is weakly taken up by cells, so that the photoproduct is non-toxic. In **Chapter 3**, we report the development of new ruthenium complexes with improved cellular uptake by increasing the lipophilicity of the complex, which was obtained by increasing the aromatic surface of the bidentate ligand. Higher cellular uptake resulted in increased cytotoxicity after light activation, while complexes are still stable in the dark. Furthermore, addition of a non-coordinating amine bridge to the bidentate ligand led to a ruthenium complex with improved photosubstitution quantum yield, therefore demonstrating that ligand alteration can be used for fine-tuning the properties of ruthenium complexes and develop compounds with better PACT properties.

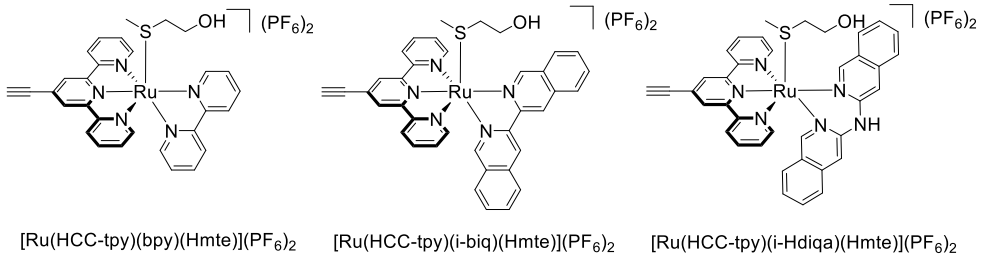


Figure 6.1. Chemical structures of the complexes described in this thesis.

6.1.5 Effect of click handle on complex properties

The purpose of an alkyne handle is the labeling of the complex with a reporter tag for visualization or isolation of the complexes in intracellular environment. To use such alkyne-functionalized complexes to study the original, non-functionalized complex, the photophysical and biological properties of the functionalized and non-functionalized complexes need to be compared with each other (**Chapter 2 and 4**). Comparison of crystal structures, photosubstitution quantum yields, singlet oxygen generation, cell uptake, and cytotoxicity revealed that the alkyne click handle has no significant effect on the complex geometry or its light activation, and all complexes remain very poor PDT agents due to their low singlet oxygen production. On the other hand, the alkyne does increase the cellular uptake of the complex: the concentrations of ruthenium in the cells was doubled after alkyne functionalization. Nevertheless, alkyne functionalization offers a great opportunity to label the complex *via* CuAAC with minimal effect on the photochemical and biological activity of the complexes.

6.1.6 Visualization of non-emissive PACT agents

Usually PACT agents are non-emissive due to the quenching by the thermally generated ^{3}MC state. Therefore, monitoring the complex in the cell by microscopy is not possible, and a fluorophore moiety is necessary to visualize the complex. The alkyne functionalization allows for the attachment of a fluorophore moiety by CuAAC reaction on the ruthenium complex. By doing so, we were able to visualize the complex after binding to model protein BSA (**Chapter 2**). Only after light activation, the complex releases its protecting ligand, resulting in a free coordination site with which it can interact with the protein. If kept inactivated in the dark, no fluorescent signal was visible. Noteworthy, the interaction could not be detected by traditional techniques such as mass spectrometry or UV-vis spectroscopy, which are suitable for the detection of covalent interactions. This indicates that the interaction is rather weak and disrupted in the latter mentioned techniques, while fluorophore

labeling in combination with gel electrophoresis is soft enough to preserve the weak metallodrug-protein interaction.

From studying the interaction of the ruthenium complexes with isolated proteins as described in Chapter 2, we moved to the use of mammalian cancer cells (**Chapter 4**). The labeling of the complex was again achieved *via* CuAAC chemistry, performed inside fixed cells. This method allowed for the preservation of the biological activity of the alkyne-functionalized complex: their uptake, distribution, and interaction within the cells resembles as much as possible that of the non-functionalized drug. A fluorescent signal was observed after attachment of the fluorophore moiety, but only for the light-activated species that can interact with a target and are therefore not washed away. Here again, this experiment is a proof for the light-controlled interaction of the complex with proteins. The results show that the complexes did not enter the nucleus. In contrast, the complexes stayed outside the nucleus in the perinuclear region. Co-staining of the cellular compartments outside the nucleus revealed that the complexes are not located in the lysosomes or in the endoplasmic reticulum. The fluorescent distribution and pattern suggest that the complexes localize in the Golgi apparatus after 24 h after activation.

6.1.7 Universal application of click handle method

In **Chapter 5**, alkyne functionalization was described for three other polypyridyl ruthenium complexes. The synthesis is challenging since the alkyne handle can react with a ruthenium center that has free coordination sites, even in presence of a TBDMS protecting group. Working with two free coordination sites and alkyne handles increases the amount of possible byproducts. In general, side reactions occur if the reaction conditions are not adapted. Fine-tuning the reaction conditions is time consuming and not straightforward, but it is usually possible. Therefore, the method of post-treatment labeling can also be applied to other complexes.

6.2 Discussion

6.2.1 Azide *vs*. alkyne: functionalization of ligands and complexes

The initial idea was to functionalize ruthenium complexes with the smallest handle possible, to keep the structure and properties of the drug derivative as similar as possible to that of the original ruthenium complex. Suitable candidates for such handles are azide (N_3) and alkyne (CCH) click handles. The functionalization of polypyridyl ligands and the binding of these ligands to ruthenium, are well reported in literature.[1, 2] An overview is given below.

Azide functionalization of polypyridyl ligands such as 2,2′-bipyridine (bpy) and 2,2′:6′,2″-terpyridine (tpy) are reported to be challenging (Table 6.1). Starting from dibromo,[3] diamino,[3] or diazido dioxide precursor[4] (Figure 6.2), functionalization of the bpy ligand resulted in the formation of the desired diazido compound, however, in low yields and sometimes as a mixture of mono-functionalized products. The best result was obtained using 4,4′-dinitro-bpy and sodium azide (95% yield).[5] For the tpy ligand, the desired azide-functionalized tpy ligand was obtained with a yield of 75% when using the 4′-nitroterpyridine precursor.[6] Noteworthy, thermal lability of the azide-functionalized ligands is frequently reported. Extension of reaction time or increase of reaction temperature led to the decomposition of the azide.[3] This instability is also put forward to explain the unsuccessful CuAAC reaction on the azide-functionalized bpy ligand.[3] So far, only the group of Elliott succeeded in this reaction.[5]

Alkyne functionalization of polypyridyl ligands has been reported more frequently, usually starting from a bromide precursor (Table 6.1). The synthesis typically proceeds using a protected alkyne intermediate to prevent the formation of side products by reaction of the alkyne with metal centers present in the catalytic reaction mixture. For the bpy ligand, the terminal alkyne is protected by a trimethylsilyl (TMS) group that can be easily removed with a base. The reactions always proceed in good yields.[3, 7, 8] For terpyridine, yields of 87% were reported for the reaction with a TMS protecting group,[9] while using the stronger triisopropylsilyl (TIPS) protecting group the ligand was obtained in 93% yield.[10] While the CuAAC reaction was difficult on the azide, the reaction on the alkyne proceeded without any problems.[3, 4]

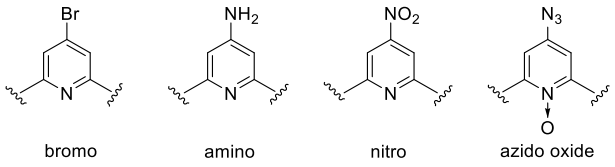


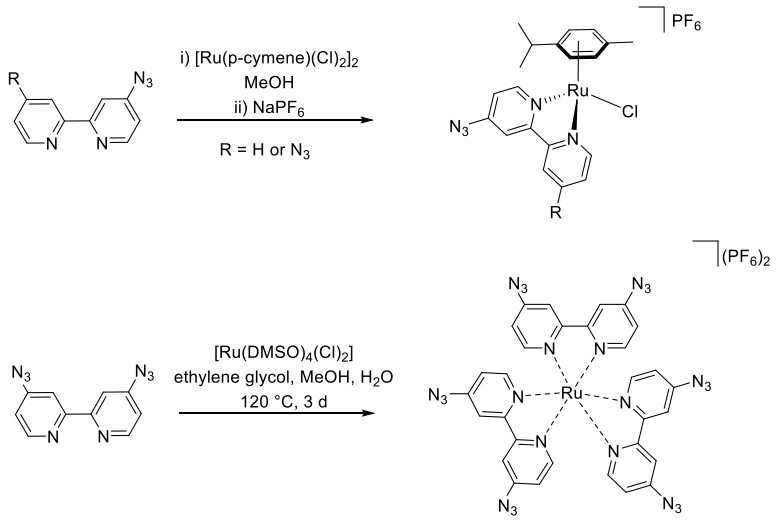
Figure 6.2. Possible starting materials for the functionalization of polypyridyl ligands.

Table 6.1. Ligand functionalization reported in literature.

handle	ligand	Starting material	conditions	yield	Ref.
azide	bpy	bromo	NaN_3, DMF, 100 °C, 18 h	27%	[3]
		amino	$NaNO_2$, HCl, NaN_3	20%	[3]
		azido oxide	PCl_3, ACN, reflux, 4 h	n.s.[a)]	[4]
		nitro	NaN_3, DMF, 100 °C, 3h	95%	[5]
	tpy	nitro	NaN_3, DMF, 100 °C, 3 h	75%	[6]
alkyne	bpy	bromo	Me_3SiCCH, CuI, $Pd(PPh_3)_2Cl_2$,	86%	[3]
			K_2CO_3	78%	
		bromo	Me_3SiCCH, CuI, $Pd(PPh_3)_2Cl_2$, THF,	63%	[7]
			MeOH, NaOH, r.t., 2 h	91%	
		bromo (mono)	Me_3SiCCH, CuI, $Pd(PPh_3)_2Cl_2$,	98%	[8]
			MeOH, K_2CO_3	68%	
	tpy	bromo	Me_3SiCCH, CuI, $Pd(PPh_3)_2Cl_2$, THF,	87%	[9]
			MeOH, KF	87%	
		bromo	TIPS-SiCCH, CuI, $Pd(PPh_3)_2Cl_2$,	93%	[10]

[a)] n.s. = not specified

With the functionalized ligands at hands, coordination to ruthenium can be undertaken. In literature, only a few examples have been reported in which coordination of the azide-functionalized ligand to a ruthenium precursor complex was successful (Table 6.2). Elliott and co-workers reported the successful complexation of a mono-azido bpy ligand at room temperature.[5] Chitre *et al*. reported a ruthenium(II) complex with three diazido-bpy ligands, but no experimental details are reported for the synthesis of the complex (Scheme 6.1).[4] Noteworthy, in general the synthesis of ruthenium complexes with azide-functionalized ligands is described as challenging and often unsuccessful.[4, 8] The higher the number of azide-substituents is, the more difficult the coordination to a ruthenium center seems to be, due to the instability of the ligand. In addition, the desired azide-functionalized complex is often reported to be unstable. A report of Aukauloo and co-workers, for example, stated: "*However, Ru-N_3 ($[Ru(bpy)_2(4\text{-azido-bpy})]^{2+}$) proved to be very unstable towards light, as well as under reductive and oxidative conditions, and could neither be fully characterized nor successfully engaged into click chemistry reactions*".[8] Coordination of the azide-functionalized tpy ligand was reported to be possible when working with a ruthenium(II) precursor, however, the exact reaction conditions or the yield of the reaction were not reported.[11]



Scheme 6.1. Coordination of azide-functionalized bpy ligands to ruthenium precursors.[4, 5]

The coordination of alkyne-functionalized polypyridyl ligands to ruthenium centers afforded higher yields than with the azide equivalents. Some research groups succeeded in the coordination of the ligand without any protection of the alkyne group,[4, 8] while Rau and co-workers showed that the absence of protecting groups leads to the formation of side products due to the coordination of the alkyne triple bond and the ruthenium center.[12] Click reaction on the functionalized complexes was successfully demonstrated many times.[8, 10] Overall, from a synthetic point of view, the functionalization of polypyridyl ligands and ruthenium complexes is more efficient with alkyne than with azide substituents. The alkyne-functionalized ligands and complexes are obtained in higher yields and the products are more stable and therefore, easier to handle in subsequent steps such as CuAAC reactions. The use of alkynes, however, requires working with an alkyne-protecting group.

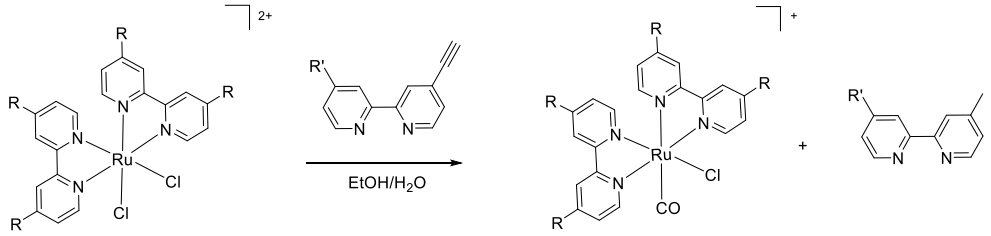
Table 6.2. Complex synthesis with azide- or alkyne-functionalized ligands reported in literature.

handle	ligand	Starting material	conditions	yield	Ref.
azide	bpy	$[Ru(DMSO)_4(Cl]_2]$	Ethylene glycol, MeOH, 120 °C, 3 d	n.s.[a]	4
		$[Ru(p\text{-cymene})(Cl_2)]_2$	MeOH, r.t., 12 h	66%	5
		$[Ru(bpy)_2(Cl)_2]$	$AgNO_3$, MeOH	-	8
	tpy	[Ru(tpy-Cl)$(Cl)_3$]	MeOH, 64 °C, 2 h	-	6
		$[Ru(Cl\text{-tpy})(MeCN)(Cl)_2]^{2+}$	MeOH	n.s.[a]	11
alkyne	bpy	$[Ru(DMSO)_4(Cl]_2]$	Ethylene glycol, MeOH, 120 °C, 3 d	n.s.[a]	4
		$[Ru(bpy)_2(Cl)_2]$	$AgNO_3$, MeOH	91%	8
		$[Ru(R\text{-bpy})_2(Cl)_2]$	EtOH/H_2O, reflux, 3 h Bis-substituted	20%	12
		$[Ru(bpy)_2(Cl)_2]$	EtOH/H_2O, reflux, 3 h mono-substituted	50%	12
	tpy	$[Ru(DMSO)_4(Cl]_2]$	DCE, 10 h	88%	10

[a] n.s. = not specified

As mentioned above and described in Chapters 2 and 5, use of a non-protected terminal alkyne may result in a lower yield due to the formation of side products. The metal center, here ruthenium, can act as a catalyst for the reaction of the click handle with alcohols, such as ethanol.[13] Moreover, the formed enol ester is also difficult to remove from the reaction mixture and isolation of the pure desired complex is challenging. In addition, Rau and co-workers reported that alkyne and ruthenium can react directly with each other, resulting in the formation of the methyl-substituted polypyridyl ligand and a complex containing carbon monoxide, if two coordination sites are free on the metal center (Scheme 6.2).[12] Protecting groups at the alkyne functionality preventing these reactions are usually silyl-based. An overview of different protecting groups and their deprotection strategies have been summarized by Greene.[14] The most common groups are trimethylsilyl (TMS), *tert*-butyldimethylsilyl (TBDMS), and triisopropylsilyl (TIPS) (Figure 6.3). The bulkier the group, the better the protection of the acidic acetylenic hydrogen. The TMS group is frequently used for the protection of the alkyne on polypyridyl ligands (Table 6.1), but in our hands, the protection was too weak and uncontrolled deprotection took place during metal coordination. The TIPS group is one of the strongest alkyne protecting groups, and deprotection was reported to be rather difficult by the group of Stahl.[10] Thus, we investigated the use of the TBDMS protecting group. It is more stable than the TMS group, but sensitive to many reaction conditions. Adaption of the reaction conditions appeared to be necessary in order to retain the group on the alkyne functionality. Other possible groups are

benzyldimethylsilyl or biphenyldimethylsilyl (Figure 6.3), but these are not commercially available as their propargyl bromides.



Scheme 6.3. Reaction between ruthenium(II) precursor and alkyne-functionalized bpy ligand in ethanol/water mixture leads to the decomposition of the alkyne, resulting in the methyl-substituted polypyridyl ligand and a complex containing carbon monoxide.[12]

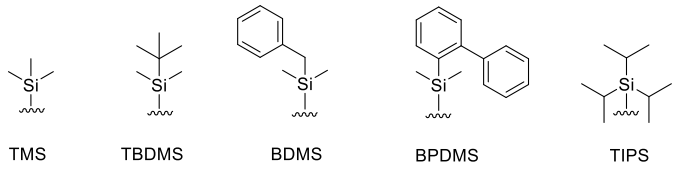


Figure 6.3. Chemical structures of common protecting groups for alkynes.[14]

6.2.2 Azide *vs*. alkyne: biological application

When choosing a click handle for ruthenium complexes, several aspects of biological applications need to be considered. Alkynes and azides are regarded to be inert under physiological conditions. However, Ovaa and co-workers reported the reaction of an alkyne with an active site cysteine in proteases.[15] Noteworthy, the reaction only took place with alkynes attached to the substrate protein of the target protease. Thiols and cysteine residues of other proteins were not affected. In addition, working with alkyne-functionalized ruthenium complexes restricts the application in living systems since the reaction with the corresponding azides requires a catalytic amount of Cu(I) which is cytotoxic due to generation of reactive oxygen species (ROS).[16] Nevertheless, there are reports of CuAAC reactions on the surface of living cells after optimization of the reaction conditions.[17-19] For example, use of chelating ligands allows for lower Cu(I) concentrations, which decreases the cytotoxicity. Cai and co-workers also tested different chelating ligands to perform CuAAC in mammalian cancer cells.[19] They demonstrated that CuAAC is possible in the cytosol of cells, unfortunately with a yield of only 0.8%. They explain the low yield with the deactivation of the catalytic amount of copper due to the presence of copper-binding ligands. Furthermore, the yield of the CuAAC reaction also depends

on the reagent concentrations present in the cell, which are limited by their cellular uptake. On the other hand, azide-functionalized complexes allow for the labeling of living systems using Cu-free reactions such as the strain promoted azide-alkyne cycloaddition (SPAAC) or Staudinger ligation.[20] These reactions do not require copper and are therefore suitable for *in vivo* experiments.[21] A comparison of the non-catalyzed reactions was published by Bertozzi.[22] They showed that SPAAC and Staudinger ligation are not as efficient as CuAAC, but that labeling on cell surfaces and inside cells is successful. An important drawback of SPAAC is the non-selective reaction of the strained alkyne such as cyclooctyne with cysteine residues. This causes a decrease in complex labeling efficiency and increase in background fluorescence.[23] In addition, attention has to be paid when working with alkyne functional groups (in particular when working with alkyne-fluorophores) since alkyne/copper complexation might affect the localization of the species and cause false fluorescent labeling.[24]

In recent years, interest grew on performing CuAAC also on ruthenium complexes for easy modulation of the complexes.[2] To the best of our knowledge, examples of CuAAC involving ruthenium complexes in cells are not reported yet. DeRose and co-workers compared the labelling efficiency of CuAAC reaction in fixed cells for their platinum-based complexes, either functionalized with an azide or alkyne.[25] The functionalization was reported to have no effect on the localization of the complexes, however, the resulting fluorescent signal from the azide-functionalized complex was more intense than with the alkyne-functionalized complex. Overall, azide as well as alkyne functionalization is suited for the use in fixed cells, while azides are easier to apply in living cells. In both cases, the limitations and drawbacks are known and should be considered when choosing one handle over the other.

6.2.3 Fluorescent labeling in comparison with other techniques

Compared to traditional fluorescent microscopy, in which fluorophore-derivatives of the drug were studied that might have different properties compared to the unlabeled drug, post-labeling allows in principle for preservation of the biological activity of the drug. In addition, in post-treatment labeling there is no drug-fluorophore processing which might lead to false fluorescent signals. Drawbacks of fluorescent microscopy are the high concentrations that are needed to obtain a signal. Here, drug concentrations were needed that are a 5-fold higher than the EC_{50} values and thus rather toxic to the cells. The complex distribution and processing in stressed cells might be different, and therefore the concentrations used

during imaging may not represent the situation in treated cells. Sample preparation requires time (here more than 24 h) and photo bleaching can cause loss of the signal. The signal-to-noise ratio depends on the background fluorescence and the accessibility of the complex for labeling and therefore efficiency of the CuAAC reaction. Overall, post-treatment fluorescent labeling is a method that can easily be applied in every lab, with moderate to good sensitivity and resolution.

Laser ablation inductively coupled plasma mass spectrometry (LA ICP MS) is a microanalytical methodology characterized by a very high sensitivity (0.01 μg / g).[26, 27] A laser beam generates fine particles on the surface of the sample. These small particles are directed into the ICP MS machine by a carrier gas. After ionization, the molecules are analyzed by the mass spectrometer detector. No sample preparation is required and the results are obtained quickly (within seconds). However, the resolution is moderate (12 – 20 μm).[26, 28] Therefore, LA ICP MS is not suited for subcellular imaging but an excellent choice for sample analysis of 2D gels.

Energy dispersive X-ray analysis (EDX) is an elemental analysis technique in which an electron beam is used to create an electron vacancy in the sample. This vacancy is quickly filled with an electron of a higher energy shell. This process is accompanied by X-ray radiation that is specific for each element. In this way, the elemental composition of the sample can be identified.[29] The sensitivity of the method is moderate due to electron scattering but the resolution is high, enabling the imaging of subcellular compartments at nanometer (nm) range resolution.[29] EDX measurements were attempted for our samples in collaboration with Jeroen Kuipers and Dr. Ben Giepmans of the department of Cell Biology at the University Medical Center in Groningen for the ruthenium complexes reported in Chapter 4 to compare the results with those obtained by confocal microscopy imaging. Unfortunately, signals corresponding to ruthenium were not found. This might be due either to the strong overlapping of the EDX signals of ruthenium and that of the chloride ion, which is abundant in cells, or to the fact that the ruthenium complexes might be washed out of the cells during EDX sample preparation steps, which are time consuming (several days).

Stimulated Raman scattering (SRS) microscopy is another intracellular imaging technique based on molecular vibrations.[30, 31] Alkynes produce Raman signals within the cell-silent region (1800 – 2800 cm^{-1}),[32] and large Raman scattering cross sections allow for strong signals even at low concentrations. It is a non-invasive method and

therefore the only technique described here that allows for imaging in live cells.[33] Whether SRS can be used depends on the intracellular ruthenium concentration.

Overall, post-treatment fluorescence microscopy (PT-FL) is neither very sensitive nor yielding high resolution, but LA ICP MS also does not provide better resolution for cell mapping and EDX on ruthenium complexes is not well established and did not allow us to obtain clear results (Table 6.3). Noteworthy, the alkyne-functionalized complexes can be used for all four imaging techniques.

Table 6.3. Overview of techniques and their properties

Technique	Sensitivity	Resolution	Sample preparation	Destructive
PT-FL	μM	μm	moderate	yes
LA ICP MS	nM	μm	easy	yes
EDX	μM	nm	challenging	yes
SRS	μM	μm	easy	no

6.3 General conclusion

In this thesis successful development is described of a new synthetic route for the alkyne functionalization of ruthenium-based complexes that allows for fluorophore labeling. The alkyne functionalization only marginally influences the properties of the ruthenium complex, therefore the alkyne-functionalized compound is a suitable model for the non-functionalized compound, with comparable biological activity. Alkyne functionalization is a powerful method to study weak interactions between ruthenium complex and isolated biomolecules. In addition, post-treatment labeling in fixed mammalian cancer cells was successful. It provided clear evidence that the complexes do not enter the nucleus and that DNA is not the main target of these compounds. Therefore, their mode of action is different from that of cisplatin, and proteins have to be considered as potential binding partners.

In order to obtain a better understanding of the complex behavior and its mode of action *in cellulo*, a combination of analytical methods has to be applied, as one technique only does not offer enough information to draw a complete picture of the localization of a compound and its mode of action. The use of different techniques that reveal different properties (oxidation state, thermal or photochemical stability, interaction with biomolecules under different conditions) may provide information on different levels (molecular, proteomic, etc.). Despite the difficulties during the

synthesis of the alkyne-functionalized ruthenium complexes, they offer a new, inexpensive way to obtain insight in the complex distribution in cells.

6.4 Outlook

The complexes synthesized in Chapter 3 are intended to be PACT agents, which work independently of cellular dioxygen levels. So far, the cytotoxicity of the complexes was tested under normoxic conditions (21% O_2). To confirm the PACT character of the ruthenium compounds, cytotoxicity assays under hypoxic conditions (1% O_2) in tumor spheroids have to be undertaken. Imaging of the PACT agent derivatives 24 h after light activation revealed the localization of the complexes outside the nucleus in the cytosol. To confirm the hypothesized accumulation in the Golgi apparatus, additional co-staining experiments are required. In addition, time-lapse experiments would give insights in the intracellular distribution of the complexes over time. This would allow for differentiation between localization of the molecular target of the drug, and simple visualization of how its trafficking is organized within the cell. In order to be able to perform drug labeling in living cells, it would be necessary to change the alkyne handle into an azide handle. However, since azide functional groups in the 4'-position of the tpy ligand are not stable, a non-conjugated spacer between the tpy and azide moieties is required. Consequently, the design and synthesis of the functionalized complex needs to be substantially revised. The shortest spacer possible, a simple CH_2 group may allow to mimic the original complex as much as possible. Examples of such complexes are reported by Guillo *et al*.[34] and Kroll *et al*.[35] (Figure 6.3). On the other hand, if the spacer needs to be expanded anyway, a flexible longer spacer can be chosen as well, which may ensure accessibility of the click handle for the CuAAC or SPAAC reaction after interaction of the drug with its target. For example, Wirth *et al*. showed that fluorescent labeling is more efficient for complexes with a linker than without.[25] Chemical and biological properties of the new derivatives would need to be investigated to guarantee the preservation of the properties of the original drug. Thus, the balance between preserving the drug biological properties and its efficient labeling requires more research.

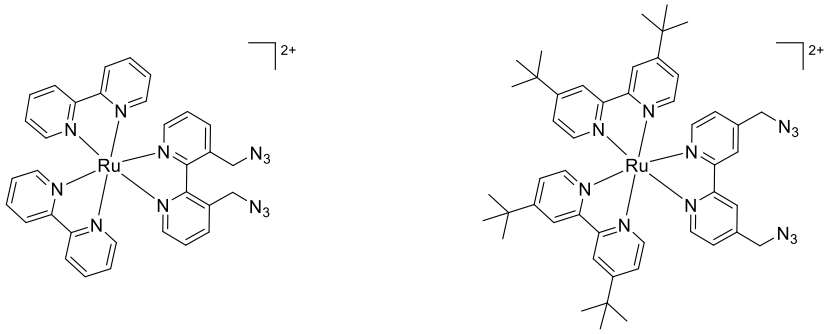
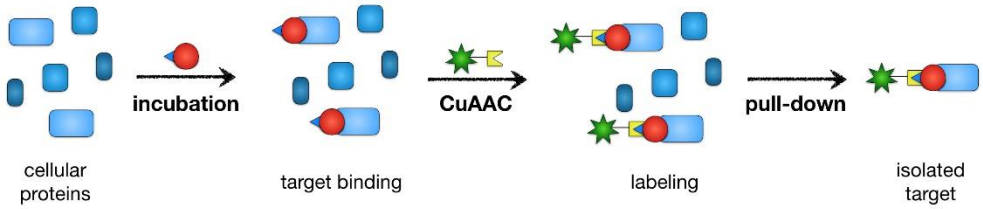


Figure 6.3. CH_2 spacer enables synthesis of stable azide-functionalized ruthenium complexes.[34, 35]

Meanwhile, the alkyne-functionalized ruthenium complexes at hand can be implemented in pull-down experiments to isolate and identify possible targets (Scheme 6.3). This would be the first example of pull-down experiments of ruthenium complexes after drug incubation in mammalian cancer cells. Different types of enrichment methods (separation by 2D gel, beads, or affinity column) can be considered and compared.[36-38] The obtained information can increase our understanding of the mode of action of these photoactivatable ruthenium complexes.



Scheme 6.3. Schematic representation of pull-down experiment using an alkyne-functionalized ruthenium complex.

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