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Lipophilic ruthenium complexes with tuned cell membrane affinity and photoactivated uptake

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ABSTRACT

Ruthenium dipyridophenazine (dppz) complexes are virtually non-emissive in aqueous solutions but show strong luminescence in hydrophobic environments, making them interesting as molecular probes in cellular imaging. We show by luminescence spectroscopy that by substituting the dppz ligand with alkyl ether chains of increasing length the complexes can be tuned from preferential intercalation into DNA to insertion in model phospholipid membranes. Confocal laser scanning microscopy (CLSM) on methanol fixed CHO-K1 cells show an analogous distribution in the cell, where the least hydrophobic complex exclusively stains the nucleus whereas the more hydrophobic ones seem to predominantly stain membrane structures in the cytoplasm. In live cells CLSM show that initially only the more hydrophobic derivatives stain the plasma membrane. However, brief further exposure to the laser light causes permeabilization of the membrane and accumulation of extracellular ruthenium complexes in internal cellular structures, similarly to the distribution found in fixed cells.

1. Introduction

Ruthenium complexes with dipyridophenazine (dppz) ligands have been frequently studied due to their strong DNA binding and their extraordinary photophysical properties [1,2]. In particular, they have raised a lot of interest due to their "light switch effect" being brightly luminescent when intercalated into DNA and virtually non-emissive in aqueous solution, which is advantageous for use in fluorescence microscopy. Barton et al. have recently shown the usefulness of this effect for studying the cellular uptake and nuclear localization of Ru(II) dppz complexes [3,4]. Similar complexes have also been used as probes for cell viability and nuclear staining [5,6]. We have previously shown that Ru(II) dppz complexes, made more hydrophobic by substitution with alkyl ether chains, are versatile as photophysical probes for phospholipid bilayers [7,8]. Due to their unique long lived charge-transfer excited state and red emission wavelengths such lipophilic ruthenium dppz complexes have potential as molecular probes in cellular imaging. Here we investigate the effect of varied lipophilicity on membrane vs. DNA binding, the intracellular localization in fixed cells, and the photoactivated uptake in live CHO-K1 cells using emission spectroscopy and confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Synthesis

Scheme 1 shows the structures of the $Ru(phen)_2dppz^{2+}$ derivatives where the dppz ligand has been substituted with alkyl ether chains of varied lengths. D4 were prepared, similarly as reported before for D2 and D6 [8], by condensation of $[Ru(phen)_2(1,10\text{-phenanthrolin-5,6-dione})]Cl_2$ with the appropriate substituted benzene-1,2-diamine (see the Supplementary data).

2.2. Cell culture

Chinese hamster ovarian (CHO-K1) cells were cultured in HAM's F12 medium supplemented with bovine calf serum (10%) and L-glutamine (2 mM) at 5% CO_2. Cells for confocal imaging were seeded on round coverslips at a density of ~80,000 cells/coverslip and cultured for 2 days. Cells were rinsed once with serum free medium before mounted in a solution chamber. Ru(II) complex was dissolved in DMSO and diluted in serum free medium to the required concentration immediately prior to adding the solution onto the coverslip. The final DMSO concentration never exceeded 1% v/v. The autofluorescence from the HAM's F12 medium is negligible and no rinsing step is needed since the unbound complex is non-luminescent. Fixation was achieved by incubating cells in methanol (−20 °C) for 15 min before addition of Ru(II) complex.

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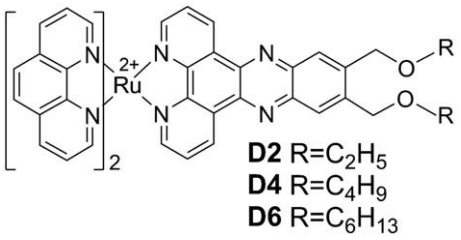

Scheme 1. Molecular structure of the Ru (II) complexes.

2.3. Preparation of large unilamellar lipid vesicles (LUVs)

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and DOPG (1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol) dissolved in chloroform were mixed at a molar ratio of 4:1, and the solvent was evaporated under reduced pressure using a rotary evaporator before being put under vacuum for at least 2 h. Vesicles were prepared by dispersion of the lipid film in buffer under vortexing. Thereafter, the vesicles were subjected to 5 freeze–thaw cycles (liquid nitrogen/37 °C) before extrusion 21 times through polycarbonate filters with a pore diameter of 100 nm using a hand held syringe LiposoFast-Pneumatic extruder (Avestin, Canada).

2.4. Luminescence spectroscopy

Steady state luminescence measurements were performed on a Cary Eclipse spectrofluorimeter (Varian) at 25 °C. The ruthenium complex hexafluorophosphate, dissolved in DMSO, was added to a final concentration of 2 μM in buffer containing preformed LUVs or calf thymus DNA. Titration with aliquots of DNA or LUVs were performed until the negative charges of the LUVs equal the charges of the nucleotides, i.e. to a final concentration of 200 μM lipids and 40 μM nucleotides. The buffer used was 150 mM sodium chloride at pH 7.4 and the final DMSO concentration was less than 0.5% in all experiments. The wavelength of excitation was 440 nm and the emission was measured between 580 and 800 nm.

2.5. Confocal laser scanning microscopy

A CLSM system (Leica TCS SP2 RS, Wetzlar, Germany) with a PL APO 63×/1.32 objective was used for acquisition of confocal fluorescence images. An argon laser (488 nm) was used for excitation of the Ru(II) complexes and a UV-laser (351 nm) was used to excite the nuclear dye DAPI (4′,6-diamidino-2-phenylindole). Illumination of cells was achieved during confocal imaging by continues scanning with the focused laser light beam in a raster pattern over a selected area of a cell culture.

3. Results and discussion

Since the emission wavelengths of the Ru(II) complexes investigated here are dependent on the immediate surroundings, luminescence spectroscopy easily determines if the complexes are bound to DNA or embedded in a lipid membrane due to a wavelength shift of 25–30 nm. D2, D4 and D6 show differences in preferred binding when both DNA and LUVs are present, which is observed by the difference in the maximum emission wavelength of the Ru(II) complex in this mixture. Fig. 1 shows *in vitro* luminescence spectra of D2, D4 and D6 initially bound to negatively charged LUVs (A) or calf thymus DNA (B), and the spectral change upon titration with aliquots of ctDNA or LUVs respectively. Red lines correspond to complex bound to pure ctDNA and green lines correspond to the final titration (200 μM lipids and 40 μM DNA). The spectra of membrane bound D4 and D6 show no change upon addition of DNA whereas for D2 there is an increase in intensity and a blue shift of the spectra (Fig. 1A). Fig. 1B shows that the wavelength maximum shift for DNA-bound D6 is immediate when adding LUVs, changing from 622 nm to 646 nm upon addition of 8 μM LUVs. For D4 the transition towards membrane binding is

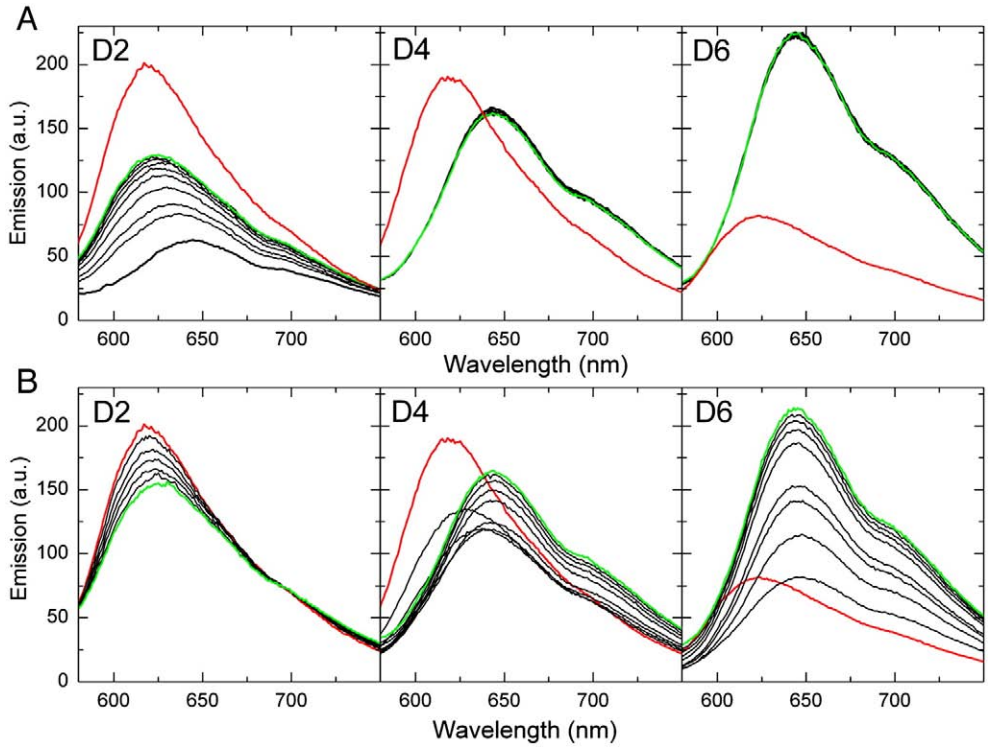

Fig. 1. (A) Emission spectra of D2, D4, and D6 (2 μM) bound to DOPC/DOPG LUVs (200 μM) and upon titration with aliquots of ctDNA to a final concentration of 40 μM (green lines). Emission spectra of the complexes in pure ctDNA are shown in red. (B) Emission spectra of D2, D4, and D6 bound to ctDNA (red lines) and upon titration with LUVs to a final concentration of 200 μM (green lines).

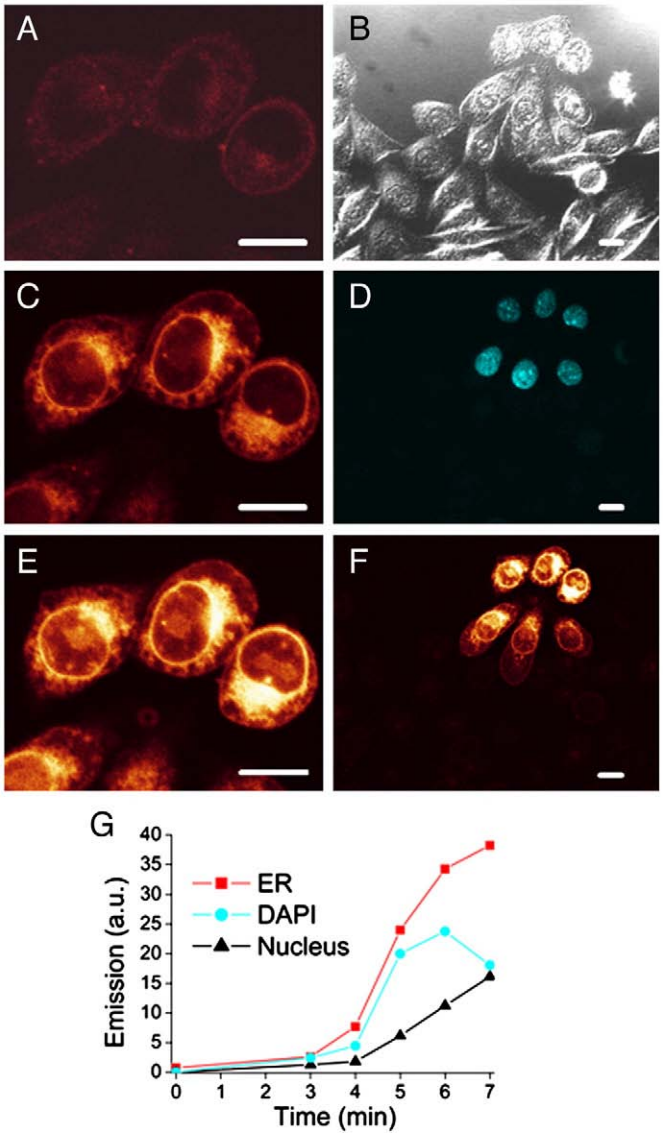

Fig. 2. Photoactivated cellular uptake of D4 (10 μM) and cellular distribution upon illumination for 3 (A), 5 (C), and 7 min (E). The right column shows a larger area of coverslip; transmitted light image (B) and DAPI nuclear staining of membrane damaged cells (D). Only illuminated cells have internalized D4 (F). Scale bars are 10 μm. The photomultiplier gain was lowered in (E) and (F) to prevent over exposure. (G) shows emission intensity increase upon illumination of D4, outside the nucleus (ER) and in the nucleus, and emission intensity increase of DAPI during illumination of D4.

somewhat slower but at a LUV concentration of approximately 30 μM the emission wavelength corresponds to that in pure LUVs. The spectra of D2 gradually shift towards equilibrium binding between DNA and membrane with a maximum emission wavelength in this mixture close to that in pure DNA. These titration experiments clearly reveal the affinity differences for the complexes towards membranes and DNA. We can conclude that the two more lipophilic complexes D4 and D6 show a preferential binding to membranes compared to DNA, while for the least lipophilic complex D2 binding to the latter is favoured as judged by the maximum emission wavelengths. The emission intensity in LUVs may depend on how deep the complexes are buried in the phospholipid bilayer, where a position more shielded from quenching water results in a brighter emission [8]. Since the luminescence properties of Ru(II) complexes are sensitive to the dppz substitutions and to the nearby environment [9], it is not surprising that the emission intensity also differ among the derivatives when bound to ctDNA. The low emission intensity of D6 bound to ctDNA can be explained by aggregate formation of D6, in agreement with some background emission of pure D6 in high salt concentrations (see Supplementary data for comparative quantum yield in high and low salt buffer).

The cell membrane binding, uptake and intracellular localization of the three complexes in CHO-K1 cells were studied with CLSM. Immediately after addition, D4 stains the cell membrane and with a very short exposure to light it remains in the plasma membrane. However, after a time threshold of 3–4 min of raster scanning with the laser, complex starts to penetrate the plasma membrane and following continued illumination the internalization increases dramatically (Fig. 2A, C and E). D4 accumulates in internal membranes, and there is also nucleolar staining. After zooming out it is clear that only cells that have been illuminated with the laser have internalized the complex, and are also the only ones stained by the nuclear staining dye DAPI (Fig. 2B, D and F) [10,11]. The illuminated cells have a compromised cell morphology and permeabilized membranes and therefore extracellular Ru(II) complex can be internalized. The graph in Fig. 2G presents the emission intensity increase inside the nucleus and outside (endoplasmic reticulum, ER) as well as DAPI emission in the nucleus with time of illumination. It should be noted that with no complex present the cells are unaffected by the laser scanning and are not stained by DAPI (Supplementary data), concluding that the uptake and membrane permeabilization effect is not a cause of heating from the laser.

When the more lipophilic complex D6 is added to live cells (Fig. 3) there is an even stronger membrane associated luminescence immediately after addition, compared to D4. After 5 min of laser illumination D6 still nicely stains the plasma membrane but luminescence is observed throughout the cytoplasm where D6 is bound to internal membranes. Interestingly, significantly less luminescence is observed in the nucleus compared to the corresponding case for D4. Due to the weak membrane binding D2 shows no sign of accumulation inside cells upon illumination (not shown). Dobrucki et al. previously showed that exposure of light results in phototoxicity and internalization of the $Ru(phen)_3^{2+}$ complex; however, the concentrations used were two orders of magnitude higher than that used here [12]. Furthermore, one could note that despite the fact that Ru(II) dppz complex is present in the medium, there is no background luminescence.

When cells are incubated with 5 μM Ru(II) complex for longer times in the dark another uptake mechanism is responsible for the internalization of the complexes. Fig. 4A, B, and C show the cellular uptake for each of the examined Ru(II) complexes after incubation at 37 °C for 24 h. Both D4 and D6 are no longer bound to the plasma membrane but are now found in punctuate structures in the cytoplasm whereas no luminescence is apparent in the nucleus. This pattern indicates uptake by endocytosis, however passive diffusion

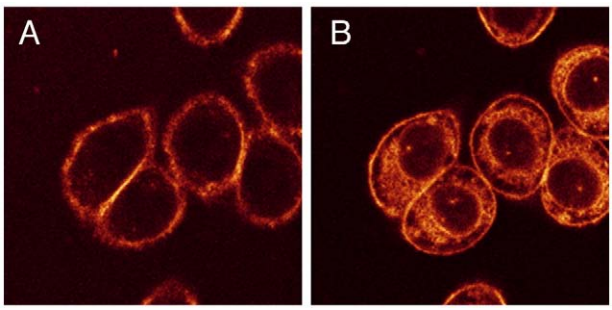

Fig. 3. Membrane binding, photoactivated uptake and cellular distribution of D6 (10 μM) in CHO-K1 cells imaged by confocal microscopy. (A) Immediately after addition, D6 stains the plasma membrane and (B) after 5 min of laser radiation, D6 accumulates mainly outside the nucleus.

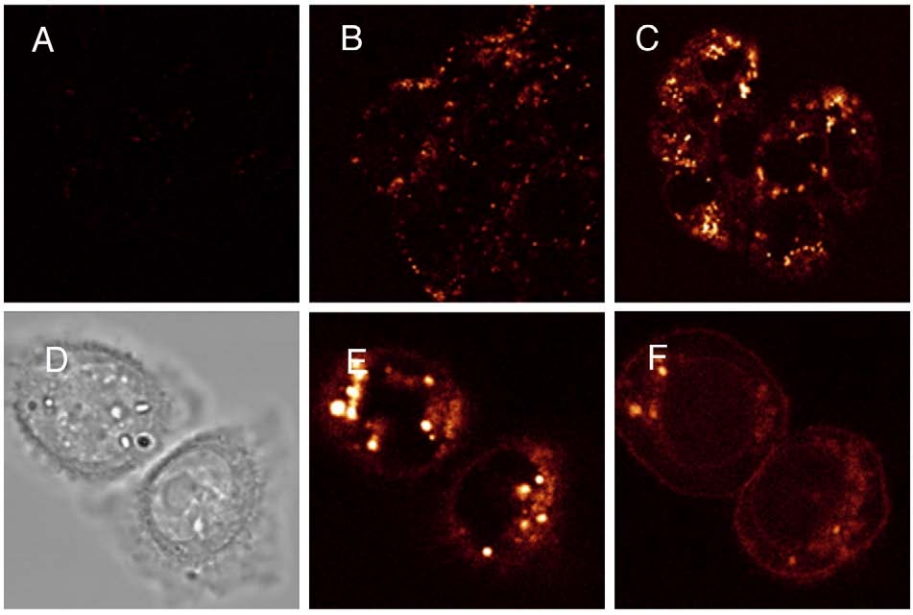

Fig. 4. Dark control experiment showing different intracellular intensities of D2 (A), D4 (B), and D6 (C) (5 μM) after incubation in the dark at 37 °C for 24 h imaged by confocal microscopy. (D) Transmitted light image of CHO-K1 cells incubated with D6 in the dark at 37 °C for 24 h. (E) Luminescence of D6 entrapped in vesicles. (F) Upon illumination for 10 min D6 are released from the vesicles, spread out in the cytoplasm and subsequently bind to membranes. The photomultiplier gain was the same in all images.

cannot be ruled out since that would also be expected to be more facile for the more lipophilic complexes [12–14]. From the luminescence intensity it can be concluded that D6 is internalized to a larger extent compared to D4. The low membrane affinity for D2 results in very low uptake of this complex. When cells are incubated with the double amount of D2 (10 μM) for 24 h some emission is observed in the cytoplasm, but compared to the corresponding experiment with D6 the intensity is significantly lower for D2 (see the Supplementary data). Moreover, when cells containing vesicles loaded with D6 are subjected to laser illumination the vesicles are ruptured and the dye is released into the cytoplasm and binds subsequently to internal membrane structures (Fig. 4D, E, and F). The final staining pattern is similar to that observed for illuminated cells (Fig. 3B).

To further investigate the distribution of all three complexes inside cells and to be able to compare with the preferred binding concluded from *in vitro* luminescence experiments, methanol fixed cells were incubated with complexes and the relative luminescence intensity inside and outside the nucleus was measured (Fig. 5; for details see Supplementary data). There is a significant difference in the localization of the complexes where a decreased length of the alkyl ether chain results in higher emission intensity in the nucleus compared to in the cytoplasm. Indeed, the least lipophilic complex, D2, is predominantly found in the nucleus (A) while D4 is more homogenously distributed in the cells with comparable emission intensities inside the nucleus and in the cytoplasm (B). The most lipophilic complex, D6, is mainly found outside the nucleus, presumably in the endoplasmic reticulum which has a large extent of membrane structure (C). This is in agreement with what was observed both in live cells and in the *in vitro* luminescence experiment where it was found that D2 has a higher preference for DNA while D4 and D6 bind more strongly to membrane structures.

4. Conclusion

In conclusion, we have shown how slight modifications in the lipophilicity of the dppz ligand result in great variations in membrane and DNA binding, and have large effect on the uptake and intracellular localization of these complexes. Indeed, the differences in preferred DNA and LUV binding observed *in vitro* correspond to the different nuclear and membrane staining in fixed cells. We further showed that upon illumination the membrane bound Ru(II) complexes cause damage to the plasma membrane and the concomitant loss of membrane integrity enables surrounding complexes to diffuse into the cell and accumulate on internal structures. Another uptake mechanism is observed when Ru(II) complexes are incubated in the dark without membrane damage and result in punctuate staining in the cytoplasm.

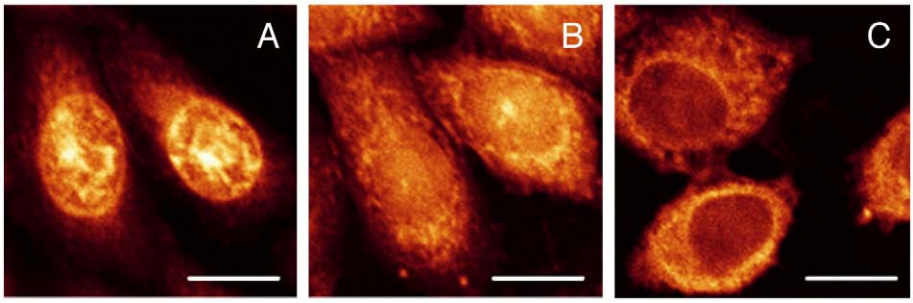

Fig. 5. Confocal fluorescence microscopy images showing the cellular distribution of ruthenium complex in fixed CHO-K1 cells. D2 (A), D4 (B), D6 (C) (10 μM) were added to cells fixed in methanol (−20 °C) for 15 min and excited at 488 nm. Scale bars are 10 μm.

Our findings show the potential of Ru(II) complexes as dyes for many different intracellular structures, just by small variations on the ligand structure, in contrast to most Ru(II) complexes that only stain the nucleus. An additional attractive feature is their long and sensitive luminescence lifetimes (~100–1000 ns dependent on environment and complex structure) [8,9] which make them very interesting for fluorescence lifetime imaging microscopy (FLIM).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2010.04.006.

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