Ruthenium polypyridyl peptide conjugates: membrane permeable probes for cellular imaging†

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Two novel polyarginine labelled ruthenium polypyridyl dyes are reported, one conjugated to five, (Ru–Ahx–R_5), and one to eight arginine residues, (Ru–Ahx–R_8); both complexes exhibit long-lived, intense, and oxygen-sensitive luminescence; (Ru–R_8) is passively, efficiently and very rapidly transported across the cell membrane into the cytoplasm without requirement for its permeablisation.

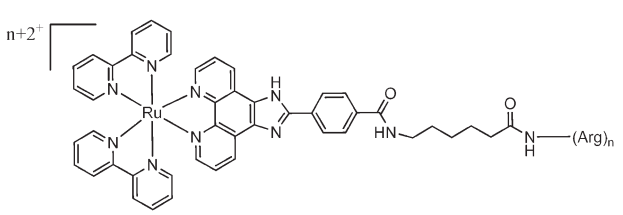
Luminescent dye molecules capable of passive cell delivery may be used as molecular probes in cellular imaging, across cell biology, molecular biology, microbiology, and flow cytometry applications.

The most widely used probes in cellular imaging are fluorescent and based on organic, typically polyaromatic, chromophores. The short luminescence lifetimes of such species, typically <10 ns, limits their environmental sensitivity, *e.g.*, towards molecular oxygen and their application in fluorescence lifetime imaging (FLIM).[1] Ruthenium polypyridyl complexes have unique photophysical properties which make them potentially invaluable as probes for cellular imaging, including intense polarised luminescence, large Stokes shifts, red emission wavelengths, and a good photostability. They are oxygen sensitive, and do not exhibit the complication of dimer or excimer formation observed for some O_2-sensitive organic probes.[2] However, there has been a longstanding barrier to their exploitation in this context because conventional complexes do not passively diffuse across the cell membrane.[3] Therefore, cells must be permeabilised by electroporation, detergent or treated with some other transfection agent.[4] Antibody conjugation has been shown to permit external labelling of cells rather than direct imaging.[5] Very recently a ruthenium complex conjugated to estradiol has been shown to be cell permeable, attributed to the lipophilicity of the steroid pendant.[6]

Herein, we report on the preparation and characterization of two novel intensely luminescent and oxygen-sensitive ruthenium polypyridyl peptide conjugates which are potentially useful targeted probes for luminescence and luminescence lifetime cellular imaging. It is important to mention that a number of elegant examples of cell permeable inorganic luminophores have been reported recently, based on Rh, Ir, Re and f block elements,[7] however this present report is, to our knowledge, the first cell-permeable ruthenium–peptide conjugate.

The two novel conjugates (Scheme 1) are based on the parent ruthenium complex $[Ru(bpy)_2(pic)]^{2+}$, where pic is 2-(4-carboxyphenyl)imidazo[4,5-*f*][1,10]phenanthroline.[8] Conjugation occurs through the terminal carboxy unit.[9] The R_n oligopeptides ($n = 5$ or 8) were obtained *via* Merrifield's solid phase peptide synthesis, according to the Fmoc/t-Bu strategy. The detailed synthesis is described in ESI.† In this protocol, the coupling efficiency of $[Ru(bpy)_2(pic)]^{2+}$ to the peptide exceeded 85%.[10] This is a significant improvement on more typical conjugations of organic fluorophores which is ascribed to the reactivity of the aryl acid pendant on the ruthenium centre. The ruthenium dyes are functionalised unequivocally through a single, reactive group, *via* reaction with nucleophilic functions on peptides. They do not contain isomers or competing functional groups which can lower the synthetic yields of the labelling step and/or require their protection.[11]

The photophysical properties of the conjugates, Ru–Ahx–R_5 and Ru–Ahx–R_8 have been investigated in phosphate buffered saline (PBS) at pH 6.7, which resembles the pH and ion concentration found in living cells. The arginine modification is relatively remote from the metal centre and is expected to have very little influence on the electronic structure of the Ru-complex. Consequently, the photophysical properties of Ru–Ahx–R_5 and Ru–Ahx–R_8 are essentially indistinguishable from each other or of the parent complex $[Ru(bpy)_2(picH)]^{2+}$.[8] As for the parent complex $[Ru(bpy)_2(picH)]^{2+}$, the electronic absorption spectra of the two arginine derivatives show an absorption band at 460 nm



Scheme 1 Structure of Ru-Ahx-R_n (n is 5 or 8). In the parent complex, $[Ru(bpy)_2(picH_2)]^{2+}$, the aryl amide pendant on the imidazole ring is replaced by an aryl acid.

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($\varepsilon \sim 16.9 \times 10^3$ L mol^{-1} cm^{-1}) which is assigned to a metal-to-ligand charge transfer (MLCT) transition. An intense emission is observed at 607 nm with a quantum yield of 0.06, which is slightly lower than that of the parent $[Ru(bpy)_2(picH)]^{2+}$ complex (Φ = 0.067), but still approximately 30% higher than the quantum yield of the well known $[Ru(bpy)_3]^{2+}$ complex. The luminescence lies in the red, well away from possible autofluorescence of biological material and the high quantum yield permits easy detection, even at low dye concentration. The presence of the Ahx-R_8 moiety slightly reduces the luminescence lifetime from 872 ± 4 ns in the parent complex (degassed aqueous solution) to 775 ± 4 ns in Ru–Ahx–R_8. This long-lived luminescence may be well suited to explore some of the longer lived, microsecond biodynamical processes, *e.g.* membrane diffusion, protein rotation or folding. The luminescence lifetime is, furthermore, oxygen sensitive. In air saturated aqueous solution the lifetime of the excited state of Ru–Ahx–R_8 decreases to 480 ns, this ~50% decrease in lifetime indicates a high sensitivity toward the concentration of dissolved oxygen.

To investigate cellular uptake of Ru–Ahx–R_5, Ru–Ahx–R_8 and $[Ru(bpy)_2(picH)]^{2+}$, we investigated myeloma cells (Fig. 1) and human blood platelets, as examples of mammalian cells (Fig. 2), using confocal laser scanning microscopy. 458 nm excitation was used, corresponding to the MLCT absorbance of the metal complex and the emission images were recorded at 610 nm. In a typical protocol, 3 μL of an aqueous solution of the Ru-complex ($[Ru(bpy)_2(picH)]^{2+}$ or Ru–Ahx–R_8 (1.2×10^{-3} M)) were added to 100 μl of the cell suspension to yield a final dye concentration of 3.5×10^{-5} M. Fig. 1 compares the ability of the parent complex and the octa-arginine derivative to transport across a myeloma cell membrane. As expected, no luminescence was observed from within the cells when they were incubated with $[Ru(bpy)_2(picH)]^{2+}$ (Fig. 1(c)), indicating the dye cannot penetrate the cell membrane and enter the cell, even after extensive incubation. In contrast, Ru–Ahx–R_8 transports passively through the cell's membrane and accumulates and preconcentrates inside the cell. Transport is rapid and relatively irreversible.[12] The migration of the dye into the cell is complete within about 12 min yielding intense well contrasted cellular images as shown in Fig. 1(a) and (b). The first 5 min of the passive transmembrane transport of Ru–Ahx–R_8 into myeloma cells is shown in the accompanying video (see ESI†).[13] During the first 2–3 min, the dye concentrates in the cell membrane. From here, the dye distributes into and throughout the cell. The distribution of the dye inside the cell is not homogenous, resulting in brighter and darker areas, according to the different cellular compartments. After between 10 and 15 min the process is complete and no further changes to the dye distribution within the cell are observed. In contrast, $[Ru(bpy)_2(picH)]^{2+}$ could only be incorporated into the cell through permeablisation of the cell membrane, in this case, on exposure to detergent (Triton) as is shown in Fig. 1(d).[14]

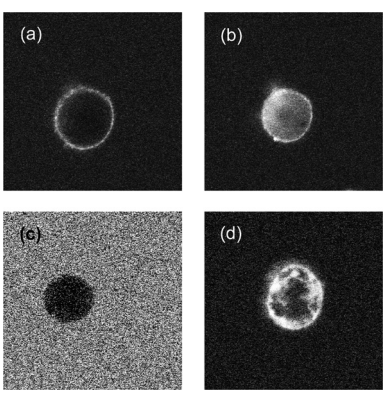

Fig. 1 Luminescence images (λ_{ex} = 458 nm, λ_{em} = 610 nm) of myeloma cell incubated with Ru–Ahx–R_8 (3.5×10^{-5} M) after (a) 3 min and (b) after 5 min. (c) Myeloma cell incubated with the parent complex $[Ru(bpy)_2picH]^{2+}$ (3.5×10^{-5} M) for 26 min and (d) for 5 min after permeablizing the cell with Triton (1% v/v).

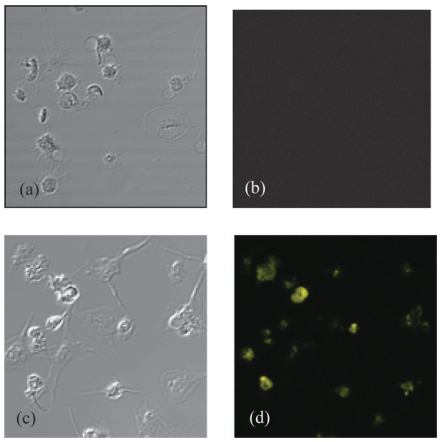

Fig. 2 (a) & (c) White-light differential-interference contrast image of human blood platelets spread on a fibrinogen-coated glass slide following 20 min incubation with 3×10^{-5} M (a) Ru–Ahx–L_5 and (c) Ru–Ahx–L_8. (b) & (d) The corresponding luminescence images (λ_{ex} = 458 nm, λ_{em} = 610 nm), on incubation with (c) Ru–Ahx–L_5 and with (d) Ru–Ahx–L_8.

The length of the polyarginine peptide (R_5 and R_8) is important in dictating the membrane transport properties of the dye, as seen in Fig. 2 for human blood platelets. Whereas platelets incubated with Ru–Ahx–R_8 show intense luminescence from their cytoplasm (Fig. 2(d)), the platelets incubated with Ru–Ahx–R_5 do not exhibit any luminescence. The cell penetrating ability of polyarginines is well known and generally thought to arise through endocytosis. This ability is polyargenine chain length dependent with R_6 to R_{11} showing best endocytosis.[15] Consistent with endocytosis, we found the dye delivery in to the cell to be temperature dependent.[17] Thus, the inability of Ru–Ahx–R_5 to penetrate the cell membrane in this instance is consistent with other peptide studies indicating the polyargenine is a useful carrier peptide for cell penetration by metal complexes.[16]

A drawback of conventional organic chromophores for laser scanning microscopy is their propensity to photobleach, which limits their use over extended periods. Under imaging conditions typically used for human blood platelets (see ESI†), under 20 min continuous irradiation, Ru–Ahx–R_8 retained more than 50% of its initial luminescence intensity from within a platelet. Such photostability is useful as it may allow the dynamics of cellular processes to be studied at timescales which are useful to the microscopist/biologist in obtaining detailed or dynamic cellular information.

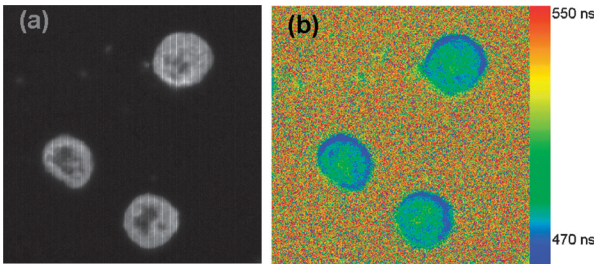

Fig. 3 (a) Fluorescence intensity image of myeloma cells after incubation for 15 min with Ru–Ahx–R_8, 3.5×10^{-5} M in aqueous PBS buffer. (b) False colour fluorescence lifetime image of the same cell (fast FLIM).

A key advantage of ruthenium polypyridyl complexes over many conventional fluorescent imaging dyes are their long lived excited states. This property renders such complexes far more sensitive to their environment, *e.g.*, dissolved oxygen concentration, pH, dielectric constant and potential. Significantly, the fluorescence lifetime is independent of luminophore concentration, the optical path of the microscope, the local excitation light intensity, as well as the luminescence detection efficiency. This makes luminescence lifetime an ideal parameter to measure in biological systems where the exact concentration of dye after cellular uptake is difficult to determine and replicate accurately. As described, the luminescence of Ru–Ahx–R_8 exhibits significant oxygen dependence. Fig. 3(a) shows a scanning confocal luminescence image of myeloma cells incubated with Ru–Ahx–R_8, Fig. 3(b) shows the false colour luminescence lifetime image of the same cells. The lifetime of the residual dye in the external buffered solution is monoexponential, however, the lifetimes of selected compartments within the cells are typically biexponential. The false colour image reflects the average lifetime of the probe.

As can be seen from the false colour coding, the average lifetime of the dye varies across the various compartments of the cells. The dye residing in the membrane of the cell, for example, exhibits the shortest lifetime. This is in agreement with the anticipated higher solubility of O_2 in the membrane[17] and demonstrates the potential of these dyes for cellular oxygen sensing.

In conclusion, we have demonstrated a high yield synthetic strategy for production of peptide labelled ruthenium luminophores. We demonstrate that an octa-arginine labelled ruthenium complex Ru–Ahx–R_8, is an oxygen sensitive luminophore that transports rapidly and passively across the cell membrane to preconcentrate inside the cell. This behaviour was demonstrated for myeloma and for human blood platelets. The Ru centre is resistant to photobleaching, is long-lived and intense, and has appropriate absorption and emission characteristics to suit most conventional confocal laser systems. Its long lifetime, makes it quantitatively sensitive to oxygen concentration and the counter ligands can be readily altered to target the environmental sensitivity of the probe. For example, permitting sensitivity to pH, water content and the rigidity of the microenvironment. Labelling of peptides assembled by solid-phase synthesis can be more broadly applied to sequences used for sub-cellular targeting and to bioactive sequences with inherent membrane translocation.[18] Overall, Ru-labelled cell-permeable peptides are likely to be an important tool in cellular imaging for probing cellular events and the cellular environment.

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12 After incubation with dye, cells were washed twice and resuspended in fresh buffer. The fluorescence intensity of the cells reduces slightly with time however, the decrease is small (< *ca.* 30% over 2 h) and even after 2 h the cells remain strongly luminescent.
13 See video—ESI†.
14 The cytotoxicity of the dye was investigated using Trypan Blue and during luminescent measurement by counterstaining with SYTOX green. On short time scales (30–40 min) at Ru complex concentrations used here the cells remained viable. At longer time scales (>2 h) there was evidence for cytotoxicity. This is currently under further investigation.
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