Rhenium *fac* tricarbonyl bisimine complexes: biologically useful fluorochromes for cell imaging applications†

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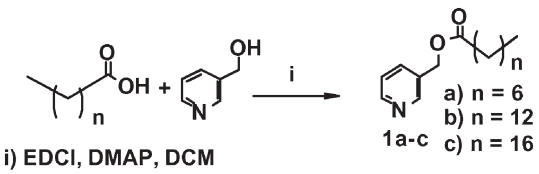
A series of lipophilic and hydrophilic *fac* tricarbonyl rhenium bisimine complexes have been prepared, their membrane-permeabilities explored in liposomes and their potential for application in fluorescence microscopy cell imaging demonstrated in the first application of MLCT-fluorescent rhenium complexes in cell imaging.

Fluorescence microscopy is a powerful, high resolution technique in biological imaging and specific fluorescent staining techniques make it especially useful for diagnostic investigations of tissue samples. One of the challenges, however, is to differentiate the endogenous fluorescence of biological species (autofluorescence) from that derived from a dye which is applied to specifically stain a target organelle or cell component.[1] There is a large literature on solving the problems associated with autofluorescence, but still it is often difficult to distinguish clearly between those areas which have accumulated the dye and those which show only autofluorescence.[1] Autofluorescence may be filtered out from the desired signal if there is an appreciable difference in wavelength between the two modes of fluorescence, and this is particularly likely if the dye has a large Stokes shift (the difference in wavelength between the absorbed and emitted light), as the typical Stokes shifts for the species involved in autofluorescence are small.[1] Alternatively, time-resolved microscopy can provide a method for eliminating autofluorescence if the fluorescence lifetime of the added dye is much longer than the short-lived autofluorescence. As both long fluorescence lifetimes and large Stokes shifts are available in certain lanthanide complexes, recently systems for cell labelling using such complexes have been developed.[2] Certain transition metal complexes also offer Stokes shifts and lifetimes which, while not as great as those of lanthanide complexes, could allow differentiation from autofluorescence. In particular, there are ruthenium[3] and rhenium[4] complexes which have well understood triplet metal-to-ligand-charge-transfer (^{3}MLCT) luminescence characterised by useful Stokes shifts. As time resolved microscopy is not as widely available as standard confocal fluorescence microscopy, and in view of the much simpler coordination chemistry of these particular transition metal complexes compared to lanthanides, these seemed appealing targets for new cell staining systems. The most studied system is probably the ruthenium trisbipyridyl, and there are studies of the cellular uptake of ruthenium complexes,[5] however the excited state can be localised on any of the three bipyridine units. Conversely, *fac* rhenium tricarbonyl bipyridines[4] and related species offer systems in which the excited state is localised on the single bipyridine unit, making them ideal for modification to allow a response to their environment. There are ruthenium complexes in which the excited state is localised on a single ligand, the bipyridyl tetracyanoruthenates, however these are highly polar anionic systems which were thought unlikely to be membrane-permeable and thus not ideal candidates for cell work.[6] The fluorescence properties of these species in simple solvents, with visible light excitation avoiding problems of UV damage to tissue, and their relatively lipophilic and monocationic nature seem suitable for cell imaging. However, this apparently useful luminescence could be subject to quenching or photobleaching as a result of interaction with species present in complex biological media, and the degree and specificity with which such rhenium complexes interact with organelles are unknown, as is their toxicity. As heavy metal toxicity is often associated with metal–DNA interactions these systems seemed appropriate as they are d^6 low spin and kinetically inert to ligand substitution. Thus, a family of *fac* $[Re(bisim)L(CO)_3]^+$ complexes have been prepared in which (bisim) represents a bisimine ligand, *i.e.* a bipyridine, phenanthroline or derivative, and L is a pyridine or derivative each of varying charges and lipophilicities, and their membrane permeabilities and suitability for cell staining tested for the first time as Re complexes previously studied in cells by fluorescence microscopy have relied upon organic fluorophores rather than ^{3}MLCT Re, the complexes being studied as models for Tc.[7]

In order to access complexes of the form *fac* $[Re(bisim)L(CO)_3]^+$ in which L is highly lipophilic a series of esters of 3-hydroxymethylpyridine, Py-3-CH_2O_2CR' (R′ = octyl, merystyl, steryl) **1a–c** were synthesised† (Scheme 1) and incorporated into the complexes following an adaptation to the literature routes[4] to such complexes. This involved initial formation of rhenium tricarbonyl bipyridyl chloride, then activation of the chloride (either exchange



Scheme 1

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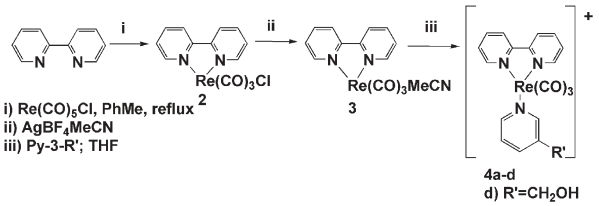
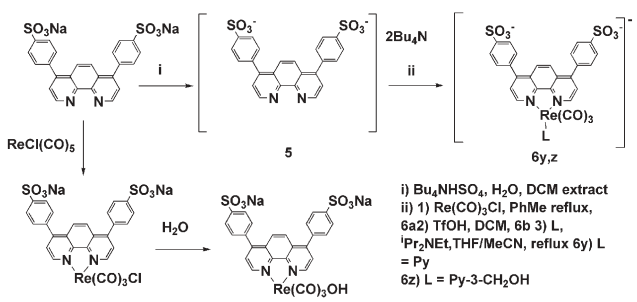


Scheme 2

to the triflate, or *via* halide abstraction to the acetonitrile complex), then finally displacement with the substituted pyridine (Scheme 2).

The more water soluble anionic systems were accessed *via* substitution of the bipyridyl unit with a commercially available dianionic analogue, bathophenanthroline sulfate.[8] This required a series of counterion exchanges, as it was found that while the sodium salt of the bathophenanthroline sulfonate reacted with $Re(CO)_5Cl$ to give the required complex, further steps were hindered by the insolubility of this species in anything but water, in which substitution of chloride by hydroxide occurred with a half life of approximately 2 hours at room temperature. No solvent or phase transfer catalyst was found which allowed chemistry to be performed on these salts, so an alternative route was developed in which the bathophenanthroline sulfate sodium salt was first treated with aqueous tetrabutylammonium hydrogen sulfate, to precipitate the tetrabutylammonium salt of the ligand as a gel, which upon drying gave a handleable anhydrous solid. This salt is soluble in normal organic solvents, and allowed the synthesis of the monoanionic rhenium complexes as the tetrabutylammonium salts **6y,z** by the normal route (Scheme 3). Ion exchange chromatography on Amberlite IR 120-H as the ammonium form gave the final complexes as their water-soluble ammonium salts.

With a variety of complexes in hand, their fluorescence behaviour and lipophilicities were examined to assay their suitability for cell microscopy applications. All the complexes **4** and **6** showed the photophysical properties expected from ^{3}MLCT based emission of rhenium bisimine complexes. The complexes containing simple bipyridines and lipophilic pyridines showed the expected photophysical properties for such species, with broad excitation maxima around 350 nm, and broad emission maxima around 555 nm. The bathophenanthroline complexes showed the expected red shifts associated with more conjugated ligands of around 20 nm for excitation to approximately 370 nm and 10 nm emission to 565 nm. These higher excitation maxima are particularly attractive as the broad excitation spectra give good excitation characteristics in the visible, avoiding problems of UV tissue damage and poor tissue penetration. These data are



Scheme 3

Table 1 Luminescent properties of complexes **4** and **6**[9]

Compound	λ_{max} excitation (nm)	λ_{max} emission (nm)
4a[a]	360	554
4b[a]	355	554
4c[a]	358	552
4d[a]	365	556
6y[b]	375	566
6z[b]	378	563

[a] Solvent: MeCN. [b] Solvent: H_2O.

summarised in Table 1. The lipophilicities of the complexes were assayed by their interactions with liposomes, lipid bilayer vesicles which are often used as models for cell membranes.[10] If a solute is encapsulated in the vesicle, then after chromatographic separation from the initial solution, the ability of other species to penetrate membranes can be studied by their addition to the liposome preparation followed by examination of the interaction with the encapsulated solute. Similarly the rate at which an encapsulated species is able to leave the liposome can be studied by the interaction with solutions of species which are known to be membrane impermeable. In this study the membrane permeability of the complexes **4** and **6** was studied by their interactions with membrane impermeable fluorescence quenchers. The complexes bearing lipophilic chains **4a–c** appeared to reside mainly in the lipid membranes, as after exposure to preformed liposomes followed by GPC isolation, all the fluorescence was localised in the fractions containing liposomes, indicating that they were associated with the liposomes. Conversely, the bathophenanthroline sulfate derived complexes **6y,z** appeared to reside solely in the aqueous phase and be membrane impermeable. Exposure of preformed liposomes to an aqueous solution of these complexes led to no entrapment, whereas liposome generation using buffer solutions containing complex led to partition of the fluorescence between the liposomes and the small molecule fractions after GPC, typical of a non-permeable species.

Addition of water soluble, non-membrane permeable quenchers to **6y,z** encapsulated in liposomes gave no loss of fluorescence, whereas addition of the same species to **4a–c** incorporated into liposomes gave a significant but not total loss of fluorescence, interpreted as indicating that the rhenium resides at both the inner and outer boundaries of the membranes, and the quenchers are partially able to quench those at the outer membrane.

In order to test the viability of these species as fluorophores in cell microscopy their ability to act as microscopy stains was tested in *Spironucleus vortens*, a parasitic flagellate which is found in many species of fish.[11] These organisms were chosen as they are of interest in their own right as important pathogens which are little understood, but also as they are prolific feeders and there was an expectation that they would actively take up the complexes, allowing a preliminary investigation of their properties *in vivo* without considering cell-specific issues of active transport. Live cells were harvested by centrifugation, incubated with solutions of each complex in DMSO for 2 h (typically 10–40 μL of a 0.5 mM solution of fluorophore incubated with 200 μL of cell suspension) before being washed in buffer (PBS, pH 7.2), mounted in methylcellulose to restrict movement and examined by confocal fluorescence microscopy using a Leica TCS SP2 AOBS spectral confocal microscope system. The samples were imaged using

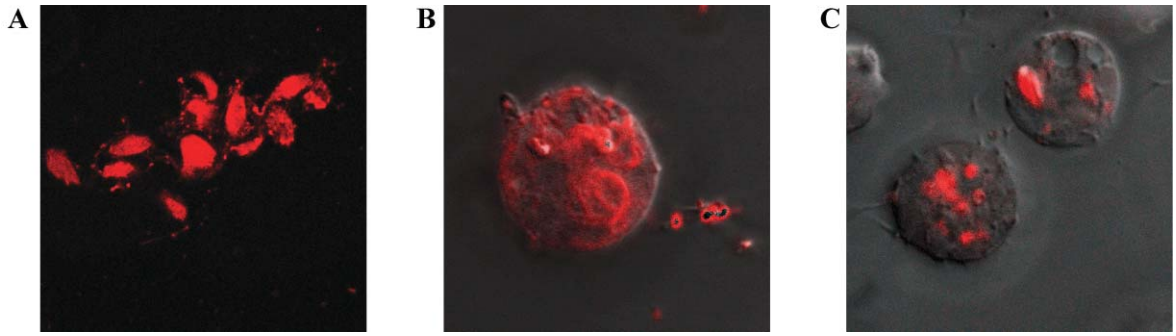


Fig. 1

excitation at 405 nm and images were recorded using detection between 520–570 nm, thus eliminating autofluorescence with a Stokes shift of < 115 nm, and giving sharp fluorescence images. The images are displayed with artificial colour fluorescence images overlaying white dispersed light images. The results of these preliminary experiments indicate that complexes of the type $[Re(bisim)L(CO)_3]^+$ may offer a viable fluorophore for cell imaging. The highly lipophilic species **4a–c** were toxic at high concentrations (40 μL of a 2 mM solution incubated with 200 μL of cell suspension), apparently disrupting the membranes and leading to cell lysis (Fig. 1(A)). The fluorescence was strongly associated with cell fragments, and at lower concentrations (20 μL of a 2 mM solution) lysis was avoided and the complexes appeared to be associated with internal membranes partitioning cell compartments and with the constituents within organelles (Fig. 1(B)). The simple bipyridyl complexes **4d** are more toxic than their sulfonated analogues, but again show accumulation in cells. It is worth noting that the neutral complex $[ReCl(phen)(CO)_3]$[12] was not only toxic but rapidly photobleached. This photobleaching is not observed *in vitro* and is not simply a result of photo-assisted hydrolysis, as the analogous aqua complexes are highly emissive. Significantly, no photobleaching was observed with any of the other dyes under the experimental conditions, indicating that this is associated with the chloride and that other Re complexes are resistant to photobleaching. The more polar sulfonated complexes **6y,z** were the most successful species but showed a difference in toxicity between themselves. The hydroxymethylpyridine complex **6z** seemed to be non-toxic or at least of low toxicity (cells were alive and mobile after 2 hours) and it appears that localisation in certain cell domains, thought to be digestive vacuoles, is occurring by phagocytosis (Fig. 1(C)). The simple pyridine analogue **6y** was also accumulated in the cells, but seems to be toxic as all the cells stained with this species were dead. This series of results implies that there is no intrinsic problem with the toxicity of rhenium complexes of the type $[Re(bisim)L(CO)_3]^+$ *per se*, however toxicity may be associated with certain ligands, and the choice of ligands will be considered carefully in future studies targeting specific organelles.

In conclusion, *fac* rhenium tricarbonyl bisimine complexes $[Re(bisim)L(CO)_3]^+$ have been shown to retain their useful fluorescence in biological systems, and in certain cases have been shown to have, at the worst, low toxicity, making them promising candidates for the design of specific cell imaging agents.

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