A covalently linked phenanthridine–ruthenium(II) complex as a RNA probe†

Naphtali A. O'Connor,[a] Nathan Stevens,[b] Diana Samaroo,[c] Marissa R. Solomon,[b] Angel A. Martí,[d] Joanne Dyer,[b] Harshad Vishwasrao,[e] Daniel L. Akins,[f] Eric R. Kandel[e] and Nicholas J. Turro*[b]

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A phenanthridine derivative covalently linked to a ruthenium complex yields an imaging probe whose fluorescence intensity and lifetime change substantially in the presence of RNA.

Time-resolved detection is emerging as an attractive approach in cellular imaging that can overcome the low signal to background (S/B) ratios caused by autofluorescence.[1] To take advantage of this technique there is a need for molecular probes exhibiting relatively long-lived emission lifetimes (>10 ns) that are longer than the lifetimes of autofluorescence typical of cells. Among probes with such lifetimes, phenanthridine derivatives, such as ethidium bromide and propidium iodide (PI), are probably the most widely used for detecting the presence of duplex nucleic acids.[2] When bound to duplex DNA or RNA, the fluorescence intensity of these probes increases greater than 20-fold and their lifetimes increase to about 20 ns.[3,4] Fluorescence lifetimes of 20 ns, though longer-lived than cellular autofluorescence, are still not sufficiently long to take full advantage of time-resolved detection techniques where lifetimes of >100 ns are desired.

However, among nucleic acid probes, very few have been shown to exhibit lifetimes of the order of 100 ns, with an important exception being ruthenium and lanthanide metal ligand complexes.[5–7] These complexes commonly exhibit lifetimes extending into the microsecond time domain. As such, they are ideal probes for time-resolved detection since virtually all autofluorescence signals have decayed at these relatively long time scales.

In this report, we describe the properties of an RNA probe, RuEth, that is composed of a phenanthridine moiety covalently linked to a ruthenium(II) isothiocyanate modified complex, RuITC (Fig. 1). RuEth was obtained by the reaction of RuITC with an amine functionalized ethidium derivative[8] (ESI†). RuEth possesses attractive advantages such as RNA binding, fluorescence intensity enhancement properties of phenanthridine derivatives, and the longer fluorescence lifetimes of ruthenium bipyridine complexes through a spin-forbidden resonance energy transfer (SF-RET) process.[9] These features make RuEth an excellent probe for RNA detection using time-resolved fluorescence spectroscopy. Using a combination of steady-state and time-resolved spectroscopic techniques, the luminescence properties of RuEth were examined and evaluated. Additionally, uptake into mammalian cells was demonstrated through confocal imaging and is shown to give the greatest signal in regions of cells where RNA is known to localize.

The absorption spectrum of RuEth in Fig. 2 shows the absorption due to the RuITC MLCT with a λ_{max} of 458 nm in the visible, with a broad phenanthridine absorption with a λ_{max} centred at 530 nm. Even though they are covalently linked, both fluorophores retain their individual absorption characteristics. The spectral overlap between the emission of RuITC (donor, Fig. 3a) and absorption of the phenanthridine intercalator (acceptor) is favourable for resonance energy transfer to take place. Such an energy transfer process to the phenanthridine intercalator has already been observed for another RNA probe that links fluorescein to the same intercalator.[10] The novelty of the RuEth probe resides in its ability to undergo SF-RET,[7] a process that occurs with a relatively slow energy transfer rate constant, making energy transfer the lifetime limiting step of the fluorescence of the phenanthridine intercalator.

In order to show the effect of RNA on the emissive properties of the uncoupled complex, RuITC, spectra were obtained in solutions with and without *Turolla* yeast RNA. The spectra of RuITC in those two environments can be seen in Fig. 3a. The spectrum of RuITC was essentially identical in both environments, except that in the presence of RNA, there was

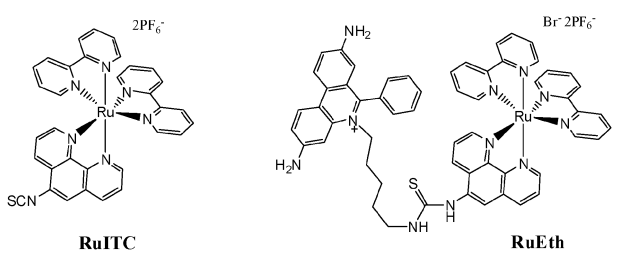

Fig. 1 The chemical structures of ruthenium isothiocyanate (RuITC) and the RNA probe RuEth.

[a] *Department of Chemistry, Lehman College of The City University of New York, Bronx, NY 10468, USA. E-mail: naphtali.oconnor@lehman.cuny.edu*
[b] *Department of Chemistry, Columbia University, 3000 Broadway, New York, NY 10027, USA. E-mail: njt3@columbia.edu*
[c] *Department of Neurology and Neuroscience, Weill Cornell Medical College, New York, NY 10065, USA*
[d] *Department of Chemistry, Rice University, Houston, TX 77005, USA*
[e] *HHMI, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA*
[f] *Department of Chemistry, City College of The City University of New York, New York, NY 10031, USA*
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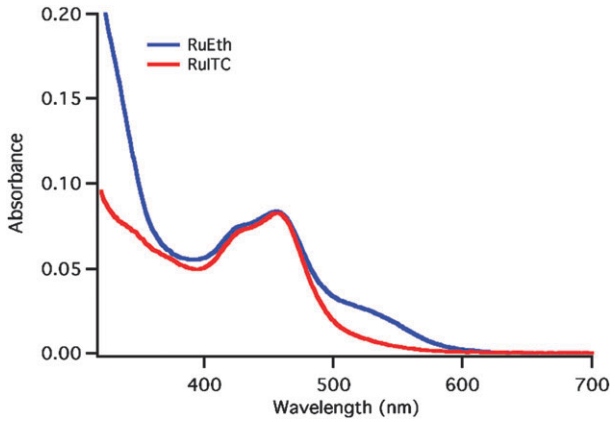

Fig. 2 Absorption spectra of RuITC (2.0 μM) and RuEth (2.0 μM) in tris buffer pH 7.5.

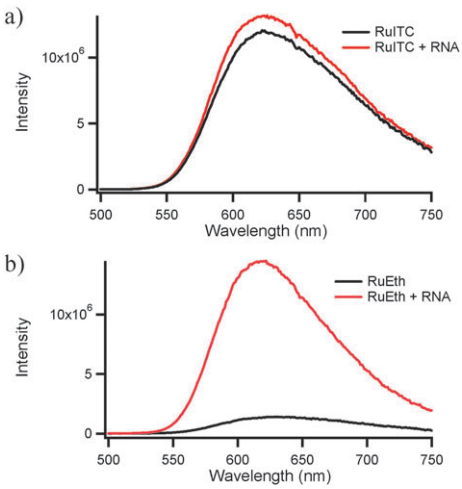

Fig. 3 Emission spectra of (a) RuITC (2.0 μM) and (b) RuEth (2.0 μM) in the presence and absence of *Turolla* yeast RNA. (λ_{exc} = 450 nm)

a slight increase in intensity. The RuEth complex, on the other hand, exhibited significant spectral changes when RNA is introduced to the sample (Fig. 3b). When no RNA is present, the emission intensity is much less intense than that of RuITC, which is a consequence of efficient SF-RET to the neighbouring phenanthridine group. Moreover, the fluorescence of the phenanthridine derivative is basically absent, since the phenanthridine excited state is efficiently quenched when in an aqueous environment. Upon addition of RNA to the solution, however, there is an order of magnitude increase in the emission intensity. The quantum yield for RuEth was found to be 0.016 ± 0.004 and 0.16 ± 0.004 in the absence and presence of RNA, respectively. Furthermore, the spectrum resembles that of the phenanthridine intercalator rather than RuITC. This result is indicative of the protection of the phenanthridine moiety from the aqueous environment by binding to the RNA polynucleotide.

To further characterize the fluorescence properties of RuEth, time-resolved emission studies were conducted. In the presence of RNA, RuITC exhibited a two-component lifetime of 347 ± 10 ns (78%) and 676 ± 10 ns (22%). RuEth also exhibited a two-component lifetime, but with values of

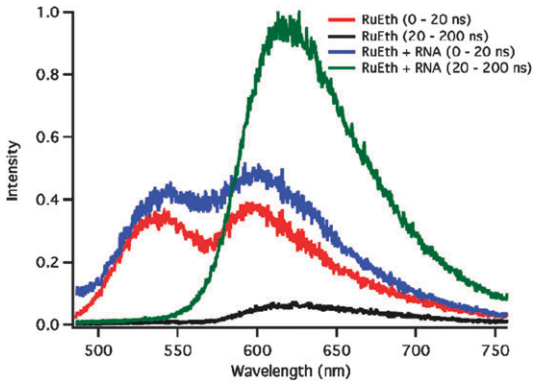

Fig. 4 Time-resolved emission spectra of RuEth (0.2 μM) in cell growth medium (CGM) with (blue, green) and without (red, black) *Turolla* yeast RNA. λ_{exc} = 450 nm.

96 ± 10 ns (88%) and 319 ± 10 ns (12%). The 96 ns component can be attributed to fluorescence from the intercalator, while the longer-lived emissive signal comes from the RuITC moiety. In solutions which contained no RNA, RuEth had a single component lifetime of 437 ± 10 ns.

The measured four-fold increase in the intercalator lifetime (96 ± 10 ns) compared to other phenanthridine derivatives (20 ns) is consistent with SF-RET from the ruthenium complex, which makes it especially useful for time-resolved detection in complex biological solutions, such as cell growth medium (CGM).[7] The autofluorescence of these solutions often overlaps with that of the probe, leading to poor S/B ratios when detected using steady-state fluorescence methods. In CGM, the steady-state S/B ratio of RuEth on addition of RNA was 3. Using the time-resolved method, which monitored the signal 20 ns after excitation, the S/B ratio was now increased to over 13 (Fig. 4). Such an increase in the S/B ratio occurs because in the time window monitored, the emission signal from the CGM has already decayed leaving only the signal from RuEth.

The affinity for duplex RNA and the ability to undergo fluorescence switching should make RuEth a suitable probe for *in vivo* imaging in cells. To test the validity of this possibility, the ability of RuEth to image mammalian breast cancer cells was evaluated. When the resulting emission of RuEth is imaged, as shown in Fig. 5b, it is apparent that fluorescence intensity is localized within certain cellular regions, when a comparison with the optical image (Fig. 5a) is done. Given the affinity of RuEth towards RNA, then

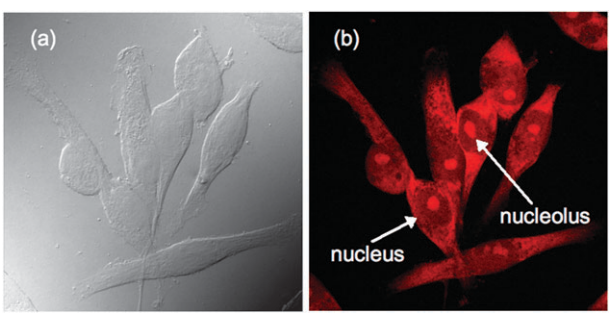

Fig. 5 Optical (a) and fluorescence (b) microscope images of RuEth uptake into mammalian cells.

regions exhibiting the greatest intensities should be those rich in polyribonucleotides. This is evident in the fluorescent image because the probe intensity is less in the nucleus, which contains DNA, than it is in the surrounding cytoplasm where mRNA is likely to be present. Furthermore, in the nucleolus (a dense region of RNA in the nucleus) the intensity of RuEth was found to be at a maximum.

In conclusion, by simply coupling RuITC to a phenanthridine fluorophore to produce RuEth we were able to create a RNA probe whose luminescence properties possibly make it superior to other probes for the detection of RNA under certain conditions. RuEth undergoes a nine-fold increase in signal intensity and has a fluorescence lifetime over times greater than other phenanthridine derivatives in the presence of RNA. Additionally, due to the relatively long-lived fluorescence, the S/B ratio can be increased from 3 to 13 using the time-resolved detection technique, in a complex biological solution. Cell imaging also shows the potential of RuEth to be employed as an *in vivo* probe for RNA. Studies on the binding of DNA with this probe are ongoing and will be shown in future work.

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