# Ruthenium Polypyridyl Peptide Conjugates: Membrane Permeable Probes for Cellular Imaging

Yann Pellegrin,[1] Ute Neugebauer,[1] Marc Devocelle,[3] Robert J. Forster,[1,2] William Signac,[4] Niamh Moran,[4] and Tia E. Keyes.[1,2]

[1] The Biomedical Diagnostics Institute, National Centre for Sensors Research, Dublin City University, Dublin 9, Ireland.
[2] The School of Chemical Sciences, Dublin City University, Dublin 9, Ireland.
[3] Centre for Synthesis and Chemical Biology, Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2.
[4] Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2.

**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 premeablisation of the cell membrane. Such ruthenium polypyridyl peptide conjugates open up the possibility for targeted cell delivery for environmentally sensitive intensity and lifetime imaging.**

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

The majority of probes used 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 for fluorescent lifetime imaging (FLIM).[1,2] Ruthenium polypyridyl complexes have unique photophysical properties which make them potentially invaluable as probes for cellular imaging. They are long lived, exhibit

polarised luminescence, have good photostability, red emission wavelengths and large stokes shifts and oxygen sensitivity. However, there has been a longstanding barrier to their exploitation in this context because conventional complexes do not typically passively diffuse across the cell membrane.[3] Therefore, the cells have to be permeabilised by electroporation, detergent or treated with some other transfection agent. [4] Recent examples include reports by Mycek et al on the $O_2$ sensitivity of $[Ru(bpy)_3]^{2+}$ in permeablised cells,[5] PEBBLEs (Probes Encapsulated By Biologically Localized Embedding) containing $[Ru(dpp(SO_4)_2)_3]^{2+}$ which are incorporated into permeablised cells,[6] and an immunological based approach reported by Tan et al on conjugation of $[Ru(bpy)_3]^{2+}$doped silica nanoparticles to antibody which permitted external labelling of cells rather than direct imaging.[7] It should be mentioned that, a very recent report on a ruthenium complex conjugated to an estradiol tag has been shown to be cell permeable attributed to the lipophilicity of the steroid pendant.[8]

Herein, we report on the preparation and characterization of a ruthenium polypyridyl peptide conjugate with an intense, long-lived emission in aqueous media which transports rapidly and passively across the cell membrane into the cell, without the requirement for potentially membrane-damaging procedures. The luminescent lifetime is sensitive to oxygen and pH and the emission wavelength shifts slightly with pH, making this material a potentially useful probe for multiplexed confocal scanning and fluorescence lifetime imaging of cells.[9] The application of solid phase peptide synthesis to ruthenium luminophores creates a highly versatile class of peptide conjugated ruthenium complexes which is open to many peptide combinations which may lead to targeted probes for fluorescence and fluorescence lifetime cellular imaging, whereas the ruthenium centre can be readily modified to target the environmental sensitivity of this probe.

Scheme 1 shows the two novel conjugates which are the focus of this investigation. In each case the parent ruthenium complex is $[Ru(bpy)_2(pic)]^{2+}$, where pic is 2-(4-carboxyphenyl)imidazo[4,5-f][1,10]Phenanthroline and conjugation occurs through the terminal carboxy unit.[10] Detailed synthesis is described in supplementary materials. Briefly, the $R_n$ oligopeptides (n = 5 or 8) were obtained via Merrifield's solid phase peptide synthesis, according to the Fmoc/*t*-Bu strategy. A hexamethylene spacer, Ahx, is inserted between the ruthenium luminophore and the polypeptide to avoid any unwanted

interaction between the peptide and ruthenium centre that could lead to quenching of the emission properties. This aliphatic linker is introduced after elongation of the peptide sequence, by N-terminal conjugation of 6-aminohexanoic acid which is fluorenylmethoxycarbonyl (Fmoc) protected. Finally, after Fmoc deprotection, the ruthenium complex, $[Ru(bpy)_2(pic)]^{2+}$, is attached to the resin immobilized peptide via amide bond formation through the terminal carboxyl functionality on the ruthenium complex, by PyBOP/HOBt/DIEA (Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate N-Hydroxybenzotriazole/N,N'-Diisopropylethylamine) coupling chemistry. After cleavage and deprotection by treatment with trifluoracetic acid, the ruthenium labelled peptide, was purified by reverse phase HPLC and its strcuture confirmed by MALDI-TOF mass spectrometry.

In this protocol, the coupling efficiency of the dye to the peptide exceeded 85%.[11] Such coupling efficiency is a significant improvement on more typical conjugation of organic fluorophores. We ascribe this efficiency to the reactivity of the aryl acid pendant on the ruthenium centre. The ruthenium dyes are functionalised through a single, reactive unequivocal group, through 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.[12]

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 has very little influence on the electronic structure of the Ru-complex, and therefore, the photophysical properties of the argenine derivatives are very similar to that of the parent complex $[Ru(bpy)_2(picH)]^{2+}$.[9] The photophysical properties of Ru-Ahx-$R_5$ and Ru-Ahx-$R_8$ are essentially indistinguishable. 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 ($\varepsilon$~$16.9*10^3$ $Lmol^{-1}cm^{-1}$) which can be 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 ($\Phi$= 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 permit 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 solutions the lifetime of the excited state of Ru-Ahx-$R_8$. drops to $\tau$ = 480 ns., which marks a good dynamic range for probing the concentration of dissolved oxygen..

Cellular uptake of Ru-Ahx-$R_5$, Ru-Ahx-$R_8$ and the parent complex, $[Ru(bpy)_2(picH)]^{2+}$, at 20$^o$C were investigated for myeloma cells as examples of mammalian cells, and human blood platelets using confocal laser scanning microscopy exciting in the metal-to-ligand charge transfer (MLCT) band of the Ru complex at 458 nm and recording the dye fluorescence around 610 nm. In a typical protocol 3µL of an aqueous solution of the Ru-complex ($[Ru(bpy)_2(picH)]^{2+}$, or Ru-Ahx-$R_8$ (1.2 x$10^{-3}$ M)) were added to 100 µl of the cell suspension to give a final dye concentration of 3.5 x$10^{-5}$ M. Figure 1 compares the ability of the plain Ru complex and the octo-arginine derivative to transport across a myeloma cell membrane. As expected, no luminescence could be observed from within the cell when incubating the cells with the parent complex $Ru(bpy)_2(picH)]^+$ (Figure 1c), indicating that the dye cannot penetrate the cell membrane and enter the cell, even after extensive incubation. In contrast, Ru-Ahx-$R_8$ transports passively through cell's membrane and accumulates and preconcentrates inside the cell. Transport is rapid, and the migration of the dye into the cell is complete within about 12 minutes yielding intense well contrasted cellular images as can be seen in Figure 1a and b. The first 5 minutes of the passive transmembrane transport of Ru-Ahx-$R_8$ into myeloma cells is shown in the accompanying video.[13] During the first two to three minutes, the dye concentrates in the cell-membrane. From the membrane, 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 about ten to fifteen minutes the process is complete and no further changes in 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 Figure 1d.

The effect of polyarginine chain length ($R_5$ and $R_8$) is demonstrated in Figure 2 with human blood platelets. Figure 2a and c show the white light images of platelets which have been incubated for 20 minutes each with Ru-Ahx-$R_5$ and Ru-Ahx-$R_8$ respectively. Figure 2b and d show the confocal scanning laser luminescence microscopy images of the same platelets excited at 458 nm and detecting the emission at 610 nm. While the platelets incubated with the octo-arginine derivative of the Ru complex show a bright luminescence from the cytoplasm (Fig. 2d), the platelets incubated with the penta-arginine derivative do not show any fluorescence from inside. This confirms that Ru-Ahx-$R_8$ can passively preconcentrate into the platelets, where it distributes in the cytoplasm. However, in contrast Ru-Ahx-$R_5$ cannot. 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.[14] Consistent with endocytosis we found the dye delivery in to the cell to be temperature dependent.[16] Thus, the inability of Ru-Ahx-$R_5$ to penetrate the cell membrane in this instant is consistent with other peptide studies indicating the peptide is indeed responsible for cell penetration.[15]

A drawback of conventional organic chromophores for laser scanning microscopy is their propensity to photobleach, which limits their use over extended periods and therefore in dynamic investigations of cellular processes. We investigated the photostability of Ru-Ahx-$R_8$ under continuous irradiation typically used in human blood platelet.[16] Under conventional imaging conditions, exciting at 458 nm, after 20 minutes continuous irradiation the dye bleached to less than 50% of its initial intensity. Such stability can permit the dynamics of cellular processes to be studied timescales which are useful to the microscopist/microbiologist in obtaining detailed or dynamic cellular information.

A key advantage of ruthenium polypyridyl complexes as 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. For example, conventional fluorescent imaging dyes are frequently relatively insensitive to $O_2$ because $O_2$ diffusion does not occur to any great extent on the short timescale of the dye's luminescent lifetime. 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 the 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 above, the luminescence of Ru-Ahx-$R_8$ exhibits significant oxygen dependence with a lifetime of 480 ns in air saturated phosphate buffered water compared with 775 ns in deaerated media, which provides a good dynamic range for probing the oxygen concentration inside living cells. Figure 3a shows a fluorescence intensity image of myeloma cells incubated with Ru-Ahx-$R_8$, indicating again that the dye has penetrated the cells. Figure 3b shows the false colour fluorescent lifetime image of the same cells. As can be seen as a first estimate from the false colour coding the lifetime of the dye varies across the various compartments of the cells. The dye residing in the membrane of the cell exhibits the shortest lifetime, which is in agreement with a higher solubility of $O_2$ in the membrane.

The lifetime of the residual dye in the external buffered solution monoexponential, however, the lifetimes of selected compartments within the cells are typically biexponential. The false colour image reflects the average lifetime of the probe.

In conclusion, we have presented two novel ruthenium polypeptide conjugates which are produced in high yield from solid phase synthesis. We demonstrated that an octoarginine 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 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 permit sensitivity to pH, water content and the rigidity of the microenvironment. Labelling of peptides assembled by solid phase synthesis can also be applied to sequences used as targeting devices and to bioactive sequences with inherent membrane translocation ability or conjugated to cell penetrating peptides.[17,18,19]

These ruthenium polypyridyl peptide conjugates open up the possibility for targeted cell delivery and dynamic lifetime imaging studies of the cellular environment.

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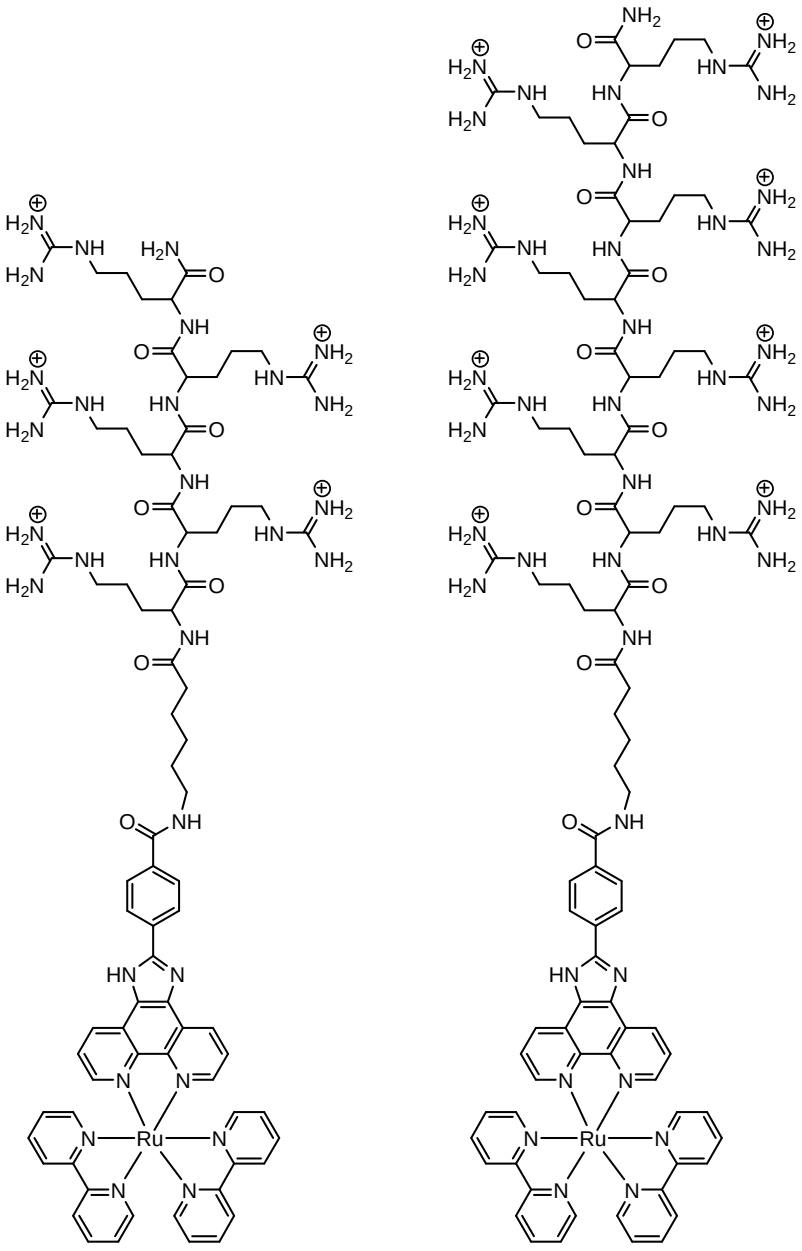


**Scheme I,** Structures of Ru-Ahx-$R_5$ and Ru-Ahx-$R_8$, the parent complex, $[Ru(bpy)_2(picH2)]^{2+}$, the aryl amide pendant on the imidazole ring is replaced by an aryl acid.

**Figure 1** (a) Myeloma cell incubated at 20$^{o}$C with Ru-Ahx-$R_8$, for 3 min in 100 µL of an PBS buffered solution of Ru-Ahx-$R_8$ 3.5 x$10^{-5}$ M (b) The same cell after 5 min, (c) Myeloma cell incubated with the parent complex $[Ru(bpy)_2picH]^{2+}$ (3.5 x$10^{-5}$ M) at 20$^{o}$C in PBS for 26 min. (d) Myeloma cell in PBS which has been permeablized with triton 1mmol prior to 5 min incubation with parent complex $[Ru(bpy)_2picH]^{2+}$.

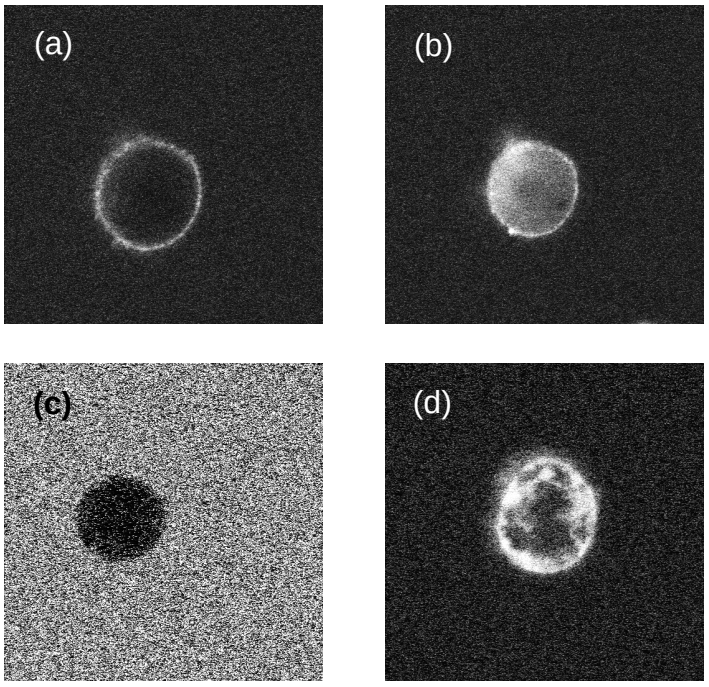


**Figure 2.** (a) & (c) White light image of human blood platelets following 20 minutes incubation with 3 $x10^{-5}$ M Ru-Ahx-$L_5$ and Ru-Ahx-$L_8$ respectively in PBS buffer, (b) Confocal luminescence image ($\lambda_{ex}$ 458 nm, $\lambda_{em}$ 610 nm) of platelets on incubation for 20 minutes with Ru-Ahx-$L_5$ (d) Confocal luminescence image ($\lambda_{ex}$ 458 nm, $\lambda_{em}$ 610 nm) of platelets on incubation for 20 minutes with Ru-Ahx-$L_8$.

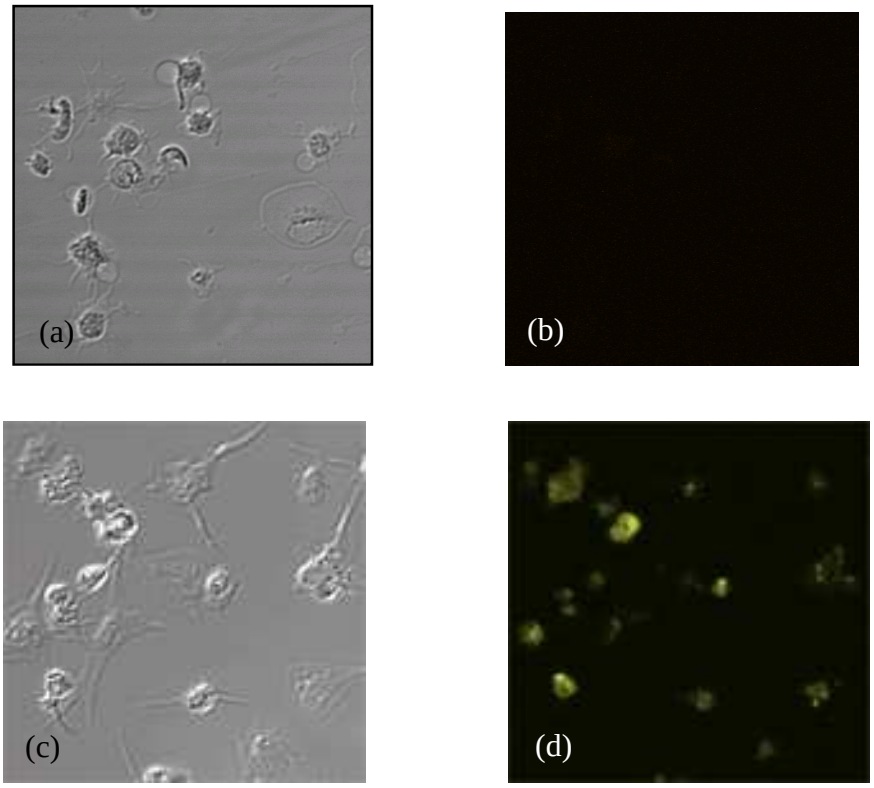
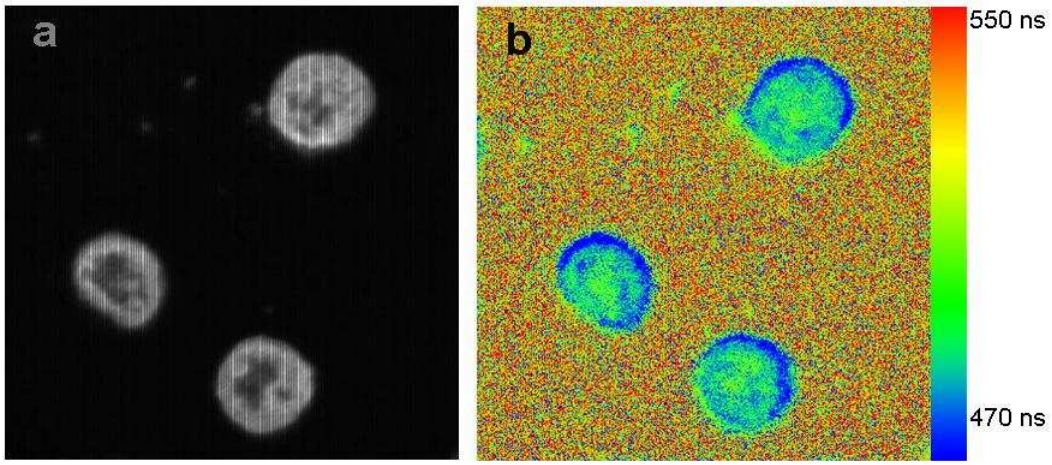


**Figure 3** a) Fluorescence intensity image of myeloma cells after incubation for 15 minutes with Ru-Ahx-$R_8$, 3.5 x$10^{-5}$ M in aqueous PBS buffer b) False colour fluorescence lifetime image of the same cell (fast FLIM)



## REFERENCES


1 W. Zhong, P. Urayama and M.-A. Mycek, J. Phys. D: Appl. Phys. 2003, 36 1689.

2 E. Musatkinaa, H. Amourib, M. Lamoureuxc, T. Chepurnykha C. Cordier, J. Inorg. Biochem., 101, 2007, 1086.

3 J R. Lakowicz, E. Terpetschnig, Z. Murtaza, H. Szmacinski, Journal of Fluorescence, 1997, 7, 17.

4 J. W. Dobrucki, J. Photochem. Photobiol. B 2001, 65(2-3), 136

5 3 references here W. Zhong, P. Urayama, M.-A. Mycek Journal of Physics D: Applied Physics 2003, 36, 1689.

6 H. Xu, J. W. Aylott, R. Kopelman, The Analyst 2002, 127(11), 1471.

7 S. Santra, P Zhang, K. Wang, R. Tapec, W Tan, Anal Chem., 2001, 73,4988.

8 K. K.-W. Lo, T. K.-M. Lee, J. S.-Y Lau, W-L. Poon, S.-H. Cheng, Inorg. Chem., 2008, 47 200.

9 Y. Pellegrin, R.J. Forster, T.E. Keyes, Inorg. Chim. Acta, 2008, 27, 6, 1690.

10 G.-Y. Bai, B. Dong, Y.-Y. Lü, K.-Z. Wang, L.-P. Jin, L.-H. Gao, J. Inorg. Biochem. 2004, 98, 2011

11 In a typical protocol, 50 μmol of resin, produced an average weight of 7 mg of the desired product after purification, with excellent reproducibility

12 R. Fischer;O. Mader; G. Jung; R. Brock Bioconjugate Chem. 2003, 14, 653-660

13 See video –supplementary material

14 S. M. Fuchs, R. T. Raines Biochemistry 2004, 43, 2438 ;
S.M. Fuchs, R.T. Raines, Cell. Mol.. Life Sci., 2006, 63, 1819.

15 S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, Y. Sugiura J. Biol. Chem.2001, 276(8), 5836-5840

16 Supplemental materials

17 Arch Immunol Ther Exp, 2005, 53, 47–60

18 L. Zhang; T. J. Falla Expert Opinion on Pharmacotherapy, 653-663, 7(6), 2006

19 S. Fulda; W. Wick; M. Weller; K.-M. Debatin Nature Medicine, 2002 8(8), 808-815