Two-photon uncaging of neurochemicals using inorganic metal complexes

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Neuroactive compounds can be photoreleased by means of two-photon excitation using a new kind of transition metal-based caged compound.

Phototriggers have increasingly been used in recent years. Their capability to release "caged" molecules through light excitation turns them into a powerful tool to deliver substances with a very high spatial and temporal resolution. One of the fields in which caged compounds are widely used is neurobiology. The possibility they offer to achieve subcellular localization without invasive electrodes or picosyringes together with the new techniques for optical imaging of neuronal activity open a new promising field of all-optical control of neuronal circuits. Caged compounds comprise two main parts: the molecule to be photoreleased and the protecting group. Nitrobenzyls are the most widely used protecting groups. In this kind of phototrigger, UV light (<350 nm) is used to break the relatively high-energy bond between the active compound and nitrobenzyl groups. For example, glutamate, GABA, glycine and other major neurotransmitters and their analogs have been caged likewise. In most studies, caged compound excitation is achieved by photon absorption at these wavelengths, with light being focused on the preparation through microscope optics. This method of excitation allows a precise delivery of the neurochemical with high lateral resolution.[1–3] Unfortunately, the depth of the excitation cannot be precisely defined. This occurs because the excitation light beam is active not only at the single focal point but also in the adjacent bi-cone that triggers photorelease above and below this point.

In some recent works, however, a new strategy has been developed in order to obtain better spatial resolution in the axial direction. By means of a high instantaneous power laser it is possible to generate two-photon absorption on the irradiated molecule, a process frequently forbidden. As the probability of two-photon excitation scales with the square of light intensity, which itself diminishes nonlinearly with increasing distance from the focal point, the two-photon excitation can only occur in a very small zone at the focal point.[4–6] Another advantage of this technique is that the excitation is generated using low energy IR photons rather than UV light. IR light scatters much less than UV-Vis light in living tissue. Two-photon release of the inorganic neurotransmitter NO has recently been achieved by Ford and coworkers.[7]

In our previous work,[8] a new kind of caged compound based on metal coordination chemistry was presented. Ruthenium bipyridyl complexes can undergo ligand substitution when irradiated with visible light, without any radical species being produced. Ligands can be entire molecules, such as the organic neurochemical 4-aminopyridine (4AP), a K^+ channel blocker. This type of compounds uses visible rather than UV photons to promote uncaging, allowing at the same time chemical, redox and photophysical tuning if changes to any of the bipyridyl ligands are introduced.[9,10]

We show in this paper that metal based caged compounds are also capable of undergoing two-photon excitation using very low energy IR photons, enabling their use as phototriggers for organic biologically-active compounds in new, highly precise, two-photon techniques.

The procedure to obtain $[Ru(bpy)_2(4AP)_2]Cl_2$ (Ru4AP) was described in our previous work.[8] UV-Vis spectra in water were obtained with U-2000 spectrophotometer (Hitachi Instruments, Parsippany, NJ). The irradiation of the samples was made by means of a Chameleon pulsed laser from Coherent (Santa Clara, CA), at an average power of 900 mW at 725 nm and 350 mW at 950 nm, providing 120 fs pulses with a 90 MHz repetition rate. The focusing required to achieve high instantaneous IR intensity was attained using a custom-made laser scanning microscope.[11] Fluorescence spectra were taken using an Ocean Optics diode array spectrofluorometer (model USB2000).

As a preliminary test, a 100 μM solution of Ru4AP was irradiated by means of the pulsed laser focused approximately at the central point of an UV-Vis cuvette. The orange fluorescence of the sample was apparent only in the focal volume, indicating effective two-photon excitation. Fig. 1a shows the fluorescence

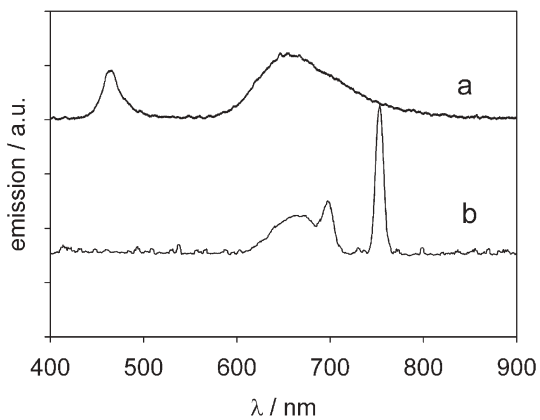

Fig. 1 Fluorescence spectra of Ru4AP in aqueous solution. (a) Excitation at 480 nm. (b) Excitation at 750 nm (two-photon absorption fluorescence). A spurious fluorescence at 690 nm from the laser is apparent.

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spectrum of Ru4AP with 1 photon excitation (λ = 480 nm). In order to take the two-photon fluorescence spectra, a glass micropipette was filled with approx. 1 μL of a 250 mM aqueous solution of the complex, attached to the microscope slide, and irradiated with 725 to 950 nm light through a 0.8 NA 40× water immersion objective. The results can be seen in Fig. 1b.

A similar experiment was performed to detect the photocleavage of the complex. The pipette was irradiated for ~30 min and the resulting solution was dissolved in 2 mL of water. Fig. 2 shows the absorption spectra of Ru4AP before and after irradiation with high power 740 nm light. The irradiated sample shows a similar change to those samples irradiated at 473 nm that yield free 4AP and the aquo complex as cleavage products.[5]

In order to test that the decomposition was effectively a two-photon process, the beam was defocused, keeping the same average power. No change in absorption spectrum was seen in this case. The irradiation at 950 nm did not show noticeable cleavage activity using our experimental set-up.

Direct irradiation of Ru4AP crystals with 750 nm light also produced photobleaching of the fluorescence emission in the solid phase. This behavior is also compatible with the photocleavage of Ru4AP yielding free 4AP and the non-fluorescent aquo complex.

In order to obtain direct proof of 4AP two-photon photorelease, a capillary containing about 5 μL of Ru4AP 500 mM in D_2O was irradiated over 1 h with a pulsed Ti–Sapphire laser through a 10× microscope objective. A NMR spectrum of this irradiated sample was taken with a Bruker 500 MHz spectrometer. A non irradiated blank was also measured. The results are shown in Fig. 3. The doublets that appear at 6.87 ppm and 8.08 after 2P irradiation are due to the hydrogens of the free 4AP. The identity of this product was verified by addition of free 4AP as internal standard. Just one pair of doublets is also seen in this case, showing that the unique photoproduct was 4AP. The slight shift in the doublets when 4AP concentration is increased is due to the change in pH of the solution.

Finally, irradiation of Ru4AP with 365 nm light was also performed to compare the two-photon process with the one-photon decomposition. A 100 W Xe lamp was filtered with a water/$CuSO_4$ filter to stop IR, a lowpass at 325 nm and a bandpass centered at 365 nm and focused on a quartz cuvette. A ferrioxalate actinometry was performed to measure the radiant power of this source, which was found to be 3.73 mW.

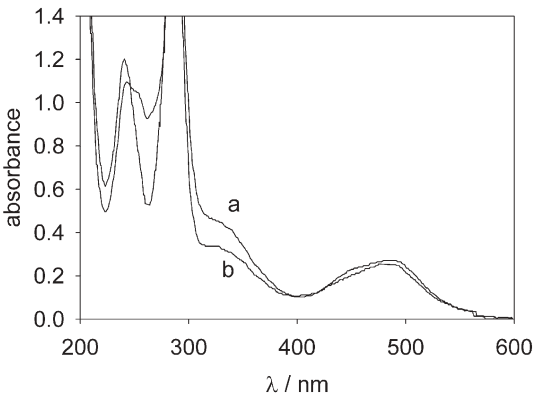

Fig. 2 Absorption spectra of Ru4AP in aqueous solution before (a) and after irradiation (b) with a 740 nm highly focused beam.

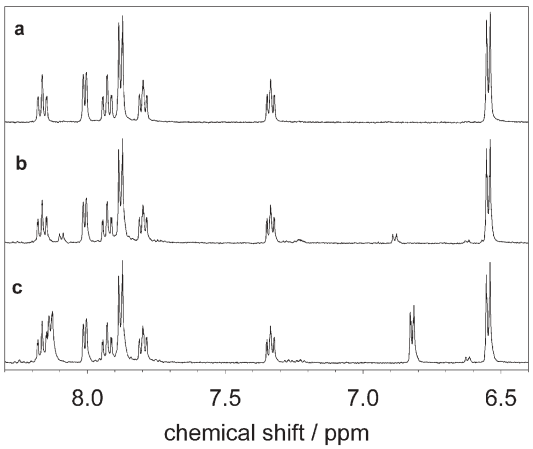

Fig. 3 (a) Ru4AP NMR spectrum before two-photon irradiation. (b) Ru4AP NMR spectrum after 1 h 2P irradiation (P_{avg} = 300 mW, λ = 800 nm, τ = 100 fs, f = 80 MHz) through a 10× objective. (c) The same spectrum after 4AP addition as internal standard. The small shift in free 4AP doublets between spectra (b) and (c) are due to the change in pH produced by the 4AP added.

A 250 mM solution of Ru4AP was irradiated using this source and the UV-Vis spectra were taken at different intervals. The photodecomposition is shown in Fig. 4. The presence of two isosbestic points suggest that only two species are involved. Although the final spectrum resembles the initial one, the differences are clearly noticeable. The final spectrum is similar to both that obtained by irradiation at 473 nm and by two-photon uncaging (Fig. 2b), all corresponding to the monoaquo complex.[8]

Complete spectral analysis allowed us to measure the degree of conversion and thus the quantum yield of photocleavage which was found to be ϕ_{365} = 0.049, slightly higher than ϕ_{473} = 0.029.[8]

In conclusion, we have demonstrated that two-photon uncaging of 4AP from its ruthenium bipyridyl complex is possible in physiological conditions, allowing the use of this new family of caged compounds with two-photon microscopes. The irradiation at 720 nm, which excites the 360 nm band, leads to photocleavage while the activity at 950 nm was not noticeable, probably due to a very low two-photon action cross section at this wavelength. Two-photon fluorescence was detected in solution or solid form at

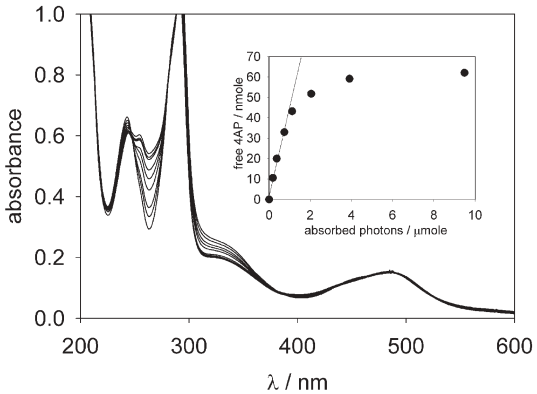

Fig. 4 Absorption spectra of 22.4 μM aqueous Ru4AP irradiated at 365 nm. Inset: Conversion plot using data from absorption spectra through complete spectral analysis.

720 nm, 800 nm and 950 nm excitation. The photoproducts are free 4AP and the complex $[Rubpy_2(H_2O)(4AP)]^{2+}$, as shown by UV-Vis and NMR spectra. The action cross section for the uncaging of 4AP was not precisely measured, but a gross estimation based in the NMR 2P photolysis indicates that it is in the order of 0.01 to 0.1 GM at 800 nm.

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Notes and references

1 E. M. Callaway and L. C. Katz, *PNAS*, 1993, **90**, 7661.
2 D. Schubert, J. F. Staiger, N. Cho, R. Kötter, K. Zilles and H. J. Luhmann, *J. Neurosci.*, 2001, **21**, 3580.
3 E. M. Callaway and R. Yuste, *Curr. Opin. Neurobiol.*, 2002, **12**, 587.
4 M. Matsuzaki, N. Honkura, G. C. R. Ellis-Davies and H. Kasai, *Nature*, 2004, **429**, 761.
5 W. Denk, J. H. Strickler and W. W. Webb, *Science*, 1990, **248**, 73.
6 S. H. Wang, L. Khiroug and G. J. Augustine, *PNAS*, 2000, **97**, 8635.
7 S. Wecksler, A. Mikhailovsky and P. C. Ford, *J. Am. Chem. Soc.*, 2004, **126**, 13566.
8 L. Zayat, C. Calero, P. Albores, L. Baraldo and R. Etchenique, *J. Am. Chem. Soc.*, 2003, **125**, 882.
9 E. S. Dodsworth and A. B. P. Lever, *Chem. Phys. Lett.*, 1986, **124**, 152.
10 D. V. Pinnick and B. Durham, *Inorg. Chem.*, 1984, **23**, 1440.
11 V. Nikolenko, B. Nemet and R. Yuste, *Methods*, 2003, **30**, 3.

