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Intracellular viscosity increases during photoinduced cell death

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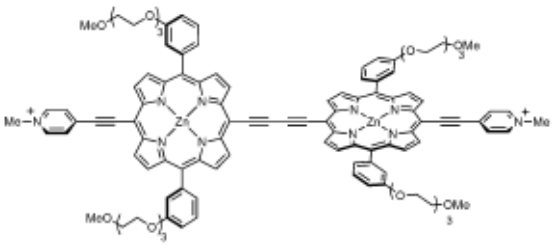
Introduction

Diffusion affects many important cellular processes, such as metabolism, signaling and mass transport. Viscosity is one of the major factors that determine the rate of translational diffusion in a cell. In addition, collisions and interactions that occur between the solute and intrinsic cellular macromolecules can play a role in determining cellular reactivty [1]. Values for the translational diffusion coefficient for both small solutes [1,2], and macromolecules [3] in mammalian cells have been identified as being 5-50 times smaller than those in pure water and we have recently demonstrated, that the local microviscosity in the hydrophobic domains of living cells can be as high as 200 cP [4]. Interestingly, and at the other extreme it has long been known that the microviscosity in the aqueous phase of the cellular cytoplasm is similar to that of pure water, 1-3 cP [5]. Such large viscosity variations within a single cell can be expected to play a major role in cell function, e.g. by influencing bimolecular reaction rates [6]. These factors might also play a role in developing strategies for drug delivery and cancer therapy.

Fluorescent molecular rotors, in which fluorescence response depends on the ambient viscosity, have emerged as promising probes for measuring the microviscosity in a biological environment [4,7]. Such rotors are capable of providing the viscosity information on a microscale, which is not available from the bulk measurements using classical rheological methods. Another advantage of studies using molecular rotors, which is due to the relatively rapid measurement times for fluorescence, is the ability to detect the evolution of intracellular viscosity during important biological processes in real time, as they occur. The simultaneous spatially- and time-resolved response of the rotors sets them aside from other frequently used methods based on excited state decay and quenching determination [6] or fluorescence recovery after photobleaching [8].

To enable accurate determination of viscosity, precise calibration of the response of fluorescent rotors to viscosity is needed. This can be achieved by either using fluorescence lifetime-based measurements [4] or a ratiometric approach [7]. Here, we use a new type of rotor, constructed as a conjugated porphyrin dimer **1**.



Recently, the group of porphyrin dimers of a similar structure has been exploited to create an efficient photosensitizer for one-photon and two-photon excited photodynamic therapy (PDT) of cancer [9,10]. In PDT, cell death and tissue eradication is achieved by irradiation of the photosensitizer and the subsequent production of cytotoxic species, in particular singlet molecular oxygen, $O_2(a^1\Delta_g)$.[21] Dimer **1** has been demonstrated to have high singlet oxygen quantum yield, high intracellular uptake, sufficient photostability, low dark toxicity and significant cell kill upon one- and two-photon irradiation [9,10].

Now, we utilize the properties of dimer **1** as both an efficient PDT photosensitizer and a fluorescent molecular rotor to monitor changes in the intracellular viscosity of light-perturbed single cells.

Results and discussion

The spectroscopic properties of **1** can be described in terms of two conformations: planar and twisted, which can interconvert by rotation of a porphyrin ring about a butadiyne linker [11]. Each conformation is characterized by distinctive absorption and emission spectra: the planar conformer absorbs and emits at lower energy and the twisted conformer emits at higher energy. The emission of **1** in solution of low viscosity, e.g. in methanol, is dominated by the lower energy planar conformation, whereas the emission of the less stable twisted conformer could be observed from glassy matrices or viscous solutions, where rotation of the two porphyrin units relative to each other is restricted.

The emission spectra for **1** in methanol:glycerol solutions of varying viscosity, ranging from 0.6 to 950 cP, are shown in Fig. 1. The following spectral changes occur with an increase in viscosity: the relative intensity of the emission maximum at 710 nm increases compared to the emission maximum at

780 nm. These observations are consistent with the formation of increasing amounts of the twisted conformer of **1**,[11] as torsional rotation about the butadiyne link becomes slower at high viscosity. Thus, we assign the emission maximum of **1** at 710 nm to the twisted conformation and the emission maximum at 780 nm to the planar conformation.

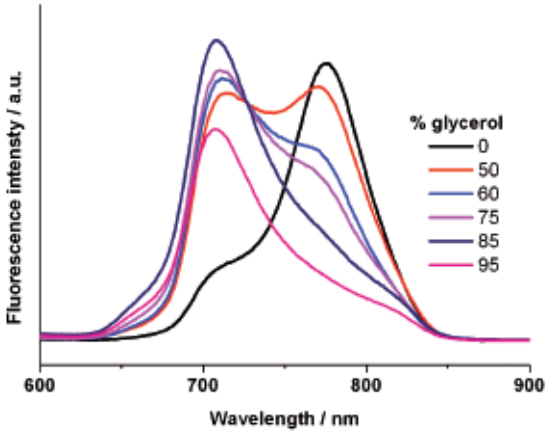


Figure 1. Emission spectra obtained upon 473 nm excitation of 1 in methanol/glycerol mixtures of different viscosity.

We have calibrated the viscosity-dependent rotor response of **1** following 473 nm excitation using a ratiometric approach, Fig. 2. The plot of the intensity ratio of the two fluorescence peaks of **1** against viscosity in double logarithmic coordinates shows a good linear correlation, as expected from theory.[23]

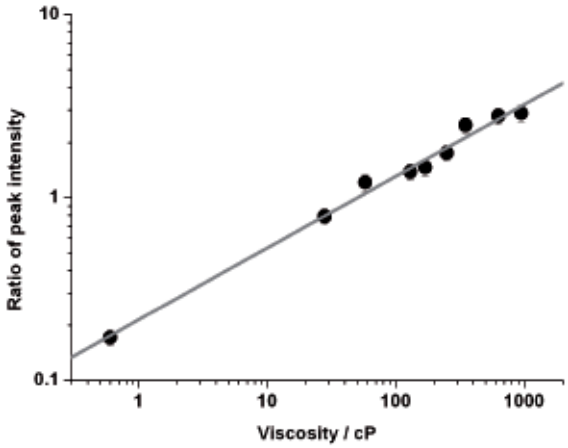


Figure 2. Double logarithmic calibration plot of the intensity ratio of the emission peaks at 710 nm and 780 nm vs the solution viscosity.

Dimer **1** is efficiently incorporated into live cells[10]. The fluorescence spectrum of **1** in a cell, Fig. 3, clearly shows two well-resolved maxima at ca. 710 and 780 nm, corresponding to emission from the twisted and planar conformations of the dimer, respectively. Using the viscosity calibration graph (Fig. 2), we ascertain that the viscosity of the intracellular compartments where **1** localizes is ca. 50 cP. A similarly high viscosity was reported for hydrophobic domains of live cells[4]. The fluorescence spectrum of **1** was also recorded in the bulk cell culture medium, where it binds to Bovine Serum Albumin (BSA)[10].

Here, the emission spectral shape shows only a slight deviation from that in non-viscous solutions. Thus, the spectrum of intracellular **1** must reflect a large ambient viscosity, not specific binding.

It is important to recognize that the emission spectrum of a fluorophore can also be influenced by factors other than viscosity. We have verified that solvent polarity does not significantly affect the shape of the emission spectra of **1**. The small blue shift in emission maximum is observed upon going from the least polar solvent dichloromethane (λ max = 820 nm) to the most polar solvent DMSO (λ max = 770 nm). The intensity ratio of the higher energy emission peak vs the lower energy emission peak, plotted against the solution viscosity in double logarithmic coordinates for solvents of different polarity, falls on the same straight line as that obtained in methanol/glycerol mixtures. It is important to realise that, in solutions of high viscosity, the ratio between the emission peaks is dependent upon the amount of light absorbed by the twisted vs the planar conformers of **1**. The spectral shift of the absorption bands of either of the conformers with polarity might result in significantly different values of apparent intracellular viscosity. Since the fluorescence maxima of **1** in cells are very close to those observed in the methanol/glycerol mixtures, and the absorption of both conformers of **1** significantly overlap following 473 nm excitation for all solvents studied, we conclude that such spectral shifts are minor contributors to ratiometric data obtained from **1** in cells.

Our data suggest that the extracellular viscosity calibration plot, obtained in methanol/glycerol mixtures, is a valid reference for the intracellular studies and thus gives the correct values of the intracellular viscosity.

The most important advantage of our work is that molecular rotors allow monitoring intracellular microviscosity in a dynamically changing environment, due to the relatively rapid fluorescence spectral measurements. We have used this advantage to record emission spectra of **1** inside cells during PDT, i.e. irradiation with light which ultimately results in cell death, Fig. 3.

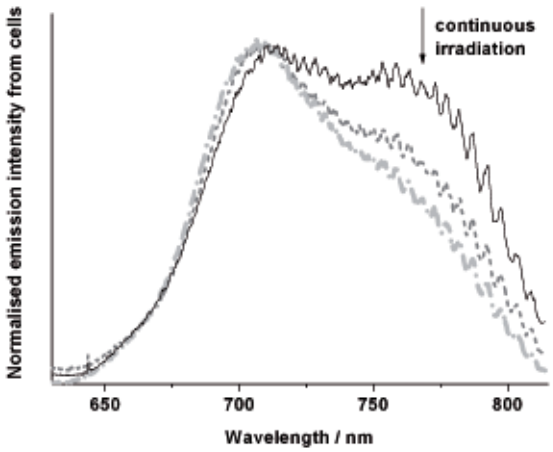


Figure 3. Emission spectra obtained upon 473 nm excitation of 1 in single cells during continuous irradiation at 473 nm (0.1 mW).

1 sensitizes the production of cytotoxic $O_2(a^1\Delta_g)$ upon irradiation with light and initiates cell death[10]. The emission spectra obtained upon irradiation of **1** in a single cell, Fig. 3, show significant increase in the emission intensity at 710 nm, which was assigned to the twisted conformation of **1**. This change corresponds to a significant increase in the viscosity of the intracellular environment surrounding **1**.

Importantly, the spectrum obtained for **1** in a cell culture medium does not change shape upon 473 nm irradiation, confirming again that the behavior observed for **1** in a cell is not a result of binding to proteins.

Using the calibration graph (Fig. 2), we determine the post-PDT viscosity of the cellular domains containing **1** as (300±50) cP. This value is significantly greater than that obtained prior to PDT, 50 cP. Such a viscosity increase could be the result of cross linking reactions, mediated by singlet oxygen or secondary reactive radicals, similar to those observed in model and intracellular protein systems[12]. Further work is required to definitively determine the reason for a marked viscosity increase in a dying cell.

Conclusions

We reported a new type of ratiometric fluorescent molecular rotor suitable for measuring intracellular viscosity in live cells. The rotor **1** enables real-time monitoring of dynamic processes in cells. The latter has been illustrated by quantifying a significant increase in intracellular viscosity during photoinduced cell death.

Acknowledgements

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References

1. H. P. Kao, J. R. Abney and A. S. Verkman, *J. Cell Biol*. **120** (1), 175 (1993).
2. M. A. O'Loughlin, D. W. Whillans and J. W. Hunt, *Radiat. Res*. **84** (3), 477 (1980); D. P. Jones, *Am. J. Phisiol*. **247** (1), C83 (1984).
3. J. W. Wojcieszyn, R. A. Schlegel, E. S. Wu and K. A. Jacobson, *Proc. Natl. Acad. Sci. USA*. **78** (7), 4407 (1981); O. Seksek, J. Biwersi and A. S. Verkman, *J. Cell Biol*. **138** (1), 131 (1997).
4. M. K. Kuimova, G. Yahioglu, J. A. Levitt and K. Suhling, *J. Am. Chem. Soc*. **130** (21), 6672 (2008); J. A. Levitt, M. K. Kuimova, G. Yahioglu, P-H. Chung, K. Suhling and D. Phillips, *J. Phys Chem. C*, DOI 10.1021/jp9013493 (2009)
5. J. A. Dix and A. S. Verkman, *Biophys. J*. **57** (2), 231 (1990); K. Luby-Phelps, *et al*., *Biophys. J*. **65** (1), 236 (1993); K. Suhling, *et al*., *Opt. Lett*. **29** (6), 584 (2004).
6. M. K. Kuimova, G. Yahioglu and P. R. Ogilby, *J. Am. Chem. Soc*. **131** (1), 332–340 (2009).
7. M. A. Haidekker and E. A. Theodorakis, *Org. Biomol. Chem*. **5** (11), 1669 (2007); M. A. Haidekker, T. P. Brady, D. Lichlyter and E. A. Theodorakis, *J. Am. Chem. Soc*. **128** (2), 398 (2006).
8. S. Seiffert and W. J. Oppermann, *Microscopy*, **220**, 20 (2005); M. J. Dayel, E. F. Y. Hom, A. S. Verkman, *Biophys. J*., **76**, 2843 (1999).
9. H. A. Collins *et al*., *Nature Photonics* **2**, 420 (2008).
10. M. Balaz, H. A. Collins, E. Dahlstedt and H. L. Anderson, *Org. Biomol. Chem*. **7**, 874 (2009); M. K. Kuimova, *et al*., *Org. Biomol. Chem*. **7**, 889 (2009); E. Dahlstedt, *et al*., *Org. Biomol. Chem*. **7**, 897 (2009).
11. M. U. Winters, *et al*., *J. Phys. Chem. C* **111** (19), 7192 (2007).
12. J. D. Goosey, J. S. Zigler and J. H. Kinoshita, *Science* **208** (4449), 1278 (1980); T. Dubbelman, A. Degoeij and J. Vansteveninck, *Biochim. Biophys. Acta* **511** (2), 141 (1978); H. Verweij, T. Dubbelman and J. Vansteveninck, *Biochim. Biophys. Acta* **647** (1), 87 (1981).