doi:10.2533/chimia.2025.259 Chimia 79 (2025) 259–262

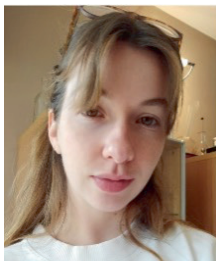
Enhancing the Brightness of Red-emitting Fluorophores in Aqueous Solution by Molecular Encapsulation

Liza Briant[§*] and Alexandre Fürstenberg[*]

[§]SCS-Metrohm Award for best oral presentation in Physical Chemistry

Abstract: Fluorescence spectroscopy and microscopy in biomolecular environments are usually performed in aqueous solution and preferably using red-emitting dyes. However, water quenches their fluorescence. We explore in this contribution how host-guest interactions between red-emitting fluorophores and macrocycles such as cyclodextrins and cucurbiturils can prevent quenching by shielding the dyes from water, thereby enhancing their brightness. We successfully apply this strategy in super-resolution imaging.

Keywords: Cucurbituril · Fluorescence · Fluorescence imaging · Super-resolution



Liza Briant is a PhD student in the Departments of Physical Chemistry and of Inorganic and Analytical Chemistry at the University of Geneva. She joined the group of Dr. Alexandre Fürstenberg in 2020 for her master's degree and decided to pursue her PhD in the same team. Her work focuses on the design and characterisation of encapsulated fluorophores for super-resolution microscopy.

1. Introduction

1.1 *Fluorescence Imaging*

Optical imaging beyond the diffraction limit, commonly termed super-resolution microscopy, has revolutionised light microscopy, enabling fine details within cells that were previously out of reach with visible wavelengths due to the diffraction limit to be resolved.[1–3] Among the super-resolution techniques, single-molecule localisation microscopy (SMLM) methods such as PALM (photoactivation localisation microscopy)[4,5] or (d)STORM ((direct) stochastic optical reconstruction microscopy)[6,7] rely on the detection of individual photo-switchable fluorescent molecules whose emissions are separated in space and time. The coordinates of the localised emitters are then used to reconstruct an image in a pointillistic manner.[8]

These fluorescent probes need to be attached to the target of interest and should ideally be photostable and as bright as possible, *i.e.* possess a large molar absorption coefficient and a large fluorescence quantum yield, as the achievable resolution directly depends on the number of photons that can be detected from every emitter.[9] In addition, in cellular environments, water-soluble red-emitting fluorophores are often a preferred choice to limit unwanted fluorescence arising from the sample or from impurities. However, it is often overlooked that water is in fact a quencher of the fluorescence of red-emitting dyes, thereby reducing their quantum yield and their brightness and potentially impacting the resolution of SMLM experiments.[10,11]

1.2 *Fluorescence Quenching by Water*

Our group has recently shown that H_2O can act as a fluorescence quencher due to the occurrence of efficient resonant energy transfer from electronically excited red-emitting dyes to harmonics and combination bands of water O-H vibrational modes (Fig. 1a).[12] Their fluorescence quantum yield is thereby divided by a factor of up to ~3 (Fig. 1b). On the other hand, the fluorescence is restored in heavy water (D_2O) or in solvents that do not possess any high-energy O-H vibrations and therefore do not display any measurable absorption below 900 nm[13] on a standard pathlength of 1 cm. This quenching mechanism is inefficient in dyes that emit at short visible wavelengths because their emission spectra do not strongly overlap with the absorption band of water (Fig. 1c). Furthermore, it was shown that the quenching efficiency was directly dependent on the number of water molecules in the first solvent sphere of the dyes, making their fluorescence lifetime a good representation of their local hydration (Fig. 1d), and explaining fluorescence enhancements observed with some of these dyes on peptide or protein surfaces.[14] Especially the oxazine dyes ATTO655, ATTO680, and ATTO700 (Fig. 2a), which all emit beyond 670 nm, were identified as promising sensors of their local hydration, compatible with single-molecule techniques such as SMLM.[15]

Although host-guest interactions were already shown to improve the photophysical properties of several dyes,[16,17] we rationalised that molecular encapsulation of red-emitting fluorophores could be a worthwhile general strategy to pursue in order to increase their brightness in fluorescence spectroscopy and microscopy experiments by physically isolating them from water.

*Correspondence: L. Briant, E-mail: liza.briant@unige.ch; Dr. A. Fürstenberg, E-mail: alexandre.fuerstenberg@unige.ch
Department of Physical Chemistry and Department of Inorganic and Analytical Chemistry, University of Geneva, CH-1211 Genève 4, Switzerland

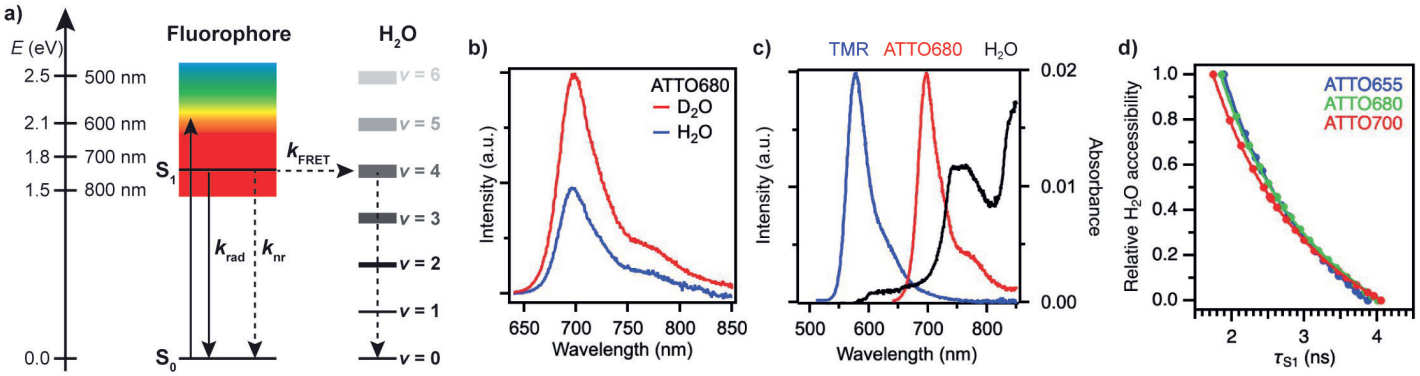


Fig. 1. (a) Scheme of the resonance energy transfer mechanism from the excited S_1 state of a fluorophore to vibrational overtones and combination bands of O-H vibrations explaining the quenching of red-emitting fluorophores by H_2O. Adapted from ref. [12]. (b) Fluorescence spectrum of ATTO680 at constant dye absorbance in D_2O and in H_2O, illustrating the quenching by H_2O. (c) Overlap between the intensity-normalized fluorescence spectra of the dyes tetramethylrhodamine (TMR, blue) and ATTO680 (red) in H_2O (left axis) and the absorption band of H_2O measured in a 1-cm cuvette (right axis, black). Adapted from ref. [12]. (d) Correspondence between the excited lifetime of a fluorophore, τ_{S1}, and the relative number of H_2O molecules in direct contact with the dye. Adapted from ref. [14].

2. Binding to Molecular Capsules

2.1 *Cyclodextrins*

Initial efforts by our group focused on interactions with cyclodextrins (CDs).[14] Cyclodextrins are cyclic macromolecules made of several glucopyranose units. They are commonly used in supramolecular chemistry for catetanes or rotaxanes, in drug delivery, as well as in pharmaceutical and food industries.[18,19]

The three natural α-, β- and γ-CDs are respectively made of 6, 7 and 8 units of glucopyranose (Fig. 2b). Only β-CD and γ-CD turned out to be large enough to accommodate the dyes ATTO655, ATTO680, and ATTO700, for which the fluorescence quantum yield and the lifetime were enhanced in their presence in aqueous solution (Fig. 3a).[14] The binding of the three fluorophores to the macrocycles was further supported by fluorescence polarisation anisotropy measurements in which the rotational correlation time of the dye significantly increased in the presence of cyclodextrins.[14]

The fluorescence enhancement could be attributed to shielding of the dyes from water and was more efficient with β-CD than with γ-CD (Fig. 3b). For the dye ATTO680, the fluorescence lifetime changed from 1.86 ns in H_2O to 2.50 ns and 2.33 ns for the dye bound to β-CD and γ-CD respectively. However, the association constant (K_a) for the formation of the complex between the dyes and cyclodextrins is in the order of 40–200, which is typical for these macrocyclic hosts, and was too low to be of practical use as saturation binding was not reached even at the solubility limit of the cages (15 mM for β-CD and 150 mM for γ-CD). In order to shield dyes from water more efficiently, other macrocyclic hosts with higher affinity for their guests were required, and cucurbiturils were identified as promising candidates.

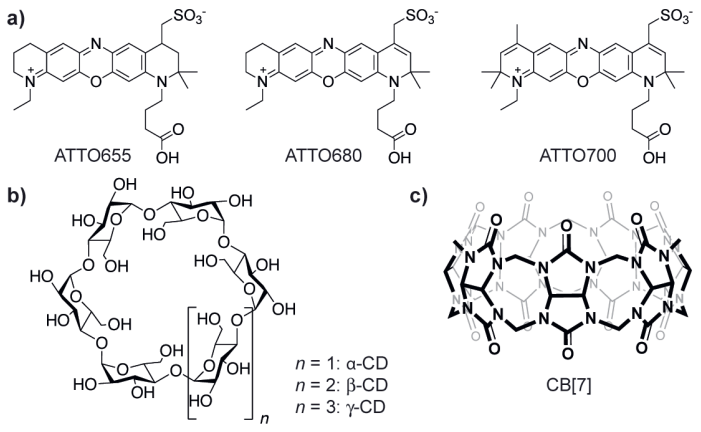


Fig. 2. Structures of the red-emitting dyes ATTO655, ATTO680, and ATTO700 (a) and of cyclodextrin (b) and cucurbituril (c) macrocycles.

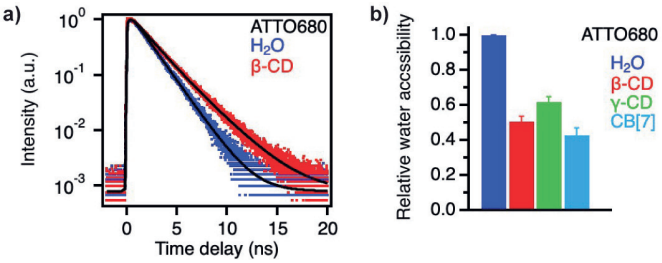


Fig. 3. (a) Fluorescence decays of ATTO680 in H_2O and in the presence of 15 mM β-CD. (b) Relative water accessibility of ATTO680 in H_2O and in the presence of 15 mM β-CD, 150 mM γ-CD, or 1 mM CB[7]. Adapted from ref. [14].

2.2 *Cucurbiturils*

Cucurbit[*n*]urils (CB[*n*]) are macromolecules made of *n* glycoluril units (Fig. 2c). Their carbonyl portals can easily accommodate cations while their hydrophobic cavities tend to bind organic compounds with high affinities ($K_a > 10^5$). In particular CB[7] has been shown to favourably interact with several dye molecules, improving their solubility or photostability in water, preventing aggregation, or enhancing their brightness.[16,17]

After the successful synthesis of CB[7] following the procedure of Nau *et al.*[20] its binding affinity to the three red-emitting dyes ATTO655, ATTO680 and ATTO700, was investigated.[21] Upon addition of CB[7], absorption and fluorescence spectra of the dyes displayed a hypsochromic shift of 4–8 nm (Fig. 4a and b), while their quantum yields relative to H_2O increased by 40–50% at a cage concentration of 1 mM (Fig. 4c), leading to an enhancement of their brightness in the complex. Also their fluorescence lifetime was enhanced by ~50% in the presence of CB[7] (2.68 ns for ATTO680, Fig. 4d).[21]

Binding of the dyes to CB[7] was supported by fluorescence polarisation anisotropy measurements, with the rotational correlation time of the dyes increasing in the presence of CB[7] (Fig. 4e). The association constants of the macrocyclic host for the dyes are equal to or larger than 2 x 10^5 and could be estimated by fitting 1:1 binding isotherms to the fluorescence lifetime data as a function of CB[7] concentration (Fig. 4f). These values however represent a lower limit as the values for the dissociation constant

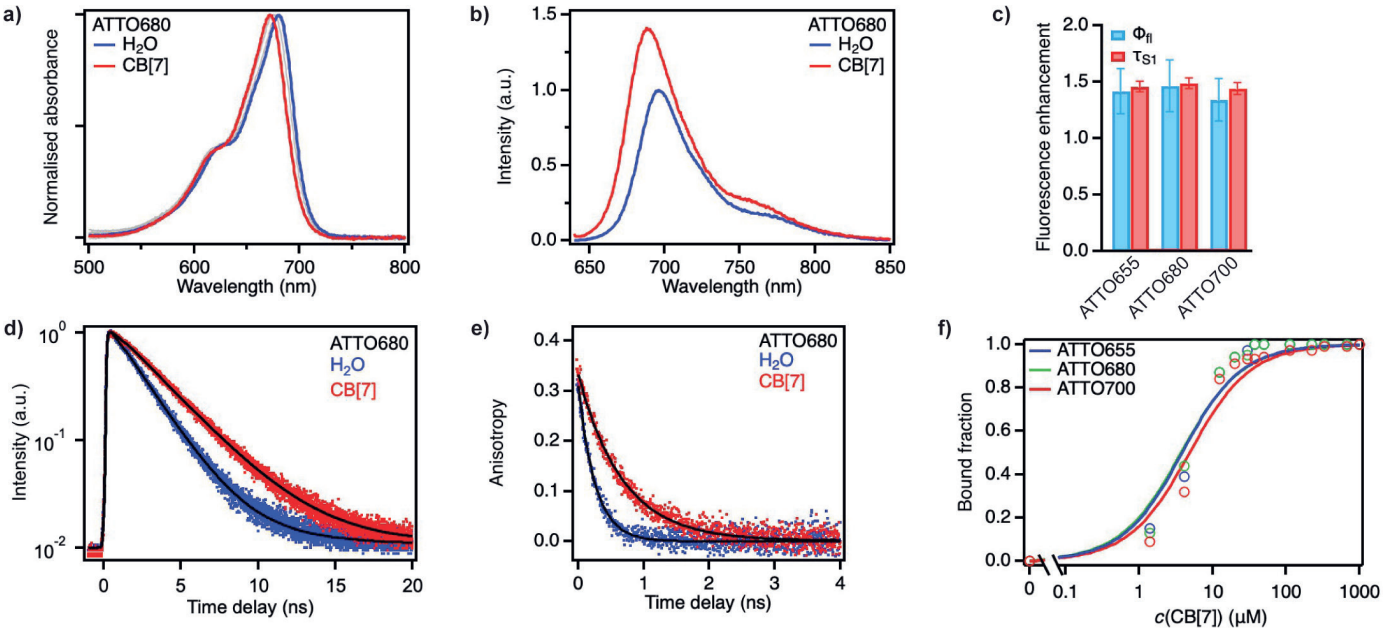


Fig. 4. (a) Intensity-normalized absorption spectra and (b) emission spectra of ATTO680 in pure H_2O and in the presence of 1 mM CB[7]. (c) Fluorescence quantum yield (Φ_{fl}, blue) and excited-state lifetime (τ_{S1}, red) enhancements of ATTO655, ATTO680, and ATTO700 when bound to CB[7] compared to the free dyes in H_2O. (d) Fluorescence intensity and (e) fluorescence polarization anisotropy decays of ATTO680 in pure H_2O and when bound to CB[7]. (f) 1:1 binding isotherms of the three investigated fluorophores in the presence of CB[7]. Adapted from ref. [21].

(K_d = 1/K_a) are quite close to the dye concentration used in the binding experiments (1–2 μM). Overall, these results show that the binding affinity of CB[7] towards these dyes is several orders of magnitude larger than for cyclodextrins. It is important to note that all measurements were performed in milliQ water, as salts hinder the binding of guests to CB[7].

Although cucurbiturils were reported to modulate the photophysics of bound dyes by changing their radiative lifetime through changes in the local polarity or polarizability,[22] the radiative lifetime of the three oxazine dyes was not affected by the cage. The fluorescence enhancement could rather be interpreted as protection of the dyes from water. From the measured fluorescence lifetime, it was estimated that 50–60 % of the contacts of the dye with water in the first solvent sphere were removed, making CB[7] the most efficient additive to shield these dyes from water described so far (Fig. 3b).

Motivated by these spectroscopic results, we aimed to assess whether CB[7] could enhance the number of photons emitted by ATTO655 in fluorescence microscopy experiments. To achieve this, we immobilised the dye on a glass surface and measured the number of photons emitted by each individual detected molecule, either in pure water or in a solution containing CB[7] at a concentration of 1 mM. The number of photons when CB[7] was added as an additive in solution (2200 ± 910 photons) turned out to be larger in comparison to pure water (1750 ± 590 photons).[21] The slightly lower enhancement in emitted photons at the immobilised glass surface (1.26) compared with the bulk could conceivably arise from the dye molecules at the surface having more limited accessibility for encapsulation.

Encouraged by this increase, CB[7] was next used in super-resolution microscopy experiments with the dSTORM technique. Microtubules of HeLa cells were stained with antibodies labelled with ATTO655 and photo-switching of the dye was induced using 50 μM of ascorbic acid, either in pure water or in a solution containing 1 mM of CB[7], but otherwise no salts. Super-resolved images could successfully be obtained (Fig. 5). The number of photons from every single-molecule in each frame was extracted, and also in this case, the distribution of photons displayed an increase of ~32% in the presence of CB[7] (3080 ± 1760 photons) compared to pure H_2O (2330 ± 1130 photons).[21] This enhancement in the brightness of ATTO655 when using CB[7] further led to an improvement of the localisation precision. Therefore, the use of the molecular cage did not affect the ability to perform imaging beyond the diffraction limit and, on the contrary, helped improve the localisation precision by inducing a fluorescence enhancement.

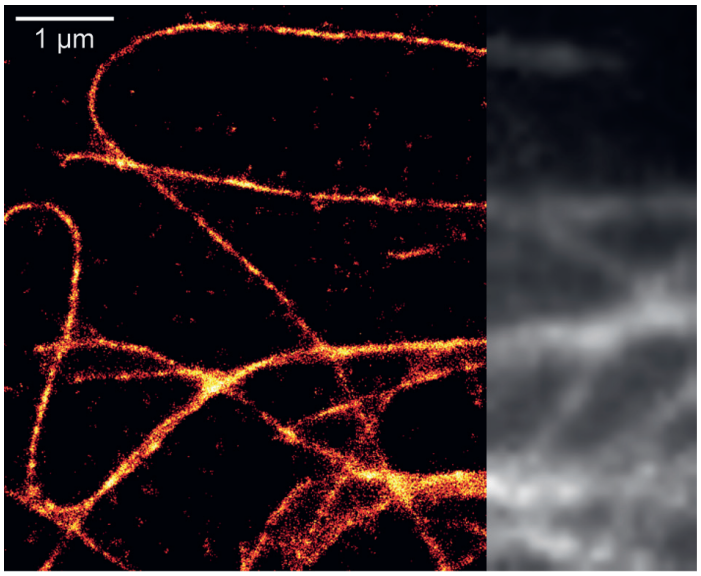


Fig. 5. SMLM dSTORM (left) and diffraction-limited (right) image of tubulin stained with an ATTO655-labeled secondary antibody in fixed HeLa cells in the presence of 1 mM CB[7]. Adapted from ref. [21].

3. Conclusions

We conclude that macromolecular cages can be used to prevent fluorescence quenching from water. Cyclodextrins usually have low affinity for guests and were shown to induce only limited protection with the three oxazine dyes ATTO655, ATTO680 and ATTO700. On the other hand, the strong binding of CB[7] to the same dyes allowed removal of 50–60% of the water molecules

from the contact sphere of the dye, improving the fluorescence properties accordingly. This cage could be used in super-resolution microscopy as an additive to increase the number of photons of red-emitting fluorophores and therefore the localisation precision. Although CB[7] has certain limitations, such as low solubility and the requirement to use pure water instead of buffers for imaging, it remains a valuable tool to fine-tune and improve the photophysics of red-emitting dyes used in fluorescence spectroscopy, standard fluorescence imaging, or super-resolution microscopy.

Acknowledgements

We are grateful to the Swiss Chemical Society and to Metrohm for the best-presentation award. We thank Claude Piguet, Eric Vauthey, Arnulf Rosspeintner and their teams for helpful discussion and experimental support. This work was supported by the University of Geneva, the Swiss National Science Foundation (project no. 205321_207482), and the Société Académique de Genève.

Received: February 5, 2025

[1] E. Betzig, *Angew. Chem. Int. Ed*. **2015**, *54*, 8034, https://doi.org/10.1002/anie.201501003.
[2] S. W. Hell, *Angew. Chem. Int. Ed*. **2015**, *54*, 8054, https://doi.org/10.1002/anie.201504181.
[3] W. E. Moerner, *Angew. Chem. Int. Ed*. **2015**, *54*, 8067, https://doi.org/10.1002/anie.201501949.
[4] E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, H. F. Hess, *Science* **2006**, *313*, 1642, https://doi.org/10.1126/science.1127344.
[5] S. T. Hess, T. P. K. Girirajan, M. D. Mason, *Biophys. J.* **2006**, *91*, 4258, https://doi.org/10.1529/biophysj.106.091116.
[6] M. J. Rust, M. Bates, X. W. Zhuang, *Nat. Methods* **2006**, *3*, 793, https://doi.org/10.1038/nmeth929.
[7] M. Heilemann, S. van de Linde, M. Schuttpelz, R. Kasper, B. Seefeldt, A. Mukherjee, P. Tinnefeld, M. Sauer, *Angew. Chem. Int. Ed.* **2008**, *47*, 6172, https://doi.org/10.1002/anie.200802376.
[8] A. Fürstenberg, M. Heilemann, *Phys. Chem. Chem. Phys*. **2013**, *15*, 14919, https://doi.org/10.1039/C3CP52289J.
[9] R. E. Thompson, D. R. Larson, W. W. Webb, *Biophys. J.* **2002**, *82*, 2775, https://doi.org/10.1016/S0006-3495(02)75618-X.
[10] S. F. Lee, Q. Vérolet, A. Fürstenberg, *Angew. Chem. Int. Ed*. **2013**, *52*, 8948, https://doi.org/10.1002/anie.201302341.
[11] K. Klehs, C. Spahn, U. Endesfelder, S. F. Lee, A. Fürstenberg, M. Heilemann, *ChemPhysChem* **2014**, *15*, 637, https://doi.org/10.1002/cphc.201300874.
[12] J. Maillard, K. Klehs, C. Rumble, E. Vauthey, M. Heilemann, A. Fürstenberg, *Chem. Sci*. **2021**, *12*, 1352, https://doi.org/10.1039/D0SC05431C.
[13] C. L. Braun, S. N. Smirnov, *J. Chem. Educ.* **1993**, *70*, 612, https://doi.org/10.1021/ed070p612.
[14] J. Maillard, C. A. Rumble, A. Fürstenberg, *J. Phys. Chem. B* **2021**, *125*, 9727, https://doi.org/10.1021/acs.jpcb.1c05773.
[15] S. Jana, O. Nevskyi, H. Höche, L. Trottenberg, E. Siemes, J. Enderlein, A. Fürstenberg, D. Wöll, *Angew. Chem. Int. Ed*. **2024**, *63*, e202318421, https://doi.org/10.1002/anie.202318421.
[16] R. N. Dsouza, U. Pischel, W. M. Nau, *Chem. Rev*. **2011**, *111*, 7941, https://doi.org/10.1021/cr200213s.
[17] W. M. Nau, A. Hennig, A. L. Koner, *Springer Ser. Fluoresc*. **2008**, *4*, 185, https://doi.org/10.1007/4243_2007_007.
[18] G. Crini, *Chem. Rev*. **2014**, *114*, 10940, https://doi.org/10.1021/cr500081p.
[19] S. A. Nepogodiev, J. F. Stoddart, *Chem. Rev*. **1998**, *98*, 1959, https://doi.org/10.1021/cr970049w.
[20] C. Marquez, F. Huang, W. M. Nau, *IEEE Trans. Nanobiosci*. **2004**, *3*, 39, https://doi.org/10.1109/tnb.2004.824269.
[21] L. Briant, J. Maillard, A. Fürstenberg, *Chem. Commun*. **2024**, *60*, 13943, https://doi.org/10.1039/D4CC05274A.
[22] J. Mohanty, W. M. Nau, *Photochem. Photobiol. Sci*. **2004**, *3*, 1026, https://doi.org/10.1039/b412936a.