Inclusion Complexes

Ultrastable Rhodamine with Cucurbituril**

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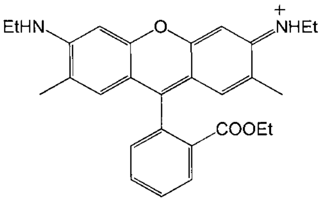
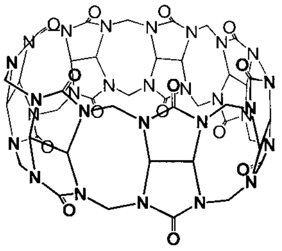
Rhodamines are arguably the most important fluorescent dyes as shown by their classical and contemporary applications,[1–8] for example, in dye lasers, as quantum counters, as photosensitizers, for spectral calibration in fluorometers, in single-molecule detection, as imaging agents for biomolecules, for scanning confocal microscopy, in fluorescence correlation spectroscopy (FCS), and in high-throughput screening assays. The various applications are made possible through the combination of their water solubility, close-to-unity quantum yields, high extinction coefficients, and very high photostability. Rhodamine 6G (Rh6G) is the most prominent derivative and has become a prototype for many reasons,[3,4] including its photostability: the first continuous-wave dye laser, for example, was based on an aqueous solution of Rh6G containing 1.5% triton X as surfactant.[9] Efforts have been made to improve the fluorescence and lasing characteristics of rhodamines, in particular in regard to their stability towards adsorption, aggregation, and photochemical decomposition, by derivatization or use of additives.[3,7] These approaches have frequently had limited success and often sacrifice other advantageous features of the chromophore.

We now describe the development of an ultrastable rhodamine derivative through the complexation of Rh6G by cucurbit[7]uril (CB7).[10,11] The additive leads to an unprecedented long fluorescence lifetime and an exceptional supramolecular stabilization, including the suppression of undesirable aggregation, the prevention of surface adsorption, and a



rhodamine 6G (Rh6G)



cucurbit[7]uril (CB7)

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substantial decrease in the quantum yield of photobleaching. These features enable the applicability of this water-soluble dye to be expanded.

Cucurbiturils are macrocyclic host molecules which can form inclusion complexes with organic guests, with the strongest complexes formed with positively charged guests.[12–14] Although their supramolecular chemistry has recently been intensively investigated, relatively few investigations have dealt with photochemical investigations such as their effect on fluorophores,[11,15–21] and their complexation behavior with classical fluorescent dyes has not yet been addressed. The addition of CB7 to solutions of Rh6G in water results in the immediate formation of a 1:1 host–guest complex (as established by Job's plots) with a high binding constant ($>50000\,\text{M}^{-1}$, by UV/Vis titration). Low concentrations of CB7 (1 mM) are therefore sufficient to ensure a virtually quantitative complexation of the fluorescent dye at the most relevant nano- to micromolar concentrations of the dye. The formation of the complex is also reflected in a decrease in the diffusion coefficient of the dye from $2.80\times10^{-6}\,\text{cm}^2\,\text{s}^{-1}$[22] to $(1.27\pm0.05)\times10^{-6}\,\text{cm}^2\,\text{s}^{-1}$, as determined by FCS. The characteristic upfield ^{1}H NMR shifts on complexation,[12,14,23] and in particular the marked change in the photophysical properties (see below), provide further evidence for the formation of an inclusion complex in which Rh6G experiences a drastically altered microenvironment. The addition of 2,3-diazabicyclo[2.2.2]oct-2-ene, which is known to form a strong inclusion complex with CB7,[11,20] leads to dissociation of the Rh6G complex through competitive binding. This result provides further experimental support that the Rh6G complex with CB7 is of the inclusion type; the size of Rh6G, however, prevents the dye from being completely immersed.

Complexation of Rh6G by CB7 (1 mM) causes a sharpening and a bathochromic shift of the absorption band (Figure 1). The latter effect is characteristic for the immersion of the guest in a less polar environment, and is similar to that observed in *n*-octanol ($\varepsilon=10.3$).[24] The shift in the fluorescence (Figure 1) is less pronounced, and the resulting smaller Stokes shift suggests that relaxation of the dye through geometrical and solvent effects is smaller, as expected for inclusion in a less polar and more-confined environment.[25] Importantly, the fluorescence quantum yield remains unchanged and close to unity (0.89 ± 0.02).[24]

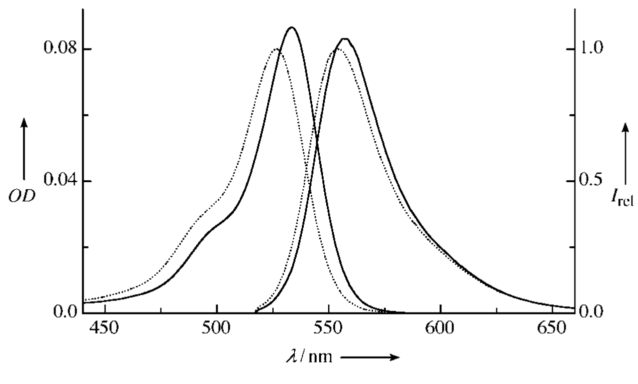

Figure 1. Absorption and fluorescence spectra of Rh6G (1 μM) in water in the absence (dotted lines) and presence (solid lines) of 1 mM CB7.

Strikingly, the fluorescence lifetime of Rh6G increases from 4.08 ± 0.03 ns in water to 4.76 ± 0.04 ns inside CB7. The latter value is the longest lifetime hitherto reported for this dye. The effect on the lifetime of Rh6G is opposite to that observed on its immersion in organic, less polar solvents, and the absolute increase is also more pronounced.[24] This special effect is related to the very low polarizability/refractive index (close to the gas phase) which a guest molecule experiences inside the CB7 cavity. We recently interpolated this low polarizability from solvatochromic effects experienced by an azo chromophore,[20,21] and the present result represents the first practically relevant example of what we have recently described as the supramolecular approach to radiative decay engineering.[21] According to the Strickler–Berg Equation,[26] the rate constant for the radiative decay of a chromophore (the ratio of the fluorescence quantum yields divided by the fluorescence lifetime) increases with the square of the refractive index, such that low environmental polarizabilities result in slower radiative decay rates and expectedly longer fluorescence lifetimes. Indeed, the rate of radiative decay decreases from $(2.18\pm0.02)\times10^8\,\text{s}^{-1}$ to $(1.87\pm0.06)\times10^8\,\text{s}^{-1}$ upon complexation of Rh6G by CB7, an observation which is in keeping with the lower polarizability inside the CB7 cavity.[20] In addition, the rate of the radiationless decay (obtained as the difference between the observed rate of fluorescence decay and the rate of radiative decay) decreases from $(2.7\pm0.1)\times10^7\,\text{s}^{-1}$ to $(2.3\pm0.1)\times10^7\,\text{s}^{-1}$ upon complexation by CB7. The slower radiationless decay of Rh6G in the CB7 complex ensures a constant fluorescence quantum yield regardless of the slower rate of radiative decay. The reduced rate of the radiationless decay can be related, among other things, to a partial protection from the bulk water, which is known to quench Rh6G fluorescence[8]—as evident by its longer fluorescence lifetime in D_2O (4.36 ns).[24,27]

The enhancement of the fluorescence lifetime of Rh6G by an additive is unique and could be of interest, for example, for assays based on fluorescence lifetimes,[28] as well as fluorescence lifetime imaging microscopy.[29] However, the addition of CB7 has benefits which go far beyond this photophysical peculiarity and are of more immediate relevance for practical use. First, CB7 reduces the photobleaching of Rh6G, a phenomenon which describes the tendency of fluorescent dyes to permanently lose their ability to fluoresce as a consequence of photon-induced chemical damage. Increasing the photostability of rhodamines either with additives or by structural modifications has remained an active field of dye research[3–5] since the photostability of a fluorescent dye is a key parameter, along with its brightness, for practical applications. This has been reinforced[3,4] in recent years by the development of single-molecule detection and scanning confocal microscopic techniques, for which chromophore stability is quintessential. Numerous additives for rhodamines have been tested to date which have in common that their photostabilizing properties are limited, that they operate either by preventing secondary photolytic steps, for example, as antioxidants to prevent oxidation, or by quenching undesired reactive triplet states, and that they have to be applied in high concentrations, which in turn may lead to undesirable fluorescence quenching effects.[3] If the use of organic solvents or surfactants are disregarded, none of the previously recommended techniques actually involves a discrete complexation of the dye and modification of the intrinsic rate constants for the primary elementary photoprocesses like CB7 does, thus establishing the current approach as a novel strategy for photostabilization in water.[3]

The photostabilization of Rh6G by CB7 is already sizable (a factor of ca. 1.5) at low levels of irradiation ($< 1\,\mathrm{kW\,cm^{-2}}$, photoreactor with lamps emitting visible light), but it becomes remarkably high at high levels of irradiation (ca. $10\,\mathrm{MW\,cm^{-2}}$, 10 Hz pulsed 2nd harmonic 532 nm Nd-YAG laser excitation, ca. 0.1 J pulse energy, pulse width ca. 10 ns), where two-step two-photon decomposition dominates.[4] As is apparent from Figure 2, there is a dramatic increase (factor of 30 ± 3) in the photostability upon addition of CB7, as quantified through absorbance-based quantum yields of photobleaching during the initial pulsed-laser photolysis period (at longer times absorbing photoproducts are formed,[25] which cause the graph to become nonlinear). The high levels of irradiation described in Figure 2 are most relevant for dye lasers, but also in scanning confocal microscopy, where irradiances of $1\,\mathrm{MW\,cm^{-2}}$ can be reached.[4] Apparently, CB7 selectively prevents the two-step two-photon photolysis, and it does not have the common drawbacks, namely, it does not act as a quencher, does not absorb in the visible region, and consequently displays no autofluorescence. These combined properties distinguish CB7 from other stabilizing additives.[3]

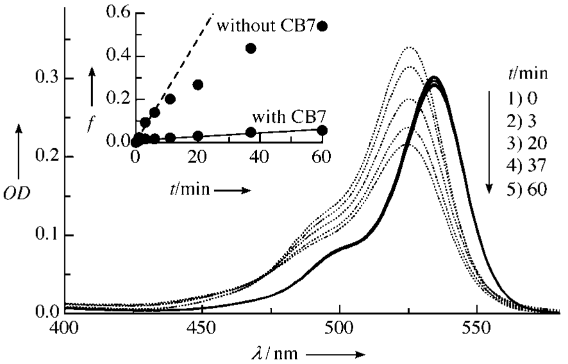

Figure 2. Photobleaching of Rh6G (ca. 4 μM) in aerated water in the absence (dotted lines) and presence (solid lines) of 1 mM CB7 followed through the decrease of the visible absorption with increasing time of pulsed 532-nm Nd-YAG laser irradiation. The inset shows the graph and correlation lines for the characteristic function $f=\log([10^{A_0}-1]/[10^{A}-1])$ versus irradiation time for the determination of the relative quantum yield of photobleaching (see Experimental Section). For the unstabilized solution, the correlation line refers to the initial 10-min time period because of the formation of absorbing photoproducts at longer times.

The higher photostability of Rh6G is presumably caused by a combination of factors. Cucurbiturils generally provide an extremely inert reaction medium; for example, it is very resistant to oxidation and even withstands ozonolysis.[30] In addition, the fluorescent dye is quite efficiently protected from water,[11] which may further reduce intermolecular follow-up (photo)chemistry,[25] including photoionization from higher excited states relevant in the two-step two-photon decomposition.[4]

There is another benefit of using CB7 as an additive to Rh6G solutions. It improves the stability of the emission by greatly increasing the thermal stability of the dye solution by preventing unspecific adsorption to glass and plastic surfaces. This was a previously documented problem for xanthene dyes.[4] The addition of CB7 to an aqueous solution of Rh6G prevents this adsorption virtually completely, such that the dye depletion can be conveniently quantified with reference to a CB7-stabilized solution. Figure 3 shows the temporal evolution of the steady-state fluorescence intensity of aqueous Rh6G solutions stored in different sample containers under ambient conditions and daylight. The CB7-stabilized Rh6G solution (1 μM) stored in a standard sample vial showed no significant loss of fluorescence intensity over a period of several weeks. In contrast, an unstabilized solution with the same concentration of Rh6G in the same vial showed a dramatic reduction in the fluorescence intensity. Some improvement (a factor of ca. 3 after 10 days) could be achieved by using quartz cuvettes instead of borosilicate vials, but the CB7-stabilized sample excelled (Figure 3). The stabilization factor can only be estimated in these cases (> 100), since the fluorescence behavior of the CB7 solution implied indefinite stability after two weeks storage, thus providing a gold standard. Additional experiments using polypropylene showed that this material was the poorest choice for handling and storage of Rh6G, unless CB7 was present; the tendency of Rh6G to adsorb to surfaces follows the order: polypropylene ≫ borosilicate glass > quartz glass. The depletion of the dye as a result of surface adsorption was, as expected, found to be most pronounced at very relevant low concentrations of the dye since at higher concentrations a surface-area-dependent adsorption–desorption equilibrium can be attained.[4] Clearly, the addition of CB7 greatly simplifies sample handling and eliminates a number of uncertainties related to the actual dye concentration studied, irrespective of the materials used. More importantly, the increased thermal (as well as photochemical) stability of CB7-containing Rh6G solutions allows the storage of aqueous Rh6G dye solutions, in particular of very dilute (nM)

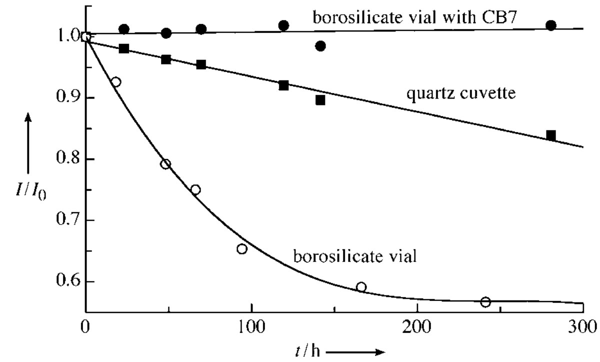

Figure 3. Temporal evolution of the fluorescence intensity of Rh6G (1 μM) in aerated water under ambient light in different sample containers.

solutions, for an extended period of time. This, therefore, allows the use of water as the solvent but eliminates the need to prepare the respective aqueous solutions freshly before each experiment, for example, for spectral calibration or as a diffusion coefficient reference in FCS. The addition of high concentrations of surfactants, which are similarly known to reduce unspecific adsorption but afford microheterogeneous solutions,[4] is also avoided.

In fact, we found that the prevention of unspecific adsorption becomes indispensable in confocal microscopy experiments (FCS mode). A routine sample preparation cycle (see Experimental Section) reduced the initial fluorescence intensity, assessed through the count rate, by nearly one order of magnitude compared to an identically handled CB7-stabilized Rh6G solution—from 96 to 13 KHz (Figure 4, $t=0$)—although the extinction coefficient of the uncomplexed Rh6G at the employed Ar/2 laser wavelength (514 nm) is about 50 % higher (Figure 1). In addition, the average number of molecules in the focal volume afforded directly by the FCS experiment decreased by a factor of 12 when CB7 was absent (from 5.6 to 0.46), thus suggesting that more than 90 % of the Rh6G molecules were actually already lost during sample preparation by adsorption to the plastic and glass walls in the absence of the stabilizer. Moreover, the count rate of the unstabilized Rh6G solution continued to deplete during the course of the on-going FCS measurements, while that of the CB7-stabilized solution remained quite stable with prolonged irradiation (Figure 4, note the logarithmic scale). The count rate after 35 minutes was nearly 50 times higher with CB7, which illustrates the importance of appropriately stabilized dye solutions for long-time FCS measurements.

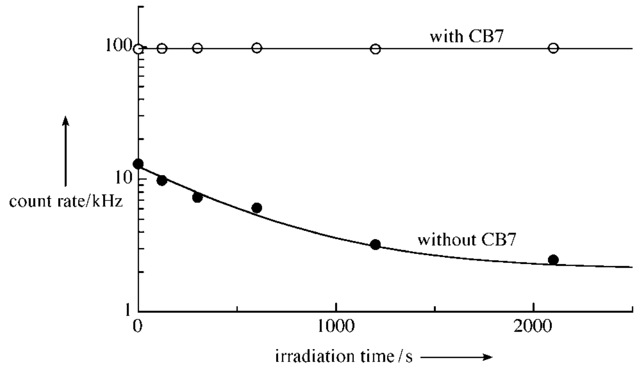

Figure 4. Dependence of the registered count rate of a Rh6G solution (10 nM) in the time course of an extended FCS measurement in a Lab-Tek 8-chambered borosilicate cover glass.

Finally, the formation of the supramolecular complex not only prevents surface adsorption, but also reduces adverse effects arising from aggregation of the dye, a phenomenon which comes into play at higher concentrations of dye that are relevant, for example, for dye lasers.[7,8] The addition of CB7 results ultimately in a stronger fluorescence of concentrated Rh6G solutions (> 10 μM), which in the absence of CB7 display a weaker fluorescence (and absorbance) than is extrapolated from lower concentrations as a consequence of Rh6G dimerization.[4] Addition of CB7 appears to suppress dye aggregation by selective formation of complexes containing only a single dye molecule. CB7 therefore plays an important role in breaking up fluorescent dye aggregates, thus providing an attractive alternative to the use of organic solvents or micellar solutions, the use of which is discouraged, for example, for dye lasers because of reduced dye photostability, poorer thermooptic performance, and economic as well as environmental considerations.[7,8,25] It should be noted that the photostabilization, adsorption, and FCS experiments (see above) were performed at low concentrations of the dye, where the deaggregating effect of CB7 is less important.

The concept of using a macrocyclic host to improve the solubility of Rh6G, enhance its photostability, and modify its photophysical characteristics is as simple as it is appealing. That the currently described supramolecular effects on the fluorescent properties of Rh6G are nevertheless nontrivial and critically dependent on the choice of CB7 as a unique host is readily seen by comparison with the effects of cyclodextrins as classical water-soluble hosts, which do not show the combination of desirable photophysical and photochemical effects. For example, their binding constants with Rh6G are very low, approximately only 200 M^{-1},[31] such that a significant fraction of dye remains uncomplexed even in solutions saturated with host.

In summary, the addition of CB7 to aqueous solutions of Rh6G has exposed a synergistic interplay of several advantageous effects, which include high brightness, increased fluorescence lifetimes, spectral shifts, increased photostability at low and in particular high levels of irradiation, and the prevention of unspecific adsorption and dye aggregation. These factors are of interest for multiple practical applications: for dye lasers, for storing dilute Rh6G solutions, in confocal microscopy, for biomolecule labeling (note that complexation of a fluorophore by CB7 in a labeled peptide has recently been reported),[11] for high-throughput screening assays, and in PCR with fluorescence read-out. Several of these applications are currently under detailed evaluation.

Experimental Section

Rh6G was obtained from Molecular Probes and CB7 was synthesized as reported.[10,11] All measurements were made in water under air. The photolysis of Rh6G solutions with and without CB7 at high levels of irradiation were carried out by using the 2nd harmonic (532 nm) output of an Nd-YAG laser (Continuum Surelite III 10 model, 0.1 J pulse energy). The absorbance of Rh6G was monitored on a Varian Cary 4000 UV/Vis spectrophotometer parallel to an optically matched unstabilized solution. For quantification of the relative photobleaching quantum yield, the decrease in the absorbance at 532 nm (A) at different irradiation times was plotted according to the pertinent function $\log([10^{A_0}-1]/[10^{A}-1])$, and the ratio of the slopes of the plots were corrected for different extinction coefficients at the irradiation wavelength to afford the relative photobleaching quantum yields.[32] The low-irradiance photolysis was performed with 1 μM solutions in a Luzchem LZC-4V photoreactor equipped with 14 cool white fluorescent tubes, and the relative quantum yield for photobleaching in the presence of CB7 was determined accordingly, but no corrections for differential absorbance were made as a consequence of the broad-band irradiation.

The long-term storage experiments under ambient conditions were performed by monitoring the decrease in the integrated steady-state fluorescence intensity (measured on a Varian Cary Eclipse fluorometer) of the stabilized and unstabilized Rh6G solutions (1 μM) in different sample containers. For the FCS experiments on a confocal microscope (Carl Zeiss LSM 510 Meta, Confocor 2), 10 nM Rh6G solutions were obtained by diluting a freshly prepared 1 μM dye solution with water or with a 1 mM solution of CB7 in an Eppendorf polypropylene standard reaction vial, and immediately transferring a 400 μL sample volume with an Eppendorf pipette/polypropylene tip into a Lab-Tek 8-chambered borosilicate cover glass (chamber walls are polystyrene). The count rate obtained in the course of prolonged 514 nm Ar/2 laser excitation (irradiance estimated as ca. 0.1 MW cm^{-2}) was then followed over time. A control experiment demonstrated that the addition of 1 mM CB7 did not significantly enhance the background count rate in the FCS mode.

The fluorescence lifetimes of 200 nM Rh6G solutions in the presence and absence of CB7 were measured by time-correlated single-photon counting on an FLS-920 fluorometer (Edinburgh Instruments) using a H_2-flash lamp ($\lambda_{ex} = 527$ nm, $\lambda_{mon} = 552$ nm) for excitation by reconvolution of the fluorescence decay with the instrument response function. The fluorescence quantum yield of the CB7-stabilized dye was determined from integrated corrected fluorescence spectra with reference to Rh6G in water ($\Phi_f = 0.89$).[24]

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