Supramolecular Chemistry

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Efficient Fluorescence Enhancement and Cooperative Binding of an Organic Dye in a Supra-biomolecular Host–Protein Assembly**

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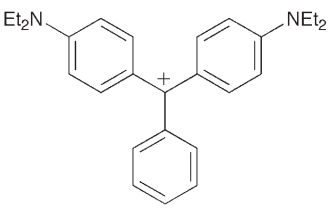
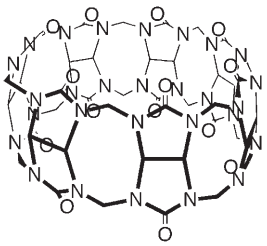
Variations in the radiative properties of fluorescent dyes through the formation of supramolecular host–guest assemblies or association with biomacromolecules such as proteins and oligonucleotides enable sensor applications and improve the understanding of the factors governing molecular recognition.[1] Macrocyclic hosts impose a structural confinement of the included chromophoric guest molecules, increase their thermal and photochemical stability, and isolate them from the bulk water, which together improves their radiative properties. The radiative lifetimes of triphenylmethane (TPM) dyes, for example, are very short (<1 ps) in low-viscosity solvents, but increase substantially upon binding with biomolecules as a consequence of altered photophysical characteristics.[2] These increases are accompanied by variations in the electron-transfer properties of the excited states,[2b,3] which are also relevant for their use as drugs in photodynamic therapy.[4] Furthermore, TPM dyes have been used as aptamer-based fluorescence sensors to assay the formation, location, or break down of RNA.[1d,5] We now demonstrate that the photophysical properties of the antimicrobial TPM dye Brilliant Green (BG) and its binding affinity to a biomacromolecule, namely bovine serum albumin (BSA), can be synergetically enhanced by the addition of a synthetic host molecule, namely cucurbit[7]uril (CB7). This finding opens up new opportunities to improve the biological and therapeutic activity of organic dyes by the addition of macrocyclic hosts as supramolecular "enhancers".



Brilliant Green (BG)



cucurbit[7]uril (CB7)

Cucurbit[7]uril, a synthetic pumpkin-shaped cation receptor, is a member of the host family of cucurbit[*n*]urils, which have recently attracted much attention because of their remarkably strong binding of organic dyes, protonated alkyl and aryl amines, and metal ions.[6–8] CB7, in particular, is known for its exceptional thermal and photochemical stabilization of fluorescent dyes.[9] In general, the binding by CB7 is thought to be driven by an interplay of hydrophobic interactions with the inner cavity as well as ion–dipole interactions with the carbonyl groups lacing the portals.

On one hand, CB7 shows a strong affinity for protonated amines, such that we expected the cationic BG dye to undergo strong interactions with CB7. On the other hand, the binding of TPM dyes with biopolymers is known to cause large enhancements in the fluorescence.[1d,2b] The aim of the present study was therefore to modify the radiative property of BG by introducing a combination of supramolecular (using CB7 as a macrocyclic host) and biomolecular (using BSA as a protein) interactions.

The complexation of BG in aqueous solution was followed by its spectral shifts upon addition of CB7. A bathochromic (ca. 5 nm) and hyperchromic shift in the wavelength and intensity, respectively, of the absorption maxima, and a hypsochromic (ca. 5 nm) shift of the emission maxima indicated the inclusion of the dye into CB7.[9] In addition, the fluorescence of BG increased about sixfold on complexation with CB7 (Figure 1). For comparison, no complexation of BG was observed when β-cyclodextrin was used as an alternative macrocycle, which has a similar capacity as CB7.

The exchange rates for guests in cucurbituril complexes are quite slow in relation to the nanosecond time scale of fluorescence.[8,9a] The fluorescence titrations (Figure 1) were consequently analyzed in terms of the contributions of the free and complexed dye in equilibrium;[9d] a Job plot confirmed a 1:1 stoichiometry and the binding constant for the corresponding BG·CB7 complex was found to be $(1.7\pm0.2)\times10^4\,\text{M}^{-1}$ (see the Supporting Information (Figure S2a)). On the basis of the dimensions of CB7 (inner diameter approximately 7.3 Å and a cavity height of about 9.1 Å)[6] and the dendritic geometry of the dye, we suggest a complexation of a single diethylamino aryl ring in CB7, which allows for favorable cation–dipole interactions. This proposal was confirmed by complexation-induced shifts of the signals in the ^{1}H NMR spectra (1 mM BG, 1 mM CB7, see the Supporting

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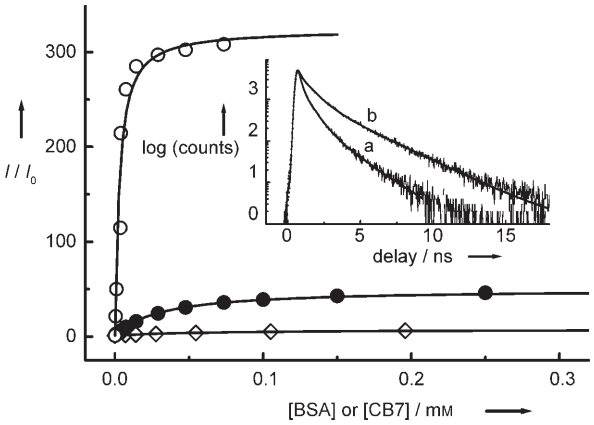

Figure 1. Plots of the fluorescence intensity ratio (I/I_0) for 1 μM BG with increasing receptor concentration: Addition of CB7 only (◇), BSA only (●), and BSA in the presence of 1 mM CB7 (○). The inset shows the corresponding time-resolved fluorescence decays for: a) 250 μM BSA only and b) 50 μM BSA in the presence of 1 mM CB7.

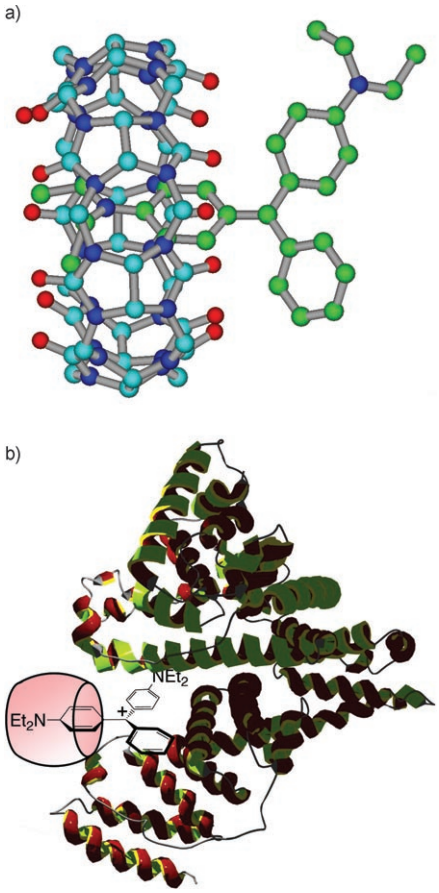

Figure 2. a) AM1-optimized geometry of the BG·CB7 complex and b) schematic representation of the fluorescent ternary complex BG·CB7·BSA.

Information (Figure S3)); shifts of about 0.6 ppm upfield were observed for one set of anilino aryl and ethyl protons, which is characteristic for inclusion in a 1:1 complex. The AM1-optimized structure of the BG·CB7 complex (Figure 2a) further supports these assignments. While the partial complexation (of a single aryl ring) may account for the moderate enhancement in the fluorescence with the synthetic receptor, it opens up the opportunity for the dye to undergo an additional interaction with a second, biomolecular receptor: BSA.

A bathochromic shift of about 10 nm was observed in the absorption and emission spectra upon complexation of BG with BSA alone (without CB7). The fluorescence intensity of BG was enhanced about 45-fold (Figure 1) and an effective binding constant of $(3.2 \pm 0.2) \times 10^4\,\text{M}^{-1}$ was determined by fluorescence titration; we employed a 1:1 binding model for fitting, in agreement with the approximate stoichiometry derived from a Job plot (see the Supporting Information (Figure S2b)). BSA consists of three homologous α-helical domains (I, II, III), which contain two isostructural subdomains (A and B) each.[10] The principal regions of guest (and dye) binding with BSA are located in the hydrophobic pockets of these subdomains. The large enhancement in the fluorescence of BG indicates a strong binding with these pockets; specifically, it reveals that the geometrical confinement hinders torsional motions of the TPM dye upon binding. These motions are known to induce rapid radiationless deactivation.[2b]

In the next step, we examined the molecular recognition between the preformed BG·CB7 supramolecular complex and BSA. For this purpose, the fluorescence intensity of BG (1 μM) was followed in the presence of CB7 (1 mM, corresponding to >94% complexation of BG) and an increasing concentration of BSA (Figure 1). As can be seen, a dramatic increase in the fluorescence yield by about a factor of 300 was observed (relative to free BG), which suggests that the fluorescence enhancements resulting from the individual components (factor of 6 for CB7 and factor of 45 for BSA) are cumulative (multiplicative) in nature, or lie even slightly above. Conversely, the fluorescence of the preformed BG·BSA complex (1 μM BG, 250 μM BSA, corresponding to >90% complexation of BG) could be further enhanced (by a factor of ca. 6) upon addition of CB7. Clearly, these results rule out a competitive binding (displacement), for which a less pronounced increase or a decrease in the fluorescence upon addition of the third component (BSA and CB7, respectively) would have been expected. Instead, the results corroborate the formation of a ternary BG·CB7·BSA complex.

We also performed time-resolved fluorescence measurements by time-correlated single-photon counting (inset of Figure 1). Although the fluorescence decays of BG in the presence of BSA were not monoexponential, as frequently observed for interactions of fluorescent dyes with proteins,[2b] the average fluorescence lifetime increased from 0.60 ns in the absence of CB7 to 1.51 ns in the presence of CB7. The fluorescence lifetimes of either free BG or the BG·CB7 complex were too short (<150 ps) to be measured with our equipment. This observation confirmed that a new supramolecular dye assembly with distinct photophysical properties had indeed been formed.

Strikingly, in the presence of CB7, the saturation of the fluorescence intensity was already reached at approximately 50 μM of BSA, whereas more than 200 μM of BSA were

required in the absence of CB7 (see Figure 1). The binding constant between BSA and BG·CB7 (assuming a 1:1 complexation because of the conditions of virtually quantitative complexation of BG by CB7) was estimated to be $(3.9 \pm 0.6) \times 10^5\,\text{M}^{-1}$, about one order of magnitude larger than in the absence of CB7 (see above). Very similar observations were made for the inverse titrations, that is, when the enhancement in the fluorescence of the preformed BG·BSA complex was studied with increasing CB7 concentration. The combined results provide evidence that the binding is positively cooperative (synergetic) in nature,[11] that is, the presence of both the supramolecular (macrocyclic) and the biomolecular component reinforce the dye complex and mutually enhance the binding constants of the other additive by an estimated factor of about 10. This effect is presumably due to the fact that the presence of CB7 causes additional favorable hydrophobic and electrostatic interactions, which visually "press" the dye even more deeply into the hydrophobic pockets of BSA. The cumulative fluorescence enhancement and cooperative binding jointly lead us to propose Figure 2b as an oversimplified representation of the strongly fluorescent BG·CB7·BSA ternary complex.

Measurements with BG and the alternative protein lysozyme (see the Supporting Information (Figure S4)) demonstrate that the observed synergy with CB7 may be more general. Thus, while the enhancement of the fluorescence with lysozyme alone amounted to a factor of only 10, it increased to a factor of 300 in the presence of CB7, which reveals that the enhancement with this protein is amplified even more strongly than with BSA.

In summary, fluorescent dyes serve as probes for biomolecular systems and in selected cases, such as the triphenylmethane (TPM) dyes studied herein, as promising drugs for photodynamic therapy and as antimicrobial agents. We were able to show that the binding strength (one order of magnitude increase) as well as the fluorescence properties (factor of 300 enhancement) of Brilliant Green, a representative TPM dye, with bovine serum albumin can be dramatically enhanced by the addition of the macrocyclic host molecule cucurbit[7]uril. The resulting cooperative binding of a dye and potential drug with biological target molecules opens up a new approach to improve medicinal activity or the sensitivity of fluorescent sensor applications by a supramolecular "enhancer" strategy. In the case of Brilliant Green, the formation of the ternary host–protein–dye complex can in part be rationalized mechanistically, because the macrocyclic host is too small to complex the entire dye, thereby leaving additional aryl sites for interaction with the protein. The effects of cucurbiturils on the binding affinities of dyes with additional proteins as well as on their antimicrobial activity and toxicity are now under investigation.

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