**pH-Responsive supramolecular assemblies of Hoechst-33258 with cucurbiturils: Modulation in the photophysical properties**

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*Received 24 December 2016*

Stimuli-responsive molecular assemblies of potential drug/guest molecules through non-covalent host-guest interaction have been found very attractive in transporting and releasing the desired form on demand. In this review article, the supramolecular interaction of a biologically important dye Hoechst-33258 (H33258) has been investigated in aqueous solutions at two different pHs (~4.5 and ~7), in the presence of macrocyclic hosts, namely, cucurbit[7]uril (CB7) and cucurbit[8]uril (CB8). The pH-dependent emission behaviour of H33258 is inherently connected with its protolytic equilibria which allows the dye to exist in different geometrical conformations. This pH-dependent structural orientation is greatly affected by the complexation with cucurbiturils. The strong ion-dipole interactions provided by the carbonyl portals of the CB7 host adequately stabilizes the CB7-H33258 complex, both in 1:1 and 2:1 stoichiometries at both the pH conditions. The non-covalently stabilized assembly brings out large enhancement in the fluorescence emission due to the unique structural orientation attained by H33258 partly within the CB7 cavity, which reduces the non-radiative relaxation pathways. The pH-dependent structural conformations of H33258 are found to be decisive in determining the stoichiometry and geometry of the supramolecular assembly. Interaction of H33258 with larger macrocycle, CB8, at pH 7 is found to be very strong. The non-covalently stabilized assembly with 2:1 (CB8: H33258) stoichiometry brings out ~26 fold enhancement in the emission yield. On the other hand, the strong ion-dipole interactions provided by the dicationic H33258 at pH 4.5 allows the CB8 to uptake two dicationic dyes in its cavity in a 1:2 stoichiometry, resulting in a quenching in the fluorescence emission. The distinct pH-mediated stoichiometric switching of CB8-H33258 complexes and the demonstrated contrasting fluorescence properties are expected to find application in the field of biomolecular imaging and exchange of included guests for a selective drug transport/release.

**Keywords**: Host-guest chemistry, Hoechst-33258, cucurbiturils, photophysical properties, stoichiometry

Molecular recognition among biomolecules through non-covalent interactions is ubiquitous in nature. In the realm of supramolecular chemistry, host-guest systems deliver the platform to study such non-covalent interactions such as hydrogen bonding, ion-dipole interactions, *etc.*, in their simplest form\(^3,5\). In contrast to traditional synthetic methods, supramolecular assemblies through host-guest strategy are being projected as a convenient approach since such non-covalent assemblies respond to external stimuli in unique and quantitative ways as an opportunity to tune the intrinsic molecular properties of the guests in the desired ways\(^6,7\). Response to various external perturbations such as a change in pH, change in the temperature or addition of metal ions/ competitive binding agents by the supramolecular host-guest systems were judiciously employed for designing novel stimuli-responsive supramolecular systems\(^5,7\). Over the years supramolecular approaches have proved to be exceedingly useful in applications such as in optical sensors\(^8\), on-off switches\(^9\), logic gates\(^9\), photo stabilization\(^10,11\), supramolecular catalysis\(^12,13\), drug delivery vehicles\(^14\), enzymatic assay\(^15\), nanocapsules\(^16\) and supramolecular architectures\(^8\). In this regard, various supramolecular systems involving preorganized synthetic receptors such as crown ethers, calixarenes, cyclodextrins and more recently cucurbiturils have been documented in the literature\(^2,17\). Interest in the family of cucurbit[\(n\)]uril (CB\(n\)) macrocycles has been growing rapidly because of their high binding affinity to a variety of guests such as metal ions, metal nanoparticles, cationic or neutral organic molecules, organometallics and even protein residues\(^1,2,18,19\). Cucurbit[\(n\)]urils (\(n = 5\)-8, 10 and 14) family of macrocyclic receptors are the methylene bridged cyclic oligomers obtained from the acid catalyzed condensation of glycoluril with formaldehyde in which the number of glycoluril units
determines the size of the cucurbituril cavity\textsuperscript{1,17,18,20,21}. CBn has two negatively polarized carbonyl laced portals and a hydrophobic cavity. Due to its unique structural features and depending on the size and charge of the guests, CBn can interact through strong ion-dipole and hydrophobic interactions\textsuperscript{1,17,18,21}. Among the CBn homologs, CB8 has the unique ability to accommodate simultaneously two guest molecules in its cavity because of its large cavity volume (479 Å\textsuperscript{3})\textsuperscript{1,17,21,22}. Contrary to CB8, while the lower homolog CB7 can bind to neutral or cationic aromatic residues, CB6 binds to cationic aliphatic molecules such as polyamino\textsuperscript{1,21,22}.

Our continuous effort in exploring the supramolecular aspects of the CBn based host-guest assemblies has resulted in deciphering some feasible such as functional materials, aqueous dye laser, fluorescent molecular capsule, on-off switches, drug delivery vehicles\textsuperscript{6,16,23-28}. Taking advantage of unique structural features of CBn family of cavatands, we have achieved enhanced photostability for well known rhodamine laser dyes, rhodamine 6G (Rh6G), rhodamine B and kiton red in aqueous solution through complexation with cucurbit[7]uril (CB7) and demonstrated the working of broad-band and narrow-band dye laser systems in aqueous solutions\textsuperscript{10,25,26}. Another advantageous feature of CBs lies in the preferential binding of different prototopic structures. Studies using dyes like neutral red\textsuperscript{29}, acridine orange\textsuperscript{30}, hydroxyphenyl benzimidazole\textsuperscript{31} and bichromophoric coumarin laser dyes\textsuperscript{32,33}, we have shown that the protolytic equilibrium can be affected to a large extent upon CB addition, bringing out desired \( pK_a \) shift for the dye in the complexed form. This feature, supported by the ability of metal ions to interact with the CB7 portals as a competitive binder allowed us to establish the effective relocation of neutral red dye from the supramolecular cavity of CB7 to a biomolecular pocket, simply by changing the ionic strength of the solution\textsuperscript{34}. Moreover, we have also demonstrated our non-covalent approach to modulate the photophysical aspects such as restricting ICT to TICT conversion in case of coumarin laser dyes, and for hydroxyphenyl benzimidazole system, the control over the excited state proton transfer reaction has been achieved with cucurbituril encapsulation\textsuperscript{31,35,36}. Recently, we have constructed a non-toxic nano-assembly of BSA protein and cucurbit[7]uril macrolcycle and established efficient loading and controlled release of a standard drug, doxorubicin (DOX) in live cells with external stimuli such as adamantylamine or \( pH \)\textsuperscript{27}. With multiple uptakes of CB7, controlled protection of the active group of a photodynamic therapy (PDT) dye, N-methyl tetrypyridyl porphyrin (TMPyP) has been illustrated which also provides a template for designing and synthesizing photo-functional materials and extended assemblies\textsuperscript{37}. In broad sense, the cucurbituril encapsulated systems offer tremendous opportunities for modulating the molecular characteristics of the guests towards their application in the aforementioned areas\textsuperscript{1}. Depending on the cavity size and solution \( pH \), CBn homologs form number of host-guest complexes with divergent stoichiometries\textsuperscript{6,38-41}. In the present review, we emphasize on the diversity of cucurbit[7]uril (CB7)/cucurbit[8]uril (CB8) assisted host-guest assemblies with a well-known DNA groove binder dye Hoechst-33258 (H33258) in an aqueous medium.

H33258 belongs to the bis-benzimidazole class of molecules having anticancer properties\textsuperscript{42}, and its derivatives are found to provide significant protection against radiation-induced DNA strand breakage and therefore its potential as radioprotector has also been the subject of detailed investigation\textsuperscript{43-47}. H33258 exists in different conformations with respect to the \( pH \) of the media displaying diverse photophysical properties. It has been widely accepted that the large fluorescence enhancement on binding to the minor grooves of DNA is due to the planar structure of the dye, which is largely devoid of the non-radiative channels\textsuperscript{46,47}. It is certain that apart from structural rigidity imposed on binding to the DNA grooves, the presence of several protophilic nitrogens and their acidity constants (\( pK_a \)s) also play a decisive role in the conformational changes and hence the emission properties of the dye\textsuperscript{48}. Having this peculiar structural features and the availability of accessible cationic sites and protophilic nitrogens, the drug H33258, offers a promising system to explore its supramolecular interactions with the versatile cucurbituril hosts, expecting large structural modifications. The chemical structure of H33258, structural formula of CBn and the structure of CB7 and CB8 are depicted in Chart 1.

**Photophysical characterization of cucurbituril-Hoechst-33258 complexes**

The photophysical properties of H33258 largely depend on the solution \( pH \) due to the presence of
several protophilic nitrogens in its structure. The dye shows two close-lying pKₐ values (3.5 and 5.5) below pH 7⁴⁹. At pH 7, the mono-cationic species (Chart 1) having a protonated methyl substituted piperazine nitrogen and undissociated phenolic OH is the predominant form, whereas, at pH 4.5, the dye exists as dicationic where the imidazolium nitrogen of the benzimidazole unit adjacent to the piperazine is also protonated. Further lowering the pH to 1.5, it is reported that imidazolium nitrogens on either ring also get protonated, making the majority of the dye tricationic⁴⁹,⁵⁰. All these protonation equilibria bring out marked differences in the chemical structure of the dye resulting in significant change in the electronic charge distribution and hence its photophysical properties. At pH ~7, H33258 displays characteristic absorption maximum at 338 nm⁴⁸,⁴⁹. In reference to the absorption profile at pH 7, the dye solution at lower and higher solution pH displayed significant hyperchromic and bathochromic shifts (~16 nm), which could be an indication of severe changes in the electronic distribution due to different protonated structure of the dye⁴⁸,⁴⁹. The intriguing intramolecular geometrical orientations of the dye due to protonation also bring out major modulation on the fluorescence characteristics with pH. At pH 7, the dye exhibited very weak fluorescence emission (Φₐ = ~0.015) with an emission maximum at ~500 nm⁴⁸,⁴⁹. However, on decreasing the pH, the emission intensity increased remarkably, reaching a maximum value at ~ pH ~4.5 having a quantum yield ~0.4⁴⁸, and the emission profile also displayed bathochromic shift of ~22 nm⁴⁸. On the contrary, on further acidifying the solution to pH ~1.5, the emission intensity once again decreased drastically with the emission yield about 0.005⁴⁸,⁴⁹. Hence, a change in the solution pH from 7 to 4.5, enhances the emission yield by ~18 fold and this fluorescence change is ~ 50 fold on changing the pH from 1.5 to 4.5⁴⁸. While a fast flipping motion among the two benzimidazole rings is considered as the most probable mechanism for the fast fluorescence decay, a more planar structure of the dicationic form at pH 4.5 having a double bond character between the two benzimidazolium groups is suggested to be the most likely fluorescent species responsible for enhanced emissions⁴⁸.

In this review, we discuss the modulations in the intriguing excited state properties of H33258 on forming supramolecular complexes with the cucurbituril macrocycles, namely the CB7 and CB8 hosts at two preset pH conditions i.e. 4.5 and 7. In one hand, being dicaticonic at pH ~4.5 and monocaticonic at ~7, CBs can provide large stabilization to the inclusion complex via ion-dipole interaction involving the carbonyl portals and the hydrophobic interaction exerted by the CB cavities. On the other hand, the notable differences in the portal charges and cavity dimensions of the CB7 and CB8 would add additional geometric/stoichiometric changes in the complex bringing out distinct spectroscopic changes. These intriguing interactions with CB7 and CB8 and the ensuing spectroscopic changes are described separately in the following sections.

**Interaction of cucurbit[7]uril (CB7) with Hoechst 33258**

A dilute solution of H33258 (~1 μM) at pH 7 provided characteristic absorption profile of the mono-cationic form having a maximum at 338 nm⁴⁸.
Titration of this solution with CB7 displayed changes both in the absorption and fluorescence spectral features. On increasing the concentration of CB7, the absorption spectrum of H33258, initially showed a reduction in the absorbance with the appearance of an isosbestic point at 368 nm (up to <2 μM of CB7), which at higher concentration of CB7 developed in to a red-shifted (~13 nm) broad band (Figure 1A)\(^5\). These stepwise spectral changes point to the existence of more than one complexation equilibrium between the dye and the CB7 within the concentration range employed. Similar way, the interaction of CB7 was examined in solutions at pH 4.5\(^6\), where the dye exists in its dicationic form. With the addition of CB7, the absorption profile displayed spectral shifts similar to that observed in the above system at pH 7, but to a lesser extent as shown in Figure 1B\(^5\). In short, at pH 4.5 also the spectral changes indicate the involvement of more than one equilibrium for the host-guest interaction of CB7 and H33258.

On the emission front, titration of the dye solution at pH 7 with CB7, the emission spectrum displayed significant enhancement in the emission intensity. As presented in Figure 2A, the addition of CB7 up to ~2 μM, resulting in a gradual increase in emission intensity along with a significant blue shift of ~20 nm compared to the emission maximum of free H33258 at ~500nm. However, the addition of CB7 thereby increased the emission intensity at 480 nm to about 80 fold without any further shift in the emission maximum\(^5\). On the other hand, the dicaticionic structure displayed distinctly different emission behavior upon complexation with CB7. On titration of the dye solution maintained at pH 4.5 with CB7, the fluorescence band centered at 520 nm displayed a gradual blue shift of ~35 nm, along with an enhancement in the emission intensity (Figure 2B)\(^5\). It is also noticed that at higher concentration of CB7, the spectral position reverted towards the free dye to some extent with an overall increase in the emission intensity at 500 nm ~3 times. This contrasting spectral shifts with host concentration observed at pH 4.5 would probably indicate a structural change in the dye due to possible variations in the stoichiometry of the complex formed\(^5\). A quantitative determination of the fluorescence quantum yields has been carried out by comparing the integrated area of the emission spectrum with that of a standard fluorophore, coumarin 1\(^5\), under similar experimental conditions. This provided fluorescence yields at pH 7 as 0.015 and 0.5 in the absence and presence of CB7, respectively, whereas at pH 4.5 the fluorescence quantum yield increased from 0.4 to 0.7 in the presence of CB7\(^5\). Here again, the fluorescence features provided strong indication for multiple complexation equilibria and is prominently seen in the case of the dicaticionic dye at pH 4.5.

![Figure 1](image1.png)

**Figure 1** — (A) Absorption spectra of H33258 (1 μM) recorded in solution at pH 7 with [CB7]/μM 0 (1); 2 (2); 5 (3); 10 (4); 15 (5); 25 (6); 40 (7); 80 (8); 100 (9); 125 (10). (B) Absorption spectra of H33258 (1.5 μM) recorded in solution at pH 4.5 with [CB7]/μM 0 (1); 2 (2); 6 (3); 10 (4); 20 (5); 30 (6); 40 (7); 50 (8). The upward arrows indicate the isosbestic points. Reproduced from Ref 51 with permission from the PCCP Owner Societies.

![Figure 2](image2.png)

**Figure 2** — (A) Steady-state fluorescence spectra of H33258 (1μM) recorded from solution at pH 7 on excitation at 365 nm with [CB7]/μM 0 (1); 2 (2); 5 (3); 10 (4); 15 (5); 25 (6); 40 (7); 80 (8); 100 (9); 125 (10). (B) Emission spectra of H33258 (1.5μM) recorded from solution at pH 4.5 on excitation at 353 nm with [CB7]/μM 0 (1); 2 (2); 6 (3); 10 (4); 20 (5); 30 (6); 40 (7); 50 (8). The arrows follow the shift in the emission maximum with [CB7]. Reproduced from Ref 51 with permission from the PCCP Owner Societies.
Determination of stoichiometry and binding constants

Following the indications of differing stoichiometric composition at different pH conditions, the composition of the CB7-H33258 complex was examined by following the Job’s continuous variation method. For this, the optical density at a wavelength with maximum absorbance change was monitored in solutions at specified pH and varying the mole fractions of the host and guest components. Figure 3A presents the Job’s plot generated from the absorbance monitored at 360 nm for the CB7-H33258 system at pH 7 and 4.5. Both the curves display maxima near to 0.66 mole fraction of the CB7, which adjudicate a 2:1 (CB7:H33258) composition for the mono-cationic as well as the dicationic H33258.

The binding constant values were evaluated following the binding curves (fluorescence titration curves) generated at pH 7 and 4.5. Though various structural arrangements for the host-guest interactions are possible, for simplification, we consider only the most probable geometries for the 1:1 and 2:1 binding interactions. The binding curves (Figure 3B) were analysed by using a modified Benesi-Hildebrand equation for the 1:1 and 2:1 stoichiometric complexes and the $K_1$ (binding constant for 1:1 complex) and $K_2$ (binding constant for 2:1 complex) values were estimated to be $(1.45\pm0.1) \times 10^5$ M$^{-1}$ and $(1.0\pm0.07) \times 10^4$ M$^{-1}$, respectively, for the system at pH 7, and $(7.0\pm0.3) \times 10^3$ M$^{-1}$ and $(9.0\pm0.7) \times 10^3$ M$^{-1}$, respectively, for the system at pH 4.5. At pH 7, the $K_1$ value does support the ion-dipole interaction for the 1:1 complex, moreover, the reasonably high value for the second binding (2:1) also indicate an ion-dipole type of interaction, which is in good support to the $pK_a$ shift due to stabilization of the dicationic form. However, at pH 4.5, the two close-lying binding constant values indicate that ion-dipole interaction is mainly responsible for both the 1:1 and 2:1 complex formation.

Effect of CB7 complexation on the fluorescence lifetime of H33258

Excited state lifetime of H33258 is known to be very sensitive to the solution conditions like solvent, p$H$, etc. It is realized that the fluorescence decays of the dye is inherently multi-exponential in nature, possibly representing different conformational structures of the dye in the solution which are sensitive to the solution pH. The significantly large change in the fluorescence properties and hence the lifetime features are directly correlated to the intramolecular rotation around the single bond connecting the two benzimidazolium rings. Since such fast torsional relaxation in the dye is expected to get largely reduced upon forming inclusion complexes with CB7, its fluorescence decay should become reasonably slow to record it in sub-nanosecond time resolution. As shown in Figure 4A at pH 7, the fluorescence decay of H33258 displayed a multi-exponential kinetics having major decay components in the range of 100-400 ps and a slower component in the range of 3.5-4 ns. Upon incremental addition of CB7 to the H33258 solution at pH 7, the faster component of the multiexponential decay profile progressively became slower and the decay profile transformed into almost a single exponential function with 98% of the decay.

Figure 3 — (A) Job plot generated by continuous variation of the mole fraction of H33258 and CB7 from solutions at pH 7 (1) and at pH 4.5 (2). The total concentration of CB7 and H33258 has been kept at 10 μM and the absorbance changes have been monitored at 360 nm in both the cases. (B) Changes in the fluorescence intensity (I) monitored at 500 nm for H33258 with varying concentration of CB7 at pH 7 (1) and pH 4.5 (2). λex = 365 nm and 335 nm respectively for (1) and (2). The solid lines in the respective figures represent the fitted line as per Benesi-Hildebrand (modified) equation. Reproduced from Ref. 51 with permission from the PCCP Owner Societies.
corresponding to the time constant of 4.1 ns$^{51}$. The trend is quite expected as the encapsulation of the dye by CB7 in both 1:1 or 2:1 stoichiometry would definitely restrict the intramolecular rotational motion and thus arrest the fast non-radiative relaxation channel. However, at pH 4.5 (Figure 4B), the decay of dicaticonic H33258 becomes nearly single exponential with 3.8 ns as the major lifetime component (97%), while the short component (~0.26 ns) contribute merely 3%. At pH 4.5, the piperazine attached benzimidazole is protonated, which allows certain degree of charge transfer (CT) from the protonated benzimidazole to the phenol substituted benzimidazole, leading to a more planar structure and is having lifetime in the range of 3.5-4 ns$^{46-48}$. As expected, addition of CB7 slowed down the decay dynamics only marginally and the decay trace corresponds to a lifetime of 4.5 ns (Figure 4B). It is observed that the decay kinetics of CB7-H33258 complex at pH 7 and that of H33258 alone at pH 4.5 shows very close agreement pointing to a similar structural orientation for the dye in these two cases. In other words, the results make it clear that the dye structural characteristics in solution at pH 4.5 (even in the absence of CB7) are attained by the dye monocation at pH 7 when it interacts with CB7. This is attributed to the shift in the dye pK$_a$ value to higher values, by 2 units or more due to complexation and hence, the dye remains as dicaticonic form even at pH 7 in the presence of CB7$^{51}$. In any case, it is true that H33258 gets encapsulated by two CB7 moieties making it bulkier. In such a case, apart from enhancing the radiative channel, the dye would also display an increase in the hydrodynamic volume which can be evaluated by anisotropy measurements.

**Time-resolved fluorescence anisotropy measurements**

It is well known that time-resolved anisotropy measurements can yield valuable information regarding the rotational diffusion time of a fluorescent species and hence can be used to corroborate the stoichiometry of the host-guest system$^{52}$. Analyzing the fluorescence anisotropy decays, the rotational correlation time $\tau_\text{c}$ can be evaluated and related to their rotational diffusion coefficients ($D_\text{c}$) and the viscosity ($\eta$) of the medium (water in the present case) according to the Stokes-Einstein relationship$^{55}$:

$$\tau_\text{c} = 1/(6D_\text{c}), \text{where, } D_\text{c} = \frac{RT}{6V\eta} \quad (1)$$

where, $V$ is the hydrodynamic molecular volume of the complex (approximated as a rigid sphere) and $T$ is the absolute temperature. Figure 5 displays the anisotropy decays measured for H33258 in the absence and presence of CB7 at pH 7 and 4.5. For the dye at both the pH conditions (pH 7 and 4.5) the

Figure 4 — (A) Decay traces of H33258 at 500 nm in solutions at pH 7 (1) with the addition of 5 μM of CB7 (2), 200 μM CB7 (3). (B) Decay traces of H33258 at 500 nm in solutions at pH 4.5 (1) with the addition of 50 μM of CB7 (2). Inset: Comparison of the decay traces recorded from the solution at pH 4.5 (a) and with CB7 at pH 7 (b). (λ<sub>ex</sub> = 374 nm). L represents lamp profile. Reproduced from Ref 51 with permission from the PCCP Owner Societies.

Figure 5 — Anisotropy decay traces monitored at 500 nm for H33258 in the absence and presence of CB7 at pH 7 (A) and pH 4.5 (B). Traces (a) and (b) represent the decay of the dye alone and in the presence of 200 μM CB7, respectively. The traces in (B) represent the decay of the dye alone (e), in the presence of 4 μM of CB7 (d) and the presence of 15 μM CB7 (e). The solid lines represent the fitted exponential curves. λ<sub>ex</sub> = 374 nm. Reproduced from Ref 51 with permission from the PCCP Owner Societies.
decay, trace fits well to a single exponential decay with a time constant of ~370 ps as shown in Figure 5A(a) and Figure 5B(c)\textsuperscript{31}. In solutions at pH 7, this time constant gets significantly increased to about 630 ps in the presence of CB7, acknowledging an increase in molecular volume due to the complex formation\textsuperscript{51}.

However, in solutions at pH 4.5, the anisotropy decay displayed remarkable changes in the rotational diffusion time with variation in the concentration of CB7. With a low concentration of CB7 (~4 μM) at a solution pH of 4.5, the anisotropy decay of H33258 corresponds to about ~475 ps, whereas at a higher CB7 concentration (~15 μM), the rotational correlation time constant increased astonishingly to 880 ps (Figure 5B)\textsuperscript{51}. These variations indicate the existence of a 1:1 and 2:1 (host: guest) complexes, having different hydrodynamic volume. At pH 7 the decay time constant (630 ps) is reasonably higher than that obtained for a similar case at pH 4.5 (~475 ps).

It may be possible that in solutions at pH 7, at higher CB7 concentration both the 1:1 and 2:1 complexes coexist, which gives an average τ\textsubscript{r} value. Thus, apparently higher τ\textsubscript{r} value obtained for the presumably 1:1 complex at pH 7, pertains to the averaged contribution of 1:1 and 2:1 complexes\textsuperscript{51}.

**Geometry optimization**

The authenticity of the proposed stoichiometries for the mono-cationic and dicationic dyes were examined by evaluating their energy optimized structures in all possible H33258-CB7 geometries at semiempirical PM3 (MM) level by using a Gaussian package\textsuperscript{39}. As shown in the geometry optimized structures (Figure 6), in a 1:1 complex for both mono-cationic and dicationic dyes, the most stable configuration is provided by the structure, which accommodates the CB7 moiety at the piperazine end in preference to that at the phenol end, providing maximum ion-dipole interaction between the host and the guest\textsuperscript{51}. Introducing the second CB7 binding, provided optimized structures with the CB7 positioned at the phenolic end. For the dicationic dye, the second CB7 binding is much stronger, and the inclusion of the phenol part into the CB7 cavity is very much apparent placing the phenolic OH at the outer carbonyl rim of the CB7. A close look at the structural orientation reveals that the twist angle between the two benzimidazole groups tends to remain more planar in the 2:1 complex, and this is seen prominently in the case of the dicationic structure (Figure 6d)\textsuperscript{51}. Obviously, manifesting the strength of interaction in these two cases, the calculated molar heat of formation (ΔH\textsubscript{f}) in the case of a dication dye provided −88 kcal/mol and −106 kcal/mol respectively for the 1:1 and 2:1 complexes whereas the values are about −47 kcal/mol and −48 kcal/mol respectively for the 1:1 and 2:1 complexes of the mono cationic dye\textsuperscript{51}.

**Interaction of CB8 with Hoechst 33258**

Spectroscopic changes are followed in H33258 solution at pH 7 and 4.5 with CB8, the higher homolog of CB7 having larger cavity size and increased portal charge density. In the presence of CB8 significant spectral changes are observed in

![Figure 6 — Geometry optimized structures of the CB7-H33258 complex in 1:1 (a) and 2:1 (b) stoichiometries of the mono-cationic structure and 1:1 (c) and 2:1 (d) stoichiometries in the case of the dicaticonic structure. Reproduced from Ref. 51 with permission from the PCCP Owner Societies.](image-url)
H33258, which are distinctly different from that observed for CB7\textsuperscript{51}. On titration with CB8 at pH \textasciitilde7, at lower concentration of CB8, the absorption spectrum of H33258 (0.9 \mu M, 5 mM Tris-HCl) displayed a hypochromic change with an isosbestic point at 365 nm as shown in Figure 7. When the CB8 concentration was increased to \textasciitilde9 \mu M, the absorption displayed \textasciitilde17 nm bathochromic shift and established a new absorption maximum at 355 nm\textsuperscript{56}. On the other hand, as presented in Figure 7B, at pH 4.5, H33258 displayed an absorption maximum at 343 nm and the addition of CB8 resulted in a hypochromic change with appearance of a prominent shoulder band at \textasciitilde375 nm and a neat isosbestic point at 362 nm\textsuperscript{56}. These distinct absorption changes in the presence of CB8 at different pH conditions not only depict the complexation induced changes in the absorption characteristics of H33258 but also points to a probable change in the CB8: H33258 structure and stoichiometry.

On recording the fluorescence changes at pH \textasciitilde7, the dye H33258 displayed very weak emission spectrum centered at \textasciitilde500 nm (Figure 8A). Upon gradual addition of CB8 to \textasciitilde9 \mu M, the emission displayed a remarkable enhancement in intensity with a concomitant hypsochromic shift (\textasciitilde15 nm) of the emission maximum to 485 nm as shown in Figure 8A\textsuperscript{56}. As discussed before, at neutral pH, the mono cationic H33258 is weakly fluorescent ($\phi_f$ \textasciitilde0.015) due to excited state energy dissipation by the fast torsional motion in the excited state with respect to the single bond joining the two benzimidazole units\textsuperscript{48,51}. Inclusion of H33258 in the rigid hydrophobic cavity of CB8 consequently restricts this fast torsional motion and also provides a hydrophobic microenvironment for the guest. These two factors

![Figure 7](image1.png)

Figure 7 — (A) Absorption spectra of H33258 (0.9 \mu M) recorded in 5mM Tris.HCl buffer at pH 7 with [CB8]/ \mu M 0 (1); 0.2 (2); 0.4 (3); 0.7 (4); 1.3 (5); 2.5 (6); 4.0 (7); 5.0 (8); 7.0 (9); 9 (10). (B) Absorption spectra of H33258 (1.5\mu M) recorded in 5mM sodium acetate buffer at pH 4.5 with [CB8]/ \mu M 0 (1); 0.2 (2); 0.9 (3); 1.4 (4); 1.8 (5); 2.7 (6); 3.7 (7); 5.0 (8); 9 (9). Reprinted with permission from ref. 56. Copyright (2013) American Chemical Society.

![Figure 8](image2.png)

Figure 8 — (A) Steady-state fluorescence spectra of H33258 (1\mu M) recorded in 5 mM, Tris.HCl buffer at pH 7 on excitation at 353 nm with [CB8]/ \mu M 0 (1); 0.2 (2); 0.4 (3); 0.7 (4); 1.3 (5); 2.5 (6); 4.0 (7); 5.9 (8); 9 (9). (B) Emission spectra of H33258 (1.5 \mu M) recorded in 5 mM sodium acetate buffer at pH 4.5 on excitation at 365 nm with [CB8]/ \mu M 0 (1); 0.2 (2); 0.4 (3); 0.9 (4); 1.8 (5); 2.7 (6); 3.7 (7); 6.8 (8); 9 (9). The arrows follow the change in the emission maximum with [CB8]. Reprinted with permission from Ref 56. Copyright (2013) American Chemical Society.
lead to the fluorescence enhancement and also to the blue shift of the emission profile, in the presence of CB8. The quantum yield of emission for the CB8-H33258 complex at pH 7 increases to 0.4, indicating an overall 26 fold enhancement in emission yield in the presence of CB8 at pH ~7.

On the other hand, H33258 at pH 4.5 shows quite contrasting emission features in the presence of CB8. The dicationic H33258 (2 μM, pH 4.5 (5 mM acetate buffer) displays strong emission (ϕ = 0.40) having an emission maximum at 505 nm \(^{38}\). However, the addition of CB8 (up to ~5 μM) resulted in quenching of the H33258 emission with a slight blue shift of the emission maximum by 5 nm. The changes are shown in Figure 8B. No further quenching was observed with increase in concentration of CB8 beyond 9 μM, and the fluorescence yield is now reduced to 0.2\(^{36}\).

These contrasting emission behaviors of H33258 in the presence of CB8 at pH ~7 and 4.5 are intriguing since at both these pH conditions, the CB7 analog having smaller cavity dimension, displayed significant enhancement in the emission intensity\(^{51}\). In our earlier studies on different chromophoric dyes such as thioflavin T (ThT)\(^{6}\), thiazole orange (TO)\(^{59}\) and neutral red (NR)\(^{57}\), it has been reported that the large CB8 cavity can encapsulate more than one guest, thereby stabilizing homo/hetero guest pairs in the cavity. However, depending on the guest characteristics, the CB8-Dye system provided emission quenching (in the case of neutral red dyes)\(^{57}\) whereas the CB8-ThT/TO systems presented strong emission bands due to an excimer formation\(^{39}\). In a recent work we have also shown contrasting emission from cucurbit[8]uril-templated H- and J-dimers of bichromophoric coumarin dyes\(^{58}\). In the present case of H33258, the above observations point to the prospect of CB8 as a template for more than one H33258, having a strong dependence on the solution pH. From the structural point of view, it is reasonable to visualize that the interaction of puckered monocationic form at neutral pH and the more planar dicationic form of H33258 at pH 4.5 with the macrocyclic receptor CB8 would be different and can support distinct host-guest complexes of different stoichiometries.

**Determination of stoichiometry and binding constant**

From the photophysical changes observed in the presence of CB8 at pH ~7 and 4.5, it is clear that interaction of H33258 with CB8 leads to distinct host-guest stoichiometric complexes specific to the solution pH, which imply the interactions of the monocationic and the dicationic forms of the dye with the CB8 host. The Job’s plot at pH ~7 (Figure 9A, 1) displayed an absorbance maximum at 0.65 mole fraction of CB8 (η\(_{CB8}\)), validating a 2:1 (CB8: H33258) stoichiometry for the complex\(^{56}\). However, with the dicationic dye at pH 4.5, the Job’s plot displayed a maximum at 0.33 mole fraction of CB8 (Figure 9A, 2), which suits a 1:2 (CB8: H33258) composition\(^{56}\).

Considering 2:1 equilibrium at pH ~7, the overall binding constant \(K_{(pH \sim 7)}\) has been evaluated from a modified Benesi-Hildebrand plot of the emission intensity at 500 nm versus the CB8 concentration (Figure 9B) and is found to be \((2.1\pm0.2)\times10^{11}\) M\(^{-2}\) (Ref 37). On the other hand at pH 4.5,
considering a 1:2 host-guest stoichiometry, the estimated overall binding constant $K_{pH 4.5}$ is $(3.2\pm0.2)\times10^{11}$ M$^{-2}$.

Effect of CB8 complexation on the fluorescence lifetime of H33258

The complex formation has been further supported by the fluorescence lifetime and anisotropy measurements at suitable solution conditions. As seen from Figure 10A at pH ~7, with the addition of CB8, the initial faster decay component seen in H33258 becomes slower and at ~1.4 µM of CB8 (~1eq of the H33258 concentration), the fluorescence decay best fitted to a bi-exponential kinetics having two time constants, 2.37 ns (35%) and 4.74 ns (65%)$^{56}$. The decay profile remains unchanged with further increase in the CB8 concentration up to ~9 µM, suggesting no further structural/stoichiometric changes, like that, observed in the case of H33258-CB7 system at similar pH conditions$^{51}$. Due to the intrinsic affinity of the cucurbituril macrocycles toward cationic guests and considering the molecular structure of the H33258 dye, inclusion complex formation through encapsulation of both the benzimidazole moieties is quite likely, which would retard the intramolecular torsional movement of H33258. As discussed above, at pH ~7 both the benzimidazole units in H33258 are unprotonated, and they exist both in non-planar and more planar conformations, having significant differences in their excited state lifetimes. In presence of CB8, it is quite expected that the proposed 2:1 (CB8:H33258) complex would stabilize these conformers independently, displaying distinct lifetime values$^{56}$. On this basis, 2.37 ns and ~4.74 ns lifetime components evaluated at pH ~7 in the presence of ~9 µM of CB8 are assigned to different planar/non-planar conformers feasible for the dye in the 2:1 complex$^{56}$.

On the other hand, H33258 at pH 4.5 (~2 µM, 5 mM acetate buffer) displayed 3.8 ns decay component as the major deactivation pathway. Addition of CB8 to this solution resulted in the appearance of a faster decay component of ~0.9 ns, which became prominent with ~26% contribution in presence of 9 µM of CB8 (Figure 10B)$^{56}$. This decrease in the average lifetime of H33258 in the presence of CB8 at pH 4.5 is appropriately seen as partial fluorescence intensity quenching in the steady-state measurement also. Since the mono-cationic and the dicationic H33258 differs in charge, and structural conformations, the absorption and fluorescence spectral changes observed here would certainly point to different modes of binding in these two cases. As compared to the mono-cationic form, the dicaticionic H33258 is more planar with increased positive charge density, and it is quite rational that two such guest dyes bind to the CB8 host by inclusion through either portals of the CB8 cavity. Such a guest dimer arrangement within the CB8 cavity can provide strong π stacking interaction among the guests in the excited state, thus introducing a faster relaxation pathway and hence the emission quenching$^{56}$.

The anisotropy decays, r(t), recorded in the absence and presence of CB8 at the specified pH conditions, clearly presented large differences in the decay profiles (Figure 11), pointing to an increase in the hydrodynamic molecular volume of H33258 in the presence of CB8. For the dye alone at pH 4.5, the

Figure 10 — (A) Fluorescence decay traces of H33258 (1 µM) recorded at 500 nm in 5 mM Tris.HCl buffer at pH 7.2 with [CB8]/ µM 0 (1); 0.3 (2); 1.4 (3); 9 (4) (B) Decay traces of H33258 (2 µM) recorded at 500 nm in 5 mM sodium acetate buffer at pH 4.5 with [CB8]/µM 0 (1); 0.9 (2); 1.8 (3); 6.7 (4); 9 (5) $\lambda_{ex} = 374$ nm. L represents excitation lamp profile. Reprinted with permission from Ref 56. Copyright (2013) American Chemical Society.
anisotropy decay fits a single exponential kinetics with a time constant ($\tau_r$) of 0.37±0.02 ns (Figure 11a) whereas, at pH ~7, the faster $\tau_r$ decay is evaluated to be 0.32±0.04 ns. However, in the presence of CB8, the $\tau_r$ gets significantly increased to 1.30±0.01 ns (Figure 11c), respectively at pH 7.2 and 4.5, conceding an increase in hydrodynamic molecular volume due to the complex formation.

Geometry optimization
The proposed stoichiometric compositions and the geometrical arrangements were further explored by the geometry optimization using Gaussian package. As shown in Figure 12, depending on the charge and planarity of the mono-cationic and dicationic forms of H33258, 2:1 (-55 kcal/mol) and 1:2 (-16.5 kcal/mol) stoichiometries were found to be energetically favorable with the respective forms. For the mono-cationic form (Figure 12A), one of the most stable configurations is provided by the structure, which accommodates the CB8 moiety at the piperazine end as well at the phenol end, providing maximum ion-dipole and hydrogen bonding interaction among the host-guest and also among the CB8 hosts. It is interesting to see that the puffed mono-cationic H33258 prefers an unusual bend structure, quite distinct from the more planar structure observed for the same guest with the CB7 host. Here, the carbonyl portals of one of the CB8 moieties find several protons from the other CB8 moiety in the hydrogen bonding distance of ~2.5 Å. However, in case of the dicaticionic H33258, the two protonated nitrogen centers in the H33258 get stabilized by the large negative charge density at the CB8 portals and allows π-stacking interaction among the two H33258s placed inside the CB8 cavity as in Figure 12B.

Summary
In summary, the supramolecular interaction of the biologically important dye Hoechst-33258 has been discussed in aqueous solutions of different proton concentrations, in the presence of cucurbituril hosts, namely, cucurbit[7]uril (CB7) and cucurbit[8]uril (CB8). The pH-dependent structural changes in the dye, especially at pH 7, were severely affected by the interaction with CB7, bringing out remarkable enhancements in the emission features. The strong ion-dipole interaction provided by the carbonyl portals of the CB7 host adequately stabilizes the CB7-H33258 complex, both in 1:1 and 2:1 stoichiometries.
The non-covalently stabilized assembly brings out an intense enhancement in the fluorescence emission, due to the structural rigidity and planarity imparted on H33258, which reduces the non-radiative channels. The close similarity of the photophysical properties of the mono-cationic dye in the presence of CB7 with the dication of the free dye is attributed to a large upward $pK_a$ shift on CB7 encapsulation. On increasing the cavity size to that of CB8, a pH-mediated stoichiometric switching of cucurbit[8]uril-Hoechst-33258 complexes, revealing distinct photophysical properties has been demonstrated. In the case of mono-cationic H33258 at pH 7, the CB8 interaction is very strong, and the non-covalently stabilized assembly brings out a 2:1 (CB8: H33258) stoichiometry with ~26 fold enhancement in the emission yield, suitable for biomolecular imaging. On the other hand, the strong ion-dipole interaction provided by the more planar dicationic H33258 at pH 4.5 supports the CB8 to accommodate two dicaticionic H33258 in its cavity in a 1:2 stoichiometry. As a result, the emission yield decreases from 0.4 to 0.2. Since the stoichiometry and thus the release/activity of drug can be controlled by regulating the protolytic equilibrium, the macromolecular encapsulation of drug can be achieved by structural manipulation with the released mechanism, where the release of the dye from the macrocyclic cavity towards a biomolecule can be achieved easily by structural manipulation with external stimuli.

References