Salt-induced guest relocation from a macrocyclic cavity into a biomolecular pocket: interplay between cucurbit[7]uril and albumin†

Mhejabeen Shaikh,[a] Jyotirmayee Mohanty,*[a] Achikanath C. Bhasikuttan,[a] Vanya D. Uzunova,[b] Werner M. Nau[b] and Haridas Pal*[a]

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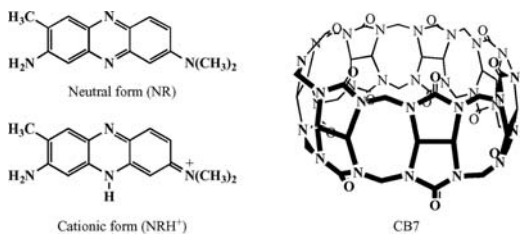
The binding affinity of Neutral Red with cucurbit[7]uril (CB7) can be fine-tuned by addition and competitive binding of metal ions, which leads also to a pK_a shift of the dye; this can be exploited to relocate the dye from the macrocyclic cavity of CB7 to the biomolecular pocket of bovine serum albumin.

Controlling the uptake and release of guest molecules, prominently those which have functional roles in biological processes, is of immense importance in drug delivery, photodynamic therapy, catalysis, and sensor applications. Among various methodologies, molecular association through host–guest complexation, where a guest molecule is encapsulated in the macrocyclic host cavity, offers a convenient approach, as it can introduce pronounced effects on as well as fine-tuning of the physico-chemical properties of the included guest. The differential affinity of a host towards the protonated or deprotonated forms of a guest can lead to a desirable shift of its acidity constant. This is an interesting aspect, as it can be exploited to control the prototropic equilibrium of the guest molecule by a supramolecular interaction. Such modification in the acidity constant (pK_a) of prototropic guests upon inclusion complex formation with macrocyclic hosts has received significant current research interest.[1–6] A striking observation has recently been reported by Pluth *et al.*, where the authors demonstrate the possibility to perform acid catalysis in a supramolecular host under alkaline condition, owing to its ability to protonate (shift the pK_a of) an included guest.[7]

Cucurbiturils (CBs), are macrocyclic container molecules composed of glycoluril monomers joined by pairs of methylene bridges.[8] Structurally, CBs constitute highly symmetrical pumpkin-shaped hydrophobic cages of low polarity and polarizability with two identical dipolar portal ends composed of carbonyl functional groups. CBs, for example cucurbit[7]uril (CB7, with seven glycoluril units), have been established as versatile and interesting host molecules, which form stable inclusion complexes with small guest molecules such as organic dyes,[8] metal cations,[8] protonated alkyl and aryl amines[8,9] and cationic dyes such as rhodamines,[10,11] triphenyl methane dyes,[12] *via* a combination of hydrophobic and ion-dipole interactions.



Neutral Red (3-amino-7-dimethylamino-2-methyl phenazine) has been extensively used as a fluorescent probe and marker for biological systems, among others, because its pK_a value lies in the physiologically most relevant region (6.8).[13] The characteristic color and fluorescence changes of the dye with pH and the differential binding affinity of the protonated (NRH^+) and neutral form (NR) towards biomolecules has enabled numerous applications of Neutral Red, in particular as intracellular pH indicator, as well as for the investigation of microheterogeneous and confined media, including micelles, microemulsions, cyclodextrins, and cucurbiturils.[2,4] In a recent study,[2] we have shown that both prototropic forms of Neutral Red undergo inclusion complex formation with CB7. However, the protonated NRH^+ form is preferentially encapsulated by CB7, which resulted in a 2 unit upward pK_a shift of the dye, effectively rendering it substantially more *basic* when included in CB7.

It was our idea, substantiated by the promising preliminary results reported herein, to exploit this large shift in the protolytic equilibrium for a controlled guest release, with the ultimate aim to demonstrate its usefulness in a biological context. Specifically, it is known that cations bind competitively to the portals of cucurbiturils,[9,14] which can cause a displacement of the included guest, particularly when its binding is driven by the coulombic interactions with the portals, *i.e.*, when the guest is positively charged. The addition of salts is then expected to lower the effective binding constants of prototropic guests and, thereby, to shift their effective pK_a values in the direction of the value of the uncomplexed guest. When working in a suitable pH region, in between the pK_a values of the uncomplexed and complexed guest, it should therefore be effectively possible to convert a charged complexed guest into an uncharged uncomplexed guest, which

[a] *Radiation & Photochemistry Division, Bhabha Atomic Research Centre, Mumbai, 400 085, India. E-mail: jyotim@barc.gov.in. E-mail: hpal@barc.gov.in; Fax: (+91) 22-25505331/25519613; Tel: (+91) 22-25593771*
[b] *School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, D-28759 Bremen, Germany. Fax: (+49) 421-200-3229*

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itself could display a high binding affinity with a hydrophobic biological assembly, for example, a protein binding pocket. Exactly this is demonstrated herein for the relocation of the dye Neutral Red from the CB7 cavity to bovine serum albumin (BSA) by the addition of salts. We will first describe a systematic study of the effect of added cations (Na^+, Cs^+ and Ca^{2+}, all as chloride salts) on the pK_a value of neural red, before we proceed to the biological interaction.

In fact, the modification of the binding interaction of Neutral Red with CB7 in the presence of salts is readily indicated from the changes in the absorption and emission characteristics of the dye. The binding isotherms, evaluated from fluorescence titrations of the dye with CB7 at varying salt concentrations, adhered well to a 1 : 1 complexation model (Fig. S1, ESI†). The binding constant for the NRH^+·CB7 complex [$K_{eq}(NRH^+) = 3.1 \times 10^6\ M^{-1}$][15] was found to decrease gradually with an increase in the concentration of the added salt, indicating a competitive electrostatic binding for the cation receptor sites of the host. The effective binding constant for the Na^+·CB7 complex (these complexes may have varying stoichiometries)[9,16] was estimated as about 80 M^{-1} (*cf.* Fig. S2, ESI†), which is very close to the value (120 M^{-1}) recently proposed by Megyesi *et al.*[16] A slight reduction in the binding constant in the presence of salt was also observed for the NR·CB7 complex, because the binding of the metal ions with the portal rims conflict also sterically with the binding of an uncharged organic guest.[3]

As reported,[2] the protolytic thermodynamic equilibrium for the present Dye·CB7 system follows a four-state model, involving the complexed and uncomplexed dye both in the protonated and unprotonated forms (Scheme A, ESI†). Accordingly, the acidity constant of the encapsulated dye, pK'_a, can be directly determined from the measured binding constants for the neutral and protonated forms, $K_{eq}(NR)$ and $K_{eq}(NRH^+)$, in combination with the pK_a value of the uncomplexed dye as; $K'_a = K_a K_{eq}(NR)/K_{eq}(NRH^+)$.[2] This provided a p$K'_a$ value as 8.8 in comparison to its normal pK_a value of 6.8 without added salt. The variation of the ionic strength of the medium by addition of NaCl allowed therefore a tuning of the pK'_a value of the complex down to the value of the uncomplexed dye. Such a ''supramolecular tuning'' of the pK_a value of Neutral Red (between 6.8–8.8) has immediate relevance for biological applications; for example, the tuning of the pK_a value of antihistaminic drugs by embedding them in water-soluble polymers is documented.[17] Also related, Lavis *et al.* have recently proposed a *synthetic tuning* of the pK_a of the dye fluorescein from 6.68 to 7.29.[18]

At low NaCl concentrations (0.01 M), the absorption spectra of Dye·CB7 complex at different pH did not show a well-defined isosbestic point (Fig. S3, ESI†), indicating the participation of the four-state protolytic equilibria.[2] However, at very high concentration of NaCl (0.5 M), the prototropic equilibrium of the dye in presence of CB7 adhered closely to a two-state model, corresponding to a typical acid–base equilibrium under normal (uncomplexed) conditions; this was confirmed by a well defined isosbestic point in the pH-dependent absorption spectra (*cf.* Fig. S4, ESI†). The pK_a curves constructed from the *OD* changes with pH at varying salt concentration are presented in Fig. 1. The titration curves for the

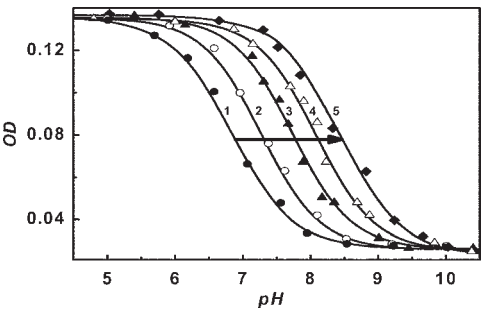

Fig. 1 pH Titration of the absorbance (λ_{mon} = 535 nm) of Neutral Red (3 μM), (1) in the absence of further additives, and (2–5) in the presence of CB7 (150 μM) with [NaCl]/M: (2) 0.5, (3) 0.1 (4) 0.01 and (5) 0.

Dye·CB7 system gradually shifted towards that of the free dye on increasing the salt concentration. This shift corresponds to a lowering of the binding constant with CB7, particularly for the protonated NRH^+ form, due to the competitive metal ion binding. The gradual decrease in the pK'_a value for the Dye·CB7 complex (Fig. 1) on addition of NaCl provides therefore not only a simple and convenient method to shift the pK_a over a large range of 2 units, but also to reduce its affinity to CB7 and thereby affect its release from the complex.

The changes in pK_a' were also examined with other salts to reveal the effects of size and charge of the metal ions. The pK_a' value decreased with increase in the concentration in all cases and tended to saturate at about 0.5 M for monovalent metal ions (Fig. S5, ESI†). In the presence of divalent metal ions, *i.e.*, Ca^{2+}, a sharper decrease in the pK_a' value was observed than in the presence of the monovalent alkali ions (*cf.* Fig. S5, ESI†). Presumably Ca^{2+} has, on purely electrostatic grounds, a higher binding affinity towards the CB7 portals, such that the binding constant of the Dye·CB7 complex decreases more readily with added Ca^{2+}. The size of the metal ions also displayed a small effect on the pK_a' value of the complex (*cf.* Fig. S5, ESI†), with the larger Cs^+ showing a marginally higher salt effect than the smaller Na^+; this can be nicely accounted for by an increased steric component in the competitive binding, which adds to a more or less constant electrostatic component. In this context, it should be noted that the apparent pK_a shift of the uncomplexed dye in the presence of salt was marginal (by ~0.3 unit).

Having demonstrated the methodology of pK_a tuning by a macrocyclic host and salt, the applicability of this method in a biological environment was evaluated in a system where the *unprotonated* dye would tend to be noncovalently bound to a protein. We selected BSA, because its binding with Neutral Red can be readily followed through the changes in the absorption and fluorescence characteristics. Though the changes in the absorption spectra are marginal, the fluorescence spectrum shows a hypsochromic shift (~50 nm for the neutral form and ~40 nm for the protonated form) along with an increase in the fluorescence intensity in the presence of BSA and the binding curves were constructed using the changes in intensity *vs.* the BSA concentration (*cf.* Fig. S6 and S7, ESI†). The fluorescence titrations established that BSA shows indeed an about one order of magnitude higher affinity for the NR

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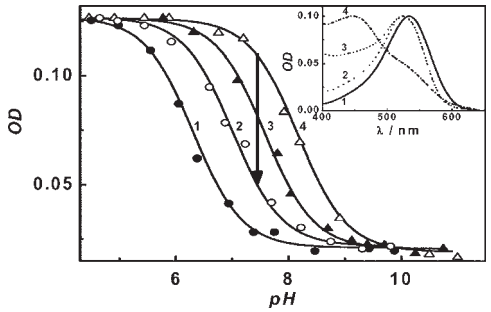
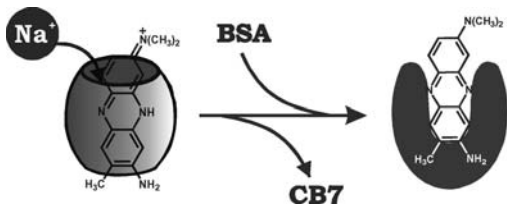


Fig. 2 pH Titration of the absorbance (λ_{mon} = 535 nm) of Neutral Red (3 μM) in the presence of 150 μM BSA (1) in the absence of CB7 and NaCl, and (2–4) in the presence of CB7 (50 μM) with [NaCl]/M: (2) 0.1, (3) 0.05 and (4) 0. Inset: Normalized absorption spectra of Neutral Red in different environments at pH 7.5: (1) Neutral Red, (2) CB7, (3) CB7-BSA and (4) CB7-BSA-NaCl.



Scheme 1 Schematic representation of the salt-induced transfer of a dye from CB7 to BSA near neutral pH.

over the NRH^+ form, with $K_{eq}(NR) = (1.0 \pm 0.1) \times 10^4\ M^{-1}$ and $K_{eq}(NRH^+) = (6.1 \pm 0.5) \times 10^3\ M^{-1}$. In the presence of BSA the pK_a of the dye was estimated as 6.3 which could again be shifted by 2 units, to 8.3, upon addition of CB7. This shift corresponds to the *relocation of the dye in the forward direction*, from BSA to CB7, where the protonated dye is more tightly bound. Following the tuning method outlined above, the pK_a value of the dye could be further tuned by addition of salt to the Dye·CB7·BSA ternary-system. Fig. 2 represents the pK_a curves thus generated at different salt concentrations, confirming a tunability essentially over the entire range, from 8.3 to 6.8. This shift corresponds to the *relocation of the dye in the backward direction*, from CB7 to BSA, where the neutral dye is more tightly bound. Interestingly, and regardless of the high complexity of the resulting four-component system, the changes can be well understood in terms of the pK_a shifts and salt effects established for the simpler model systems. The net effect of the addition of metal salt is illustrated by the vertical arrow in Fig. 2, which illustrates how the addition of salt—at a constant pH near 7.5—converts the protonated (CB7-complexed) form (top curve) into the unprotonated (BSA-complexed) form. Supporting evidence for this guest relocation came from the absorption spectra (Fig. 2 inset), whose maxima shifted, upon addition of NaCl, from *ca.* 526 nm, (characteristic for the protonated form included in CB7), to 450 nm, (characteristic for the neutral form in BSA).

Although ^{1}H NMR spectroscopy was well-suited to independently monitor the displacement of dye from the CB7 cavity upon addition of salt (Fig. S8 and Table S1, ESI†), the relocation into BSA could not be conclusively demonstrated by this technique due to the required higher dye concentration and the limited solubility range of BSA.

The net effect of the salt-induced transfer of Neutral Red from CB7 to BSA is visualized in Scheme 1. While the transferability of this case study to drug delivery is far fetched, it deserves conceptual attention. Moreover, since the stability and activity of drugs depend on their protonation state,[17] the macromolecular encapsulation into CB7 could provide an interesting tool to improve drug stability through a host-assisted guest protonation.[1,2,5] The addition of salts could then provide a simple stimulus for the controlled release of a potential drug, as well as for its activation owing to accompanying deprotonation. These applications will be interesting to explore, especially since drug delivery applications of CB7 are presently under investigation.[19,20]

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