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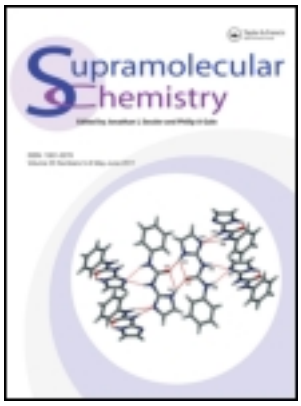

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Effect of cucurbit[*n*]urils on tropicamide and potential application in ocular drug delivery

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Effect of cucurbit[*n*]urils on tropicamide and potential application in ocular drug delivery

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The supramolecular interactions of the ocular drug tropicamide (**TR**) with cucurbit[7]uril (CB7) and cucurbit[8]uril (CB8) were investigated in aqueous solutions by using ^{1}H NMR, ESI-MS and UV–vis spectroscopic techniques. The results indicate a 1:1 binding stoichiometry of **TR** with CB7 and CB8. The binding constants of **TR** in its protonated form were higher (e.g. $K = 4 \times 10^6\,M^{-1}$ with CB8) than in its neutral form (e.g. $K = 1.4 \times 10^4\,M^{-1}$ with CB8), which led to a complexation-induced increase in its pK_a value of ca. 0.5 and 2 units with CB7 and CB8, respectively. In the presence of about 1% (*w*/*v*) CB8, the ionisation degree of 0.1% (*w*/*v*) **TR** was increased from 2% to 62% at neutral pH. The increase in the pK_a value and thus stabilisation of the protonated **TR** species at neutral pH is discussed in the context of supramolecular drug delivery of ophthalmologic drugs.

Keywords: cucurbiturils; tropicamide; drug ionisation; pK_a shifts; host–guest complexes

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1. Introduction

The primary purpose of drug delivery systems is to despatch the necessary amount of drug to the target site for a necessary period of time, both efficiently and in a well-defined manner (*1*). Besides conventional drug formulation techniques, encapsulation of the drugs inside supramolecular host molecules by non-covalent interactions of the host–guest type has become increasingly important in the design of intelligent delivery systems (*1*, *2*). For example, cyclodextrins (CDs), substituted calixarenes and, most recently, cucurbiturils are promising water-soluble macrocyclic drug containers because of their ability to encapsulate and transport the targeted drug molecules without altering their chemical properties (*1–3*). Cucurbit[*n*]urils (CBs), readily synthesised by condensation of glycoluril with formaldehyde under acidic conditions (*4*), possess very low if not negligible *in-vitro* and *in-vivo* toxicity (*3c*, *5*) and they stand out owing to their strong binding affinities (binding constants $>10^4\,M^{-1}$) as well as selectivity towards many neutral and cationic species (*3*, *4c,d*, *6*). Although the encapsulation, stabilisation and delivery of different drugs (e.g. platinum-containing anti-cancer drugs) have already been reported (*5d–f*, *7*), their formulation into actual dosage forms is unfolding only now (*8*).

Another unique fascinating feature of CBs, which has only recently been documented in the literature, is their ability to shift the pK_a values of protonated guests upon binding (*3*, *6e*, *9*). This is an implicit consequence of their cation-receptor properties that render cucurbituril-encapsulated guests protonated at pH values, at which the uncomplexed guests would remain unprotonated in aqueous solution, suffering from a low solubility in their neutral form (*3*). Others and we demonstrated that such complexation-induced pK_a shifts can lead to interesting photophysical effects such as fluorescence enhancements (*9b,e,g*, *10*, *11*) as well as potential applications, including acid-promoted hydrolysis reactions (*12*). With respect to the binding of drugs, cucurbituril-induced pK_a shifts have been shown to facilitate the solubilisation and increase the stability of agriculturally important benzimidazoles (*3a,c,d*) and to activate and transport proton pump inhibitor drugs (*3b*).

It is of interest to investigate other benefits of cucurbituril-induced pK_a shifts in drug formulations. We selected the drug tropicamide (**TR**; see Scheme 1), which is used to dilate the pupils during eye examination (*13*). Cationic **TR** molecules, at the ocular pH of 7.4, exhibit good permeation across the negatively charged corneal epithelium (*1a*). The drug was a good model compound as **TR** has a pyridine group, which acts as a weak base in water with a pK_a value of 5.2. The other functional groups have no notable acid–base properties (i.e. are neutral in aqueous media). This means that only 2% of the drug is ionised at pH 7, which sets a critical limitation to its bioavailability. The ionisation degree of **TR** should become strongly increased upon complexation by cucurbit[7]uril (CB7) and cucurbit[8]uril (CB8; Scheme 1), which could ultimately improve the drug bioavailability.

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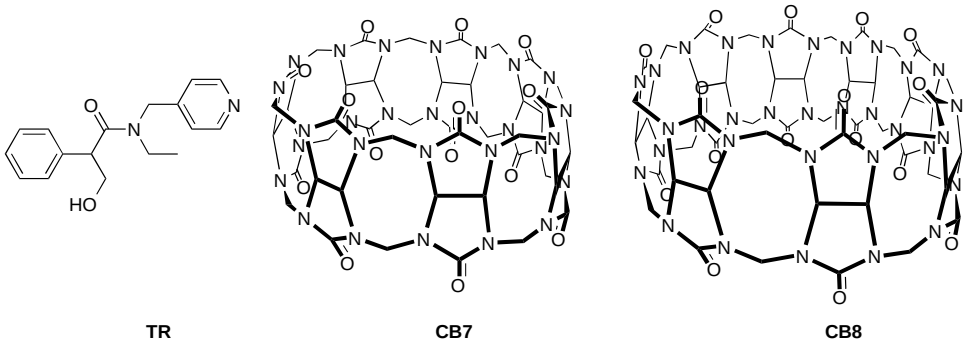

Scheme 1. Chemical structures of **TR**, CB7 and CB8.

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2. Results

2.1 Determination of binding constants

Upon addition of CB7 and CB8, characteristic changes in the UV absorption spectra of **TR** with the occurrence of isosbestic points were observed (Figure 1 and Figure S1 in the Supporting Information available online), which can be attributed to a 1:1 host–guest complexation due to the inclusion of the pyridine ring of **TR** inside the macrocyclic cavities [see also below, NMR and mass spectrometric (MS) measurements]. The corresponding binding constants of **TR** (Table 1) were derived from UV titrations, which were nonlinearly fitted according to a 1:1 binding model (in Pro Fit or Sigma Plot software) by using the formula previously reported (*14*). Note that the binding constants of the protonated forms of **TR** were determined 2 pH units below the pK_a value of the uncomplexed **TR**, whereas the binding constants of the neutral form of **TR** were determined above the pK_a' value of the complexed **TR** (see below, pH titrations) to ensure that the protonation state of **TR** did not change during the course of the host–guest titrations. As expected, the binding constants of **TR** with both host molecules are higher in its protonated than in its neutral form.

2.2 NMR measurements to determine binding modes

NMR measurements were used to determine the binding modes of **TR** with the host molecules. For example, in the presence of CB7 at pH 2.0 (pD 2.4), the pyridine protons (region from 8.0 to 8.5 ppm) of **TR** were up-field shifted by up to 0.8 ppm, whereas the protons from the benzene ring (region from 7.0 to 7.6 ppm) did not show any significant change (Figure 2). In analogy to the complexation-induced shifts of other guest molecules complexed to CBs (*3a*), these characteristic shifts can be attributed to a preferential inclusion of the pyridine moiety of **TR** inside CB7. In addition, the protons of the ethyl group (around 1 ppm) were also up-field shifted by 0.3 ppm, which suggests that this group is also encapsulated inside the CB7 cavity. Unfortunately, the NMR spectra of the CB8 complex were not accessible owing to the poor solubility of CB8 in water (less than 100 μM) (*4c*).

2.3 Mass spectrometric measurements

To further examine the binding of **TR** with the hosts, we also carried out MS measurements. **TR**, CB7 and CB8

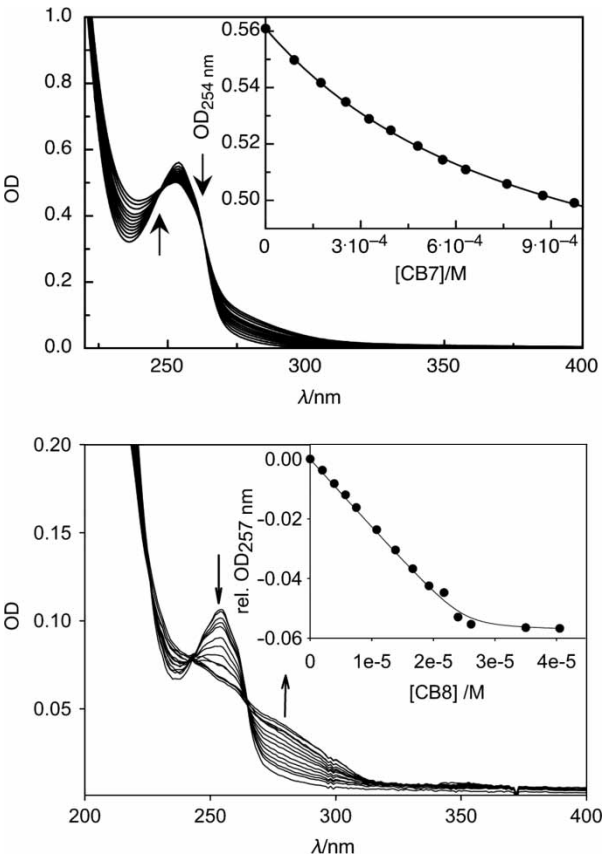

Figure 1. UV–vis host–guest titrations of protonated **TR** (115 μM for CB7 and 25 μM for CB8) with CB7 (top graph) and CB8 (bottom graph) at pH 3.0. The insets show the nonlinear fitting according to a 1:1 complexation model.

Table 1. Binding constants of **TR** in its neutral and protonated forms with CB7 and CB8 and complexation-induced pK_a shifts.

Host	$K_{\mathbf{TR}}/M^{-1}$ [a,b]	$K_{\mathbf{TRH}^+}/M^{-1}$ [a,c]	ΔpK_a [d,e]	ΔpK_a [e,f]
CB7	–	1.3×10^3	–	0.5
CB8	1.4×10^4	4.0×10^6	2.5	1.5

Notes:
[a]Determined by nonlinear least-squares fitting of the UV–vis host–guest titrations according to a 1:1 complexation model; error ± 10%.
[b] Binding constant with neutral **TR**.
[c] Binding constant with protonated **TR**.
[d] pK_a shift determined from thermodynamic cycle according to Equation (1).
[e] The pK_a value of free **TR** is 5.2 (± 0.2).
[f] pK_a shift determined from pH titrations, cf. Figure 4.

showed peaks at *m*/*z* of 285, 582 and 665, respectively, which can be attributed to their corresponding pseudo-molecular ion peaks (monoprotonated for **TR** and diprotonated for CB7 and CB8). Mass spectra of a mixture of 50 μM **TR** and 100 μM CB8 showed a peak at *m*/*z* of 808 (diprotonated), which was attributed to the host–guest complex, **TR**·CB8. Upon collision-induced decomposition (CID) of this complex peak, the pseudo-molecular ion peak of CB8 was observed, and additionally a peak at *m*/*z* of 1465, which was confirmed to be a fragmented complex of CB8 under retention of the pyridine-containing fragment (Figure 3). The latter fragmentation pattern was also observed upon decomposition of uncomplexed **TR**. The fact that the pyridine residue remains complexed to CB8 after CID provides strong circumstantial evidence for the intrinsic affinity and preferential inclusion of the pyridine ring to CB8, as independently revealed by ^{1}H NMR experiments for the corresponding CB7 complex (see above). For CB7, the complex peak was observed at *m*/*z* of 1447 (monoprotonated), which afforded the pseudo-molecular ion peak of CB7 upon CID (see Figure S2 in the Supporting Information available online). In addition, a peak assigned to a 2:1 CB7·**TR** complex (*m*/*z* = 869) was detected in the gas phase. Such higher order complexes did not need to be invoked in solution.

2.4 *Complexation-induced pK_a shifts*

The absorption spectra of **TR** change markedly with pH, which can be attributed to the protonation of the pyridine nitrogen. The pK_a value of **TR** was accordingly determined from the change of the absorbance with pH at a selected wavelength. We obtained a value of 5.2, in good agreement with the reported value of 5.4 (*13b*). Encapsulation inside CB7 and CB8 shifted the pK_a of **TR** towards higher values, which can be attributed to the stabilisation of the protonated form of **TR** in the presence of hosts. The obtained pK_a shifts were 0.5 for CB7 and 1.5 units for CB8 (Figure 4 and Figure S3 in the Supporting Information available online). This additional stabilisation

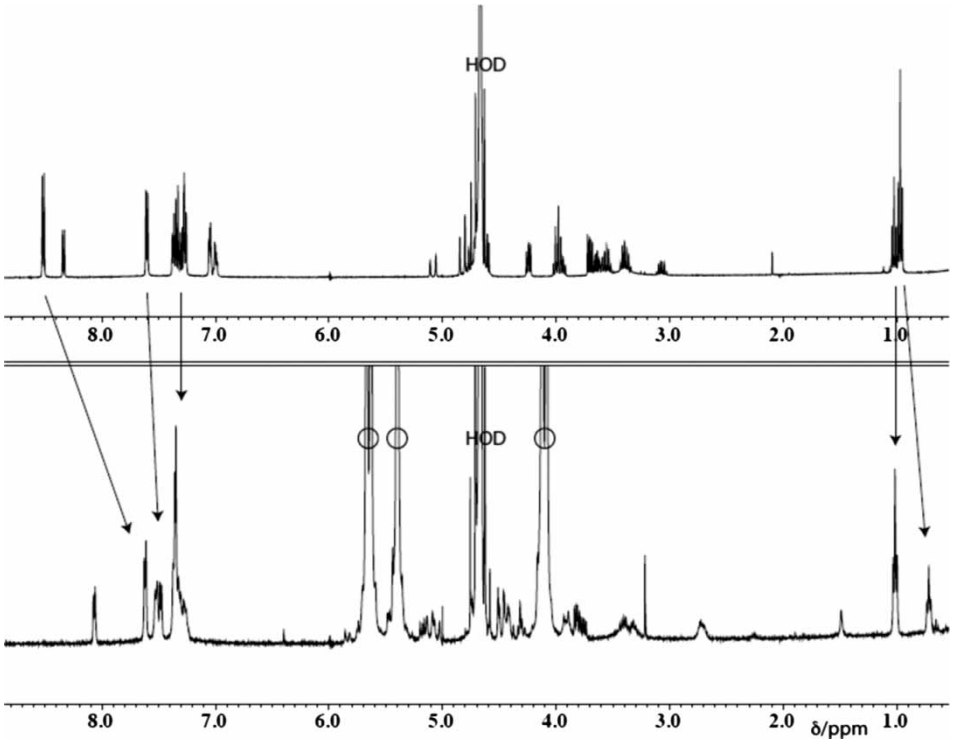

Figure 2. ^{1}H NMR spectra of 500 μM **TR** with 3 mM CB7 in D_2O at pD 2.4. Arrows indicate the complexation-induced shifts of the proton signals of **TR**.

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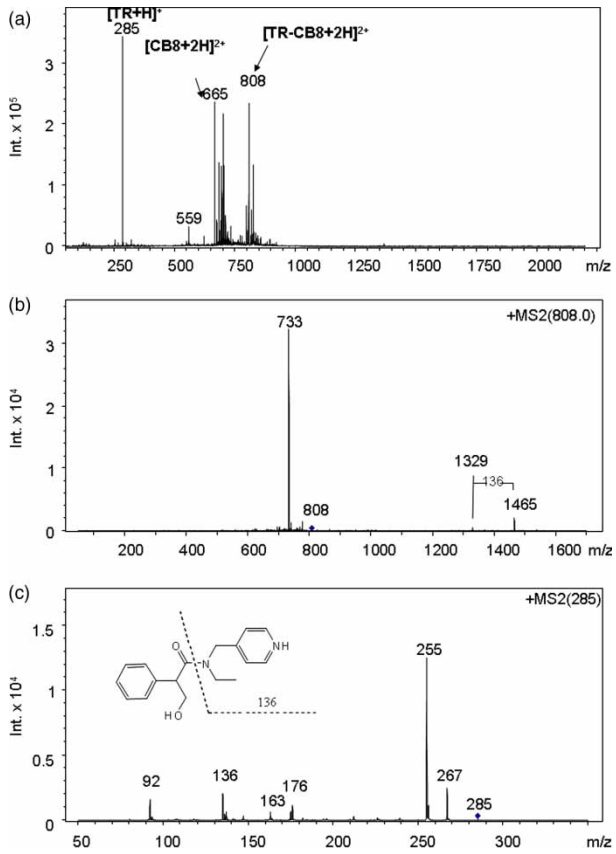


Figure 3. Mass spectra of **TR** and its inclusion complex with CB8 (a). Figures (b) and (c) show the tandem MS measurements for the **TR**·CB8 complex ($m/z = 808$) and **TR**, respectively.

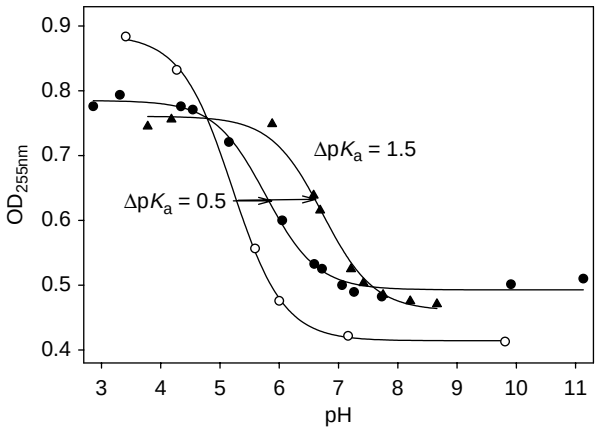


Figure 4. pH titrations monitored by UV absorption spectrophotometry of 215 μM **TR** drug in the absence of hosts (empty circles), of 215 μM **TR** in the presence of 3 mM CB7 (filled circles) and of 40 μM **TR** drug with 80 μM CB8 (filled triangles). The concentrations of the hosts were sufficient to ensure virtually quantitative complexation of **TR** over the investigated range of pH range.

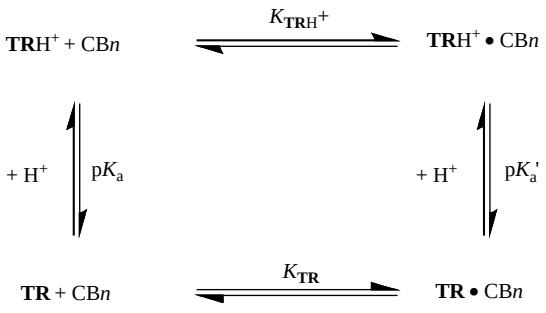
was previously rationalised by the ion–dipole interactions between many cationic guests and the carbonyl portals of CB7 or CB8 (*9*). Nau et al. (*3a*, *9c*) have developed fitting algorithms for the pH titration in the presence of host based on a four-state model to determine the pK_a values of complexed guests. For **TR**, this involves as the four states: (1) the uncomplexed unprotonated **TR**, (2) the uncomplexed protonated **TR**, (3) the unprotonated host–**TR** complex and (4) the protonated host–**TR** complex, which are all connected through a thermodynamic cycle, as illustrated in Scheme 2 (*3a*, *9c*). The binding constant values of protonated and neutral **TR** with the host molecules are related to the acidity constants of free and complexed **TR** according to Equation (1):

$$K_{\mathbf{TR}H^+} = K_{\mathbf{TR}} \times \frac{K_a}{K_{a'}}. \quad (1)$$

2.5 *Fluorescence enhancement*

With the inclusion inside the cavity of host molecules, changes in the fluorescence properties of **TR** were also observed (see Figure 5), namely a blue shift in the emission spectra and an enhancement in the fluorescence quantum yield of **TR** (ca. two-fold for CB7 and three-fold for CB8). These changes are not relevant from a practical point of view, because the fluorescence of **TR**, which to the best of our knowledge has not yet received any attention, is very weak. However, they provide additional spectroscopic evidence for the host–guest interaction (*9b*). The difference in the absolute magnitude of the fluorescence effects between CB7 and CB8 can be further rationalised by the stronger affinity of CB8 *versus* CB7 (*11g*).

We tentatively assign the observed weak fluorescence as originating from the pyridine chromophore, which is also the one included in the cavity according to NMR and mass spectrometry (see above). The observed blue shift and the enhancement of the fluorescence of **TR** are consistent with previous reports on the complexation of fluorophores by CB7 (*9b,e,g*, *11a,c,d,f–i*) and CB8 (*11e,g*), namely anilinonaphthalenesulphonate (*11a,b*),



Scheme 2. Four-state complexation model of neutral and protonated **TR** with CB*n* ($n = 7$ or 8).

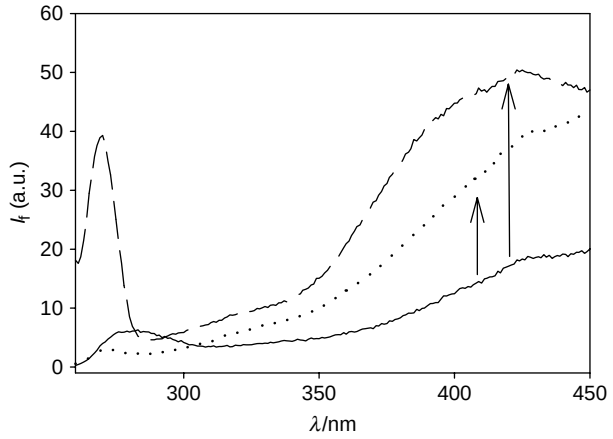


Figure 5. Fluorescence enhancement ($\lambda_{exc} = 250$ nm) of **TR** (50 μM, at pH 3) in the presence of 1.25 mM CB7 (dotted line) and 90 μM CB8 (dashed line). The fluorescence enhancements were determined by calculating the total area under the spectra, without and with the hosts, in the depicted range.

carbendazin (*10b*, *11c*), berberine (*11d*), 2,7-dimethyldiazapyrenium (*11e*), sanguinarine (*11f*), coptisine (*11g*), palmatine alkaloid (*11h*), acridine orange (*9g*, *11i*), curcumin (*6h*) and others. The fluorescence changes have been invariably rationalised in terms of either hydrophobic effects (polarity effects) (*10b*, *11a*) or geometrical confinement effects (*11b*) within the cucurbituril cavity, in addition to the role of ion–dipole interactions with the cucurbituril portal (host-assisted protonation effects) (*9b*).

3. Discussion

TR is an anti-cholinergic drug routinely applied as eye drops to cause a mydriatic response (pupil dilation) in preparation for ophthalmological examinations and surgery (*13*). It is formulated in aqueous solutions with a pH between 4 and 5 similar or below the pK_a value of the drug (5.2–5.4). As this is far below the physicological ocular pH (7.1–7.5), topical application of the drug results in significant, even if temporary, discomfort for patients. The associated irritation of the cornea leads, furthermore, to increased lacrimation, which promotes a fast washout of the instilled drug and, thereby, decreased ocular bioavailability. This, in turn, requires excessive concentrations of the drug to be applied in the strongly acidified solution, with frequently repeated administration of the eye drops being required to achieve the desired physiological response.

Several attempts to improve drug bioavailability have been reported in the literature. The idea is to increase the contact with the absorptive surface of the eye, thus improving drug penetration across the cornea. Aside from the use of acid to increase the drug's ionisation degree, and of benzalkonium chloride (also acting as preservative) to disrupt the corneal epithelium, one alternative approach aimed at an increase in the residence time of the drug in the precorneal area by using liposomes (*15*). Unfortunately, liposomes turned out not to be retained in the eye for a sufficiently long time to exhibit the desired prolonged drug action. Yet, other methods used polymer systems (*16*) to improve the viscosity of ophthalmic solutions. However, if the polymer is formulated with the drug, the intrinsic ability of the drug to penetrate biological membranes will be adversely affected.

We are also not the first to test macrocycles as molecular containers and additives to develop new formulations for **TR**. Specifically, the stability of **TR** has been shown to increase using CDs (*2c*) under physiological condition, but without affecting the cationic populations (*2d*). However, the method has the major drawback of a very weak binding (K ca. 35 M^{-1}) (*17*) of the resulting complexes, which would require excessive CD concentrations to achieve even fractional binding. Also, the absorption of the drug/CD complex itself in the eye surface is weak (*18*). It transpires that it would be highly desirable to (1) develop aqueous formulations of **TR** with comparable concentrations of the drug, but without lipidic or polymeric adjuvants and (2) to formulate the drug at neutral pH by (apparently) increasing its pK_a value by 2–3 units. The supramolecular approach with CBs appears to be a viable strategy to achieve both goals, as it is known that the complexation of basic guests by CBs increases the pK_a values of their conjugate acids by up to 4.5, typically 2 units (*3a*).

As shown by UV–vis host–guest and pH titrations, CB7 and CB8 bind the protonated form of **TR** strongly (Table 1), and its neutral form with distinctly lower affinity (although still much stronger than CDs). This results in an apparent pK_a shift of the drug in its complexed *versus* its uncomplexed form. The pK_a shift is particularly large for complexation by CB8, for which values of 1.5 and 2.5 were obtained by direct pH titrations and from the thermodynamic cycle for the independently determined binding constants, respectively (see Equation (1)). The difference between the two values is quite large, but at least partially attributable to experimental error related to, among others, the low solubility of CB8. Consequently, we report the pK_a shift for **TR** by CB8 conservatively as amounting to 2.0 ± 0.5 units. The variation of the pK_a shifts of **TR** with different hosts (CB7 *versus* CB8) is not understood in theoretical detail, but it is well known that small structural variations can lead to large changes in pK_a shifts (*3a*). For the **TR**·CB8 *versus* **TR**·CB7 complexes, no detailed structural comparisons can be made, as only the CB7 complex was amenable to NMR spectroscopic measurements. However, the host–guest absorption titration plots as well as the MS characterisations indicate

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the preferential inclusion of the pyridine group and the formation of 1:1 complexes by both CB7 and CB8.

Protonation of **TR** occurs at the pyridine nitrogen with a pK_a value of 5.2 (*13b*). The degree of ionisation at a particular pH is defined as in Equation (2):

$$\alpha = \frac{1}{1 + 10^{\mathrm{pH}-\mathrm{p}K_a}}. \quad (2)$$

For **TR** in its uncomplexed form, only 2% of the drug is protonated near pH 7 according to Equation (2), but more than 94% is protonated at pH 4. For **TR** in its complexed form, assuming a pK_a shift by 2 units upon complexation by CB8, one can project 62% to be protonated at pH 7 and more than 94% to be protonated at pH 6. This reveals that complexation-induced pK_a shifts have strong effects on the degree of protonation of the encapsulated guest molecule.

The increase in the pK_a value and, thus, stabilisation of the protonated form of **TR** has potential for application in ophthalmology in terms of drug penetration through the corneal endothelium. First, our results clearly show that encapsulation of about 0.1% (*w*/*v*) **TR** drugs complexed by about 1% (*w*/*v*) CB8 increases significantly the cationic populations of **TR** in pure water at the ocular pH (ca. 30-fold). This should facilitate the drug to display a prolonged action at the anionic eye surface, possibly without the need to add acid, solubilisers, penetration enhancers, buffer salts, polymers, gels or any other ingredients that may have adverse effects. Second, it is well known that complexation as well as decomplexation of host–guest complexes of the higher cucurbituril homologues is sufficiently fast (microseconds–milliseconds, as judged by the fast exchange in the ^{1}H NMR measurements) such that the immediate availability of **TR** is ensured by the chemical equilibrium.

4. Conclusions

CB7 and CB8 form strong 1:1 host–guest inclusion complexes with the ocular drug **TR** and shift the pK_a of this drug towards higher values. Such a combination of supramolecular effects (reversible encapsulation and host-assisted guest protonation) is beneficial in enhancing the cationic percentages of hydrophobic basic drugs by protonation without the necessity of lowering the pH into physiologically unacceptable regions. Cucurbiturils are consequently promising delivery vehicles for slightly basic drugs, particularly when topical administration is indicated as for ophthalmological applications.

5. Experimental details

TR and CB8 were purchased from Sigma-Aldrich (purity >98%) and CB7 was synthesised according to our previously reported procedure (*4d*). Millipore water and D_2O (99.5%, purchased from Applichem) were used for the measurements. The UV–vis spectra were measured on Cary 50 or Cary 4000 instruments (Varian). ^{1}H NMR spectra were measured using Jeol or Varian instruments at 400 MHz in D_2O. The pH values of the solutions were adjusted (±0.2 units) by adding adequate amounts of HCl (DCl) or NaOH (NaOD) and recorded using a pH meter (WTW 330i equipped with a WTW SenTix Mic glass electrode). MS measurements were carried out by an Agilent instrument with an electrospray ionisation ion trap in positive polarity mode. Fluorescence spectra were measured using a Cary-Eclipse instrument with a slit width of 10 nm for both excitation and emission monochromators and an excitation at 250 nm.

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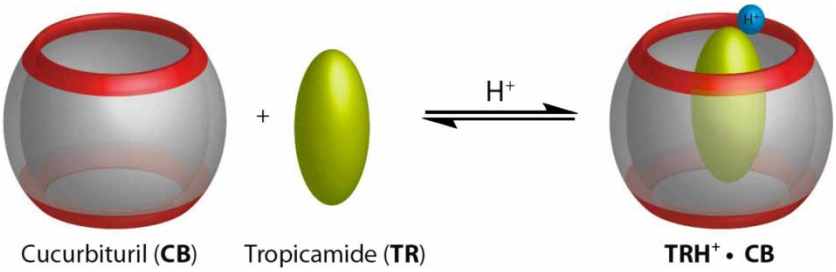

Cucurbituril induces protonation of tropicamide drug at neutral pH.

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