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Complexation-Induced Fluorescence and Acid-Base Properties of Dapoxyl Dye with γ-cyclodextrin: A Drug-Binding Application Using Displacement Assay

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Abstract: Host-guest complexation of Dapoxyl Sodium Sulphonate (DSS), an intramolecular charge transfer dye with water-soluble and non-toxic macrocycle γ-cyclodextrin (γ-CD) has been investigated in a wide range of pH. Steady-state absorption, fluorescence and time-resolved fluorescence measurements confirm the positioning of DSS into the hydrophobic cavity of γ-CD. Large fluorescence enhancement *ca.* 30 times, due to 1:2 complex formation and host-assisted guest-protonation have been utilised for developing a method for utilisation of CD based drug-delivery applications. A simple fluorescence-displacement based approach is implemented at physiological pH for the assessment of binding strength of pharmaceutically useful small drug molecules, (Ibuprofen, Paracetamol, Methyl Salicylate, Salicylic acid, Aspirin, and Piroxicam) and six important antibiotic drugs (Resazurin, Thiamphenicol, Chloramphenicol, Ampicillin, Kanamycin, and Sorbic acid) with γ-CD.

1. Introduction

Formulation of water-insoluble drug with the help of water-soluble and non-toxic macrocyclic host molecule has received a substantial interest.[1-10] Encapsulation of small drug molecule by water-soluble macrocyclic host due to non-covalent interactions can reinforce bioavailability and solubility by modulating their physical and chemical properties.[5-8] Encapsulation of drug molecules inside hydrophobic cavity leads modulations of their solution properties and this triggers activation of

drug from its proactive form to its active state.[11-14] Supramolecular drug-macrocycle complex has already been shown great promise for their usefulness in targeted drug delivery applications.[15-17]

Naturally occurring, non-toxic and water-soluble Cyclodextrins (CDs) can encapsulate drugs in their hydrophobic cavity. CDs are a family of cyclic compounds composed of sugar units connected by 1,4-glycosidic linkage and depending on the number of sugar unit in the cyclic structure they are dubbed with different name. Alpha-, beta- and gamma-cyclodextrin consists of six, seven and eight sugar units respectively.[18] CD cavity provides an access to a "new phase of matter" to the encapsulated guest molecule, which is very different from the exterior environment.[19-21] This unique cavity can modulate solution properties and optical properties of encapsulated fluorescent guest. Due to non-covalent interactions in the complex the physical and chemical properties of the encapsulated drug molecule can be modulated such as protection against photochemical and thermal decomposition, oxidation or hydrolysis.[22, 23] The larger cavity size together with higher water-solubility compared to its lower homologue, [24, 25] prompt us to select γ-CD (see Fig 1a), which is sufficiently spacious to accommodate moderate-to-large drug molecules for assessing its utility for drug-delivery applications. The same drug-delivery application is not always possible to be achieved using lower homologue of γ-CD (*i.e.* α-CD and β-CD) as their cavity volume is not enough to accommodate drug molecules with larger size and in most cases results a partial encapsulation. Consequently, such partial encapsulation often provides an insignificant modulation of properties and not suitable for aforementioned applications. Furthermore, the water-solubility of γ-CD is 10 times higher than that of β-CD and this allows better drug solubility upon encapsulation.

Generally, in pharmaceutical industries water-insoluble drugs are formulated using hydrophilic polymer, small molecule additives or *via* macrocyclic encapsulation.[26, 27] CDs are a well-known host molecule for solubilising drug and there are quite a few numbers of CD formulated drugs are already available commercially.[28] Spectrophotometry, thermogravimetric analysis (TGA), XRD, differential scanning calorimetry (DSC) and NMR are commonly used methods to validate the suitability of CDs as a co-solute for drug molecule.[29] Although, these established methods are time taking and needs quite a significant amount of drugs and solubiliser for it evaluation.

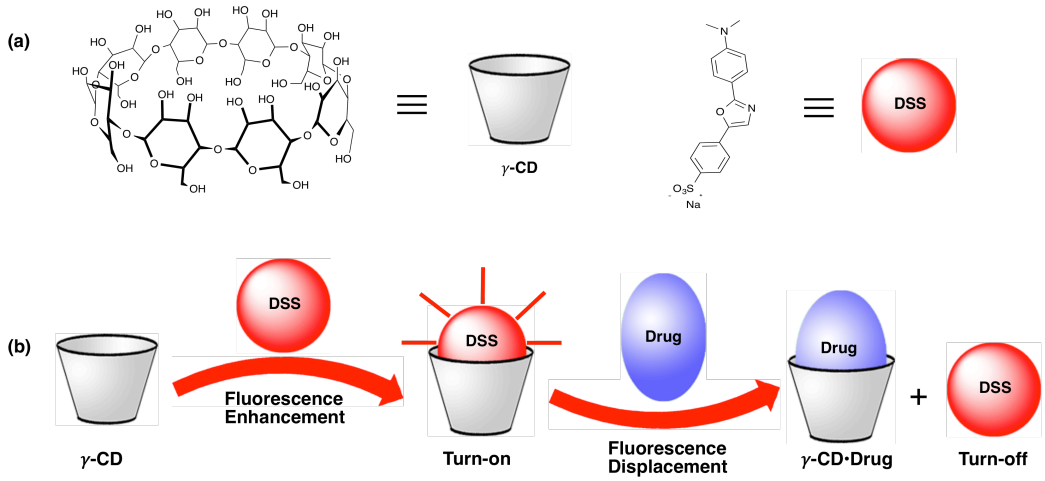

Figure 1. (a) Structure of γ-cyclodextrin and DSS, (b) schematic presentation of complexation-induced fluorescence enhancement of DSS, and switch-off fluorescence displacement principle based on displacement principle to determine drug binding constant.

Fluorescence-based indicator displacement assay [30-32] can be an alternative and easy-to-implement method to test the suitability of CD in general as a solubiliser for water-insoluble drugs. In a displacement assay a competitive analyte is introduced to a solution containing a host-dye complex and upon displacement of the dye from receptor causes a significant change in optical signal, which in turn allows one to evaluate analyte-binding ability of the receptor. To develop a displacement assay we need a fluorescent molecule having differential optical properties in complex and in its free form. An environment sensitive fluorophore will be an ideal choice for such method. Considering this fact, Dapoxyl Sodium Sulphonate (DSS, Fig 1) was selected as a fluorescent dye, which is an intramolecular charge transfer (ICT) dye having both electron rich and deficient group.[20, 33-36] Immediately after the excitation of an ICT dye it reached to the Franck–Condon state or locally excited state (LE) and upon solvation a relaxed ICT state is achieved. Due to large excited state dipole moment a solvent relaxation exhibits a red-shifted fluorescence spectrum as the polarity of the solvent increases.[37, 38] Likewise, DSS exhibits solvent polarity dependent fluorescence properties.[33, 34, 39, 40] A recent study by us was performed to validate the solvent polarity and pH dependent photophysical properties of DSS.[40] A 1:1 complex formation of DSS with α-CD and β-CD cavity results a large fluorescence enhancement along with a blue-shift in emission maxima.[40] This finding prompts us to investigate further the binding of DSS with larger cavity *γ-CD* with an anticipation of novel photophysical properties of DSS, which can be implemented to develop a displacement assay for assessing the binding of sparingly-soluble drug

with *γ-CD* (Fig 1b). In general, it is very difficult to find a suitable fluorescent dye, which can form a 1:1 host-guest complex with spacious *γ-CD* cavity and exhibits novel photophysical properties for displacement applications.[5] Moreover, in most cases large guest molecule results into 2:1 complex which makes the appropriate analysis of the displacement assay more challenging.[41] Positively charged surfactants and DNA have been used as a competitor for fluorescent-based displacement method using β-CD.[42, 43] Ueno and co-workers reported a fluorescent-based displacement method by implementing covalently attached dye with CD surface.[44] Recently, a NMR-based displacement method has been reported to test drug-binding ability of CD.[45] A highly sensitive method such as fluorescence-based turn-on or turn-off method will be well suited for such application. Best of our knowledge; there is no report on fluorescence-based displacement assay using CD to validate drug binding.

Herein, we report pH-dependent fluorescence properties of DSS upon its 1:2 complex formation with γ-CD. Further, the modulated properties have been used to foster a novel, fluorescence-based, an easy-to-perform turn-off fluorescence displacement method for assessing drug-binding ability.

2. Experimental Section

2.1. Materials: DSS was purchased from Invitrogen (USA), γ-cyclodextrin (γ-CD) and Na_2HPO_4 were purchased from Spectrochem (India), Paracetamol, NaOH, NaCl, KCl and HCl were purchased from SDfine (India), Salicylic acid was from Merck (India), Methyl Salicylate from Loba Chemie (India), Ibuprofen was obtained from Alfa-Aesar (India), Piroxicam, Aspirin, Resazurin, Sorbic acid, and Thiamphenicol were purchased from Sigma-Aldrich (India), Chloramphenicol, Ampicillin, Kanamycin were purchased from BR Biochem Life Science (India). KH_2PO_4 was obtained from CDH chemical (India). All the chemicals were used as they were received without performing any further purification.

2.2. Steady-state absorption and fluorescence measurement:

Steady-state absorption measurement was done by Shimadzu UV-Spectrophotometer 1800 (UV probe 2.42 software) using 1 cm path length quartz cuvette. All steady-state fluorescence measurements were carried out using HORIBA Jobin Yvon Fluoromax-4 fluorimeter using Origin 8 software provided with the instrument. 5 μM solution of DSS was taken for all the measurement to keep the

absorption value at such that we can avoid inner filter effect. The experiment was carried out using Mili-Q grade water using Milipore water purification set up with resistivity 18.2 MΩ•cm at 298 K. pH of water solutions was adjusted by using HCl and NaOH solution. All the *γ-CD* stock solutions were prepared in the same DSS solution and pH was adjusted. Displacement assay was performed at physiological pH *ca.* 7.4 with *γ-CD* with different concentration of drugs varying from 0-100 μM. Fluorescence spectra were recorded using 1 cm path length quartz cuvette from 355 nm to 680 nm range by exciting at 340 nm and keeping both excitation and emission slit at 2 nm. All the experiments were carried out at ambient temperature (298 K).

2.3. Time-resolved measurement:

Time-resolved fluorescence measurements were performed using a Hamamatsu MCP photomultiplier (R-3809U-50). The time-correlated single photon counting (TCSPC) setup consists of an Ortec 9327 pico-timing amplifier and using pulse Diode laser (NanoLED, N-375) for excitation (λ_{ex} =375 nm) with fwhm ~167 ps with a setup target 10,000 count. The instrument response function (IRF) was measured before and after fluorescence lifetime measurement using a dilute suspension of Ludox (purchased from Sigma) colloidal silica. The emission polarizer was positioned at magic angle (54.7 º) with respect to excitation polarizer. Single and multi-exponential fitting function was employed by iterative deconvolution method using supplied software DAS v6.2. General form of the fitting function was:

$$I(t)/I(0) = \sum a_i exp(-t/\tau_i)$$

Where I (t) and I (0) are the fluorescence intensity at time t and 0 respectively, t is time and a_i and τ_i are the contributing amplitude and it's corresponding lifetime. The quality of the fitted data was judged from the reduced chi-squared value (χ^2), calculated using the IBH software provided with the instrument. All the measurements were carried out at ambient temperature (298 K).

2.4. Drug binding experiment:

Displacement assay was performed in aqueous solution at pH *ca.* 7.4 containing 5 μM DSS and 5 mM *γ-CD* in a 1 ml fluorescence cuvette. Then the fluorescent intensity was measured at 500 nm upon excitation at 337 nm with subsequent addition of pH adjusted concentrated drug solution in the same *γ*-CD•$(DSS)_2$ solution to avoid dilution effect and pH. Binding constants of *γ-CD*•drug were calculated using 1:2 and 1:1 equation for the small-molecule drugs and for antibiotic respectively. The

concentration of γ-CD•DSS complex was calculated from the binding constant of γ-CD•DSS complex at pH 7.4.

2.5. pH titration, binding titration and their analysis:

pH-dependent absorbance of DSS dye was measured in to determine it's pK_a value using a two-state model [40, 46] (by considering protonated and non-protonated dye) and in case of the γ-CD•DSS complex a four-state model [40, 46] was used to find out the pK_a value in the complex state. The pH dependent binding constants of DSS dye with *γ-CD* was fitted with 1:2 binding equation using a nonlinear fitting procedure of the Pro Fit 6.2.9 software as reported earlier by Nau and co-workers.[47] In all the measurements, the error was calculated after fitting the experimental data using an appropriate equation with the help of Pro Fit 6.2.9 software. The software provides the goodness of fit as the difference of the mean value as an error after fitting and which was within ±10%.

2.6. Computational study of complexation:

To investigate the mode of complexation between *γ-CD* and DSS we have performed the computational study. In this regard, we employed Density Functional Theory (DFT) based molecular module DMol3 in Material Studio 6.1 programme package.[48] Perdew-Wang generalised-gradient approximation functional (PWC) was used for all the geometry optimisation.[49] To make our computational study consistent we used the Conductor-like Screening Model (COSMO) for applying the solvent effect on complexation in water. The electrostatic energy during the optimisation is:

$$E_{elec}^{COSMO} = -{}^{1}/_{2}\ \langle Z|D|Z\rangle - \langle \rho|D|Z\rangle - \langle \rho|D|\tilde{\rho}\rangle + {}^{1}/_{2}\ \langle \tilde{\rho}|D|\tilde{\rho}\rangle$$

Where Z is the nuclear charge, D is the dielectric operator, *r* is the density and $\tilde{\rho}$ is the auxiliary density; here auxiliary density is introduced to solve the Poisson equation for the electrostatic potential of the solute.

3. Results and Discussion

The present study deals with the pH dependent complexation of DSS with γ-CD and determination of drug binding ability of γ-CD. DSS binding with γ-CD causes large fluorescence enhancement and a shift in the acid dissociation constant and this helped us to design a turn-off indicator displacement assay. UV-Vis and fluorescence titration of DSS with γ-CD were performed at different pH and complexation-induced fluorescence change was monitored to calculate the binding

constant of DSS with γ-CD. Job's plot at pH 7.0 by monitoring the fluorescence intensity changes confirms 1:2 complexation between γ-CD and DSS. Differential binding affinity at different pH indicated a shift in the acid dissociation constant and one unit negative shift was measured by the pH titration of DSS and its γ-CD•$(DSS)_2$ complex. Further, binding of small molecule and antibiotic drugs with γ-CD was estimated using turn-off displacement principle. The result and discussion section is subdivided into subsections detailing DSS binding to γ-CD, host-induced pK_a shift, lifetime, anisotropy properties and drug-binding study using displacement principle.

3.1. pH-dependent binding studies of DSS with γ-CD using absorption and fluorescence spectroscopy

Host-guest titration at pH 7.0 using 5 μM DSS was performed by successive addition of a concentrated stock solution of γ-CD in the same concentration of DSS. The titrations were monitored by both absorption and fluorescence spectroscopy. Absorption spectrum of DSS was characterised by a broad non-structured band spanning in the region between 300–480 nm (Fig 2). Similarly, emission spectrum has a broad range of 350–650 nm. A significant red shift of *ca.* 27 nm in the absorption spectra was observed apparently due to H-bonding interactions mediated complexation [50] of DSS and γ-CD. The emission maximum of DSS has a considerable blue shift from 582 nm to 488 nm and large fluorescence enhancement upon complexation with γ-CD (Fig 3a). FWHM of both the absorption and fluorescence spectra of DSS in the presence of γ-CD get narrower compared to the same in water due to its positioning in hydrophobic environment and higher-order complex formation (*vide infra*).

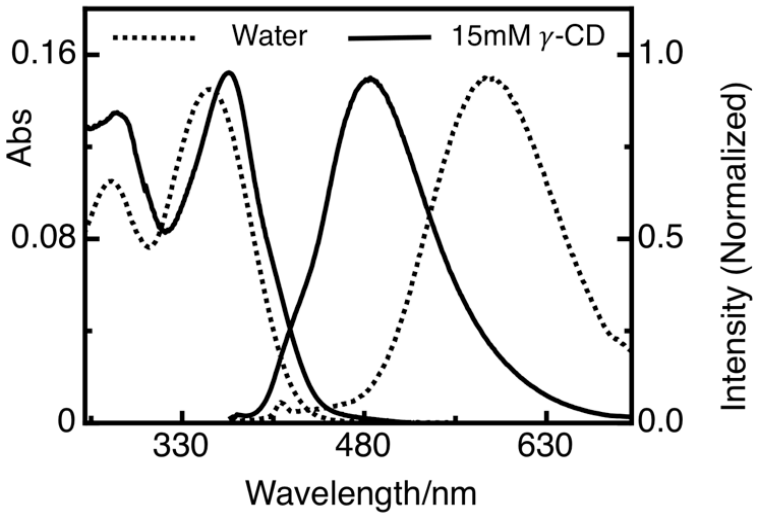

Figure 2. Absorption and fluorescence spectra of DSS in water and with 15 mM γ-CD at *ca.* pH 7.0

Complexation-induced photophysical properties of DSS from the steady-state and time-resolved measurements at pH 7.0 are summarised in Table 1. Upon γ *-CD* complexation we observed 3.6 and 3.2 times increase in QY and fluorescence lifetime, respectively.

Table 1. Photophysical properties of DSS in water and in γ-CD complex

Medium	λ_{abs} (nm)	λ_{em} (nm)	Stokes Shift (cm^{-1})	QY[b]	τ [c,d] (ns)	$k_r \times 10^8$ (s^{-1})	$k_{nr} \times 10^8$ (s^{-1})[f]
Water	342	383[a], 582	12058	0.19	1.9	1.00	4.26
γ-CD	369	488	6608	0.68	6.1	1.11	0.52

[a] obtained at pH 2.0 and 4.0 due to LE state [b] QY was calculated using 8-Anilinonapthalene-1-sulphonic acid in methanol (QY=0.21) as a standard [51] [c] average fluorescence lifetime [d] lifetime measured at pH 7.0 and lifetime of DSS in other pH without and with γ -CD complex are given in Table S1

Notably, large complexation-induced spectral shift, fluorescence enhancement and spectral feature of γ-CD•$(DSS)_2$ complex at pH 7.0 prompt us to find out the stoichiometry of the host-guest complex. The fluorescence peak observed for the complex is very different compared to the maximum of DSS in water (LE band: 383 nm, CT band: 580 nm) or of DSS in β-CD•DSS complex (CT band: 553 nm)[40]. Job's plot in the inset of Fig. 3a with the maximum around 0.40 indicates a γ-CD•$(DSS)_2$ complex formation.

To characterise further and evaluate the binding strength, pH-dependent fluorescence titration of DSS was performed with γ*-CD* as the pK_a value of DSS is *ca.* 4.1.[20, 40] Protonated form of DSS ($DSSH^+$) shows emission maxima originating from both LE as well as from the CT state. In contrast, the spectrum only due to CT state is visible from the non-protonated state. At pH, lower than the acid dissociation constant $DSSH^+$ binds weakly with the hydrophobic cavity of γ*-CD* due to protonation of *N,N*-dimethyl aniline part of the guest molecule. On the contrary, DSS has significantly higher binding strength due to hydrophobic interaction with aromatic

rings as well as hydrogen bonding interactions with sulphonate part (see Fig S2). Binding affinities of DSS and *γ-CD* were calculated from the change in fluorescence intensity of DSS upon addition of increasing amount of *γ-CD* to a fixed concentration of DSS (see Fig. 3b). We also performed a computational study employing DFT based molecular module DMol3 in Material Studio 6.1 program package[48, 49] to test the binding mode of the host-guest complexation. As it can be seen from Fig S2 that 1:1 γ-CD•DSS complex shows a significant distortion in the *γ-CD* structure but in contrary, γ-CD•$(DSS)_2$ complex shows retention of symmetric structure of γ-CD. pH-dependent binding constant were calculated using 1:2 fitting function[47] and the values are summarised in Table 2.

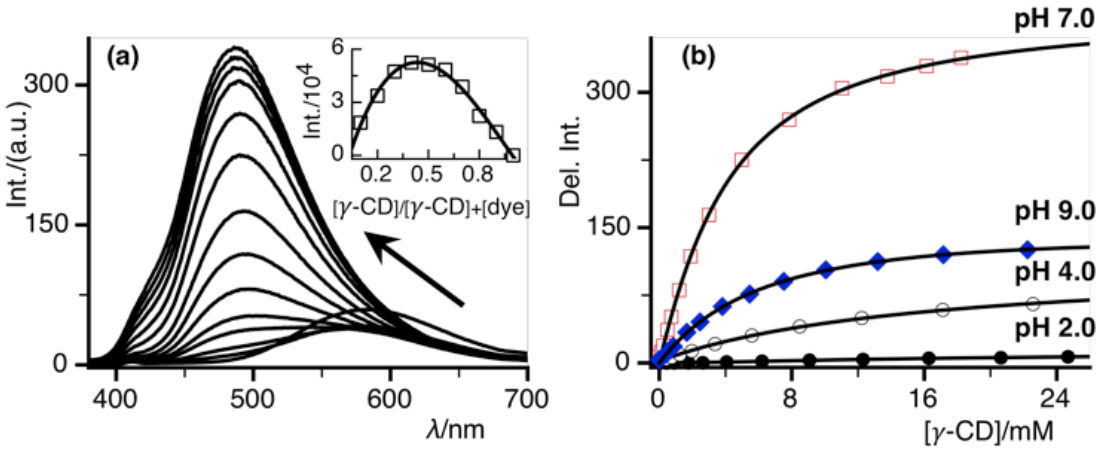

Figure 3. (a) Fluorescence titration of DSS with γ-CD at pH 7.0 shows shift in the emission maxima with gradual increase in the fluorescence intensity, inset shows Job's plot and it confirms 1:2 complexation of γ-CD and DSS at pH 7.0, (b) pH dependent fluorescence enhancement of DSS with γ-CD monitored at 480 nm, data fitted with 1:2 binding model.

Table 2. pH dependent binding constants of DSS with γ-CD and fluorescence nhancement and average fluorescence lifetime upon complexation

pH	K_1 $(M^{-1})^a$	K_2 $(M^{-1})^a$	$K_1 K_2$ $(M^{-2})^a$	Fluorescence Enhancement factor[c]	Average Lifetime (ns)[d]
2.0	12	5	60	1.59	5.5 (1.5, 2.0)[e]
4.0	55	20	1100	20	5.9 (1.6, 1.9)[e]
7.0	185	25	4625 (2700)[b]	57	6.1 (1.9)[e]
9.0	155	15	2325	36	5.7 (2.1)[e]

[a] binding constant values are obtained from 1:2 fitting equation [47]. [b] binding constant in 100 mM phosphate buffer solution. [c] fluorescence enhancement factor was calculated from the intensity ratio of the γ-CD•$(DSS)_2$ complex band at *ca.* 485 nm after full complexation (except for pH 2.0 at 570 nm) compared to without γ-CD, note a reduction of intensity at 380 nm at pH 2.0 titration. [d] average fluorescence lifetime of DSS with 35 mM γ-CD, [e] values in parenthesis show average fluorescent lifetime of only DSS dye in aqueous solution when monitored at 430 nm and in 530 nm at pH 2.0 and 4.0 and 530 nm at pH 7.0 and 9.0.

The differential binding affinity of DSS and $DSSH^+$ with *γ-CD* prompts us to investigate the complexation-induced shift in acid dissociation constant of DSS. A pH titration of DSS and γ-CD•$(DSS)_2$ using spectrophotometry reveal a 0.9 unit negative pK_a shift (Fig 4a). Inset of Fig. 4a shows a typical pH titration of DSS in the presence of 15 mM of γ-CD. Binding titration of DSS and *γ-CD* at pH 4.0, which is close to the pK_a of DSS, shows decrease in fluorescence intensity of the LE band and concomitant fluorescence enhancement from CT band (Fig. 4b).

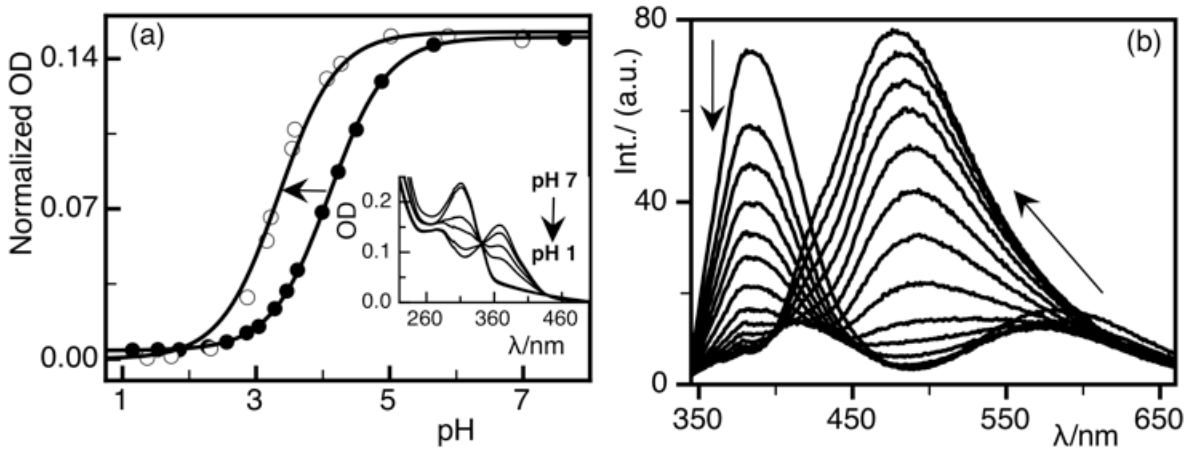

Figure 4. (a) pH titration of DSS using UV absorption of DSS without (closed circle) and with (open circle) 15 mM γ-CD by monitoring at 340 nm shows *ca.* 1 unit pK_a shift; inset shows absorption sepctra of γ-CD•DSS in different pH using 15 mM γ-CD, (b) binding titration of DSS with γ-CD at pH 4.0, decrease in 385 nm band and commencing of 1:2 complex band around 500 nm.

pH-dependent fluorescence titration of DSS in four different pH with and without γ-CD shows a visible change (*c.f.* Fig 5a) in the fluorescence outcome due to the formation of *γ-CD*•$(DSS)_2$ (Fig. 3a). At pH around 3.0 the protonated form of DSS is weakly fluorescent upon excitation at 354 nm hand-held UV lamp whereas at same

pH *γ-CD*•$(DSS)_2$ shows high fluorescence. Moreover, at pH 9.0 the deprotonated form of DSS has a significant change in fluorescence emission maxima after *γ-CD* complexation (see Fig. S1).

3.2. Effect of *γ-CD* complexation on the fluorescence lifetime of DSS and $DSSH^+$

The fluorescence lifetime of DSS is known to be sensitive towards the solvent environment.[40] In aqueous solution, for both DSS and $DSSH^+$ have approximately 2.0 ns fluorescence lifetime, which has been related to strong intermolecular hydrogen bonding and associated with non-radiative deactivation pathways.[52] In the present study, the fluorescence decay of DSS and its corresponding *γ-CD* complex were investigated in aqueous solution at pH 2.0, pH 4.0, pH 7.0, and pH 9.0. The fluorescence lifetime of DSS and its *γ-CD* complex show bi-exponential decays (Fig 5b, Table 2).

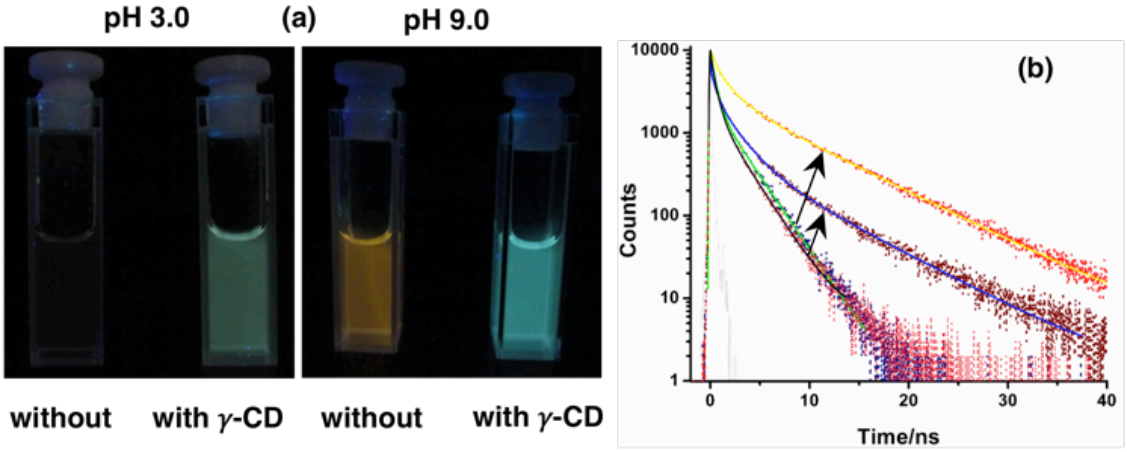

Figure 5. (a) Digital images showing distinct fluorescent colour and intensity change of DSS (10 μM) without and with 50 mM γ-CD at pH 3.0 (left) and pH 9.0 (right), (b) fluorescence lifetime enhancement (shown using arrow) of DSS (5 μM) at pH 4.0 (dark-brown) and pH 9.0 (red) without and with 35 mM γ-CD.

3.3. Fluorescence anisotropy studies of DSS and its *γ-CD* complex

Time-resolved fluorescence anisotropy measurements were carried out in aqueous solutions of DSS in the absence (Fig 6a) and presence (Fig 6b) of 15 mM *γ-CD* at pH 7.0. In both cases, the anisotropy decays appeared to be mono-exponential in nature. A rotational correlation time (τ_r) of 0.5 ns for DSS was obtained at pH 7.0.

The anisotropy decay for *γ-CD*•$(DSS)_2$ was sufficiently slower compared to DSS, and it gave τ_r values of about 1.3 ns. The initial anisotropy (r_0) for both DSS and its corresponding *γ-CD* complex was also estimated to be quite high (*ca.* 0.4). The reduced rotational diffusion times are fully consistent with the formation of inclusion complex. The rotational correlation time (τ_r) can be related to its rotational diffusion coefficient and the viscosity of the surrounding environment according to the Stokes-Einstein relationship: $\tau = 1/6D_r = \eta V/kT$

Here, *V* is the hydrodynamic molecular volume of the complex, *η* is the viscosity of the medium, *k* is Boltzmann constant, and *T* is the absolute temperature. For the inclusion complex between DSS and γ-CD, a rough estimation of the hydrodynamic molecular volume can be obtained by considering the complex as an effective sphere and the hydrodynamic diameter similar to the outer diameter of *γ-CD* molecule (17.5 Å) plus the volume corresponds to the part outside the γ-CD cavity. The volume (*V)* of γ-CD can be calculated as 1690 cm^3mol^{-1}. Thus, the total volume of the 1:2 complex can be calculated based on our theoretically calculated optimised structure (Figure S2), where part of *N,N*-dimethyl aniline group is outside of the *γ-CD* cavity. For single *N,N*-dimethyl aniline group a total of ~74 cm^3mol^{-1} (~43 cm^3mol^{-1} for benzene ring and ~31 cm^3mol^{-1} of $–N(CH_3)_2$ group) was estimated.[53] Therefore, by considering a *γ-CD*•$(DSS)_2$ complex a total volume of ~1838 cm^3mol^{-1} (*cf*. SI for detail calculation) has been estimated. Hence, from this volume and *η* value of bulk water at 25 °C we calculate τ_r (0.72 ns) of the complex, which is smaller presumably due to underestimate of micro–viscosity as a result of hydrogen bonding network between the complex and solvent molecules. Noteworthy, the slower rotational correlation time for the DSS suggest formation of a tight inclusion complex, which appear to rotate as a whole in aqueous solution; *i.e.* the independent motion of the dye inside γ-CD cavity is highly restricted.

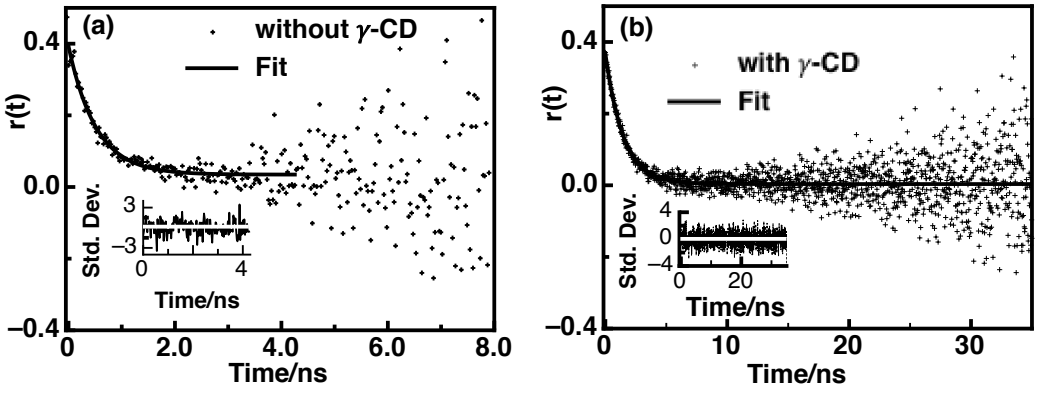

Figure 6. Time-resolved anisotropy decay curves at pH 7.0 in water at 25 ºC for (a) 5 μM DSS without γ-CD monitored at 580 nm and (b) the 5 μM DSS and 15 mM γ-CD complex monitored at 480 nm; inset shows residual of the fitting

3.4. Drug-binding affinity of *γ-CD* by fluorescence displacement assay

Based on the modulation of optical properties of DSS upon complexation with moderately large *γ-CD* cavity, we have designed a fluorescence turn-off-assay to evaluate drug-binding propensity of it's unique hydrophobic nanocavity, which can be used as an efficient carrier of sparingly water-soluble drugs to enhance their bioavailability. Twelve structurally different commodity drugs (*viz.* Salicylic acid, Methyl salicylate, Aspirin, Ibuprofen, Piroxicam, Paracetamol, Ampicillin, Kanamycin, Chloramphenicol, Thiamphenicol, Sorbic acid and Resazurin, *c.f.* Fig. 7a) were selected to assess the complexation with *γ-CD* cavity.

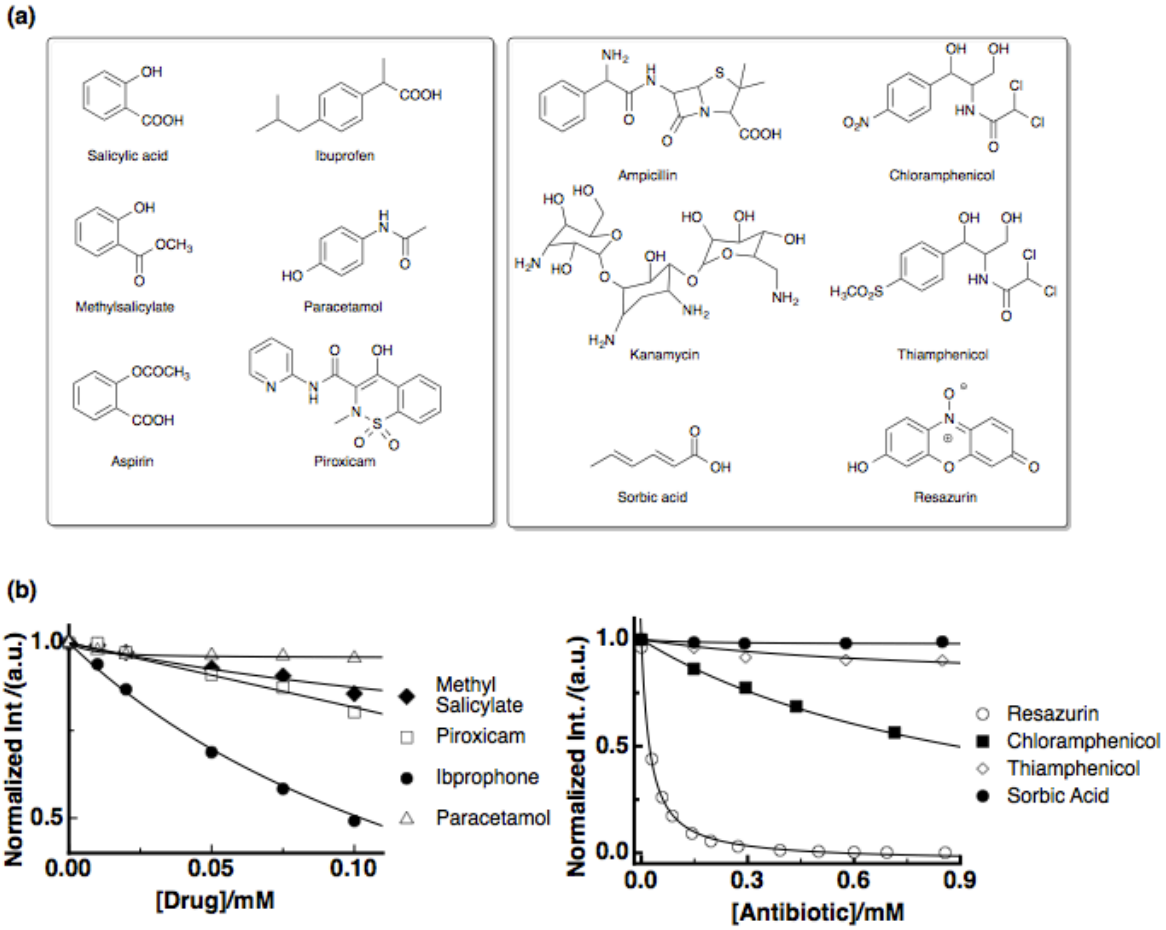

Figure 7. (a) Structure of the drugs used to validate our principle; small molecule drugs (left) and antibiotic drugs (right) (b) relative change in fluorescence intensity and their

corresponding fitted plot (solid line) upon displacement of DSS from the γ-CD cavity to quantify the binding affinity of small-molecule drug (left) and for antibiotic drugs (right)

A γ-CD•$(DSS)_2$ fluorescent complex was pre-formed at pH 7.4 using 5 μM DSS and 5 mM *γ-CD* to get a significant effect of fluorescence enhancement. A binding titration of DSS with γ-CD in phosphate buffer solution (100 mM, pH 7.4) was performed to test suitability of the assay in physiological condition. We have observed a significant fluorescence enhancement (Fig. S3) even in 100 mM PBS suggests a negligible competition from phosphate ions. As the guest exchange equilibria between the guest and host are dynamic and rapid (nanosecond to millisecond) in nature and allows performing a displacement assay using a high concentration of host molecule.[30] Subsequently, a gradual addition of drug solution, which was prepared in the same γ-CD•$(DSS)_2$ solution to avoid dilution effect, was performed to displace fluorescent dye from γ-CD cavity. Indeed, such displacement causes a gradual loss in fluorescence intensity due to the relocation of DSS dye into the aqueous solution. Such decrease in fluorescence is an indirect indication of the binding strength of drug molecules. Binding affinity of small-molecule drugs and relatively larger antibiotic drugs with γ-CD; (Fig. 7b, Table 3) was calculated by fitting the data (*c.f.* experimental section) for the change in fluorescence in terms for drug concentration. Among the studied small-molecule drugs Ibuprofen shows highest binding affinity towards *γ-CD* cavity followed by Methyl salicylate, Piroxicam and others. On the other hand, Resazurin shows highest affinity (independently measured by UV and fluorescence titration; as shown in Figure S4 and UV-Vis spectra of the drugs are provided in Fig. S5) among the antibiotic drugs followed by Chloramphenicol and others. The binding constant of the drugs with *γ-CD* was calculated from the fitting (Table 3). Binding constant of the encapsulated drug within γ-CD is really important for its *in vivo* implication. Higher binding strength (*ca.* 10^4-10^5 M^{-1}) of the drug•CD complex will provide appreciable bioavailability as reviewed comprehensively.[54] Fluorescent-based, easy-to-perform and quick assay can be useful to other class of drugs as well as to improve their chemical and physical properties to enhance bioavailability for drug delivery application.

Table 3. Binding constant of drugs with 5 mM γ-CD at pH *ca.* 7.0 determined using 1:1 and 1:2 binding equation [a,b]

Entry	Drugs	Binding constant (M^{-1})
1	Ibuprofen	5400
2	Piroxicam	560
3	Salicyclic acid	<20
4	Methyl Salicylate	2400
5	Aspirin	<10
6	Paracetamol	<10
7	Ampicillin	<50
8	Kanamycin	<10
9	Chloramphenicol	970
10	Resazurin	48000
11	Thiamphenicol	140
12	Sorbic Acid	<20

[a] Drug binding constants were evaluated using 1:2 equation for small-molecule and 1:1 equation for large antibiotic molecules respectively. [b] DSS binding with γ-CD is a dynamic equilibrium process and as the binding constant is related to rates ($K_1K_2=k_{on}/k_{off}$) and the displacement of DSS from γ-CD can be realised as a dynamic process too.

4. Conclusions

In conclusion, a γ-CD•$(DSS)_2$ complex was characterised using absorption, steady-state and time-resolved fluorescence spectroscopy and anisotropy measurements. pH-dependent binding affinity of DSS with *γ-CD* shows preferential binding of neutral form over protonated form. Further, a simple fluorescence displacement assay was developed to validate the effectiveness of drug-binding ability of *γ-CD* cavity for small-molecule drugs and antibiotic drugs. This assay is operable at the physiological condition and useful to any hydrophobic, neutral and negatively charged drugs. Our method will allow pharmaceutical industries to quickly assess and analyse the suitability of CD for drug formulation application. The same assay can be performed to test thermal and chemical stability of the formulated drug using high-throughput fashion. Such fluorescence-based displacement assay will allow us

to quickly validate the suitability of *γ-CD* as a drug carrier for sparingly water-soluble drugs.

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Supporting information: Electronic supplementary information (ESI) available.

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