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Detailed Scenario of the Acid–Base Behavior of Prototropic Molecules in the Subdomain-IIA Pocket of Serum Albumin: Results and Prospects in Drug Delivery

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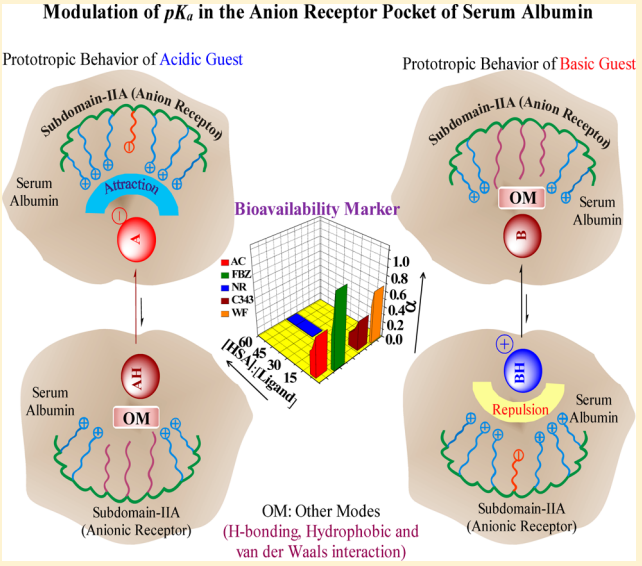
Supporting Information

ABSTRACT: The protein pocket performs magically in controlling, inhibiting, or optimizing various biochemical processes. The elegant 3D disposition of different side chains in the cavity is a key point in accommodating specific ligands. Anion receptors in the subdomain-IIA pocket of serum albumin (SA) prefer to home anionic ligands. Acid–base behavior is an important property that relates to bioavailability and action of prototropic molecules/drugs. The present study provides a comprehensive understanding of the effect of subdomain-IIA pocket-specific interaction on the acid–base equilibrium of housed guests. The pK_a of subdomain-IIA binder basic drugs decreases due to unfavorable interaction with the cationic drug species, while the decrease in the pK_a of acidic drugs is due to favored binding of the deprotonated species presumably via electrostatic interaction with anion receptors. Acidity-shifting efficacy of albumins is introduced for the first time using the pK_a-shifting index (α), a unique parameter for a given prototropic-drug-host pair to assess bioavailability. The acidic drug warfarin and the basic drug fuberidazole, showing a high α-value, should be efficient in drug-SA cocktail, and those with low α should be less efficient. Use of the pK_a-shifting index for prototropy-based drugs should enable the drug efficacy to be evaluated smartly for similar systems. Shifting of the pK_a of protein-encapsulated drugs stems the possibility of albumin-based delivery systems for extracting the therapeutically active species.



1. INTRODUCTION

The implication of noncovalent interactions between macrocyclic host and different guest molecules has attracted considerable interest in biophysical research in recent times.[1] Cylcodextrins, calixarenes, and cucurbiturils (CBs) are the by and large explored macrocyclic hosts.[2,3] Interestingly, cucurbiturils, with unique structural features,[4] can bind nonpolar molecules through hydrophobic forces and cationic ligands by ion–dipole interactions. However, their known primary role is to take part in strong host–guest complexation with cationic ligands.[5] The unique feature of host–guest interaction is that it results in pronounced modification of molecular properties like solubility, stability, prototropic equilibrium, etc., of the encapsulated guest.[6,7] Among these modifications, host-induced pK_a shift of guest has been one of the important research themes due to their various applications,[8−11] the latest accession being in the area of pharmacokinetics.[3,12−14] Activity of prototropic drugs with either acidic or basic functional groups strongly depends on the population ratio of two different protomers in the human body system.[3] Thus, any factor which can alter the ratio will in turn have a direct impact on the therapeutic activity. The preferential binding of the cationic form of basic guest with cucurbiturils (CBs) results in a large positive pK_a shift, and this has been exploited toward the designing of supramolecular hosts to promote the active promoter of basic drugs.[3,15−17] The primary advantage of host–guest interaction is that these are responsive to external stimuli like salt, temperature, or pH jump which can be very useful for the controlled release of prototropic drugs from the macrocyclic cavity to blood serum.[18−21] However, cytotoxicity of cucurbiturils has been a major issue of concern. These CBs are found to be nontoxic only up to a certain concentration level which is sometimes below that required for drug delivery applications.[3] The even-numbered CBs are too poorly soluble in water[3] to become practically useful, which demands for its improved derivatization.

In recent years, serum albumin-based drugs have attracted considerable attention for diagnosing and treating diabetes,

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cancer, and infectious diseases.[22,23] Due to the small size and high abundance of albumin in blood plasma, a major fraction of the administered drug is bound to plasma albumin proteins. Researchers have used these insights to develop drugs with high binding affinity for albumin to either improve the pharmacokinetic profile or bioavailability of drugs. Drugs either complexed or conjugated with albumin have improved pharmacokinetic profiles, like enhanced half-life, enhanced solubility and stability, as well as enhanced targeting properties. Herein, for the first time, we would like to invoke the possibility of employing albumin-based delivery systems for extracting the therapeutically active species of prototropic drugs. The activity of a large number of prototropic drugs is very much dependent on the ratio of its active to inactive forms in the human system, and hence, enhancement of the population of the active form to increase the bioavailability of a drug has always been a major challenge to researchers. Thus, it will be very much useful to explore if binding of prototropic drugs in the albumin pocket has any effect on the prototropic ratio and thereby extracting more efficiency out of the drugs.

In a recent report,[24] we have highlighted that the ground-state acid−base equilibrium of (acidic) drugs like warfarin and structurally similar coumarin dye, coumarin 343, is considerably shifted toward the anionic form (i.e., the basic form). The role of anion receptors (amino acid residues with a positively charged side chain) available in the biosupramolecular cavity (in subdomain-IIA) of HSA has been shown to stabilize the basic (anionic) form of warfarin which is also the major active component for showing its anticoagulant activity.

The major subsequence to our first report[24] is whether we can extrapolate our understandings with basic guests as well and arrive at some generalized model. A basic guest also has two different prototropic forms, namely, cationic and neutral. Since the subdomain-IIA cavity in serum proteins is reported to act primarily as an anion receptor, the binding of cationic ligand could be unfavorable here and hence it should affect the prototropic equilibrium of basic guest also.

Thus, to have a solid and conclusive understanding of the role of the subdomain-IIA cavity of HSA on the modulation of the ground-state acid−base equilibrium of both acidic and basic guests, we have chosen three basic guests, fuberidazole (FBZ), neutral red (NR), and acridine (AC) in our study. Fuberidazole is a derivative of benzimidazole which is extensively used in the preparation of anthelmintic drugs.[25] Several research groups have reported that benzimidazole has a high affinity for cucurbit[7]uril (CB7).[12,15,17] Koner et al. have reported that encapsulation of benzimidazole derived drugs by CB7 results in stabilization of the protonated form, thereby shifting the pK_a toward a higher value.[13] Recently, it has been reported elsewhere that both solubility and bioactivity of albendazole are enhanced due to encapsulation in acyclic cucurbiturils.[26]

Neutral red (NR) is another basic guest which, depending upon the pH of the medium, can exist in two prototropic forms.[19,27] NR has been extensively used as a fluorescence probe for the investigations of different microheterogenous and confined media.[28,29] Since its pK_a (=6.8) lies close to physiological pH conditions, this has also been extensively used as a marker for biological systems, especially as an intracellular pH indicator.[19] It is reported to form inclusion complexes with cyclodextrins and cucurbiturils.[30] Mohanty et al. have reported that the pK_a of neutral red is up-shifted in the cation receptor cavity of CB7 and down-shifted (decreased to a lower value) in the hydrophobic cavity of cyclodextrins.

Acridine derivatives are very important molecules due to their many pharmaceutical applications.[31,32] These are also used as a fluorescence probe in investigating different micro-heterogenous systems[33] and also used as a model system for studying both drug−protein and drug−DNA interactions.[32] Several research groups have explored the interaction of acridine (AC) with β-cyclodextrin (β-CDx).[34,35] Shaikh et al. have recently investigated the inclusion of acridine in two different cyclodextrins (β-CDx and γ-CDx).[36] They have reported that the protonated (cationic) form of acridine does not show any interaction with either β-CDx or γ-CDx, but the neutral form of acridine (AC) strongly binds with both β-CDx and γ-CDx. This differential binding affinity of cyclodextrins with cationic and neutral forms of acridine results in the negative pK_a shifting.

Benzimidazole derivatives are known to bind the warfarin binding site of HSA.[37] The neutral form of NR is also known to bind subdomain-IIA of BSA.[38] HSA is the major (multifunctional) transporter of various exogenous molecules like drugs and prodrugs, and hence, the investigation of the position of the prototropic equilibrium of these basic guests in the presence of serum protein can be worthwhile, since the outcome may put us one step forward to argue in favor of albumin-based drug delivery systems. The results can indeed furnish a new dimension to pharmacokinetics.

A comparison of the amino acid sequence of HSA with its homologous serum protein BSA reveals that 15 amino acid residues in the subdomain-IIA cavity of the former has been replaced in the latter.[39] Binding of anionic ligand in the subdomain-IIA cavity of BSA is found to be solely guided by electrostatic interaction, while that in HSA is a combination of both electrostatic and nonelectrostatic forces.[39] Hence, their affinity for other ligands (cationic and neutral) is also expected to be different and ought to have some distinct effect on the prototropic behavior of guests roomed in their subdomain-IIA cavity. Thus, in order to explore the possible role of such differential binding affinity on pK_a, we have also investigated the prototropic behavior of guests encapsulated in BSA also. The outcomes will throw light on the apparent competition between these two homologous biomolecules and can suggest whether HSA or BSA will be the preferred encapsulate for different prototropic drugs.

2. EXPERIMENTAL SECTION

2.1. Materials. HSA (>98%), BSA (~99%), neutral red (>90%), fuberidazole (analytical standard), and acridine (>97%) are purchased from Sigma-Aldrich, and warfarin (>97%, HPLC) (analytical grade) is purchased from TCI chemicals, Japan. Coumarin 343 (laser grade) from Exciton, USA, is also used as received. Sodium chloride (analytical grade), phosphoric acid, NaH_2PO_4, Na_2HPO_4, and ethanol (UV spectroscopic grade) from E-Merck are used as received. Other chemicals are of analytical reagent grade, and ultrapure water is used throughout the study for preparation of solutions.

2.2. Solution Preparation. Stock solutions of fuberidazole, warfarin, coumarin 343, and acridine are prepared in ethanol. The stock solution of neutral red is prepared in water. A very small volume of these stock solutions is added to get the final concentration. All pH solutions are 5 mM phosphate buffer. A EUTECH pH 510 ion pH-meter is used for the measurement of pH.

2.3. Instrumentations and Methods. The UV−vis absorbance and the steady state fluorescence spectra are

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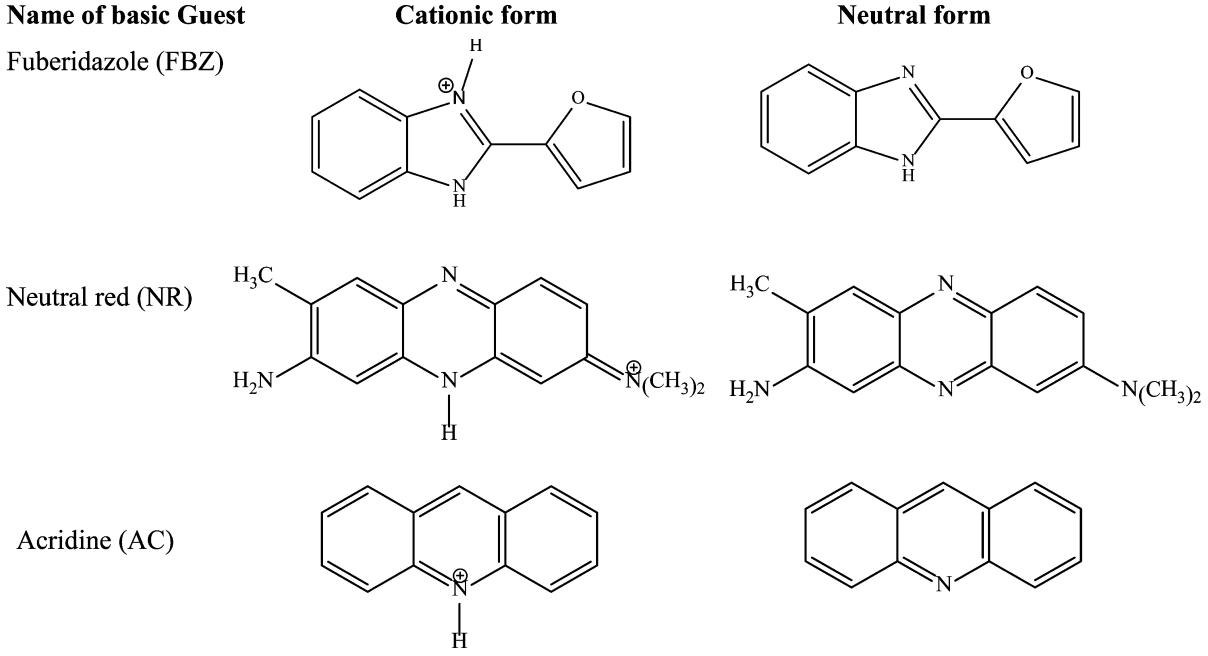
Scheme 1. Chemical Structures of Prototropic Forms of Basic Guests (FBZ, NR, and AC) in an Aqueous System



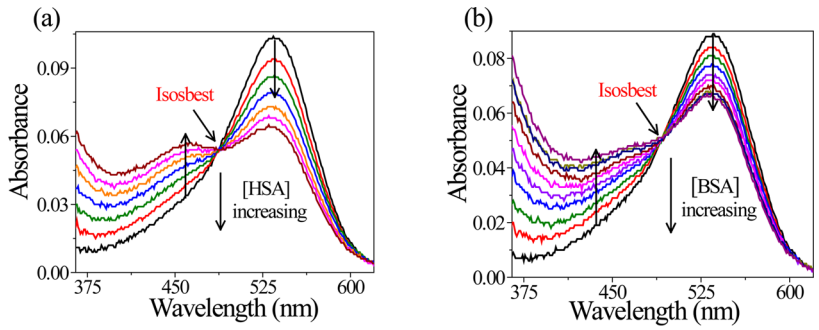


Figure 1. Absorbance spectra of NR at pH 5.0 with increasing concentration of HSA (a) and BSA (b). [NR] = 2 μM. [HSA] is varied from 0 to 174 μM, and [BSA] is varied from 0 to 155 μM.

recorded on a Shimadzu UV-2450 spectrophotometer and Jobin Yvon - Spex Fluorolog-3 spectrofluorimeter equipped with a temperature controlled water cooled cuvette holder, respectively, and a quartz cuvette of 1 cm path length has been used. All spectral measurements have been performed at 298 K.

Fluorescence lifetimes of coumarin 343 are measured by means of a single photon counting apparatus where the samples are excited at 408 nm using a picosecond diode laser (IBH, Nanoled), and the signals are collected at the magic angle 54.7° using a Hamamatsu microchannel plate photomultiplier tube (R3809U), where the instrument response is 100 ps. Fluorescence lifetimes of acridine and neutral red are measured by means of a single photon counting apparatus (Horiba, Jobin Yvon, IBH Ltd., Glasgow, Scotland) equipped with a nanosecond LED excitation source. Solutions of acridine are excited at 376 nm, and solutions of neutral red are excited at 460 nm. The decay analyses are done with IBH DAS, version 6 software. The perfectness of fitting is judged in terms of a χ^2 value and weighted residuals.

Molecular Docking Simulation. The binding location of two different protomers of both acidic and basic guests in HSA and BSA has been investigated using molecular docking simulation. A particular protomer of a ligand has been docked into the crystal structure of HSA (PDB ID: 1AO6)[40] and BSA (PDB ID: 4F5S).[41] The methodology of the docking simulation is similar to that mentioned in our earlier report.[39] Free proteins and their docked conformations (poses) with ligand with lowest energy are used for the calculation of solvent-accessible surface area (SASA) using the Discovery Studio Visualizer 2.5 (Accelrys, Inc., San Diego, CA). The change in SASA for a residue is calculated as $\Delta SASA = SASA_{protein} - SASA_{protein-ligand}$.

3. RESULTS AND DISCUSSION

3.1. Modulation of Photophysical Properties in the Presence of SA. The chemical structures of two different protomers of basic guests (FBZ, NR, and AC) used in our study are shown in Scheme 1.

The prototropic behavior of FBZ, NR, and AC in the presence of serum protein has been investigated at two different pH's. In the case of NR, the study is performed at pH 5.0 (when the cationic form predominates) and 8.0 (when the neutral form predominates). For FBZ and AC, the experiments are performed at pH 3.0 (the cationic protomer predominates) and 7.5 (the neutral form predominates). The pH's in all of these cases are purposely kept at two extreme points with

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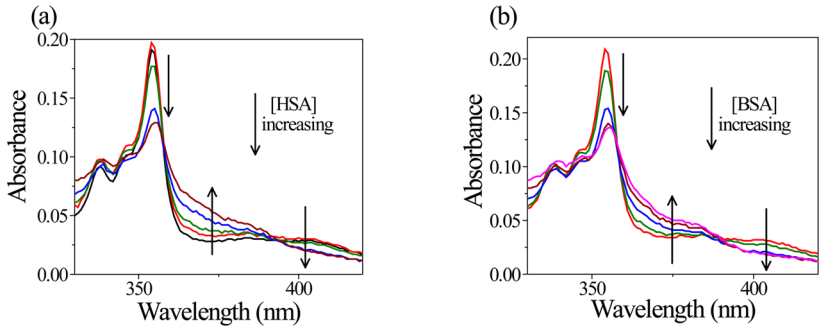

Figure 2. Absorbance spectra of acridine (AC) in the absence and presence of different concentrations of HSA (a) and BSA (b) at pH 3.0 and 298 K. [AC] = 12 μM. [HSA] is varied from 0 to 93 μM, and [BSA] is varied from 0 to 94 μM.

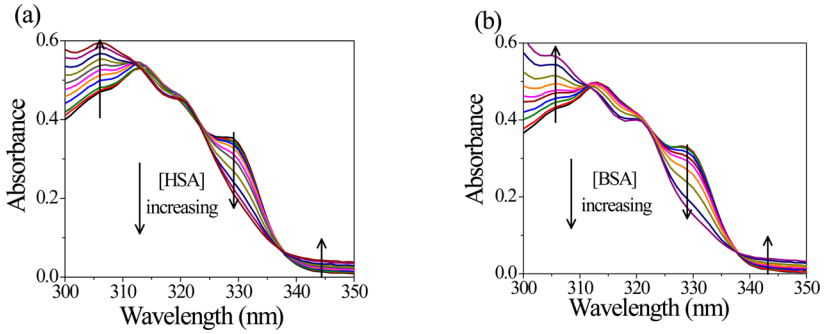

Figure 3. Absorbance spectra of fuberidazole (FBZ) in the absence and presence of different concentrations of HSA (a) and BSA (b) at pH 3.0 and 298 K. [FBZ] = 15 μM. [HSA] is varied from 0 to 53 μM, and [BSA] is varied from 0 to 75 μM.

respect to their pK_a values (pK_a of NR is 6.8, pK_a of FBZ is 4.8, and pK_a of AC is 5.4) as a midpoint so that the absolute interaction of either the cationic form or neutral form can be investigated independently in the presence of serum protein. At two extreme pH values (pH 3 and 8), the net charge of SA varies largely.[42,43] However, in a recent report,[44] we have shown that for the site-specific binding of ligand in the subdomain-IIA cavity of HSA the pocket charge rather than the overall or surface charge of the macromolecule seems to have a paramount role in determining the strength of interaction.

Since the solubility of the cationic form of FBZ, NR, and AC is higher than their neutral protomer, the experimental concentration has been kept at least 1/10th the minimum solubility of their neutral form in aqueous buffer (pH > pK_a), and hence, complication due to alteration in solubility does not arise here. Also, such a low concentration of guests rules out the possibility of their aggregation in aqueous buffer.[13,45]

The absorbance maximum of the cationic form of NR appears at 535 nm, and that of the neutral form appears at 452 nm.[19,30] The absorbance spectra of NR at pH 5.0 in the presence of serum protein are shown in Figure 1.

From Figure 1, it is observed that with increasing concentration the absorbance at 535 nm is decreased along with the rise of a new band around 458 nm for both HSA and BSA. This blue-shifted new band corresponds to the absorbance maximum of the neutral form of NR.[19] The absorbance spectra also show an isosbestic point (isobest) which is an indication of equilibrium between two different species. The changes in the absorbance spectra of NR at this pH are higher in the presence of HSA than with BSA, which indicates that the former is more effective in altering the prototropic equilibrium of NR. At pH 8.0, the absorbance of the neutral form of NR at 452 nm (Figure S1, Supporting Information) is found to be increased by the addition of serum protein without appearance of any new spectral band.

Depending on the pH of the medium, acridine can exist as different protomers, namely, cationic and neutral forms. Both the cationic and neutral forms of AC show absorbance maxima around 355 nm, but the cationic form has stronger absorbance at this wavelength than the other form.[36] Apart from these, the absorbance spectrum of the neutral form of AC shows a hump at around 380 nm arising out of the intramolecular charge transfer from the nitrogen lone pair to the anthracenic ring, while the cationic form of AC shows a relatively weak but broad absorbance band showing a maximum around 400 nm arising from the localization of the positive charge over the anthracenic ring.[36] The absorbance spectra of AC at pH 3.0 in the presence of serum protein are shown in Figure 2.

From Figure 2, it is clear that in the presence of serum protein the absorbance maximum at 355 nm is sharply decreased. Also, the hump observed in the absorbance spectra of the cationic form around 400 nm is blue-shifted to 380 nm which corresponds to its conversion to the neutral form. The absorbance spectra also show an isosbestic point which indicates that two different species remain in equilibrium in the protein-bound state, and this will be discussed latter. Similar changes in absorbance spectra of AC are observed with an increase of pH where the cationic form transforms to the neutral one.[36] In pH 7.5, the absorbance of the neutral form of AC at 355 nm is found to increase in the presence of serum protein, and also, the absorbance maximum is slightly red-shifted (Figure S2, Supporting Information). Similar red-shifting in the absorbance spectra of AC was previously observed when the neutral protomer forms an inclusion complex with both β-CDx and γ-CDx by means of hydrogen bonding interactions.[36]

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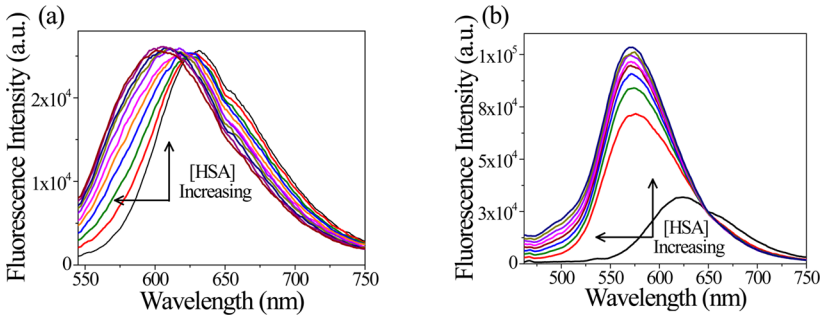

Figure 4. Emission spectra of neutral red (NR) in the absence and presence of different concentrations of HSA at pH 5.0 (a) and at pH 8.0 (b) at 298 K. At pH 5.0, [NR] = 2 μM and [HSA] is varied from 0 to 174 μM. At pH 8.0, [NR] = 3 μM and [HSA] is varied from 0 to 212 μM.

The absorbance spectra of the cationic form of FBZ show two peaks around 313 and 328 nm, while those of the neutral form appear at 305 and 320 nm. Also, the absorbance of the cationic form at these two peaks is more compared to that of the neutral form.[13] The absorbance spectra of FBZ at pH 3.0 in the presence of serum protein are shown in Figure 3.

Figure 3 shows that at pH 3.0 two absorbance peaks at 313 and 328 nm are blue-shifted to 305 and 320 nm, respectively, in the presence of serum protein and, in addition, a decrease in absorbance is observed. This observation is similar to the case when the cationic form of FBZ gets deprotonated and converted into the neutral form with an increase of pH. On the other hand, the absorbance spectra of FBZ at pH 7.5 in the presence of serum protein (Figure S3, Supporting Information) is enhanced by the addition of protein without any considerable shift in peak position.

An important observation to be noted here is that the absorbance spectra of FBZ and AC at pH 3.0 and that of NR at pH 5.0 in the presence of SA show an isosbestic point which indicates equilibrium between two different prototropic species.[24,46] Here two different species refer to the cationic and neutral forms of said basic guests. For FBZ and AC at pH 3.0 and NR at pH 5.0, the population ratio of the cationic to neutral forms is considerably high. Thus, it may be assumed that under such pH conditions the solution effectively contains only one species, the cationic form. However, in the presence of SA, the cationic form gets converted into the neutral form and hence at this situation the solution should contain two species, cationic and neutral protomers, which is clearly reflected by the appearance of an isosbestic point in the absorbance spectra.

On the other hand, the absorbance spectra of FBZ and AC at pH 7.5 and that of NR at pH 8.0 in the presence of SA do not show any isosbestic point. For FBZ and AC at pH 7.5 and NR at pH 8.0, the population ratio of the neutral form to the cationic form is considerably high. Thus, under such pH conditions, the solution practically contains only one species, the neutral form. Also, in the presence of SA, the solution should have the neutral form predominantly; otherwise, we should have observed the isosbestic point in the absorbance spectra.

We have also recorded the steady-state fluorescence emission spectra of NR and AC in the presence of serum protein. As the fluorescence emission spectra of FBZ considerably overlap with that of serum protein, the fluorescence measurements are not possible in these systems and hence not attempted.

In aqueous solution, the protonated (cationic) form of AC shows a fluorescence maximum at 482 nm, while the emission maximum of the neutral form appears at 430 nm.[36] The fluorescence intensity of the neutral form of AC measured at pH 7.5 and 430 nm is substantially decreased with increasing concentration of protein with a slight blue-shifting of the emission maxima by 3−4 nm (Figure S4, Supporting Information). The fluorescence intensity of the cationic form at 482 nm is decreased with the rise of another blue-shifted band around 450 nm in the presence of protein, which is further blue-shifted upon addition of more SA (Figure S5, Supporting Information). The ratio of fluorescence intensity at these two wavelengths (I^{450}/I^{482}) at pH 3.5 (Figure S6, Supporting Information) is increased with protein concentration, which indicates growth of the population of the neutral form; i.e., prototropic equilibrium is shifted toward the deprotonated state.

The emission spectra of NR in the presence of serum protein show that the emission maxima of the cationic form of NR, in pH 5.0, are shifted to the lower wavelength region by about 25 nm in the presence of BSA (Figure S7a, Supporting Information) and 33 nm in the presence of HSA, respectively (Figure 4a). The fluorescence intensity of NR at this pH is slightly increased in the presence of BSA, while the change in the presence of HSA is insignificant. At pH 8.0, the emission maximum of the neutral form of NR is blue-shifted by about 44 nm in the presence of BSA (Figure S7b, Supporting Information) and 55 nm in the presence of HSA, respectively (Figure 4b). Also, at this pH, in both SA cases, the fluorescence intensity of NR is greatly increased. The changes in fluorescence intensity (ΔI) of NR as a function of protein concentration at different pH's, shown in Figure 5, reveal that a larger change in the fluorescence intensity of NR is observed at high pH when its neutral form binds the serum protein. It is also clear from Figure 5 that at pH 8.0 the observed ΔI is

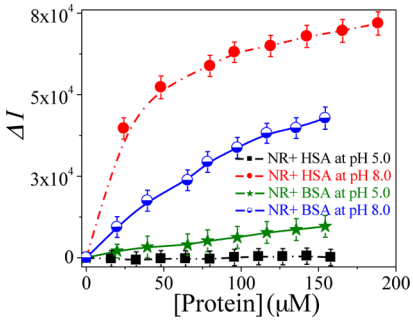

Figure 5. Change in fluorescence intensity ($\Delta I = I_{Final} - I_{Initial}$) of neutral red in the presence of HSA and BSA at its emission maxima at two different pH's, 5.0 and 8.0.

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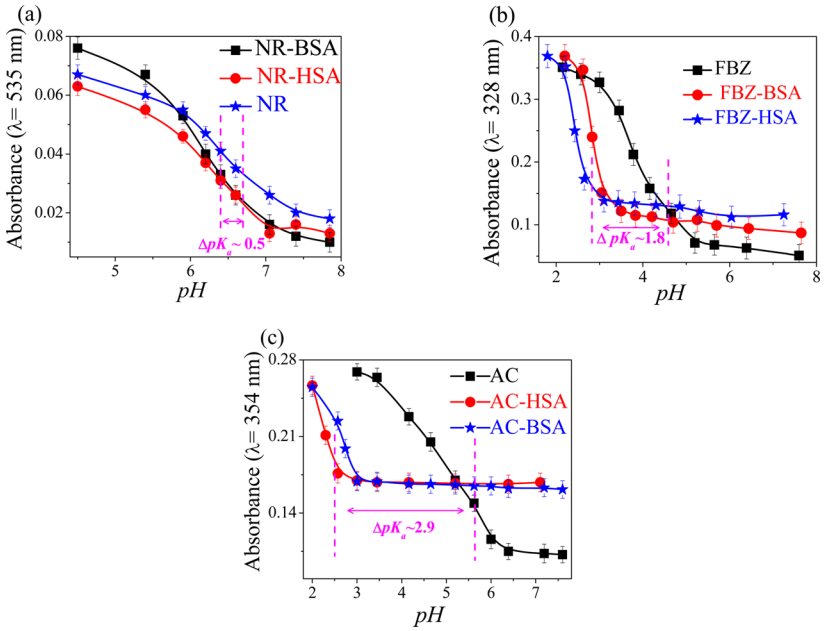

Figure 6. Determination of pK_a: Absorbance at 535 nm of NR (a), absorbance at 328 nm of FBZ (b), and absorbance at 354 nm of AC (c) as a function of pH in the absence and presence of serum proteins at 298 K.

higher with HSA than BSA, indicating stronger interaction of the neutral NR with the former.

Observations, so far discussed, clearly indicate that both serum proteins more strongly bind the neutral form than the cationic form of the basic guests under investigation. In a recent report, we have highlighted that preferential binding of the anionic form of both WF and C343 in subdomain-IIA of HSA results in a negative pK_a shift.[24] Here we have explored the interaction of another serum protein, BSA, with WF and C343 at pH 3.5 when both ligands should assume the neutral form. The pH of the solution is purposely kept at 3.5 so that any alteration in prototropic equilibrium due to interaction with BSA can be conveniently monitored. The UV−vis absorbance spectra of C343 and WF at pH 3.5 in the presence of BSA are shown in Figures S8 and S9, Supporting Information, respectively. In the presence of BSA, the absorbance maximum of C343 is strongly blue-shifted by about 24 nm from 454 nm along with the appearance of an isosbestic point at 417 nm. Absorbance of the anionic form of WF at 308 nm is found to be enhanced by the addition of BSA. This indicates that shifting of the prototropic equilibrium of both C343 and WF toward the anionic form is due to binding with BSA.[24] We could not monitor the neutral form of WF at 270 nm due to strong overlap with the absorbance maxima of protein at 278 nm. Thus, the experimental results reveal that BSA prefers to bind the anionic form of the studied acidic guests. Although the individual role of both of the serum proteins toward the modulation of prototropic activity of guests is found to be very similar, their strength can be compared only after the determination of pK_a of these guests in the protein-bound state.

The experimental concentration of FBZ, AC, and NR in all measurements is kept very low compared to the concentration of SAs, and consequently, a 1:1 stoichiometry is expected.[36,47] The stoichiometry of ligand−protein complexation has been determined with the help of UV−vis absorbance spectra. The changes in absorbance of all studied ligands (FBZ, AC, and NR) in the presence of SA at two different pH's have been used to generate the Benesi−Hildebrand (B−H) plot[48,49] which are shown in Figures S10, S11, and S12 (Supporting Information). The B−H plot for all ligands is found to be linear, which indicates 1:1[48,49] complexation with SAs. The stoichiometry of complexation has also been confirmed by a modified Job's method of continuous variation.[50] The Job plots generated with the help of UV−vis absorbance spectra as described elsewhere[51] are shown in Figures S13, S14, and S15 (Supporting Information), which also confirm 1:1 complexation[49,51] for all guest−SA pairs. Since the ligand is present in very low concentration compared to SA, the possibility of nonspecific binding interaction, which is generally observed for excess ligand concentration, can be ruled out and ensures site-specific binding of ligand in the subdomain-IIA cavity of SA.

3.2. Comparison of pK_a Values in the Absence and Presence of Serum Albumin. In supramolecular drug delivery systems, the choice of the macromolecular host for any prototropic drug is made on the basis of the strength of the interaction of the former with the two different protomers of the latter. The supramolecular host which can bind strongly, and thereby substantially alter the prototropic equilibrium toward the active form of the drug, can be the best vehicle for its delivery to the target. From our earlier report[24] and the experimental results so far presented, it is evident that albumin preferentially binds the anionic form of acidic guests (WF and C343) and the neutral form of basic guests (NR, FBZ, and AC). Such preferential binding ought to have strong effects on the position of prototropic equilibrium of these guests which can be easily realized after the determination of their pK_a value in the protein-bound state, and this is crucial from the point of view of clinical impacts of albumin as a carrier on drug delivery.

We have, therefore, determined the pK_a of NR, FBZ, and AC in the presence of HSA and BSA. All pK_a measurements in the presence of protein are performed in the physiological ionic strength condition (~150 mM NaCl). The plots of a change in absorbance as a function of the pH of NR, FBZ, and AC, in both the absence and presence of serum protein, are shown in Figure 6.

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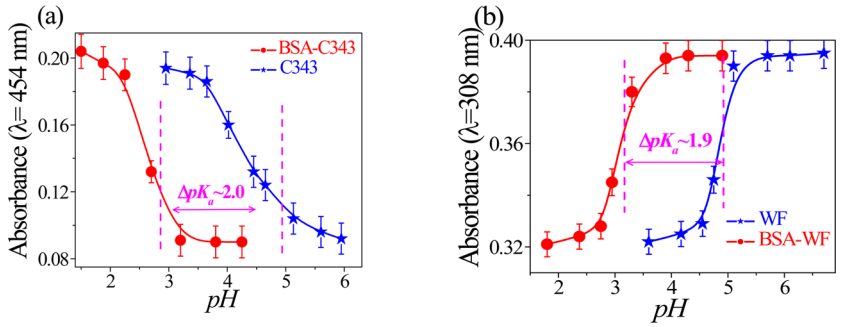
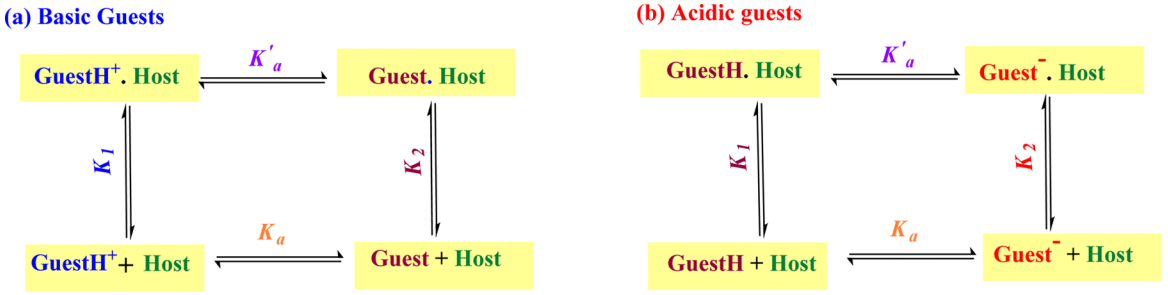


Figure 7. Determination of pK_a: Absorbance of C343 at 454 nm (a) and of WF at 308 nm (b) as a function of pH in the absence and presence of BSA at 298 K.

Scheme 2. Four-State Thermodynamic Equilibrium Cycle of Basic Guests (a) and Acidic Guests (b) in the Presence of a Host



Absorbance of the cationic form of NR (at 535 nm), AC (at 354), and FBZ (at 328 nm) is monitored here for the determination of pK_a. From Figure 6, the pK_a of NR, FBZ, and AC in buffer is found to be 6.7 ± 0.1, 4.6 ± 0.05, and 5.5 ± 0.1, respectively, which are close to the value reported in the literature.[13,19,36] In the presence of serum protein, the pK_a of all these basic guests is found to be decreased. The pK_a of NR (3 μM) in the presence of BSA (180 μM) is 6.3 ± 0.06, and that in the presence of HSA (180 μM) is 6.2 ± 0.06. The measured pK_a values of FBZ (15 μM) are found to be 2.80 ± 0.04 and 3.11 ± 0.02 in the presence of HSA (60 μM) and BSA (60 μM), respectively. The pK_a of AC (18 μM) in the presence of BSA (180 μM) is 2.9 ± 0.07, and that in the presence of HSA (180 μM) is found to be 2.5 ± 0.08. Thus, the ground-state prototropic equilibrium of subdomain-IIA binder basic guests is strongly decreased, except for NR. The plots of absorbance of C343 and WF in both the absence and presence of BSA, as a function of pH, are shown in Figure 7.

The absorbance maximum of C343 at 454 nm corresponding to the neutral form and that of WF at 308 nm corresponding to its open chain deprotonated form are monitored. From Figure 7, it is found that the pK_a of both C343 and WF is shifted in the protein-bound state. In the presence of BSA, the pK_a of C343 is decreased by about 2.2 units, while that of the WF is decreased by about 1.9 units. This reveals that BSA also prefers to bind the anionic form of these acidic guests and thereby shifts their pK_a to the acidic pH region.

The shift of the pK_a of a guest within the cavity depends on how strongly the selected host binds one of the two protomers, and it can be easily understood with the help of the four-state equilibrium model of pK_a analysis. Four-state thermodynamic equilibrium cycles[3,13,30] for both basic and acidic guests are shown in Scheme 2.

Here, GuestH$^+$ and Guest are the cationic and neutral forms of the basic guest, respectively, whereas GuestH and Guest$^-$ are the neutral and anionic forms of the acidic guest, respectively. K_a (calculated from the pK_a value as $K_a = 10^{-pK_a}$) and K_a' are the dissociation constants of acidic/basic guests in the absence and presence of host, respectively. For basic guests, K_1 and K_2 are the binding constants of host with the cationic and neutral forms, respectively, and can be determined from the changes in the absorbance or emission spectra at two different pH's using a B−H plot. The estimated values of K_1 and K_2 for the NR−SA system are shown in Table T1 (Supporting Information).

For acidic guests, K_1 and K_2 are the binding constants of host with the neutral and anionic forms, respectively. Now using the above thermodynamic equilibrium cycle,[3] one can write the following eq 1.

$$K_a' = K_a \times \left(\frac{K_2}{K_1}\right) \quad (1)$$

It is observed in eq 1 that the magnitude of K_a' depends on the ratio of K_2 and K_1. The higher the difference in binding constant of any host with two different protomers, the higher will be the shift of pK_a. Therefore, a high negative pK_a shift of basic guests like AC and FBZ in the presence of serum protein indicates that the binding of serum protein with the neutral form (K_2) of these basic guests is stronger compared to the cationic form (K_1). A similarly larger negative pK_a shift of acidic guests like C343 and WF in the presence of serum protein indicates that the binding constant of the anionic form of these acidic guests (K_2) is substantially higher with serum protein compared to that for the neutral form (K_1).

An increase of the pK_a of basic guests is generally observed in the presence of cation receptors or hydrogen-bond-acceptor hosts (as with cucurbituril and sulfonatocalixarene), and a decrease of pK_a is observed in the presence of anion receptors (e.g., calixpyridine, C4P) or hydrogen-bond-donor host molecules or molecules which offer nonpolar cavities (e.g., cyclodextrin).[3] On the other hand, the pK_a's of acidic guests are expected to be increased in the presence of a hydrogen-bond-

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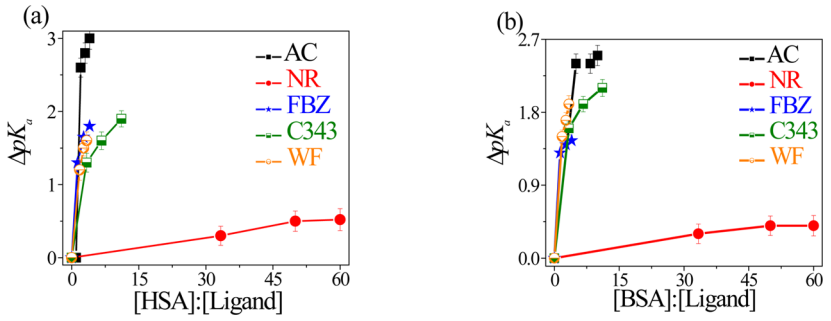

Figure 8. Shift in pK_a (ΔpK_a) of guests as a function of concentration of (a) HSA:ligand and (b) BSA:ligand at 298 K.

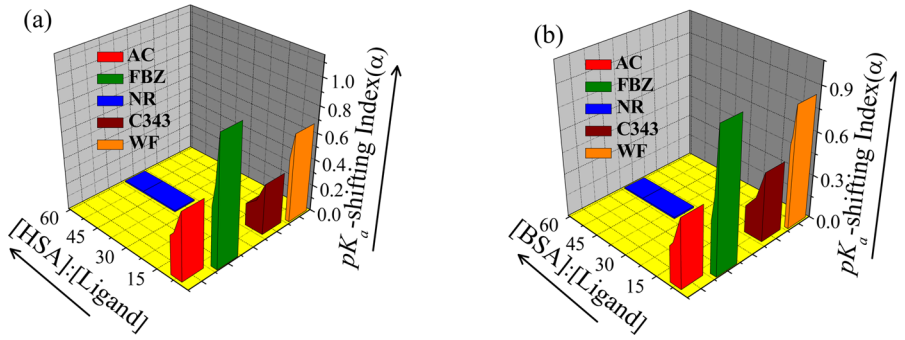

Figure 9. pK_a-shifting index (α) of guest as a function of (a) [HSA]:[ligand] and (b) [BSA]:[ligand].

acceptor host or molecules with nonpolar cavities (e.g., cyclodextrin) or cation receptors, and the pK_a is expected to be decreased in the presence of anion receptors or a hydrogen-bond-donor host. In our earlier report, we concluded that the pK_a of acidic guests, C343 and WF, is decreased in the presence of HSA due to the availability of anion receptors (positively charged side chain of amino acid residues like Arg, Lys, and His) in subdomain-IIA which strongly binds the anionic form of these acidic guests by electrostatic interactions.[24] Contribution of a hydrogen bond donor is ruled out here because residues like tryptophan, alanine, tyrosine, leucine, and glutamine available in the binding pocket can act as a hydrogen bond acceptor but not as a donor. NR, FBZ, and AC are found to bind the same pocket, i.e., the subdomain-IIA cavity, and hence, their prototropic equilibrium is also expected to be altered there. Between the two prototropic forms of these basic guests, the cationic form is likely to be less favored in the anion receptor cavity. The neutral form is expected to bind the side chain of residues like glutamine, tryptophan, alanine, tyrosine, and leucine,[24] which are known to bind neutral ligands by weak nonelectrostatic forces. Thus, the observed large decrease in pK_a of these basic guests is primarily due to the presence of anion receptors in the said cavity which do not like to bind the cationic protomer, and is presumably due to electrostatic repulsion between (two) positive charges. On the contrary, binding of the anionic form of the acidic guests is favored due to strong electrostatic interaction with the positively charged amino acid side chains (anion receptor). Hence, electrostatic interaction plays a dominant role in the binding of the said guests in the subdomain-IIA binding pocket of SA.

We have measured the pK_a of drugs/guests as a function of the concentration of serum proteins (as the encapsulator). The pK_a shift of both acidic and basic guests is found to be very much dependent on the concentration of the encapsulator. The plot of pK_a shift (i.e., ΔpK_a) with protein to ligand ratio is shown in Figure 8.

From Figure 8, it is obvious that, except for NR, the ΔpK_a of other acidic and basic guests is sharply raised with an increase of protein to ligand ratios. The ΔpK_a of the basic guest is higher in the presence of HSA, and that of the acidic guest is higher with BSA. With increasing concentration of serum protein, the guest is partitioned more into the protein cavity from bulk water. Since the subdomain-IIA cavity of serum protein prefers the anionic form of acidic guests and the neutral form of basic guests, with increasing protein concentration, the population of the preferred protomer is considerably enhanced, resulting in more pK_a shifting. After a certain concentration of encapsulate is reached, the pK_a shift levels off. Therefore, the pK_a value at this point is the true one, while those recorded below the saturation concentration are apparent values (due to incomplete pK_a shift) in the protein environment. This dependency of pK_a shift on serum concentration can be smartly exploited in the designing of some albumin based drug delivery systems to extract more efficacies out of the prototropic drugs. It is also found that the efficiency of serum protein in shifting the pK_a of guests is quite distinctive depending on the host−guest pair (Figure 8) and also the extent of pK_a shift depends on the host as well as on the nature of the guest. We can quantitatively express this pK_a shifting by a parameter named the pK_a-shifting index (α) as follows

$$\alpha = \frac{\Delta \mathrm{p}K_a}{\Delta C_r} \tag{2}$$

where ΔC_r is the change in protein to ligand ratio and ΔpK_a is the shift of pK_a. Hence, from eq 2, it may be written, if $\Delta C_r = 1$, then $\alpha = \Delta \mathrm{p}K_a$. Thus, the p$K_a$-shifting index ($\alpha$) may be defined as the magnitude of pK_a shift when the protein to ligand ratio is unity. The higher the value of the pK_a-shifting index (α), the lower will be the concentration of protein

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required to extract the concerned drug-protomer and hence will be a more efficient albumin-based delivery system.

We have estimated α values of the guest molecules from Figure 8 using eq 2, and a plot of its variation with protein to ligand ratio is shown in Figure 9. This plot represents the bioavailability marker for prototropic drugs. The height and width of the plot for a given drug can provide important information about its efficacy in the albumin-based delivery systems. The greater the height, the higher should be the efficacy of the drug. On the other hand, the lesser the width of the plot, the lower will be the concentration of protein required to bring a sharp change in pK_a and hence should be more efficient delivery systems to extract more out of the guest. Interestingly, here, drugs like WF and FBZ are found to have a high α value and hence will be highly efficient in such delivery systems. Since α of NR is found to be very low, drugs with a similar structure are not expected to be effective in albumin-based delivery systems. Hence, the determination of pK_a-shifting index (α) for any prototropic drug with respect to a given carrier is important and attempts on other similar systems would get us a better picture of the anticipated efficacy of new drugs as well.

One unit decrease in pK_a of an acidic drug like WF results in an increase of the ratio of anionic to neutral forms by a factor of ~10 both in physiological conditions (in pH 7.4) as well as at pH 5.0 (like the acidic pH environment in cancer cells). Similarly, one unit decrease in pK_a of a basic drug like FBZ results in the ratio of the neutral to cationic form increasing by a factor of ~10 both at pH 7.4 and at pH 5.0. Thus, the shifting efficiency (α) value of a guest in a protein environment is an important and unique parameter which in turn quantitatively expresses the effectiveness of the albumin-based delivery system in giving a roadmap to extract more efficiency out of the concerned prototropic drug. Thus, by playing with the concentration of serum proteins (host) in a protein−drug complex (cocktail), one can have control over the bioavailability of the prototropic drugs.

The pK_a measurements also show that, although the role of two homologous serum proteins (HSA and BSA) in the modulation of prototropic equilibrium of both acidic and basic guests is very similar, their relative strength is found to be somewhat different. The decrease in pK_a of acidic guest molecules is higher in the presence of BSA than HSA, while the decrease in pK_a of basic guests is higher in the presence of HSA than BSA.

We have estimated the equilibrium constant (K_a) for the deprotonation of acidic and basic guests and the associated free energy changes (ΔG^0) in the absence and presence of serum protein, as highlighted in Table 1. The protonated form of these guests is found to be more stable than the corresponding deprotonated form and is reflected by the positive value of ΔG^0. From Table 1, it is observed that deprotonation of both basic and acidic guests becomes more favorable (ΔG^0 becomes less positive) in serum protein.

A schematic representation of the free energy change for the deprotonation of basic and acidic guests is shown in Figure 10 which does not represent the actual magnitude of ΔG^0 listed in Table 1 but their trend in a qualitative manner.

From Figure 10, it is observed that the subdomain-IIA cavity of BSA stabilizes the deprotonated form of the acidic guest (i.e., anionic form) more favorably compared to HSA and hence ΔG^0 becomes less positive in the presence of BSA. In both proteins, the cationic form of the basic guest is strongly

Table 1. Equilibrium Constant (K_a) and Change in Free Energy (ΔG^0) for the Deprotonation of Basic and Acidic Guests in the Absence and Presence of Serum Proteins

system	K_a	ΔG^0 (kJ/mol) (for deprotonation)
FBZ(cationic)	2.5×10^{-5}	26.2
FBZ(cationic) + HSA	1.6×10^{-3}	15.9
FBZ(cationic) + BSA	7.1×10^{-4}	17.9
AC(cationic)	3.2×10^{-6}	31.4
AC(cationic) + HSA	3.1×10^{-3}	14.3
AC(cationic) + BSA	1.0×10^{-3}	17.1
NR(cationic)	1.9×10^{-7}	38.2
NR(cationic) + HSA	6.3×10^{-7}	35.3
NR(cationic) + BSA	5.0×10^{-7}	35.9
WF	1.3×10^{-5}	27.9
WF + HSA	5.0×10^{-4}	18.8
WF + BSA	1×10^{-3}	17.1
C343	3.2×10^{-5}	25.7
C343 + HSA	2.5×10^{-3}	14.8
C343 + BSA	3.9×10^{-3}	13.7

destabilized due to repulsion with anion receptors in the binding pocket, but the deprotonated form of the basic guest (i.e., neutral form) is stabilized more favorably by HSA than BSA, which is reflected by a less positive ΔG^0 in the former. Since the subdomain-IIA cavity of these two serum proteins shows, by and large, an anion receptor property, it prefers to bind the anionic ligands. However, in a recent study, we have shown that due to a remarkable conformational feature the subdomain-IIA pocket HSA is also able to bind neutral ligands.[39] As the solution structure of HSA is more flexible, it is capable of adopting a favorable conformation to bind a neutral protomer of basic guests also. Contrary to this, the solution structure of BSA appears rigid and is less effective to take up a favorable conformation. Hence, interaction with a neutral protomer of the basic guest is weaker with BSA.

A very low pK_a shift of NR in the presence of albumin is quite contrasting to other systems. The ratio of K_2 and K_1 (shown in eq 1) is not very high for NR, which is quite different from the other two cases. We have determined the binding thermodynamics of the cationic form of NR with serum protein at pH 5.0 by isothermal calorimetric titration (ITC). The thermodynamic parameters extracted from the fitted titration profile [$\Delta H^0 = 3.25 \pm 0.95$ kJ mol^{-1} and $\Delta S^0 = -11 \pm 1.54$ J mol^{-1} K^{-1}] indicate that the binding interaction is guided by hydrogen bonding and hydrophobic forces. The only possible way of binding of the cationic form of NR in the anion receptor pocket of albumin is via hydrogen bonding or hydrophobic forces. As mentioned in the earlier section, a few residues exist in the subdomain-IIA cavity of serum protein which are known to bind neutral ligand by comparatively weak hydrogen bonding or hydrophobic forces. Thus, a low negative pK_a shift could be due to the fact that, although subdomain-IIA repels the cationic form of NR due to electrostatic repulsion with the anion receptors, it ought to have other nonelectrostatic modes of interaction, as mentioned above, which weakly allows the binding of its cationic form in the pocket. Since in general the binding in this anion receptor pocket is primarily guided by electrostatic interaction,[44] the strength of hydrogen bonding or hydrophobic forces should be relatively weak in nature and hence the difference between K_2 and K_1 is not high enough to warrant a substantial pK_a shift. A similar observation of low pK_a shift is also found in the case of the subdomain-IIA binder

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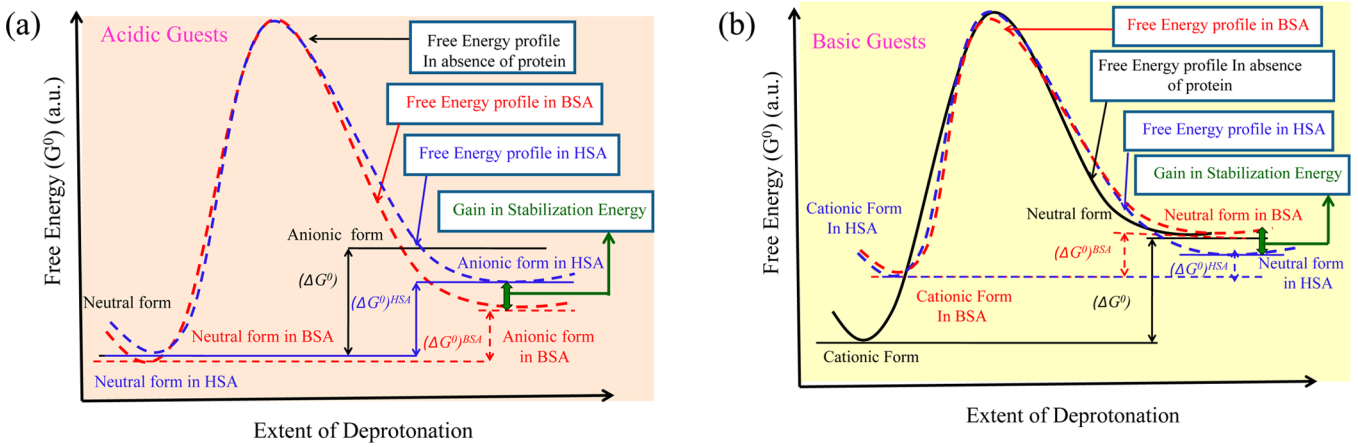

Figure 10. Schematic of free energy change for the deprotonation of acidic (a) and basic (b) guest in the absence and presence of serum proteins.

acidic guest, carmoisine (CM), which was reported to show a decrease of pK_a (due to a phenolic-hydroxyl group) by only 0.5 units in the protein-bound state.[49] We have previously investigated the binding thermodynamics of carmoisine with serum protein at pH 7.4, which is below the pK_a of its hydroxyl group, and found that binding is primarily guided by electrostatic interaction.[49] Actually, carmoisine has two negatively charged sulfonate groups that bind the positively charged amino acid side chains (anion receptor) available in this binding pocket and can substantially stabilize the complex. This is possibly why deprotonation of a hydroxyl group, to generate an anion, is not compulsory for its binding and consequently pK_a shift is feeble. Recently, Douhal and co-workers have studied the excited state intermolecular proton transfer (ESPT) interaction of 1-hydroxypyrene (1-HP) and 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) with HSA.[52,53] They have reported that the time constant of ESPT for the HSA−1HP system is longer compared with that for HSA−HPTS systems. It is also observed from their report that in the presence of HSA the excited state prototropic equilibrium of HPTS is shifted toward the neutral form while that of 1-HP is shifted toward the anionic form. This is very similar to the carmoisine case that due to the presence of sulfonate groups the deprotonation of the hydroxyl group in HPTS is not very essential, while the absence of a sulfonate group in 1-HP demands the deprotonation of its hydroxyl group for binding and consequent stabilization in the serum protein pocket. In another two reports, it is found that the excited state prototropic equilibrium of two basic guests, harmane[54] and norharmane,[55] is also shifted to the neutral form like our present observations with AC, FBZ, and NR. Hence, this shifting of the acid−base equilibrium of the protein-bound guest, whether a ground state or an excited state phenomenon, is dependent on the stabilization of the preferred protomer in the protein cavity. All of these observations reveal the existence of some exquisite role of the albumin pocket in modulating the prototropic equilibrium of acidic and basic guests both in the ground and the excited state as well.

3.3. Time-Resolved Fluorescence Measurements. Time-resolved fluorescence study is an important and very sensitive technique to monitor the prototropic changes of fluorescent probes. We have measured the fluorescence lifetime of C343 in the absence and presence of BSA under different pH conditions. A representative decay plot is shown below.

Analysis shows that the fluorescence decay profile (Figure 11) of C343 in the absence of any host at pH 3.5 and 7.5 can be

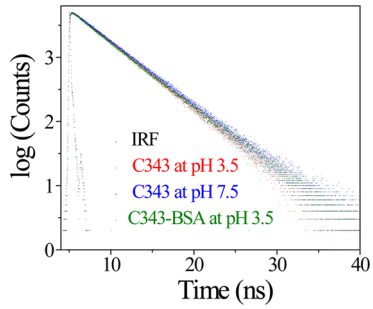

Figure 11. Fluorescence decay profiles of C343 at pH 7.5 and in the absence and presence of BSA at pH 3.5. λ_{ex} = 408 nm and monitored at the emission maxima. [C343] = 1.5 μM.

fitted to a single exponential and the corresponding decay times are 4.18 ± 0.05 and 4.48 ± 0.04 ns, respectively, which should correspond to the lifetime of neutral and anionic forms of C343, respectively. In the presence of BSA at pH 3.5, the decay plot becomes biexponential with two components having lifetimes of 4.41 ± 0.02 ns (~97%) and 1.99 ± 0.06 ns (~3%). Here the lifetime of the major decay component is found to be lower than that of the unbound anionic form (4.48 ± 0.04 ns) of C343 at pH 7.5 but is higher than the lifetime of its unbound neutral form (4.18 ± 0.05 ns) at pH 3.5. Therefore, the major decay component in the presence of BSA at pH 3.5 corresponds to the lifetime of the anionic form of C343 and its lifetime decreases due to binding and consequent quenching by the protein. The steady-state fluorescence quenching of C343 in the presence of BSA (Figure S16, Supporting Information) is also supportive of this. Thus, encapsulation of C343 within the receptor cavity of BSA results in shifting of the acid−base equilibrium, and thereby C343 gets deprotonated. The biexponential nature of fluorescence decay of similar probes in the presence of serum protein is found in many similar systems. The other shorter lifetime component in the presence of BSA may correspond to the bound neutral form of C343. However, importantly its percentage contribution is significantly less.

The fluorescence decay of AC and NR has been measured in the absence and presence of serum protein at different pH's, and the fitted results are tabulated in Table 2. In the absence of

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Table 2. Fluorescence Decay Parameters of AC and NR at Different pH's in the Absence and Presence of Serum Albumins[a]

system	τ_1 (ns)	a_1 (%)	τ_2 (ns)	a_2 (%)	χ^2
AC at pH 3.5	31.27	100			1.21
AC + 40 μM HSA at pH 3.5	30.13	79.4	5.77	20.6	1.20
AC + 94 μM HSA at pH 3.5	29.53	65.4	5.74	34.6	1.17
AC + 40 μM BSA at pH 3.5	31.14	80.7	7.24	19.3	1.14
AC + 94 μM BSA at pH 3.5	29.61	66.8	7.13	33.2	1.13
AC at pH 8.0	8.39	100			1.18
AC + 40 μM HSA at pH 8.0	8.37	81.1	0.96	18.9	1.13
AC + 94 μM HSA at pH 8.0	7.77	70.8	0.87	29.2	1.21
AC + 40 μM BSA at pH 8.0	8.67	83.3	1.12	16.7	1.14
AC + 94 μM BSA at pH 8.0	8.48	71.6	1.06	28.4	1.16
NR at pH 5.0	0.31	100			1.03
NR + 75 μM HSA at pH 5.0	0.40	41.7	2.97	58.3	1.21
NR + 200 μM HSA at pH 5.0	0.66	34.3	3.29	65.7	1.15
NR + 75 μM BSA at pH 5.0	0.51	54.2	2.91	45.8	1.14
NR + 200 μM BSA at pH 5.0	0.55	41.9	3.21	58.1	1.13
NR at pH 8.0	0.75	100			1.01
NR + 75 μM HSA at pH 8.0	1.14	26.6	3.7	73.4	1.06
NR + 200 μM HSA at pH 8.0	1.42	16.6	3.89	83.4	1.02
NR + 75 μM BSA at pH 8.0	1.01	25.7	3.41	74.3	1.21
NR + 200 μM BSA at pH 8.0	1.22	21.4	3.54	78.6	1.15

[a]The error in fitting/measuring lifetime is ±5%.

serum protein, the fluorescence decay of the cationic form (measured at pH 3.5) and the neutral form (measured at pH 8.0) of AC is monoexponential with lifetimes of 31.27 and 8.39 ns, respectively, similar to that reported elsewhere.[36]

At both of the studied pH's, in presence of serum protein, the decay profiles of AC become biexponential in nature, with short and long components. As seen from Table 2, the lifetime of the long component is slightly decreased, although its contribution is gradually decreased. On the other hand, the lifetime of the short component remains almost unaltered and its contribution is increased with increasing protein concentration. In steady-state fluorescence measurements, for both of the studied pH's, it is observed that the fluorescence intensity of AC gets quenched with increasing concentrations of serum protein. Thus, the long component corresponds to the unbound form while the short component corresponds to the bound form. At pH 8.0, the lifetime of AC is ~10- and ~7.9-fold decreased in the presence of HSA and BSA, respectively (8.39 ns in the free state to 0.87 ns in HSA and 1.06 ns in BSA). At pH 3.5, the lifetime of AC is ~5.4- and ~4.4-fold decreased in the presence of HSA and BSA, respectively (31.27 ns in the free state to 5.74 ns in HSA and 7.13 ns in BSA). Thus, fluorescence decay measurements indicate that the binding of the neutral form of AC is stronger with serum protein and it leads to a large change in its lifetime compared with the cationic form.

In the absence of serum protein, the fluorescence decay of the cationic form (measured at pH 5.0) and the neutral form (measured at pH 8.0) of NR is single-exponential in nature with decay times being 0.31 and 0.75 ns (Table 2), respectively, which are close to that reported in the literature.[30] For both of the serum proteins, the decay profile of the cationic form and the neutral form of NR becomes biexponential, with short and long lifetime components. The lifetime of the longer component of both of the protomers remains almost unchanged, although its contribution is gradually increased with protein concentration. In steady-state fluorescence measurements, it is observed that the fluorescence intensity of both of the protomers of NR is enhanced with protein concentration also. Thus, the longer and shorter components correspond to the bound and unbound forms of NR, respectively. It is interesting to note from Table 2 that the contribution of the bound form is substantially higher at pH 8.0 than at pH 5.0. This indicates that binding of NR is higher at pH 8.0 when predominantly the neutral form interacts with serum protein.

3.4. Analysis of Docking Simulation. The preferred binding site of a selected protomer of both basic and acidic guests has been located by performing their docking study into the 3D crystal structure of HSA and BSA (available in RSCB PDB) using Auto Dock 4.2. Our experimental results reveal that the anionic form of the acidic guest and the neutral form of the basic guest are the preferred protomers for binding in the subdomain-IIA cavity of serum proteins. In order to have a qualitative understanding of the experimental results, we have analyzed the results of docking simulations by considering the lowest energy docked pose and estimated the change of SASA between the ligand-docked protein and the free protein to assess the possibility of interaction.

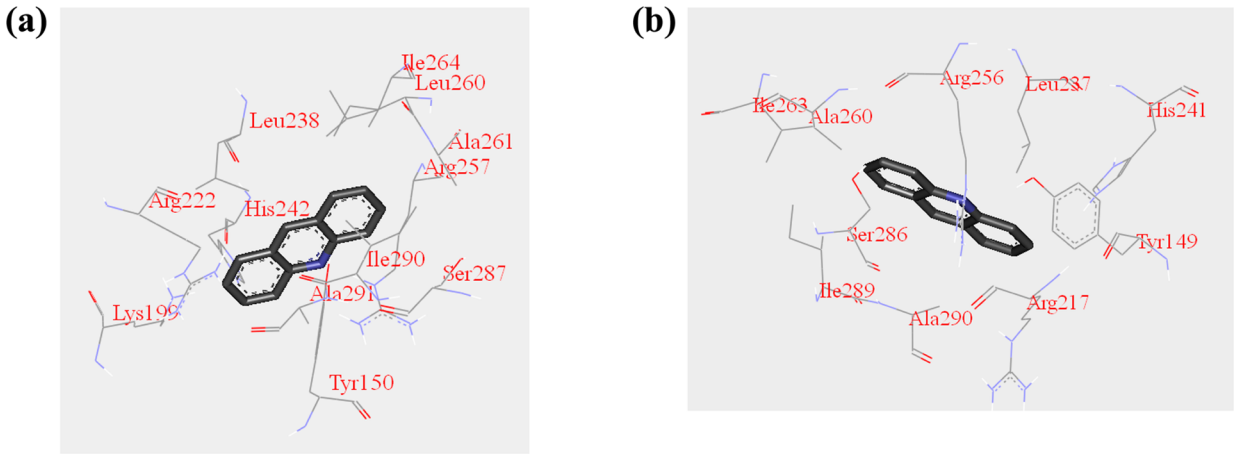

Figure 12. Interacting amino acid residues present in the binding site of the docked neutral form of AC (at a distance of 4 Å from AC) in (a) HSA and (b) BSA.

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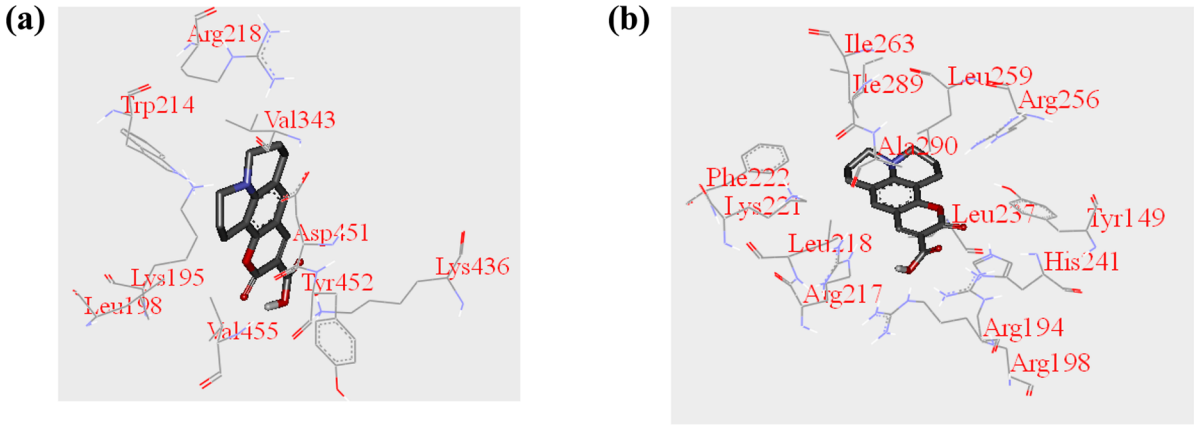

Figure 13. Interacting amino acid residues present around the binding site of the docked neutral form of C343 (at a distance of 4 Å from C343) in (a) HSA and (b) BSA.

The results of docking simulation can be summarized into the following points:

(i) It is observed experimentally that the cationic form of both AC and FBZ does not bind in the subdomain-IIA cavity of serum proteins. The docked poses of the neutral form of AC and FBZ are shown in Figure 12 and Figure S17 (Supporting Information), respectively, which indicates that these molecules dock in the subdomain-IIA cavity. The changes in the solvent accessible surface area (SASA) of amino acid residues available in the subdomain-IIA cavity when the cationic and neutral forms of both AC and FBZ dock in serum protein are given in Tables T2–T5 (Supporting Information). From these tables, it is evident that the solvent accessible surface area (SASA) of those concerned amino acids in subdomain-IIA remains unchanged during the interaction of the cationic form with protein, while the SASA is substantially decreased when the neutral form docks. A considerable loss in the solvent accessible surface area of those amino acid residues in the subdomain-IIA cavity indicates their stronger interaction with the neutral form. From experimental results, it has also been found that the binding of the cationic form is very weak and this is also reflected in the ΔSASA of the cationic drug–protein complex.

(ii) The anionic form of C343 is known to bind in the subdomain-IIA cavity of serum albumin.[39] The crystal structure of the HSA–WF complex is already available in the literature.[56] From this crystal structure, it is found that the anionic form of WF binds in the subdomain-IIA cavity of serum albumin. A considerable change in the solvent accessible surface area (SASA) of nearby amino acid residues available in the binding site of the anionic form of both C343 and WF[39,44] reveals the strong complexation between the anionic form and serum albumin.

(iii) From the docked pose of the neutral form of WF (Figure S18, Supporting Information) and C343 (Figure 13), it is found that they also bind the subdomain-IIA cavity. The changes in the solvent accessible surface area (SASA) of local amino acid residues available in the binding site of the neutral form of C343 and WF are highlighted in Tables T6 and T7 (Supporting Information). A comparison of these SASA values with those reported for the anionic form of acidic guests[39,44] reveals that ΔSASA is higher when the anionic form of C343 and WF docks with serum albumin. Thus, docking simulation results also indicate that serum albumin binds more strongly with the anionic form compared to the neutral form of the acidic guest (C343 and WF).

3.5. Bioavailability Correlation. Here, in the present scenario, it is very important to discuss the possible implications of pK_a shift on the supramolecular and albumin-based drug delivery systems and correlate our experimental results with the bioavailability of drugs which can be helpful in predicting a more appropriate vehicle for the drug. A correlation chart is shown in Figure 14.

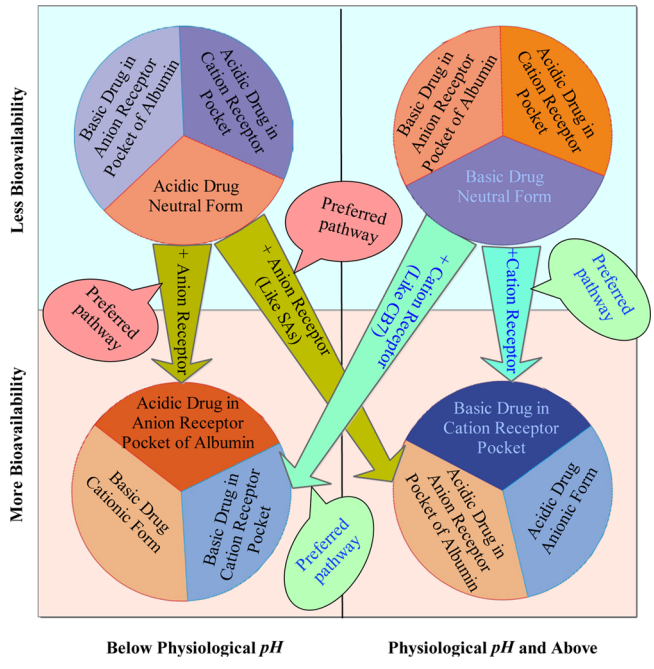

Figure 14. Bioavailability of drugs in free state and encapsulated either in supra- or biosupramolecular host cavity. Arrows indicate only the preferred pathway to enhance bioavailability of basic and acidic drugs.

Assuming the neutral form of any prototropic drug (acidic or basic) to be less bioactive than the corresponding ionic (deprotonated form of acidic guest and protonated form of basic guests) species, we can make some correlations. Figure 14 shows that prototropic activity of acidic drugs/guests (e.g., WF and C343) in the anion receptor cavity of albumin is strongly modified in ways which favor the formation of the active component and thereby increase their bioavailability. On the other hand, the prototropic activity of basic drugs/guests (e.g., NR, FBZ, and AC) is shifted toward the neutral form, thereby

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reducing their bioavailability under physiological conditions. Hence, encapsulation of basic drugs in supramolecular hosts with cation receptors (such as cucurbituril and sulfonatocalixarene), which shifts their prototropic equilibrium toward the cationic form, appears to be essential to enhance their bioavailability. Practically, it is found to be true and is evident from several literature reports.[2,3] As the bioavailability of basic drugs is increased in the presence of cation receptor hosts, thinking in the same line, we can forecast that acidic drugs should be more bioavailable when encapsulated in the anion receptor cavity. However, the literature reports on the modulation of prototropic activity of acidic guests especially in the presence of an ionic receptor are less common and have been an unresolved area of research. Thus, our present study can be useful and importantly it may be predicted that, although binding of cationic ligands in the anion receptor cavity of subdomain-IIA is very weak, it can certainly be improved by the proper functionalization of the drug so that it can bind alternatively by the nonelectrostatic mode to avoid electrostatic repulsion.

The present results are highly encouraging for albumin-based drug delivery systems where site-specific anchoring of acidic drugs (e.g., warfarin) will enhance the bioavailability. Due to binding with serum protein, the therapeutic window of the drug can be substantially increased, which can now work in acidic pH conditions that could be highly demandable for different treatments, e.g., treating the cancer patients.

4. CONCLUSION

The results of the study reveal that the anion receptor cavity of SAs prefers the neutral form of the basic guest and the anionic form of the acidic guest via formation of pocket−inclusion complexes. The preferential binding of the anionic form of the acidic guest by electrostatic interaction leads to the shifting of the prototropic equilibrium toward the deprotonated species. On the other hand, electrostatic repulsion of the cationic form of basic guests with the anion receptors available in the subdomain-IIA cavity of SAs results in a decrease of pK_a. The present study anticipates that, although binding of cationic ligands in the anion receptor cavity of subdomain-IIA is very weak, it can be substantially improved by availing of alternative nonelectrostatic modes using suitable derivatives of the drug.

For the first time, we have nurtured the prospect of using an albumin based delivery system for extracting the therapeutically active species of prototropic drugs. The pK_a shifting ability of serum proteins has been expressed by introducing a unique parameter, the pK_a shifting index (α), which represents the ΔpK_a value when the protein to ligand ratio is unity. The α value of a drug in a protein environment can be an important parameter which in turn quantitatively expresses the effectiveness of the delivery system in giving a road map to extract more efficiency out of the prototropic drug. Thus, by playing with the concentration of serum proteins in a protein−drug complex, one can have control over the bioavailability of the prototropic drugs. Since the pK_a shifting index (α) is a composite parameter to account for the effect of the host and nature of the guest, in future studies, it can be nicely implemented to assess bioavailability of protein-cocktailed prototropic drugs.

Although a supramolecular carrier system is found to alter the population ratio of physiologically active to inactive protomers of drugs, its application as a vehicle for prototropic drugs is restricted due to its limited solubility and less biocompatibility. In this respect, serum albumin can be a far better delivery system. Importantly, the whole decade of the 2000s has been involved in developing various nanoparticles for targeted drug delivery to tumors, but the outcome as a whole is not very encouraging.[57] In this issue, the results of an albumin-based delivery system are highly prospective because of its nonimmunotoxicity, long circulation time in blood, and high tumor accumulation.[57] Hence, the future of the targeted delivery of the active protomer of prototropic drugs to tumors should be on the successful implementation of albumin-based vehicles.

■ ASSOCIATED CONTENT

Supporting Information

Absorbance and emission spectra of basic guests (FBZ, AC, and NR) and acidic guests (WF and C343) at different pH's in the absence and presence of serum protein at 298 K, *B*−*H* plots and modified Job plot for ligand−protein complexes, plot for the determination of pK_a of NR, WF, and C343, docked poses of the neutral form of FBZ and WF, table with K_1 and K_2 values, and table representing change in SASA of protein−ligand complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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