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ORIGINAL ARTICLE

Intermolecular interactions and binding mechanism of inclusion complexation between sulfonate calix[*n*]arenes and ethidium bromide

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Abstract
In this work, the interactions between ethidium bromide (ET) and water-soluble sulfonate calix[*n*]arenes (*n*: 4, 6, and 8) were investigated by NMR, FT-IR, and fluorescence spectroscopic methods. The aim was to evaluate both the stoichiometry and the mechanism of the possible complex structure between sulfonate calix[*n*]arenes and ET. The spectroscopic data revealed that a 1:1 binding mode between calixarene and ET was occurred. Furthermore, thermodynamic parameters and fluorescence titration experiments were studied at different temperatures to determine both the quenching mechanism and the type of intermolecular forces in complex formation. Host–guest complexation of sulfonate calix[*n*]arenes and ET could be used to overcome some adverse effects related to the using of ethidium bromide during biological applications as a DNA marker treatment.

Keywords Ethidium bromide · Calixarene · Inclusion complexation · Fluorescence · DNA marker

Introduction

Ethidium bromide (ET) is a well-known cationic dye and molecular probe for double helical RNA and/or DNA chains in gel electrophoresis applications [1]. It is also used in prophylaxis and treatment of animal trypanosomiasis in affected areas [2]. Despite its intense use in gel electrophoresis applications, it must be carefully handled because of its mutagenic and moderately toxic properties [3]. For example, its powder form is harmful if swallowed and very toxic by inhalation, and at higher levels may cause irritation to the upper respiratory system, the eyes, and skin [4]. Based on all of these factors, careless using of ethidium bromide can cause serious health effects in the laboratory as well as an environmental hazard during disposal [5]. If an untreated ethidium bromide waste is poured into a drain or placed in the trash, waste of ethidium bromide may create a potential risk for the environment. Therefore, ethidium bromide solutions must be prepared and handled with little portions in a fume hood and its waste must be evaluated as a special waste [6].

Supramolecular complex formation is a new and efficient method for both monitoring and removal of many hazardous substances such as toxic, mutagenic, teratogenic, or radioactive neutral or charged species arising from industrial or clinical applications. This intermolecular complex formation is usually fast and reversible due to prevailing non-covalent interactions. For example, the production of smart new materials as biosensing material-based supramolecular complexation is promising for monitoring or eliminating bio-hazard molecules [7, 8]. In this field, calixarene compounds are important host molecules. Calixarenes are macrocycle compounds made of some phenolic units via methylene bridges [9, 10]. These compounds have been widely used as a host molecule in the supramolecular chemistry, due to their ability to encapsulate organic compounds into their hydrophobic cavities [11, 12]. Owing to the calixarene skeleton, these molecules are able to act as "molecular baskets" for neutral or charged guests [13]. Calixarene molecules are more soluble compounds in general organic solvents but most importantly calixarene compounds containing hydrophilic units are water-soluble. For instance, sulfonate calixarene derivatives are more favorable host molecules to encapsulate small charged guest molecules in their cavity owing to their better water solubility properties, especially in biomedical applications [14].

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Due to several advantages of water-soluble sulfonate calixarene such as easily synthesis, solubility, and inclusion complexation ability, the objective of this study was to investigate and compare the use of sulfonate calix[*n*]arenes (*n*: 4, 6, or 8) for possible host–guest applications between calixarene and ethidium bromide in biological or biochemical areas. The stoichiometry, association constants, and thermodynamic parameters for the inclusion complexes were determined to obtain a quantitative data for the encapsulation of ethidium bromide with calix[*n*]arenes by fluorescence quenching [15, 16]. Furthermore, these parameters may provide some insights regarding the kind of achieved interaction, to understand the mechanism of the highly toxic ethidium bromide action in the cavity of calixarene skeleton.

Experimental

Apparatus

Shimadzu UV 1800 spectrophotometer with matched 1-cm quartz cells was used for the all spectrophotometric measurements. All fluorometric measurements were made with a Hitachi F-7100 fluorescence spectrophotometer. FT-IR spectra for both free and complex structures were obtained on a Bruker Vertex 70 ATR-FTIR instrument. Agilent 600 MHz spectrometer was used for the NMR spectra of free and inclusion complex structure of the calixarene compounds.

Synthesis

The starting materials *para*-t-butylcalix[*n*]arenes (*n*: 4, 6 or 8) were prepared by following procedure described by Gutsche [17]. After preparation of the starting materials, preparation of water-soluble sulfonate calix[*n*]arenes (*n*: 4, 6, or 8) SC4, SC6, and SC8 was carried out by using modified literature procedures via *ipso* sulfonation method (Fig. 1). Briefly, 3 mmol of corresponding *para*-t-butylcalix[*n*]arenes (*n*: 4, 6, or 8) was mixed with 10 mL of concentrated sulfuric acid and then solution was heated to 60 °C for 24 h. After completion of reaction, solution was mixed with diethyl ether to remove excess amount of sulfuric acid and obtained precipitated was filtered. Obtained analytical data for clarification of calixarene structures SC4, SC6, and SC8 were identical with previously published results [18].

Reagents and procedure

For the stock standard solution of ethidium bromide ($2.5\ 10^{-3}$ mol/L), 0.045 g of ET was dissolved in water,

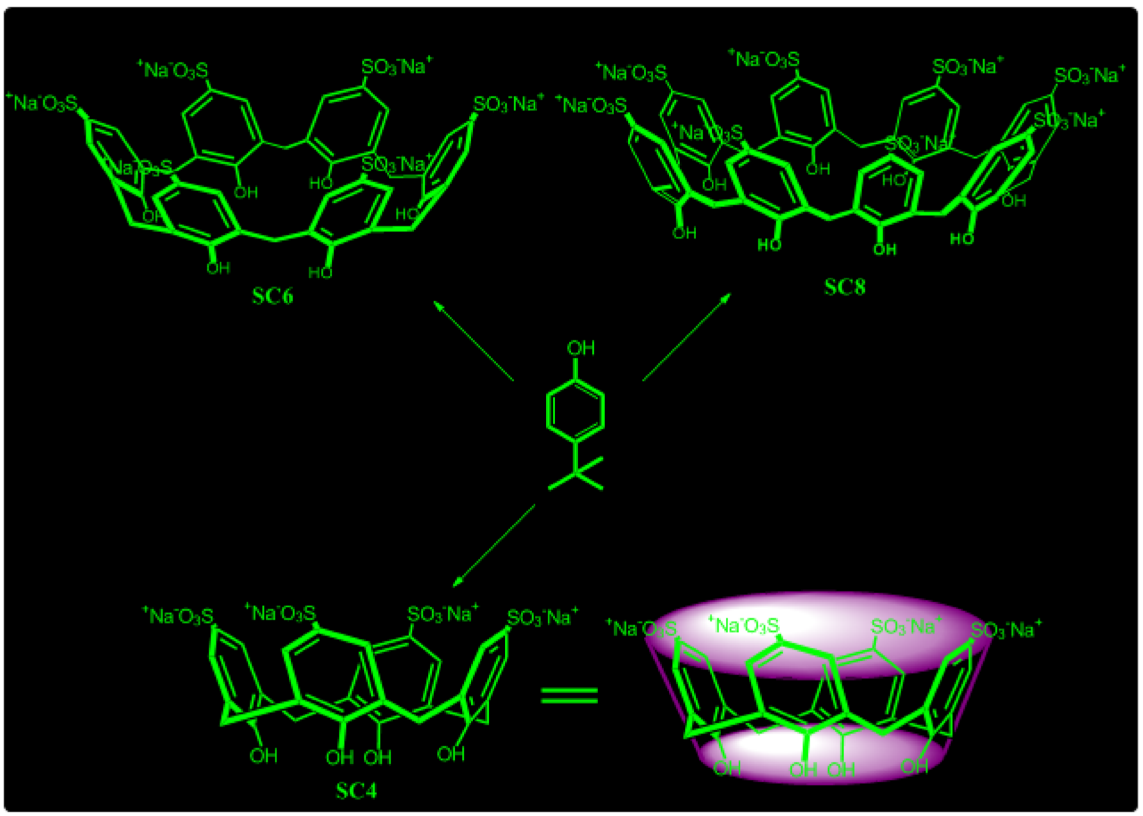

Fig. 1 Synthesis of water-soluble sulfonate calix[*n*]arenes (*n*: 4, 6, or 8)

transferred into a 1000-mL standard flask and diluted to the mark with water, and mixed well. For the stock calixarene solutions (0.5 mol/L), 0.42, 0.62, and 0.75 g of calix[*n*]arene (*n*: 4, 6 or 8) were weighted and dissolved in double-distilled water, respectively. A 1.0-mL portion of ET (1 mM) diluted appropriately with double-distilled water was transferred accurately into a 1-cm quartz cell containing different concentrations of calixarene hosts (100–1000 µM) while the concentration of ET was fixed constant at 100 µM. The resultant mixture was subsequently incubated at 25, 35, and 45 °C for 15 min. Absorption spectra of ET in the presence and absence of calix[*n*]arenes were recorded in the range 200–600 nm in a UV–Vis spectrophotometer. The solutions were scanned (1200 nm/min) with 400 W of PMT voltage in a spectrofluorometer with the wavelength range 550–750 nm. The widths of slit for both the excitation and emission were adjusted at 10 nm. The fluorescence intensity at 618 nm was determined under the excitation at the wavelength of 525 nm.

Results and discussion

The UV–Vis spectra of ET in the presence of different amounts of calixarene molecules SC4, SC6, and SC8 in water solution are shown in Fig. 2. The maximal absorption intensity of ET varies markedly upon the addition of different amounts of SC4. These changes in absorption spectra show an interaction and complex formation between ET with SC4. ET indicates the absorption maximum at the wavelength of 212 and 282 nm in aqueous solution. It may be due to the n–σ* and π–π* corresponds to a benzene moiety of ET [19]. The strong absorption peak of ET (100 µM) at wavelength 496 nm decreases and shifts after addition of SC4 and SC8 (0-1000 µM). Similar changes in the spectra of ET are observed for SC6. The strong hypsochromic shift is observed only for SC4 when the concentration ratio of ET:SC4 is 1:4 or higher. An approximately 44 nm strong hypsochromic shift at 496 nm band of ET is observed for SC4 while powerful hypochromic shift is seen at all studied concentrations for SC8. All shifts in absorption bands of ET are probably related to mobilization of ET molecule from the polar environment to the non-polar hydrophobic micro-environment in a cavity of calixarene skeleton. Both hypsochromic and hypochromic shifts at different guest concentrations mentioned above support the possible host–guest complex formation between fluorescence ET and non-fluorescence SC4. Additionally, disappearance of a strong peak of guest ET at the wavelength 212 nm attributable corresponding π–π* transition after addition of calixarene host may also evidence for the possible host–guest complex formation via hydrophobic attractions as π–π interactions. Furthermore, color change of ET from a strong pink to yellowish tone under daylight is also an evidence to support the possible interaction of ET with SC4 (Fig. 3).

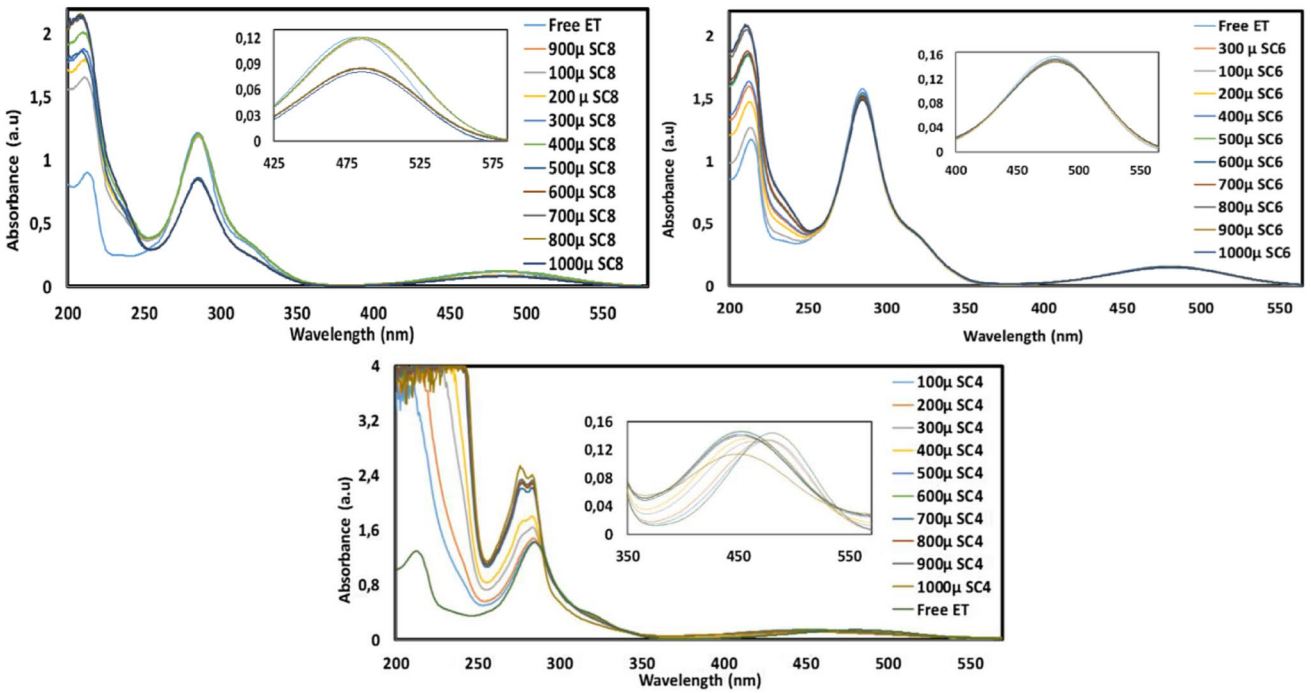

Fig. 2 Absorption titration spectra of ET in H_2O in the presence of increasing concentration of SC4, SC6, and SC8 (100–1000 µM)

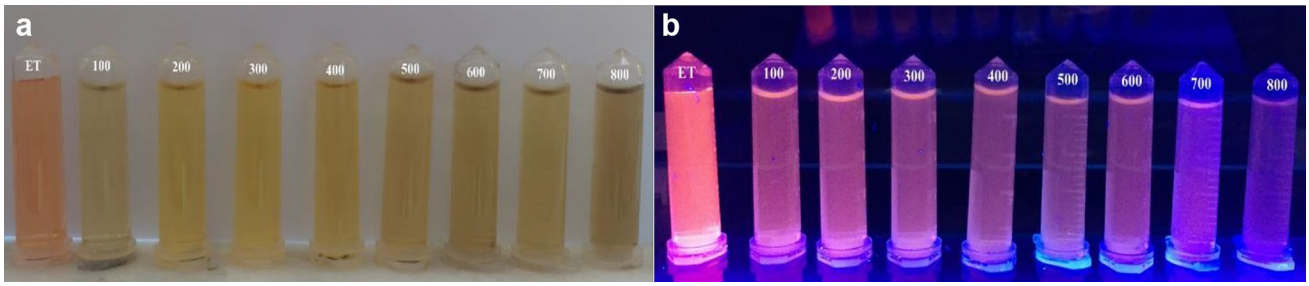

Fig. 3 Color changes of ET (100 μM) in the presence of SC4 (100–800 μM) (**a** under daylight, **b** under UV-light)

Intermolecular interactions between ET and SC4, SC6, and SC8 are investigated by fluorescence spectra. ET molecules contain a fluorophore group as phenanthroline which exhibits strong fluorescence signal. ET and calixarene molecules SC4, SC6, and SC8 are found to react forming a complex that results in quenching of the fluorescence of ET. The quenching of ET is measured at 614 nm after excitation at 525 nm (Fig. 4). The interaction of ET with SC4, SC6, and SC8 is evaluated by monitoring an intrinsic fluorescence intensity changes of ET upon addition of calixarene molecules SC4, SC6, and SC8. Fluorescence quenching spectra of ET in the presence of different concentrations of SC4, SC6, and SC8 are shown in Fig. 4. Under experimental conditions, fluorescence spectrum of ET (100 μM) is gradually decreasing with increasing concentrations of studied calixarene molecules (0–1000 μM). This decrease in emission spectra of ET indicates that there is an interaction between ET and SC4, SC6, and SC8. However, calixarene molecules SC4, SC6, and SC8 do not show any fluorescence intensity under the same conditions.

This fluorescent decrease is also supported by UV image of ET under UV light. Intensity light of ET gradually becomes pale and then disappears as shown in Fig. 2. It just causes a concentration-dependent quenching of the fluorescence of ET. From the fluorescence quenching experiments, it is clearly observed that the most powerful binding interaction is monitored in fluorescence spectra for SC4 molecules as compared with other studied calixarene molecules such as SC6 and SC8 (Fig. 5). This situation is probably due to the reason that SC4 has the best platform for ET molecule such as rigidity or flexibility of calix [4] arene skeleton as well as suitable cavity diameter of calix [4] arene backbone. Furthermore, the conformation of calix [4] arene platform can be influenced by the interactions between rigid hydrophobic cavity or aromatic rings of calixarene platform with ET molecules by means of the π-π interactions or van der Waals attractions [20]. ET molecule has a dual binding mode such as charged ammonium group and aromatic moiety. In literature, it is well known that sulfonate calixarenes can complex with the charged aromatic ammonium derivatives by electrostatic attractions or aromatic ring interaction in a cavity of calixarene skeleton with respect to the anchoring groups such as carboxylic or sulfonate leading to a more stable inclusion complex [21]. As a result, it is seen that obtained binding data for ET as a positively charged aromatic methyl ammonium derivative are accordance with the literature results [22, 23]. This interpretation is strongly supported by the ^{1}H NMR results showing that guest ET is more deeply included into the cavity of SC4.

In order to clarification of the quenching mechanism of ET-SC4 complex formation, temperature-dependent fluorescence spectra of ET with SC4 are studied by drawing of

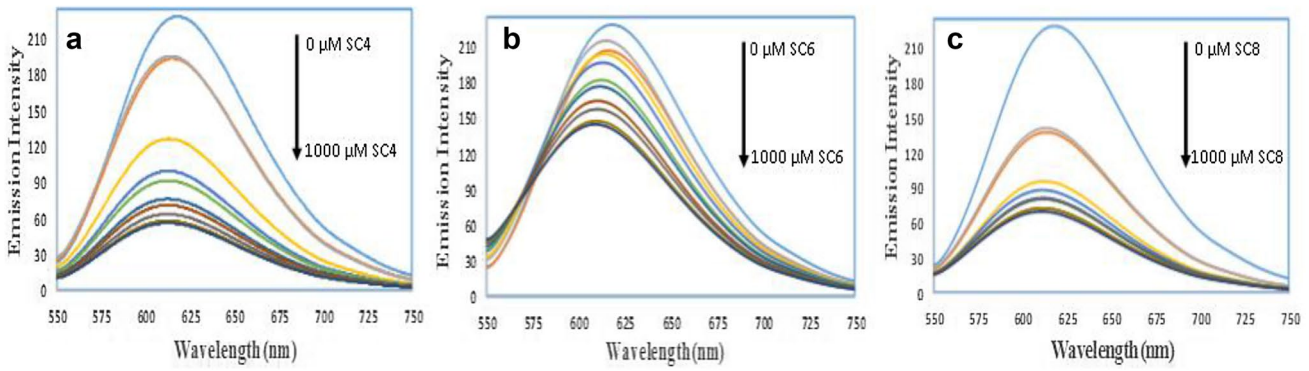

Fig. 4 Fluorescence titration of ET in the presence of increasing calixarene concentrations (**a** SC4, **b** SC6, and **c** SC8)

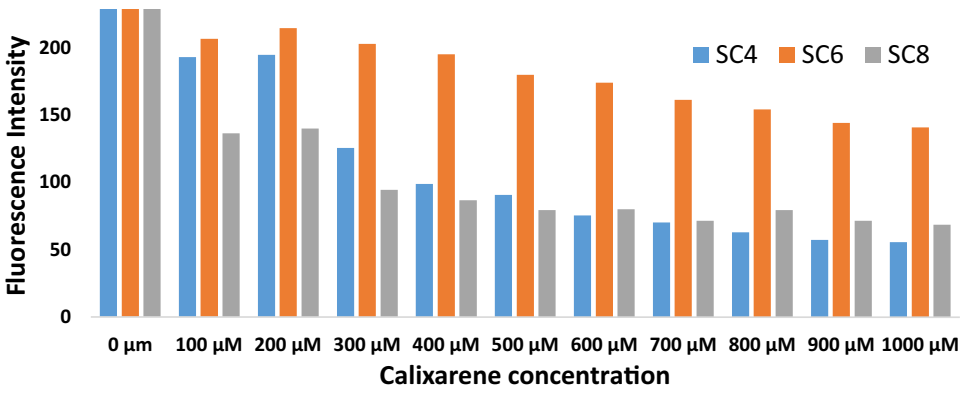

Fig. 5 Fluorescence intensity of ET with increasing concentration of SC4, SC6, and SC8

the Stern–Volmer plots (Fig. 6a). Fluorescence quenching is described by a Stern–Volmer equation as mentioned below [24]:

$$F_0/F = 1 + K_q \cdot \tau_0[Q] = 1 + K_D \cdot [Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of ET in the absence and/or in the presence of SC4 (Quencher), while K_q is the bimolecular quenching constant; τ_0. If the quenching is known to be dynamic, the Stern–Volmer constant will be represented by K_D. Otherwise this constant will be described as K_{SV}. K_{SV} represent the Stern–Volmer constant; τ_0 is the average lifetime of ET without SC4 (Quencher), and $[Q]$ is the concentration of SC4 (Quencher). The Stern–Volmer plots for ET-SC4 complex formation are given in Fig. 6a. The resulting graph with straight line that provides the determination of the quenching rate constant (K_q) will have an intercept of 1 and a slope so-called the Stern–Volmer constant (K_{sv}). Based on these results, linear plots supporting the static quenching mechanism are observed in the interaction between ET and SC4 [24]. With respect to Eq. (1), the corresponding quenching constants (K_{sv}) for the interaction between ET and SC4 are 1.8×10^4 (25 °C, $R=0.9904$), 1.6×10^4 (35 °C, $R=0.9970$), and 1.4×10^4 L/mol (45 °C, $R=0.9911$), respectively. The quenching constants K_q are determined as 1.06×10^{13} at 25 °C, 0.95×10^{13} at 35 °C, and 0.83×10^{13} L/mol·s at 45 °C by using the natural radiative lifetime of ET (about $1.7\cdot10^{-9}$ s), and calculated the slope from Eq. 1, respectively [25]. In literature, it is well known that the maximum scatter collisional quenching constant of various quenchers is around 2.0×10^{10} L/mol·s [26]. Since values obtained in this study are greater than the maximum collisional quenching constant (2.0×10^{10} L/mol·s.), it can be concluded that the quenching mechanism does not include dynamic quenching, but probably static quenching is predominant [27].

In addition, careful examination of the absorption spectra of fluorophore is performed to distinguish static and dynamic quenching. Collisional quenching only affects the excited states of fluorophores, and thus no changes in the absorption spectra are expected. Because collisional quenching just influences the excited state of fluorescent molecule, there is no any respectable changes in the absorption spectra of ET, whereas ground-state complex formation and subsequent static quenching typically cause mentionable changes in the absorption spectrum. As shown in Fig. 2, these changes are seen apparently as blue-shifts with a decrease in absorption intensity of ET upon addition of SC4 at 496 nm. This suggests that the interaction between ET and SC4 is mainly a static quenching

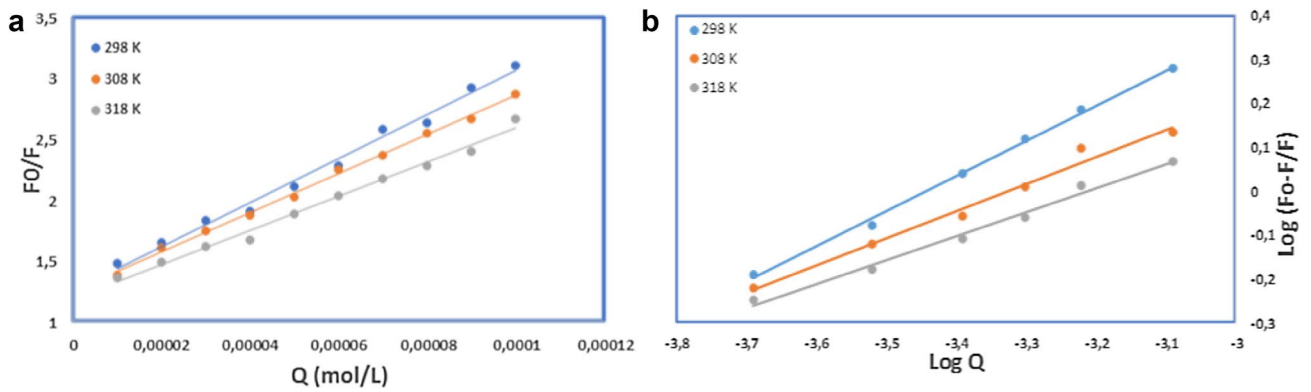

Fig. 6 **a** The Stern–Volmer plots; **b** double reciprocal plots of ET with SC4 at 298, 309, and 318 K

process [27]. When small molecules bind dependently to a set of equivalent sites on a macromolecule, the binding equilibrium between free and bound molecules can be represented by an equation. The number of ET molecules bound to calixarene skeleton (n) and binding constant (K) for ET-SC4 system is calculated from the intercept and slope of the plot of $\log[F_0-F/F]=\log K+n\log Q$ for the static quenching, respectively (Fig. 6b) [28]. The n value from the slope of the straight-line plot is 0.9 in ET-SC4 complex formation. It is noticed that the binding constant values decreased with an increase in temperature due to reduction of stability of ET-SC4 complexes. The binding constant values (K) are found as 5.8×10^2 (25 °C, $R^2=0.9970$), 1.1×10^2 (35 °C, $R^2=0.9824$), and 0.5×10^2 K^{-1} (45 °C, $R^2=0.9882$) at different temperatures. Since a benzyl part of ET molecule enter probably to a hydrophobic cavity of calixarene skeleton, stoichiometric ratio of inclusion complex formation between ET and SC4 should be theoretically 1:1. This theory can be proved if a linear relationship is obtained from the reciprocal plot of $1/F$ vs. $1/[Q]$ based on the modified Hildebrand-Benesi Equation [29]. Figure 7a shows reciprocal plots that determine a stoichiometric ratio of an inclusion complex formation. A very good linear relationship is obtained for $1/F$ vs. $1/[Q]$ with $R^2=0.9913$. This reciprocal plot clearly indicates that there is a strong binding force between ET and SC4 and the stoichiometry ratio for an inclusion complex formation between ET and SC4 is 1:1 [30].

The binding forces between ET and SC4 molecules may contain hydrophobic, van der Waals, electrostatic interaction, hydrogen bonds, dipole–dipole attractions or combination of these forces. For clarification of possible binding modes between ET and SC4, thermodynamic parameters such as an enthalpy (ΔH), free energy (ΔG), and entropy (ΔS) data estimated from the following Eqs. 2–4 can be useful [31].

$$(\Delta G) = -2.303RT\log K, \tag{2}$$

where R is the gas constant; T is temperature, and K is the binding constant at suitable temperature (T).

An enthalpy change (ΔH) and entropy (ΔS) can be calculated from the following Eqs. 3 and 4;

$$\log K_2/K_1 = \left(1/T_1 - 1/T_2\right)\Delta H/2.303R \tag{3}$$

$$\Delta G = \Delta H - T\Delta S. \tag{4}$$

A negative ΔG (− 15762.4 J/mol) value shows that the interaction process between ET and SC4 is spontaneous and negative ΔH (− 138.30 kJ/mol) and ΔS (− 411.2 J/mol·K) quantities indicate that hydrogen bonds and van der Waals attractions are basically forces for this interaction process [32, 33]. Furthermore, additional experiments as FT-IR and ^{1}H-NMR analysis were carried out to clarify the binding mechanism of ET with SC4 and to support the obtained physicochemical and analytical data mentioned above for ET-SC4 complex formation.

In IR spectra of inclusion complex of ET and SC4, broad bands of calixarene molecules overlap basic molecular bands of ET (Fig. 8). Nevertheless, ET characteristic bands can be detected in FT-IR spectra of 1:1 complex formation. IR spectrum of pure ET shows the presence of a peak at 3297 cm^{-1} (Fig. 8b), assigned to N–H stretching vibration while a spectra of pure SC4 is characterized by intense bands at 3183 cm^{-1} due to O–H stretching vibration. These sharp peaks become broad and move towards higher or lower wavenumbers after formation of an inclusion complex. Upon complexation, amine absorption peak of ET at 3297 cm^{-1} is shifted to 3342 cm^{-1} in IR spectra and a phenolic hydroxy band of calixarene skeleton is slightly shifted towards a lower wavenumber, due to an appearance of host–guest complexation. From FT-IR data, it is seen that the possible hydrogen bond formations occur between hydroxyl groups of calixarene skeleton and amine group of ET. Additionally, in FT-IR spectra, we do not observe any appearance of new bands or disappearance of an existing characteristic bands which was evidence for the possible chemical bond formation between SC4 and ET via covalent bond formation. This

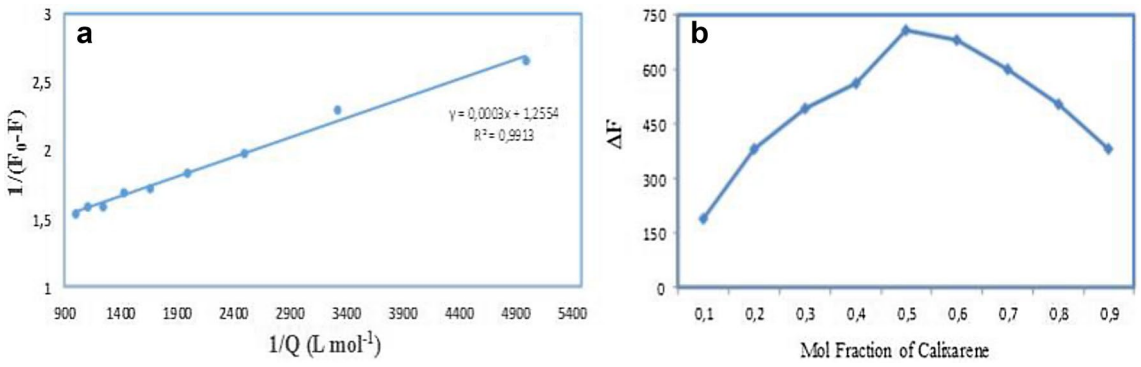

Fig. 7 **a** The Benesi–Hildebrant plot and **b** Job plot for the binding stoichiometry of ET with SC4 plotted from fluorescence spectra at 614 nm with total concentration maintained at 100 μM

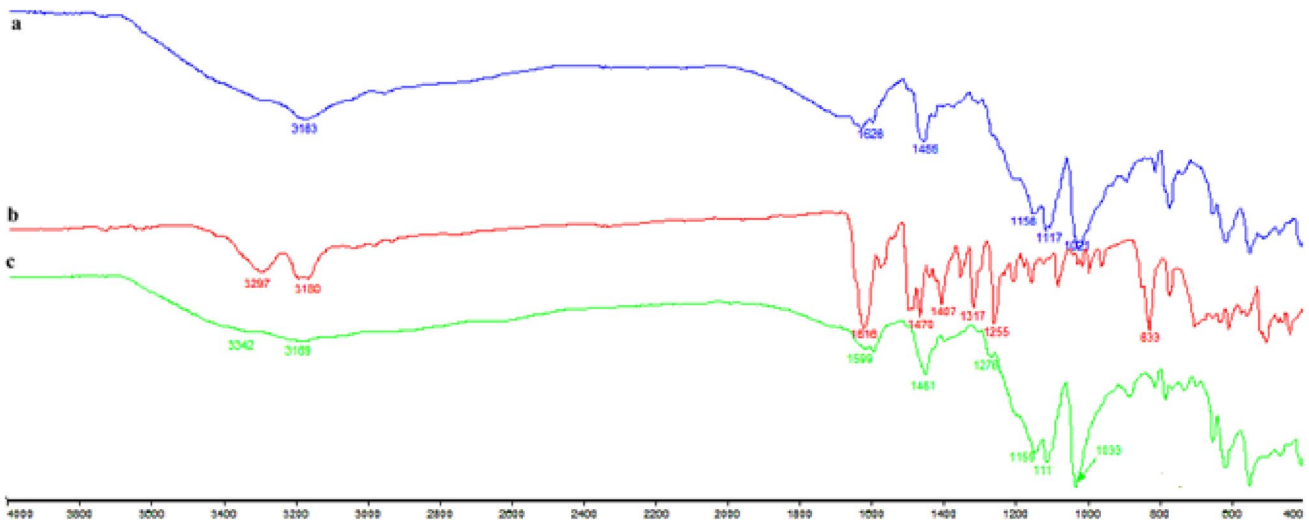

Fig. 8 FT-IR analysis of free SC4 (*a*), free ET (*b*), and inclusion complexation of SC4-ET (1:1) (*c*)

situation also supports the possible non-covalent interactions such as hydrogen bonds or van der Waals attractions between calixarene skeleton and ET.

To better understand the interactions of SC4 with ET in water, ^{1}H NMR experiments are performed for SC4 in D_2O (Fig. 9). NMR is a technique which provides the most evidence for an inclusion of a guest molecule into the hydrophobic cavity of calixarene skeleton in solution. An inclusion of ET in the calixarene cavity is indicated by changing in chemical shift of guest and host protons in a complex structure, compared with chemical shifts of the same protons in pure host or guest. ^{1}H NMR spectra of SC4 and SC4-ET complex structures are presented in Fig. 9. From ^{1}H NMR data, it is indicated that basic aromatic hydrogen peaks of calixarene skeleton as singlet observed around 7.39 ppm for SC4 is shifted to downfield as 7.58 ppm for SC4-ET (1:1) complex structure. This shift is probably due to the π–π interaction between aromatic rings of both calixarene and ethidium molecules inserted in the cavity. The same peak also shifts to 7.44 ppm for SC4-ET (1:2). When NMR spectrum was recorded with 2-equivalent of added ET (2:1 stoichiometry), signals already altered by 1 equivalent ET are not shifted further considerably. This observation reveals that there is a possibility of only 1:1 binding stoichiometry. On the other hand, as seen from Fig. 9, chemical shift values of aromatic protons in ET change upon complexation in ^{1}H-NMR spectrum. From all spectroscopic data for the complex formation of the calixarene skeleton and ET, the possible

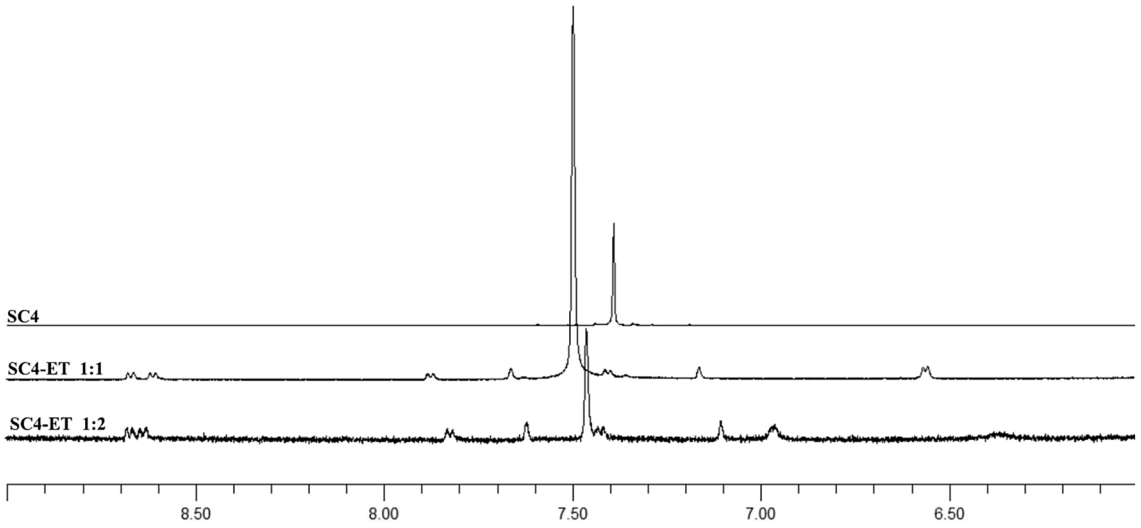

Fig. 9 ^{1}H-NMR analysis of free SC4, SC4-ET 1:1 and SC4-ET 1:2 complex structures

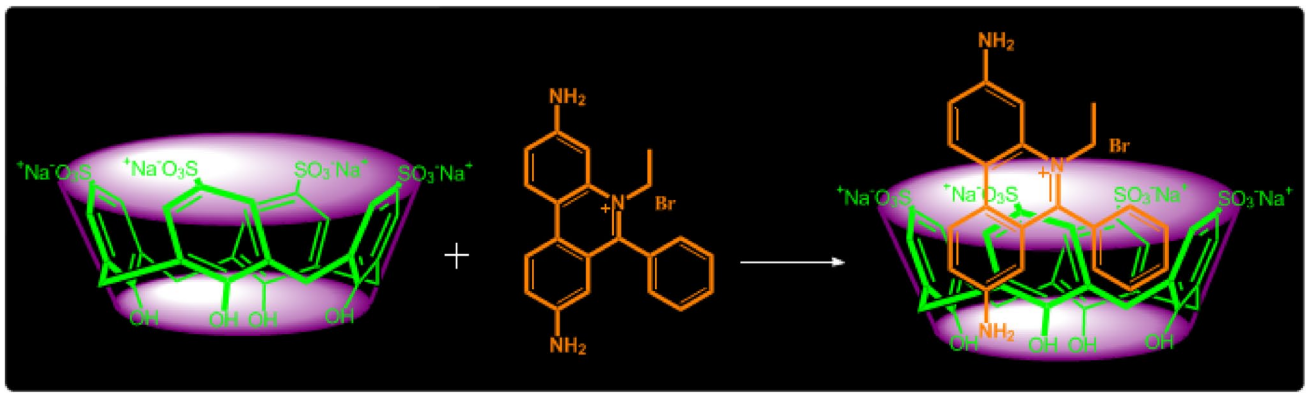

Fig. 10 The proposed inclusion pattern between ET and SC4

proposed inclusion mechanism between ET and SC4 is given in Fig. 10. It is well known that an inclusion complexation between a host and guest molecule is generally occurred by weak forces such as hydrogen bonding, π–π interaction, dipole–dipole or van der Waals. Generally, in the process of an inclusion complex formation, the key force depends on structure, charge and/or functional group of guest and host.

Conclusion

In conclusion, the binding behaviors of SC4 with ET are investigated by spectrophotometric and spectrofluorimetric titrations in water. An inclusion complex formation is confirmed by FT-IR and ^{1}H-NMR. It has been shown from NMR data that ET is inserted partially into the cavity of SC4 due to favorable formation constant of π–π interactions with benzene rings and hydrogen bonding with polar groups. Besides, various binding parameters have been evaluated. The decreasing *K* values with an increasing temperature from 298 to 318 K show that static quenching mechanism is occurred between SC4 and ET. Thermodynamic parameters indicate that hydrogen bonding and weak van der Waals interaction are predominant intermolecular forces in complex formation. All findings support an inclusion complex formation and thus the current work describes its appropriateness towards miscellaneous applications as a controlled delivery system or binding probe towards charged or neutral organic molecules in fields related to modern biomedical sciences.

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