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Alexidine and Pentamidine Fold Inside the Bowl-Shaped Cavity of *p*-Sulfonato-calix[4]arene

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Dedicated to the Institute of Physical Chemistry Polish Academy of Sciences on the occasion of its 70th anniversary

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Abstract: We report on the U-shaped folding of flexible guest molecules of medicinal interest upon their inclusion into macrocyclic cavity of *p*-sulfonato-calix[4]arene in aqueous media. Alexidine and pentamidine are FDA-approved drug compounds currently rediscovered as potent membrane-targeting antibiotic adjuvants helping restore antibiotic activity against multidrug resistant bacteria pathogens. We have adopted host-guest and crystal engineering approach to study these drugs with a view of potential supramolecular formulations and/or crystal forms. We focus on the host-guest conformational and structural behaviour of alexidine and pentamidine under macrocyclic confinement conditions benefitting from single crystal X-ray diffraction analysis, self-assembly studies in solution by NMR spectroscopy, dynamic light scattering and atomic force microscopy, and ion mobility mass spectrometry (IM-MS) analysis complemented by theoretical calculations. Our findings show that the simple bowl-shaped host promotes conformational fixing and crystallization of these guest molecules of high conformational freedom that are otherwise challenging to crystallize. The IM-MS structural studies of *p*-sulfonato-calix[4]arene complexes with pentamidine and alexidine revealed significant guest reorganization in the solution/gas phase, compared to the binding modes observed in the crystal structures. Despite these changes, the host-guest complexation remained consistent, with new interactions highlighting the increased role of electrostatic forces in the gas phase.

Introduction

The inner space of macrocyclic hosts enables the recognition and confinement of various guest molecules due to the non-covalent interactions and hydrophobic effect in aqueous media.^{1,2} For highly flexible guests, e.g. comprising aliphatic chains, inclusion into the hydrophobic cavity of a host can result in conformational fixing.³ Such host-guest ensemble formed *via* (mutual) induced fit mechanism is usually more prompt to crystallize, paving the way to the structural characterization benefitting from single crystal X-ray diffraction analysis.⁴ The mode and degree of compression of the flexible guest would be defined by the geometry and chemical identity of the host molecule serving as a confinement vessel.⁵ The advantages here are in (i) gaining structural information on the host-guest systems integrating macrocyclic host as additive to promote crystallization,⁶ (ii) host-guest assembly and crystallization as a mean to change/lock the conformation *via* non-covalent interactions⁷ and, in the next step, to modulate properties or function of a molecule of interest; (iii) insight into conformational behavior of the active molecule under macrocyclic confinement conditions mimicking biological confinement.^{8,9} Moreover, the inner space of host molecules can promote chemical reactions¹⁰ and/or stabilize reactive intermediates.¹¹

Of the available abiotic macrocyclic hosts operating in water, the family of anionic *p*-sulfonato-calix[*n*]arenes is

particularly attractive due to the rich palette of molecular recognition properties and biocompatibility making them potentially useful for life sciences and pharmaceutical applications.^{12, 13, 14} In terms of providing confinement for hydrophobic folding, the smallest *p*-sulfonato-calix[4]arene (**C4S**) of rigid bowl-shaped cavity has been well explored towards organic ammonium and amino acid guests. The early structural work of Coleman and de Rango on the host-guest complex of **C4S** with L-lysine showed the inclusion of folded amino acid into the macrocyclic cavity and additional traversing of **C4S** bilayer by L-lysine in extended conformation.¹⁵ Later, other amino acids have been studied to form inclusion complexes based on partial encapsulation of their side chains within **C4S**.^{16, 17} Systematic study by Barboiu *et al* on the progressive folding of biomimetic guests showed how molecular compression occurs inside **C4S** cavity.¹⁸ In that study cationic 1, ω -diammonium-alkanes and 1, ω -amino-acids have been screened in the presence of **C4S** host and metal ions. The X-ray crystal structures revealed coiling behavior of the guests with compression up to 40 % compared with their linear conformations. Peter Crowley and his team took *p*-sulfonato-calix[*n*]arenes to a higher level of molecular recognition, namely using macrocycles as scaffolds that mold to cationic surfaces of proteins promoting assembly and crystallization.^{19,20}

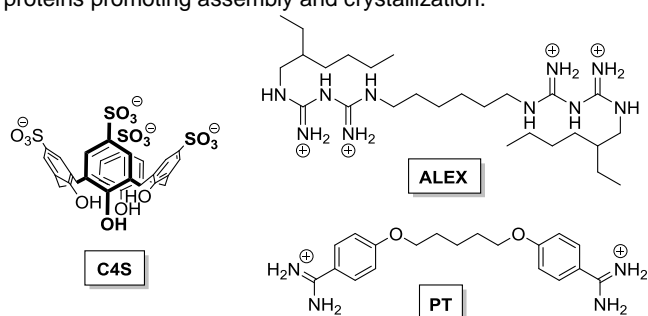


Figure 1 Chemical structure of *p*-sulfonato-calix[4]arene (**C4S**), alexidine (**ALEX**) and pentamidine (**PT**).

In the current work we describe conformational fixing of flexible guest molecules, alexidine and pentamidine upon their inclusion into macrocyclic cavity of **C4S**, Figure 1. Our interest in these FDA-approved drug compounds stems from their current rediscovery as membrane-targeting antibiotic sensitizers helping restore antibiotic activity against multidrug resistant bacteria pathogens.²¹ The main challenge in treating Gram-negative bacteria is the impermeability of outer membrane layer, linked to the presence of the lipopolysaccharide (LPS) – an amphipathic glycolipid acting as a barrier for the antibiotics.²² Alexidine and pentamidine belong to the same structural class of non-steroid small molecule sensitizers of C_{2h}-symmetric di-cationic structure provided by two biguanidinium (alexidine) or two amidinium (pentamidine) groups.^{23,24} Both these chemicals could assist the antibiotics, favoring their passage through the membrane into the cells.²⁵ Unfortunately, as for now this phenomenon has limited potential for clinical translation mainly because of poor pharmacological and toxicological profiles of the sensitizers.^{26,27}

We have decided to focus on these cationic drug molecules adopting host-guest²⁸ and crystal engineering²⁹ approach. Despite some initial literature reports^{30, 31, 32} the biguanide and amidine pharmaceuticals are certainly understudied from the supramolecular and structural perspective. The objective of this work was to study host-guest conformational and structural behavior of highly flexible drug molecules in the confined space of *p*-sulfonato-calix[4]arene. The investigation of the host-guest chemistry of these assemblies would bring insight for the development new supramolecular formulations and/or crystal forms with controlled pharmacological kinetics and improved toxicological profiles.³³

Results and Discussion

Crystallization Behavior

The cocrystallization of *p*-sulfonato-calix[4]arene with alexidine (**ALEX**) and pentamidine (**PT**) was challenging because of the immediate precipitation induced by host-guest charge neutralization. The combination of aqueous solutions of host and guest resulted in the significant clouding and rapid formation of white suspensions. The precipitation occurred at mM **C4S** concentration suggesting strong host-guest interactions leading to a product of much lower solubility than the starting host and guest components. Obviously, when anionic sulfonate groups of the macrocycle encounter cationic binding partners bis-biguanidinium (**ALEX**) or bis-amidinium (**PT**) the formation/precipitation of the low-soluble products is almost instant. The complementarity of the electrostatic charges is strengthened by the ditopic nature of the guest molecules and possibility of multiple charge-assisted hydrogen bonds between biguanidinium or amidinium donors and sulfonate acceptors at the upper rim of the macrocycle. The behavior of the anionic calixarenes to afford precipitates when meeting certain guest molecules in aqueous solutions is generally known among supramolecular community,^{34, 35} but is rarely described or discussed because of the significant obstacles in the characterization of such host-guest systems.³⁶ One of the strategies to overcome the rapid precipitation is the chemical modification of the binding partners with additional solubilizing groups. For example, the complementarity between tetra-anionic sulfonate and tetra-cationic amidinium calix[4]arenes render the capsular assembly insoluble in water.³⁷ The replacement of sulfonate groups by carboxylates and introduction of glycol-type substituents at the lower rims of both calix[4]arenes provide aqueous solubility to the molecular capsule based on ionic interactions.³⁸ Another strategy to study the rapidly precipitated host-guest systems (at least in terms of obtaining crystal structure), is to slow down the nucleation and crystallization of the low-soluble product. We have recently reported that the problem of charge neutralization induced precipitation in the system of cationic pillar[4]pyridinium and anionic *p*-sulfonato-calix[4]arene can be overcome by changing the crystallization medium from aqueous solution to agarose gel.³⁹ The crystallization in the U-tube set-up involving diffusion of each macrocyclic component through the gel succeeded in the

formation of single crystals of the product of good quality for home diffractometer. We have tried the same approach in order to crystallize host-guest complexes of *p*-sulfonato-calix[4]arene with **ALEX** and **PT**, however without any success. Crowley *et al* observed the immediate precipitation upon complexation studies of **C4S** with arginine-rich protein lysozyme and obtained single crystals of the desired complex from heavy precipitates.⁴⁰ Taking into account their experience, we simply left the obtained suspensions to stand for the time being at ambient conditions. To our delight, after several weeks we perceived small amount of single crystals suitable for single crystal X-ray diffraction analysis within sedimented precipitates.

C4S-PT host-guest complex

C4S-PT complex crystallizes in the triclinic *P*-1 space group. Asymmetric unit comprises one calix[4]arene macrocycle, two pentamidine guests and eight water molecules, Figure 2A,B. One of the pentamidine molecules is partially included in the host cavity with its central pentandiol chain. Second pentamidine molecule is complexed outside interacting with external skeleton and anionic sulfonate rim of the macrocycle. Both pentamidines are in the form of dications with their amidine functionalities protonated. Thus, the charge balance is satisfied to compensate the tetra-anionic nature of calix[4]arene.

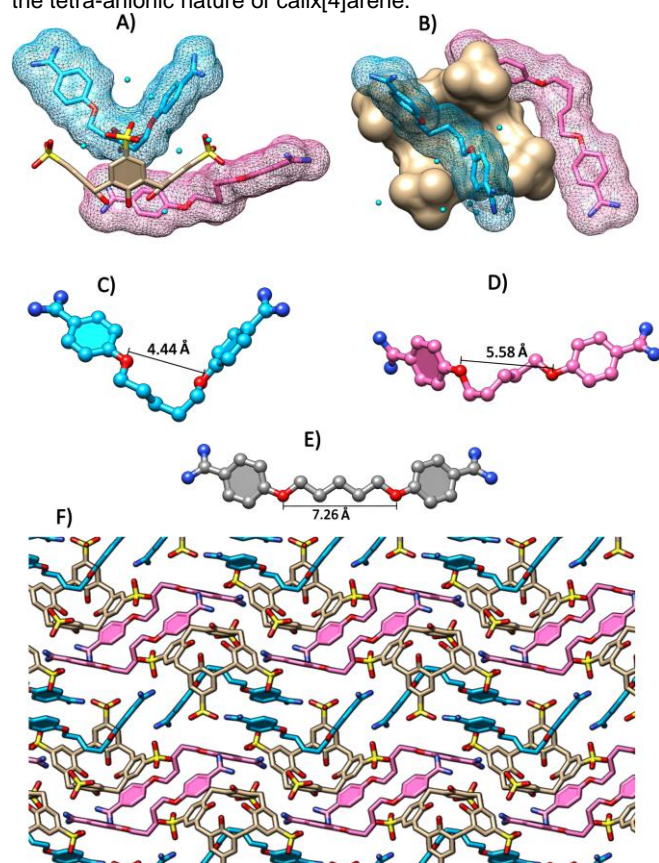


Figure 2. Host-guest complex of *p*-sulfonato-calix[4]arene with pentamidine: (A) side view; (B) top view. (C) Molecular conformation of pentamidine showing O...O distances between oxygen atoms bound to the central pentyl chain for endo **PT** (blue) subjected to U-folding; and (D) exo **PT** (pink) in the crumpled conformation; (E) extended conformation of **PT** in the inclusion complex with carboxylated pillar[5]arene, previous work (refcode BAWNIA). (F) Crystal packing viewed along a direction; water molecules omitted for clarity.

Two pentamidine guests have different conformations – the endo guest adopts an U shape, while the exo guest is "crumpled" (not fully extended). The distances between O...O atoms directly bound to the central aliphatic chain are of 4.44 and 5.58 Å, for the endo and exo guests, respectively, as shown in Figure 2C,D. We have previously reported host-guest inclusion complexes of **PT** with carboxylated pillar[5]arene⁴¹ and pillar[6]arene.⁴² Due to rigid prismatic cavity and two equivalent rims of pillar[5]arene the pentamidine adopts extended rod-shaped geometry with O...O distance of 7.26 Å, Figure 2E. Compared to bowl-like calix[4]arene, the pillar[5]arene better complements the extended pentamidine, also because of favourable charge-charge interactions between the amidine groups and the carboxylates at either rim of the pillarene. If we compare pentamidine conformation in calix[4]arene and pillar[5]arene host-guest complexes, the estimated compression are of about 39 and 23 %, for the endo and exo pentamidine molecules, respectively. In the PDB database⁴³ various conformational states of pentamidine in its complexes with biomacromolecules can be found. Pentamidine is known to bind in the minor groove of the DNA adopting extended conformation.^{44, 45} The U-folding of pentamidine has been revealed in its complexes with different classes of enzymes.^{46,47} The search of CSD⁴⁸ (version 5.46) on the pentamidine structures gives only two hits on the diisethionate salt of pentamidine^{49,50} and two hits on the pentamidine host-guest complexes with carboxylated pillar[5 and 6]arenes reported by our group.^{42, 42} All small-molecule crystal structures reported up to now comprise pentamidine in the extended conformation.

The coiled pentane aliphatic linker of the endo pentamidine molecule is involved in C-H... π and C-H...O interactions with cavity interior and sulfonate oxygen atoms of calix[4]arene host. Two benzamidinium moieties of the endo pentamidine point away from the cavity due to the imposed U-shaped geometry. There is no direct hydrogen bonding between amidinium groups of the endo pentamidine and sulfonate rim of the parent macrocycle. Instead, amidiniums form charge-assisted hydrogen bonds (salt bridges) to the sulfonate groups of adjacent **C4S** in the crystal environment, Figure S4. Crystal packing shows alternate up and down orientation of calix[4]arenes, but bilayer organization is diminished due to the extensive insertion of pentamidine molecules between macrocycles, Figure 2F.

The **C4S-PT** complexation studies in aqueous solution (D_2O) by ¹H NMR spectroscopy were hampered by low solubility of the host-guest complex. The addition of pentamidine isethionate to **C4S** unbuffered solution results in the instant formation of precipitate and no proton resonances of the guest can be observed in the spectrum. Despite some precipitation in the acetate buffer the host-guest complexation is manifested through the upfield shifts of all proton resonances of pentamidine, Figure 3. The largest shifts are observed for the proton resonances of the central aliphatic linker ($\Delta\delta$ of 0.38 and 0.35) of the guest indicating its location in the macrocyclic cavity as in the crystal structure model. The ¹H NMR experiments in the methanolic solution (CD_3OD) show very small shifts of **PT** aliphatic signals in the presence of **C4S**, Figure S8. The addition of 10% methanol to aqueous solution improves the solubility but

also hinder the inclusion of pentamidine aliphatic chain into **C4S** cavity, Figure S9. We determined the crystal structure of **C4S-PT-EtOH** complex obtained from water-ethanol solvent mixture, and confirmed that ethanol molecule is included in the cavity forming O-H...O hydrogen bond towards sulfonate oxygen atom of calix[4]arene, Figure S5. Two pentamidine guests reside exo embracing outer surface of the macrocycle and forming amidinium-sulfonate hydrogen bonds. Obviously, the host-guest complexation of pentamidine is solvent-dependent. Both ethanol and methanol successfully compete for the intracavity space, changing the supramolecular regime of pentamidine complexation from endo to exo. The aromatic proton resonances of pentamidine experience upfield shifts noting on the inclusion probability of terminal benzamidine groups in the solution, that was not observed in the solid state complex. Our recent work on the structural landscape of **C4S** complexes with simple benzamidine ligand supports the aromatic ring entering the macrocyclic cavity while amidinium groups engaging in the charge-assisted hydrogen bonding with sulfonate groups at the calix[4]arene upper rim.⁵¹

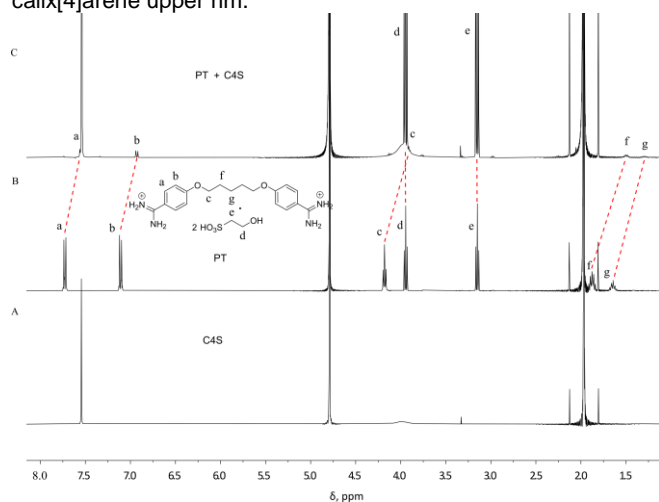


Figure 3. ¹H NMR spectra of **C4S** (A), pentamidine isethionate (B) and host-guest complex – in the presence of the precipitate (C) recorded on Agilent 400 MHz instrument at room temperature in 0.3 M acetate buffer, pH = 5.1 (D₂O). C(**C4S**) = 4 mM, C(**PT**) = 4 mM.

C4S is known to effectively modulate supramolecular aggregation of certain aromatic and amphiphilic molecules.^{52, 53} We evaluated the capacity of **C4S-PT** complex to trigger supramolecular assembly into nanoparticles in aqueous solutions and thus, to serve as a potential drug carrier for pharmacological formulations. For this, we have prepared colloidal suspension of the host-guest system in water by the solvent diffusion method.⁵⁴ The physico-chemical characterization of the colloid by Dynamic Light Scattering (DLS) revealed the presence of particles of a mean hydrodynamic diameter of 334 nm, with a polydispersity index of 0.3 (Figure S11). The morphology of the supramolecular aggregates was evaluated by Atomic Force Microscopy (AFM). Smooth spherical particles of a mean diameter of 600 nm and a tendency to coalesce are observed, (Figure 4). The difference in the size

with respect to DLS measurements is explained by the flattening of the particles during water evaporation. The average particle height is estimated to be 88 nm, confirming their flattening.

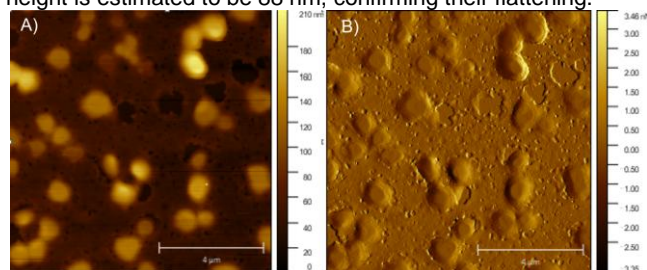


Figure 4 AFM morphological characterization of **C4S**-pentamidine assembly- (A) topographic and (B) error image; scale bar is 4 μ m.

Solution/gas-phase properties of C4S-PT host-guest complex. The transfer of the solution of **C4S** and pentamidine to the gas phase upon electrospray ionization followed by simple analysis using mass spectrometry brought insight on the type of aggregates formed between these molecules. The mass spectrum recorded in negative ion mode (Figure 5A) has revealed the formation of two types of complexes between **PT** and **C4S** of stoichiometry 1:1 and 2:1. These complexes appear in the spectrum as ions of different charge states (from 1- to 3-) and forms (deprotonated, with exchanged H \rightarrow Na). The peaks corresponding to the ions of **C4S** are also visible.

Complement insight into the nature and structure of **C4S-PT** host-guest complex in the solution/gas phase has been obtained by ion mobility mass spectrometry (IM-MS) supported by computational modelling. The ion mobility spectrometry is based on the measurements of the mobility of ions as they travel in a buffer gas under the influence of an electric field. The ions of different sizes and shapes experience different resistance upon migration through the gas and hence differ in the arrival times at the detector. Based on the experimental conditions and arrival time distribution, the value of collision cross section (CCS) can be determined, representing the rotationally averaged surface area of the ion available for the collision with buffer gas. The experimentally derived CCS values can be used to extract information on the three-dimensional structure of the studied ions, even for large supramolecular assemblies.^{55, 56, 57} The ion mobility spectrum of **C4S-PT** host-guest system shows a single Gaussian-shaped arrival time distributions (AT) of the complex ions with mean arrival times AT of 8.6 and 12.3 ms for 1:1 and 2:1 complexes, with corresponding experimental collision cross sections ^{TW}CCS_{N₂} of 291 \pm 1 and 344 \pm 1 \AA^2 , respectively (Figure 5b). In contrast to the single AT distributions of the complexes, the **C4S** anion shows well-resolved two AT distributions, with mean arrival times AT = 4.9 and 5.4 ms, which correspond to experimental values of ^{TW}CCS_{N₂} = 226 \pm 1 and 236 \pm 1 \AA^2 . Hence, the existence of two distinguished conformations of **C4S** anion are anticipated.

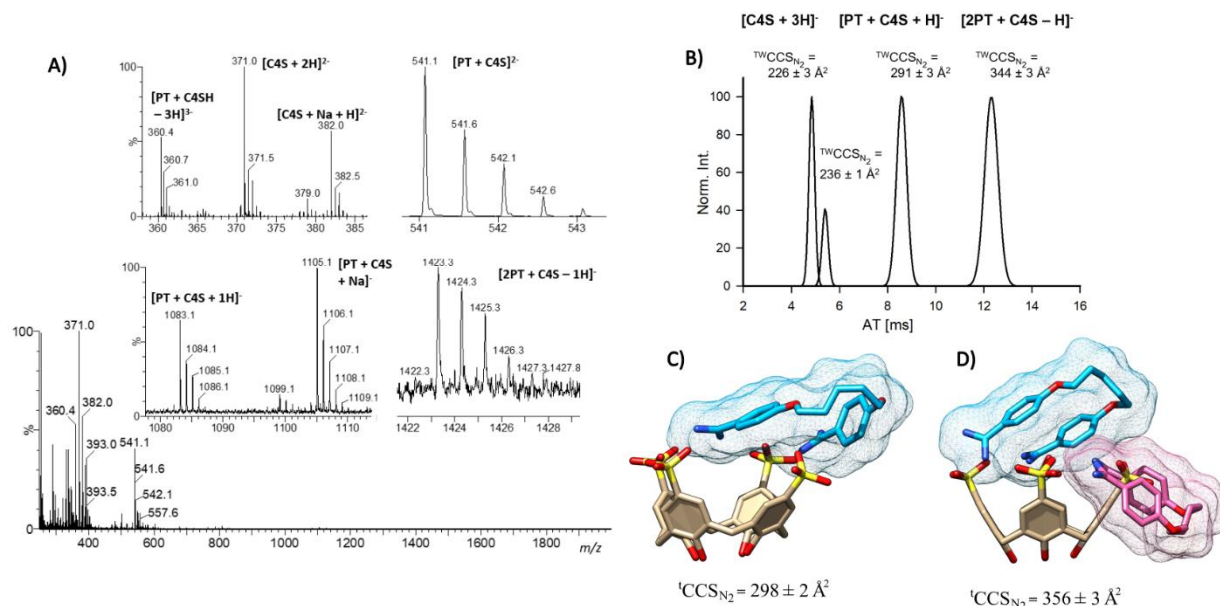


Figure 5 (A) Full range ESI mass spectrum recorded in negative ion mode for an equimolar mixture of pentamidine (**PT**) and **C4S** ($c = 0.03$ mM) in water. Selected regions of the spectrum are zoomed in to be clearly visible. Relevant peaks corresponding to ions of **C4S** and its associates with **PT** are assigned. (B) Gaussian-fitted AT distributions of selected and extracted ions corresponding to singly deprotonated **C4S**, 1:1, and 2:1 complexes of **C4S** and **PT** recorded at wave velocity (WV) = 450 m/s, wave high (WH) = 26 V along with the determined experimental $^{TW}CCS_{N_2}$ values (collision cross section values in nitrogen drift gas determined by traveling wave ion mobility). (C) The lowest energy conformations of singly charged 1:1 and (D) 2:1 complexes of pentamidine and **C4S**. The $^{t}CCS_{N_2}$ are the Boltzmann averaged values ($T = 300$ K) over the found conformers ($\Delta G \leq 10$).

The structural information is then elucidated from the comparison of experimental ($^{TW}CCS_{N_2}$) and theoretical ($^{t}CCS_{N_2}$) collision cross sections calculated for 1:1 and 1:2 **C4S-PT** model structures. The structure of the ion of interest is ultimately assigned, when both CCS values are closely matched. The initial structures extracted from **C4S-PT** crystallographic data were subjected to an intensive Monte Carlo conformational search, followed by semi-empirical (PM7) and final DFT calculations (details in the SI). Only singly charged ions were considered in our theoretical calculations. For the most stable and dominant model structures theoretical collision cross sections ($^{t}CCS_{N_2}$) were calculated by trajectory method implemented in MobCal-MPI software. The lowest energy conformers for 1:1 and 1:2 **C4S-PT** complexes generated from the theoretical search along with their computed collision cross sections are presented in Figure 5C,D. The theoretical CCS values show good correlation with the experimental values, for 1:1 complex $\Delta CCS_{N_2} = 2.4\%$, and for 1:2 complex $\Delta CCS_{N_2} = 3.5\%$.

Both complexes show **PT** molecule in the U-shaped conformation residing above the bowl cavity in order to fulfill the charge-assisted hydrogen bonding between its amidinium groups and anionic sulfonate substituents of calix[4]arene. Such structural alteration of the gas phase complex respectively to the crystal structure is dictated by strengthening the host-guest non-covalent interactions (electrostatic and hydrogen bonding) in the absence of solvent.^{58, 59} Besides, all amidinium-sulfonate hydrogen bonds in the crystal complex are realized towards neighboring calix[4]arene molecules, thus packing effect is strongly responsible for the host-guest binding mode in the solid state.

C4S-ALEX host-guest complex

The complex **C4S**-alexidine crystallizes in the triclinic *P*-1 space group, with asymmetric unit comprising one **C4S** molecule, one **ALEX** guest partially included into macrocyclic cavity, and five water molecules (disordered). Alexidine is in the U-shaped folded conformation with its central hexamethylene chain positioned inside the bowl cavity of calix[4]arene, Figure 6A,B. The anionic sulfonate rim of the macrocycle serves as an anchor for the cationic biguanide groups of the **ALEX** due to electrostatic interactions and charge-assisted hydrogen bonding. It can be deduced that **ALEX** molecule is in the form of tetracation due to four anionic sulfonate groups of calix[4]arene and 1:1 host-guest stoichiometry in the crystal complex. Thus, both biguanide groups of alexidine are diprotonated and overall **ALEX** is a tetra-cation to satisfy charge balance upon host-guest complexation.

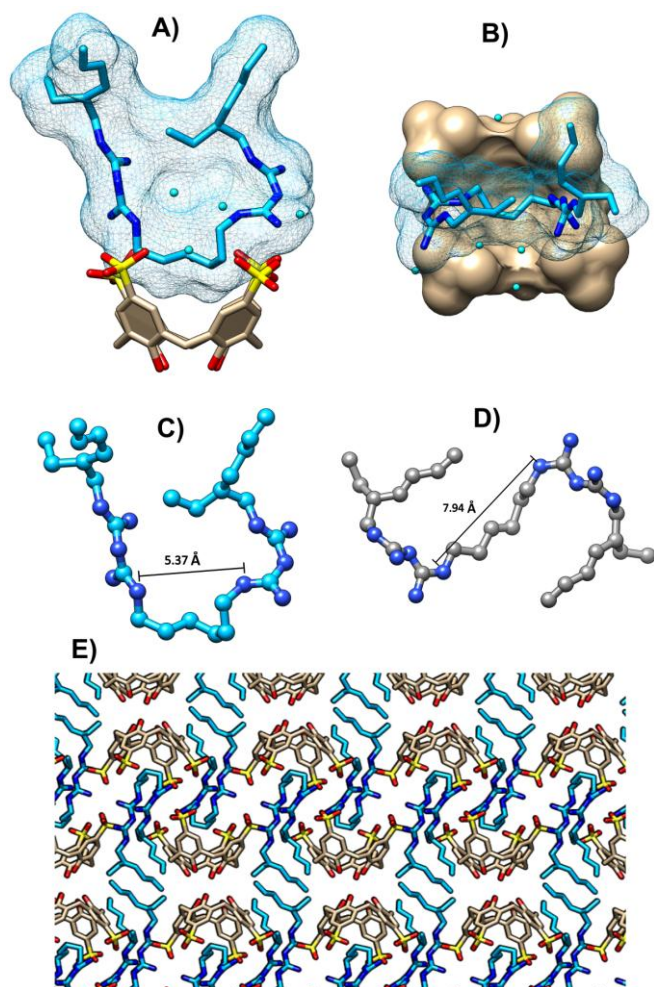


Figure 6 Host-guest complex of *p*-sulfonato-calix[4]arene with alexidine: (A) side view; (B) top view; water molecules in cyan. (C) Molecular conformation of alexidine showing N...N distances between nitrogen atoms bound to the central hexyl chain for inclusion complex with *p*-sulfonato-calix[4]arene, this work; and (D) exclusion complex with carboxylated pillar[5]arene, previous work (refcode TODLAD). (E) Crystal packing viewed along a direction; water molecules omitted for clarity.

The angle between two guanidinium subunits within two biguanides are 52.6 and 55.3°. The turning is somewhat more pronounced than in the case of **ALEX** with monoprotonated biguanides (50.1° for two symmetry-equivalent biguanides) in the carboxylated pillar[5]arene host-guest complex recently reported by our group, that was actually the first and only structural elucidation of the **ALEX** in the crystal form.⁶⁰ All C-N distances of the dicationic biguanides are in the range of 1.29 - 1.38 Å, being intermediate between C-N single (1.42 Å) and double bonds (1.28 Å). Comparing to monocationic form of biguanide, there is a small increase in the C-N bridge bond lengths and corresponding decrease in the C-N terminal bond lengths, Figure S6. Indeed, in the case of monoprotonated biguanide in the **ALEX** complex with pillar[5]arene (refcode TODLAD), the similarity of all C-N distances is more pronounced (1.32 – 1.35 Å) due to the extensive delocalization. In the case of diprotonated biguanide in the current complex, the C-N bond lengths formed by the central N atoms are longer 1.37-1.38, while C-N terminal bond lengths are shorter 1.30-1.33 Å. The

differences in the geometric parameters between monoprotonated and biguanides are in agreement with other structures described in the literature and deposited in the CSD.^{61,62}

The central hexamethylene chain of the endo **ALEX** molecule takes unfavorable *gauche* conformation with the torsional angle around the central C-C bond of -58.4°. The corresponding torsional angle in the exo **ALEX**-pillar[5]arene complex of the extended central linker is of 180.0° typical for the energetically preferred *anti* conformation. The distance between N...N atoms directly bound to the central aliphatic chain of the U-folded **ALEX** molecule is of 5.37 Å, Figure 6C. For comparison, the same N...N distance in the S-shaped conformation of **ALEX** in its complex with pillar[5]arene is 7.94 Å, Figure 6D. The compression of the central part of the **ALEX** molecule in the calix[4]arene host-guest complex can be estimated as *app.* 32 % with respect to the extended shape of the hexamethylene chain in the exclusion complex with pillar[5]arene. The folding of **ALEX** is additionally secured by charge-assisted hydrogen bonds of its guanidinium groups with sulfonate oxygen atoms of the host with N-H...O distances of 2.77 and 2.85 Å, Figure S7. Each di-cationic biguanide is a potential donor of seven hydrogen bonds. Of altogether fourteen hydrogen bonds, ten are realized towards sulfonate oxygen atoms of calix[4]arenes, and four towards water molecules. The crystal packing represents a bilayer character well-known for this macrocycle and its complexes,^{63,64} with the bilayer additionally perforated by ethyl-hexyl terminal groups of the **ALEX** guests, Figure 6E.

The low solubility and immediate precipitation of the **C4S-ALEX** complex precluded its characterization by ¹H NMR spectroscopy in aqueous solution (both in the unbuffered D₂O and 0.3 M acetate buffer). The replacement of the host-guest complexation medium to methanol (CD₃OD) improved the solubility and revealed the significant change in the **ALEX** proton resonances in the presence of the macrocyclic host. Some of the proton resonances of **ALEX** experience upfield shifts (signals **e**, **i**, **j**) indicating inclusion of the central hexyl chain into the macrocyclic cavity, Figure 7. In the presence of **C4S** several conformers of **ALEX** are evident from the ¹H NMR spectrum. 2D correlation NMR spectroscopy (Figure S10 - COSY spectrum, Supporting information) has been used in order to assign signals of different conformers of **ALEX** after the complexation.

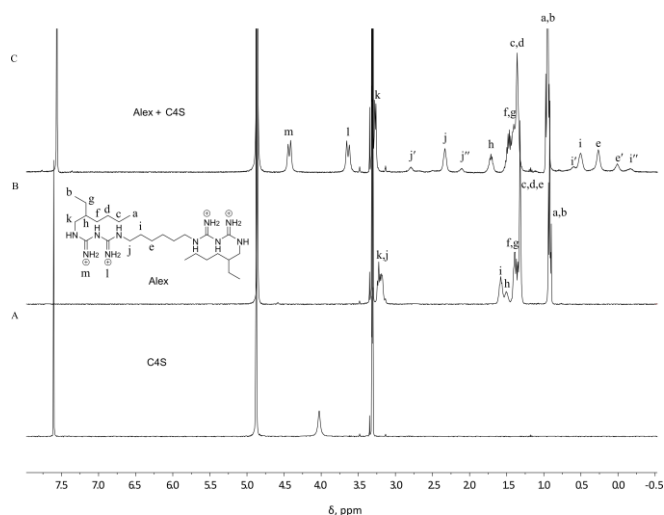


Figure 7 ^1H NMR spectra of **C4S** (A), alexidine hydrochloride (B), and their host-guest complex (C) recorded on Agilent 400 MHz instrument at room temperature in CD_3OD . $C(\text{C4S}) = 4 \text{ mM}$, $C(\text{ALEX}) = 4 \text{ mM}$.

The capacity of **C4S** to complex alexidine in aqueous environment generating supramolecular self-assemblies was verified by DLS analysis. A monodisperse population of nanoparticles with a mean hydrodynamic diameter of 222 nm and a low polydispersity index of around 0.1 is observed (Figure S11). AFM imaging of the formulation reveals spherical nanometric particles along with some sporadic aggregates, Figure 8. These aggregates could be due to coalescence of particles upon dehydration. The mean diameter of the particles was estimated to be of 65 nm. The larger size measured by DLS could be explained by the presence of larger hydration layer that is eliminated while drying, potentially contributing to a shrinking of the particles

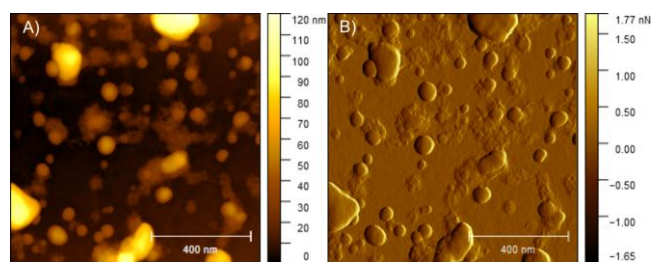


Figure 8 AFM morphological characterization of **C4S-ALEX** assembly-(A) topographic and (B) error image; scale bar is 400 nm.

Solution/gas-phase properties of C4S-ALEX host-guest complex. Adding equal amounts of alexidine and **C4S** to water ($c = 0.03 \text{ mM}$) results in a slightly cloudy solution, for which the recorded spectrum is shown in Figure 9A. The main product formed in the mixture corresponds to 1:1 complex of **C4S** and **ALEX** observed in the spectrum as singly, doubly and triply negatively charged ions. The addition of methanol to the initial aqueous solution of **ALEX** and **C4S** improved the solubility and resulted in a clear solution. IM-MS analysis showed that the addition of methanol contributed to an overall improvement in the ion intensities corresponding to the complexes (25-fold increase for singly charged, less pronounced for higher charge states ions). Additionally, both profile of the ion corresponding to 1:1 complex (Figure 9B) and its AT distribution indicated the presence of the doubly charged dimer complex (Figure 9C). Apparently, the addition of methanol promotes non-specific aggregation, which can be concluded from the observation of another type of aggregate, the 1:2 **C4S-ALEX** complex (Figure S12), not observed in water as well as in the crystal structure.

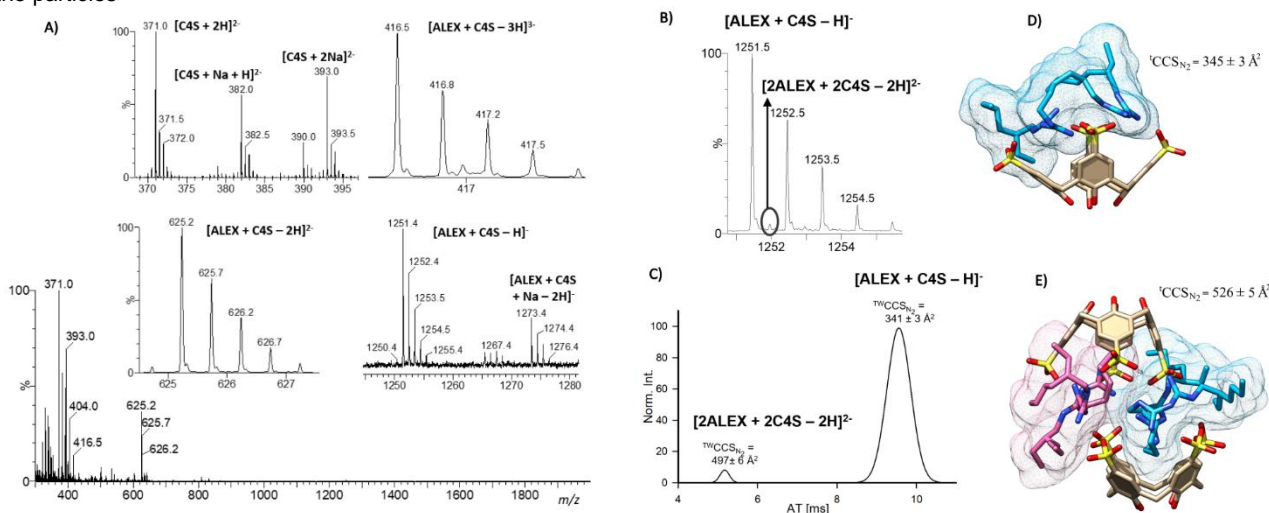


Figure 9 (A) Full range ESI mass spectrum recorded in negative ion mode for an equimolar mixture of **ALEX** and **C4S** ($c = 0.03 \text{ mM}$) in water. Selected regions of the spectrum are zoomed in to be clearly visible. Relevant peaks corresponding to ions of **C4S** and its associates with **ALEX** are assigned. (B) Isotopic profile of the overlapping ions corresponding to the 1:1 complex of **C4S** with **ALEX** and its doubly charged dimer (2:2). (C) Gaussian-fitted AT distributions of selected and extracted ion at 1251.5 Da corresponding to singly deprotonated 1:1 and doubly charged 2:2 complex of **C4S** and **ALEX** recorded at wave velocity (WV) = 450 m/s, wave high (WH) = 29 V along with the determined experimental $^{TW}\text{CCS}_{N_2}$ values (collision cross section values in nitrogen drift gas determined by traveling wave ion mobility). (D) The lowest energy conformations of singly charged 1:1 and (E) doubly charged 2:2 complexes of **ALEX** and **C4S**. The $^{TW}\text{CCS}_{N_2}$ are the Boltzmann averaged values ($T = 300 \text{ K}$) over the found conformers ($\Delta G \leq 10$).

Extensive theoretical computations (detailed in the Supporting Information) were conducted for **C4S-ALEX** complex, resulting in the structure displayed in Figure 9D. Similar to **C4S-PT** case, the obtained model differs from the crystal structure, in terms of more effective electrostatic interactions involving hydrogen bonds between positively charged guanidinium groups of **ALEX** and the negatively charged sulfonate groups of **C4S**. The lack of solvent resulted in the rearrangement of the complex to meet the dominant electrostatic forces in the gas phase. The theoretical value of collision cross section computed for the structure shown in Figure 9D (${}^t\text{CCS}_{\text{N}_2} = 345 \pm 3 \text{ \AA}^2$) agrees with the experimental value ($\Delta\text{CCS}_{\text{N}_2} = 1\%$). The structure of the dimeric complex reflects the crystal structure with U-shaped **ALEX** and inclusion of its central chain into the macrocyclic bowl, Figure 9E. The experimental CCS value of doubly charged dimer has reasonable agreement with the theoretical value (${}^t\text{CCS}_{\text{N}_2} = 526 \pm 5 \text{ \AA}^2$) obtained for model structure ($\Delta\text{CCS}_{\text{N}_2} = 5.8\%$). The lower experimental value compared to the theoretical one indicates a higher compaction and shrinkage of the dimeric structure than predicted by theoretical computations.

Conclusion

The simple bowl-shaped calix[4]arene macrocycle framed with sulfonate groups effectively mediates U-shaped folding of ditopic drug molecules alexidine and pentamidine. The combination of C_4 symmetric bowl of calix[4]arene and C_{2h} symmetric flexible guests results in the clamping down the central aliphatic linker (hexyl or pentyl) of the guest by its inclusion and coiling inside the macrocyclic cavity. The biguanide and amidine cationic groups support complexation and assembly in terms of charge-assisted hydrogen bonding with sulfonate groups of calix[4]arene. The synergy of hydrophobic effect and multiple charge-assisted hydrogen bonds sustains conformational fixing of these highly flexible guest molecules.

The host-guest studies in the solution and solution/gas phase confirm the capacity of **C4S** to complex alexidine and pentamidine. The formation of host-guest supramolecular assemblies has been identified by NMR spectroscopy, dynamic light scattering and AFM studies. Host-guest colloidal self-assemblies induce the formation of homogeneous particles of several hundreds of nanometres in diameter. The IM-MS structural studies in the gas phase of the host-guest complexes were challenging. The partially exclusion nature of the complexes facilitated guest reorganisation upon transfer from solution to the gas phase due to increased role of electrostatic interactions. Thus, the primary non-covalent structures in the precrystallization solutions were strongly affected by the ionization step, which brought about a modification in the complexation mode compared to the crystal structures. The difference between pentamidine exclusion complexes reported here and deep inclusion complexes described earlier for carboxylated pillar[5]arene and pillar[6]arene^{42,42} suggests the potential to control the guest conformation by the appropriate choice of the macrocyclic host. Further studies addressing this issue are currently underway in the laboratory.

Supporting Information

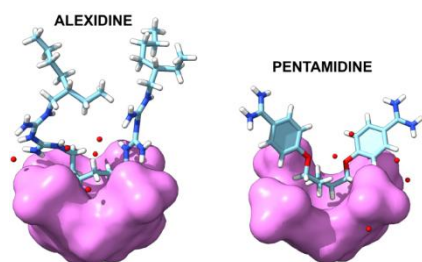
The authors have cited additional references within the Supporting Information.^[66-74]

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Keywords: host-guest • crystal structure • macrocycle • mass spectrometry•

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The simple bowl-shaped macrocyclic host promotes conformational fixing and crystallization of the guest molecules of high conformational freedom in aqueous media. The binding site in both crystal structures is the central aliphatic chain enabling guest folding through adopting *gauche* conformation upon entering the calix[4]arene cavity.

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