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Superstructure based on β-CD self-assembly induced by a small guest molecule[†]

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Abstract

The size, shape and surface chemistry of nanoparticles play an important role in cellular interaction. Thus, the main objective of the present study was the determination of the β-cyclodextrin (β-CD) self-assembly thermodynamic parameters and its structure, aiming to use these assemblies as a possible controlled drug release system. Light scattering measurements led us to obtain the β-CD's critical aggregation concentration (cac) values, and consequently the thermodynamic parameters of the β-CD spontaneous self-assembly in aqueous solution: $_{agg}G^o = -16.31$ kJ mol^{-1}, $_{agg}H^o = -26.48$ kJ mol^{-1} and T $_{agg}S^o = -10.53$ kJ mol^{-1} at 298.15 K. Size distribution of the self-assembled nanoparticles below and above cac was 1.5 nm and 60–120 nm, respectively. The number of β-CD molecules per cluster and the second virial coefficient were identified through Debye's plot and molecular dynamic simulations proposed the three-fold assembly for this system below cac. Ampicillin (AMP) was used as a drug model in order to investigate the key role of the guest molecule in the self-assembly process and the β-CD:AMP

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supramolecular system was studied in solution, aiming to determine the structure of the supramolecular aggregate. Results obtained in solution indicated that the β-CD's cac was not affected by adding AMP. Moreover, different complex stoichiometries were identified by nuclear magnetic resonance and isothermal titration calorimetry experiments.

1. Introduction

The design of nanosized architectures with predictable and uniform size and shape is an ongoing challenge in supra-molecular chemistry. The binding and assembly mechanisms of these systems have received increasing attention as the number of practical applications for nanomaterials continues to expand.[1,2] One exciting use for nanoparticles is to deliver poorly water-soluble drugs. Spontaneous self-assembly of amphiphilic molecules is a promising high-efficiency technique employing biodegradable polymers, liposomes and cyclodextrins (CDs) as building blocks to fabricate nanostructures with desired properties.[3–6]

CDs are toroidal polysaccharide molecules with a hydrophobic cavity. A variety of organic and inorganic guest molecules can be inserted within the cavities to form inclusion complexes (ICs).[7–11] Additionally, CDs have also been used as building blocks to construct nanostructured functional materials, especially bioactive ones.[12–16] Common CD molecules include the α-, β-, and γ-cyclodextrins containing six, seven, and eight glucopyranose units, respectively.[7,17] In these structures, the primary and secondary hydroxyl groups render the outer surface of the molecule hydrophilic, making the CDs water-soluble.[7] Although CDs have been studied for more than 100 years, the self-assembly of natural and modified CDs in water has been recently investigated with the improvement in analytical technologies.[5,18–23] Even with abundant data obtained from techniques such as light scattering, the thermodynamic study of the natural CD self-assembly is still limited. Considering CDs aggregated as colloids, the stability can be described as a result of a delicate balance of weak attractive and repulsive forces.[19,24,25] Furthermore, the aggregation process depends on the surrounding environment, including the solvent, and if the solvent is changed the supramolecular structure may collapse.[15] Additionally, the use of physical processes such as filtration or centrifugation prior to β-CD particle size measurement may alter the solution composition and the aggregate structure.[23]

Because of the sensitivity of CD assembly processes to external parameters, a precise understanding of the self-assembly mechanism is necessary for the design of future drug delivery and solubilization systems.[26,27] Supramolecular CD assemblies may provide several inherent advantages over natural CDs, including multiple cavities that enhance guest molecule binding through a great number of binding sites.[12]

In this article, we report thermodynamic parameters ($_{agg}G^o$, $_{agg}H^o$, and T$_{agg}S^o$) for β-cyclodextrin (β-CD, Fig. 1a) self-assembly in aqueous solution. These parameters were obtained based on critical aggregation concentration (cac) values. The correlation between the size and number of β-CD molecules per cluster in aqueous solution and the second virial coefficient (A_2) were evaluated using dynamic and static light scattering (DLS and SLS). Theoretical calculations based on classical molecular dynamics (MD) were used to predict the three-dimensional structure of the supramolecular assemblies below cac. Understanding

the β-CD self-assembly process and, obtaining thermodynamic parameters may aid the construction of supramolecular assemblies with predictable three-dimensional structures using a variety of guest molecules. Moreover, the determination of the β-CD cac at different temperatures demonstrates the spontaneous behavior and thermodynamic conditions of the self-assembly process.

The effect of a water-soluble guest molecule on the self-assembly process was investigated in aqueous solution using Ampicillin (Fig. 1b). UV-visible spectroscopy was used to investigate the β-CD self-assembly process in the presence of AMP using the guest molecule absorbance as a probe. The supramolecular complex was characterized using 1D and 2D magnetic nuclear resonance (NMR) experiments and isothermal titration calorimetry (ITC). The NMR results confirm that the inclusion of AMP into the β-CD cavity occurs mainly through interactions with the aromatic ring. The thermodynamic parameters for the inclusion process obtained using ITC indicate spontaneous complex formation (G^o <0) with a favorable enthalpy contribution and increasing entropy change. Based on the experimental findings, MD simulations were performed to gain insights into the spatial arrangement of the host–guest complex, its molecular assembly, and the stabilization forces occurring in this system, demonstrating that the presence of AMP is crucial to stabilizing the linear structure through electrostatic forces.

2. Results and discussion

2.1 Dynamic light scattering measurements

Titration of β-CD aqueous solution at 12 mM in Milli-Q® water was used to obtain the particle size distribution and also the cac at different temperatures. This titration led us to two distinct regions, when monitoring scattering intensity as a function of β-CD concentration at 293.15, 298.15, and 303.15 K (Fig. 2a–c), indicating two domains arising from the interaction between β-CD molecules in aqueous solution. Moreover, the intersection of the linear regression curves for the two regions in each titration indicates the β-CD cac point (Table 1). The cac values and size distribution measured in this work are smaller than those proposed by Bonini *et al.*, who reported structures 90 nm in size at 3 mM concentration aqueous solution.[18,20] The first portion of the titration curve (*i.e.* below cac) represents the non-aggregated form of β-CD (β-CD *"monomers"*) in solution, or at least smaller aggregates than those observed in the second portion of the curve.

These assumptions were confirmed using size distribution analysis, in which a maximum particle size of approximately 1.5 nm was observed below the cac at 298.15 K (Fig. 3a). The second portion of the titration curve indicates the presence of β-CD aggregates of 60.0 nm and 120.0 nm in size, confirmed by the size distribution curve appearing in Fig. 3b. Similar size distributions were observed in experiments carried out at 293.15 and 303.15 K (data not shown). These larger β-CD aggregates are similar to those reported previously for the self-assembly of unmodified CDs in aqueous solutions of various concentrations, in which the size distribution ranged from 100 to 300 nm.[5,18,21,23]

In order to obtain insight into the physicochemical aggregation process, the thermodynamic parameters for the β-CD self-assembly were determined using the pseudophase separation

model (Table 1). The negative values for $_{agg}G^o$ at all three temperatures demonstrate the spontaneous self-assembly behavior of β-CD in aqueous solution. The $_{agg}G^o$ values were similar to those described in the literature for a modified β-CD in which the hydroxyl groups were substituted with oligo(ethylene glycol),[28] demonstrating that DLS experiments are suitable for measuring cac values. The enthalpic change for the assembly process was $_{agg}H^o$=−26.48 kJ mol^{-1}, suggesting that aggregates form through non-covalent interactions.[29]

Finally, the entropic change observed at all three temperatures (Table 1) may arise from the β-CD conformational restriction after the molecular self-assembly. This unfavorable entropy contribution is counteracted by weak non-covalent interactions with low activation energies for formation, the assembly process being driven by enthalpy. As the number of components in a supramolecular system increases, the enthalpy or desolvation contribution must increase to counteract the inherent negative entropy due to association.[30]

2.2 Static light scattering measurements

SLS experiments were carried out to identify the number of β-CDmolecules per cluster in solution at concentration below cac. The Debye plots obtained by plotting Rayleigh's ratio as a function of β-CD concentration at 293.15, 298.15, and 303.15 K are depicted in Fig. 4a–c. The number of β-CD molecules per cluster was obtained by extrapolating the curves to zero concentration. The results obtained at 293.15 and 298.15 K indicated that the clusters had weights of approximately 3378 g mol^{-1}, which is equivalent to that of three β-CD molecules. At 303.15 K the assembly weight was higher at 5307 g mol^{-1}, corresponding to 4–5 β-CD molecules.

Evidence in support of our hypothesis for β-CD self-assembly below cac includes the values of the second virial coefficient A_2, which represents solute and solvent attractive and repulsive forces. The A_2 values were determined from the slopes of the Debye curves (KC/R_θ *versus* C) in Fig. 4a–c.[31,32] The second virial coefficients were negative at the three temperatures studied for the β-CD system: -9×10^{-5} mol mL g^{-2} at 293.15 and 298.15 K and -5×10^{-5} mol mL g^{-2} at 303.15 K. These negative values reflect the weak interactions between the β-CD molecules and water, favoring the aggregation process. Correlation of the SLS and DLS measurements suggests that self-assembly below the cac involves three β-CD molecules and produces an aggregate 1.5 nm in diameter.

2.3 Theoretical calculations of β-CD self-assembly

Based on the experimental results, theoretical calculations were carried out to propose the three-dimensional structure of the β-CD self-assembled system. Fig. 5 is a graph of the average assembly size ($2R_g$) as a function of simulation time for all arrangements studied. The radius of gyration (R_g) is taken as a reference parameter, which describes the massweighted distance of atom "i" from the center-of-mass of the molecular aggregates according to eqn (1).

$$R_g = \sqrt{\dfrac{\sum_i m_i R_i^2}{\sum_i m_i}} \quad \therefore \ R_i = |r_i - r_{cm}| \quad (1)$$

After the warming process ($t = 0$ ps in Fig. 5) the geometries were almost linear with $2R_g$ ranging between 1.60–1.86 nm, larger than the experimentally observed 1.5 nm. The stability of the aggregates was tested at the equilibrium temperature of 298.15 K, as carried out in the experimental measurements. The sizes of structures A, B and C did not appreciably change after $t = 5000$ ps, remaining at 1.6 nm for A and C and 2.0 nm for B (Fig. 5). On the other hand, arrangement D became larger than other ones (3.2 nm) at the end of simulation ($t = 5000$ ps). The result for the 3P aggregate is also included in Fig. 5. This arrangement was proposed based on the very stable structure reported in our previous paper for (α-CD)^3, which does not possess any HH local association. [33] As shown in Fig. 5, the 3P structure for (β-CD)^3 is also stable throughout the simulation time, and the final structure size of 1.53 nm agrees well with the experimentally observed value.

Analysis of the 3P structure using MD simulation provided us with insight into the role of local association (HH, HT, or TT) in the stability of (β-CD)^3 aggregates. Fig. 6a and b depict the structures after $t = 5000$ ps for A (ht–th–ht) and B (th–th–ht) starting associations (see Scheme 1 for nomenclature definitions). Both arrangements have a local HH association that remains almost unaltered throughout the simulation. However, configurations A and B both have additional local associations (TT for A and HT for B). In structure B the β-CD monomer (represented by th) assumes a perpendicular arrangement relative to the remaining HH dimer, enlarging the size of the aggregate. This was not observed in structure A, in which the ht–th–ht arrangement was maintained until the end of the simulation (Fig. 6a). It is worth mentioning that this behavior is distinct from (α-CD)^3, which exhibits a significant change in the linear arrangement for structures A and B.[33]

The linearity of empty small CD aggregates depends on the number of glucose units in the macrocyclic molecule, as described for dimeric associations in aqueous media.[34] The structures of C and 3P aggregates are depicted in Fig. 6c and d. They display similar spatial forms, equivalent to three perpendicular local associations. The $2R_g$ values for these molecules were 1.57 and 1.53 nm, respectively. The aggregation energies have not been calculated in the present work, once it is difficult to evaluate how accurately the method will describe energies in solution. In our previous work, the molecular mechanics/Poisson–Boltzmann surface area (MM–PBSA) approach was applied to estimate the relative stability of the (α-CD)^3 aggregate.[33] The results for that system revealed that the 3P structure is at least 10 kcal mol^{-1} more stable than other possible forms in solution.

Two main conclusions can be drawn from the MD results, in addition to the experimental findings: (i) four distinct forms of the (β-CD)^3 aggregate were proposed, named B, A, C, and 3P with sizes of 1.93, 1.58, 1.57, and 1.53 nm, and (ii) the 3P-type non-linear arrangement probably represents the average structure of β-CD assembly in aqueous solutions at lower concentrations, as the size is in agreement with that observed in DLS measurements.

2.4 Characterization of a β-CD-ampicillin supramolecular system

In order to characterize the effect of a guest molecule at sub-and supra cac, the absorbance values of AMP ($_{Abs}$) at λ = 215 nm were plotted against β-CD concentration (see ESI 1†). An inflection point was observed at 1.43 mM of β-CD, which is close to that obtained for titrations of β-CD using DLS. These results indicated that the β-CD aggregation point could be monitored using a physical property of the guest molecule. This inflection point suggests that aggregates of β-CD might be formed in solution at concentrations above 1.43 mM. A similar behavior was observed for the *trans*-2-[4(dimethylamino) styryl]benzothiazole with β-CD, in which fluorescence measurements revealed a blue shift of the maximum fluorescence band of approximately 11 nm at 10 mM β-CD concentration.[35]

The supramolecular interaction between β-CD and AMP molecules was confirmed in a 2D ROESY NMR experiment using a 1 : 1 molar ratio host: guest system. The 2D ROESY spectrum in Fig. 7 indicates dipolar correlations between the β-CD cavity and all of the hydrogen atoms on the AMP molecule. These multiple cross-peak correlations suggest that supramolecular complexes of β-CD:AMP might form in solution at 1 : 1, 2 : 1, or higher molecular ratios, as reported previously for other systems in the literature in which an equilibrium between different complexes was confirmed.[8,26,36–38] Qualitatively comparing the NOE intensities, the aromatic hydrogens in AMP exhibit stronger cross-peak correlation with the β-CD internal hydrogens than all other ones. This observation indicates preferential inclusion of the aromatic moiety of the AMP molecule into the β-CD cavity, this result is in accordance with previous work reported in the literature.[39] When comparing the ^{1}H spectra of pure AMP and the supramolecular complex a chemical shift of hydrogens Ha and Hb can be observed (see ESI 2†). This result suggests a variation in the electron density of these hydrogens upon their inclusion,[40] confirming that theAMP molecule is interacting with the β-CD.

The thermodynamic parameters of the interaction between β-CD and AMP were obtained using ITC, titrating AMP solution in β-CD aqueous solution and diluting AMP solution in water (see ESI 3†). Subtracting the AMP dilution curve from its titration curve in β-CD aqueous solution yielded the stoichiometry (n), the enthalpic change (H^o), and the binding constant ($K = 1.260 \pm 99$ M^{-1}). A stoichiometry $n = 0.83 \pm 0.05$ was observed for this system, suggesting that more than one type of supramolecular complex may exist in equilibrium in solution, and corroborating the 2D NMR results. Similar stoichiometry coefficients have been observed for other supramolecular systems involving CDs and have been ascribed to the existence of equilibria between different complexes in solution.[8,26] The inclusion of AMP is spontaneous ($G^o = -19.4$ kJ mol^{-1}) and enthalpically favored ($H^o = -4.8$ kJ mol^{-1}), with an increasing entropic contribution ($T S^o = 14.6$ kJ mol^{-1}). These results obtained from NMR and ITC experiments suggest equilibrium between different supramolecular systems in solution.

2.5 Theoretical calculations of β-CD:AMP complexes

The potential energy surface (PES) for ICs may have a very complex landscape with many local minima. For a CD host, which has a well-defined cavity, the interaction energy profile might be tracked by following the average force to keep the guest molecule at the constraint

distance along the main CD axis. Thus, the integration of the force–distance curve would yield the potential of mean force (PMF) from which attractive and repulsive arrangements could be located. This procedure gives the complete description of the host–guest interaction pathway and could be used to get insight into the main features responsible for the complex stability. However, calculation of PMF is a computationally demanding task and was not performed in this work, once our main goal is to find out the overall structures, those representing the equilibrium arrangements, and the role of AMP in the alignment of the CD clusters.

Assuming only systems with a 1 : 1 stoichiometry of β-CD:AMP, MD simulations revealed only two stable arrangements, in which the AMP was included in the β-CD cavity through the aromatic ring (Fig. 8a and b, structures A1 and A2). The energy difference between A1 and A2 obtained from average potential energies was 22.1 kJ mol^{-1} in favor of the A2 isomer, suggesting that this form of inclusion complex should represent the majority of the 1 : 1 stoichiometry species observed in solution. The distances between H3 and H5 (inside the β-CD cavity) to various hydrogen atoms of AMP were monitored for these complexes. The H3/H5· · ·H distances were within 4 Å for hydrogen atoms of the aromatic ring and H_{am} (see ESI 4†). The distance was approximately 7 Å for the other hydrogen atoms of the AMP molecule except for methyl hydrogen atoms, for which the distance was nearly 11 Å. These results support the 2D ROESY experiment that suggested dipolar correlation between the H3/H5 signals and the hydrogen atoms of AMP mainly with those of the aromatic ring.

Several attempts were made in order to identify an inclusion mode for the polar moiety of AMP. However, the proposed complexes were not stable along the simulation time of 2.5 ns, reverting to structure A2 or to complexes in which the AMP molecule interacted with hydroxyl groups outside the β-CD cavity (structures not shown). This result is also in accordance with previous MD simulations,[41] in which the AMP guest gradually slipped out of the CD cavity after 20 ps when the interactions occurred through its polar moiety.

In addition to this previous work, MD simulations for ICs with 2 : 1 and 2 : 2 stoichiometries for all possible local associations of a β-CD dimer (HH, TT, and HT) were proposed. In these complexes the available cavity is enlarged and may be more suitable for accommodating an AMP molecule. Among all possibilities, only the HH arrangement survived longer than 2.5 ns. The initial and final structures from the MD simulation for 2 : 1 and 2 : 2 complexes are depicted in Fig. 9a and b, respectively. The common behavior in which the polar moiety of AMP was always found outside the cavity at the end of simulation was also observed for the 2 : 1 and 2 : 2 ICs keeping the β-CD HH local dimer structure. The AMP molecule in the 2 : 1 β-CD:AMP complex moves along the main cavity axis, leading to an IC similar to structure A2 with the phenyl ring inside the β-CD cavity and close to the narrow rim. The average H3/H5···H distances were the same as discussed for the A2 structure, with short distances (o 5 Å) observed for aromatic hydrogens and approximately 6 Å for H3/H5···Ha/Hb. The results for the 2 : 2 complex are quite interesting (Fig. 9b). When two guest molecules are included in the HH cavity formed by the β-CD dimer, only one remains inside the cavity after 2.5 ns. The other AMP molecule exits the β-CD cavity and assumes a parallel arrangement relative to the CD narrow rim that is stabilized by two weak

hydrogen bonds. This complex A4 (Fig. 9b) is quite stable and may also contribute to the equilibrium mixture, representing the basic unit for large aggregates.

2.6 Theoretical calculations for the linear assembly

In an attempt to understand the forces contributing to the formation of the β-CD:AMP superstructures, MD simulations were conducted for larger aggregates containing four β-CD units and four AMP guest molecules, abbreviated as (AMP@β-CD)[4]. The spatial arrangement of the 2 : 2 complex represented in Fig. 9 was used as a molecular building block. The production phase in the MD simulation was extended to 5.0 ns at 298.15 K to ensure the stability of the nanostructure. The (β-CD)[4] structure without the guest molecule was also simulated under the same computational protocol for comparison with (AMP@β-CD)[4]. The results are illustrated in Fig. 10a, in which the normalized average potential energy is plotted against the simulation time in the production phase at a constant temperature of 298.15 K. The distinct behaviors represented in Fig. 10a indicate the existence of different structural events for each molecular association. Both structures are nearly linear at the beginning of the simulation. However, a dissociation process for the (β-CD)[4] linear aggregate begins near 10% of the trajectory (indicated by the arrow in the Fig. 10a), yielding two HH dimers and leading to a decrease in potential energy. When the AMP IC is used as a building block to construct the superstructures, the system remains stable through 100% of the trajectory considered (total simulation time of 5.0 ns), which is indicated by an almost constant average potential energy. The initial and final structure of the (AMP@β-CD)[4] system are depicted in Fig. 10b. As expected from our previous simulations of smaller aggregates, only one AMP remains inside the β-CD cavity of each dimer unit at the end of the simulation, yielding a local arrangement similar to structure A4 (Fig. 9b). Interestingly, the entire structure is kept nearly aligned due to the interaction between AMP molecules in the TT moiety. Although no hydrogen bonding was predicted between AMP molecules, the attractive forces are all electrostatic in nature.

It has been reported that rod-like nanoparticles persist in the circulation considerably longer than spherical particles, highlighting the effect of shape in biological systems at the nanoscale.[42] Thus, the β-CD:AMP superstructures may affect the physical and chemical properties of the guest molecule (AMP) in a manner distinct from the spherical nanoparticles observed below cac. Overall, the supramolecular self-assembled arrangements formed between a β-CD and a guest molecule could affect the host–guest properties in at least two different ways. A guest molecule with a low binding constant could have insufficient energy to disrupt the CDs supramolecular aggregate, and therefore would not form an IC. On the other hand, once a highly ordered supramolecular structure is formed between several CDs and guest molecules, additional energy would be required to break these clusters, resulting in an additional reason for their modified release profile, since, not only would the CD–drug conjugated need to be disrupted, but also a large number of intra- and intermolecular hydrogen bonds and other attractive forces. Thus, supramolecular systems may act as drug delivery nanoparticles, depending on the environment and also CD concentration.

3. Materials and methods

3.1 Dynamic and static light scattering measurements

DLS and SLS measurements were performed in a Zetasizer ZS Nano Series (Malvern Instruments Ltd, United Kingdom) using a square quartz cell. The samples were subjected to scattering using a monochromatic light (10 mW He–Ne laser, wavenumber 632.4 nm) with a scattering light angle of 173°. The β-CD (Xiamen Mchem, Xiamen, China) solutions used for titrations in the DLS and SLS measurements were prepared using Milli-Q® water filtered through Durapore filters (0.1 μm). Titrations were carried out at 293.15, 298.15, and 303.15 K.

DLS and SLS titrations were used to investigate the process of β-CD self-aggregation in solution. Specifically, the DLS measurements were carried out to identify the β-CD critical aggregation concentration (cac), at the respective size of these aggregates. Titrations of β-CD solution (12.0 mM) were carried out and the intensity of the light scattering signal measured in counts s^{-1} (kcps)[43] was plotted as a function of β-CD concentration. The concentration of β-CD in these experiments ranged from approximately 0.2 to 3.0 mM. A pseudophase separation model was used to investigate the thermodynamic aggregation process once cac values were obtained at several temperatures. In this approach, the aggregates were considered a separate phase at the cac, and the standard free-energy change ($\Delta_{agg}G^{o}$) is given by eqn (2):[44]

$$\Delta_{agg}G^{o} = RT \cdot \ln(X_{cac}) \quad (2)$$

in which R is the universal gas constant, T is the absolute temperature, and X_{cac} is the critical aggregation concentration in molar fraction units.[44] Applying the Gibbs–Helmholtz equation (eqn (3)) the enthalpy standard energy ($\Delta_{agg}H^{o}$) can be expressed as:[44]

$$\Delta_{agg}H^{\circ} = R \left[\frac{\partial \ln(X_{cac})}{\partial \frac{1}{T}} \right]_{P} \quad (3)$$

Lastly, the standard entropy of aggregation per mol of surfactant ($\Delta_{agg}S^{o}$) was obtained using eqn (4):

$$\Delta_{agg}S^{o} = (\Delta_{agg}H^{o} - \Delta_{agg}G^{o})/T \quad (4)$$

The aggregate size was obtained by measuring the particle size 10 times for each concentration during 30 s, in which correlation function was used to get size information. The average of each point of the titration process and its standard deviation were plotted as a function of percentage. In addition, SLS experiments were conducted to calculate the number of β-CD molecules per cluster (M) and the second virial coefficient (A_2) below cac, according to eqn (5) and (6):[24,32]

$$\frac{KC}{R_\theta} = \frac{1}{M} + 2A_2 c \quad (5)$$

$$K = \frac{2\pi^2 n_o^2 \left(\frac{dn}{dc}\right)^2}{\lambda_0^4 N_A} \quad (6)$$

where C is the solute concentration, R_θ is the Rayleigh ratio, K denotes an optical constant in which n_o is the refractive index of the solvent and dn/dc is the refractive index increment of the solution per unit mass concentration increase of β-CD (0.139 mL g^{-1}, determined previously[45]), λ_o is the wavelength of the incident light, N_A is Avogadro's number. The β-CD concentration ranged from 0.5 to 1.5 mM in these titrations.

3.2 Molecular dynamics simulations

In our recent paper,[33] we described MD simulations of α-CD assemblies containing three molecules, referred to as (α-CD)^3, spatially arranged in head–head (HH), head–tail (HT), and tail–tail (TT) local associations. The conclusions were interesting, predicting a new stable aggregate not possessing any HH local association. In this new structure named 3P (three perpendicular), all of the α-CD units are arranged in a perpendicular profile.

In the first part of the present work, addressing the assembly of β-CD in aqueous media, the simulation protocol was similar to that previously applied.[33] The MD simulations were performed in aqueous solution for trimeric β-CD associations, (β-CD)^3, in linear and 3P spatial arrangements. The linear input geometries were identified by capital letters (A, B, C, and D) according to the relative orientation of each β-CD unit as depicted in Scheme 1. The GAFF force field (Generalized AMBER force field) as implemented in the AMBER 10[46] package was used. The atomic charges employed in the condensed phase simulations were obtained using the BCC[47] approach with the aid of the *antechamber* software, an AMBER 10 accessory module. In order to include explicit solvent molecules, the TIP3P water model was chosen. The model consists of a periodic solvent octahedral box, in which an average of 1800 water molecules are included to perform the simulations.

The simulations were initiated at 5 K, warmed to 50 K for 10 ps, then equilibrated for 100 ps. Subsequently, five similar consecutive MD steps were executed to achieve a final temperature of 298.15 K. An NVT ensemble was used in the warming process and a NPT ensemble was used during the production period with a simulation length of 5.0 ns. Throughout the MD simulation, a nonbonding cutoff of 10.0 Å was chosen and the particle mesh Ewald (PME) method[48] applied to treat long-range electrostatic interaction.

In the second portion, ICs containing β-CD and AMP were simulated in aqueous solution using the Generalized Born (GB/SA) model[49] with a continuum dielectric approach for the solvent (ε=78.39). The simulations were carried out using the AMBER*[50,51] force field as implemented in the Macro- Model® suite.[52] Cutoff radii for van der Waals and Coulombic electrostatic interactions were 8 and 20 Å, respectively. The C–H and O–H bond lengths were fixed using the SHAKE algorithm.[53] The warming protocol was the same as used for the (β-CD)^3 aggregate with a final temperature of 298.15 K. The total length of the

simulations was 2.5 ns (5.0 ns for the largest aggregate 4 : 4 β-CD:AMP) with a time step of 1.5 fs. Distinct structures were proposed as starting guess for simulations accounting for β-CD:AMP stoichiometries: 1 : 1, 2 : 1, 2 : 2, 3 : 2, and 4 : 4 (ESI 4†).

3.3 Spectroscopic measurements of a β-CD-ampicillin system

AMP (Sigma-Aldrich) was used as a model compound to investigate how the self-aggregation of β-CD is affected by the supramolecular interaction between host and guest species. A solution of AMP (6.7×10^{-2} mM) was prepared using water previously filtered through a 0.2 μm membrane. Titrations of β-CD at 12.0 mM, using successive 10 μL additions, were carried out in solutions containing AMP for which concentrations range from 6.7×10^{-2} to 4.8×10^{-2} mM and β-CD concentrations range from 0.0 to 3.3 mM. Absorbance measurements were obtained using a Varian Cary 50 UV-visible spectrophotometer at 215 nm at room temperature. The absorbance values of AMP (Abs = $Abs_{AMP} - Abs_{AMP:\beta CD}$) at 215 nm were plotted against β-CD concentration.

3.4 Nuclear magnetic resonance

The IC was prepared by the freeze-drying method at a 1 : 1 molar ratio at a concentration of 2 mM. NMR spectra were obtained in D^2O (Cambridge Isotope Laboratories, Inc –99.9% isotopic purity) to investigate the inclusion of AMP into the β-CD cavity. NMR spectra were recorded at 30 °C on a Bruker DRX 600–AVANCE spectrometer (Bruker BioSpin) operating at 600 MHz and equipped with a 5 mm TXI cryo-probe. Rotating frame Overhauser Effect Spectroscopy (ROESY) experiments were performed using the standard protocols contained in the spectrometer library with $p15 = 300$ μs.

3.5 Isothermal titration calorimetry

Calorimetric titrations were performed in duplicate using a VP-ITC Microcalorimeter (Microcal Company) at 30.0 °C. Each titration experiment consisted of 51 successive injections of an aqueous solution of AMP (20.0 mM) into the reaction cell charged with 1.5 mL of β-CD aqueous solution (1.0 mM). An interval of 260 s was used for the signal to return to the baseline. The first injection of 1 μL was discarded to eliminate the effects of material diffusion from the syringe to the calorimetric cell. Subsequent injections containing a constant volume of 5 μL of AMP were performed over an injection time of 10 s.

The AMP dilution process was carried out in water to evaluate the heat dilution of the AMP molecule. The heat dilution curve was subtracted from the titration curve (AMP in βCD) to eliminate interactions between AMP and water. Binding enthalpies, stoichiometries, and binding constants were obtained using nonlinear fitting (Wiseman isotherm),[54] incorporated in the software, which assumes a single set of identical binding sites. The ITC data were analyzed using the software supplied with the calorimeter (Microcal Origin 7.0 for ITC).

4. Conclusions

In this study the thermodynamic parameters of β-CD self-assembly were investigated in solution and using MD simulations. An AMP guest molecule was added to the assembled system to identify the effect of the guest molecule on the β-CD aggregation process. DLS

measurements were used to determine the cac of β-CD and the size distribution of the assemblies below and above the aggregation point. Nanostructures of approximately 1.5 nm in size were verified below cac and assemblies between 60 to 120 nm were observed above this point. The thermodynamic parameters of the β-CD self-assembly were calculated based on the cac values and indicated that the spontaneous behavior was driven by enthalpy. SLS measurements were used to identify the number of β-CD molecules per cluster (three β-CD molecules) below cac and revealed a negative value for the second virial coefficient A_2, indicating a repulsive force between the water solvent and the β-CD aggregates. Based on the DLS and SLS results, assemblies of three β-CD molecules were present even below the identified cac, indicating that the cac values represent a second aggregation point for β-CD. The supramolecular arrangement of the three β-CDs was simulated using MD calculations in solution and a perpendicular arrangement was proposed for this system.

The titration process of β-CD in AMP solution was evaluated using UV-visible spectroscopy. An inflection point was observed near the cac previously identified using DLS. NMR and ITC techniques were used to obtain insight into the supramolecular interactions and the thermodynamic parameters of the AMP inclusion process, indicating that high order complexes with a variety of stoichiometries may form in equilibrium in aqueous solution. MD simulations were carried out for several β-CD:AMP stoichiometries, demonstrating that a complex with the hydrophobic moiety of AMP included through the narrow rim of β-CD is the preferred arrangement for the 1 : 1 complex. Proposed structures with the polar moiety of AMP inside the cavity were not stable, even when two CDs were considered in a 2 : 1 complex. Interestingly, for the 2 : 2 complex only one AMP guest molecule remained inside the cavity at the end of the trajectory, with the AMP molecule outside the β-CD cavity assuming a parallel arrangement close to the primary hydroxyl groups. This might be considered as the basic unit for β-CD:AMP species in solution at low concentrations.

MD simulations were used to obtain a molecular explanation for this linear nanostructure. The 4 : 4 β-CD:AMP nanostructure was stable and retained the linear arrangement for the entire simulation trajectory at 298.15 K. Two AMP guest molecules were included, while the other two were located outside the cavity. The linear (β-CD)[4] structure survived only for 10% of the MD trajectory at 298.15 K. The theoretical results suggest that the guest molecules act as glue between the TT local arrangements, stabilizing the linear β-CD tube.

Acknowledgments

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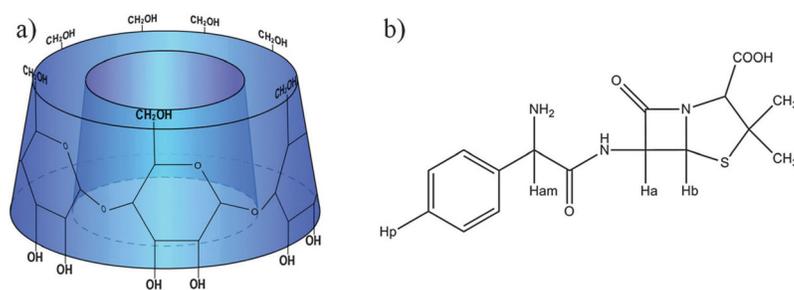54. Turnbull WB, Daranas AH. J Am Chem Soc. 2003; 125:14859–14866. [PubMed: 14640663]

Fig. 1.
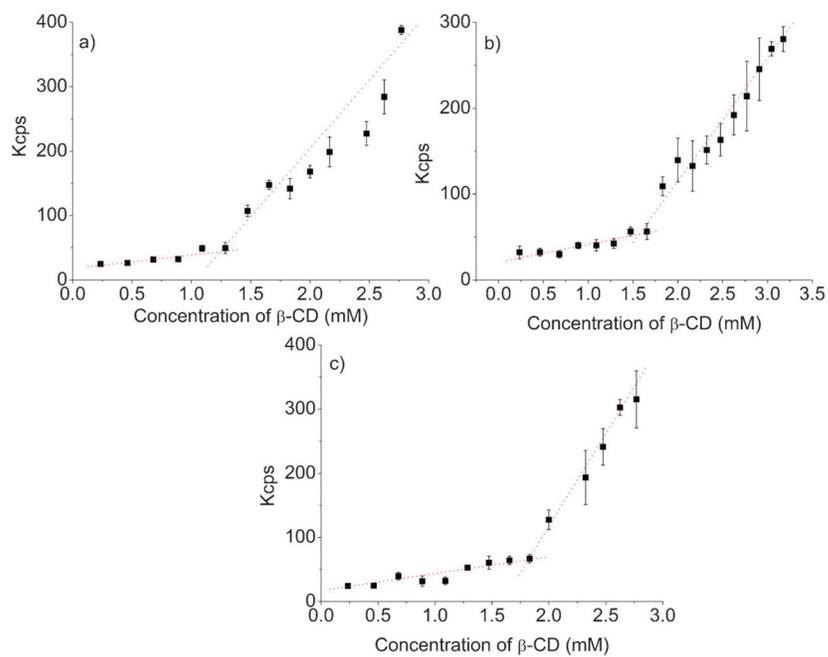(a) Schematic structure of the β-cyclodextrin and (b) molecular structure of Ampicillin.

Fig. 2.
Critical aggregation concentration curves for β-CD in water at: (a) 293.15 K, (b) 298.15 K and (c) 303.15 K.
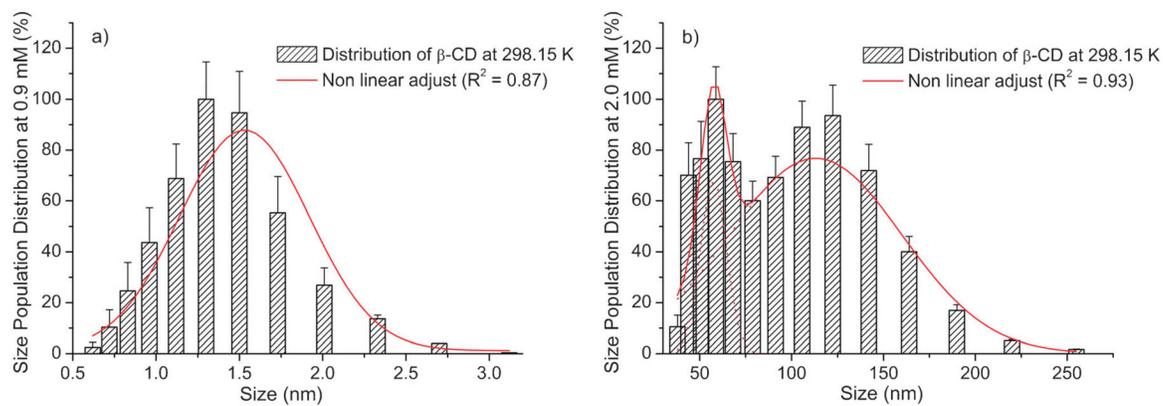
Fig. 3.
Size distribution of a β-CD aggregate at 298.15 K: (a) before the cac at 0.9 mM and (b) after the cac at 2.0 mM.
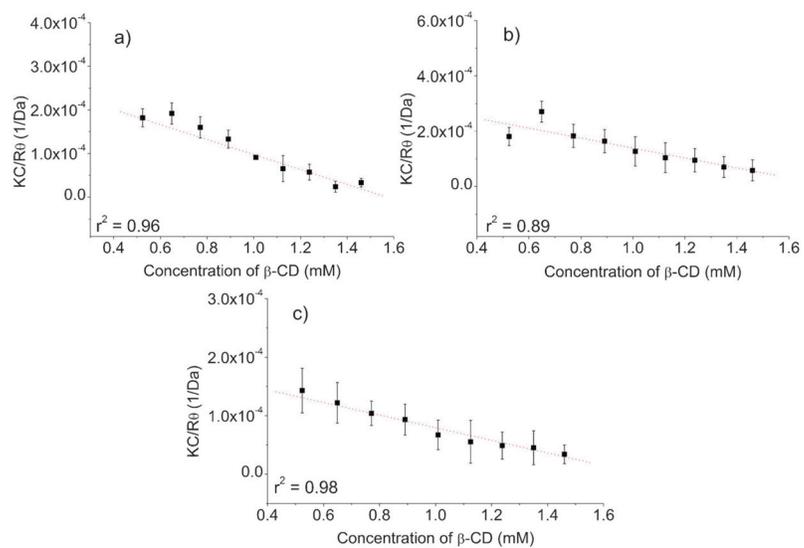
Fig. 4.
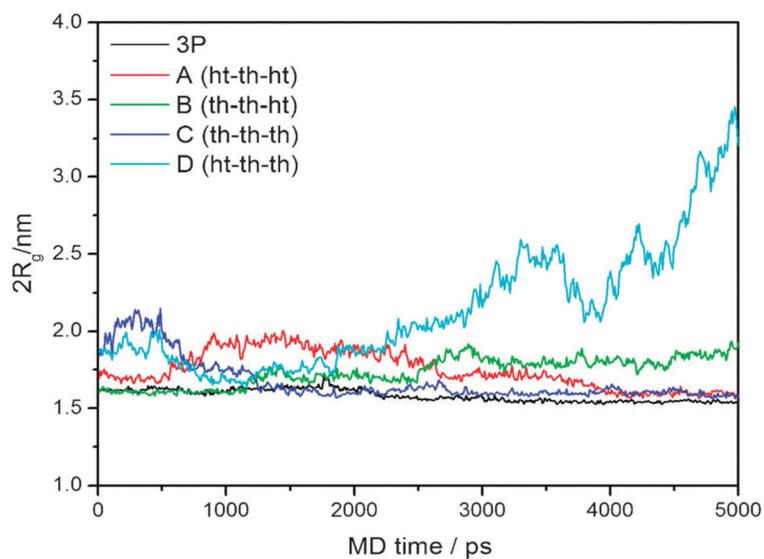Debye plot curves for a β-CD in water before cac at: (a) 293.15 K, (b) 298.15 K and (c) 303.15 K.

Fig. 5.
Variation of the size of the aggregate with the simulation time in aqueous solution at 300 K. The quantity R_g is the gyration radius calculated as given by eqn (5).
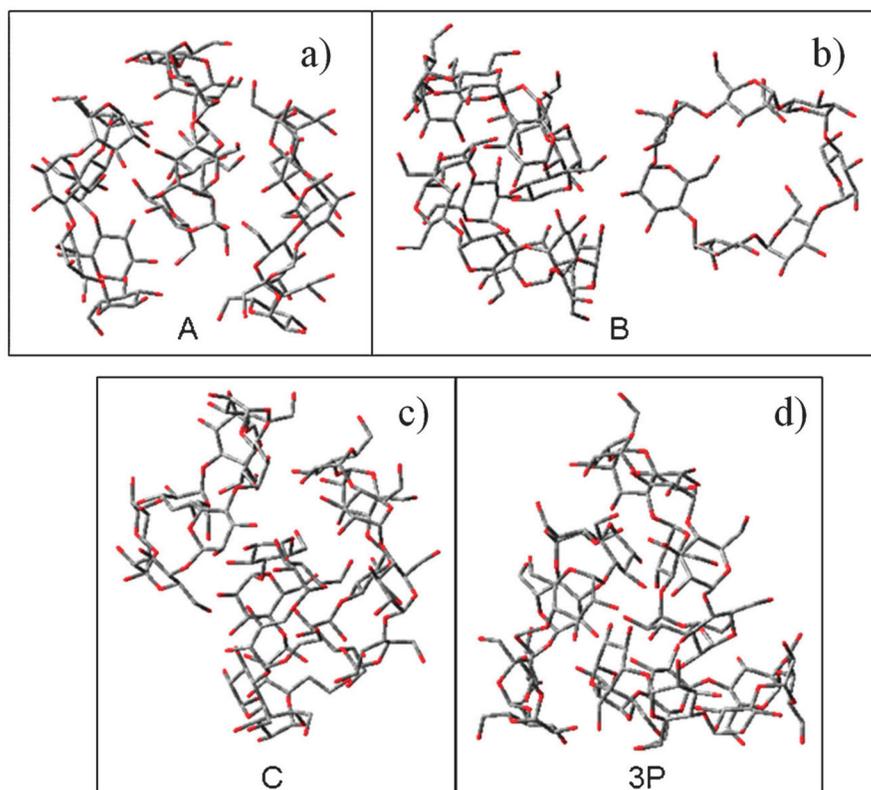
Fig. 6.
Structures obtained from MD simulation for a (β-CD)$_3$ aggregate. The distinct arrangements were found at the end of simulation (t = 5000 ps) using different starting points.
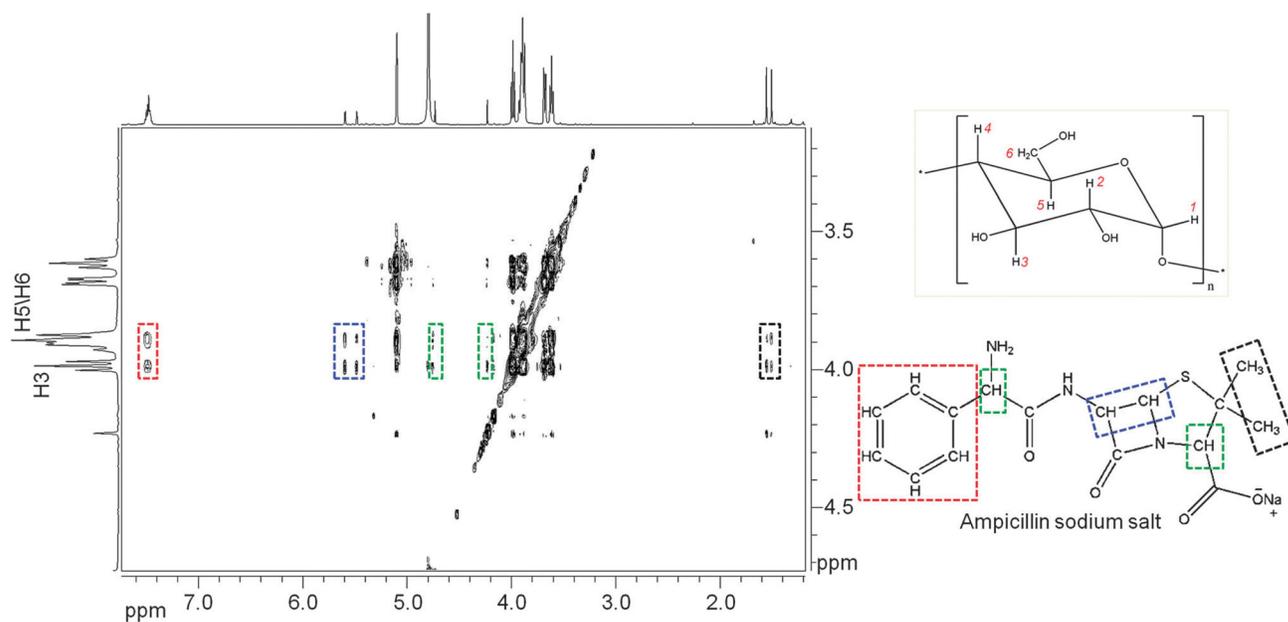
Fig. 7.
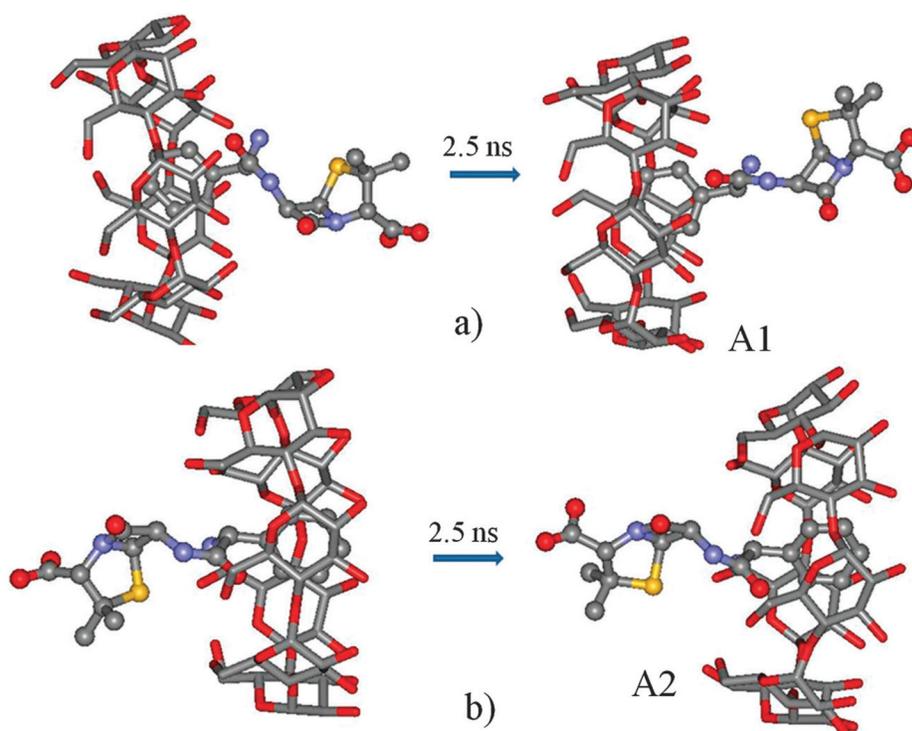Expansion of β-CD:AMP at 1 : 1 molar ratio in NMR 2D ROESY spectrum at 600 MHz in D₂O at 30 °C.

Fig. 8.
Initial (left) and final (right) structures from molecular dynamics trajectories. The starting arrangements for β-CD:AMP complexes (1 : 1) have the AMP included by the wider rim (a) and narrow rim (b).
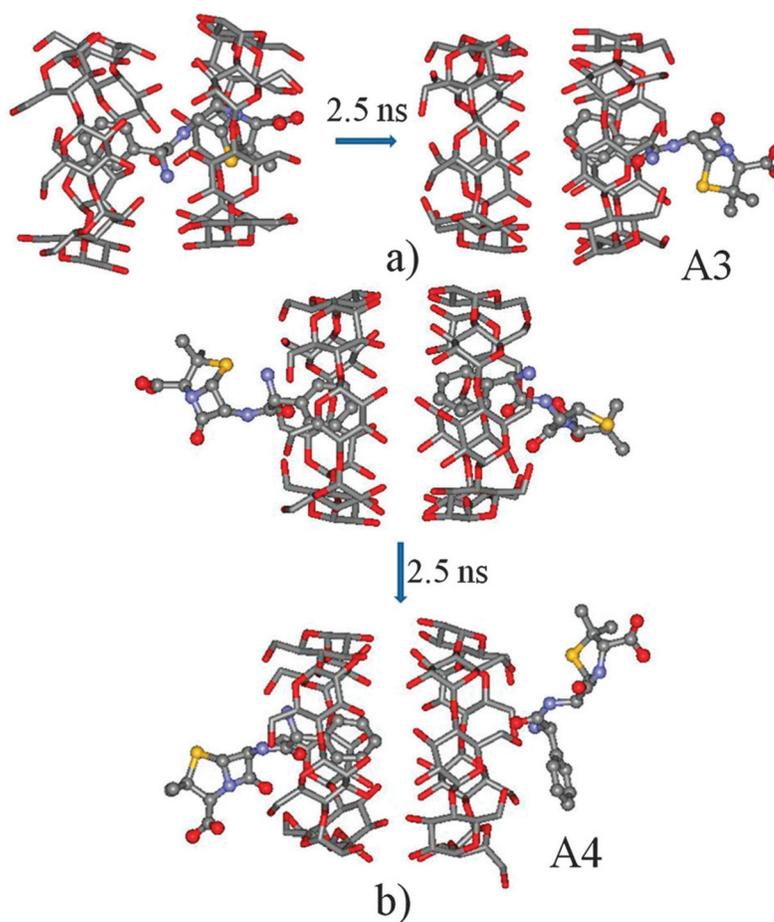
Fig. 9.
Initial and final structures from molecular dynamics trajectories. The starting arrangements for β-CD:AMP complexes have the AMP included by the narrow rim. (a) 2 : 1 complex and (b) 2 : 2 complex.
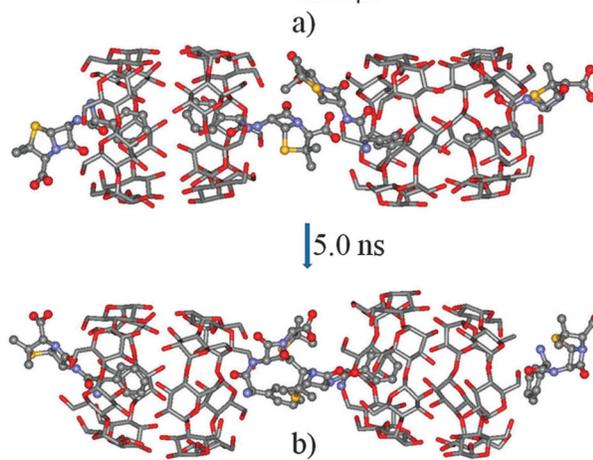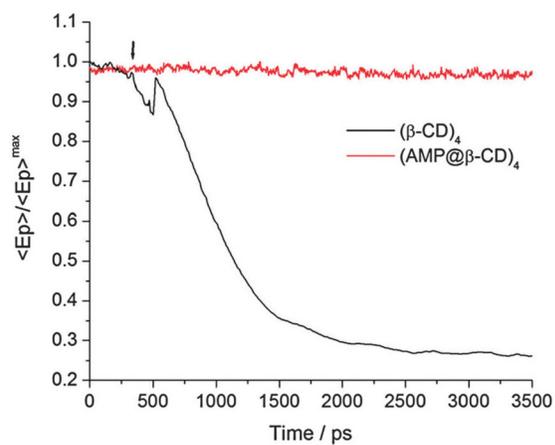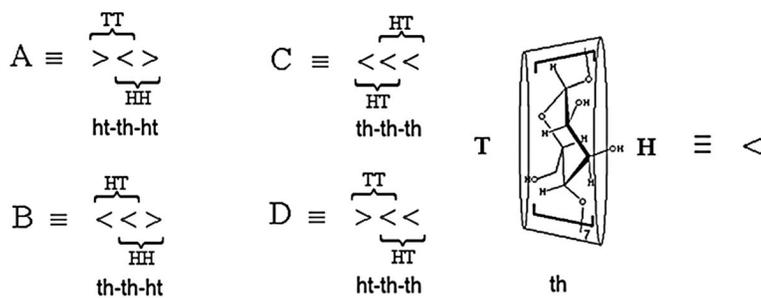
Fig. 10.
(a) Normalized average potential energy as a function of MD simulation time. (b) First and last structures from MD trajectory for the complex (AMP@β-CD)$_4$.

$$A \equiv \overbrace{>}^{TT} < \underbrace{>}_{HH}$$
ht-th-ht

$$C \equiv \overbrace{<}^{HT} < \underbrace{<}_{HT}$$
th-th-th

$$B \equiv \overbrace{<}^{HT} < \underbrace{>}_{HH}$$
th-th-ht

$$D \equiv \overbrace{>}^{TT} < \underbrace{<}_{HT}$$
ht-th-th

T   H $\equiv$ <

th

Scheme 1.
Nomenclature scheme used for a (β-CD)$_3$ aggregate.

Table 1

Thermodynamic parameters for the β-CD self-assembly in water observed by DLS and SLS

Temperature/K	cac/mM	$_{agg}G^o$/kJ mol^{-1}	$_{agg}H^o$/kJ mol^{-1}	$T\,_{agg}S^o$/kJ mol^{-1}
293.15	1.24	−16.31	−26.48	−10.17
298.15	1.60	−15.95	−26.48	−10.53
303.15	1.82	−15.90	−26.48	−10.58