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Unusual self-assembly of a hydrophilic β-cyclodextrin inclusion complex into vesicles capable of drug encapsulation and release†

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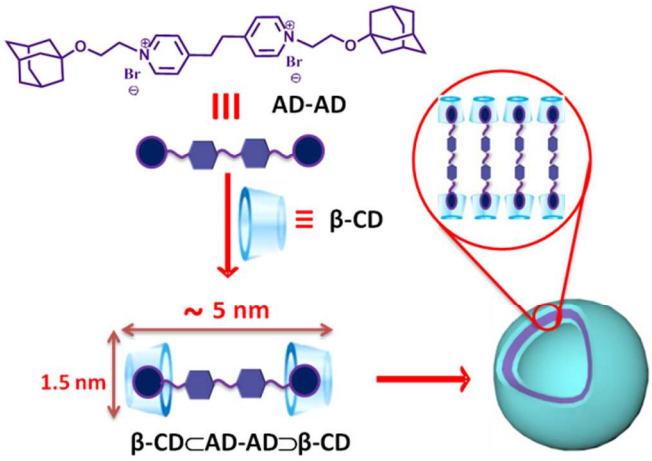
Formation of a hydrophilic β-cyclodextrin bis-inclusion complex and it's self-assembly into supramolecular vesicle are described. The vesicles can be loaded with the anti-cancer drug doxorubicin and the loaded drug can be released upon addition of a competitive inclusion binder such as adamantane carboxylate.

Self-assembly of carefully designed cyclodextrin (CD) inclusion complexes into higher order structures is an important aspect of current CD research. For example, self-assemblies of CD inclusion complexes into supramolecular polymers are well documented in the literature.[1] Recently several groups have reported self-assembly of CD inclusion complexes into supramolecular vesicles.[2] CDs are nontoxic and biocompatible and hence CD based vesicles would have potential uses as drug/gene delivery agents.[3] Vesicles intended for use as drug or gene delivery agents must undergo dispersion or dissociation into molecular components in response to some external stimuli. It has been shown that grafting of stimuli responsive sites on the included guest or CD host can lead to CD based vesicles capable of dispersion in response to chemical,[2b, 4] electrochemical,[5] light,[6] thermal[7] or pH stimuli.[5c, 8] In most of such studies chemically modified CDs were used for the construction of vesicles. Due to the large number of chemically equivalent hydroxyl groups, controlled modification of CDs is challenging and hence construction of vesicles using modified CDs is a difficult task. There are only very few reports dealing with self-assembly of host-guest complexes of native CDs into vesicles.[2a,15] In this paper we report the unusual and novel self-assembly of a hydrophilic, native β-CD bis-inclusion complex into vesicles. We also show that these vesicles can be loaded with the anti-cancer drug doxorubicin (DOX) and the loaded drug can be released upon addition of a competitive binding agent such as adamantane carboxylate (ADC).

Adamantane derivatives exhibit a very high tendency to form inclusion complexes with β-CD[9] and hence the bis-adamantane derivative AD-AD studied here is expected to bind to two β-CD molecules to give the bis-inclusion complex β-CD⊂AD-AD⊃β-CD as shown in Scheme 1. We observed that the bis-inclusion complex undergo spontaneous stacking, ultimately leading to formation of vesicles as shown in Scheme 1. Data from isothermal titration calorimetry (ITC), NMR titration, atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and dynamic light scattering (DLS) experiments supported the self-assembly processes shown in Scheme 1.



Scheme 1: Graphical illustration of chemical structure of AD-AD and its host-guest complexation induced self-assembly into vesicles.

Synthesis and characterization of AD-AD are presented in the electronic supplementary information (ESI†). Fig. S5 (ESI†) shows the ITC titration curve for the β-CD/AD-AD interaction. The experiment was carried out by taking β-CD (2 mM, 200 μL) in the cell and AD-AD (10 mM, 40 μL) in the syringe and gave values of $K_a = 6.4 \times 10^4$ M^{-1}, $\Delta H = -8.19 \times 10^4$ J mol^{-1} and $\Delta S = -178$ J mol^{-1} deg^{-1} and $n = 0.45$. When the titration was carried out with AD-AD in the cell and β-CD in the syringe, value of n obtained was close to 2, suggesting that AD-AD interacts with two β-CD molecules. In an earlier study we reported $K_a = 6.09 \times 10^4$ M^{-1}, $\Delta H = -3.47 \times 10^4$ J mol^{-1} and $\Delta S = -22.9$ J mol^{-1} deg^{-1} and $n = 1.0$ for a similar system with only one adamantane unit.[10] The fact that $-\Delta H$ more than doubled and $-\Delta S$ increased several fold for AD-AD suggested that both adamantane units in AD-AD are included in the β-CD cavity as shown in β-CD⊂AD-AD⊃β-CD (Scheme 1).

^{1}H NMR spectra of AD-AD in the presence of varying amounts of β-CD, where the β-CD concentration changed from 0 – 2 equivalent, are shown in Fig. 1, with the lower panel showing the proton assignments of AD-AD. In Fig. 1 the dotted vertical lines indicate the original peak positions and the change in peak positions is indicated by arrow in the top panel. Signals due to the adamantyl protons (H_a, H_b and H_c) appear in the δ 1.4 – 2.0 ppm

range and all these peaks shifted down field in the presence of β-CD indicating encapsulation of the adamantyl moieties into the CD cavity (2D-ROSEY spectrum of AD-AD/β-CD (1:2) system (Fig. S6 ESI†) showed cross-peaks of adamantane H_a, H_b and H_c protons with the interior H-3 and H-5 protons of β-CD, which further confirmed inclusion of the adamantane moiety of AD-AD inside the β-CD cavity). A surprising observation in Fig. 1 was the down field shifting of almost all protons of AD-AD which are not involved in the encapsulation process. We attributed the chemical shift changes observed for the H_e – H_h protons in the β-CD/AD-AD system to secondary interactions involving β-CD⊂AD-AD⊃β-CD. In order to probe such secondary interactions we have subjected the β-CD/AD-AD system to TEM, AFM, SEM and DLS studies.

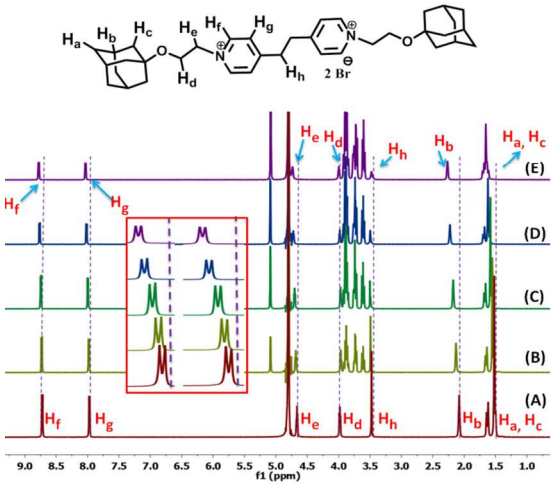


Fig. 1. ^{1}H NMR titration spectra of AD-AD (10 mM) in the absence (A) and presence (B-E) of β-CD (0.5 – 2 eq.) in D_2O. Inset shows the shifts of H_f and H_g protons of AD-AD.

In the β-CD/AD-AD system we observed formation of small particles even in very dilute solutions. Fig. 2a shows the TEM images of the particles obtained upon drop casting an aqueous solution containing AD-AD (5×10^{-5} M) and β-CD (1×10^{-4} M). Spherical structures ranging from 70 – 500 nm diameters could be seen in the TEM. A distinguishable feature of each of these particles is a narrow dark particle skin and a central light region, which is typical for vesicle type structures.[11] The dark particle skin, which most probably corresponds to the vesicle membrane, had a thickness of 10 nm for a 250 nm sized particle. The TEM images also showed that the interiors of some of these particles contain dark objects which most probably are small aggregates of the inclusion complex which are captured inside the vesicles during their formation. Formation of vesicles in the β-CD/AD-AD system was confirmed by AFM studies as well (Fig. 2b). The AFM height image and height profile showed particle heights less than 10 nm and width in the range 80-250 nm, suggesting that these vesicles are sufficiently flattened on the mica surface.[12] Fig. 2c shows the SEM images of the vesicles. We were also successful in obtaining SEM images of destroyed or opened particles (Fig S8b ESI†) which confirmed that the particles are vesicles possessing hollow interior cavities. Fig. 2d shows the DLS profile obtained for the β-CD/AD-AD system in water. The study showed a narrow size distribution of 150 - 400 nm for the particles with an average hydrodynamic diameter corresponding to 256.7 nm. Presence of nano aggregates in the β-CD/AD-AD solution is further confirmed by the observation of Tyndall effect (Fig. 2e) upon passing a laser beam through the above solution. Tyndall scattering will occur only if the solution contained particles that can scatter the laser light.[4b]

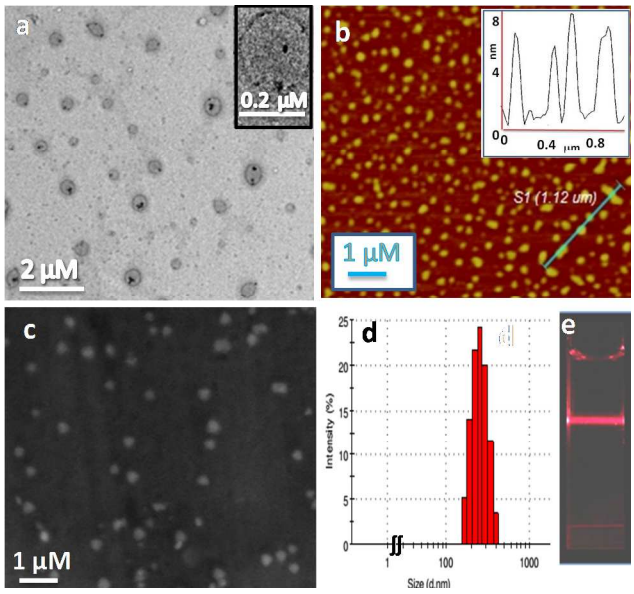


Fig. 2. (a) TEM image, (b) AFM image, (c) SEM image (d) DLS profile and (e) Tyndall effect of β-CD/AD-AD system in water. [β -CD] = (1×10^{-4} M), [AD-AD] = 5×10^{-5} M for a-e. (See ESI† for additional TEM, AFM and SEM Images).

Formation of vesicles from native or functionalized CD inclusion complexes have been reported by several groups. For example, Hao and co-workers have recently reported inclusion complex formation between β-CD and curcumin[2m] as well as β-CD and *N,N'*-bis(ferrocenylmethylene)diaminohexane[2c] and self-assembly of the inclusion complexes into vesicles. Tao *et al.*[2n] and Jing *et al.*[2l] also reported vesicles from supramolecular complexes of cyclodextrins. In all these cases one can identify the inclusion complex as a 'supramolecular amphiphile' or 'supramolecular bola-amphiphile', with the CD acting as the hydrophilic head group and the alkyl or aryl residue acting as the hydrophobic tail or hydrophobic core. The AD-AD molecule is water soluble and its hydrophilicity can be attributed to the presence of two positive charges in the molecule. When the adamantane units of AD-AD are included in the β-CD cavity the bis-inclusion complex β-CD⊂AD-AD⊃β-CD is formed, which has hydrophilic β-CD at both ends and hydrophilic, positively charged pyridinium groups at the core. Thus β-CD⊂AD-AD⊃β-CD is not a conventional bola amphiphile and charge repulsion may act against close packing. Hence we did not expect β-CD⊂AD-AD⊃β-CD to self-assemble to give vesicles as shown in Scheme 1. The fact that stacking occurs and vesicles are formed suggests that the repulsive forces do not play a dominant role here and that the core unit may be sufficiently hydrophobic to drive the self-assembly process. The outer diameter of β-CD is 1.53 nm and geometrical requirements suggest that the core units in successive layers (in Scheme 1) are also separated at least by the same distance. Thus the distance between adjacent pyridinium

moieties is sufficiently large and hence the repulsive forces could be very weak.[13] A few water molecules can also be present near the charged sites leading to screening of the repulsive interactions. Alternatively, the positive pyridinium ions and the counter bromide ions may form tight ion pairs, which also can eliminate repulsion between successive layers. The molecular length of the bis-inclusion complex is ~5 nm and hence a vesicle membrane thickness of ~10 nm (from TEM) suggested that the vesicles are formed by bilayer assemblies of the inclusion complex. The two layers may be held together by hydrogen bonding interactions involving the hydroxyl groups at the narrow rims of the β-CD. There are seven hydroxyl groups at the narrow rim of the β-CD and these can be engaged in interlayer hydrogen bonding and stabilize the bilayer structure.

Formation of vesicles from AD-AD/β-CD mixture has their origin in the inclusion binding of adamantane moiety of AD-AD in the β-CD cavities. It is well known that guests included in the CD cavity can be displaced using guest molecules with higher binding constants. ADC is most commonly used for this purpose as it exhibits a very high β-CD binding constant of 2.9×10^5 M^{-1}.[2e, 14] We observed that addition of 2.25 eq. of ADC to the β-CD⊂AD-AD⊃β-CD vesicle led to disappearance of the vesicle structures in few hours as evidenced from the absence of these structures in AFM images. ^{1}H NMR spectrum obtained after addition of ADC suggested that the adamantane groups of AD-AD are pushed out of the CD cavity by ADC (Fig. S9, ESI†).

Vesicles can be used as carriers for controlled drug delivery and in such cases stimuli responsive, slow disruption of the vesicle is an essential requirement for drug release.[3a, 3b, 3d, 4b, 15] The fact that β-CD/AD-AD vesicles can be disrupted by ADC suggested that these vesicles may have potential applications as drug carriers. In order to see if the β-CD/AD-AD vesicles can actually carry drug molecules, we have attempted to load the anti-cancer drug DOX into these vesicles. In a typical experiment, DOX hydrochloride solution (1×10^{-3} M) was mixed with the vesicle solution (1×10^{-3} M of β-CD and 5×10^{-4} M of AD-AD) and kept aside overnight followed by dialysis to remove the uncomplexed DOX.

Confocal laser scanning microscopy (CLSM) images of the DOX loaded vesicles are shown in Fig. 3a,b. The bright red luminescence due to DOX inside the vesicles can be clearly seen in Fig. 3, which confirms that these vesicles are loaded with DOX. The DOX loading efficiency of the vesicles (see ESI† for procedure) was found to be 7.06%. This value compares very well with the reported 5.02% loading efficiency of pillar[6]arene based supramolecular vesicles.[16] Both the empty and DOX-loaded vesicles were found to be stable for at least 3 weeks which was confirmed by SEM and DLS studies (Fig. S8c ESI†).

We observed that the DOX loaded into these vesicles can be released by dis-assembling the vesicles using ADC. For the release study, a solution of ADC (2.25 eq.) was added to the DOX-loaded vesicle and the solution was kept aside for 2 h. CLSM images of the solution after vesicle dis-assembly are shown in Fig. 3c,d. In Fig. 3c,d vesicles are not observed and the fluorescence of the dye is seen in most of the viewing area indicating that the vesicles are dispersed into its constituents. This finding suggests that the supramolecular vesicle formed in β-CD/AD-AD system can have potential as a drug delivery agent.

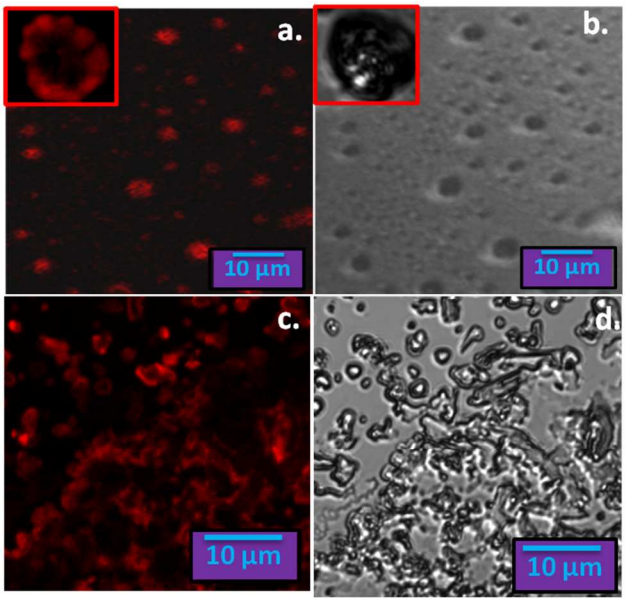


Fig 3. CLSM (a) and transmission (b) images of DOX loaded β-CD/AD-AD vesicles. Insets show enlarged image of a DOX loaded vesicle. CLSM (c) and transmission (d) images of the same after DOX release. (See ESI† for additional Images)

In conclusion we have prepared supramolecular vesicles by the spontaneous self-assembly of a bis-inclusion complex. Formation of the vesicular structures was proved by TEM, SEM, AFM, and DLS studies. These novel vesicles may have potential applications as drug delivery systems.

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† Electronic Supplementary Information (ESI) available: Synthesis and detailed characterization of molecules, ITC graph, additional AFM, and TEM images, 2D-ROSEY NMR of AD-AD – β-CD interaction, ^{1}H NMR showing vesicle disassembly, and additional CLSM images.See DOI: 10.1039/b000000x/

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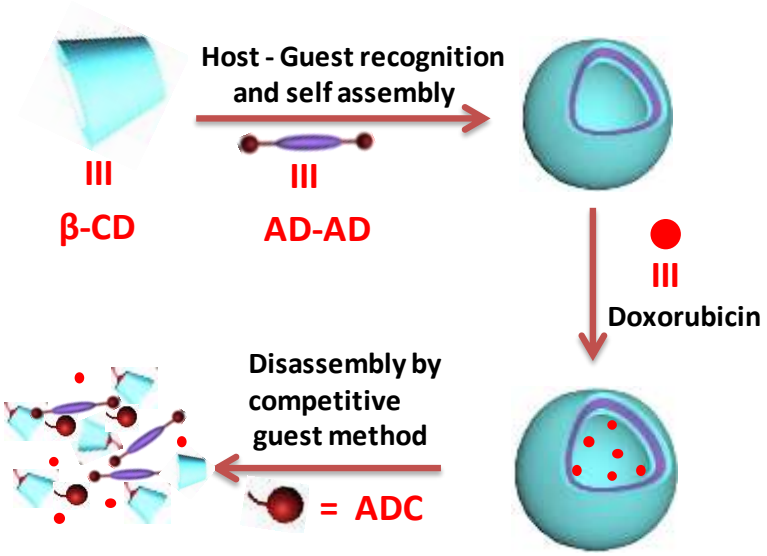

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