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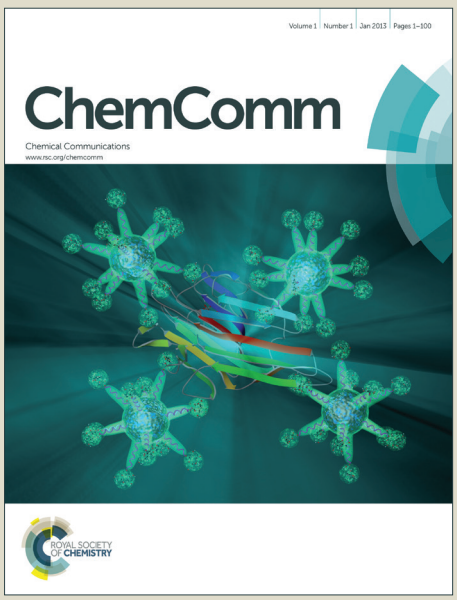

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ARTICLE TYPE

Self-assembly of lipidated pseudopeptidic triazolophanes to vesicles

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A series of designer lipidated pseudopeptidic triazolophanes was synthesized using a copper-catalyzed azide-alkyne cycloaddition reaction. These 32-membered cyclophanes form sturdy vesicles and pot-like supramolecular structures as demonstrated by ultramicroscopic studies.

Design and synthesis of molecules programmed with the ability to self-assemble into definite supramolecular structures is a challenging task. Control and predictability of self-assembling molecules to nano and micro dimensions are not yet fully realized at the molecular level.[1] The bottleneck for the specific supramolecular design is the lack of adequate knowledge about the non-covalent interactions at a finite molecular level. In order to understand the complex nature of non-covalent forces, as well as predict and harness these forces for the design of specific supramolecular structures; chemists often study molecules with the inherent ability to self-assemble.[2] Studies of several self-assembling systems may unearth the fundamentals of molecular self-assembly and that can be utilized for the design of new functional supramolecular materials.[3]

Despite these hurdles, however, a wide spectrum of synthetic molecules has been designed and synthesized over the last few decades that form a variety of self-assembled structures such as nanotubes,[4] nanorods,[5] coils[6] and vesicles.[7] Among them, vesicles have attracted particular interest primarily due to their resemblance to protocells[8] and their potential application as vehicles for delivery of genes and pharmaceuticals.[9] Supramolecular structures often show interesting yet unexpected properties that can be valuable for chemical, biological, material and other technological applications.[10] In this context, peptidic systems deserves special attention due to their wide utility as promising biomaterials and pharmaceuticals.[11]

Herein we report the design and synthesis of pseudopeptidic triazolophanes and their unprecedented self-assembly to vesicles in organic solvents. Amino acid based macrocyclic design incorporating desirable structural moieties is a powerful approach for generating self-assembling macrocycles. The inherent chirality of amino acids and their easily functionalizable amine, carboxylic acid, and side chain provide a multitude of possibilities for design. In this work, we chose amino acid serine (Ser) as a building block for the construction macrocycles since the hydroxyl group on its side chain is easily modifiable and hence can be incorporated in to a macrocyclic framework. We envisioned that incorporation of an aromatic unit, amide bond and lipid units in a cyclic framework would ensure self-assembly, therefore, we designed and synthesized triazolophanes **S1**-**S2**. Triazolophanes **S1** and **S2** consist of four serine residues that are placed between phenyl and triazole units (Fig. 1). **S1** has a symmetric architecture with four hexyl chains on the periphery, while **S2** contains two hexyl chains and two N-methoxy-N-methyl (Weinreb amide) units. Acyclic compounds **S3** and **S4** are synthesized to serve as control compounds.

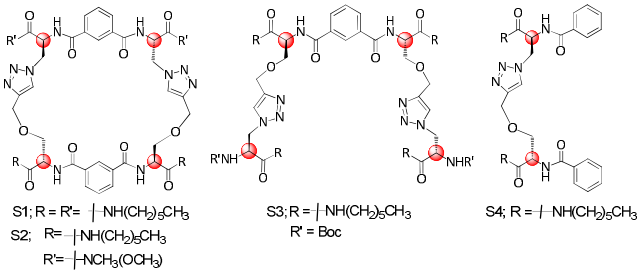

Fig. 1 Structures of pseudopeptidic triazolophanes **S1**-**S2** and the acyclic analogs **S3**-**S4**

Triazolophanes were synthesized by the Copper-catalyzed azide–alkyne cycloaddition reaction (CuAAC) between dialkyne **6** and diazides **4a**/**4b** (Scheme 1). Boc-Serine was coupled with *N*-methoxy *N*-methylamine or hexylamine under standard coupling conditions to afford **1a**/**1b**. Tosylation of **1a**/**1b** using tosylchloride yielded **2a**/**2b**, which was further reacted with NaN_3 to yield azide derivatives **3a**/**3b**. The alkyne unit was introduced by reacting **1b** with propargyl bromide in the presence of NaOH to yield **5a**. Boc deprotection of **3a-b**/**5a** followed by reaction with benzenedicarbonyl dichloride yielded diazide precursors **4a-b** and dialkyne **6**. Benzoyl derivatives **3c**/**5b** were synthesized from their respective Boc-protected compounds **3b**/**5a** by removing the Boc group followed by reaction with benzoyl chloride. The diazide derivatives **4a**/**4b** were reacted with the dialkyne derivative **6** in the presence of $CuSO_4$/Sodium ascorbate under pseudo high dilution conditions yielded 32-membered cyclophanes **S2** and **S1** respectively. Similarly **S3** was synthesized by the reaction of **6** and **3b**. Compounds **5b** and **3c** were reacted in the presence of CuI to give **S4**.

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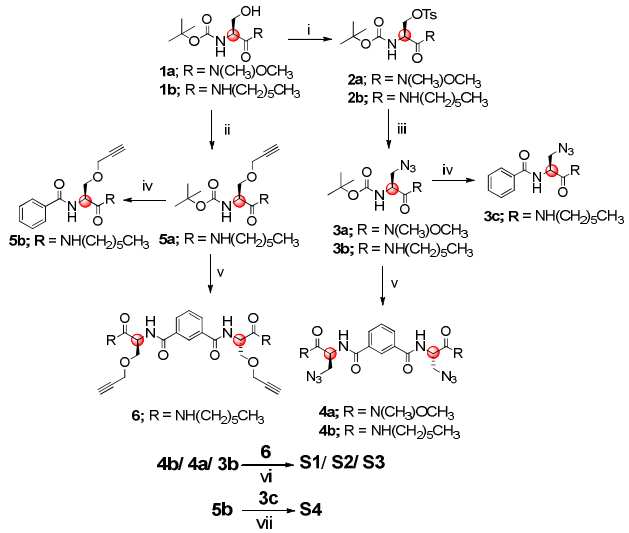
†Electronic supplementary information (ESI) available: Synthesis, procedure and data of all compounds. SEM and TEM images and CD data. See DOI



(i) p-TsCl, NEt_3, DMAP, 0°C-RT, 8 h (CH_2Cl_2) (ii) NaOH(Conc.), Propargyl bromide, $(nBu)_4NBr$, CH_2Cl_2, 0°C-RT, 12 h (iii) NaN_3, 50°C (DMF) (iv) TFA/CH_2Cl_2, 4 h; Benzoyl chloride, NEt_3, 0°C-RT, 24 h (dry CH_2Cl_2) (v) TFA/CH_2Cl_2, 4 h; Benzene 1,3 dicarbonyl dichloride, NEt_3, 0°C-RT, 24 h (dry CH_2Cl_2) (vi) $CuSO_4.5H_2O$, sodium ascorbate, DIEA, 0°C-RT, 40 h (EtOH:$PhCH_3$:H_2O (6:3:1)) (vii) CuI, DIEA, RT, 72 h (CH_3CN)

Scheme 1 Synthesis of triazolophanes **S1**-**S2** and acyclic analogs **S3**-**S4**

The semi-rigid cyclic architecture of **S1** and **S2** is expected to favor supramolecular assembly by π-π stacking, hydrogen bonding and CH….O type interactions as evident from our previous studies.[12] This is clearly evident from the NMR spectra of **S1**-**S2** in $CDCl_3$, as broad signals were observed indicative of aggregation (Fig. S1a, ESI†).[13] Well defined peaks were observed in highly polar solvents like DMSO-d_6 since DMSO can break the intermolecular hydrogen bonding due to its strong hydrogen bonding ability. This notion was further supported by FT-IR studies in chloroform (Fig. S1b-c, ESI†). Circular Dichroism (CD) spectra of compounds **S1**-**S3** showed a minimum at 202-206 nm, indicative of the absence of secondary structure (Fig. S2, ESI†).

Self-assembling properties of triazolophanes were studied by scanning electron microscopy (SEM), high resolution transmission electron microscopy (HR-TEM) and atomic force microscopy (AFM). A 0.5 mM solution of compound **S1** in $CHCl_3$:CH_3OH was drop-casted on a glass surface displayed spherical vesicles ranging from 150-600 nm in diameter (Fig. 2a). A histogram of 80 vesicles collected from several samples of **S1** show an average diameter of 419 nm (Fig. S3, ESI†). HR-TEM imaging after staining the sample resulted in uniformly darkened vesicles (Fig S4, ESI†). Therefore, HR-TEM imaging without staining was attempted, which revealed a clear contrast between the interior and periphery of the vesicles, indicating that vesicles are hollow (Inset Fig. 2a and Fig. S4a, ESI†).[7a] The vesicles are of ~200-500nm in size with a peripheral thickness of ~3nm (Fig. S4, ESI†), which is close to the dimension of **S1**. Concentration dependent SEM experiments on **S1** showed no considerable change in the size of the spherical vesicles upon increasing concentration (0.05-5 mM), though a few spherical vesicles with large diameters (2-5 μm) were observed at higher concentration (Fig. S5, ESI†). AFM images of **S1** also showed vesicles (Fig. 3a). AFM cross sectional analysis of vesicles uncovered that the diameter of the vesicles is seven times larger than the height of the vesicles (Fig 3a). The observed deformation of spherical vesicle is due to flattening as a result of adsorption on to the surface. This suggests that the vesicles are robust and soft. Interestingly, bis-lipidated **S2** showed spherical vesicles and pot-like structures in SEM and HR-TEM. The imaging by HR-TEM without staining showed a peripheral wall thickness of ~3 nm (Fig. S6, ESI†) matching well with the molecular dimensions. Careful analysis using Field Emission-SEM (FE-SEM) revealed that these pot-like structures have an average diameter of 500 nm and have a single orifice with well defined edges. The diameter of the orifice ranges from ~60-150 nm (Fig. 2b and Fig. S7, ESI†). Though **S2** showed pot-like structures in SEM and TEM, the AFM imaging and cross-sectional analysis showed only vesicles (Fig. 3b). Absence of pot-like structures in AFM implies that the hole on the vesicle is caused by flux of evaporating solvents in the high vacuum condition of SEM and TEM. These holed vesicles appear as pots in the SEM images, as also observed by other researchers.[14] The absence of pot-like structures in **S1** is plausibly due to more stable organization of the vesicles. The presence of four lipid chains in **S1** might enforce stronger interactions compared to **S2** with only two lipid chains leading to stable and stronger vesicles.

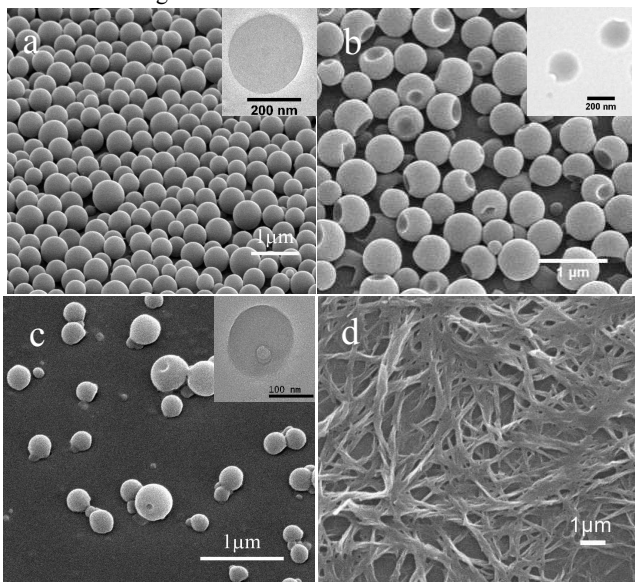


Fig. 2 FE-SEM images of (a) **S1** showing spherical vesicles (b) **S2** showing the pot-like morphology (c) **S3** showing pot-like morphology. (d) SEM image of **6** showing fibres. All samples were prepared by the evaporation of 0.5 mM solution of the respective compounds in $CHCl_3$:CH_3OH (1:1) on glass. Inset shows the HR-TEM image of the respective samples on copper grid.

Focused ion beam-SEM (FIB-SEM) was used for further investigating the nature and robustness of vesicular assembly.[15] FIB-SEM allows the sample to be tilted to a particular angle, viewed, and bombarded with gallium ion beam within selected target areas of the sample. FIB milling of vesicles using a Ga^+, 16 keV beam with a 4 pA current revealed that the vesicles of **S1** and **S2** are sturdy with a hollow interior (Fig. 3c-e and Fig. S8, ESI†). FIB milling on the vesicles of **S1** by outlining a square box resulted in vesicle with a square inscribed on it without rupturing the vesicle. Fig. 3d shows FIB-SEM of **S1** after slicing off a portion of the vesicle. Similarly, pot-like structure of **S2** was

milled to remove some part of it to reveal its hollow interior (Fig. 3e). The results of the FIB-SEM clearly show the robustness of the self-assembly and the hollow nature of the vesicles and pots.

In order to study the mode of self-assembly, control compounds **S3** and **S4** were synthesized. **S3** can be considered as an acyclic analog of **S1**, whereas **S4** is structurally equivalent to half of **S1**. **S3** showed spherical vesicles and pots (Fig. 2c and Fig. S9, ESI†) with a single well-defined circular orifice (~50 nm), and was also confirmed by HR-TEM studies (Inset, Fig. 2c). The HR-TEM images show a wall thickness of ~3 nm (Fig. S10, ESI†), which is close to the dimensions of **S3** (2.4 nm). We believe that **S3**, with 1, 3-phenyl unit and triazole give adequate surface and curvature leading to 3D vesicular assembly. The other control compound **S4**, resembling half of **S1**, containing a 1, 3-triazole but not 1, 3-phenyl does not show vesicular assembly (Fig. S11, ESI†) implying that the adequate curvature is not attained in **S4** for vesicular assembly. In order to see the role of molecular geometry on self-assembly, we studied other simple precursors like **4b** and **6**. The diazide **4b** and dialkyne **6** formed organogels, but no vesicles were observed. The vial inversion method confirmed the gel formation of **4b** in $CHCl_3$:hexane and **6** in $CHCl_3$:hexane as well as in EtOAc:hexane (Fig. S12, ESI†). SEM analysis of **6** showed long thin fibres of ~100 nm thickness (Fig. 2d), whereas **4b** showed thicker fibres with 0.7 μm thickness (Fig S13, ESI†).

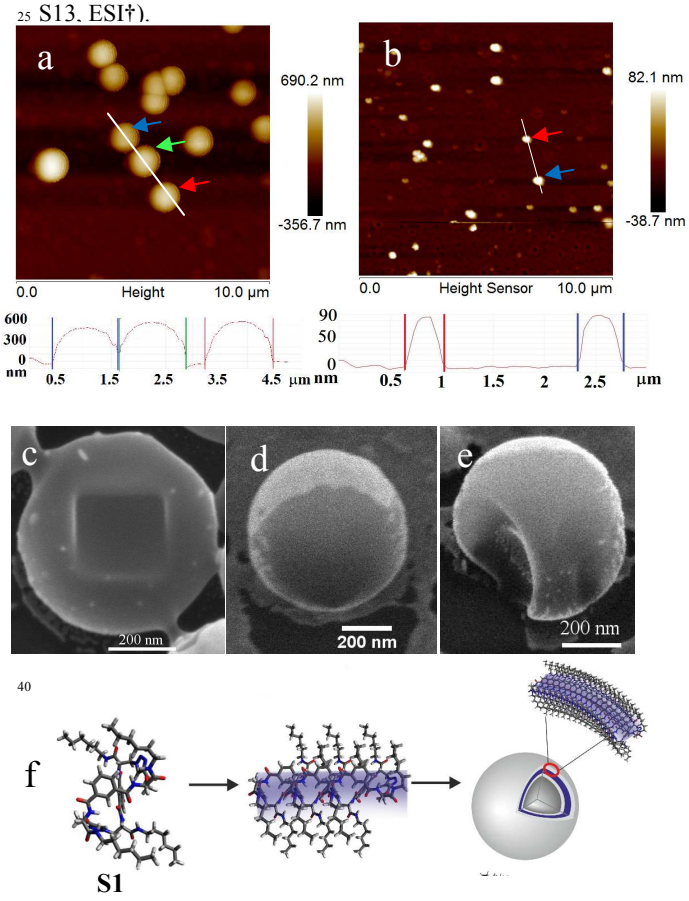


Fig. 3 AFM images of (a) **S1** in CH_3OH:$CHCl_3$ (3:1) below is the cross sectional analysis of the vesicles. (b) 0.5 mM solution of **S2** in CH_3OH:$CHCl_3$ (1:1) (c-d) FIB milled vesicles of **S1** (e) FIB milled **S2** .White layer on the surface of the sphere shows the gold coating (f) MD simulated structure of **S1** and schematic representation of their self-assembly to vesicles.

Solvent has a critical influence on the vesicular self-assembly, as vesicles were observed in methanol or a mixture of methanol and chloroform, and not in samples made from $CHCl_3$ alone. Increasing amounts of chloroform resulted in distorted vesicles (Fig. S14, ESI†). Protic solvent like methanol affects self-assembly, as methanol can take part in hydrogen bonding interactions and therefore can mediate efficient self-assembly.

To gain more insight and to rationalize the self-assembly of **S1**, **S2** and **S3**, we performed Molecular Dynamics (MD) simulations using the AMBER 12 package. The MD simulated structure in methanol shows that **S1** adopts a bent shape with hexyl groups projecting out of the ring (Fig. 3f). The hexyl chains are arranged such that one pair of the long chains are opposite to the other pair. In a similar way, **S3** adopts a structure with hexyl chains projecting outwards (Fig. S15, ESI†). The bent conformation of the macrocycles **S1** and **S2**, with their diverging alkyl chains induce a curvature to the assembly and thus favor a spherical vesicular assembly. The fact that the peripheral wall thickness of all the observed vesicles as evident from the HR-TEM studies (Figs. S4, S6 and S10, ESI†) matches with the molecular dimensions implies a one molecule thick self-assembly to form vesicles. We postulate that the lipid-mediated assembly as shown in Fig. 3f may be the plausible mechanism of the assembly. The rigid open analogs **6** and **4b** adopt a flat conformation and therefore favor a 2D extended assembly leading to the fibrillar morphology.

In conclusion, we transformed serine amino acid into click substrates by modifying the side chain. These substrates were converted to self-assembling 32-membered cyclophanes by CuAAC reaction. The click strategy outlined in this work opens up multitudes of possibilities for the synthesis of higher membered designer macrocycles. The demonstrated self-assembling properties of these novel molecules to vesicles, fibres, and gels will stimulate much work in this area. The vesicular self-assembly exhibited by the triazolophanes opens up possibilities for encapsulation of guest molecules.

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