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Study of the nano-morphological versatility by self-assembly of a peptide mimetic molecule in response to physical and chemical stimuli†

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A small peptide mimetic molecule can form diverse nanostructures such as nano-vesicles, nano-tubes and nano-ribbons/fibrils by self-assembly, in response to various physical and chemical stimulations.

Peptides have long been recognized as an important class of molecules in biochemistry, medicinal chemistry, physiology, and bionanotechnology.[1–3] At a fundamental level, proteins are the combination of several small peptide fragments. Therefore an understanding of how small peptides respond to physical and chemical stimuli is very important for understanding protein interaction behaviour in biological systems. Although morphological changes of small peptides, by varying self-assembling parameters, for model study of various biological phenomenons, have been studied by many groups in the last few decades,[4–9] *e.g.* Zhang *et al.* have studied the self-assembly of surfactant-like peptides with variable glycine tails which form nanotubes and nanovesicles in neutral aqueous solution,[4] yet the behaviours such as surface-induced morphological transformation of peptide-based nanostructures and their solvent induced inter-conversion have not been focused. In addition, study of morphological diversities of very small peptide mimetic molecules (PMMs), constituting only un-natural amino acids, has not been noticed yet.

Herein, we synthesized a new PMM, Boc-*m*-ABA-Aib-OMe (*m*-ABA: *m*-aminobenzoic acid, Aib: α-aminoisobutyric acid), and investigated the morphological transformations of this peptide-based nanostructure under various stimulations. Interestingly this newly synthesized terminally protected dipeptide molecule self-assembles to generate various nano-structures such as nano-vesicles, nanotubes and nano-ribbons/fibers depending upon the solvent effect, substrate functionality and thermal treatment. Such morphological diversities make it an attractive building block for model study and possible applications in nanobiotechnology. Moreover, this salt responsive PMM based vesicular structure has high efficiency of encapsulation, indicating the possibility of its use in model study for carrying drugs. The morphological diversity of the self-assembled nanostructures of the PMM was examined by various microscopic techniques. Field emission scanning electron microscopy (FE-SEM) reveals that the methanolic solution of the PMM generates highly dense, well-structured, spherical objects having size distribution with diameters of approximately 500 nm (Fig. 1(a) and (b)). The chemical structure of the PMM is demonstrated in the inset of Fig. 1(f).

The number density and the diameter of the spheres vary with respect to the PMM concentrations in methanolic solution (Fig. S1 in ESI†). The porous nature of vesicles is also clear from the SEM image (Fig. S2 in ESI†). Several larger spherical aggregates were observed which formed through the fusion of smaller ones (Fig. S3 in ESI†). The driving forces behind the fusion process may be the release of strain in the initially formed vesicles, which have

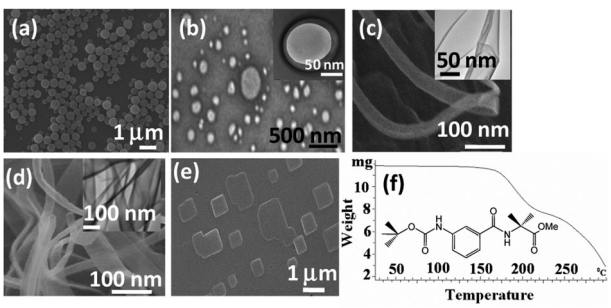

Fig. 1 FE-SEM image of vesicles generated by PMM (a), TEM image showing the hollow nature of vesicles generated by PMM (b), one enlarged TEM image of a vesicle has been shown in the inset of (b); FE-SEM image of PMM after thermal treatment for 1 h at a constant temperature of (c) 50 °C, (d) 100 °C, and (e) 150 °C, and (f) the chemical structure and the TGA plot of PMM.

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a high degree of curvature. By fusion into larger vesicles, the curvature energy decreases, leading to a thermodynamically more stable state.[10] Transmission electron microscopic (TEM) studies were performed to investigate the internal structure of these self-assembled spherical objects. It is found that the spheres obtained from the methanolic solution of the peptide have hollow spaces inside, indicative of the vesicle configurations (Fig. 1(b) and Fig. S4 in ESI†). The dynamic light scattering (DLS) experiment was performed in the methanolic solution of the PMM in order to assess the formation of self-assembled structures in solution. The result showed that the nanostructures display hydrodynamic diameter centered at 320.25 nm with polydispersity index, 0.30 (Fig. S5 in ESI†). The hydrodiameter values that are higher than the average diameters observed in TEM indicate extensive solvation of the nanoscopic structures or fusion of smaller vesicles to larger ones in the solution phase.

To examine the thermal effect on the nano-vesicles, we performed a comparative study by aging them in a convection oven at a constant temperature of 50, 100 and 150 °C for 1 h each. The SEM and TEM results indicate that a morphological change occurred from vesicular structures to fibrils and to plates (Fig. 1(c)–(e)) due to rise of temperature. Such morphological changes due to temperature variation might serve as a scaffold for construction of various nano-devices where different process temperatures are required.[11] A complete destruction of all the nanostructures was observed when they were heated above 150 °C (Fig. S6, ESI†), which can be further confirmed by the thermogravimetric analysis (TGA) result (Fig. 1(f)).

To understand the precise role of solvents in generating different morphologies of the new peptide, solvent dependency experiments were carried out.[5,6,12] We discovered that the PMM forms elongated nano-tubular morphologies with a diameter of ~200 nm and having several micrometer length from chloroform–methanol (1 : 1 v/v) solvent medium (Fig. S7(a), ESI†), whereas from toluene, the peptide forms short nanotubes (Fig. S7(b), ESI†). In the presence of ethyl-acetate, well aligned nano-fibrils were found to be generated (Fig. S7(c), ESI†). Therefore our PMM could offer a model for the investigation of peptide fibrillation.[8] On the other hand, the PMM forms flat tape or ribbon like structure when grown from $CHCl_3$–petroleum ether (1 : 1 v/v) solvent medium, acetone and dimethylformamide solvents, individually (Fig. S7(d)–(f), ESI†). From the results, it is found that there is a clear-cut impact of different solvent systems on the fabrication of morphological diversity, which may be due to the influence of different functional groups present in each solvent on the various noncovalent interactions responsible for self-assembly of the peptide molecule.[5]

Furthermore, we demonstrated the influence of surface functionality[13] on the self-assembly of the PMM using self-assembled monolayers (SAMs) generated on the glass surface as a model system. We utilized four different SAMs, namely, 3-aminopropyltriethoxysilane (–NH_2), 3-mercaptopropyltriethoxysilane (–SH), propyltriethoxysilane (–CH_3) and octyltriethoxysilane (–C8). On both –NH_2 and –SH SAMs, the PMM showed the formation of vesicles (Fig. S8(a) and (b), ESI†). However, formation of nano-ribbons/fibrils of the PMM was observed on both –CH_3 and –C8 SAMs (Fig. S8(c) and (d), ESI†). Thus, we commented that the functionality on the surface, not the chain length, has a great effect on the self-assembly pattern of peptides. It was previously proved by DLS study that in methanol medium the peptide forms nano-vesicles. Therefore we can assume that the self-assembly pattern of the peptide is not disturbed when the methanolic solution of it is dropped on –NH_2 or –SH SAMs. Because in methanol, the vesicles are surrounded by the polar hydroxyl (–OH) group of methanol and while the vesicles are dropped on –NH_2 or –SH SAMs, the outer environment around the vesicles does not change to a great extent. Therefore the vesicular structures remain unaltered. However, when methanolic solution of the peptide is dropped on different –CH_3 SAM surfaces, having different chain lengths of alkyl silanes, the outer environment around the vesicles was drastically changed. The PMM molecules tend to interact with –CH_3 functional groups in a way that causes an alteration in the higher order self-assembled packing of the peptide molecules. This experiment may provide insights for understanding the interaction of peptide/protein with different functional groups at biological interfaces. Moreover the solvent induced interconversion of these nano-fibers to nano-vesicles in the presence of methanol was also investigated (Fig. S9, ESI†). This experiment supports the fact that the influence of functional groups present in solvent or on substrates are very important for the higher order self-assembly pattern of PMM. This interconversion of nano-structures may facilitate biological, therapeutic and cosmetic applications and may have a great impact for several nano-technological applications.

To investigate the secondary structure in self-assembled peptide vesicles, solid state FT-IR spectroscopy was performed. The important absorption bands of the vesicles have been compared with the as-synthesized compound (Table S1 and Fig. S10, ESI†). No peak at around 3430–3440 cm^{-1} was observed indicating that all the –NH groups are involved in intermolecular hydrogen bonding.[14] The peaks at around 1745.73 cm^{-1} and 1745.56 cm^{-1} for the as synthesized compound and the vesicles, respectively, indicate the C=O stretching vibrations of saturated non-H-bonded methyl ester. From the position of the amide I band at around 1644 cm^{-1} and the amide II band at around 1558 cm^{-1}, it is assumed that the PMM forms β-sheet like structure in the solid state.[15–18] The IR result also reveals that the supramolecular β-sheet like structure is retained while forming fibrils on –CH_3 SAM or generated from different solvents or after thermal treatment and during formation of tubes from aromatic solvent.

Single-crystal X-ray diffraction (XRD) results (Fig. S11 and Tables S2–S4, ESI†) revealed that the PMM forms β-sheet like layers through molecular self-assembly (Fig. 2(a)), in which the *m*-ABA mediated aromatic π-staking interactions play an important role (Fig. S12, ESI†). Various noncovalent interactions such as intermolecular hydrogen bonding and hydrophobic interactions play the key roles in the formation of the β-sheet layer like structure. These structures take part in higher order self-assembly through non-specific van der Waals force and hydrophobic interactions (Fig. 2(b)). From the wide angle X-ray diffraction (WAXD) analysis (Fig. S13 and Table S5, ESI†) it is supposed that a similar

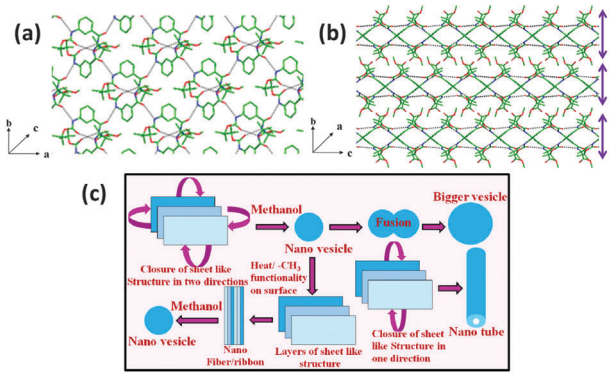

Fig. 2 Single crystal XRD results of (a) supramolecular β-sheet-like structure of PMM in the *ab* plane; (b) higher order self-assembly of the β-sheet layers in the *b*-direction. Each sheet-like layer is indicated by a double headed arrow, and (c) proposed schematic model of formation of various nano-structures.

type of crystal packing is responsible for the formation of vesicle, tube and fibril/ribbon-like structures.[8,19] The formation of nano-vesicles may be envisaged by considering the wrapping of the β-sheet-like layers in two different directions simultaneously[20] as depicted in the schematic model in Fig. 2(c). We assume that due to thermal treatment or interaction with $-CH_3$ functional groups on different SAM surfaces or the presence of different solvents, the two-way wrapping of β-sheet layers opens up and they are arranged side by side to form the fibrils/ribbons (Fig. 2(c)). Again in a chloroform–methanol solvent mixture (1 : 1 v/v) and aromatic solvent like toluene, β-sheet-like layers may fold in only one direction to form the nano-tubes (Fig. 2(c)).[8,20] A simple co-relation with the crystal structure and the TEM images of PMM nanostructures has been provided in Fig. S14 of ESI,† which also supports our assumption.[21]

Thus, the self-assembly of *m*-amino benzoic acid and α-amino isobutyric acid mediated peptide molecules, without any electrostatic interactions, supports the fact that various non-covalent interactions can facilitate the formation of such well-ordered nano-morphologies.

The design and construction of nano-vesicles from the self-assembling peptide and pseudo peptides are being considered as excellent vehicles for encapsulating and carrying drugs,[22,23] but the entrapment capability of stable nano-vesicles generated from small peptide-mimetic molecules is yet to be explored. Thus, we investigated the efficiency of the PMM based nano-vesicles to encapsulate the anti-cancer drug Methotrexate (MTX).[24] TEM images of the drug and drug after encapsulation by the nano-vesicles clearly depict the encapsulation of the MTX drug by the nano-vesicles formed by PMM (Fig. 3(a) and (b)). Moreover, the entrapped drug can be released easily by simple addition of a biocompatible metal salt, such as KCl, into the drug-loaded vesicles, which was confirmed by fluorescence microscopic images (Fig. 3(c) and (d)). This encapsulation–release process was further confirmed by fluorescence emission study (Fig. 3(e)). The fluorescence of the MTX drug is found to drop enormously after encapsulation of it by the peptide nano-vesicles. After incubation of the drug-encapsulated peptide

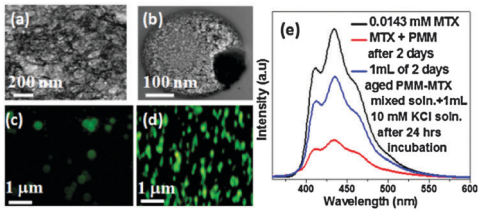

Fig. 3 TEM image of (a) methanolic solution of 0.014 mM methotrexate solution and (b) methotrexate loaded PMM vesicles after incubation for 2 days; fluorescence microscopic images showing (c) green fluorescence of the methotrexate loaded PMM vesicles after incubation for 2 days and (d) rupture of methotrexate loaded PMM vesicles after 24 h incubation with 10 mM KCl salt solution; (e) fluorescence emission spectra showing encapsulation and release of the methotrexate drug at an excitation wavelength of 350 nm.

solution with 10 mM KCl solution (1 : 1 v/v mixing) for 24 h, the fluorescence of MTX again increases, confirming effective drug-release. In Fig. S15 (ESI†), we tried to provide a probable mechanism of MTX drug penetration through the nano-pores on the surface of the hollow PMM vesicles. Interestingly, the vesicles can entrap the drug in its cavity for several days, even up to 2 months (Fig. S16, ESI†), which indicates the stability of self-assembly of the nano-vesicles. Notably, the morphological pattern of the day or month aged MTX drug directly reflects the morphology of the drug inside the peptide-vesicle (Fig. S16, ESI†), depicting that there is no chemical interaction of the PMM with the drug due to protection of the peptide molecule at both ends and thereby highly recommending it to be utilized as a drug carrier in therapeutics.

In summary, we have demonstrated the self-assembly of a new di-peptide mimetic molecule, constituting only un-natural amino acids, into various nanostructures under various stimulations and its possible application in drug delivery. This newly described small peptide mimetic molecule not only is attractive for the model study of peptide interaction behaviours in biological systems, but also offers novel scaffolds for the future design of new functional biomaterials.

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