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pH Responsive Soft Vesicles through Self-assembly of Peptide β-Turns

Sudeshna Kar and Animesh Pramanik*
Department of Chemistry, University of Calcutta, 92, A. P. C. Road, Kolkata-700 009, India

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The *meta*-aminobenzoic acid-inserted peptide β-turns are found to generate well-structured vesicles from methanol/water solution through molecular self-assembly. Fluorescence microscopy and UV studies reveal that the nanovesicles can encapsulate dye molecules which can be released by addition of acid (at pH 2.0).

Reverse turns play important roles in facilitating intermolecular recognition in biological systems such as enzyme–substrate interactions and protein–ligand binding.[1] Therefore, designing nanomaterials through the self-assembly of reverse turns may allow biomedical engineering where enzyme-turn or protein-turn recognition can be utilized in targeted drug delivery. Studies on vesicles are very important because they are crucial building blocks for all living systems and have various applications in drug and gene delivery, as nanoreactors and as artificial cell membranes.[2] In continuation of our research on peptidomimetic design of β-turns[3] we have shown that the *m*-aminobenzoic acid (*m*-ABA)-inserted acyclic peptides can adopt β-turn structures.[3a] It is well known that aromatic π-stacking interactions provide energetic as well as directionality for self-assembled protein/peptide aggregates.[4] Several bioinspired designs of soft supramolecular assemblies based on aromatic π–π interactions have been reported recently.[5] So we assumed that *m*-ABA, present in peptide β-turns, may invoke aromatic π–π interactions in self-assembly and may help to create nanomaterials under suitable conditions. Moreover, the conformationally rigid *m*-ABA in the peptide backbones may help in the formation of well-structured supramolecular assemblies by restricting the conformational entropy[6] and may provide proteolytic stability to the self-assembled supramolecular structures.

Here we have chosen three structurally related β-turn forming peptides Boc–Leu–Aib–*m*-ABA–OMe (**1**), Boc–Leu–Aib–*m*-ABA–OH (**2**), and Boc–Leu–Aib–*m*-ABA–Leu–Aib–*m*-ABA–OMe (**3**) (Aib: α-aminoisobutyric acid) (Figure 1) containing *m*-ABA, to examine the formation of nanomaterials through molecular self-assembly. It has been shown that in peptide **1** the centrally placed helicogenic Aib[7] helps to attain a β-turn structure (Supporting Information; SI).[3a,17] Peptide **2** is the carboxylic acid of peptide **1**, generated through the base-catalyzed hydrolysis to make the peptide β-turn more hydrophilic in nature. The hexapeptide **3** is a dimer of peptide **1** and expected to adopt a double turn conformation due to suitably placed Aib residues along the peptide backbone. The presence of conformationally rigid and geometrically extended *m*-ABA in the middle will help to form turn structure in **3** rather than helix. The peptides **1**–**3** were synthesized by conventional solution-phase methodology (SI).[8,17]

The ultrastructural characterization by scanning electron microscopy (SEM) shows that peptides **1**–**3** generate spherical

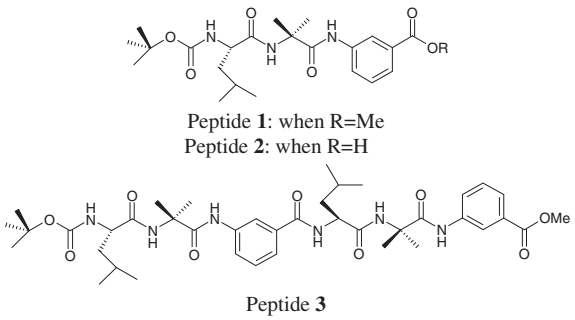

Figure 1. Schematic representation of peptides **1**–**3**.

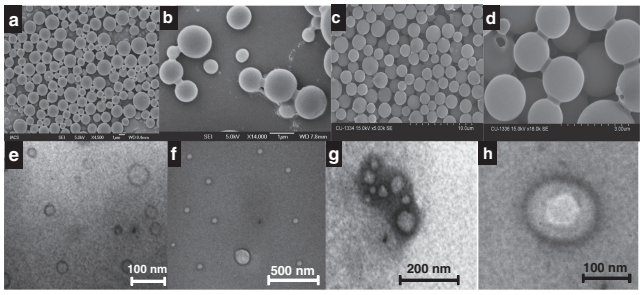

Figure 2. FE-SEM images of (a) peptide **1**, (b) peptide **2**, and (c) peptide **3** show the formation of microvesicles in the solid state. FE-SEM image (d) shows the porous vesicle together with fused vesicles of peptide **3**. The solid materials of the peptides were grown slowly from methanol/water (9:1 by v/v) (10.0 mM solution), were dried and gold coated. TEM images of (e) peptide **1**, (f) peptide **2**, and (g) peptide **3** show the formation of nanovesicles in methanol/water (9:1 by v/v) (10.0 mM). TEM image (h) clearly indicates the layer-like boundary and hollow nature of self-assembled structure of peptide **3**.

objects of an average diameter 0.5–2 μm, when freshly prepared methanol/water (9:1 by v/v) solution of the peptides (10.0 mM) were dried on glass surfaces by slow evaporation for SEM imaging (Figure 2). Interestingly the SEM image of peptide **3** reveals opened-up hollow spheres which confirm the hollow nature of the spherical structures (Figure 2d). These porous structures are formed when the trapped volatile solvent such as methanol escapes from the vesicle during the drying process.[9] Again the SEM images of the peptides **1**–**3** show that some of the vesicles are fused with each other, indicating that the larger vesicles are formed through the fusion of smaller vesicles while growing on the glass surfaces (Figure 2).

The driving force behind the fusion process may be the thermodynamic stability due to decrease of curvature energy.[10] On the other hand the TEM studies reveal that all the peptides **1**–**3** generate nanovesicular structures from 10.0 mM solutions of peptides in methanol/water (9:1 by v/v) with an average

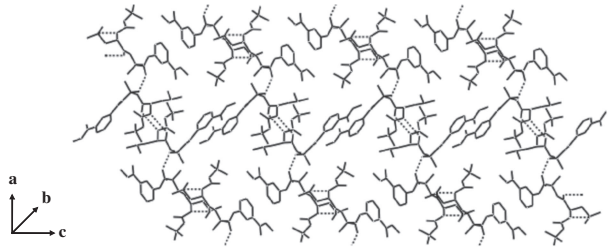

Figure 3. Formation of sheet-like layer in *ac* plane through hydrogen bonding and π stacking induced self-assembly of β-turns of peptide **1**.

diameter of 10–100 nm (Figures 2e–2h), which is much less than the average diameter observed in SEM studies indicating the absence of vesicle fusion while growing on carbon-coated copper grids. The TEM images clearly demonstrate the layer-like boundary and hollow nature of the self-assembled spherical structures of the peptides (Figures 2e–2h). Dynamic light-scattering (DLS) experiments were carried out to measure the size distribution of the vesicles in methanol/water (9:1 by v/v), which show that peptide **1** generates vesicular structures from a 10.0 mM solution with a distribution of hydrodynamic diameters centered on 321.25 nm with polydispersity index (fractional dispersity) of 0.305. The distribution of hydrodynamic diameters of peptide **2** vesicles was centered at 351.45 nm with polydispersity index, 0.823, and that of peptide **3** at 122.58 nm with polydispersity index, 1.00 (i.e., monodispersed) (SI[17]). Formation of vesicles with higher hydrodynamic diameters may be due to extensive solvation or aggregations.

The peptide Boc–Leu–Aib–*m*-ABA–OMe (**1**) has been shown to adopt a type II β-turn structure (SI[17]).[3a] Further studies with ^{1}H NMR solvent titrations in $(CD_3)_2SO/CDCl_3$ and CD measurement in methanol confirm the existence of β-turn conformation of peptide **1** in the solution phase also (SI[17]). The crystal packing analysis reveals that the β-turns of peptide **1** are interconnected by two types of intermolecular hydrogen bonds Leu–CO···HN–Leu (O···H: 2.14 Å, O···N: 2.982 Å, O···H–N: 164.7°, $1-x$, $1-y$, $-z$) and Aib–CO···HN–Aib (O···H: 2.12 Å, O···N: 2.890 Å, O···H–N: 148.9°, $0.5-x$, $0.5+y$, $-0.5-z$) to create a layer of β-sheet (*ac* plane) through molecular self-assembly (Figure 3). This molecular packing is further stabilized by aromatic π–π interactions where the average distance between two closely placed phenyl rings is ca. 3.8 Å, which is well within the range of π stacking. Gazit et al. have suggested that a closure of sheetlike structure along two axes may cause the formation of vesicles.[11] Many other examples are there where β-sheet-mediated self-assembly gives rise to formation of vesicles.[12a] C_3-symmetric peptide conjugates also form vesicles by self-assembly.[12b,12c] The existence of β-turn conformations of peptides **2** and **3** in methanol is confirmed from CD measurements which show a positive ellipticity at 205.2 nm in case of peptide **2** and at 201.6 in case of peptide **3**, which are characterestic of a β-turn conformation (SI).[13,17] Therefore, β-turns are the building blocks in the self-assembly of peptides **1**–**3** to form supramolecular vesicular assemblies.

The existence of aromatic π-stacking interactions in the solid state of vesicles was further probed by UV studies. In methanol the concentration dependent UV-studies (starting from 0.015 to 10 mM) of the peptides **1**–**3** showed absorption maxima

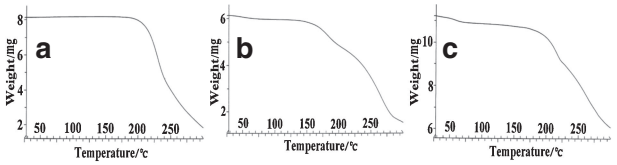

Figure 4. Thermogravimetric analysis (TGA) plots of (a) peptide **1**, (b) peptide **2**, and (c) peptide **3** (flow rate = 30 $cm^3\,min^{-1}$). The samples were heated in an alumina crucible at a rate of 5 °C min^{-1}.

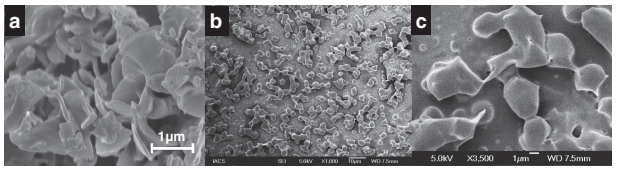

Figure 5. FE-SEM image of ruptured vesicles of (a) peptide **1**, (b) peptide **2**, and (c) peptide **3** at pH 2.0.

around 312 nm due to aromatic chromophore of *m*-ABA (SI[17]). The results show that the π-stacking interactions are not present among the peptide molecules in methanol. But in the solid state of vesicles the peptides **1** and **2** show absorption maxima around 388 nm and peptide **3** at 425 nm. The substantial red shift of absorption maxima, ca. 76 nm in case of peptides **1** and **2** and ca. 113 nm in case of peptide **3**, and significant broadening of the absorption band on going from solution to solid state may be attributed to the π-stacking interactions among the aromatic rings of *m*-ABA in the solid state.[14]

The TGA thermogram shows that the nanovesicles of peptide **1** are stable up to 200 °C, after which the peptide **1** decomposes (Figure 4). The nanovesicles of peptides **2** and **3** are found to be thermally stable up to 160 and 200 °C, respectively (Figure 4). The low thermal stability of the nanovesicles of **2** compared to that of peptides **1** and **3** may be due to free carboxylic group. Probably the loss of entrapped methanol and water from the nanovesicles of peptide **2** causes low thermal stability by disturbing the solvent-mediated self-assembly.

In order to investigate the extent of softness of the vesicular structures, a pH-triggered disruption study was carried out. In this experiment a methanolic solution of HCl was added to the freshly prepared methanol/water (9:1 by v/v) solutions of peptides **1**–**3** (10.0 mM) so that the final pH was maintained at 2.0. Then the solutions were incubated for 12 h followed by scanning electron microscopic imaging. The SEM images of the above experiment show complete rupture of vesicles (Figure 5). The loss of structural integrity of the vesicles may be attributed to the protonation of the peptide molecules in acidic medium, followed by electrostatic repulsion between the positively charged molecules. The loss of Boc groups of the molecules in acidic condition may also cause the disruption of vesicular structures (SI[17]).

The potentiality of the nanovesicles of peptides **1**–**3** to encapsulate natural and unnatural molecules was explored by an experiment involving the encapsulation of dye.[5a,15] In order to perform this experiment, a aqueous methanolic solution of rhodamine B was added to methanol/water (9:1 by v/v) solutions of peptides **1**–**3** separately so that the final concen-

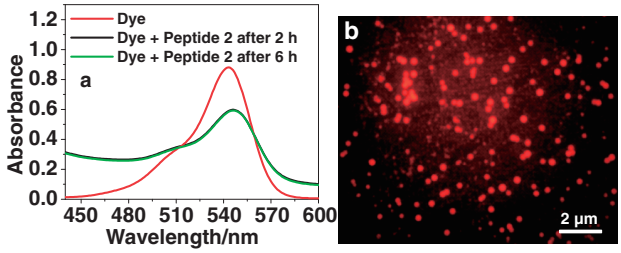

Figure 6. (a) UV-absorption spectra showing the encapsulation of rhodamine B dye by the vesicles of peptide **2** in methanol/water (9:1 by v/v). (b) Fluorescence microscopic image of rhodamine B dye-entrapped vesicles generated from a 6 h aged solution of peptide **2** (ca. 10^{-4} M).

tration of each substance is maintained at ca. 10^{-4} M. Initially the UV absorption spectra of a ca. 10^{-4} M solution of pure rhodamine B in methanol was recorded, which showed an absorption maximum around 543 nm (Figure 6). Then the UV spectra of methanolic solutions of rhodamine B with peptides **1**–**3**, where the concentration of each substance was maintained at ca. 10^{-4} M, were recorded after 2 and 6 h (Figure 6 and SI[17]). The decrease of absorption intensity of rhodamine B indicates the entrapment of the dye molecules by the vesicles of peptides **1**–**3**. After 6 h, one drop of each of the above solutions was loaded on a glass slide, dried at room temperature, and washed with water several times to remove free rhodamine B followed by drying in air and then in vacuum. The slide was then examined under a fluorescence microscope. The visualization of the rhodamine B-entrapped vesicular structures of peptides **1**–**3** strongly supports the encapsulation phenomenon (Figure 6 and SI[17]). Interestingly, when after 6 h, the methanolic solutions of peptides **1**–**3** with rhodamine B were acidified with methanolic HCl at pH 2.0, all the entrapped dye molecules got released, and the intensity of the absorption spectrum regained almost its initial value. The release of entrapped dye molecules in the acidic medium was further confirmed by the breakage of the dye-loaded vesicles (SI[17]). Among the three peptides **1**–**3**, the vesicles of peptide **2** show the most effective encapsulation property of rhodamine B dye (Figures 6a and 6b). Peptide **2** is hydrophilic in nature due to the presence of free carboxylic groups which helps to form complexes with the dye molecules during entrapment, preferably through the polar interactions.

In summary, we have shown that *meta*-aminobenzoic acid-inserted peptide β-turns can generate vesicular structures through molecular self-assembly. The formation of vesicles in the solid state has been facilitated through the participation of various noncovalent interactions such as aromatic π stacking, hydrogen bonding, and hydrophobic interactions. Since in UV studies no characteristic red shift due to π stacking has been observed in methanol, probably hydrophobic interaction is the main driving force of self-assembly to form vesicles in solution. The incorporation of conformationally rigid *meta*-aminobenzoic acid and Aib in the peptide backbones helps to form well-structured vesicles by restricting the conformational entropy. Fluorescence microscopy and UV studies reveal that the peptide nanovesicles can encapsulate dye molecules which can be released by addition of acid. These pH-responsive soft vesicular structures may find applications in entrapment of natural and unnatural molecules and release of them. Currently extensive research interest has been directed toward studies of vesicle fusion.[16] In the present study it has been observed that larger vesicles are formed through fusion of small vesicles. These β-turn-based vesicles may be utilized for the model study to gain more insights about the formation and targeted fusion of endosomal vesicles in cell-free conditions.

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