Self-assembly and potassium ion triggered disruption of peptide-based soft structures†

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This report describes formation of soft vesicular structures by a tetrapeptide and its disruption triggered by potassium ions.

Soft molecular self-assemblies that respond to external stimulus, leading to predictable alteration in physico-chemical properties, are increasingly being sought in diverse applications such as drug delivery systems, (bio)chemical sensors, cell adhesion mediators, and microfluidics.[1] External stimuli including temperature, pH, light, electric field, chemicals and ionic strength are expected to manifest observable change in responsive systems leading to modifiable shape and surface properties, solubility characteristics, ability to self-assemble or to exhibit sol-to-gel transitions.[2]

Synthetic polymers and biological macromolecules have been extensively used for the development of signal responsive materials.[3] Carefully crafted polymers and biomolecules have the ability to deliver materials with varying response mechanisms for tailored biomedical requirements. Of the various possibilities, peptides are attractive building blocks for soft structure design owing to their inherent ability to self-assemble in nanoscopic dimensions.[4] Consequently, several examples of peptide-based responsive materials sensitive to external stimuli including light, pH, salt concentration and temperature have been described in contemporary literature.[2,5]

Herein, we report self-assembly of a simple tetrapeptide into vesicular structures and its ultrastructural characterization by microscopic techniques. PWWP tetrapeptide (**SV1**), derived from the antimicrobial peptide indolicidin sequence, spontaneously formed vesicles in the solution phase which could be disrupted by the simple addition of KCl. This peptide represents a unique example of cation-responsive soft material which undergoes facile rupture in the presence of a physiologically relevant cation.

SV1 was synthesized *via* solution phase peptide synthesis techniques (see the Supporting Information).[6]‡ A closer inspection of its MM+ optimized structure suggested an inherent curvature in the molecule and that the two tryptophan indole moieties were positioned in an almost parallel displaced mode thus allowing π–π stacking interactions to stabilize the starting conformation towards eventual formation of spherical patterns (Fig. 1b). Due to solubility constraints, the aggregation behavior of **SV1** (0.25 mM) was studied in 50% aqueous methanol, where a random coil-like

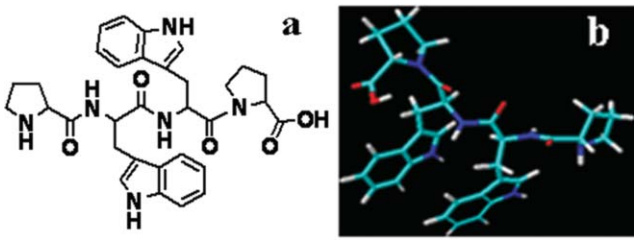

Fig. 1 (a) Molecular structure of **SV1**; (b) MM+ optimized structure of **SV1**.

structure was confirmed from CD studies (data not shown). The choice of solvent system was dictated by the solubility of **SV1**.

Light microscopic studies revealed instantaneous formation of vesicular structures in **SV1** solution (data not shown). The appearance of vesicular structures was confirmed by scanning electron microscopy (SEM) where uniformly circular patterns (Fig. 2a) were evident in the fresh solution with very little change in the morphology when aged for a week (data not shown). A transmission electron micrograph (TEM) and atomic force micrographs (AFM) also revealed the presence of punctated, circular structures (Figs. 2b and c) with an average diameter of ~1 μm. An environmental SEM image (E-SEM) revealed a swollen vesicular structure, under moist native-like conditions,

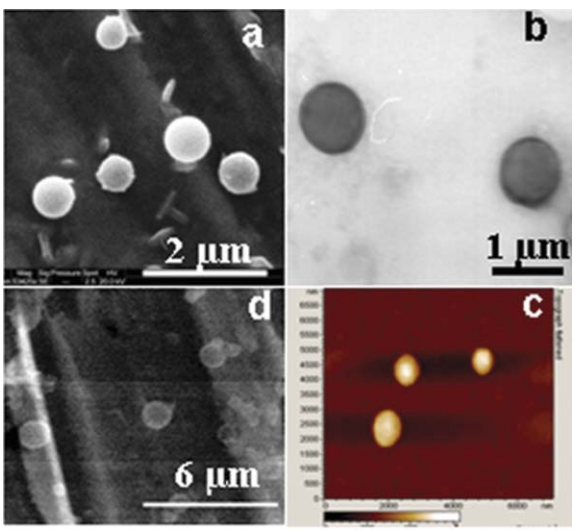

Fig. 2 Ultrastructural microscopic analysis of **SV1**. (a) SEM image of **SV1** solution after 5 min of incubation; (b) TEM micrograph of **SV1** vesicles in fresh solution; (c) AFM image of **SV1** solution; (d) E-SEM image of **SV1** vesicles.

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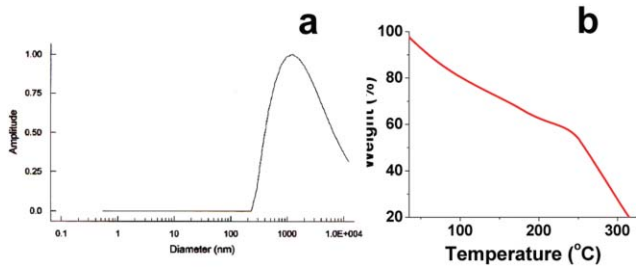

Fig. 3 (a) Average size distribution curve by intensity shows the average size of the vesicles ~1.5 μm; (b) **SV1** vesicles show thermal stability up to ~285 °C in TGA analysis.

with an average diameter of ~1.4 μm (Fig. 2d), which suggests that these spherical structures do not result as drying artifacts. The size of vesicles under native conditions matched well with dynamic light scattering size measurements which revealed a size distribution of ~1.5 μm from the intensity data (Fig. 3a).

Further evaluation of these vesicles confirmed their stability towards 45 min of ultrasonication.[6] The TGA thermogram of **SV1** vesicles showed ~40% weight loss between room temperature and ~285 °C (Fig. 3b). This may be attributed to the loss of methanol and water from the confines of **SV1** soft vesicular structures.

Interestingly, control experiments using the aromatic core of ditryptophan dipeptide alone did not exhibit formation of vesicular structures,[6,7] while truncated PWW and WWP tripeptides afforded ill-defined structures,[6] thus demonstrating a crucial role of PWWP tetrapeptide in the self-assembly process. Moreover, it was possible to dry these vesicles and rehydrate them without destroying the morphology of vesicular structures.

Having demonstrated the formation of vesicular structures, we became interested in determining the conditions for the disruption of these soft vesicular structures. Interaction of cations with the aromatic indole ring of a tryptophan residue is a well-studied phenomenon in artificial and natural systems.[8] Moreover, alkali metal ions have also been shown to interact favorably with amino acids such as proline and serine leading to cluster formation.[9] We reasoned that incubation of vesicles with alkali metal ions, such as potassium ion, may influence self-assembled morphology *via* cation–π interaction, thereby altering the gross morphology of vesicles. The premise of such interaction prompted us to use potassium ions as an external stimulus for altering **SV1** peptide structure.

Interestingly, vesicular structures ruptured subsequent to their incubation with KCl solution (0.25 mM) at ambient temperature. SEM and TEM micrographs revealed drastically altered morphology of **SV1** with a complete loss of vesicular structure (Fig. 4). Cation interaction and microscopic analysis suggested the possibility of using **SV1** for guest entrapment and release in the presence of potassium ions.

Encapsulation properties of **SV1** were confirmed by using rhodamine B dye as a guest molecule. Peptide was dissolved in 1 μM dye solution and incubated for optimal guest entrapment. Visualization under a fluorescence microscope confirmed that intensely red fluorescence was contained within vesicular structures (Fig. 5a). Prolonged incubation resulted in extensive aggregation of dye-trapped vesicles without any change in the gross morphology of the vesicular structures. Dye-loaded vesicles were incubated with

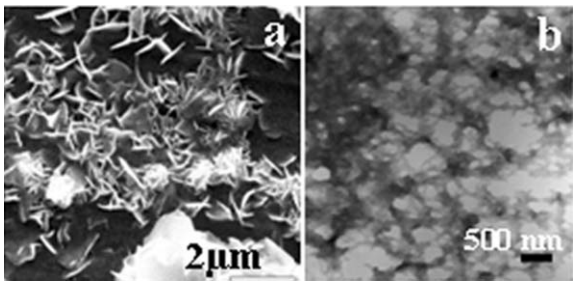

Fig. 4 Rupturing of **SV1** vesicles with potassium ions. (a) SEM image; (b) TEM image.

KCl solution (0.25 mM) and once again, the salt solution caused release of rhodamine B by disrupting guest entrapped vesicles (Fig. 5b). Similar disruption of peptide vesicles was also observed on the addition of NaCl solution.[6] This approach presents an interesting design paradigm for cation-responsive peptide architectures which release guest molecules in the presence of physiologically relevant potassium ions.

We propose that π–π stacking of the tryptophan side-chain plays a crucial role in the self-assembly process, which is ably supported by the curvature in the molecule and backbone interactions. Future work will deal with replacement of tryptophan with other aromatic amino acid residues and *N*-methylation of the indole ring. Interestingly, the role of aromatic π-stacking interactions in providing energetic contributions as well as directionality has been proposed for self-assembled protein/peptide aggregates.[10] As expected, the addition of potassium and the possibility of cation–π interaction leads to altered morphology of vesicles.

In conclusion, we have described the formation of self-assembled structures by PWWP tetrapeptide, guest entrapment studies and its disruption in the presence of potassium ions. We perceive that such potassium-responsive vesicular structures may find applications as delivery vehicles for entrapment and transport of natural and unnatural molecules. These efforts are currently underway.

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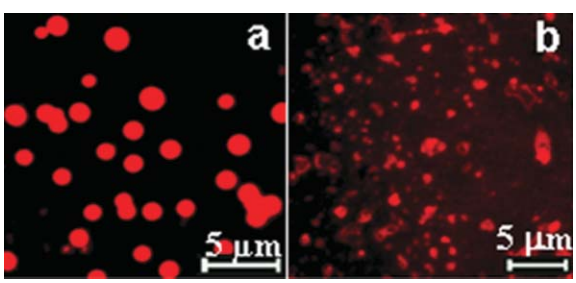

Fig. 5 Visualization of rhodamine B entrapped **SV1** vesicular structures as observed by fluorescence microscopy. (a) 2 days aged solution of dye-loaded vesicles; (b) KCl-mediated disruption of dye-loaded **SV1** vesicles.

Notes and references

‡ **Synthesis of *N*-(*tert*-butyloxycarbonyl)-L-prolyl-L-tryptophanyl-L-tryptophanyl-L-proline methyl ester (X)**: The compound was synthesized through routine solution-phase peptide synthesis by DCC–HOBT mediated coupling reaction. **Synthesis of L-prolyl-L-tryptophanyl-L-tryptophanyl-L-proline (SV1)**: Compound **X** (0.1 g, 0.15 mmol) was stirred with 1 M HCl in EtOAc (3 mL) for 3 hours. After 3 hours the solid was separated out by filtration and washed with diethyl ether and dried. Then, the dry solid was dissolved in 50% methanol–water and passed through an ion exchange column chromatograph and the solvent was concentrated under reduced pressure. Next, the solid was dissolved in methanol and reprecipitated by diethyl ether; this process was repeated three times and the solid was dried in a high vacuum pump to get **SV1** (0.03 g, 0.06 mmol). M.P. = decomposes at 195 °C, R_f [10% methanol in dichloromethane] = 0.3, $[\alpha]_D^{25} = -00.05$ [c 0.22, methanol]. ^{1}H NMR (400 MHz, CD_3OD, TMS, δ ppm): 1.66–1.88 (m, 6 H, proline β H and γ H); 2.02–2.12 (m, 1 H, proline β H); 2.12–2.25 (m, 1 H, proline β H); 2.85–2.92 (m, 2 H, proline γ H); 3.01–3.08 (m, 4 H, tryptophan β H); 3.16–3.29 (m, 2 H, merged signal for proline γ H and CD_3OD peak); 4.06–4.07 (m, 1 H, chiral); 4.13–4.16 (m, 1 H, chiral); 4.60–4.64 (m, 1 H, chiral); 4.77 (1 H, merged signal for chiral proton and CD_3OD peak); 6.83–7.07 (m, 6 H); 7.17–7.24 (m, 2 H); 7.32–7.34 (m, 1 H); 7.42–7.43 (m, 1 H); ^{13}C NMR (100 MHz; CD_3OD, δ ppm): 25.01, 26.08, 26.21, 29.1, 30.06, 31.04, 33.85, 47.61, 56.0, 60.42, 60.93, 112.39, 119.19, 119.9, 122.31, 124.8, 128.7, 137.86, 171.87, 175.25; FT IR (KBr, cm^{-1}): 1553 (amide II); 1635 (amide I); 3304 (–NH str); FAB MS (M + 1): 585; Anal. Calcd. for $C_{32}H_{36}N_6O_5$, C, 65.74; H, 6.21; N, 14.37; found, C, 65.19; H, 5.93; N, 13.91%. Analytical HPLC purity: > 97%. **Procedure for potassium ion interaction**: Both **SV1** (2 days aged, 0.25 mM) and KCl were taken 1 : 1 (mol/mol) in 50% aqueous methanol and stirred for 24 hours. After this time, solvent was lyophilized. **Fluorescence microscopy**: **SV1** was dissolved in rhodamine B solution (1 μM) in 50% aqueous methanol to make the final concentration 0.25 mM and incubated for 2–20 days at 37 °C. After 2 and 20 days 20 μL of the solution were loaded on the glass slide and dried at room temperature. These dye entrapped vesicular structures were examined on a fluorescent microscope (Zeiss Axioskop 2 Plus) provisioned with an illuminator (Zeiss HBO 100) and a rhodamine filter (absorption 540 nm/emission 625 nm). This filter optimized visualization of rhodamine-treated (positive resolution) compared with untreated (negative resolution) vesicles that are virtually invisible to this light. Images were electronically captured utilizing the Zeiss AxioVision (version 3.1) computer program. For KCl and KNO_3 triggered disruption studies 0.25 mM KCl and KNO_3 were added to a 2 days aged solution and incubated for a further 24 h followed by fluorescence microscopic imaging. **Scanning electron microscopy**: Sample was coated atop metal slides. A gold coating was applied to the top of the sample to make it conductive for analysis. Fresh samples and samples aged for 7 days were analyzed by SEM. SEM measurements were performed on a FEI QUANTA 200 microscope equipped with a tungsten filament gun. The micrograph for the short mutant was recorded at WD 10.6 mm, magnification 40000×. The concentration of the samples used was 0.25 mM. For KCl, KNO_3 and NaCl triggered disruption studies 0.25 mM KCl was added into the 2 days aged solution and incubated for a further 24 hours followed by scanning electron microscopic imaging. **Environmental scanning electron microscopy**: The peptide solution was placed on a metal stand made from aluminium for good thermal conductivity and viewed using a FEI QUANTA 200 microscope equipped with a field emission gun operating at 20.0 kV in a wet mode and the pressure was 1.0 torr. **Atomic force microscopy**: Fresh and aged peptide samples were imaged with an atomic force microscope (Molecular Imaging, USA) operating under Acoustic AC mode (AAC), with the aid of a cantilever (NSC 12(c) from MikroMasch). The force constant was 0.6 N m^{-1}, while the resonant frequency was 150 kHz. The images were taken in air at room temperature, with the scan speed of 1.5–2.2 lines s^{-1}. The data acquisition was done using PicoScan 5® software, while the data analysis was done with the aid of visual SPM. **SV1** (0.25 mM) was incubated for 0–7 days in 50% aqueous methanol and micrographs were recorded for selected incubation periods. 10 μL of sample solution were transferred onto a freshly cleaved mica surface and uniformly spread with the aid of a spin-coater operating at 200–500 rpm (PRS-4000). The sample-coated mica was dried for 30 min at room temperature, followed by AFM imaging. **Transmission electron microscopy**: A solution of **SV1** (100 μL, 0.25 mM) in 50% aqueous methanol was aged for 7 days. This solution (100 μL) was sonicated (TPC-25) for 15 seconds. 4 μL of this solution were transferred onto Formvar (Fluka, Switzerland) coated and carbon coated copper grids (SPI supplies, West Chester, USA, 200 mesh) and dried. These grids were negatively stained with 2% uranyl acetate, dried and subsequently examined under a JEOL 2000FX-II electron microscope at an operating voltage of 100 kV. For KCl and KNO_3 triggered disruption studies 0.25 mM KCl and 0.25 mM KNO_3 were added into the 2 days aged solution and incubated for a further 24 hours followed by transmission electron microscopic imaging. **Light scattering measurements**: Freshly prepared 0.25 mM sample solution in 50% aqueous methanol was used for the light scattering measurements using a MALVERN HPPS (1.00) instrument. **Thermogravimetric analysis**: A solution of **SV1** (5 mg mL^{-1}) in 50% aqueous methanol was aged for 7 days and lyophilized. This sample was analyzed in a Perkin-Elmer Pyris6 thermogravimetric analyzer. The rate of heating was 10 °C min^{-1} under nitrogen atmosphere.

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