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Pseudopeptosomes: non-lipidated vesicular assemblies from bispidine-appended pseudopeptides
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We report a novel molecular topology-based approach for creating reproducible vesicular assemblies in different solvent environments (including aqueous) using specifically designed pseudopeptides. Deviating from the classical "polar head group and hydrophobic tail" model of amphiphiles, we showed (reversible) self-assembly of synthesized pseudopeptides into vesicles. Naming these new type/class of vesicles "pseudopeptosomes", we characterized them by high-resolution microscopy (scanning electron, transmission electron, atomic force, epifluorescence and confocal) along with dynamic light scattering. While accounting for hydrophility index of the constituent amino acids (side chains) of pseudopeptides, we probed molecular interactions, resulting in assembly of pseudopeptosomes by spectroscopy (fourier-transform infrared and fluorescence). Molecular characterization by X-ray crystallography and circular dichroism revealed "tryptophan (Trp)-Zip" arrangements and/or hydrogen-bonded one-dimensional assembly depending on specific pseudopeptides and solvent environments. Our data indicated that pseudopeptosomes are formed in solutions by self-assembly of bispidine pseudopeptides (of Trp, leucine and alanine amino-acid constituents) into sheets that transform into vesicular structures. Thus, we showed that assembly of pseudopeptosomes utilizes the full spectrum of all four weak interactions essential in biological systems. Our findings have direct implications in chemical and synthetic biology, but may also provide a new avenue of investigations on origins of life via pseudopeptosome-like assemblies. We also showed that these designer peptides can act as carriers for cellular transport.

Introduction

Investigation of the formation of cell membranes from fundamental molecular building blocks has a unique place in contemporary scientific pursuits.1,2 The "protocells" that might have formed in primordial conditions are believed to be the harbinger of life on Earth.3 Therefore, development of a protocell model for studying and investigating innumerable issues in developmental biology is a challenging problem.

The building blocks of the early membrane of a protocell is not known. Therefore, the investigation of vesicular self-assembly from biologically relevant molecules such as peptides deserves special attention because the vesicular structure has a strong resemblance to the structure of primitive biological cells. A small molecule that assembles into a vesicular structure provides relevant clues on the origin-of-life problem.4–6 Most vesicle-forming structures are based on a single-chain or double-chain lipidated amphiphiles.7–9 Vesicles with a non-amphiphilic structure as part of the "primordial soup" have not been investigated.

Spherical vesicles are formed as a result of the spontaneous organization of molecules. Typically, the chemical structure of a vesicle-forming molecule consists of a charged head group and a long hydrocarbon tail. Phospholipids and sphingolipids are archetypical amphillic molecules that can assemble spontaneously into vesicles.10 The chemical, physical, and biological aspects of vesicular assembly are being investigated because vesicles serve as prototypes of living cells. The lipophilic unit is present in the cell membrane as long lipid chains. These long lipid chains are conserved in the cell membrane, as well as the liposomes that we prepare in the laboratory.

The first example of a completely synthetic vesicular assembly was reported more than four decades ago.11 Substantial efforts in this direction have been reported in the past decade.
Formation of vesicular structures with different components, exhibiting “functional” features (e.g., response to light), environmental variables (e.g., redox agents) and pH along with chemical interactions, are opening up new avenues for exploring biomimicry. This work reports a vesicular assembly that we call a “pseudopeptosome”, which is derived from the self-assembly of pseudopeptides. The latter have no structural similarity to “classical” amphiphiles.

The possibility of utilizing a lipophilic unit in structural formats other than a long lipid chain for vesiculation has not been investigated. We investigated the self-assembly of a series of pseudopeptides, wherein the hydrophobic part was in the form of a bicyclic unit reminiscent of a folded long chain, and the polar peptide bonds acted as the charged unit. Previously, we showed that the bispidine scaffold could nucleate the β-strand and β-arch. Here, we report the ability of bispidine to induce vesiculation in organic and aqueous media. Moreover, these “designer peptides” can penetrate cells. Additionally, we demonstrated a “TrypZip” structure in the tryptophan (Trp)-appended compound.

The seven-carbon bicyclic structure (bispidine) was reminiscent of a seven carbon-long chain but folded into a compact “double chair” bispidine structure. These designer pseudopeptides containing lipophilic bispidine could penetrate cells. These bispidine-containing cell-penetrating pseudopeptides (CPPPs) were biocompatible according to the Resazurin assay. We also demonstrated that our designer pseudopeptides assembled into bio-friendly pseudopeptosomes that had several potential applications. Interestingly, unlike phospholipid vesicular assemblies of size ~250 to ~1000 nm, which require 4000–80 000 molecular constituents, our pseudopeptosomes of size ~500 nm showed many more molecular constituents (~10^6) with relatively “thicker” vesicular boundaries/walls. This indicated: (a) the involvement of more weak interactions towards vesicular stability; (b) possibly better control of the balance between morphological rigidity and flexibility in terms of more constituents from the perspective of law of mass-action governing self-assembly. Our design and synthesis of pseudopeptides, followed by their controlled assembly into novel vesicular structures called pseudopeptosomes, advances the scope of synthetic biology significantly, but may also open a new line of investigations towards various applications.

Results and discussion

Design and synthesis of pseudopeptides

Utilizing the tools of synthetic organic chemistry, but inspired by the utilization of naturally occurring molecules, we first explored creation of a lipophilic unit in non-phospholipidic structures. Thus, we created a series of pseudopeptides, wherein the hydrophobic part was in the form of a bicyclic unit (reminiscent of a folded long chain) and the polar peptide bonds acted as the charged unit. Fig. 1 shows examples of a seven-carbon system of a bicyclic structure. Clearly, it is unlike classical amphiphiles having a charged head group and one or two long (acyl or acyl-like) chains. Specifically, we chose the bicyclic unit bispidine as a spacer between two amino acids (Fig. 1). Compounds 1–4, in which two amino-acid units are linked via a rigid bicyclic bispidine, were synthesized from Boc-protected piperidone, benzyamine, and formaldehyde through a double Mannich reaction, followed by Wolff-Kishner reduction of bispidinone. The Boc and benzyl-protected bispidine was converted to the designer systems 1–4 (Schemes S1 and S2†).

Pseudopeptosomes from a self-assembling pseudopeptide

To investigate the self-assembling properties of synthesized molecules, compound 1 was dissolved in methanol (1.43 mM) and the resulting assemblies analyzed by high-resolution microscopy (scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM)) (Fig. 2), along with dynamic light scattering (DLS). SEM images of 1 show spherical vesicular aggregates of
size 200–1100 nm (Fig. 2a, Fig. S4a†). Concentration-dependent studies on 1 showed initial formation of sheets (Fig. S1a†), followed by toroid formation at a concentration of 0.715 mM (Fig. S1b†) and, on further increase in concentration, formation of vesicular assemblies (Fig. S1c†). Compound 1 also showed vesicular assembly in water: ethanol (1:1) (Fig. S1e†). A spherical “hollow” or “encapsulable” nature of the formed vesicles on 1 was evident from TEM, which displayed a clear contrast in Fig. 2b and Fig. S2.† The volume of a hollow sphere is given by \( V = \frac{4}{3} \pi R^3 - \frac{4}{3} \pi r^3 \), where \( R \) is the outer radius and \( r \) is the inner radius. TEM data showed \( R \) to be \( \sim 500 \) nm and \( r \) to be \( \sim 350 \) nm (Fig. S2†). Furthermore, the volume of one molecule of compound 1 (calculated from its crystal structure), \( V_{\text{mol}} \), was \( \sim 1.5 \) nm \( \times 1.5 \) nm \( \times 1.5 \) nm = \( \sim 3.375 \) nm\(^3\). Therefore, the number of molecules resulting in the observed vesicular self-assembly (i.e., a pseudopeptosome) = \( V/V_{\text{mol}} \sim 10^8 \).

The critical aggregation concentration (CAC), which is analogous to the critical micellar concentration and critical vesicular concentration, of 1 was determined to be 175 \( \mu \)M based on DLS (Fig. S3b†). AFM imaging of 1 (Fig. 2c), while showing circular vesicles, also revealed the ratio of diameter : height larger than 1:1. However, as noted earlier, sample preparations for AFM may result in flattening of spherical vesicles, presumably due to evaporation of the solvent from the vesicle and interaction with the surface. DLS studies of 1 revealed vesicle size to be 200–1100 nm (Fig. S3a†), which was consistent with AFM measurements (Fig. 2d) and SEM data (Fig. S3 and S4a†).

**Pseudopeptosomes from different self-assembling pseudopeptides in different solvent environments**

Next, the effect of bispidine on the nucleation of vesicular self-assembly with different amino acids and in different solvents was analyzed. Compounds 3 and 4 were synthesized to observe the effect of the “hydrophathy index” of the amino acids (side chain) on self-assembly using SEM and epifluorescence microscopy (Fig. 3).

SEM of compounds 3 and 4 again showed vesicular assembly (Fig. 3a and b, Fig. S4c and d†) implying that bispidine plays a part in vesicular assembly rather than the hydrophathy index of amino acids (i.e., their side chains). The water-soluble compound 2 was also synthesized to see the solvent effect on the formation of vesicular assembly (Scheme S1†). Interestingly, compound 2 in water also formed vesicles of similar sizes (Fig. 3c) and the population of vesicles increased in 10% solution of methanol and water (Fig. S3f), which again showed that a bispidine scaffold had a significant role in forming the self-assembled structures. Compound 2 was a versatile molecule because it could form vesicles in methanol, water and mixture of water and methanol (Fig. 3c, Fig. S3f).

To obtain further insights into the mechanism of formation of vesicular assembly, fluorescence microscopy was undertaken using two “location-sensitive” measurements. Tryptophan (in compounds 1 and 2) has fluorescence properties, but rhodamine B (RB) dye was chosen to probe the polar environment of vesicles. Fig. 3d shows an epifluorescence microscopy image at 100× magnification of 1 + 0.02-equivalents of Rhodamine B dye at a UV filter. The length of the scale bar is 2 \( \mu \)m.

**Pseudopeptosomes have biocompatible compartments**

Confocal microscopy was used to overcome the observational limitations presented by epifluorescence microscopy. Targeted Z-optical sections (100 nm) were observed using specific laser sources for excitation (Fig. 4). Fig. 4a clearly shows RB to be encapsulated in the pseudopeptosomes formed by 1 but also carrying 0.02-equivalents of RB. Tryptophan fluorescence clearly showed a difference between fluorescence on the periphery and in the center of the vesicle.

Confocal microscopy of NR-stained pseudopeptosomes, showed NR on the periphery (Fig. 4b, Fig. S6b). Confocal microscopy of NR-stained pseudopeptosomes, including their Z-stack analyses, showed NR on the periphery of pseudopeptosomes of 1 (Fig. 4b, Fig. S6f). Interestingly, SEM data showed a slight increase in the vesicular sizes of 1 upon encapsulation with RB (Fig. S1df†). This result indicated the: (a) key roles of weak interactions in assembly of pseudopeptosomes similar to classical vesicular assemblies; (b) possibility of stoichiometric control of “cargo-dependent” size of pseudopeptosomes (but without any direct interaction with pseudopeptides, as demonstrated in subsequent results). Hence, we obtained conclusive evidence of pseudopeptosomes having appropriate compartments with hydrophobic shells encapsulating polar materials.
In addition, we also noted interesting vesicular properties of 1, such as “fusion” and possible morphological transitions, as represented by fused vesicles and possible toroids (though the latter may have been a result from optical sectioning). These data showed that shells, despite being relatively thick, were also flexible. The expansion of vesicles in the presence of a guest such as RB indicates the plasticity of the vesicular assembly. RB molecules made several non-covalent interactions between the aromatic chromophores of tryptophan.

To further expand the application of pseudopeptide 2, we used a fluorescein isothiocyanate (FITC)-tagged peptide: corneal targeting sequence 1 (CorTS1). This peptide has been reported to have an activity against methicillin-resistant Staphylococcus aureus (MRSA) and Fusarium dimerum.

We used two cargos (RB and CorTS1) and carried out a cell-uptake (monitored by measuring fluorescence using confocal microscopy) experiment in the presence of 2. At a concentration of 10 μM, FITC fluorescence of CorTS1 peptide was not observed in cells (Fig. 5). However, when RAW264.7 cells were treated with CorTS1 mixed with pseudopeptide 2 (200 μg ml\(^{-1}\)) and RB (5 μg ml\(^{-1}\)), higher transport of CorTS1 to cells was noted. These results supported the notion of a cell-penetrating ability of pseudopeptide 2 and its ability to carry cargo.

We wished to obtain some mechanistic information on the self-assembly of our pseudopeptides into pseudopeptosomes. Fourier transform infrared (FT-IR) spectroscopy of compound 1 showed an intense band at 3418 cm\(^{-1}\) indicating non-hydrogen-bonded NHs. IR absorption bands at ~1634 cm\(^{-1}\) (amide I) revealed a β-strand arrangement (Fig. S9†). A band at 1696 cm\(^{-1}\) also indicated β-strand structure. The chemical shift of C\(^\text{H}\) of the amino acids in all compounds was moved downfield as compared with that of a random coil, thereby confirming a β-strand structure (Table S1†).

Circular dichroism (CD) of compound 1 showed a negative band at 222 nm and positive bands at 232 nm and 205 nm (Fig. 6a). The band at 232 nm indicated interactions between the aromatic chromophores of tryptophan. The negative band at ~222 nm indicated β-strand formation.

The powder XRD (PXRD) patterns of compound 1 exhibited a reflection peak in the wide-angle region, which is characteristic of a typical π–π stacking distance ~3.35 Å (Fig. 6b), and indicated that hydrogen bonding and π–π stacking interactions were involved in vesicular self-assembly. The CD spectrum of compound 2 showed a band at ~232 nm (which indicated interactions between the indole units of the Trp residue) (Fig. 6c) and a negative band at ~222 nm (which indicated β-strand formation). The CD spectra of compounds 3 and 4 (Fig. 6c) also showed β-strand formation.

The single-crystal X-ray structure of 1 (Fig. 7) also revealed a Trp-zip arrangement which supported the PXRD data. The crystal structure of 1 (Fig. 7a, Table S2†) showed bispidine in a chair–chair conformation, and two carbonyls are anti to each other. The anti-arrangement allowed the molecules to pack bidirectionally through hydrogen bonding. The indole NH of Trp hydrogen-bonded with neighboring molecules through the amide carbonyl, thereby forming a one-dimensional string (Fig. 7b). The other indole took part in hydrogen bonding with a carbonyl group of the neighboring molecule through a water molecule (Fig. 7c). The Trp involved in water-mediated hydrogen bonding further associated to form a Trp-zipper (Fig. 7d).
The Leu-appended compound 3 was also crystallized from DMSO (Fig. 7e, Table S3†). The crystal structure reported here is very similar to that of a crystal from chloroform.\textsuperscript{19} Compound 3 organized to a one-dimensional string through hydrogen bonding of amide (Fig. 7f and g). Despite subtle differences in the hydrogen bonds in 1 and 3, both self-
assembled to form vesicles in solution. The crystal structures of 1 and 3 suggested that both formed extended packing that became bent eventually to form vesicles. Interestingly, vesicles could be made from various amino acids containing peptides. The common feature in all of these peptides was bispidine, so it had a nucleating effect for vesicular assembly. The pseudo-peptide design was versatile because it allowed for the design of vesicles in an organic solvent (organo-vesicles) in the case of 1 and aqueous-vesicles and organo-vesicles in the case of 2. These vesicles were stabilized in polar solvents such as methanol and water.

A concentration-dependent nuclear magnetic resonance (NMR) study of 1 showed an upfield shift in aromatic protons and downfield shift in indole NH (Fig. S10†). These data supported the role of aromatic interactions and hydrogen bonding have important roles in vesicle formation. Thus, it appears that, as opposed to classical vesicular assemblies that rely on specific dominance of hydrophobic interactions operating only in aqueous solution, the pseudopeptosomes we prepared utilized the full spectrum of all four weak interactions that are essential in biological systems.

Considering that pseudopeptosomes are formed by self-assembly of pseudopeptides and utilize the full spectrum of the four weak interactions, we wanted to test them for reversible self-assembly in different solvents. The removal/addition of chloroform in the solvent environment of compound 1 was carried out. Remarkably, Fig. 4c shows the solvent-dependent reversibility of pseudopeptosome formation of 1 as observed by confocal microscopy. The reversible behavior in pseudopeptosome assembly allows exploration of applicational avenues such as creation of reversible sub-micron reaction centers.

**Solvation effects the assembly of pseudopeptosomes**

Inspired by the discovery of the solvent-dependent reversible vesicular self-assembly of 1, we carried out detailed high-resolution microscopy studies to test the effects of polar/non-polar environments on vesicular assembly of compound 1. The effects of varying the solvent environment created by titrating...
methanol and chloroform on pseudopeptosome formation are shown in Fig. 8. Vesicular assemblies were not observed in pure chloroform, but methanol : chloroform at 1 : 5 favored vesicular formation. Furthermore, methanol : chloroform at 3 : 1 showed partially formed or partially formed vesicles.\textsuperscript{35,36} Complete vesicles were formed in methanol : chloroform at 1 : 1. An increase in the percentage of methanol resulted in an increase in vesicle size (Fig. 8). These results clearly showed that methanol favoured, whereas chloroform disrupted, vesicular assembly.

Diffusion ordered spectroscopy (DOSY) of compound 1 at different concentrations in methanol showed that diffusion-coefficient values decreased upon increasing concentration (Fig. S11\textsuperscript{†}) and that the diffusion coefficient in chloroform : methanol at 1 : 1 was lower compared with that of chloroform solution (Fig. S12\textsuperscript{†}), again confirming self-assembly in methanol (molecules of compound 1 were expected to show a lower diffusion coefficient in self-assembled forms compared with being free).

Fluorescence spectroscopy showed that the intrinsic fluorescence of compound 1 at 339 nm first increased until 0.130 mM (Fig. S13a\textsuperscript{†}), and then fluorescence was quenched upon increasing the concentration (Fig. S13b and S13c\textsuperscript{†}). Interestingly, the concentration at which quenching was observed was equivalent to the CAC calculated by DLS (Fig. S3\textsuperscript{†}). Addition of 0.02-equivalent of RB to compound 1

![Fig. 7](image-url) (a) and (e) are the X-ray crystal structures of 1 and 3 with a 50% probability level of atomic displacement ellipsoids. (b) One-dimensional extended structures of 1 showing hydrogen bonding between carbonyl oxygen and indole ring of one Trp unit (purple). (c) Indole ring of the Trp unit takes part in hydrogen bonding to the carbonyl group of the neighboring molecule through a water molecule (cyan). (d) The Trp-Zip arrangement mediated by C–H···π interaction (spacefill model) between the π-system of Trp and C–H from neighboring indole (distance = 2.87 Å and angle = 157.2°). (f) One-dimensional stringing amide hydrogen bonding. (g) Structural representation of amide hydrogen bonding in 3 observed in the solid state.
An excellent TURN ON fluorescence was observed upon the gated with H$_2$PO$_4^-$, supporting a multilayer arrangement (model III) (Fig. 9a(iii) and b). Hence, model III is most preferred in our system.

The self-assembly of 1 is influenced by the concentration and solvent system. At 0.35 mM, morphology is a sheet. At 0.72 mM, morphology is half-toroids and toroids, and vesicles are observed mainly at 1.43 mM. Similarly, methanol favors vesicles, while chloroform does not favor vesicular assembly, as shown in the phase diagram (Fig. 9c).

The anion-sensing property of compound 1 was also investigated with H$_2$PO$_4^-$, F$^-$, Br$^-$, HSO$_4^-$ and I$^-$ as their TBA salts. An excellent TURN ON fluorescence was observed upon the addition of some anions (Fig. S14a†), while such changes were not prominent with other anions. Among all anions (F$^-$, Cl$^-$, Br$^-$, I$^-$, HSO$_4^-$, H$_2$PO$_4^-$) tested, only F$^-$ and H$_2$PO$_4^-$ caused maximum fluorescence enhancement. Binding of H$_2$PO$_4^-$ resulted in a 17 nm red-shift in fluorescence maxima. Job plots showed a 1 : 1 stoichiometry (Fig. S14b†). These results indicated that, while pseudopeptides designed and created by us assembled into pseudopeptosomes, they also have the potential to be developed into sensors.

Conclusions

In general, it is thought that vesicular assemblies, mimicking biological cells and subcellular compartments, are formed due to the hydrophobically-driven self-assembly of amphiphilic molecules in aqueous environments. At the same time, manifestations of biological functions result from the action of polymeric structures (predominantly proteins) within particular ranges of environmental variables. Vesicular assemblies utilize the full spectrum of four weak interactions (hydrogen-bonding, ionic interactions, van der Waals forces and hydrophobic interactions). Clearly, a balance between maintaining morphological rigidity vs. flexibility along with retention of reversible vesicular self-assembly is a hallmark of molecular assemblies relevant to biological systems. We took inspiration from: (a) amino acids as building blocks of proteins that allow a myriad of functional structures realized through various physicochemical interactions; (b) amphiphilic assemblies resulting in the formation of whole cells and subcellular compartments; (c) molecular topology-based concepts found in biological assemblies and protein folding. Our molecular-topology based design of pseudopeptides, with amino acids appended on rigid bispidine, deviates from the classical “polar head group and hydrophobic tail” model of amphiphiles. Providing net polarity to one part of a designer molecule by orienting dipoles within structures that have inherent carbon–chain arrangements for hydrophobicity obviates the need for a separate “polar head” or “long tails” for vesicular self-assembly. We described vesiculation in pseudopeptides containing three amino acids (Ala, Leu, and Trp). This design allowed fabrication of vesicles in organic and aqueous solutions. More importantly, controllable formation of pseudopeptosomes allowed full exploration of all possible weak interactions in self-assembling systems rather than relying heavily on just one vs. the other. For example, pseudopeptosomes formed by compound 1 in MeOH and MeOH:water at 1 : 1 indicated that hydrophobic interactions were not the predominant mode of vesicular assembly, implying that an entropy-driven process was not the favored one. Addition of MeOH favored hydrogen-bonding, hence implying an enthalpy-driven assembly. The hydrogen bond-assisted “sheets” eventually became curved (possibly due to other weak interactions, including hydrophobicity), resulting in vesicles. Important parts were also played by other local interactions, such as Trp-zippers. Thus, we showed that a combination of dipoles and hydrophobic parts in molecular entities (and not just charged units and long-chain hydrocarbons) could be generic struc-
tural requirements for molecules self-assembling into vesicles. Our work has direct relevance to chemical and synthetic biology, and also provides applicational ideas for designing biocompatible delivery vehicles.

Author contributions

VH conceived the study. VH and AM designed experiments and assisted in data analyses. HS participated in designing experiments, carried out the experiments, collected the data, prepared the figures and analyzed the data. PP assisted in carrying out epifluorescence and confocal-microscopy experiments as well as carrying out cytotoxicity studies, collecting and analyzing relevant data. HS, VH and AM wrote the final version of the manuscript with input from PP.

Data availability

All synthetic procedures, microscopic analysis, and NMR data supporting this article are in the ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

VH is grateful to the Department of Science and Technology, Government of India, for support. HS thanks the Council of Scientific and Industrial Research, New Delhi, for a fellowship. PP thanks University Grants Commission, New Delhi, for a fellowship. AM is grateful for financial support from the Kusuma Trust (UK), which allowed him to establish the confocal-microscopy facility at IIT Delhi. The authors are grateful to the Department of Chemistry, Central Research Facility, and the Kusuma School of Biological Sciences at IIT Delhi. We thank Upanshu Gangwar for helping in DOSY NMR experiments. We thank Professor Archana Chugh for providing the FITC-labelled peptide.

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