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**Lipid-like Self-Assembling Peptide Nanovesicles for Drug Delivery**

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**ABSTRACT:** Amphiphilic self-assembling peptides are functional materials, which, depending on the amino acid sequence, the peptide length, and the physicochemical conditions, form a variety of nanostructures including nanovesicles, nanotubes, and nanovalves. We designed lipid-like peptides with an aspartic acid or lysine hydrophilic head and a hydrophobic tail composed of six alanines (i.e., ac-AK-CONH$_2$, KA$_6$-CONH$_2$, ac-A$_6$D-CONH$_2$, and DA$_6$-COOH). The resulting novel peptides have a length similar to biological lipids and form nanovesicles at physiological conditions. AFM microscopy and light scattering analyses of the positively charged lipid-like ac-A$_6$K-CONH$_2$, KA$_6$-CONH$_2$ peptide formulations showed individual nanovesicles. The negatively charged ac-A$_6$D-CONH$_2$ and DA$_6$-COOH peptides self-assembled into nanovesicles that formed clusters that upon drying were organized into necklace-like formations of nanovesicles. Encapsulation of probe molecules and release studies through the peptide bilayer suggest that peptide nanovesicles may be good candidates for sustained release of pharmaceutically active hydrophilic and hydrophobic compounds. Lipid-like peptide nanovesicles represent a paradigm shifting system that may complement liposomes for the delivery of diagnostic and therapeutic agents.

**KEYWORDS:** lipid-like peptides, designer peptide surfactants, liposome alternatives, tunable peptides, controlled release

## INTRODUCTION

Molecular self-assembly has enabled the fabrication of nanostructures and the development of advanced functional materials. The design and synthesis of biologically inspired molecules with self-assembling properties has significantly advanced the field of biomaterials and includes peptide, oligonucleotide, and polysaccharide systems. Depending on the amino acid sequence, self-assembling peptides have varying properties and have been tested in biomedicine as permissive materials, which, depending on the amino acid sequence, balance and strength of the intermolecular interactions between the monomers, which determine the formation of hierarchical supramolecular structures of different morphologies and properties. Since their discovery in 2002, self-assembling lipid-like peptides have been studied by many groups; these studies improved our understanding and highlighted the importance of the system. The development of lipid-like self-assembling peptides with surfactant properties has opened new avenues for applications in biotechnology for the stabilization of membrane proteins more effectively than commercial detergents and in nanotechnology for the construction of energy conversion devices.

Lipid-like self-assembling peptides are amenable to molecular design allowing modifications in the number, type, and order of amino acids on the peptide chain as well as incorporation of pH of the medium. These factors affect peptide alignment, packing density, and strength of the intermolecular interactions between the monomers, which determine the formation of hierarchical supramolecular structures of different morphologies and properties. Since their discovery in 2002, self-assembling lipid-like peptides have been studied by many groups; these studies improved our understanding and highlighted the importance of the system. The development of lipid-like self-assembling peptides with surfactant properties has opened new avenues for applications in biotechnology for the stabilization of membrane proteins more effectively than commercial detergents and in nanotechnology for the construction of energy conversion devices.

Lipid-like self-assembling peptides are amenable to molecular design allowing modifications in the number, type, and order of amino acids on the peptide chain as well as incorporation of pH of the medium.
active peptide sequences to facilitate cell penetration or reactive chemical groups such as fluorescent dyes or biotin. The ease of production and the wide scope of modification allow for the synthesis of designer sequences with “tailor-made” tunable properties. In this work, we set out to investigate the physicochemical of cationic and anionic lipid-like peptides, and determine the release kinetics of model drug compounds through peptide formulations as a first step towards the development of a peptide-based drug delivery system.

**MATERIALS AND METHODS**

**Lipid-like Self-Assembling Peptides.** Ac-A,K-CONH₂, K₆-CONH₂, ac-A,D-COOH, and D₆-A-COOH were received in powder (SynBioSci, Livernmore, CA). The purity of the peptides was 90–94% as determined by electrospray ionization-quadrupole-time-of-flight (ESI-Q-TOF) mass spectrometry. Peptides were dispersed in PBS pH 7.4, probe sonicated for 10 min to facilitate dispersion, and equilibrated for 15 min to allow for self-association of the monomers.

**CMMC of Lipid-like Peptides.** DLS was used to determine the peptides’ CMMC (PDDLS/Batch setup, Precision Detectors, Franklin, MA). Solutions of different peptide concentrations in PBS pH 7.4 were filtered through 0.45 μm pore size filters prior to measuring. Scattered light was detected at 90° and recorded and processed by Precision Deconvolve. The solvent viscosity of the suspended particles were determined using the Stokes formula (for a given concentration of Nile red in water), where

\[ \tau = \frac{2\pi D}{k_B T} \]

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was the distribution of relaxation times (which is equal to \( \tau \) is the product of the particle size and the solvent viscosity, and \( \tau \) is the apparent hydrodynamic radii of the suspended particles were determined using the Stokes–Einstein relation \( R_g = k_B T/6\pi D \eta \), where \( k_B \) is the Boltzmann constant, \( \eta \) is the viscosity of the solvent, and \( D \) is the diffusion coefficient of the particle which is equal to \( D = (1/\tau)q^2 \), where \( q \) is the relaxation time of g(2)(q,t).

**AFM of Peptide Nanovesicles.** 3 μL of the peptide vesicle dispersions (20 mg/mL of peptides in PBS filtered through 0.45 μm filters) were deposited on mica (G250-2, rms roughness 8 nm, Veeco Instruments SAS, France). Images were collected by the Siegert relation:

\[ g(2)(q,t) = \frac{1 + f^2 q^2 g(2)(q,t)}{2} \]

where \( f = \frac{2\pi D}{k_B T} \) is an instrumental factor (in our system \( f = 0.95 \)). Hence, \( g(2)(q,t) \) (for simplicity we drop the q-dependence) was analyzed as a weighted sum of independent exponential contributions using the CONTIN algorithm. The apparent hydrodynamic radii of the suspended particles were determined using the Stokes–Einstein relation \( R_g = k_B T/6\pi D \eta \), where \( k_B \) is the Boltzmann constant, \( \eta \) is the viscosity of the solvent, and \( D \) is the diffusion coefficient of the particle which is equal to \( D = (1/\tau)q^2 \), where \( q \) is the relaxation time of g(2)(q,t).

**RESULTS AND DISCUSSION**

A class of self-assembling peptides (i.e., ac-A,K-CONH₂, K₆-CONH₂, ac-A,D-COOH, and D₆-A-COOH) was designed to mimic lipids of biological membranes. These lipids have a hydrophobic tail composed of six alamines, a hydrophilic head, which is an amino acid with charged side group and a length of ~2.5 nm (Figure 1). As lipid-based systems, addition of lipid-like peptides to water or an electrolyte solution results in formation of a turbid suspension due to self-assembly of the peptide monomers to minimize the interaction between hydrophobic domains and polar environment. We determined the CMMC of lipid-like peptides in phosphate buffer saline (PBS, 100 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl at pH 7.4) to be 0.12 mg/mL for K₆-CONH₂, 0.09 mg/mL for K₆-A-CONH₂, 0.08 mg/mL for ac-A,D-COOH, and 0.06 mg/mL for D₆-A-COOH.

**Atomic Force Microscopy (AFM) Imaging.** We used AFM to study the morphology of lipid-like peptide assemblies. Previously, we reported that self-assembly of lipid-like peptides results in uncontrolled formation of various supramolecular structures including vesicles, micelles, and nanotubes. Herein, the experimental conditions typically resulted in nanovesicle formation (Figure 2). Size distribution analyses of the peptide nanovesicles showed that self-assembly of the lysine-containing, positively charged ac-A,K-CONH₂ and K₆-CONH₂ peptides results in larger particles (126 and 169 nm, respectively) compared to those observed from association of encapsulated CF was removed by centrifugation at 11 000 rpm for 20 min. Then the vesicles were re-suspended in PBS pH 7.4 to a final volume of 1 mL and were incubated at 25 °C. CF released through peptide nanovesicles (n = 4) was collected by centrifugation of the membrane tubes at 11 000 rpm for 20 min, the fluorescence intensity of the filtrate was measured, fresh PBS pH 7.4 was added to a final volume of 1 mL, and the suspension was incubated. This process was repeated at specific time points, and a graph was created to show the time course of released CF through peptide nanovesicles. All measurements were carried out in a PerkinElmer LS-50B spectrophotometer using 1 cm quartz cuvettes. The excitation wavelength was at 470 nm, and the emission maximum was observed at 520 nm.
the negatively charged ac-A<sub>D</sub>-COOH and DA<sub>C</sub>-COOH (28
and 44 nm, respectively) (Table 1, Figure 2 insets). This is
due to the smaller side chain of aspartic acid compared to lysine,
which allows for better packing of ac-A<sub>D</sub>-COOH and DA<sub>C</sub>-
COOH peptides in the nanovesicle bilayer as suggested
previously by molecular modeling studies.\(^9\)

AFM imaging of ac-A<sub>D</sub>-COOH and DA<sub>C</sub>-COOH peptide
assemblies revealed dispersed nanovesicles as well as necklace-
like ultra-structures (Figure 2 C–D). Such formations are
observed at the micrometer scale in colloidal systems, when a
drop of the suspension evaporates on a solid surface and have
been described by two dimensional crystallization laws,\(^22\)
or the coffee-ring effect\(^23\). It may be that such phenomena are
reproduced at the nanometer scale due to evaporation of the
solvent and repulsion of like-charged peptide nanovesicles. The
diameter of necklaces composed of ac-A<sub>D</sub>-COOH and DA<sub>C</sub>-
COOH nanovesicles is 200 and 159 nm, respectively (Table 1).
Image analysis showed that ac-A<sub>D</sub>-COOH necklaces have 20 ±
5 individual (average diameter 28 nm) ac-A<sub>D</sub>-COOH
nanovesicles per necklace whereas necklaces composed of
larger (average diameter 44 nm) DA<sub>C</sub>-COOH nanovesicles have 9 ± 3 nanovesicles per necklace.

These observations prompted inquiry about the mechanism
of necklace formation. Changing the sample drying conditions
(from quick nitrogen gas drying to slow air drying for 1 h)
revealed metastable nanovesicle clusters (Figure 2 E–F)
suggesting that prior to necklace formation ac-A<sub>D</sub>-COOH
and DA<sub>C</sub>-COOH peptide nanovesicles formed loosely bound
clusters with diameter 113 and 135 nm, respectively. It is likely
that during quick sample drying under nitrogen gas the
generally charged peptide nanovesicle clusters disassemble
and spread on the like-charged mica surface leading to nanovesicle
necklace-like formations. Theoretical calculations assuming
tight packing of hard spheres, revealed that 20 ac-A<sub>D</sub>-D
COOH nanovesicles with diameter 28 nm and 9 DA<sub>C</sub>-
COOH nanovesicles with diameter 44 nm form clusters with
diameter 97 and 120 nm, respectively.\(^24\) These values correlate
well with the AFM determined sizes of the ac-A<sub>D</sub>-COOH (i.e.,
113 nm) and DA<sub>C</sub>-COOH (i.e., 135 nm) nanovesicle clusters.

Dynamic Light Scattering (DLS). The hydrodynamic
radii, \(R_\text{h}\), of the peptide nanovesicle suspensions were
determined by DLS from which the intensity-time correlation
functions and the corresponding inverse Laplace transform
analyses of lipid-like peptide nanovesicles in PBS were
calculated (Figure 3). Analysis of the light scattering data
yielded monomodal peptide vesicle size distributions except in
the case of KA<sub>C</sub>-CONH<sub>2</sub> suspensions in which peak analysis
showed the presence of particles with average diameter of 164
and 906 nm; the latter probably represent aggregates of
individual nanovesicles.

DLS measurements of the ac-A<sub>D</sub>K-CONH<sub>2</sub> and KA<sub>C</sub>-CONH<sub>2</sub>
nanovesicle diameter are in agreement with the size of mica
deposited nanovesicles as determined by AFM. However, DLS
analysis of ac-A<sub>D</sub>D-COOH and DA<sub>C</sub>-COOH peptide nano-
vesicle suspensions revealed the presence of particles with
diameter 99 and 137 nm, respectively (Figure 3). These values
deviate from the nanovesicle diameter determined by AFM for
dispersed ac-A<sub>D</sub>-COOH and DA<sub>C</sub>-COOH nanovesicles but
 Table 1. Size of Individual Lipid-Like Peptide Nanovesicles, Peptide Nanovesicle Necklace-Like Formations and Peptide Nanovesicle Clusters As Determined by AFM (Dry State) and DLS Curve Fitting (Solution)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>AFM analysis</th>
<th>DLS analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vesicle diam. (nm)</td>
<td>necklace diam. (nm)</td>
</tr>
<tr>
<td>ac-A_6K-COONH_2</td>
<td>126 ± 23</td>
<td>122 (30–173)</td>
</tr>
<tr>
<td>KA_6-COONH_2</td>
<td>169 ± 29</td>
<td>164 (65–350)</td>
</tr>
<tr>
<td>ac-A_6D-COOH</td>
<td>28 ± 9</td>
<td>200 ± 11</td>
</tr>
<tr>
<td>DA_6-COOH</td>
<td>44 ± 11</td>
<td>159 ± 26</td>
</tr>
</tbody>
</table>

Figure 3. Inverse Laplace transform analysis of the time correlation functions of lipid-like peptide nanovesicles in PBS. Open circles, experimental data; solid lines, best fit curve obtained by inverse Laplace transform; solid squares, volume distributions of the hydrodynamic radii. The hydrodynamic diameters for each distribution are also shown.

Figure 4. Release kinetics of (A) carboxyfluorescein, CF and (B) Nile red through (●) ac-A_6K-COONH_2, (▲) KA_6-COONH_2, (♦) ac-A_6D-COOH and (⧫) DA_6-COOH peptide nanovesicles in PBS. (B Inset) Nile Red emission spectra upon interaction with the lipid-like peptide bilayer in PBS. All data points represent the average of four samples.

The hydrodynamic diameter of the nanovesicles (Table 1) appears to be slightly smaller than that measured by AFM which may be due to slight deformation of the nanovesicles caused by adsorption on the mica surface and/or AFM tip broadening effects. Therefore, the size of the nanovesicles measured by AFM (dry state) correlates well with the hydrodynamic diameter determined by DLS. This suggests that nanovesicle shrinking is negligible during drying that precedes AFM scanning.

Release of Hydrophilic Molecules through Peptide Nanovesicles. To determine whether peptide nanovesicles can be used in drug delivery applications, we measured the release kinetics of the hydrophilic fluorescent probe CF through the vesicle bilayer. Encapsulated CF is strongly quenched, and therefore, only the released CF contributes to the fluorescence emitted. Figure 4A shows that positively charged ac-A_6K-COONH_2 peptide nanovesicles retained CF entrapped for more than 6 h whereas negatively charged ac-A_6D-COOH nanovesicles released CF in ~3 h. Nanovesicles composed of the positively charged KA_6-COONH_2 and the negatively charged DA_6-COOH peptides released CF within ~1 h.

This suggests that electrostatic interactions do not affect CF encapsulation and release through the nanovesicles because (i) the less positively charged ac-A_6K-COONH_2 nanovesicles retained the negatively charged CF better than the more positively charged KA_6-COONH_2 nanovesicles (KA_6-COONH_2 carries two positive charges while ac-A_6K-COONH_2 peptide has one positive charge, Figure 1) and (ii) the negatively charged DA_6-COOH nanovesicles did not retain CF better compared to the more negatively charged ac-A_6D-COOH nanovesicles (DA_6-COOH overall carries one negative charge at the C-terminal whereas ac-A_6D-COOH carries two negative charges at the C-terminal). These results suggest that peptide nanovesicles can be used for encapsulation and controlled release of hydrophilic compounds.

Hydrophobic Molecule Uptake and Release through Peptide Nanovesicles. We next studied the hydrophobic character of the peptide nanovesicle bilayer using Nile red as probe molecule, which is hydrophobic and often used to assess liposome bilayer stability. The fluorescence emitted by Nile red in water is weak and shows maximum at ~660 nm. The intensity increases and the emission maximum is blue shifted when Nile red is buried in a hydrophobic environment shielded from the polar solvent. Figure 4B, inset, shows the emission spectra of Nile red incorporated into lipid-like peptide nanovesicles. Depending on the peptide formulation emission maxima were observed between 621 and 633 nm, which suggests that assembly of the peptides’ hydrophobic tails results in the formation of a bilayer with hydrophobic properties. The fluorescence intensity was higher in the case of Nile red.
interacting with ac-A,D-COOH nanovesicles compared to that emitted by Nile red incorporated in ac-A,D-K-CONH₂ nanovesicles. This result suggests that the ac-A,D-COOH bilayer accommodated more Nile red molecules with better shielding from water compared to Nile red inside the ac-A,D-K-CONH₂ bilayer. Ac-A,D-COOH and ac-A,D-K-CONH₂ have similar CMCs and therefore, the observed differences in the emitted fluorescence of nanovesicle incorporated Nile red is likely due to better packing of the ac-A,D-COOH peptide monomers in the bilayer. This results in the presentation of a more hydrophobic environment for incorporating Nile red in the ac-A,D-COOH bilayer compared to the ac-A,D-K-CONH₂ bilayer. The fluorescence intensity of Nile red interacting with K,A,C-CONH₂ and D,A,C-COOH nanovesicles was significantly lower suggesting less Nile red incorporation in these peptides’ bilayers. Furthermore, we studied the release kinetics through peptide nanovesicles, and we found slow Nile red release through ac-A,D-COOH and ac-A,D-K-CONH₂ nanovesicles reaching significant levels after 6 and 4 h, respectively (Figure 4B). K,A,C-CONH₂ and D,A,C-COOH nanovesicles appeared to be less stable and released Nile red within 2 and 1 h, respectively. Notably, the release kinetics of Nile red through the peptide nanovesicles follow the order of Nile red fluorescence emission incorporated inside the hydrophobic environment of the peptide bilayer (Figure 4B and inset). As in the case of CF release, we observed that ac-A,D-K-CONH₂ and ac-A,D-COOH nanovesicles retain Nile red for prolonged periods of time suggesting that these peptide nanovesicles can be used for sustained delivery of hydrophilic and hydrophobic compounds.

**Cell Viability.** To determine the effect of lipid-like peptides on Caco-2 cell viability, we used the MTT assay. Figure 5 shows a constant increase in cell numbers after 3 and 24 h incubation with and without 0.2 or 1.0 mg/mL lipid-like peptides (P < 0.05). These results suggest that ac-A,D-K-CONH₂, K,A,C-CONH₂, ac-A,D-COOH, and D,A,C-COOH lipid-like peptides do not affect Caco-2 cell proliferation compared with the control.

### CONCLUSIONS

Lipid-like peptides were designed to mimic natural lipids having a hydrophilic head and a hydrophobic domain. Although all peptides form nanovesicles some of them do not retain model drug probe molecules for prolonged times. Peptide design by altering amino acid sequence and charge distribution provides a means to control the loading capacity and the drug release kinetics through the nanovesicles. Of the two leading lipid-like peptide nanovesicle systems (i.e., ac-A,D-K-CONH₂ and ac-A,D-COOH), the latter may be more suitable for drug delivery. Negatively charged drug carriers, such as the ac-A,D-COOH nanovesicles, are preferable for intravenous administration because they result in electrostatic repulsions with the like-charged surface of blood cells and vessel walls, which allows for prolonged circulation in the bloodstream.

Liposomes were proposed as drug delivery carriers in the 1970s. A significant amount of work has been done to increase liposomal stability in serum, prolong drug release and reduce side effects associated with immunogenicity and toxicity of liposomes. Lipid-like peptides are nontoxic, nonimmunogenic and may encapsulate or incorporate and slowly release both hydrophilic and hydrophobic drug molecules and may present a platform to append to existing liposomal drug release systems.

Peptide self-assembly is similar to that of lipids and fatty acids. However, peptides differ from these systems because the peptide bilayer is stabilized by a combination of hydrophobic interactions of the hydrophobic amino acids’ side groups and hydrogen bonding of the peptides’ polar backbones. Therefore, the bilayer’s internal chemistry differs between liposomes and peptide nanovesicles. However, lipid-like peptides readily mix with lipids to form hybrid peptide/lipid liposome systems. The incorporation of lipid-like peptides in liposomes conferred functionality and modulated the liposome bilayer curvature and stability of the formulation. Furthermore, lipid-like peptides can be easily modified and tailored to incorporate other molecules such as sugars and functional motifs, including cell signaling and cell penetrating peptides to allow the synthesis of cell targeting drug delivery systems.

Depending on their sequence peptide nanovesicles can retain and slowly release both hydrophilic and hydrophobic compounds. We believe that these simple, inexpensive, and nontoxic peptides will open new paths in the field of vesicle-mediated drug delivery systems.

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**Notes**

The authors declare no competing financial interest.

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### ABBREVIATIONS:

ac; acetyl group; AFM; atomic force microscopy; CMC; critical micelle concentration; DLS; dynamic light scattering; CF; 5,6-carboxyfluorescein
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