Self-Assembly of Model Collagen Peptide Amphiphiles

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We have used cryo-transmission electron microscopy (cryo-TEM), small-angle neutron scattering (SANS), differential scanning calorimetry (DSC), and circular dichroism (CD) for microstructural characterization of amphiphiles that have a model collagen peptide headgroup. Single-tail amphiphiles and double-tail amphiphiles with short tails such as C12 and C14 formed spherical micelles. Further increase in tail length of the double-tail amphiphiles led to the formation of disklike micelles that aggregated to form a strandlike structure. SANS curves for double-tail amphiphiles were obtained at different contrasts by using different fractions of D2O in the D2O/H2O mixture. SANS data analysis using the sphere method confirmed the structures imaged by cryo-TEM and provided a detailed structural characterization. CD data showed that the peptide's capacity to intertwine into a triple helix by intertwining with two neighboring molecules can be affected by increasing the tail length. Double-tail amphiphiles with tails such as C18 and C20 that are crystalline at room temperature disrupt the association of the triple helix at room temperature. Increasing the temperature to melt the crystalline tails helps restore the triple-helical conformation in the headgroups.

Introduction

Amphiphiles self-assemble into aggregates in water and can be cast into monomolecular films on certain substrates.1 The amphiphile tail anchors the headgroup to the hydrocarbon–water interface of the aggregates and to the thin films, and as a result, the interfacial properties of such assemblies are governed by the headgroups. Using amphiphiles with different headgroup functionalities is a method of modifying interfacial properties.

Kunitake and Okahata2 developed a procedure for synthesizing amphiphiles that have been designed to have specific tails and headgroups. Synthetic amphiphiles have also been made by attaching various molecules to natural phospholipids, in particular to distearoyl phosphatidylethanolamine (DSPE). Such synthetic amphiphiles can influence the biological activity of the peptide by presenting the peptide’s capacity to intertwine into a triple helix by intertwining with two neighboring molecules. Increasing the temperature to melt the crystalline tails helps restore the triple-helical conformation in the headgroups.

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Properties to control cell–biomaterial interactions. Such amphiphiles can also be used to target drug-loaded vesicles to specific cells with which the peptide interacts. Appropriate presentation might become important for preserving the biological activity of the peptide and involves control of the peptide conformation and accessibility of the peptide to the receptor with which it interacts. Rational amphiphile design can lead to effective control of peptide presentation.

Recently, it has been shown that diacyl tails attached to (Gly–Pro–Hypro,–IVH1 (Gly–Val–Lys–Gly–Asp–Lys–Gly–Asp–Pro–Gly–Trp–Pro–Gly–Ala–Pro) peptide increased the stability of the peptide triple-helical conformation.3 IVH1 is a 15-amino-acid peptide sequence found in the triple-helical domain of type V collagen. IVH1 mediates melanoma cell adhesion and migration3 and is biologically active only in the triple-helical form.4 Previously, triple-helical structures have been stabilized by attaching (Gly–Pro–Hypro) repeat units to the peptide,5 by linking the three peptide termini by a linker,6,11 or by using a Kemp triacid (KTA) template to link the C termini of the three strands.12 Triple-helical stabilization in amphiphiles is thought to be due to the tight alignment of the three termini achieved by tethering the peptide to the self-assembled aggregates.

Amphiphilic self-assembled aggregates can have a variety of geometrical shapes.1 The shape of the aggregate can influence the biological activity of the peptide by affecting its presentation and conformation. Kunitake13 showed that the geometry of the aggregates affects the
optical activity of a chiral headgroup of a synthetic amphiphile. Lee et al. synthesized peptide amphiphiles with a peptide that had a site susceptible to trypsin cleavage. The cleavage rate of the peptide by trypsin was found to be higher when the amphiphiles were in micellar aggregates than when they were in bilayer aggregates. It was proposed that the crowding of the peptides when incorporated in bilayers prevented trypsin from accessing the cleavage site. Thus, understanding the self-assembly properties of peptide amphiphiles might allow for better design of amphiphiles that can be incorporated into vesicles for drug delivery purposes.

In this work, we have used cryo-transmission electron microscopy and small-angle neutron scattering to characterize the geometry of aggregates formed by peptide amphiphiles made by attaching monoalkyl and dialkyl tails to (Gly-Pro-Hyp)₄-IVH₁.

**Materials and Methods**

The synthesis procedures for the peptides and peptide amphiphiles are described elsewhere. Dialkyl tails required for double-tail amphiphiles were synthesized using the procedure described by Berndt et al. For single-tail amphiphiles, fatty acids of appropriate chain length were used as tails. The chemical structures and abbreviations of the peptide amphiphiles are shown in Figure 1. The peptide (Gly-Pro-Hyp)₄-IVH₁ is abbreviated as G4. The amphiphile molecular weights vary with the peptide amphiphile concentration was determined by UV absorbency analysis.

**Figure 1.** Chemical structure and nomenclature of single- and double-tail peptide amphiphiles.

Triple-helical structure

"Basic Unit" representation of the triple-helix for SANS data analysis

microscope and a Gatan model 626 transfer station. Images were recorded using a Gatan CCD camera and processed using Digital Micrograph 3.1. Some specimens were observed using a Philips CM-120 instrument fitted with a Gatan CCD 741 Multiscan camera and an Oxford model CT-2500 specimen holder and transfer station.

Samples for DSC were made in large-volume (60-µL) stainless steel pans fitted with an O-ring for volatile samples. Forty microliters of solution at a concentration of ca. 10 mg/mL was added to the pan, which was then crimped using a hand vise. DSC scans were run on the Perkin-Elmer Pyris DSC in the temperature range of 4–65 °C at a rate of 10 °C/min. The data were baseline-corrected. Solutions for CD were prepared the previous day and equilibrated overnight. CD spectra were collected with a JASCO 710 spectropolarimeter. Water-jacketed cylindrical cells of path length 0.01–1 cm were used depending on the signal intensity. The melting curves were obtained by measuring the CD signal at λ = 225 nm as a function of time. The temperature of the sample was changed at a rate of 0.2 °C/min for heating and cooling cycles.

Small-angle neutron scattering was carried out on the 30 m beamline (NG-7) at the National Institute of Standards and Technology (NIST), Gaithersburg, MD. The wavelength of radiation, λ, was on average 6 Å, with a spread in wavelength of 10%. Data were collected with the detector at distances of 1.0, 4.0, and 13.7 m from the sample. Samples were held in 1.0-mm-thick quartz cells in a sample chamber that was temperature-controlled to 25.0 ± 0.1 °C unless otherwise stated. A total of 10⁶ or more counts were collected for each sample. To place the data on an absolute scale, corrections were made for detector efficiency, the presence of background radiation, and the scattering of the empty cell. The corrected data were then circularly averaged to obtain curves of the differential scattering cross section of the sample dσ(q)/dq, as a function of the scattering vector q = 4π sin θ/λ, where 2θ is the scattering angle.

As mentioned in the Introduction, the headgroup of the IVH₁ peptide amphiphile folds into a triple-helical conformation. This type of secondary structure induces the formation of aggregates of three molecules, which will be referred to as the "basic units" (Figure 2). Conceptually, we viewed the self-assembly process in these systems as an aggregation of basic units whose tails are packed close together to form a hydrophobic core. The "headgroups" of the basic units, which surround the core, are stiff, cylindrical structures, about 86 Å long, with cross-sectional areas of 133 Å².

The scattering intensity from a dilute system of particles of identical shape (which is overall isotropic) can be regarded as the sum of the intensities scattered by the individual particles. If the particle size can be represented by a single size parameter...
R, then the total intensity (differential scattering cross section) of the sample \(d\Sigma(q)/d\Omega\) is given by the general relation\(^{18}\)

\[
\frac{d\Sigma(q)}{d\Omega} = \int_0^\infty D_n(R) \frac{d\Sigma(q,R)}{d\Omega} dR
\]  

(1)

where \(D_n(R)\) dR is the number of particles whose size is between \(R\) and \(R + dR\) and \(d\Sigma(q,R)/d\Omega\) is the differential scattering cross section of a single particle of size \(R\). Equation 1 can be easily generalized to describe particles having more than one characteristic size parameter (e.g., ellipsoids).

The calculated scattering curves shown in this paper were computed from eq 1, assuming a normal distribution of the particle sizes, using a self-written Visual Basic program. The calculated curve was fitted to the experimental data via a trial-and-error procedure to extract the average size and the standard deviation. Because of the complexity of particle geometry, explicit evaluation of \(d\Sigma(q,R)/d\Omega\) required numerical calculation. We employed the sphere method\(^{19}\), a widely used method for such computations. The particle is approximated by a finite number of homogeneous spherical elements, which do not necessarily describe realistic subunits of the particle but must be smaller than the smallest structural detail of interest. The scattering intensity is computed from the Debye formula\(^{18}\)

\[
\frac{d\Sigma(q)}{d\Omega} = \sum_i [V_i \eta_i \Phi_i(q)]^2 + 2 \sum_{i \neq j} V_i V_j \eta_i \eta_j \Phi_i(q) \Phi_j(q) \frac{\sin(qd_{ij})}{qd_{ij}}
\]

(2)

where \(V_i\) is the volume of the \(i\)th element; \(\eta_i\) is the fluctuation of the \(i\)th element, i.e., the difference between the element and the solvent scattering length densities; \(d_{ij}\) is the distance between the centers of the \(i\)th and the \(j\)th elements, and \(\Phi_i\) is the shape factor of the \(i\)th element, given by

\[
\Phi(qR) = \frac{3 \sin(qR) - qR \cos(qR)}{(qR)^3}
\]

(3)

The hydrophobic core was viewed as a solid body and was represented by closely packed spherical elements of equal size. To simplify the determination of their coordinates, the individual elements were placed on a cubic lattice with a spacing of 5 Å. The volume of the core (and thus that of the individual spheres) was determined from the aggregation number, assuming that the density of the hydrocarbon tails is identical to that of alkanes.

Figure 3. Cryo-TEM micrographs of single-tail amphiphiles: (a) sC12G4, 3 mM; (b) sC14G4, 3 mM; (c) sC16G4, 2 mM. The black areas in a are frost particles due to water condensation on the cold film, and part of the polymer support film is seen in b.
of the same chain length. The scattering length density of the core was determined from the chemical composition. Each headgroup of the basic units was represented by a 86 Å-long cylinder composed of eight spheres. The volume of the cylinder, 11,494 Å$^3$, was found by fitting to the entire set of experimental data. The scattering length density of the spheres representing the headgroup was taken as $2.6 \times 10^{-6}$ Å$^{-2}$. It should be noted that careful test calculations have shown that the sensitivity of the fit to variations in scattering length density is low.

Results and Discussion

Effect of Tail Length. Figures 3 and 4 show the micrographs of all of the amphiphiles examined at room temperature. Aggregates were studied in the amphiphile concentration range of 1–4 mM. No change was observed in the aggregate structure over that concentration range. The micrographs with the best resolution are presented here.

All single-tail amphiphiles formed spheroidal micelles that appear as black spots in the cryo-TEM micrographs. The micelles appear to be close-packed and organized on a lattice. Such organization has been observed before in other systems and is probably an artifact caused by blotting of the sample during sample preparation.

The aggregates formed by dC12G4 and dC14G4 are seen as spheroidal structures in the micrographs. Similar to the micelles formed by single-tail amphiphiles, these micelles appear to be aggregated and organized on an ordered lattice.

The SANS data of 1.5 mM dC12G4 solutions at three different contrasts, obtained by varying the D$_2$O concentration in the D$_2$O/H$_2$O mixture used to prepare the solution, are shown in Figure 5. The fits to the experimental data, shown as solid lines, were calculated from the sphere model shown in Figure 6. The SANS analysis supports the spheroidal micelle model. The aggregation number was found to be 78 basic units. The core is an oblate spheroid with major axes of 50 and 16 Å. The average outer radii of the spheroids were 101 and 134 Å, with a standard deviation of ca. 7 Å. It should be noted that the spheroid model does not fit the experimental data at low $q$ values. In particular, this model does not account

for the pronounced peak at \( q = 0.0159 \) Å\(^{-1}\), which corresponds to a Bragg distance of 66 Å. We suggest that the deviation of the model and the presence of the peak are due to interparticle interference. This claim might be surprising at such low concentrations. However, the length of the headgroup leads to the formation of large aggregates that are forced to pack closely. A simple calculation based on the aggregation number shows that, if the particles are to be arranged on a cubic lattice, this lattice should have a spacing of 64 Å, i.e., the distances between the particles are on the same order of magnitude as the particle size. Moreover, the calculated lattice size corresponds to \( q = 0.0162 \) Å\(^{-1}\), in close agreement with the experimental observation.

The SANS data for 4 mM dC14G4 solutions at three different contrasts are shown in Figure 7. The fits to the experimental data are shown as solid lines. From the SANS analysis, we concluded that the best geometric model is spheroidal micelles, in agreement with the TEM observation. The aggregation number was found to be 82 basic units. The core dimensions were 50 and 18 Å. The slight increase in the core size reflects the increase in the tail length. The average outer radii of the spheroids were 103 and 134 Å, with a standard deviation of ca. 8 Å. Again, a pronounced peak arising from interparticle interference appears at low \( q \) values.

Increasing the tail length to C16 resulted in a significant change in the aggregate structure. As seen in the micrograph (Figure 4), the aggregates are strands having diameters of about 200 Å. The strands exhibit a pattern of alternating black and white bands of 50-Å thickness that are separated by a distance of 50 Å from one another. Helical strands such as tobacco mosaic virus and the tail of bacteriophage T4 produce such patterns in TEM projection images.\(^{21,22}\) However, extensive simulations of various helical strand models failed to give a reasonable fit to the experimental SANS data (Figure 8). Another possible model that could explain the formation of a striped pattern is a stack of micelles. We examined models of spheroidal and disklike micelles. The best-fit model to the SANS data for four different contrasts (Figure 9) was found to be stacked disklike micelles, as shown in Figure 10. The hydrophobic core is disk-shaped, with an average spherical cross section radius of 50 Å and a standard deviation of 1 Å. The height of the core was 40 Å, which corresponds to bilayer formation. The aggregation number was found to be 114 basic units. The distance between the disks was found to be 197 Å, with a standard deviation of 14 Å. The outer dimensions of the disks were 206 and 240 Å, with a standard deviation of 14 Å.

Amphiphiles with longer tails such as C18 formed strandlike aggregates similar to those formed by dC16G4

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\(^{21}\) De Rosier, D. J.; Klug, A. Nature 1968, 217, 130.
but with a larger diameter. The SANS data for a 4 mM dC18G4 solution (Figure 11) could be fitted with a model of stack of disklike micelles. The height of the core was found to be 44 Å. The distance between the disks was 192 Å, and the standard deviation was 12 Å. However, this model was not sensitive to the disk diameter and could be fitted with any core diameter larger than 100 Å.

Increasing the tail length drove the structure to change from spheroidal micelles to bilayer aggregates. Several theoretical treatments predict such a progression. Surfactant number theory is the simplest model used to explain aggregate geometry in self-assembled structures.²³

The surfactant number is defined as \( \frac{v}{a} \), where \( v \) represents the volume occupied by the hydrocarbon tail, \( l \) the length of the tail, and \( a \) the optimal area occupied by the headgroup. Therefore, the ratio \( v/l \) is the cross-sectional area of the tails. Thus, the surfactant number can be considered as the ratio of the tail cross-sectional area to the headgroup cross-sectional area. The tail cross-sectional area becomes a constant after a certain tail length \([\text{CH}_2]_n\) and thus is independent of the tail length for the molecules that we studied. According to surfactant number theory, the aggregates formed by all of the amphiphiles we observed should be the same, which is not the case. This shortcoming of the surfactant number approach arises from the fact that it considers the contribution of the tails only on a geometrical basis.

Ben-Shaul and co-workers²⁴,²⁵ developed a theory that considers the configurational entropy of the tails in different aggregate geometries. Different geometries require space to be filled in different ways. A spherical structure has most of its volume close to the interface, whereas a bilayer aggregate has a uniform distribution of volume. Thus, chains in a spherical aggregate have to bend to occupy the volume close to the surface, whereas chains in a bilayer aggregate can extend straight into the layer. Using this construct, the theory predicts that longer tails pack favorably in low-curvature aggregates.

Other interesting observations in our system are the presence of disklike micelles as a transition structure between spheroidal micelles and bilayers and the association of the disks to form extended strands. Disklike micelles are a rarity in single-component systems. The structure of disklike micelles compels molecules to be located at interfaces with two different curvatures, as shown in Figure 12. Few examples exist of single-component systems that form disklike micelles,²⁶–²⁸ of which cesium perfluorooctanoate, a cesium salt of perfluorooctanoic acid, is the most extensively studied.²⁹

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reasons for the formation of finite bilayer aggregates such as disklike micelles are not clear. Because the hydrophobic edges of a finite aggregate are exposed to water, intuitively, one can argue that the bilayer aggregate should either extend up to infinity or fold over to form a vesicle and seal the exposed hydrophobic edges. Finite bilayer aggregates are predicted to exist if repulsive forces between the aggregates prevent them from aggregating and coalescing to form extended bilayers or if the edge energy of the disklike micelle is less than the bending penalty incurred during formation of a vesicle.30-32

The aggregation of disks into strands can be driven by a combination of enthalpy and entropy effects. Enthalpic factors such as the hydrophobic attraction between exposed (Gly-Pro-Hyp), repeat units at the center of the micelles might cause the aggregation. Measurements of the forces between two Langmuir-Blodgett deposited layers of dC16G4 using a surface force apparatus (SFA) detected electrostatic repulsion between the layers,33 suggesting that the above proposition about existence of hydrophobic forces cannot be true. The structure of the layers studied in the SFA was different in terms of the orientation of the headgroups from the surface of the disklike micelle; therefore, the results from the SFA experiments need not be valid in this system. Ordered phases have been observed in dilute solutions (1-3 wt %) of spherical, disklike, and rodlike particles that interact only through repulsive forces.34,35 In the absence of any attractive forces, aggregation must be driven by entropic factors. Theoretical treatments of such aggregation phenomena confirm that ordering is caused by an overall increase in the entropy of such systems.36 Because of the ordering of a fraction of the particles, the remaining particles in the disordered state have more volume to occupy, leading to an increase in the entropy of the disordered phase and in the total entropy of the system. Ordered phases in such dilute solutions have a characteristic spacing of several hundred nanometers.36 This spacing is 10 times larger than the spacing observed in the strands formed by aggregation of disklike micelles. Such a large difference indicates that entropic effects are not sufficient to explain the aggregation phenomenon. Further study of the aggregation phenomenon is required to thoroughly understand all of the responsible causes.

Effect of Temperature. dC18G4 and dC20G4 exhibit nonreversible chain melting transitions at 42 and 50 °C, respectively, according to the DSC data presented in Figure 13. The other double-tail amphiphiles did not show a chain melting transition in the temperature range investigated (4-65 °C). The nonreversible nature of the chain melting can be understood by comparing the CD melting curve of dC16G4, an amphiphile that does not exhibit a chain melting transition, with those of dC18G4 and dC20G4 (Figure 14). The melting curves for dC16G4 were reversible, and hence, only the heating curve is shown. At room temperature, the headgroups of dC16G4 are in a triple-helical configuration, as shown by the high value of the mean residual ellipticity (MRE). The decrease in the MRE value with an increase in temperature

33 Schneider, J. W. In Chemical Engineering and Materials Science University of Minnesota; Minneapolis, MN, 1998.
indicates that the triple helix denatures. For dC18G4 and dC20G4, the low value of the MRE implies that the headgroups are not in a triple-helical configuration at room temperature. The triple helicity increases sharply at a temperature that is very close to the chain melting temperature of the respective amphiphile. Such an increase suggests that, at temperatures below the chain melting temperature, the crystalline packing of the tails is not conducive for the formation of the triple helix, most probably because the crystalline packing of the tails does not allow the three constituent peptide strands to align in the correct register to fold into a triple helix. During the cooling process, as the helix refolds, the tails cannot approach sufficiently close to each other because the cross-sectional area of the triple helix (133 Å²) is larger than that of three double tails (123 Å²), and consequently, the tails cannot crystallize.

At temperatures above 50 °C, dC16G4 and dC18G4 formed spheroidal aggregates (Figure 15), whereas the geometry of dC12G4 and dC14G4 aggregates did not change up to 45 °C (data not shown). At higher temperatures, the triple helix denatures, leading to an increase in the area per headgroup, thus allowing for the formation of high-curvature aggregates. The SANS data recorded for dC16G4 at 55 °C (Figure 16) and for dC18G4 at 60 °C (data not shown) exhibit patterns similar to that for data obtained from spheroidal micelles of dC12G4 and dC14G4 (Figures 5 and 7). However, an exact fit was not possible because of our inability to model the molten triple-helical headgroup.

Surprisingly, the geometry of aggregates formed by dC16G4 and dC18G4 was not reversible, and cryo-TEM and SANS analyses confirmed that aggregates retained their spheroidal shape upon cooling to room temperature (data not shown). As discussed earlier, the peptide triple-helical conformation of dC18G4 goes through a significant change as a result of a heat–cool cycle. However, the CD melting curves for dC16G4 are reversible (Figure 14), indicating that the headgroup conformation at room temperature is independent of the thermal history of the solution. Therefore, changes in headgroup conformation cannot be a possible driving force for the observed phenomenon. These observations suggest that the geometry of dC16G4 and dC18G4 aggregates depends on the initial conditions of the solutions. If the solutions are made

![Figure 15](image1.png)

**Figure 15.** Aggregate structures at high temperatures. (a) dC16G4, 2 mM, 55 °C; (■) dC18G4, 3 mM, 60 °C.

![Figure 16](image2.png)

**Figure 16.** SANS curves for dC14G4 (4 mM) at 55 °C. The curves are at different mole percents of D₂O in the D₂O/H₂O mixture: (●) 100% D₂O, (■) 70% D₂O, (▲) 44% D₂O, (△) 0% D₂O.

at room temperature, long aggregates of disklike micelles are formed, whereas heating and cooling the solution results in the formation of spheroidal aggregates.

**Conclusions**

We have characterized aggregates formed by amphiphiles having a model collagen peptide headgroup with cryo-TEM and SANS. The aggregate geometry was sensitive to the tail length and, for certain amphiphiles, to temperature. Single-tail amphiphiles aggregated into spheroidal micelles. Double-tail amphiphiles with C12 and C14 tails also formed spheroidal micelles, but amphiphiles with longer tails aggregated into disklike micelles that stacked up to form extended strandlike structures. Only amphiphiles with C18 and C20 tails showed a chain melting transition, and the crystalline organization of the tails in such amphiphiles disrupted the triple-helical structure of the peptide headgroup. An increase in temperature affected the aggregate geometry of amphiphiles with C16 and C18 tails. The aggregate geometry of the above amphiphiles also depended on the thermal history of the solution. The aggregate geometries of the peptide amphiphiles studied in this work follow some of the trends seen for other more common surfactants;
however, such systems also show interesting behavior, such as the dependence of aggregate geometry on thermal history and the formation of extended strands, which might be a result of subtle interactions between the peptide headgroups.

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