

Self-assembling Behavior of Designer Lipid-like Peptides

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(Received 14 December 2005; Accepted 24 February 2006)

Even though enormous progress has been made in our understanding of soluble proteins and enzymes, our knowledge of membrane proteins, particularly integral membrane proteins, still lags far behind. Although a large number of chemical detergents and lipids are available, so far they are inadequate for tackling the problem of solubilizing and stabilizing membrane proteins outside of the lipid bilayer. Thus, the development of new detergents and lipid-like molecules is an important prerequisite for solving those problems. We have designed a class of lipid-like peptides that are less than 10 natural amino acids in length and structurally amphiphilic. Each peptide consists of a hydrophilic head-group composed of charged or polar residues and a hydrophobic tail consisting of a string of hydrophobic amino acids. In aqueous solutions, they self-organize to form different supramolecular structures such as vesicles, nanotubes or membranes. It has been shown that they stabilize the functions of several membrane proteins against environmental stresses such as heat and drying. We here report the self-assembling behavior of several designer lipid-like peptides at different peptide concentrations, using dynamic light scattering and fluorescence measurements. These peptides exhibit self-assembling behavior akin to some lipids, with distinct critical aggregate concentration (CAC) values and sequestration of the hydrophobic tails away from water. These values depend on the peptide sequence, with more hydrophobic peptides having lower CAC values. This information will be essential in using the lipid-like peptides for membrane protein stabilization and structural studies.

Keywords: Self-assembly; Lipid-like peptides; Proteins; Enzymes; Surfactant peptides; Membrane proteins

INTRODUCTION

It is estimated that about one third of total genes in the genomes of most organisms code for membrane proteins [1]. This reflects the indispensable roles membrane proteins play in the functioning of an organism, as it is through these membrane proteins

that cells convert energy, sense their environments, transport molecules and communicate with their surroundings. Furthermore, many metabolic enzymes including energy producing proteins and protein complexes are membrane bound. Yet, little is known about most of their structures let alone the detailed mechanisms of their functions. Although ~33,000 three-dimensional protein structures have been elucidated (December 2005), only 183 (97 unique ones) of these are of membrane proteins. This disparity stems from the technical challenge of solubilizing and crystallizing proteins that are membrane-bound, particularly integral membrane proteins. Often the membrane proteins aggregate once taken out of their natural environment. This problem is compounded by the lack of reliable and high throughput methods to systematically screen different solubilizing agents (i.e. detergents and surfactants). Significant cost and effort would thus be expended analyzing different detergents per protein without much promise of knowing why some of them work while some do not. Nevertheless, considerable efforts have been made to solubilize, stabilize and crystallize membrane proteins using diverse detergents [2–6].

Most of the surfactants that have been used to solubilize and stabilize membrane proteins are small molecules with a biological moiety including sugar based hydrophilic moiety as the polar head and 6–18 carbon chain tails [2,3,6,7]. It has been proposed that these detergents interact with membrane protein or lipid molecules that co-purify with them. In general, non-ionic surfactants are better at maintaining the function of the membrane proteins [4], perhaps because they do not destabilize the structure by electrostatically binding to the outer cellular or cytoplasmic domains of the proteins. However, it is

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certain that the basic science on membrane proteins will benefit greatly from the development of new detergent-like or lipid-like molecules that are able to stabilize them outside of their natural cell membrane milieu.

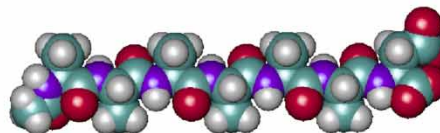
We previously described a class of short lipid-like amphiphilic peptides (peptide detergents) that incorporated segments of homopolymeric, natural amino acids (Fig. 1) [8–10]. We discovered that these peptides have the ability to solubilize and stabilize various membrane proteins and membrane protein complexes [11,12]. Each peptide in this class has a tail consisting of consecutive hydrophobic amino acids and a polar head consisting of one or two charged residues. The hydrophobic tail starts at the acetylated N-terminus of the peptide. For negatively charged head group, the C-terminal end of the peptide is uncapped and thus carries its inherent negatively charged carboxylic acid group. Therefore aspartic acid based amphiphilic peptides, such as A_6D and V_6D , consisting of six alanines (A) and valine (V), respectively, followed by a negatively charged aspartic acid (D), carry a maximum of -2 charge at a pH well above the pK_a 's of the two carboxylic acids ($pK_a \sim 4$), one from the C-terminus and one from the side chain of aspartic acid.

Similar to common surfactants such as dodecyl maltoside (DM) and octyl glucoside (OG), this class of lipid-like peptides can stabilize the functions of membrane proteins outside of the natural cellular membrane. Lipid-like peptides, however, may have different stabilizing mechanism than common detergents with alkane tails due to the possibility of protein–protein interactions and intermolecular hydrogen bonding between the detergent molecules themselves.

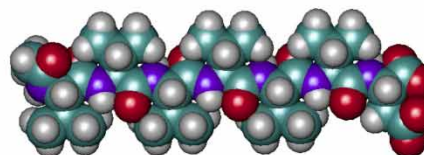
When using chemical detergents to stabilize membrane proteins, it is essential to know the critical aggregation concentration (CAC), also commonly referred to as the critical micelle concentration (CMC), a value at which the energetically favorable release of water molecules around the hydrophobic region(s) of the detergents become more significant than the electrostatic repulsion of the head groups, causing the molecules to aggregate and form micelles. The types of structures formed depend on many different factors, such as the shape of the monomers and chemical identities of each molecule [13,14]. Solubilization and stabilization experiments of membrane proteins are usually conducted at or slightly above the CMC of the detergents. This allows for the membrane proteins to be solubilized inside the pre-formed micelles. Concentrations significantly higher than the CMC are usually not used as these chemical detergents may also interfere with membrane protein structures.

In order to comprehend further how the lipid-like peptides self-assemble in water, we carried out

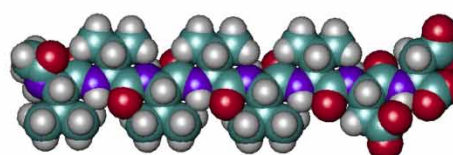
A) A_6D , Ac-AAAAAAD



B) V_6D , Ac-VVVVVVD



C) V_6D_2 , Ac-VVVVVVDD



D) V_6D peptide nanotubes

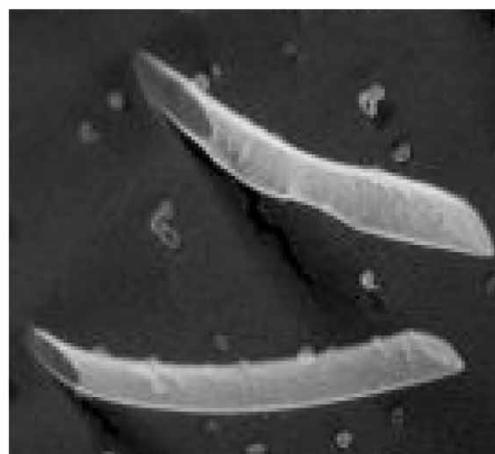


FIGURE 1 Molecular models of the three lipid-like peptides are presented (N \rightarrow C). (A) A_6D with two negative charges at the C-terminus, one from the terminus COO^- and one from the side chain COO^- of aspartate; (B) V_6D with 2 negative charges; and (C) V_6D_2 with 3 negative charges (one additional side chain COO^- of aspartate). The positive charge at the N-terminus is blocked by an acetyl group. Color coding: turquoise (carbon), blue (nitrogen), red (oxygen) and white (hydrogen). Note that all charges are located at the C-termini of the lipid-like peptides; (D). Transmission electron microscopy image of nanotubes formed by V_6D peptides in solution. The peptide sample was prepared using quick freeze deep etch technique (QFDE) to retain the three-dimensional structures. The diameter of the tubes is approximately 30–50 nm.

experiments that elucidate their behavior. We here describe the measurement of critical aggregate concentrations of several lipid-like peptides and correlate them to structural factors. These results are essential in order to use these peptides for stabilization studies of membrane proteins.

MATERIALS AND METHODS

Peptide Synthesis

Each peptide was synthesized using standard F-moc chemistry for peptide synthesis and was custom-made from SynPep Corporation (www.synpep.com), or the Biopolymers Laboratory at MIT. The N-terminal amino acid is the end of the hydrophobic valine tail and the primary amine there was acetylated with acetic acid and thus was uncharged. A Wang resin was used for the C-terminal aspartic acid residue. The peptide was analyzed using mass spectrometry (MALDI-TOF) to ascertain for homogeneity of synthesis.

Sample Preparation

Each peptide sample was resuspended in HPLC grade water in glass scintillation vial at 95% (volume) of the final concentration. The peptide solution was then mixed by gently swirling the vial. Each solution was sonicated in a sonicating water bath operating at 45 W average power (Model 50HT, VWR) for 10 min. All of the peptide powder would be mixed into the solution and large aggregates would have been dispersed completely. The pH of the solution was then adjusted to the desired pH by a stepwise addition of 1 N and 0.1 N NaOH, with swirling after each addition of the base. Accuracy in the pH of the solution was within 0.02 pH units from the desired pH. The volume of the sample was then brought up to 100%, accounting for the volume of the NaOH that was added. The sample was then filtered through a 0.8 μm polyethersulfone syringe filter (Pall Gelman, Acrodisk with Supor[®] membrane, 13 mm diameter) and extruded into a new scintillation vial. Each sample was incubated for approximately 18 h before an analysis was carried out.

Dynamic Light Scattering (DLS)

Dynamic light scattering readings were performed on a Precision Detector PDDLs/Batch with a 680 nm laser operating at 30 mW. The scattered light was collected at a 90° angle, and the intensity-fluctuations were processed by an auto-correlation unit. Data was acquired and displayed by the proprietary Precision Deconvolve program. For a particular DLS reading, 200 μl of a peptide solution was slowly pipetted into a clean quartz micro-cuvette that has been dried under a stream of nitrogen. The concentration of the peptide solution was prepared so that the intensity of the scattered light did not go higher than the limit of the photon counter of the instrument.

CAC Measurement Using Fluorescence Dyes

DPH (1,6-diphenyl-1,3,5-hexatriene) and pyrene were obtained from Sigma and were dissolved in acetone and dimethyl formamide, respectively. For

DPH fluorescence assay, 5 μL of 1 mM DPH solution were pipetted into 5 ml of peptide solution at varying concentrations and incubated at room temperature overnight. Fluorescence excitation was 350 nm. For pyrene treated samples, 1 μl of 1 mM pyrene was pipetted into 1 ml of 1 mg/ml V₆D and 1 ml of 1 mM Triton X-100. For the dilution experiment, the same pyrene treated V₆D sample was diluted ten-fold and incubated overnight at room temperature. For pyrene, the fluorescence excitation was 335 nm.

RESULTS

Effect of Molecular Parameters on the Critical Aggregation Concentration

DPH is often used to investigate the CMCs of amphiphilic molecules. It is found that above the CMC value of each surfactant, the dye fluoresces due to alignment of the dye in the organized micelles or vesicles isolated from water, while below that value, the fluorescence is quenched due to nonalignment. This phenomenon is attributed to the effect of hydrophobic environment on the fluorescence of DPH. In water, the DPH fluorescence is quenched due to the randomness of the orientation of the dye molecules [15,16]. Only when hydrophobic environments are formed and the dye molecules are able to insert themselves into that milieu, does fluorescence occur.

Figure 2 shows the fluorescence emission intensity at 425 nm of DPH in different concentrations of A₆D and V₆D and confirms that A₆D and V₆D self-assemble to form hydrophobic regions. At low concentrations, the peptides did not present a hydrophobic environment to incorporate the hydrophobic dye. At a certain concentration, the fluorescence intensity of DPH started to rise linearly as the dye molecules inserted itself into the newly formed hydrophobic milieu.

The intersection point of two straight lines fitting the low intensity region and the linearly increasing intensity region is typically considered the CAC of the molecule. This point corresponded to approximately 0.5 mM for V₆D and 1.6 mM for A₆D in pure water (see Table I for a tabulation of these values). For comparison, the CMC of TritonX-100, a commonly used non-ionic detergent, is 0.24 mM to 0.3 mM and that for sodium dodecyl sulfate (SDS) is 8.0 to 8.2 mM. The difference of more than threefold in CAC between A₆D and V₆D can be explained by the higher hydrophobicity of valine relative to alanine. That is, for the same head group, a lipid-like peptide having consecutive valine residues as its hydrophobic tail will assemble at a lower concentration than if it had alanine. It should be noted that the absolute fluorescence intensities of DPH in these two peptide solutions were widely

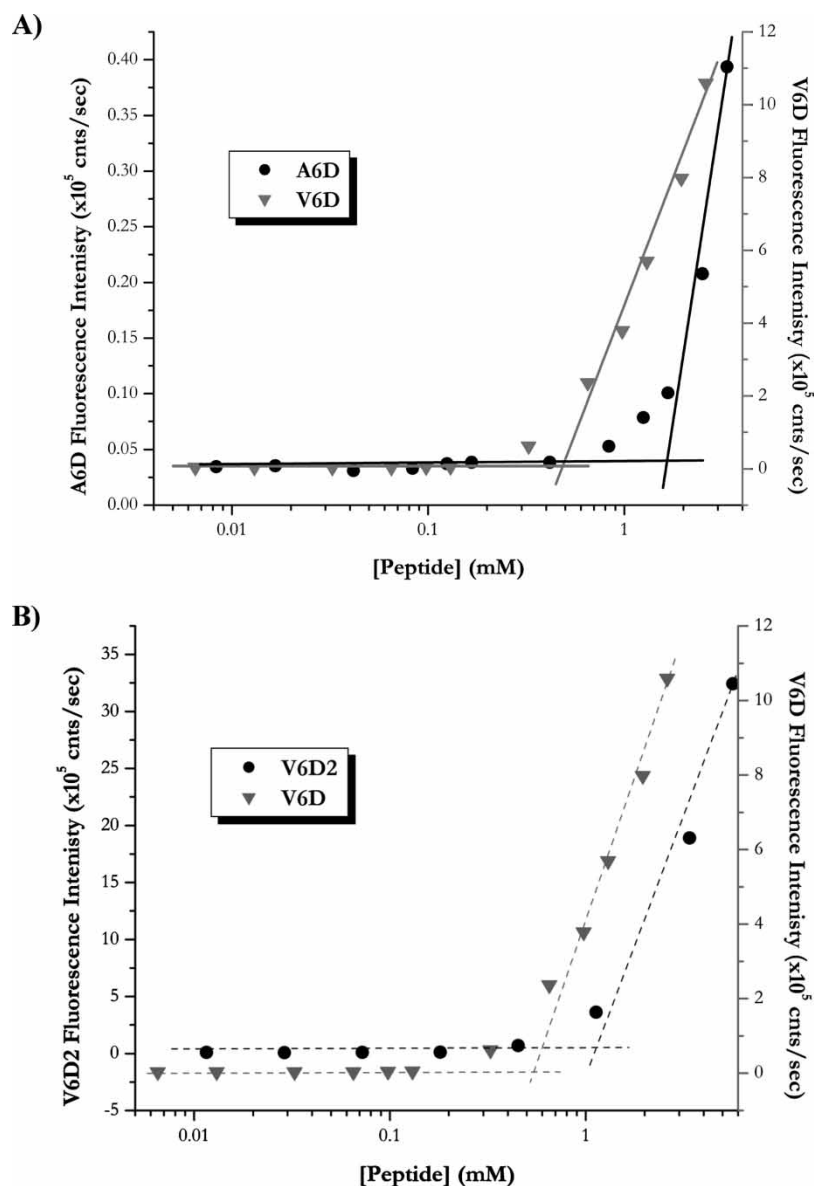


FIGURE 2 CAC measurement in water for A_6D , V_6D and V_6D_2 using DPH. A_6D and V_6D lipid-like peptides have different hydrophobicity due to their side chains. V_6D is more hydrophobic since valine has 3 carbons while alanine has only one. (A) DPH fluorescence intensities of different concentrations of A_6D and V_6D . The lines on each graph intersect at a point that is traditionally recognized as the CAC. (B) CAC measured using DPH fluorescence intensities of different concentrations of V_6D and V_6D_2 . V_6D_2 is less hydrophobic since it has 2 aspartic acids, whereas, V_6D has one.

different. For example, for both peptides, the fluorescence intensities at concentrations much below the CAC were approximately 5000 counts/s. Starting at the CAC, the absolute magnitude of DPH fluorescence in V_6D was greater than in A_6D by a factor of approximately 10. It is plausible that this

difference in absolute intensity signal was caused by a tighter packing of the valine hydrophobic tail with each other, allowing the presence of much less water molecules than in A_6D . For A_6D the looser packing contained more water molecules hydrating the peptide backbone and caused an increased quenching effect.

TABLE I Various lipid-like peptides and their CACs in water, compared with two commonly used detergents

Molecule	CAC (mM)	Method
A_6D	1.6	Fluorescence
V_6D	0.5	Fluorescence, light scattering
V_6D_2	1.1	Fluorescence
Triton X-100	0.28	literature
SDS	8.0	literature

In the figure, the CAC curve for A_6D also shows an intermediate region that did not fit into sharp intersecting lines. Such gradual increase in the fluorescence intensity has been commonly attributed to the formation of various pre-micelle assemblies for some amphiphilic surfactants.

The CAC of the other valine-based peptide, V_6D_2 , which was more soluble, was also determined by

DPH fluorescence. The result and comparison to V_6D is shown in Fig. 2b. The CAC of V_6D_2 was ~ 1 mM of peptide, higher than V_6D by a factor of two. This is likely due to the extra negative charge on V_6D_2 that resulted in an increased electrostatic repulsion between the peptide monomers, not only making it more soluble, but also rendering the same length of valine-tail slightly less effective in assembly. Such behavior was also observed in the assembly of other amphiphilic molecules [17]. This is perhaps the reason why electrostatic shielding afforded by stronger ionic strength in solution can promote aggregation at lower concentrations.

It is interesting to note also that the absolute intensity of the fluorescence emission was two-fold higher in V_6D_2 than in V_6D . This may reflect the presence of some subtle structures that may incorporate and retain the DPH dye more efficiently. However, electron microscopy studies did not reveal any striking structural difference (results not shown). Detection of finer differences in structures will require other spectroscopic or scattering techniques.

Comparison of CAC Value with Light Scattering

Light scattering has also been used to determine the CACs of lipid-like peptide detergents. It is known that the amount of light that is scattered by a solution is dependent on the number and size of particles [18]. The intensity of scattered light will therefore increase sharply after the critical aggregate concentration. In order to confirm the CAC reading from DPH fluorescence with another independent technique, we therefore repeated a similar experiment using

light scattering. Figure 3 shows that the CAC derived from light scattering experiment for V_6D was between 0.5 to 0.6 mM, in good agreement with the CAC result obtained from fluorescence. It should also be known that critical aggregation readings would differ slightly between different techniques [15,19,20]. Thus, light scattering method can now be conveniently used to measure CAC of lipid-like peptide detergents.

Measuring the Hydrophobic Environment with Pyrene

We wanted to ascertain the formation of a hydrophobic environment when the amphiphilic peptides self-assemble, and thus we chose to study the fluorescence of another dye, pyrene. The pyrene dye has a long fluorescence lifetime and has the advantage of being able to indicate the polarity of the microenvironment around it. Pyrene fluorescence yields 5 predominant peaks (Fig. 4). The first emission peak (called I_1 , appears around 375 nm) increases in intensity in polar solvents, while the third peak (I_3 , appears around 385 nm) shows minimal intensity variation with polarity [21]. The ratio of I_1/I_3 is therefore is dependent on the polarity of the environment of pyrene and is approximately 1.7 in water and 0.58 in cyclohexane [22]. For micellar structures that contain a hydrophobic environment, the ratio is in between the two extremes. For example, it is 1.32 for TritonX-100 [23] and 1.2 for SDS [21]. These values also reveal that the probes still contact water even when “buried” inside the interiors of the micelles. This is due to the

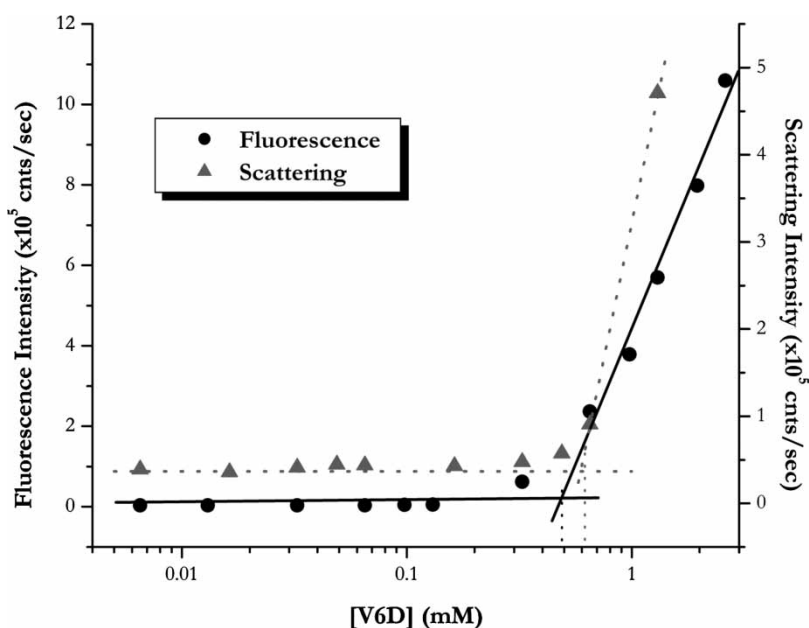


FIGURE 3 A comparison of V_6D CAC measurement in water by dynamic light scattering and intensity of DPH fluorescence. The points of the intersection of the lines showing the CAC are shown. The slopes are different reflecting differences in the parameters being measured. However, the CAC values from the two experiments are similar within experimental error.

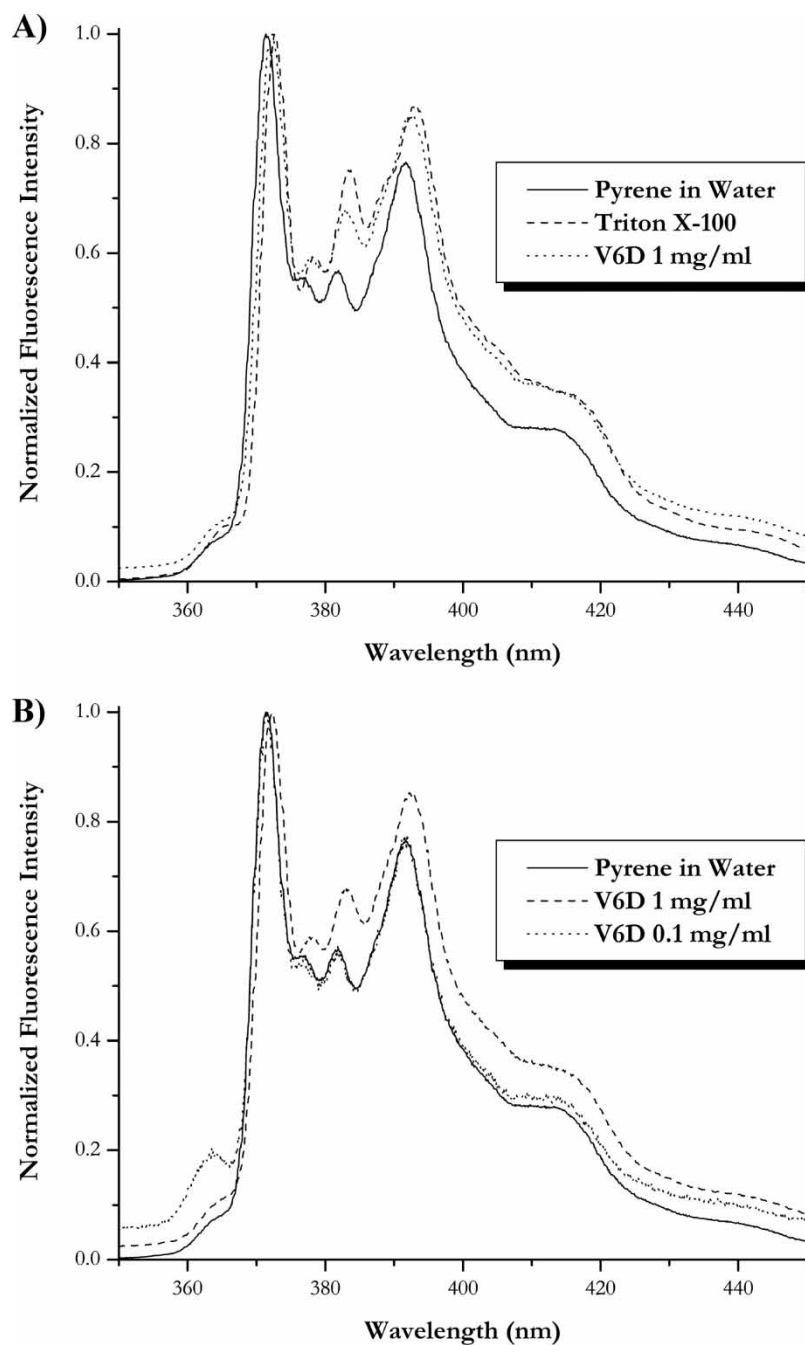


FIGURE 4 Pyrene incorporated into the structure of lipid-like peptide V_6D . (A) Characteristic pyrene fluorescence spectrum in three different conditions: water, 1 mM Triton X-100 and 1.3 mM (1 mg/ml) V_6D . Each graph is normalized to the first vibronic peak or I_1 . (B) Characteristic pyrene fluorescence spectrum of V_6D upon dilution to a concentration value below the CAC.

penetration of water and pyrene molecules into the interfacial layer between the hydrophobic tail and the hydrophilic head group.

Figure 4A shows the fluorescence of 1 μ M pyrene in three different solutions: water, 1 mM Triton X-100 and 1 mg/ml (1.3 mM) V_6D . The fluorescence intensities were normalized to the first vibronic peak at approximately 372 nm for easier interpretation. As can be readily observed, I_3 was the lowest for water, yielding the highest I_1/I_3 ratio. The value was 1.76, in good agreement with the literature value. The highest I_3 intensity in the series was Triton

X-100, which yielded a ratio of 1.32. V_6D produced a ratio of 1.47. For V_6D the ratio further showed that a hydrophobic microenvironment was presented to the pyrene molecules. However, this environment was not as hydrophobic as the one in TritonX-100, which suggests a looser packing of the hydrophobic tail in the self-assembled structures. Thus, the same analysis carried out with A_6D and V_6D_2 will most likely produce a higher ratio for A_6D and a similar ratio for V_6D_2 .

Figure 4B shows pyrene fluorescence after a solution of 1 mg/ml V_6D and 1 μ M pyrene was diluted by a

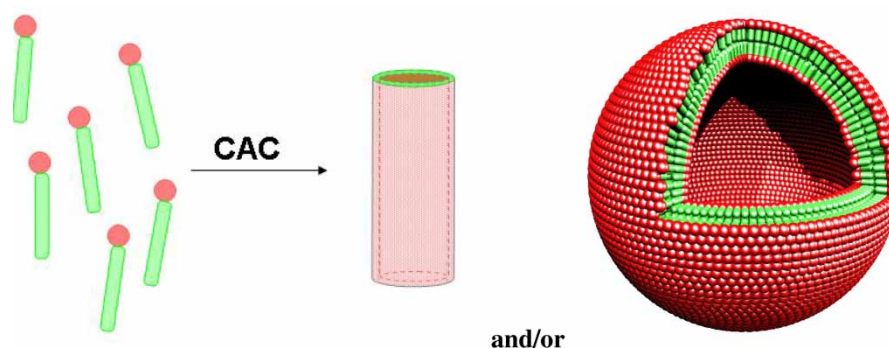


FIGURE 5 Proposed schematic models of lipid-like peptides and their self-assembly at the critical aggregation concentration (CAC) to form nanostructures. At concentrations lower than the CAC, the lipid-like peptides exist mostly as monomers or very small clusters undetectable by the instruments and methods used here. At concentration values higher than their respective CAC, these peptides form supramolecular assemblies such as micelles, nanovesicles and nanotubes.

factor of 10 and incubated for several hours at room temperature. The concentration value is below the CAC point of V_6D . If the structures remained after dilution, then the observed I_1/I_3 ratio should remain the same. However, if the hydrophobic environment was abolished due to dilution, then the ratio should increase and approaches that of pyrene in water. Interestingly, the ratio for diluted V_6D was similar to that of water. This suggests that after dilution the hydrophobic region was substantially diminished.

We propose a schematic model of lipid-like self-assembling peptides and their possible assembled nanostructures (Fig. 5). When concentrations of these peptides are below their CAC, they do not form detectable assemblies using the methods described in our experiments. At their CACs, under the appropriate conditions, they undergo self-assembly to form dynamic nanostructures. These structures could be measured, analyzed and observed experimentally. It must be emphasized that these nanostructures are not static, but are rather dynamic and undergo self-assembly, disassembly and reassembly [8].

DISCUSSION

New materials and tools always have had a significant impact in biology. Different biological disciplines depend on materials that are now commonly used, for example agar in bacteriology, agarose and acrylamide in molecular biology and different fluorescent chromophores in cell biology and neurobiology imaging, including single molecular imaging [24,25]. Indeed some current biological problems are intractable due to the lack of effective tools to study them. One of these problems, we believe, is the systematic study of the structure and functions of membrane proteins. As mentioned previously, one of the limiting steps is the lack of appropriate detergents or surfactants to solubilize and stabilize these membrane proteins.

Some efforts have also been made to design peptide and protein based detergents, due to their potential to

be easily modified and screened by simply changing single amino acid. Stroud and colleagues designed the first alpha-helical peptide detergent, which they called "peptitertgent." Each molecule has both a hydrophilic and a hydrophobic, and formed a four helical bundle in solution when not interacting with membrane proteins [26]. They showed that this peptitertgent can solubilize 85% bacteriorhodopsin and ~60% bovine rhodopsin for a period of 2 days, although they also noted that the peptide was not able to solubilize a membrane protein with a different structure, for example, porin. However, this peptide also first needs to be folded correctly into a stable helical structure before they exert the detergent effect. Furthermore, this peptide is rather long and expensive, not affordable for wide spread use.

Privé and colleagues also designed a monomeric alpha helix coupled with two alkyl chains at both N- and C-termini [27]. This hybrid molecule has two faces, one hydrophilic face from the charged residues on one side of the helix that interacts with water and the hydrophobic face on the other that interacts with transmembrane domains of membrane proteins. They showed that this type of hybrid peptide-alkyl detergents solubilized and stabilized several diverse types of membrane proteins. However, it is very difficult to obtain large quantity of high purity hybrid peptide-alkyl detergent products. Furthermore, these hybrid-peptides are expensive to produce and are not easy to make for large-scale productions.

Our short, lipid-like peptides mentioned here are easy to synthesize and to scale up for producing large quantity. Because of this, screening different sequences for effectiveness in solubilization and stabilization can be achieved in a rapid and cost-effective manner. We have preliminary results suggesting this system can stabilize membrane proteins such as photosystem complex [11], and glycerol-3-phosphate dehydrogenase [12] that was also crystallized in the presence of the surfactant peptide and structural analysis is currently in progress, as well as a G-protein coupled receptor bovine rhodopsin.

These examples are just the beginning of a concerted effort in our laboratory and others to tackle the problem of membrane protein solubilization, stabilization and crystallization using the lipid-like peptide detergents. The advantages of using this designed short lipid-like peptide system include: ease of synthesis, amenability to large scale screening, and economy of scaling up for widespread use.

Acknowledgements

We thank members in of our laboratory for helpful and stimulating discussions. This work is supported in part by grants from the Multidisciplinary University Research Initiative (MURI)/AFOSR, and the National Science Foundation to the Center for Bits & Atoms to the Massachusetts Institute of Technology.

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