Peptide self-assembly into lamellar phases and the formation of lipid-peptide nanostructures

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ABSTRACT

Lipids exhibit an extraordinary polymorphism in self-assembled mesophases, with lamellar phases as the most relevant biological representative. To mimic lipid lamellar phases with amphiphilic designer peptides, seven systematically varied short peptides were engineered. Indeed, four peptide candidates (V₄D, V₄WD, V₄WD₂, I₄WD₂) readily self-assembled into lamellae in aqueous solution. Small-angle X-ray scattering (SAXS) patterns revealed ordered lamellar structures with a repeat distance of ~ 4–5 nm. Transmission electron microscopy (TEM) images confirmed the presence of stacked sheets. Two derivatives (V₃D and V₄D₂) remained as loose aggregates dispersed in solution; one peptide (L₄WD₂) formed twisted tapes with internal lamellae and an antiparallel β -type monomer alignment. To understand the interaction of peptides with lipids, they were mixed with phosphatidylcholines. Low peptide concentrations (1.1 mM) induced the formation of a heterogeneous mixture of vesicular structures. Large multilamellar vesicles (MLV, *d*-spacing ~ 6.3 nm) coexisted with oligo- or unilamellar vesicles (~ 50 nm in diameter) and bicelle-like structures (~ 45 nm length, ~ 18 nm width). High peptide concentrations (11 mM) led to unilamellar vesicles (ULV, diameter ~ 260–280 nm) with a homogeneous mixing of lipids and peptides. SAXS revealed the temperature-dependent fine structure of these ULVs. At 25 °C the bilayer is in a fully interdigitated state (headgroup-to-headgroup distance d_{HH} ~ 2.9 nm), whereas at 50 °C this interdigitation opens up (d_{HH} ~ 3.6 nm). Our results highlight the versatility of self-assembled peptide superstructures. Subtle changes in the amino acid composition are key design elements in creating peptide- or lipid-peptide nanostructures with richness in morphology similar to that of naturally occurring lipids.

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1 Introduction

The remarkable ability of phospholipids to self-assemble into various lamellar structures is essential for life, as they create the fundamental structural component of biological membranes [1–5]. Lipid-like designer peptides are promising candidates to mimic this feature. A fundamental characteristic is their amphiphilicity, which derives from an antagonistic arrangement of charged (hydrophilic) amino acids and hydrophobic tail residues [6]. Above a critical aggregation concentration (CAC), amphiphiles self-assemble into supramolecular structures similar to lipid mesophases. Several one-dimensional (1D) and two-dimensional (2D) structures have been reported, including micelles, cylinders, and lamellar or hexagonal phases [6–17]. Not only do peptides structurally resemble lipid molecules, but they also exhibit similar surfactant-like properties. Several earlier studies focused on their application as surfactants in membrane protein stabilization [18–23]. Their use as antimicrobial agents has also been considered [24, 25]. Recently, peptide vesicles were tested for their capability as drug- or gene-delivery systems [26–29] or as bioactive scaffolds in tissue engineering applications [30–32]. All applications involve peptides either mimicking lipid molecules or coming in close contact with biological lipid membranes.

We therefore aimed to relate those features that make amphiphilic designer peptides “lipid-like” to their self-assembling behavior. We intended to create peptide-based lamellar phases and study how peptides react upon interaction with lipid systems. In order to achieve this, we recently investigated the self-assembly and lipid-interaction propensity of two short amphiphilic designer peptides (V_4WD_2 and A_6YD). V_4WD_2 has shown a high propensity to interact with artificial as well as biological membrane systems [33]. In the present work we attempted to gain a better understanding of which features of the peptide sequence are critical for self-assembly and what drives the interaction with membranes by combining information from small-angle X-ray scattering (SAXS), transmission electron microscopy (TEM), and attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR). We systematically designed six derivatives of the peptide V_4WD_2 (Fig. 1). In particular, three regions of

Figure 1 Molecular structures of the 7 investigated amphiphilic designer peptides. All peptides are acetylated at their N-terminus. The hydrophobic parts of the molecules are highlighted in green; tryptophan residues are shown in yellow; and residues that are charged at pH 7 are labeled blue.

the peptide were modified. First, the hydrophobicity level was varied by replacing the four hydrophobic valine tail residues with either leucine or isoleucine. Second, the influence of tryptophan was investigated by comparing V_4WD_2 and V_4WD to their tryptophan-lacking derivatives. Third, by varying the number of charged headgroup residues and by designing the extremely short peptide V_3D , we aimed to elucidate the influence of hydrophile:lipophile ratio and peptide length. A list of all peptides and their physicochemical characteristics is provided in Table 1. By addressing the influence of systematical changes in the amino acid composition, we found relationships between details at the molecular level and both peptide structure development and lipid-peptide interactions. We demonstrate that subtle changes in peptide sequence can be utilized as key design elements to create a diversity of peptide- or lipid-peptide nanostructures.

2 Experimental

2.1 Chemicals

The amphiphilic designer peptides ac- V_3D , ac- V_4D , ac- V_4D_2 , ac- V_4WD_2 , ac- V_4WD , ac- I_4WD_2 , and ac- L_4WD_2 were custom synthesized and purified by Peptide 2.0 (Chantilly, VA, USA). Dipalmitoyl phosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were purchased either from Sigma-Aldrich (Vienna, Austria) or Carl Roth GmbH (Karlsruhe, Germany) and were of analytical grade.

Table 1 Summary of the 7 investigated peptides and their chemical characteristics

	Number of amino acids	Charge at pH 7	M_w (g/mol)	Hydrophobicity (kcal/mol) [34, 35]	$\sim$ CAC ^a (mM)
V ₃ D	4	−2	475.5	+10.16	>> 20
V ₄ D	5	−2	571.7	+9.70	> 2
V ₄ D ₂	6	−3	686.8	+13.34	n.d. ^b
V ₄ WD	6	−2	757.9	+7.61	4.8
V ₄ WD ₂	7	−3	873.0	+11.25	7.5
I ₄ WD ₂	7	−3	929.1	+8.61	4.1
L ₄ WD ₂	7	−3	929.1	+8.09	1.9

^aThe CAC was determined by fluorescence spectroscopy (Fig. S1 in the ESM).

^bThis peptide does not form supramolecular assemblies and did not show a fluorescence signal over the investigated concentration regime.

2.2 Peptide sample preparation

Samples were prepared by mixing the lyophilized peptide with double-distilled water and by a stepwise addition of 1 M or 0.1 M NaOH until the samples appeared as clear, colorless solutions. Concentrations ranged from 0.1–75 mM with resulting pH values between 9 and 12. Samples were left to equilibrate at 20–25 °C 1 week prior to CAC determination (Fig. S1 in the Electronic Supplementary Material (ESM)), SAXS, ATR-FTIR, and TEM measurements.

2.3 Calculating the peptide average hydrophobicity

To calculate a peptide's overall hydrophobicity, a decision has to be made regarding the hydrophobicity scale to be used. Several different scales are used [36]; however, owing to different experimental or calculation approaches, they are comparable only to a certain extent. With respect to subsequent peptide-lipid partitioning experiments, we decided to use the Wimley–White whole-residue scale [34] for our peptides (see Table 1).

2.4 Preparation of lipid-peptide mixed systems

DPPC was dissolved in chloroform/methanol (2:1 v/v) and mixed, and organic solvents were evaporated under a gentle stream of nitrogen, followed by drying under vacuum overnight in order to completely remove residual organic solvents. The dry lipid-film was hydrated by adding either double-distilled water (reference) or freshly prepared aqueous solutions of the peptides to finally obtain two different molar ratios: 50:1 and 5:1 DPPC/peptide (mol/mol). These ratios correspond to peptide concentrations of 1.1 and 11 mM,

with a constant DPPC concentration of 55 mM. The samples were incubated above the lipid's phase transition temperature at 50 °C for 2 h, intermitted by vigorous vortexing every 15 min. Samples were incubated with gentle rotation for 24 h at room temperature before SAXS and TEM measurements were performed.

2.5 SAXS

Synchrotron X-ray scattering data were collected at the Austrian SAXS beamline at ELETTRA (Trieste, Italy) [37]. Measurements were conducted at a wavelength of 0.154 nm and a sample-detector distance of 1.1 m. The photon energy was 8 keV. The X-ray images were recorded with a Pilatus detector (Pilatus3 1M, DECTRIS Ltd., Villigen PSI, Switzerland) calibrated with silver behenate. The scattering intensity was measured as a function of the scattering vector q where

$$q = 4\pi(\sin\theta)/\lambda \quad (1)$$

with 2θ being the scattering angle and λ being the wavelength. Samples were measured in a 1.5-mm glass capillary. Data analysis was done with Fit2D [38] and Igor Pro (Version 6.22A, WaveMetrics Inc., Portland, OR, USA). Experimental intensities were background corrected, and absolute calibration was performed with water as the secondary standard using the method described by Orthaber [39].

2.5.1 SAXS data analysis of pure peptide structures

(a) Power law of the initial slope

In a double logarithmic plot, the slope of the form factor at small angles (Guinier regime) allows for a rough

classification into globular (q^0), cylindrical (q^{-1}), or lamellar (q^{-2}) shapes. The power law of the form factor in the linear region was determined from the 75-mM peptide scattering profiles between $q = 0.1$ and 0.25 nm^{-1} .

(b) Determination of the lamellar spacing
Many of the scattering curves contained quasi-Bragg peaks. The lamellar spacing d was determined by

$$d = 2\pi h/q(h) \quad (2)$$

where h is the diffraction order and $q(h)$ is the respective position of the peak maximum.

2.5.2 SAXS data analysis of lipid-peptide binary mixtures

The following form factor and structure factor models were applied:

(a) Bilayer form factor model

Samples with no positional correlation (50 °C samples) were fitted with a bilayer form factor model. We used a Gaussian representation of the electron density profile [40, 41]. Four parameters had to be adjusted: z_H and σ_H , the center and width of the headgroup Gaussian respectively; σ_C , the width of the hydrocarbon chain Gaussian; and ρ_R , the ratio of the methyl-terminus electron density amplitude to the headgroup amplitude ($\rho_R = \rho_C/\rho_H$).

(b) Structure factors

Depending on the temperature, two different structure factor models were applied. For samples measured at 25 °C, a paracrystalline theory (PT) structure factor was applied, whereas for 50 °C samples, a modified Caillé theory (MCT) was used [40, 41]. Fitting parameters were as follows: the estimated number of layers within the scattering domain N , the lamellar repeat distance d , and a model-dependent disorder parameter, i.e., either the mean square fluctuations of the bilayer Δ (PT) or the Caillé parameter η (MCT).

2.6 ATR-FTIR spectroscopy

Infrared spectra were recorded between 4,000 and 600 cm^{-1} on a Bruker ALPHA-T FTIR spectrometer equipped with an ATR accessory (Bruker Optics Inc., Billerica, MA, USA). The spectra were the averages of 128 scans with a resolution of 4 cm^{-1} . Spectra were water corrected, and data analysis was done with OPUS

(Bruker Optics Inc.) and Igor Pro (Version 6.22A, WaveMetrics Inc.).

2.7 Negative staining TEM

Samples were dropped onto a glow-discharged carbon-coated copper grid and allowed to settle for 1 min. Excess fluid was carefully removed with filter paper and immediately replaced by 5 μL of a 2% (w/v) uranyl acetate staining solution, which was centrifuged for 6 min at 10,000 rpm prior to usage in order to precipitate potentially present aggregates. The uranyl acetate solution was allowed to settle for 30 s, blotted, and replaced by another 5 μL of fresh solution. After 30 s of incubation, the staining solution was removed, and the samples were air-dried. Visualization of the samples was achieved with a Fei Tecnai G² 20 transmission electron microscope (Eindhoven, the Netherlands) operating at an acceleration voltage of 120 kV. Digital images were acquired using a Gatan US1000 CCD camera at $2k \times 2k$ resolution and the Digital Micrograph software (Version 1.93.1362, Gatan Inc., Pleasanton, CA, USA). Brightness and contrast were adjusted globally.

3 Results

3.1 Structural characterization of self-assembled pure peptide structures (75 mM samples)

To study whether the designed amphiphilic designer peptides are capable of forming lamellar structures, their self-assembling behavior was investigated at extremely high peptide concentrations. Peptides were prepared as 75 mM samples, as structural details at this concentration usually appear very pronounced, yet the samples remain in their liquid phase and do not display gel-like characteristics. Figure 2 reveals that all investigated samples can be roughly divided into three classes: (1) peptides that show no superstructural features, (2) peptides with pronounced lamellar characteristics, including an inter-lamellar structure factor, and (3) one peptide with a combination of lamellar and tape-like characteristics.

More precisely, (1) two peptides (V_3D and V_4D_2) did not self-assemble into ordered structures, even at those high peptide concentrations. The SAXS patterns

Figure 2 Supramolecular structure formation. The SAXS patterns (left panel) highlight fits of the initial slopes (green curves) and the positions of the lamellar peaks (green and grey asterisks). ATR-FTIR spectra (right panel) show the amide I regions in light green. V_3D and V_4D_2 (a) show no supramolecular structures at 75 mM. V_4D (b), V_4WD (c), V_4WD_2 (d), and I_4WD_2 (e) all have lamellar characteristics. L_4WD_2 (f) shows a slightly different scattering pattern, hinting at a mixed system (likely lamella/tape-like structures). All recorded ATR-FTIR spectra show β -sheets as their main characteristics. Samples depicted in (a)–(e) most probably contain parallel β -type structures, whereas (f) shows an antiparallel arrangement.

showed a very moderate rise in intensity at low q -values, which indicates the onset of peptide aggregation (Fig. 2(a)). ATR-FTIR data showed β -sheet characteristics (amide I peak at $1,635\text{ nm}^{-1}$), which suggests some degree of order, but not on a long-range scale. (2) The majority of peptides (V_4D , V_4WD , V_4WD_2 , and I_4WD_2) showed a lamellar arrangement, evident by an initial slope of q^{-2} in the SAXS pattern, and repeating peaks, which indicate quasi-long-range order. The associated d -spacing covered a range between 3 and 6 nm (highlighted with asterisks in Figs. 2(b)–2(e), data in Table 2). All of them showed an ATR-FTIR signal at

positions typical for β -sheet arrangement (Table 2). We therefore assume that the peptides arrange in a parallel way, creating flat sheets with the thickness of only one peptide monomer ($\sim 3\text{ nm}$) plus its hydration layer. We believe that these sheets then stack into several layers, which results in the regular lamellar spacing observed with SAXS (see schematic representation in Fig. 3(a)).

This behavior could be best visualized for the peptide I_4WD_2 (Figs. 2(e), 3(b) and 3(c)). TEM images confirmed the presence of multilayered stacks, where each individual layer was around 4–5 nm thick. (3) One peptide stood out of the crowd: L_4WD_2 . It showed a scattering profile that could not be assigned to a classical structure easily. It showed cylindrical characteristics, but at the same time the Guinier region showed features of lamellae. This peptide was also the one with a different ATR-FTIR spectrum. We observed splitting of the amide I peak into two peaks at $1,678$ and $1,626\text{ cm}^{-1}$, which indicates that the peptide assembled into antiparallel β -type structures (Fig. 2(f)). TEM images revealed twisted tape-like structures (Fig. 4). The majority of structures showed diameters in the range of 14–20 nm. Lengths extended to a few hundred nanometers. A closer look into the individual tapes revealed that they were also internally organized in a lamellar way (Fig. 4(c)).

3.2 Lipid-peptide interplay induces a variety of nanostructures

Next, we studied lipid–peptide interplay. For this, we prepared DPPC-peptide binary mixed systems of two different compositions. The DPPC fraction was kept constant (55 mM), whereas peptides were present either in low (1.1 mM) or high (11 mM) concentration. Pure DPPC formed multilamellar vesicles (DPPC MLVs) and served as a reference.

3.2.1 The impact of low peptide concentrations (1.1 mM) on a lipid system

At concentrations of 1.1 mM, all peptides were present in a state below their CAC (see Table 1). Figure 5 clearly shows that different peptides interacted differently with DPPC and that the majority of curves significantly deviated from the pure DPPC reference. The scattering of pure DPPC MLVs showed characteristic Bragg peaks

Table 2 SAXS parameters and ATR-FTIR peak assignment of 75 mM peptide samples

Peptide (75 mM)	SAXS, initial slope	SAXS, lamellar spacings (nm) ^a	ATR-FTIR peaks (cm ⁻¹)	ATR-FTIR band assignment
V ₃ D	-2.5	—	1,635	β-Sheet
V ₄ D ₂	-2.3	—	1,637	β-Sheet
V ₄ D	-1.9	4.7, 4.7	1,635	β-Sheet
V ₄ WD	-1.9	3.1, 3.5, 4.2	1,615 1,626 Plateau (1,649–1,684)	Aggregated strands β-Sheet Antiparallel β-sheet, loops & turns
V ₄ WD ₂	-2.6	6.6, 4.7, 6.3, 6.4 ^b	1,618 1,628	Aggregated strands β-Sheet
I ₄ WD ₂	-2.8	3.9, 4.0, 4.0	1,614 1,622 Plateau (1,653–1,683)	Aggregated strands β-Sheet Antiparallel β-sheet, loops & turns
L ₄ WD ₂	-1.5	—	1,678 1,626	Antiparallel β-sheet, or turns & loops β-Sheet

^aLamellar *d*-spacing is derived from the SAXS peaks marked with asterisks in Fig. 2.

^bGrey asterisks and *d*-spacing label a second lamellar phase ($d_{av} = 6.4$ nm), coexisting with the primary lamellar phase ($d = 4.7$ nm).

Figure 3 The lamellar arrangement of peptide superstructures. The scheme (a) illustrates the parallel alignment of peptide monomers into flat sheets, which stack into layers. Negative staining TEM images of the self-assembled peptide I₄WD₂ at 75 mM (b) and (c) show tapes with lamellar arrangement (black arrows). The line profile in (c) shows distances of 4–5 nm for each lamella.

Figure 4 Negative staining TEM images of the self-assembled peptide L₄WD₂ (a)–(c). At 75 mM, the peptide forms twisted tapes. (c) shows the lamellar internal structure. We assume that monomers are arranged in an antiparallel way (based on ATR-FTIR results).

Figure 5 SAXS analysis of DPPC in the presence of 1.1 mM peptide at 25 °C. Pure DPPC and DPPC:L₄WD₂ show the same scattering pattern, which is typical for MLVs. All the other peptides show a significant amount of positionally uncorrelated bilayers, with only the signature of DPPC diffraction peaks at their original positions remaining. Black curves are the experimentally obtained scattering patterns; green curves are the respective fits (bilayer form factor model combined with a PT structure factor).

up to the 5th order with a lamellar repeat distance d of 6.38 nm. We could fit the scattering pattern by applying a full q -range global fitting model, combining a Gaussian model representation of the bilayer electron density profile with a PT structure factor [40, 41]. The PT structure factor was chosen because at 25 °C DPPC is in its gel phase and shows a high bending rigidity. All obtained structural parameters are presented in Table 3 and are in accordance with Ref. [42].

Once DPPC is mixed with peptides (1.1 mM), it depends on the peptide sequence whether the multilamellar arrangement is maintained. Only DPPC:L₄WD₂

Table 3 Fit results for pure DPPC, DPPC:L₄WD₂, and DPPC:V₄D at low peptide concentrations

Parameter	Pure DPPC	DPPC:L ₄ WD ₂	DPPC:V ₄ D
z_H (nm)	2.07	2.08	2.11
σ_H (nm)	0.3	0.3	0.3
ρ_R (a.u.)	0.85	0.87	0.86
σ_C (nm)	0.39	0.37	0.39
L_E (nm)	50	50	3.3
Δ (nm)	0.001	0.001	0.2
d (nm)	6.4	6.4	6.4
d_{HH} (nm)	4.1	4.2	4.2
d_B (nm)	5.3	5.4	5.4
d_W (nm)	1.1	1.0	1.0

showed the same scattering pattern as pure DPPC and yielded the same structural parameters after fitting (Table 3). The derived electron density profiles of DPPC and L₄WD₂ are illustrated in Fig. 6. The similarity in their profiles might be explained by the fact that L₄WD₂ does not interact at all with DPPC molecules. This means that L₄WD₂ monomers have a higher affinity toward each other than toward DPPC and thus build a separate phase, coexisting with the DPPC MLVs. However, due to the low peptide concentration, this fraction is almost negligible and not represented in the scattering pattern. Another option might be that the peptide molecules are integrated into the whole lipid system without changing the overall or the fine structure of MLVs.

L₄WD₂ is the exception—all the other peptides resulted in a significant disturbance of the DPPC multilamellar arrangement, as can be seen in Fig. 5. For DPPC mixed with V₃D, V₄D, V₄D₂, V₄WD, V₄WD₂, and I₄WD₂, Bragg peaks were dramatically reduced to only the signatures of quasi-Bragg reflections. In return, the contribution of diffuse scattering was increased, which suggests that the positional correlations between the layers were lost. This goes in line with the observation that the estimated average domain size (L_E) decreased. It happens when the number of

Figure 6 Modulation of the electron density across the bilayer. The profiles of pure DPPC, DPPC:L₄WD₂, and DPPC:V₄D are superimposable. The light blue area shows the basic structure of one bilayer. In the case of multilamellar structures, this basic building block is repeated. The peaks (ρ_H) at the positions $\pm z_H$ represent the high electron density of the phosphate groups in DPPC, whereas the central Gaussian (ρ_C) represents the hydrocarbon chain region.

layers is reduced or the number of defects within the bilayers increase. In addition, the mean square fluctuation of the bilayer (Δ) increases when set in reference to pure DPPC. DPPC:V₄D was chosen as a representative example for fitting, because it takes a central position among all investigated samples. Fitting results are presented in Table 3. Altogether we only observed changes in the parameters that refer to the structure factor (L_E & Δ), whereas parameters describing the bilayer form factor did not change (z_{H_V} , σ_{H_V} , σ_C , and ρ_R). We conclude that the bilayer structure of the membrane remained, but the positional correlation between the individual bilayers changed. Subtle differences could be observed in the group of peptides that contain tryptophan residues versus the ones without. Peptides with tryptophan (I₄WD₂, V₄WD₂, V₄WD) showed more distinctive trends toward a unilamellar arrangement than the others. In contrast, peptides without tryptophan (V₄D, V₃D, and V₄D₂) showed a plateau at $q = 1.1\text{--}1.8\text{ nm}^{-1}$. This plateau can be attributed to unspecific assemblies, and an exact assignment was not possible. SAXS analysis did not allow us to draw sophisticated conclusions on the origin of the large diffuse scattering contribution we observed in all samples, except DPPC:L₄WD₂. We therefore picked DPPC:V₄D again to perform a detailed TEM characterization. As can be seen in Fig. 7, TEM revealed a surprisingly heterogeneous mixture, nevertheless with three very well-defined main components: first, large quasi-spherical structures confined by lamellae (labeled Lam). We attribute these structures to the original fraction of MLVs (Fig. 7(a)). The lamellae

were highly ordered, with the majority of diameters between 4.6 and 4.8 nm (statistical analysis is provided in Fig. S2 (in the ESM)). Second, small vesicles (V) that showed a clearly defined spherical shape but were significantly smaller than MLVs were detected (Fig. 7(b)). The diameters of these vesicles ranged from as small as 30 to 200 nm and even larger, but the majority lied between 45–50 nm (Fig. S3 in the ESM). Steric considerations suggest that these vesicles contained a limited number of bilayers, and thus they explain the origin of the large diffuse scattering contribution in SAXS. Most surprisingly, we found another fraction of structures, which was highly defined and present in large amounts throughout the sample: bicelles (Fig. 7(b), labeled Bc). They exhibited a disk-shaped morphology, with lengths of 42–46 nm and widths around 18 nm (Figs. S4 and S5 in the ESM). The conventional way to prepare bicelles is by mixing long- and short-chain lipids [43]. We believe that in our case the peptides took over the task of short-chain lipids. One sterically possible conformation is presented in Fig. 7(c).

3.2.2 The impact of high peptide concentrations (11 mM) on a lipid system

Samples with a peptide concentration of 11 mM contained peptides above their CAC, except for V₃D and V₄D₂, which were shown to remain in their monomeric state even at very high peptide concentrations (see Fig. 2). Thus, for all the other peptides, we expect not only a contribution from lipids but also from supramolecular peptide assemblies. Indeed, when

Figure 7 TEM images of DPPC in the presence of 1.1 mM V₄D. The sample shows a high heterogeneity: (a) demonstrates the regular arrangement of lamellae (Lam) within MLVs, and (b) reveals the presence of smaller vesicles (V) and bicelles (Bc). (c) shows a schematic summary of the observed morphologies and their approximate size regimes.

investigating the DPPC:V₄D mixture, TEM confirmed the presence of self-assembled supramolecular peptide structures. As can be seen in Fig. 8(a), V₄D assembled into long tapes, where 2–5 single tapes were frequently aligned in a parallel way to compose even larger structures (similar to pure V₄D superstructures, as can be seen in Fig. S6 in the ESM). Single tapes showed diameters of 50–60 nm and extended to several micrometers in length. A closer look into one single tape (Fig. 8(b)) revealed a layered arrangement also on a smaller scale: Approximately 6 parallel-aligned layers comprised one single tape, with each individual layer being around 15 nm in width. Obscured by this network of supramolecular tapes laid a different kind of nanostructure, which appeared very crowded in the original sample (Fig. 8(a)) but revealed its full quality in a 1:10 dilution (Fig. 8(c)). Morphologically highly homogeneous spherical structures were observed, with their appearance resembling classical lipid ULVs. The majority of vesicles showed diameters of 260–280 nm (Fig. S7 in the ESM). This indicates that the original multilamellar DPPC lipid system experienced dramatic morphological changes in the presence of the peptide V₄D. It can be understood that this behavior is not restricted solely to V₄D when looking at the SAXS patterns of all 7 investigated peptides (Fig. 9). Depending on peptide sequence and temperature, we observed different types of patterns. Therefore, in a next step we aimed to obtain information on the internal organization of the vesicles by analyzing the SAXS patterns with respect to two different temperatures: 25 °C (below the

lipid's phase transition temperature) and 50 °C (above the lipid's phase transition temperature).

3.2.2.1 SAXS analysis of lipid:peptide mixed systems at high peptide concentrations (11 mM) and 25 °C

All samples exhibited scattering curves largely distinct from pure DPPC MLVs (Fig. 9). They showed the general characteristics of positionally uncorrelated bilayers, most likely ULVs. In addition, we observed the emergence of a new lamellar phase in almost all samples. The amplitudes of these peaks were quite weak, suggesting that only a very limited fraction of the sample exhibits this shape. However, the lamellar spacing was well conserved throughout the different samples ($d = 4.48$ nm). In general, the scattering curves could be again divided into three main classes, which only partly overlapped with the three classes we observed when investigating pure peptide structures (which were: (1) no superstructures, (2) lamellar structures, and (3) twisted tape superstructures). First, we would like to have a closer look at the class comprising V₄D, V₄WD, V₄WD₂, and V₄D₂. TEM revealed the presence of vesicular structures in DPPC:V₄D. In accordance with this, the corresponding scattering curve could be fitted to a ULV model by using a single bilayer electron density model (see Fig. 10). Compared with pure DPPC, DPPC:V₄D showed a significantly reduced headgroup-to-headgroup distance (d_{HH}). The ρ_R and σ_C values, which are associated with the tail region, also experienced a significant change (Table 4). The electron density profile shown in Fig. 10 reveals

Figure 8 TEM images of DPPC in the presence of 11 mM V₄D. The peptide forms long tubes (a), which are composed of layered structures (b). As soon as the sample is diluted below the peptide's CAC, the vesicular structures come to the fore (c). Their appearance resembles classical ULVs.

Figure 9 Summary of peptide-membrane interactions at high peptide concentrations. SAXS patterns of DPPC in the presence and absence of 11 mM peptide were investigated. The black solid curves represent the experimentally obtained scattering pattern at 25 °C; the black dotted curves were measured at 50 °C; and the green curves highlight the fits of the 50 °C samples. Fitting was achieved by using a bilayer form factor model. For pure DPPC, an additional MCT structure factor was added. At 25 °C, results allow a rough classification into three different classes: first, peptides that induce the formation of fully interdigitated ULVs (V_4D , V_4WD , V_4WD_2 , and V_4D_2). Second, peptides that induce the formation of ULVs with partially interdigitated, partially non-interdigitated characteristics (V_3D and I_4WD_2). Third, L_4WD_2 that most probably assembles into cylinders in the presence of DPPC with 2D packing into a squared lattice. At 50 °C, all samples exhibit similar ULV characteristics.

the origin of these changes: The peptide induced the formation of an interdigitated phase. Because d_{HH} was only 2.9 nm, and peptides as well as DPPC molecules were in the same order of magnitude, we hypothesize that the bilayer was fully interdigitated. This means that the terminal methyl groups of DPPC are located in close proximity to the polar interface of the opposite membrane's leaflet [44]. The second class

Figure 10 Electron bilayer density distribution of DPPC: V_4D at a peptide concentration of 11 mM. At 25 °C, we see a strong interdigitation. At 50 °C, higher flexibility is induced and the interdigitation opens up.

Table 4 Fitting results for pure DPPC (using a bilayer form factor and a PT structure factor) and DPPC: V_4D (using a single bilayer form factor model)

Parameter	Pure DPPC	DPPC: V_4D
z_H (nm)	2.07	1.47
σ_H (nm)	0.3	0.3
ρ_R (a.u.)	0.85	0.49
σ_C (nm)	0.39	1.0
L_E (nm)	50	—
Δ (nm)	0.001	—
d (nm)	6.4	—
d_{HH} (nm)	4.1	2.9
d_B (nm)	5.3	4.1
d_W (nm)	1.1	—

comprises the peptides I_4WD_2 and V_3D (Fig. 9). The first order maximum was shifted to lower q values, which already indicates larger structures. The overall morphology of the curve suggests only partial interdigitation. The vesicles exhibited a predominant non-interdigitated phase, in combination with a small portion of interdigitated structures [44]. In addition, we see that these two samples contained a peak at the very small-angle regime ($q = 0.3$ – 0.4 nm $^{-1}$). This might be due to interparticle interactions. However, whether this contribution arises from peptide superstructures or lipid-peptide mixed vesicles remains elusive.

A scattering curve that clearly stood out is DPPC mixed with L_4WD_2 (Fig. 9, top). It displayed quasi-Bragg peaks up to high orders. As can be seen in Fig. 11,

Figure 11 Relating the scattering curve of DPPC:L₄WD₂ (11 mM) to a square-packed cylinder lattice. The green arrows show the expected peak positions, which overlap with the Bragg peaks of the experimentally determined scattering curve.

a first try to relate the structure to a lattice type resulted in a very good match with cylinders packed in a square lattice ($P4/mm$) [45]. The predicted peak positions (Fig. 11, green arrows) overlapped with maxima at the experimentally determined scattering curve. However, this structure needs to be characterized in detail in a subsequent study.

3.2.2.2 SAXS analysis of lipid:peptide mixed systems at high peptide concentrations (11 mM) and 50 °C

Above the lipid phase transition temperature, the scattering curves showed in general a higher uniformity, nevertheless with subtle differences between the individual peptides (see Fig. 9, where the experimental

scattering curves are black dotted lines and the respective fits are represented by light green curves). The first order maximum was shifted to smaller angles, which indicates larger structures. Indeed, when fitting the curves with a single bilayer form factor model, we saw that the tight interdigitation seen at 25 °C opened up and d_{HH} values increased. Nevertheless, we still expect a small contribution from interdigitated parts, similar to DPPC:L₄WD₂ and DPPC:V₃D samples at 25 °C. Table 5 summarizes the obtained fitting parameters.

4 Discussion

The molecular interactions that lead to the formation of self-assembled supramolecular peptide structures include hydrophobic interactions between tail residues, electrostatic interactions between charged headgroups, chiral dipole–dipole interactions, π – π stacking, hydrogen bonds, nonspecific van der Waals interactions, and repulsion due to steric hindrances. Even though extensive research has been carried out to relate peptide sequence to structure, it is still challenging to derive general rules for peptide self-assembly, as all the aforementioned forces are balanced amongst each other in a fine interplay. Understanding the peptides' interaction with lipids is even more difficult.

In this systematic study, we aimed to elucidate which sequence or structure components are essential for self-assembly into supramolecular structures and if variations of these components are directly linked to

Table 5 Fitting results for pure DPPC (using a bilayer form factor and a MCT structure factor) and DPPC:peptides (using a single bilayer form factor model) at 50 °C

Parameter	Pure DPPC	DPPC:V ₄ D	DPPC:V ₄ WD	DPPC:V ₄ WD ₂	DPPC:V ₄ D ₂	DPPC:L ₄ WD ₂	DPPC:V ₃ D	DPPC:L ₄ WD ₂
z_H (nm)	1.87	1.77	1.76	1.85	1.86	1.91	1.81	1.69
σ_H (nm)	0.30	0.42	0.40	0.34	0.30	0.24	0.45	0.37
ρ_R (a.u.)	0.97	1.15	0.73	1.07	0.92	0.77	1.09	0.73
σ_C (nm)	0.66	0.73	1.14	0.65	0.66	0.68	0.82	1.20
L_E (nm)	108	—	—	—	—	—	—	—
η	0.081	—	—	—	—	—	—	—
d (nm)	6.5	—	—	—	—	—	—	—
d_{HH} (nm)	3.7	3.6	3.5	3.7	3.7	3.8	3.6	3.4
d_B (nm)	4.9	5.2	5.1	5.0	4.9	4.8	5.4	4.9
d_W (nm)	1.6	—	—	—	—	—	—	—

interactions with lipid molecules. First, we have a closer look at pure peptide systems and features that drive the self-assembly process.

4.1 Hydrophile–lipophile ratio

Electrostatic repulsion of charged residues is the most important counteracting force to the hydrophobic attraction of tail residues. The hydrophile–lipophile balance determines whether peptide self-assembly is generally possible or restricted. This was best observed when comparing the peptides V_3D , V_4D_2 , and V_4D . V_3D and V_4D_2 do not self-assemble into supramolecular structures for a wide concentration regime. We suggest that three hydrophobic valines are not able to balance the electrostatic repulsion of one aspartic acid residue (V_3D), and likewise, four valines are not able to balance the electrostatic repulsion of two aspartic acid residues (V_4D_2). In contrast, V_4D has only 20% charged residues. The peptide is still soluble, and in addition it is able to self-assemble into supramolecular structures.

Thus, for these simple structures exclusively composed of hydrophobic valines and hydrophilic aspartic acids, the following rule of thumb was established: Not more than 20%–25% of the peptide's total number of residues may have the same charge because then self-assembly is hindered due to electrostatic repulsion.

4.2 Hydrophobicity and steric considerations

By replacing all valines in V_4WD_2 with the amino acids leucine and isoleucine, we were able to compare three peptides that were very close in sequence but varied in their level of hydrophobicity. Despite their similarity on the molecular level, they displayed different characteristics in their supramolecular structures as well as in their lipid-interaction profile. These differences likely arise from their differences in hydrophobicity. As a rule of thumb, the more hydrophobic a peptide is, the lower its CAC is. We have seen that this general statement does not strictly apply anymore when the variations in hydrophobicity are as subtle as in our experiments. V_4WD_2 has the highest CAC amongst the three peptides but is also the most hydrophobic one. Earlier studies have shown that this peptide assembles only into loosely packed aggregates [33] and at very high concentrations (50–75 mM) into ordered lamellar

structures. Many hydrophobicity scales [34, 36, 46] rank isoleucine and leucine as less hydrophobic than valine, and structure formation appeared easier for peptides containing these two residues. The steric arrangement of the amino acid side-chains also might influence structure formation. The only difference between leucine and isoleucine is the position of one methyl group. Hence, this little variation has a huge impact on the supramolecular level. However, this characteristic cannot be seen uncoupled from hydrophobicity. Therefore, no general statement on the sole influence of sterics can be given.

4.3 Tryptophans

We posed ourselves the question whether tryptophan-containing peptides form different supramolecular structures than peptides without tryptophan due to their ability of π – π stacking. Neither with SAXS nor with ATR-FTIR spectroscopy could structural characteristics be solely attributed to either tryptophan-containing peptides or tryptophan-lacking peptides. However, an influence of tryptophan could be observed when peptides were mixed with DPPC. These peptides showed a small trend towards facilitated induction of ULV formation at low concentrations (1.1 mM). Tryptophan has a known propensity to intercalate into bilayer interfaces [47] and thus might facilitate the peptide's interaction with DPPC.

4.4 Peptides induce the formation of ULVs, which show temperature-dependent interdigitation

When looking at the power of individual peptides to interact with DPPC, we have seen that the majority of peptides at high concentrations lead to the formation of ULVs. The fine structure of the derived ULVs depends on peptide sequence and temperature. The transformation of pure DPPC into various phases as a function of temperature is well known, and the concept may be partly applied also to lipid-peptide mixed systems. At 25 °C, pure DPPC displays a gel phase (L_{β}'), with tilted hydrocarbon chains. Upon heating, DPPC finally changes into the L_{α} phase, with hydrocarbon chains in a molten state, leading to an increase in the lamellar repeat distance of MLVs but a decrease in the bilayer thickness d_{HH} [42]. However,

the presence of peptides leads to a highly rigid packing at 25 °C and interdigitated structures, which might change the tilting and packing of hydrocarbon chains [44]. Upon heating, we suggest a peptide- and temperature-induced higher flexibility and motility of the hydrocarbon chains, which leads to the observed increase in bilayer thickness.

4.5 Peptide-induced bicelle formation

Samples of 55 mM DPPC with 1.1 mM V₄D contained bicelle-like structures. Conventionally, bicelles are discoidal particles composed of long-chain lipids (e.g., 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)) and short-chain lipids (e.g., 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC)). DMPC forms the bilayer region, whereas DHPC forms a surrounding belt [43, 48]. Instead of DHPC, detergents and bile-salt derivatives have also been used [49, 50]. To the best of our knowledge, this is the first time that peptide-induced bicelle formation is reported. When speaking of lipid-peptide-mixed systems, similar structures are often described by the term “nanodisc”. However, what distinguishes our structures from nanodiscs? Nanodiscs are defined as patches of a lipid bilayer surrounded by amphipathic helices of proteins or peptides [51]. Because V₄D contains only 5 amino acid residues, we assume that it does not attain the critical length that renders helix formation possible. Thus, we believe that the term “bicelle” is more appropriate to describe our derived structures. Whether they are equally suited for membrane protein crystallography applications or NMR studies [49, 50, 52] (and whether they are also magnetically orientable) could be approached in future experiments.

4.6 The DPPC environment changes the local peptide concentration

It appears that the DPPC environment influences the local peptide concentration and thus structure. When mixed with DPPC, 11 mM V₄D exhibited clearly pronounced long tapes. Similar structures within the pure peptide samples were only observed by TEM at concentrations above 20 mM. Therefore, we assume that DPPC acts as a solubility-reducing agent on peptides. It changes the local microenvironment by removing solvent from the peptide and thus leads to

an effective higher local peptide concentration, which facilitates the peptide self-assembly process.

4.7 Peptides elicit the formation of a conserved new lamellar phase when mixed with DPPC

The majority of peptides induced the formation of oligo- or unilamellar vesicles when DPPC was mixed with 11 mM peptide solution. Besides the ULV characteristics, SAXS patterns revealed an additional lamellar phase, which showed a highly conserved lamellar spacing of 4.48 nm throughout the samples. The amplitudes of these peaks were quite weak, so only a very limited fraction of structures was involved. We posed ourselves the question of how this new lamellar phase can be explained and whether it originates from the peptide-, lipid-, or a mixed phase. For many of the samples, we expect supramolecular peptide structures to be present, as 11 mM exceeds the CAC of the peptides. The distance of 4.48 nm comes very close to the values observed for pure peptide samples (~ 4–4.7 nm, Table 2). These were interpreted as the lamellar distances between the individual supramolecular peptide tapes. It is likely that the same or similar peptide superstructures are also present in the peptide-lipid mixed systems. Moreover, for many samples the 4.48-nm lamellar phase remained present when samples were heated to 50 °C, although some peaks were slightly reduced in their amplitude. However, two peptides did not assemble into supramolecular structures, even at high peptide concentrations. Nevertheless, when these two peptides were mixed with DPPC, they also displayed the 4.48-nm lamellar phase. If this lamellar phase is attributed to a supramolecular peptide structure, it means that the presence of DPPC triggered the self-assembly process for these two peptides. Another explanation for the emergence of the 4.48-nm lamellar phase considers the lipid fraction. One case assumes the formation of lipid-peptide mixed vesicles that show compositional fluctuations within the bilayer. Lipid-rich domains might be alternating with peptide-rich domains. However, a thinning within the same bilayer from 6.35 to 4.48 nm (e.g., V₄D₂ and V₄WD₂) seems very unlikely with respect to bending rigidity. More likely, the samples contained two different fractions of vesicles: a large number of ULVs in

coexistence with fewer oligolamellar vesicles, both of which showed interdigitation.

5 Conclusion

Despite intensive research over the last decades, it is still a challenge to predict the self-assembled supramolecular structure from a given peptide sequence. We designed seven peptides with systematic sequence variations, and although they differed only in a few amino acid residues, they exhibited different self-assembled supramolecular morphologies. We have shown that lamellar structures with high similarity to lipid membranes can be designed and that these peptide membranes offer exciting possibilities for being applied as successfully as their lipid counterparts. Peptide-lipid mixed systems also spontaneously form a variety of nanostructures: We here described interdigitated and non-interdigitated vesicles, bicelles, and cylindrical structures packed in a 2D squared lattice. All of these structures might be promising candidates for next-generation drug- or gene-delivery systems. Our results highlight that amphiphilic designer peptides that were designed to mimic lipid molecules indeed show similar assembly characteristics. In analogy to the plethora of lipid mesophases, many peptide- or lipid-peptide mixed phases are yet to be discovered.

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