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On the design of supramolecular assemblies made of peptides and lipid bilayers‡

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Peptides confer interesting properties to materials, supramolecular assemblies and to lipid membranes and are used in analytical devices or within delivery vehicles. Their relative ease of production combined with a high degree of versatility make them attractive candidates to design new such products. Here, we review and demonstrate how CD- and solid-state NMR spectroscopic approaches can be used to follow the reconstitution of peptides into membranes and to describe some of their fundamental characteristics. Whereas CD spectroscopy is used to monitor secondary structure in different solvent systems and thereby aggregation properties of the highly hydrophobic domain of p24, a protein involved in vesicle trafficking, solid-state NMR spectroscopy was used to deduce structural information and the membrane topology of a variety of peptide sequences found in nature or designed. ^{15}N chemical shift solid-state NMR spectroscopy indicates that the hydrophobic domain of p24 as well as a designed sequence of 19 hydrophobic amino acid residues adopt transmembrane alignments in phosphatidylcholine membranes. In contrast, the amphipathic antimicrobial peptide magainin 2 and the designed sequence LK15 align parallel to the bilayer surface. Additional angular information is obtained from deuterium solid-state NMR spectra of peptide sites labelled with $^{2}H_3$-alanine, whereas ^{31}P and ^{2}H solid-state NMR spectra of the lipids furnish valuable information on the macroscopic order and phase properties of the lipid matrix. Using these approaches, peptides and reconstitution protocols can be elaborated in a rational manner, and the analysis of a great number of peptide sequences is reviewed. Finally, a number of polypeptides with membrane topologies that are sensitive to a variety of environmental conditions such as pH, lipid composition and peptide-to-lipid ratio will be presented. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: oriented solid-state NMR spectroscopy; circular dichroism (CD) spectroscopy; p24; magainin; LAH4; CRAC domain; designed model peptide; supported lipid bilayers; membrane topology; peptide-membrane interactions; transmembrane domain

Introduction

Peptides are used to design new materials, drug delivery systems and analytical devices [1–4]. They can be prepared in quantitative amounts in a reproducible manner and under stringent conditions of quality control. With the advent and development of solid-phase peptide synthesis as well as methods for bacterial over expression, it has become feasible to prepare peptide libraries where the characteristics of the resulting sequences can be easily modulated by amino acid exchange using not only the 20 common amino acids but also non-natural, chiral and even non-peptidic building blocks almost at will [5–7]. Furthermore, labels can be included or added for fluorescence, EPR or NMR spectroscopic measurements, which provide a convenient means to investigate and modulate the design features, or to be used as sensors for practical applications. Consequently, as can be seen by the diverse contributions to this Special Issue, the practical applications of peptides are wide spread, and in this paper, we will focus on their use in lipid membrane environments.

Apart from their relative ease in preparation and their versatility, peptides have the added advantage that when designed in the appropriate manner, they form or can be included into self-associating systems [1–4,8]. Biological or model membranes are such supramolecular complexes that self-assemble into bimolecular layers of high Ohmic resistance, and these can be doped with sensors, linked to solid supports, and connected to electrodes just to mention a few examples [9–11]. In particular, lipid vesicles or supported lipid bilayers have found their way into a number of applications such as drug delivery or sensor devices [12,13]. A virus or a vesicle for drug delivery that at the same time carries a receptor-recognition sequence represents a setting where the barrier forming properties of lipid membranes and the selective recognition of targets have successfully been combined with each other [13,14].

During the design and assembly of such supramolecular assemblies, a number of methods are required that allows one to monitor and control if the desired structural elements fold and behave as anticipated. Therefore, in this paper, we will present

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Abbreviations: *CD, circular dichroism; DPC, dodecyl phosphocholine; CP, cross polarization; CRAC, cholesterol recognition/interaction amino acid consensus; di-C12:0-PC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; di-C20:1-PC, 1,2-dieicosenoyl-sn-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; FID, free induction decay; HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; MAS, magic angle spinning; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SM, sphingomyelin; SUV, small unilamellar vesicles; TFE, 2,2,2-trifluoroethanol; TMD, transmembrane domain.*

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a few examples how biophysical approaches have helped us to follow the membrane reconstitution process and which information can be obtained from such measurements from the final assembly. In particular, we will demonstrate how the reconstitution process was optimized for a highly hydrophobic membrane anchor using CD spectroscopy and how solid-state NMR spectroscopy can be used to determine the topology and alignment of the membrane constituents.

Materials and Methods

Materials

Organic solvents were from Sigma Aldrich (Saint Louis, MO, USA). The lipids were from Avanti Polar Lipids (Alabaster, AL, USA). Fmoc-amino acids were from NovaBiochem Merck-Millipore (Darmstadt Germany).

Peptide Synthesis

The peptides were prepared by solid-phase peptide synthesis using a Millipore 9050 automatic synthesizer and the Fmoc-chemistry. The [^{15}N-Leu23, $^{2}H_3$-Ala20]-p24TMD peptide has the sequence KKTNS RVVLW SFFEA LVLV**A** MT**L**GQ IYYLK R-CONH2 where a ^{15}N-labelled leucine and $^{2}H_3$-alanine (Cortecnet, Voisins les Bretonneux, France, or Aldrich, Saint Louis, MI, USA) were incorporated at the positions shown in bold. Another transmembrane model sequence, which was extended by a CRAC motif was designed carrying a $^{2}H_3$-alanine at position 24 (KKNIT NWLWY LKLFI MIALA LAL**A**L ALALA LKK-CONH$_2$). The magainin 2 (GIGKF LHSAK KFGKA FVGEI MNS-CONH$_2$), hΦ19W (KKKAL LALLA LAWAL ALLAL LAKKK) and KL15 (KKLLK ALKKL LKKLK) sequences have been presented previously [15,16]. Table 1 provides an overview of the peptide sequences discussed in this paper.

The peptides were purified by reversed phase high-performance liquid chromatography (HPLC) (Bischoff Chromatography, Leonberg, Germany) or (Gilson Chromatography, Villiers-Le-Bel, France) on Prontosil 300-6-C4 5.0-μm (Bischoff, Leonberg, Germany) or Luna 100-C18 5.0-μm columns (Phenomenex, France) using an acetonitrile/water gradient. The gradient was established with solvent A (acetonitrile/water/TFA, 10/90/0.1, v/v) and B (acetonitrile/TFA, 100/0.1, v/v), and the peptides eluted at the solvent B concentration as indicated in the succeeding text. When tested by analytical HPLC, the purity of p24TMD was 96%. This being the most difficult to dissolve and resolve, it can be safely estimated that all peptides are $>$ 90% pure. The identity of the peptides was verified by matrix-assisted laser desorption ionization mass spectrometry. For the peptides used here, the theoretical masses and the elution (in % B) from the HPLC columns are the following: CRAC-TM (MW 3769.8, 65% B), hΦ19W (2673.5, 40%), KL15 (1793.4, 19%), magainin 2 (2465.9, 26%) and p24TM (3674.4, 44%). The measured experimental masses are in general within 1–2 units of the expected values when taking into consideration that ^{15}N and $^{2}H_3$ labels increase the mass by +1 and +3 units, respectively.

Circular Dichroism Spectroscopy

A 1 mg/mL stock solution of the p24TMD sequence was prepared in water. The concentration of peptide was determined by absorbance at 280 nm by use of an extinction coefficient calculated from the content of tryptophan, tyrosine and cysteine residues [17] (cf. http://www.basic.northwestern.edu/biotools/proteincalc.html). This stock solution was dried and dissolved in 300 μL of the respective solvents to give a 30 μM concentration.

In another set of experiments, the p24TMD peptide was reconstituted into membranes by dissolving the peptide and POPC in HFIP/water 50/50 (v/v). The solvent was removed first under a stream of nitrogen to form a thin film and then by exposure to high vacuum. Thereafter, the sample was hydrated with 10 mM phosphate buffer and tip sonicated to form small unilamellar vesicles.

The CD spectra were recorded on a Jasco J-510 spectropolarimeter (Tokyo, Japan) with a 50 nm/min scan speed, and data points were collected from 250 to 190 nm at 25°C using a quartz cell of 1 mm path length. The spectra were processed using the spectra manager software of the instrument, and the baseline corrected for solvent contributions (H_2O) by subtraction. Secondary structure analyses were performed with the CDpro Web server using the CONTINLL algorithm [18].

Sample Preparation for Solid-State NMR Spectroscopy

The preparation steps for the reconstitution of peptides into uniaxially oriented samples are documented in detail and illustrated in references [19,20]. In short, selectively labelled [^{15}N-Leu23, $^{2}H_3$-Ala20]-p24TMD was first dissolved in HFIP, and the solvent was removed under a stream of nitrogen. The

Table 1. Amino acid sequences of the peptides discussed in this paper

Alamethicin[a]	Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phl						
CRAC-TM	KKNIT	NWLWY	LKLFI	MIALA	LALAL	ALALA	LKK-CONH$_2$
hΦ19W	KKKAL	LALLA	LAWAL	ALLAL	LAKKK		
Htt17[b]	MATLE	KLMKA	FESLK	SF			
ICP47[c]	SWALE	MADTF	LDNMR	VGPRT	YADVR	DEINK	RGR
KL15	KKLLK	ALKKL	LKKLK				
LAH4	KKALL	ALALH	HLAHL	ALHLA	LALKK	A-CONH$_2$	
Magainin 2	GIGKF	LHSAK	KFGKA	FVGEI	MNS-CONH$_2$		
PGLa	GMASK	AGAIA	GKIAK	VALKA	L-CONH$_2$		
p24TMD	KKTNS	RVVLW	SFFEA	LVLVA	MTLGQ	IYYLK	R-CONH$_2$

The one-letter code is used except for the peptaibol alamethicin, which caries a considerable number of unusual amino acids.

[a]The F50/7 isomorph is shown.′ Aib: α-aminoisobutyric acid, Phl: L-phenylalaninol.

[b]Htt17 represents the most amino-terminal residues of the huntingtin protein.

[c]Residues 2-34 of this 88 residue early gene product of Herpes simplex virus.

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peptide was then dissolved in HFIP/water 50/50 (v/v), which had been shown to result in stable helix formation (Figure 1B). Thereafter, the peptide was added in a step-wise manner to POPC in HFIP. This was performed in such a manner that the water content remained low, and peptide aggregation was avoided.

Thereafter, the solvent was partially evaporated under a stream of nitrogen to reduce its total volume to about 0.5 mL. At this point, the clear and viscous sample was deposited onto 20 ultrathin glass plates (thickness 00 (about 80 μm), 6 × 11 mm or 8 x 22 mm for some of the other peptides, Paul Marienfeld, Lauda-Königshofen, Germany), slowly dried in air and the residual of solvent evaporated under high vacuum. Sample hydration was achieved at 98% humidity and the glass plates stacked on top of each other. The sample was stabilized by Teflon tape and sealed in plastic wrapping to avoid dehydration. The final peptide-to-lipid (P/L) molar ratio is 1%.

The oriented samples made of 5 mole% [^{15}NLeu17]-hΦ19W in POPC, 2 mole% [^{15}N-Ala15]-magainin2 in di-C12:0-PC, 2 mole% [^{15}N-Leu7]-KL15 in di-C20:1-PC and 2 mole% [$^{2}H_3$-Ala24]-CRAC-TM in POPC were prepared in an analogous manner by initially co-dissolving the peptide and lipid in TFE (hΦ19W, KL15), methanol/chloroform 2:1 (v/v, CRAC-TM) or methanol/chloroform 1:1 (v/v; magainin 2).

Non-oriented samples were prepared in an analogous manner except that a film of 0.13 mg peptide and 2 mg of deuterated lipid was formed in a glass tube, which was then resuspended in 200 μL of 10 mM phosphate buffer by vortexing and sonication in a water bath followed by 10 of freeze/thaw cycles. The resulting vesicle suspension was pelleted in a bench-top centrifuge (Biofuge Pico, Hearaeus Instruments, Hanau, Germany) at 13 000 rpm for 1 min directly into a tube for microcentrifuges (VWR Radnor, PA, USA), which was directly used for the NMR measurements.

Solid-State NMR Spectroscopy

The samples were inserted with the normal parallel to the magnetic field of a Bruker Avance NMR spectrometer operating at 9.4 Tesla (Rheinstetten, Germany) and the NMR spectral acquisitions calibrated as described in detail in reference [19]. All spectra were recorded at 295 K.

Proton-decoupled ^{31}P solid-state NMR spectra were recorded using a Hahn echo pulse sequence [21] with an echo time of 40 μs, a repetition time of 3 s, a ^{31}P B_1 field of 80 kHz and a spectral width of 40 kHz. An exponential line broadening of 150 Hz was applied prior to Fourier transformation. The spectra were referenced relative to 85% H_3PO_4.

Proton-decoupled ^{15}N solid-state NMR spectra were recorded using a CP pulse sequence [22] with a CP time of 800 μs, a repetition time of 3 s, a B_1 field of 31 kHz (^{15}N and ^{1}H channel) and a spectral with of 38 kHz. The FIDs were subject to an exponential apodization with line broadening of 100 Hz prior to Fourier transformation. The spectra were calibrated relative to external ammonium chloride (40 ppm), which places NH_3 at 0 ppm [23].

Deuterium solid-state NMR spectra of samples made of POPC labelled with d_{31} along the palmitoyl chain were measured using a quadrupolar echo sequence [24] with a repetition delay of 0.3 s for the lipid and 0.6 s for the peptide spectra, an echo time of 100 μs, a B_1 field of 40 or 62 kHz for the lipid and peptide spectra, respectively. The FID was oversampled with a spectral with of 100 kHz enabling the precise adjustment of the echo by left-shifts after the acquisition. The processing included an exponential apodization with line broadening of 200 Hz for the lipid and 500 Hz for the peptide spectra, respectively. The spectra were referenced relative to D_2O.

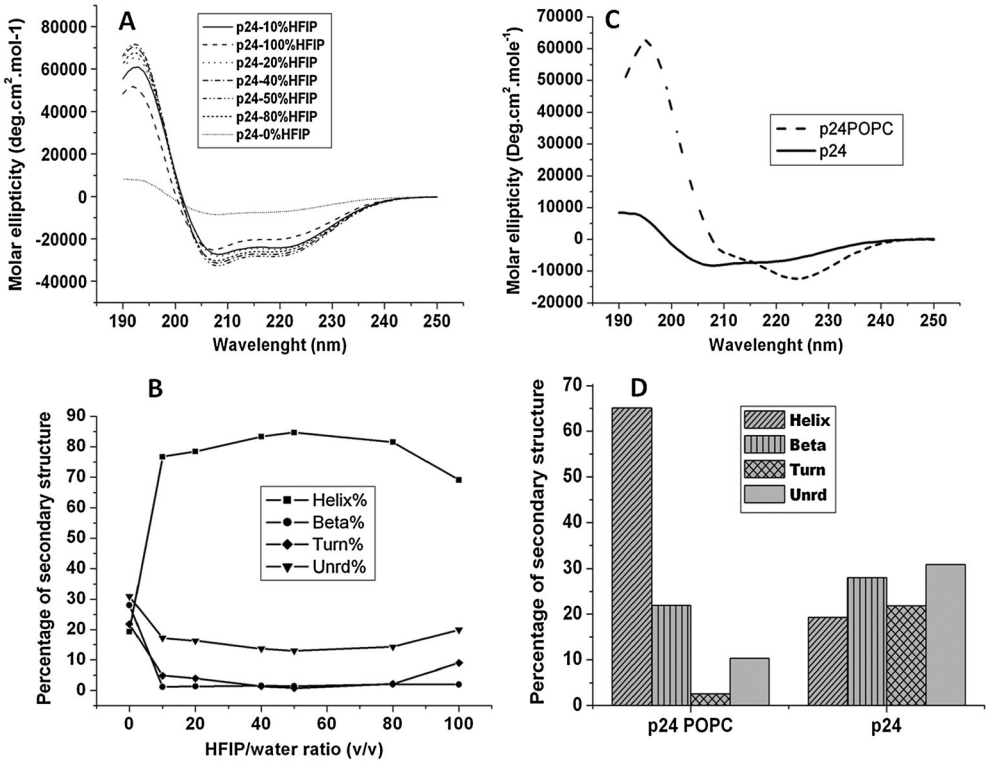

Figure 1. Circular dichroism data to optimize p24TMD reconstitution. (A) p24TMD folding in solvents of different HFIP/water composition. (B) The secondary structure preferences of p24TMD as a function of HFIP concentration. (C) CD spectra of p24TMD in POPC liposomes (dashed line) or in aqueous buffer (solid line). The spectra were recorded in 10 mM phosphate buffer, pH 7 at 25°C. (D) The secondary structure composition of p24TMD in the presence or absence of POPC as obtained from the CD spectra using the CDPro software.

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Results and Discussion

Developing a Membrane Reconstitution Protocol for Hydrophobic Peptides

Cellular membranes consist of a wide variety of lipid species varying in head group and fatty acyl chain composition. The lipid composition not only varies between organelles, but they are also distributed unevenly between the inner and outer leaflets of the cellular or organelle membranes [25,26].

Whereas many membrane lipids and/or their precursors are synthesized in the endoplasmic reticulum of eukaryotic cells, they are then transported either monomolecularily by lipid transport proteins, or with the membrane in intracellular transport vesicles [27]. There is a continuous flow of lipids between the endoplasmic reticulum the Golgi, the plasma membrane and the endomembrane system, and during the transport the lipids are processed and sorted to assure the differing lipid compositions of individual subcellular membranes. In this context, a highly specific interaction of a SM lipid carrying a C18 fatty acyl chain with the (predicted) transmembrane domain of p24, a protein of the COPI transport vesicles has been described [28–30]. Such observations are strongly suggestive that the lipids provide more than merely the 'solvent' for the proteins and the 'bricks' of the membrane barrier; however, when compared to protein or nucleic acid interactions, little is known about specific protein–lipid interactions [31].

In order to analyse this interaction of p24 with membranes of different composition, a 31 amino acid polypeptide, which includes the hydrophobic domain of the p24 protein, was designed. The sequence of the peptide is KKTNS RVVLW SFFEA LVLV**A** MT**L**GQ IYYLK R-CONH2 , where the underlined residues make up a domain of high hydrophobicity. The doubly underlined positions have been identified as being of high relevance for the specificity of interactions with SM C18 [30], and charged amino acids were added at the N-terminus and C-terminus to act as anchor sequences and to improve peptide solubility and handling. Alanine-20 and leucine-23 (in bold) were labelled with stable ^{2}H and ^{15}N isotopes, respectively, for NMR spectroscopic investigations. It should be noted that the study of membrane-associated peptides can be a difficult task as they tend to aggregate, and their preparation and purification are often difficult.

In order to investigate the interaction of different lipids with p24TMD (Table 1), a reconstitution assay was performed where, in a first step, the synthetic peptide was dissolved in a variety of solvents including TFE, dichloromethane, HFIP and aqueous solutions as well as in the presence of detergents and investigated by CD spectroscopy.

Interestingly, for most of the solvents used to initially dissolve the peptides and lipids, the CD spectra are either characterized by low intensities and/or characteristic of high β-sheet/random coil conformations (not shown) suggesting aggregated structures during the reconstitution process [32,33]. Depending on the peptide finding a good solvent where both the lipids and the peptides are dissolved can be difficult, but in our and others hands, but HFIP has proven a versatile solvent for this purpose [20,34–36]. HFIP exhibits a combination of strong hydrogen bonding and at the same time hydrophobic properties with structurating capabilities on some polypeptides [37]. As a consequence, it has been shown to dissolve hydrogen bond acceptors such as organic polyamides, polyacrylonitriles, polyesters and even β-sheet aggregated polypeptides [38,39].

Indeed, when the secondary structure was tested upon exposure to HFIP/water mixtures, for which the composition was varied in a systematic manner, the peptide adopts a largely α-helical secondary structure in the presence of even small amounts of HFIP suggesting that, under these conditions, it can be maintained in a reconstitution-competent state (Figure 1A). A more detailed analysis indicates that p24TMD is ≥80% helical in HFIP/water 50/50 (v/v) (Figure 1B).

Therefore, the peptide was reconstituted into liposomes starting from such an HFIP/water mixture, which is also suitable to dissolve many phospholipids. After co-dissolving peptides and POPC, the solvents were evaporated in such a manner to form a film along the glass walls of the test tube, resuspended in buffer, vortexted, sonicated, and the thus prepared sample analysed by CD spectroscopy (Figure 1C). The CD spectrum of 3 mole% p24TMD reconstituted in POPC SUV is shown in Figure 1C and compared with the spectrum of 30 μM p24TMD in aqueous buffer. Whereas the high helix-forming propensity is maintained in the membrane environment, much of the spectral intensity is lost in the absence of lipids (Figure 1D).

This example illustrates how optical spectroscopy can help to scan a large number of solvents and experimental conditions with the goal to develop a protocol for efficient membrane reconstitution. Optical techniques such as CD- and fluorescence spectroscopy require orders of magnitude less peptide than, for example, NMR spectroscopy. The semi-quantitative information that they provide about the global secondary structure of the peptide in a wide variety of conditions provides a good screening opportunity to test a large number of sample conditions in a single or a few days. Similar approaches can be used in order to find a suitable detergent for concentration for multidimensional solution NMR investigations [40–42].

Investigating the Membrane Interactions of Polypeptides by Solid-State NMR

In a next step, the p24TMD was investigated by solid-state NMR spectroscopy, a technique which can provide quite detailed information about the structure, topology and interactions of membrane-associated peptides [43,44]. The method requires several milligrammes of isotopically labelled peptides per sample and many hours of spectrometer time. It is therefore advantageous that essential steps of the membrane reconstitution protocol were already established using CD spectroscopy (Figure 1). In solid or semi solid samples, the NMR parameters are anisotropic, that is, the chemical shifts and dipolar and quadrupolar couplings all depend on the molecular alignment relative to the magnetic field of the spectrometer [44]. In samples where all spatial orientations are present, this results in very broad spectral line shapes with little or no spectral resolution [45]. In order to re-introduce high-resolution into these spectra either very fast sample spinning around the MAS can be applied and results in spectral averaging and narrow lines [43], or static oriented samples are investigated that reveal the NMR parameters of just one molecular orientation in space [44]. The latter approach has been applied to membrane-associated peptides, which have been labelled with ^{13}C, ^{15}N and/or ^{2}H [44,46].

Whereas MAS solid-state NMR follows similar concept as multidimensional NMR in solution by providing isotropic chemical shift spectra and correlations representative of distance or intra-molecular angles [43], uniaxially oriented samples reveal valuable highly complementary information about the alignment

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of bonds and consequently whole molecules relative to the magnetic field direction [44]. These data can be used to reconstruct the structure of biomacromolecules and/or the relative alignment of molecules within supramolecular complexes [41,47]. A successful approach consists in labelling a peptide bond with ^{15}N and reconstituting the peptide into lipid bilayers that are oriented with the normal parallel to the magnetic field direction. From such samples, alignment information of the ^{15}N-H vector and thus the helical domain are obtained in a straightforward manner [44,45]. Notably, the natural abundance of ^{15}N is only 0.4% of all nitrogen atoms, therefore, in general, only the signal from the isotopically enriched sites (typically >95%) is observed. For helical domains labelled with ^{15}N at their backbone chemical shifts around 200 ppm correlate with transmembrane helices, whereas values <100 ppm are indicative of domains oriented parallel to the bilayer surface [45]. The tilt and azimuthal angles of the helices can be determined quite accurately when it is possible to combine the ^{15}N chemical shift information with angular restraints that are obtained from $^{2}H_3$-labelled alanines, where the resulting deuterium quadrupolar splitting is correlated to the relative alignment of the Cα–Cβ bond, and this combination has provided valuable information on a variety of polypeptides that were prepared by solid-phase peptide synthesis [15,48–50].

When the ^{15}N solid-state NMR spectrum of p24TMD labelled at the leucine-23 position was investigated, a chemical shift of 217 ppm was recorded (Figure 2A). This measurement, together with the high helical content measured by CD spectroscopy, is indicative that the peptide spans the membrane in a transmembrane helical fashion.

Whereas natural transmembrane helical sequences are of complex compositions, they all share a high hydrophobicity and a length of typically 16–20 residues. Several of these have been investigated by ^{15}N solid-state NMR spectroscopy and a transmembrane alignment confirmed (e.g. [34,51–54]). These general features can be used to design much simpler domains such as sequences made solely of leucines or of mixtures of alanines and leucines [55–61]. One such reductionist peptide, hΦ19W (Table 1), has been designed in our laboratory, with an additional tryptophan for fluorescence studies [15,16]. For [^{15}N-Leu17]-hΦ19W, a ^{15}N chemical shift is observed at 202 ± 2.5 ppm when reconstituted into oriented POPC bilayers thereby confirming the transmembrane helical design of the sequence (Figure 2B). Notably, dimerisation or higher order oligomerisation of such transmembrane sequences can be controlled by small modifications of their primary sequences [62–64].

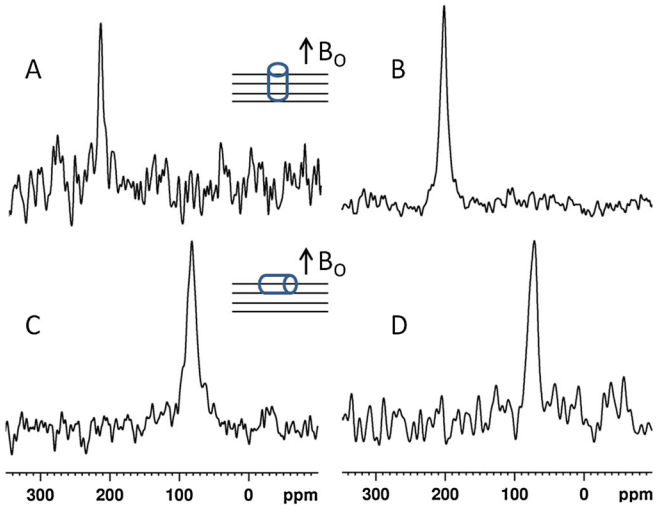

Figure 2. Proton-decoupled ^{15}N cross polarization NMR spectra of peptides labelled with ^{15}N at a specific position and reconstituted into oriented phosphatidylcholine membranes: (A) 1 mole % [^{15}N-Leu23]-p24TMD in POPC, (B) 5 mole% [^{15}N-Leu17]-hΦ19W in POPC, (C) 2 mole % [^{15}N-Ala15]-Magainin2 in di-C12:0-PC and (D) 2 mole % [^{15}N-Leu7]-KL15 in di-C20:1-PC. The corresponding helix orientation for each row is sketched in the inserts.

The solid-state NMR spectrum of another designed transmembrane helical domain is shown in Figure 3A. Here, the peptide was prepared with an alanine carrying a deuterated methyl group, reconstituted into oriented phospholipid membranes and investigated by ^{2}H solid-state NMR spectroscopy. Due to fast rotation around the Cα–Cβ bond, all three deuterons are equivalent and exhibit identical NMR spectra. The deuterium nucleus is quadropolar (spin 1), characterized by three energy levels with two allowed transitions. Therefore, the spectrum of the deuterated alanine methyl group is characterized by two lines, which can be separated by up to 72 kHz, its exact value depending on the dynamics of the deuterated site and its alignment relative to the magnetic field of the NMR spectrometer (for additional details cf. reference [44]). For the transmembrane model sequence labelled at position Ala-24, the frequency difference between the two peaks is 12 ± 1 kHz and represents the quadrupolar splitting, which is directly correlated to the average angle of the Cα–Cβ bond relative to the magnetic field direction/membrane normal (Figure 3B).

In contrast, many amphipathic sequences exhibit a high hydrophobic moment and consequently they align at the membrane interface parallel to the surface. One of the early examples investigated by the ^{15}N solid-state NMR technique is magainin 2, which orients parallel to the membrane surface under many different conditions so far investigated [65]. This observation is confirmed by the experiment shown in Figure 2C, where the peptide was reconstituted into a very thin membrane made of di-C12:0-PC and a chemical shift of 82 ± 4 ppm is measured. Notably, membranes of small hydrophobic thickness have been shown to promote a transmembrane alignment of other peptide sequences [55,66,67], and this experiment thereby provides a stringent test for the propensity of a sequence to traverse the bilayer. In-plane alignments have also been observed for many other sequences found in nature including antimicrobial peptides [40,49,68–76], signal sequences [52], surfactant peptides [77], the amino-terminal membrane anchor of huntingtin [41] or a domain of the Herpes virus protein ICP47 [48] (Table 1). Furthermore, peptide sequences have been designed to orient along the membrane surface [78–81], and this design has been confirmed by solid-state NMR spectroscopy, for example, for [^{15}N-Leu7]-KL15 [15] (Table 1), which exhibits a chemical shift of 71 ± 5 ppm in di-20:1-PC lipid bilayers (Figure 2D).

Furthermore, some polypeptides exhibit a more dynamic behaviour where transitions from the in-planar configuration to more inserted and even transmembrane alignments can be triggered by environmental changes such as pH [61,82–85], lipid fatty acyl [67,72,86,87] or head group composition [72,88,89], hydration [34,90], buffer [89], peptide concentration [87,91] or the presence of other peptides. Notably, domain realignment in the presence of other polypeptides could be of importance for the regulation of membrane proteins [67,86]. Even alamethicin (Table 1), a long-standing paradigm for the formation of pores by transmembrane helical bundles [92] has been found

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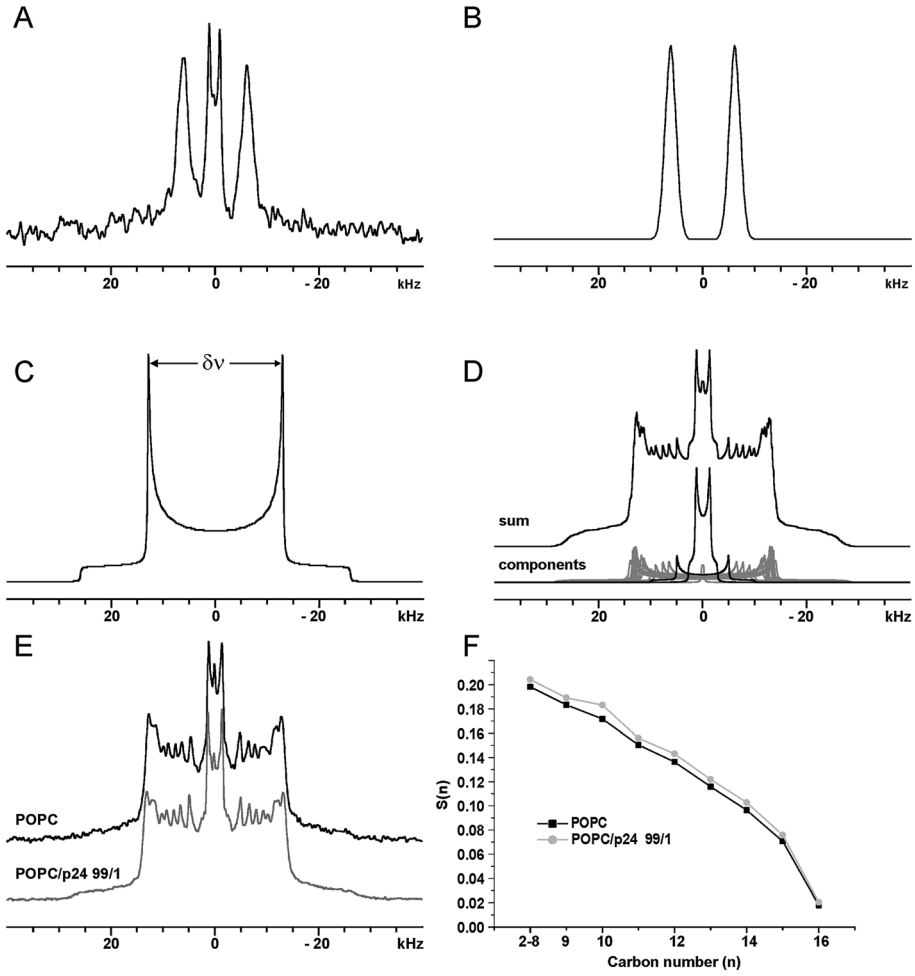

Figure 3. (A) ^{2}H spectrum of the [$^{2}H_3$-Ala24] CRAC-TM model sequence reconstituted into oriented POPC bilayers. The central doublet corresponds to water molecules interacting with the oriented membrane. (B) Simulated spectrum of $^{2}H_3$-alanine with an angle of 48° ± 1° between the Cα–Cβ bond and the magnetic field when a static quadrupolar coupling constant of 72 kHz is taken into a consideration. (C) Simulated spectrum of a ^{2}H solid-state NMR spectrum obtained from a non-oriented sample carrying a single site labelled with ^{2}H (powder pattern of a quadrupolar nucleus). (D) Simulated ^{2}H solid-state NMR spectrum of POPC-d_{31} where the signals from many sites with different quadrupolar splitting add up. (E) Experimental ^{2}H solid-state NMR spectra of POPC-d_{31} in the presence (grey) or absence (black) of [^{15}N-Leu23]-p24TMD. (F) Deconvolution of the spectra shown in E provides position-dependent quadrupolar splittings and the corresponding deuterium order parameters S_{CD}. The p24TMD peptide causes a small increase in several of the POPC-d_{31} order parameters. The estimated error bar is <0.004 for S_{CD}, which fits within the outline of the symbols used in panel E.

to switch between transmembrane and in-planar alignments under certain conditions [87,88]. In a similar manner, magainin 2 and PGLa (Table 1) have been considered to exhibit stable in-planar alignment. Interestingly, whereas magainin 2 has shown in-planar orientations under all conditions so far investigated [65], this is not the case for PGLa. The latter has been found to be able to adopt a multitude of topologies in a manner that depends on environmental factors such as lipid composition, peptide-to-lipid-ratio and the presence of magainin 2 [67,86].

The question remains which structural features cause the different physico-chemical behaviour of PGLa and magainin 2, both being linear cationic amphipathic peptides found in the skin of the African clawed frog. At the present time, we can only speculate, as to our knowledge, no experimental data have been accumulated to answer this question. However, the structures of a number of amphipathic helical peptides have been determined in DPC micelles, and their alignments tested in oriented lipid bilayers. On the one hand, these include magainin 2 [93,94] and the 17-residue N-terminal membrane anchor of huntingtin, Htt17 [41,50], both exhibiting stable orientations along the membrane surface. On the other hand, PGLa [95,96] and LAH4 [84,89,97] undergo transitions in more tilted and even transmembrane alignments more easily (Table 1). When the Edmundson wheels are constructed of the domains that are found to be helical in micellar environments, the following differences become apparent (Figure 4). First, the helical domain of LAH4 PGLa ends with a lysine, and including this residue in the analysis enlarges considerably the hydrophilic face of the α-helices. It seems well possible that this residue at the very end of the helical structure is in dynamic exchange between helical and non-helical conformations, while in the latter conformation, it can interact more easily with the aqueous phase. Whereas the occurrence of lysine in the helical domain may be stabilized by its possibility to snorkel to the membrane surface, it should also be mentioned that, in a highly curved micelle, the energetic penalty of keeping a lysine at the C-terminus of the helix is less pronounced than in a planar lipid bilayer. Second, when the helical wheel is analysed by assuming that this C-terminal lysine is in a non-helical conformation and exposed to the water face the hydrophobic angles of LAH4 and PGLa are much increased and considerably larger

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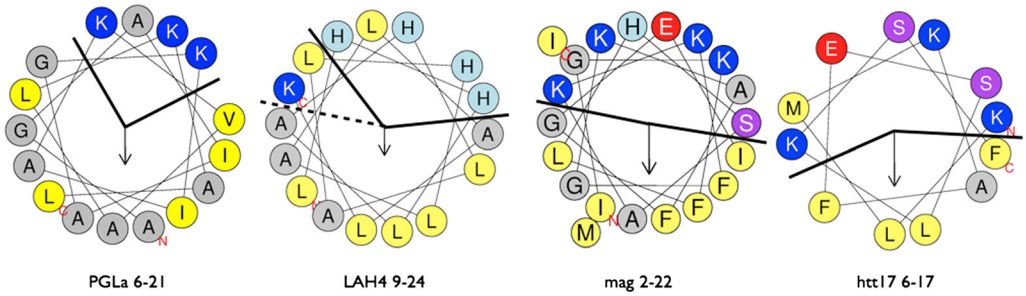

Figure 4. Helical wheel representations of PGLa, LAH4 at acidic pH, magainin 2 and Htt17. The outlines of the helices are taken from NMR structures that have been determined in DPC micelles [41,93,95,97]. The charged residues are shown in red (negative), dark and light blue (positive), the polar ones in magenta, hydrophobic residues in yellow and the glycines and alanines which exhibit little preference for the interface or the hydrophobic interior in grey [82]. The very N-terminal and C-terminal residues of the helix domains are marked by red letters. The black lines indicate the separation of predominantly charged/polar face from the hydrophobic side, where the dotted lines include the most C-terminal lysine of LAH4 whereas the continuous lines exclude this residue. The arrows represent the calculated hydrophobic moments. The helices were created using http://heliquest.ipmc.cnrs.fr [131].

when compared with Htt17 or magainin 2 (Figure 4). Third, the hydrophobic moments of the helical domains with less stable topologies are considerably smaller than those of magainin 2 or Htt17 (Table 1). Whereas a strong hydrophobic moment combined with a large hydrophobic angle suggest that the helices that penetrate more deeply into the membrane hydrophobic core exhibit less stable alignments, the resulting topological changes may be coupled to conformational rearrangements. In this context, it is noteworthy that small energetic differences can tip the balance between different topologies, and the exchange of a single amino acid can cause a shift from an in-planar to a transmembrane propensity [56,57].

Peptide domains that monitor specific environmental conditions, including, for example, a different membrane composition or pH in cancerous tissues, or that accumulate in the presence of specific receptor molecules have a high potential to be useful in materials developed for biomedical applications [8,98,99]. Other environmental factors that have been suggested or experimentally proven to influence the membrane topology of polypeptides are transmembrane electric fields [92,100], peptide-peptide interactions [67,101] and/or membrane hydration [34,67,87].

Figure 2 shows the NMR spectra of two sequences isolated first from natural sources and two peptides designed to adopt a particular topology. Clearly, with our present knowledge, it is possible to design peptides that interact with a lipid matrix in a defined manner [84,102] and to use oriented solid-state NMR spectroscopy (Figure 2), ATR FTIR [103,104] or oriented CD spectroscopy [105,106] to validate such design features. Whereas the latter two techniques can follow the topology in a semi-quantitative manner, the analysis by solid-state NMR spectroscopy in combination with isotopic labelling can be applied in a residue-specific and/or domain-specific manner. When required, the NMR approach has the potential for a full structure determination using larger sets of isotopic labels and/or more elaborate NMR experiments [107–109]. Importantly, the aforementioned examples and the published data also illustrate that the technique can be applied to membranes of very different fatty acyl chain (Figure 2) or lipid head group composition [65] and to a wide variety of peptides and proteins [107–111]. Therefore, when a material or a devise is to be developed, the NMR methods described are by far not limited to systems that closely resemble the aforementioned examples. Notably, more recent developments in the field of solid-state NMR even allow a topological analysis for peptides in non-oriented membrane systems [112], and the characterization of the dynamics and thereby the oligomerization of membrane-associated peptides using the same samples that helped in the topological analysis [41,113]. Such experiments provide additional useful information to fine-tune the design and preparation of peptide-based materials and devices.

So far, the discussion of the design features of the peptide-lipid supramolecular assemblies focused on particular characteristics and functionalities of the peptides as well as methods to analyse their structure, topology and dynamics in membranes. However, the lipid membranes are soft and adapt to the environment, therefore, the lipids can much contribute to the properties of the final result and need to be taken into consideration. As the ^{31}P nucleus is one of the more sensitive NMR nuclei and the only isotope of the phosphorous atom, no isotopic enrichment is necessary to record quite sensitive ^{31}P solid-state spectra of the phospholipids, which in addition are typically present in tenfold to 1000-fold molar excess when compared with peptides. The ^{31}P solid-state NMR spectra of the oriented phosphatidylcholine sample containing 2 mole% KL15 is shown in Figure 5A. This is the same sample for which the ^{15}N solid-state NMR spectra are shown in Figure 2D. The spectrum exhibits a broad spectral line shape between 30 and −15 ppm with its major intensity around 30 ppm. Thereby, the ^{31}P chemical shift of the phosphatidylcholine headgroup in the liquid crystalline bilayer shows an anisotropy of 45 ppm in a related manner as outlined for the ^{15}N chemical shift of the peptide bond (cf. above). Whereas the phospholipids that are aligned with their long axis parallel to the magnetic field direction resonate at 30 ppm (Figure 5B), those perpendicular exhibit a chemical shift at −15 ppm. A spectrum with all lipid alignments present at random is shown in Figure 5C. In Figure 5A, the sample has been aligned in such a manner that the glass plate normal coincides with the magnetic field of the spectrometer, and the spectrum is therefore indicative that a large proportion of the lipids are aligned parallel to the membrane normal. The spectrum shown in Figure 5A can be explained by the addition of contributions such as the ones represented in Figure 5B and C, where the smaller intensities <30 ppm are due to misaligned sample. However, phospholipids where the molecular orientation is rearranged in the vicinity of the p24 domain or a range of head group conformations, which may, for example, be a result of electrostatic interactions [114] should also be considered.

Notably, macroscopic alignment of pure POPC lipid bilayers has been achieved in the past where only a 30 ppm signal intensity becomes visible suggesting that a high degree of sample alignment can be obtained by orienting pure phospholipid bilayers between glass plates [19,40,115]. In contrast, the ^{31}P line shapes observed in the presence of antimicrobial peptides are

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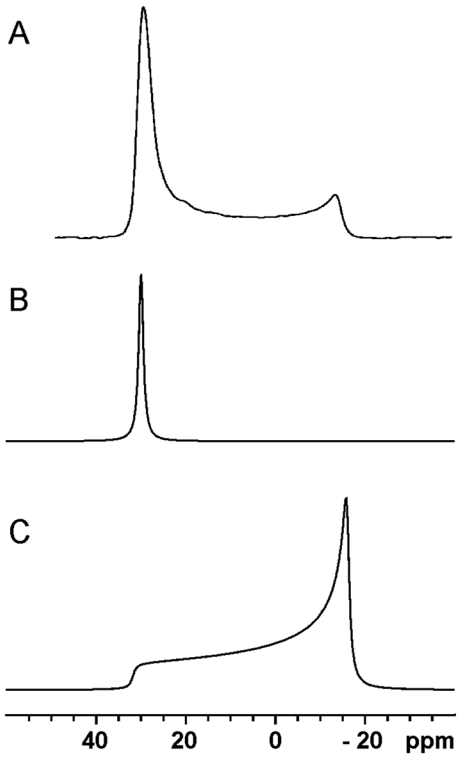

Figure 5. (A) Proton-decoupled ^{31}P solid-state NMR spectrum of di-C20:1-PC in the presence of 2 mole% [^{15}N-Leu7]-KL15. Simulated ^{31}P solid-state NMR spectra of liquid crystalline phosphatidylcholine bilayers (B) oriented with the lipid long axis parallel relative to the magnetic field direction and (C) exhibiting a random distribution of alignments.

suggestive for membrane thinning or regions of high local curvature [116]. Such membrane deformations may ultimately result in membrane pore formation and lysis [117]. Therefore, the ^{31}P-NMR spectra provide additional valuable information with regard to the interactions and/or mechanisms of action of the polypeptides. Importantly, antimicrobial peptides or peptide-derived mimetics have been included into the design of materials, for example, to confer antibacterial resistance to implants and surfaces [118–120]. Notably, for the design of novel, more efficient or cheaper to produce compounds or for the correct insertion into a new material, knowing the peptides' membrane insertion properties and their mechanism of action makes a considerable difference. For example, in the case of antimicrobial peptides, realizing that they interact in an interfacial fashion has resulted in the design of short peptides, alkylated tripeptides or amphipathic polymers that are unsuitable to span the membrane [121–123]. Their properties would be difficult to explain without the knowledge that the antimicrobial action of a number of peptides is better when aligned parallel to the membrane surface, rather than in the configuration of a transmembrane helical bundle as was initially suggested (reviewed in [100]).

Whereas the ^{31}P solid-state NMR spectra monitor the conformational and dynamic properties at the level of the headgroup of the phospholipids including local conformational rearrangements [114] and macroscopic phase transitions [124,125], the packing of the lipid fatty acyl chains can be analysed by ^{2}H solid-state NMR spectroscopy of liposomes prepared with deuterated lipids [126]. Many of those are available commercially and have been used to follow the effects of the peptides on the lipid fatty acyl chain order parameters [73,116,127].

The ^{2}H solid-state NMR spectrum of a single non-oriented deuterated site is represented in Figure 3C and the corresponding quadrupolar splitting $\Delta\nu_Q$ indicated. When many sites with different quadrupolar splittings overlap, spectra as the ones simulated in Figure 3D are obtained. Finally, Figure 3E compares the ^{2}H solid-state NMR spectra of pure POPC (deuterated throughout the palmitoyl chain) with that in the presence of p24TMD. Each deuterated CH_2 and CH_3 segment results in a deuterium quadrupolar powder pattern from which the frequency separation of the two main intensities, that is, the deuterium quadrupolar splitting $\Delta\nu_Q$, can be extracted (Figure 3C). The comparison of the measured segment-specific deuterium quadropolar splitting with that of a static sample can be directly represented by the deuterium order parameters, and these are shown in Figure 3F in a position-dependent manner. Whereas the intercalation of in-plane aligned helices has in many cases resulted in a significant decrease of the order parameter [128–130], the transmembrane p24 has only a small effect on the palmitoyl chain packing (Figure 3E and F). Whereas here the modifications of the membrane packing occur on a more local level changes in the overall morphology, macroscopic phase properties, membrane thickness and curvature are also reflected in the ^{2}H NMR line shapes [116] and in combination with the corresponding ^{31}P spectra provide a valuable global view on the lipid system. Together with the spectra recorded from the peptides, a comprehensive analysis of the supramolecular assembly is thereby obtained (Figures 1, 2 and 3A).

In conclusion, here, CD spectroscopy has been used to test solubility, secondary structure preferences and to refine the membrane reconstitution protocol for peptides into membranes. Solid-state NMR spectroscopy provides a valuable tool to analyse the topology of membrane-associated peptides, the packing and morphology of the lipid membranes and thereby control of the design of the peptide sequences. A considerable number of peptides have been analysed using these techniques and some exhibit interesting features for applications in materials in particular those that 'sense' the environmental conditions.

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