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PAPER

The interaction of lipid modified pseudopeptides with lipid membranes†

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We have studied the structure of two lipopeptides based on the simple dipeptide building block L-Phe-D-Oxd. These peptides have been reported previously to form fiber-like materials. The lipopeptides synthesized here had the structures $C_n{}^2H_{(2n+1)}$CO-L-Phe-D-Oxd-OBn or $C_n{}^2H_{(2n+1)}$CO-D-Phe-L-Oxd-OBn with $n = 5$ or 11. Addition of the N-terminal lipid modification did not cause a major disturbance of the structures these molecules form. The lipid modifications themselves showed highly rigid structures as inferred from solid-state ^{2}H NMR. The peptide backbone showed ^{13}C NMR chemical shifts in agreement with β-sheet secondary structure. Addition of a lipid modification to the N-terminus is a common motif in biology to attach proteins to the membrane. Therefore, we also investigated the lipopeptides in the presence of synthetic POPC bilayers. Two different molecular species were detected under these circumstances: (*i*) lipopeptide monomers that showed chain order parameters similar to those of the host membrane, (*ii*) lipopeptide aggregates that exhibited very similar structures and dynamics as the crystalline aggregates. Overall, the lipopeptides showed a well defined and rigid secondary structure that is in agreement with fibrillar aggregates previously detected for those peptides without the lipid modification.

Introduction

The self-assembly of small peptides represents a key structural motif for the development of new materials and nanoscaled objects.[1,2] Various applications of small peptides in all fields of chemistry and material science have already been demonstrated. Further, peptide aggregation is of great importance in biochemistry and medicine as many severe diseases are caused by the aggregation of small peptides forming amyloid structures.[3] Medical and pharmacological approaches to develop drugs to cure amyloid diseases are underway, which heavily depend on structural knowledge about the fibrillation intermediates and final products. The exact pathway by which neurons degenerate in Alzheimer's disease is still poorly understood and is subject to intense research. However there is a general consensus that protein aggregation is a major element of cell toxicity.[3,4]

The search for small-molecule inhibitors of amyloid fibril formation is of fundamental importance for both academic and industrial research.[5,6] From the chemical point of interest, several studies of short peptides have demonstrated that peptides made of only a few amino acids can form stable aggregates with β-sheet- or α-helix-like structures that are held together by 2–3 hydrogen bonds.[7] Interesting results have been obtained in the area of self-assembling peptide nanotubes (SPNs)[8,9] formed by stacking cyclic peptides and stabilized by hydrogen bonds. These compounds are interesting because of their potential applications in several fields, among them chemotherapy and drug delivery.

We have recently shown that a simple dipeptide L-Phe-D-Oxd can form fibrous structures that are only stabilized by a single hydrogen bond forming both parallel or antiparallel β-sheet structures.[10,11] In accordance with our results, other groups have demonstrated that similarly simple compounds like L-Phe-L-Phe can form complex structures such as helices or carbon nanotubes.[12–14]

Here, we extend our investigations on the structures the building block L-Phe-D-Oxd can form by adding lipid chain moieties to the N-terminus of the peptides. Lipid modifications are known to play the decisive role in the formation of micelles, membranes, and other supramolecular structures due to the hydrophobic effect.[15] In addition, lipid modification is a common motif for membrane binding of peptides and proteins.[16–18]

Thus, we study whether structure formation of the lipopeptides can be influenced by the presence of lipid membranes as well by means of solid-state NMR spectroscopy under magic-angle spinning (MAS) conditions, which has proven to successfully characterize peptide aggregates[3] and membrane-associated peptides.[19] In particular lipidated peptides are well studied in their backbone[20–22] and lipid[23–25] structure and dynamics using ^{13}C or ^{2}H NMR, respectively. We found that β-sheet-like nanostructures are formed by the lipidated dipeptides in the absence of membranes, which

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also exist when simple lipid bilayers are present. However, some of the dipeptides are also dissolved in the membrane environment and are detected as highly mobile monomers that are immersed in the bilayer.

Results

Synthesis

The compounds analyzed in this work (Fig. 1) were synthesized in solution by a standard coupling reaction in the presence of HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate) and Et_3N (triethylamine) in dry acetonitrile. Both Boc-L-[U-^{13}C,^{15}N]-Phe-D-Oxd-OBn and Boc-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn have been easily prepared following a previously reported method,[10] as described in the Experimental Section. These compounds are enantiomers, as fully labeled amino acids are available only in the L form as far as we know. Lipopetides **A** and **B** have been prepared by acylation of the Boc-L-[U-^{13}C,^{15}N]-Phe-D-Oxd-OBn, with fully deuterated hexanoic acid-d_{11} and dodecanoic acid-d_{23}, respectively, after removal of the Boc moiety with trifluoroacetic acid. With the same approach, lipopeptides **C** and **D** were prepared by acylation of the Boc-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn, with the fully deuterated hexanoic and dodecanoic acid. The four compounds were purified by flash chromatography and fully characterized.

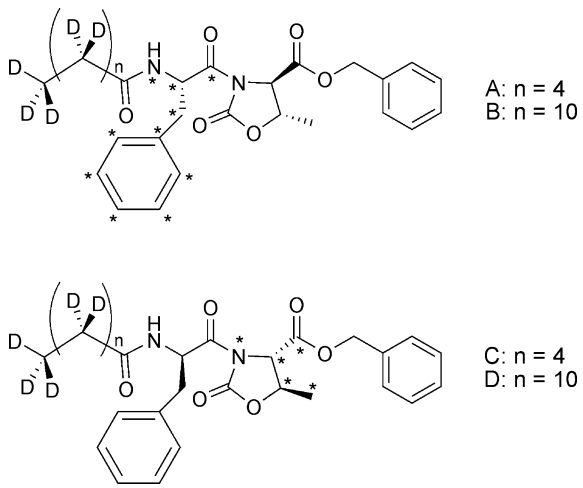

Fig. 1 Molecular structure of the investigated lipopeptides in the different ^{13}C and ^{15}N labeled forms (labeled atoms are marked by asterisks), note the -L-Phe-D-Oxd- and -D-Phe-L-Oxd- versions. D: deuterium.

Investigation of the lipopeptides in the crystalline state

We first investigated the properties of the peptide's lipid chains in the crystalline state using solid-state ^{2}H NMR. Typical NMR spectra are shown in Fig. 2. In particular for the C6 peptide chain, the spectra can be well understood from a superposition of two Pake doublets, one with a quadrupolar splitting of ~111 kHz and one with a quadrupolar splitting of ~27 kHz. The large Pake doublet can be assigned to the methylene groups of the chain, which experience an almost fully rigid conformation. For a rigid methylene group, the quadrupolar splitting is 125.25 kHz. Therefore, the chain methylene groups exhibit an

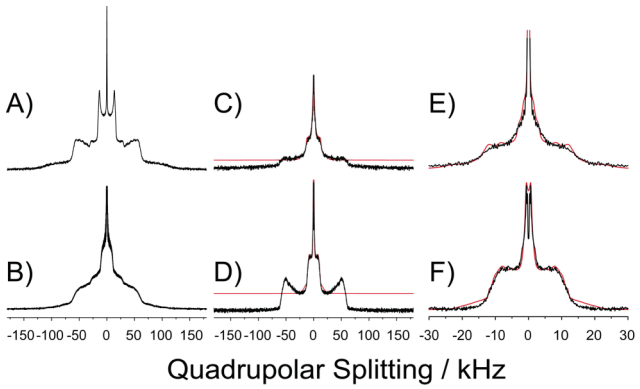

Fig. 2 115 MHz ^{2}H NMR spectra of lipopeptide **A** (A, C, E) and **B** (B, D, F) in the crystalline state (A, B), in POPC membranes (C, D) and of the central part of the spectrum of the lipopeptides in POPC membranes (E, F) to highlight the membrane bound part of the lipopeptides. A numerical simulation of the membrane bound fraction of the lipopeptide spectrum as described in the text is given in red. The spectra were measured at a temperature of 310 K.

order parameter of 0.89 indicating that the lipid chain modifications of crystalline molecule **A** are almost fully rigid and well ordered. The smaller quadrupolar splitting can be assigned to the terminal methyl group undergoing fast rotation about the C5–C6 bond leading to motional averaging of the quadrupolar interaction by a factor of 1/3, as well described in literature.[26] This would result in a quadrupolar splitting of 41.75 kHz. The fact that we measure ~27 kHz means that additional tensor averaging takes place, yielding an order parameter of 0.65, which is still rather rigid but indicates that the chain ends already undergo some more significant motional averaging. The motions that lead to this kind of averaging are fast on the NMR timescale, *i.e.* the correlation times would be shorter than microseconds. The small isotropic peak indicates a very small fraction of isotropically mobile lipid chain most likely due to packing defects in the crystalline material.

Molecule **B**, featuring the longer C12 lipid modification, shows a more complicated ^{2}H NMR spectrum. While the general width of the NMR spectrum is similar to that of peptide **A**, we no longer detected the two well resolved Pake doublets. In contrast, the spectral intensity of each set of the two quadrupolar splittings is redistributed towards the center. Such ^{2}H NMR spectra are in agreement with intermediate timescale motions in the μs time window.[26,27] Apparently, the long C12 lipid modification of the peptide cannot form a rigid and well packed assembly allowing molecular motions in the μs correlation time window to take place.

To gain more understanding about the secondary structure of these molecules, we also carried out ^{13}C CP MAS NMR studies on these crystalline materials. ^{13}C CP MAS NMR spectra of molecules **A**, **B** and **C**, **D** are shown in Fig. 3 and 4, respectively.

All signals from the ^{13}C labeled amino acids are well resolved showing typical line widths of ~1 ppm, indicative of a rather high structural homogeneity. The chemical shifts for the Oxd amino acid were assigned by proton driven spin diffusion experiments[28] with varying mixing times to follow the magnetization transfer from the CH_3 group to the Cα and Cβ carbon of Oxd. Due to its dipolar nature the magnetization transfer is more efficient towards the Cβ carbon, resulting in faster cross peak build up, which allowed assignment of the peaks in the spectrum. Since the

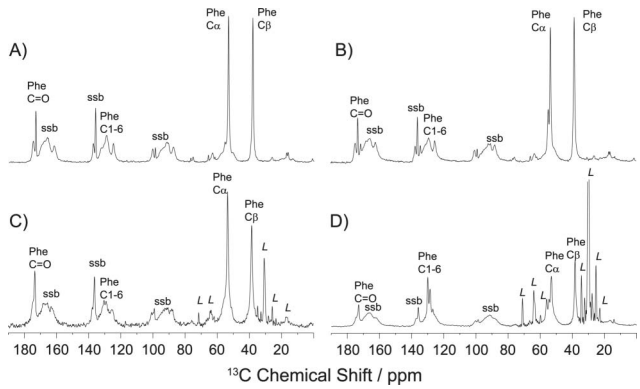

Fig. 3 Proton decoupled 188 MHz ^{13}C CP MAS NMR spectra of ^{13}C-Phe labeled version of lipopeptide **A** (A, C) and **B** (B, D) in the crystalline state (A, B) and bound to POPC membranes (C, D). Peaks arising from phospholipid molecules are marked with "*L*"; ssb denotes spinning side bands. The spectra were measured at an MAS frequency of 7000 Hz and temperature of 310 K.

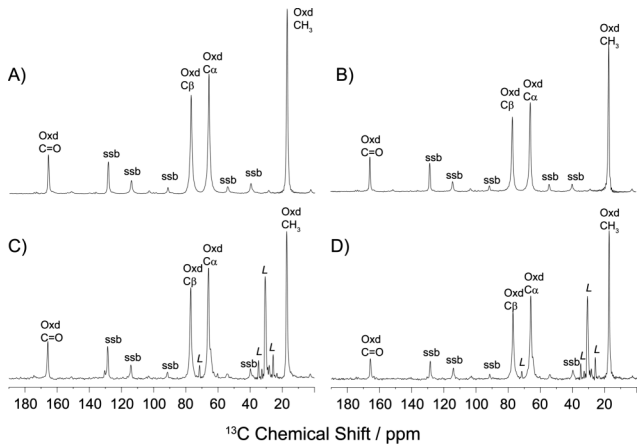

Fig. 4 Proton decoupled 188 MHz ^{13}C CP MAS NMR spectra of ^{13}C-Oxd labeled version of lipopeptide **C** (A, C) and **D** (B, D) in the crystalline state (A, B) and bound to a POPC membrane (C, D). Peaks arising from phospholipid molecules are marked with "*L*", ssb denotes spinning side bands. The spectra were measured at a MAS frequency of 7000 Hz and temperature of 310 K.

isotropic ^{13}C chemical shift values are known to be sensitive to the secondary structure,[19,29] it could be confirmed that the Phe residue of molecules **A** and **B** is in a β-sheet conformation in agreement with previous results on non-lipid-modified analogs[10,11] of these compounds (chemical shifts are given in Tables 1 and 2). Note that the Phe Cα peak also has a minor contribution at lower field, which is indicative of a small portion of peptide in random coil conformation. For the Oxd amino acid, no chemical shift reference data are available; therefore, no information about secondary structure could be deduced from the ^{13}C NMR spectra.

^{1}H–^{13}C dipolar coupling measurements confirmed the high rigidity of the molecules in the crystalline state already inferred from the ^{2}H NMR data. Order parameters well above 0.8 were determined for the backbone and sidechain sites of the labeled amino acids, except for the methyl group of Oxd (Tables 1 and 2).

Investigation of the lipopeptides bound to POPC membranes

As discussed in the introduction, lipid modification is a common motif for membrane binding of peptides and proteins.[16–18] Here, we were particularly interested in the question whether the insertion of the lipopeptide into the membrane would take place and, if so, whether it occurred as a monomer or as the fiber-like aggregates that were detected in the absence of membranes. To this end, we allowed the peptides to mix with synthetic POPC molecules and to form lipid membranes with inserted peptide molecules. To confirm that intact lipid membranes in the biologically relevant liquid-crystalline phase state were formed, we carried out static ^{31}P NMR measurements of the samples. Typical static ^{31}P are shown in Fig. 5. The ^{31}P NMR spectra showed the characteristic anisotropic line shapes with a $\Delta\sigma$ on the order of 42–44 ppm,[30,31] confirming that all preparations formed stable POPC membrane stacks with the lipopeptide incorporated. Only in the spectrum of POPC in the presence of the short chain lipopeptide **A** a small isotropic peak is visible, which accounts for ~10% of the spectral intensity.

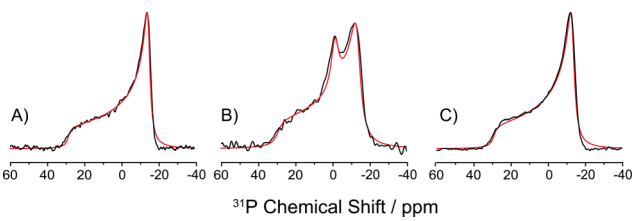

Fig. 5 Static ^{31}P NMR spectra (black) of (A) pure POPC, (B) lipopeptide A in POPC and (C) lipopeptide B in POPC and the respective numerical simulations of the lineshape (red). The spectra were measured at 310 K.

Table 1 ^{13}C Chemical shift and C–H order parameter for lipopeptides **A**, **C**

	^{13}C Chemical Shift Crystalline/ppm	CH Order Parameter *S*, Crystalline	^{13}C Chemical Shift in POPC Membranes/ppm	CH Order Parameter *S* in POPC Membranes
Phe-Cα	53.4[a]	0.86	53.5	0.99
Phe-Cβ	38.2	0.91	38.6	0.92
Phe-CO	173.4	—	173.5	—
Phe-C1–C6	125–130	0.52	125–130	0.30
Oxd-Cα	66.0	0.89	66.2	0.86
Oxd-Cβ	77.0	0.88	77.2	0.91
Oxd-CH_3	17.4	0.31	17.2	0.28
Oxd-CO	165.7	—	165.8	—

[a] Minor contribution at 55.4 ppm.

Table 2 ^{13}C Chemical shift and C-H order parameter for lipopeptide B, D

	^{13}C Chemical Shift Crystalline/ppm	CH Order Parameter *S*, Crystalline	^{13}C Chemical Shift in POPC Membranes/ppm	CH Order Parameter *S* in POPC Membranes
Phe-Cα	53.3[a]	0.82	53.1[b]	0.84
Phe-Cβ	38.5	0.97	38.4	0.64
Phe-CO	173.4	—	173.4	—
Phe-C1–C6	125–130	0.58	125–130	0.28
Oxd-Cα	65.8	0.88	66.0	0.93
Oxd-Cβ	77.0	0.89	77.0	0.96
Oxd-CH_3	17.1	0.31	17.3	0.19
Oxd-CO	165.6	—	165.9	—

[a] Minor contribution at 54.7 ppm. [b] Minor contribution at 55.5 ppm.

To further probe the interaction of the lipopeptides with the POPC membrane, we carried out spin diffusion experiments.[32] In this experiment, magnetization is prepared that solely resides on the lipid molecules. During mixing, this magnetization can diffuse into the peptide component. In the case of membrane inserted peptide segments, the magnetization build up is fast.[32] For surface associated peptide segments, a slower magnetization build up is observed, while no magnetization transfer is observed when no membrane interaction takes place.[22] Fig. 6 shows the spin diffusion build up curves for the Oxd signals of lipopeptide **D**. As can be seen, there is magnetization transfer from the lipid into the peptide, however, the magnetization build up is rather slow and within the 900 ms of magnetization transfer does not reach a plateau value. Such spin diffusion curves are characteristic for surface-associated peptides as for instance shown for the surface bound peptide GCAP.[22] Therefore, we can conclude that the lipopeptides are associated with the membrane surface. It should be noted that the lipid modification of the lipopeptides was deuterated in the ^{1}H spin diffusion experiment so the magnetization transfer is accomplished directly from the membrane lipids into the peptide.

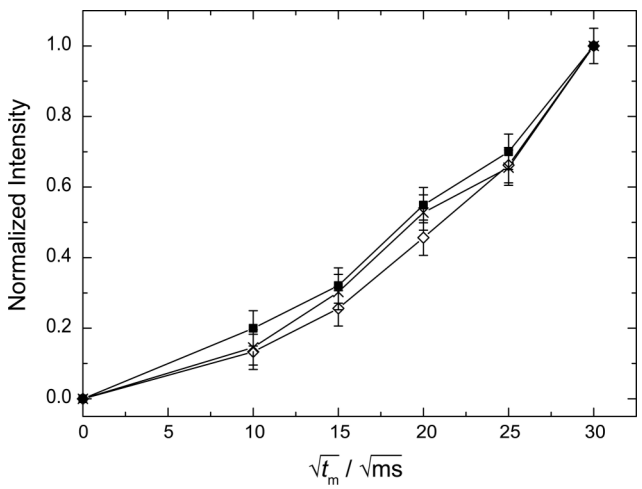

Fig. 6 Spin diffusion build up curves as a function of mixing time (t_m) for lipopeptide **D**. Magnetization transfer origins at the membrane phospholipids and is detected for the Cα (■), the Cβ (◇), and the CH_3 (×) signals of ^{13}C labeled Oxd in the lipopeptide. Error bars represent the noise level in the ^{13}C NMR spectra.

In order to study if the lipid chains are actually also inserted into the membrane, we carried out ^{2}H NMR measurements of the lipid modifications of the lipopeptides. Fig. 2 shows the ^{2}H NMR spectra of molecules **A** and **B** in the presence of POPC (molar mixing ratio 1 : 5). Rather drastic alterations of the spectral line shapes compared to those of the crystalline material were observed. We still observed the broad Pake spectra with a quadrupolar splitting of ~104 kHz at the basis of the NMR spectra. However, in addition to this line shape, we also detected a narrower ^{2}H NMR spectrum with maximal quadrupolar splittings of about 27 kHz for lipopeptide **B** and 18 kHz for lipopeptide **A** and that is reminiscent of the superposition of motionally averaged Pake spectra as observed for chain deuterated phospholipid molecules.[33–35] This part of spectral intensity could be assigned to the C6 and C12 peptide chains that are highly mobile and truly inserted into the liquid crystalline membrane environment stemming from monomeric membrane inserted lipopeptides.[36–38] This can be appreciated in a magnification of the spectra shown in Fig. 2C and F and would suggest that part of the lipopeptide remains aggregated most likely in a fibrillar state, while the other part is monomeric and rather mobile in the liquid-crystalline membrane.

We attempted to quantify the relative portions of the two states of the peptides by integrating the intensity in the ^{2}H NMR spectra. The line shapes for the membrane bound component were simulated,[24] so that the amount of the membrane bound lipopeptides could be estimated to be ~48% for lipopeptide **A** and ~29% for lipopeptide **B**. It has been reported before, that high peptide-to-lipid mixing ratios can lead to unwanted peptide aggregation.[39] Therefore, we also studied samples of the lipopeptides in a highly diluted mixture (1 : 50, mol/mol). Interestingly, the ^{2}H NMR spectra remained nearly identical, suggesting that peptide aggregation/fibril formation was not affected by the preparation and, therefore, not an artifact of the preparation.

For the membrane bound component of the ^{2}H NMR spectra of the membrane bound lipopeptides, smoothed chain order parameter profiles[40] were calculated and are shown in Fig. 7. As a reference, pure POPC-d_{31} membrane order parameters recorded under the same experimental conditions (*i.e.* water content, temperature) are plotted. Due to the different chain length of the deuterated chains (C16 in POPC, C12 in molecule **B**, and C6 in molecule **A**) order parameters of these chains vary quite significantly. However, if one plots the chains with a common starting point at the terminal methyl group (ω position), it can be seen that the peptide chain order parameters nicely follow those of the host membrane. This suggests that the acyl chains of the non-aggregated lipopeptides are well incorporated into the lipid membrane.

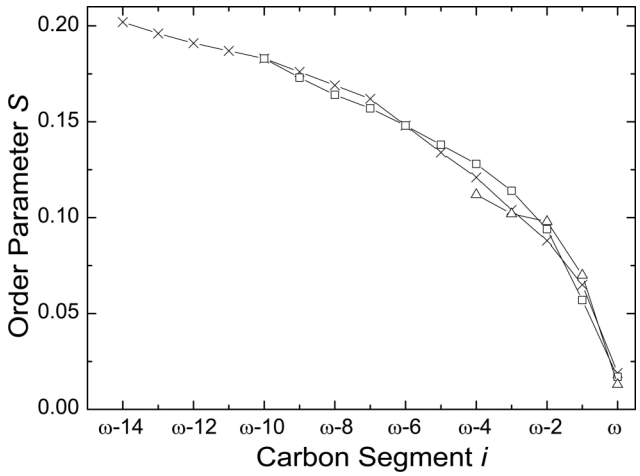

Fig. 7 ^{2}H NMR smoothed order parameter plots derived from the spectra of the lipid bound lipopeptides shown in Fig. 2. Data are shown for a pure POPC-d_{31} membrane (×), and of the deuterated hexanoic acid chain of lipopeptide **A** (△) and the deuterated dodecanoic acid chain of lipopeptide B (□).

We also detected ^{13}C CPMAS NMR spectra of molecules **A**–**D** in POPC lipid membranes (the spectra are shown in Fig. 3 and 4). There are only marginal chemical shift differences (see Tables 1 and 2), indicating that the secondary structure of the molecules does not change upon membrane insertion. This also supports the previous conclusion that a large portion of the molecules remains in the fibrillar aggregate, which it forms in the crystalline state. Further, even those molecules that are immersed in the membranes as putative monomers show a very similar structure. The Phe peaks show some broader base and a minor contribution with a Cα chemical shift more indicative of random coil structure. This contribution is also seen in the crystalline form of the dipeptide. However, the Oxd residues exhibit narrow and well defined NMR signals without indication for a different secondary structure.

Finally, the C–H order parameter of the labeled Phe and Oxd amino acids of the lipopeptides incorporated in POPC membranes from the C–H dipolar coupling were measured using DIPSHIFT experiments. For all labeled carbons relatively high order parameters were observed. Such a behavior, which is typical for fibrillar peptides/proteins, shows that there is not much space for conformational flexibility and motion in the backbone of the molecules.[4,41,42] This behavior has to be expected on the basis of the strong tendency of Boc-L-Phe-D-Oxd-OBn to self aggregate to form a fiber-like material.[10] Indeed, it spontaneously forms fibers consisting of infinite linear chains, where the parallel dipeptide units are connected only by a single hydrogen bond of the Phe residue, since the second amino acid of the dipeptide (Oxd) is not involved in further intermolecular interactions. The role of the Oxd moiety is to impart a rigid conformation to the whole molecule, as the Oxd nitrogen atom is connected both to an endocyclic and to an exocyclic carbonyl that always adopt the *trans* conformation. As a consequence of this locally constrained disposition effect, Boc-L-Phe-D-Oxd-OBn is forced to fold in a β-sheet conformation.

Discussion

We have recently described a series of studies on the material properties of aggregated peptides made from the general building block [-L-Phe-D-Oxd-]$_n$[10,11] Depending on the number of building blocks, these molecules can form nanofibers with β-sheet secondary structure or more amorphous aggregates. Further, it could be established that the stereochemistry has a profound impact on the overall structure of these molecules.[43] Here, we study the influence of an N-terminal acylation on the dipeptide comprising either 6 or 12 carbons. First, peptides were investigated in the crystalline state; the secondary structure of the peptide backbone was evaluated from the chemical shifts of the Phe residue in the peptide determined from ^{13}C CPMAS NMR experiments. It could clearly be seen that the backbone structure of both peptides represents a β-sheet, as indicated by the reference independent chemical shift difference between the Cα and the Cβ signals.[44] For this quantity, we measured values of 15.2 ppm and 14.8 ppm for molecules **A** and **B**, respectively, which agree very well with the reference value for ideal β-sheet secondary structure (14.7 ppm[45]). A similar value of 15.9 ppm was also determined for the prototype peptide Boc-L-Phe-D-Oxd-OBn.[10] Apparently, the N-terminal lipid modification does not seem to interfere with the general property of this peptide to form highly ordered nanofiber-like materials. On the contrary, the ^{2}H NMR results suggest that the lipid chains also appear to be well aligned, which may provide an additional intermolecular stabilization in addition to the single hydrogen bond that has been found before.[10]

The situation changes partially when the peptides are allowed to interact with lipid membranes. As lipid modifications represent a common motif for membrane binding of membrane-associated proteins and peptides,[16–18] we studied the interaction of the lipidated dipeptides with POPC membranes. Spin diffusion experiments showed that the peptides were indeed associated with the membranes. While we did not detect any major secondary structure changes in the peptide backbone associated with membrane binding, the ^{2}H NMR spectra of the lipid chains of the peptide allowed us to detect two populations of peptide molecules. One portion of oligomeric lipopeptides was found to closely resemble the crystalline nanofibers detected in the absence of any membranes and another portion of monomeric dipeptides were identified that are immersed into the lipid membrane.

The membrane propensity of the lipopeptides can be explained by the hydrophobic effect.[15] Partitioning of lipidated peptides into the lipid water interface of a membrane can be understood from thermodynamic principles. It has been found that the insertion of a methylene group into a phospholipid bilayer releases –3.45 kJ mol^{-1} free energy,[46,47] amounting to a sizable energetic contribution for long lipid chains. However, association of a peptide to the lipid surface involves an entropy penalty, which has been estimated to be on the order of ~14 kJ mol^{-1}.[46,47] There is also some energetic contribution to membrane binding arising from the hydrophobic Phe sidechain, which would amount to –4.73 kJ mol^{-1}.[48] For the Oxd sidechain, no literature values are available; however, this amino acid carries a rather polar character and most likely does not contribute free energy for membrane binding. Summing up these contributions, a theoretical ΔG^{0} for the transfer of the peptide from aqueous environment into a POPC membrane of ~–8 kJ mol^{-1} can be calculated for the lipopeptide with the C6 modification and ~–29 kJ mol^{-1} for the lipopeptide with the C12 modification. Clearly, these peptides show a tendency for membrane binding, however, the fraction of bound peptide varies for the two molecules. Assuming a lipid concentration of 1 mM,

it can be calculated that less than 1% of the lipopeptide with the C6 chain would be membrane associated, while for the peptide with the C12 chain approximately 60% of the peptide should bind the membrane. From the quantification of the ^{2}H NMR spectra, we estimated about 50% and 30% of the peptide to be membrane bound, respectively, which clearly does not correspond well to these theoretical estimates. What the estimate does not consider is the fact that the peptides are not soluble in aqueous environment and that the aggregation also involves free energy changes. An estimate of the amount of free energy gained in peptide aggregation is much more difficult and goes beyond the scope of this paper. However, the fact that no more than ~50% of the peptide is found in monomeric form suggests that the free energy gain of membrane binding is comparable to the free energy gain of peptide aggregation.

This consideration may explain why more than half of the lipidated dipeptides remain aggregated even in the presence of membranes and only a smaller portion forms monomers that are inserted into the membranes. This suggests that the intermolecular forces that lead to fiber formation of the peptides must be relatively strong and are not easily dissolved in the case where the membrane becomes the solvent. However, it should be stressed that lipidated peptides do not generally tend to aggregate in the membrane environment.[49]

We previously reported that the dipeptide Boc-L-Phe-D-Oxd-OBn is stabilized only by a single hydrogen bond.[10] This would certainly not suffice to keep the peptides aggregated in the presence of membranes. Clearly, the highly ordered lipid modifications also contribute to the intermolecular forces that keep the peptides in the nanostructured form. Only part of the peptide aggregates are dissolved in the membrane and will most likely occur in a monomeric form. These molecules are also relatively mobile in the membrane, the lipid modifications undergo fast motions comparable to those of the lipid chains of the phospholipids. Here, the intermolecular forces between adjacent peptides, which stabilize the nanofibered structure of the dipeptide in the absence of membranes are replaced by interactions of the peptide with the membrane that in particular occur in the lipid–water interface of the bilayer.[50]

The structural findings of this study are summarized in a cartoon shown in Fig. 8. In the crystalline state, the peptides form parallel β-sheets and the N-terminal lipid modifications are highly ordered in the all *trans* state (A). In the lipid membrane (B), two populations of lipopeptides are observed. First, aggregated nanofibers remain undetected and may due to their hydrophobicity be buried in the liquid crystalline membrane or could occur clustered on the membrane surface. Second, also monomeric molecules have been detected that are inserted in the membrane, these molecules show higher mobility and are characterized by highly mobile lipid modifications that undergo fast *trans-gauche* isomerizations just as the lipid chains of the POPC molecules in the host membrane.

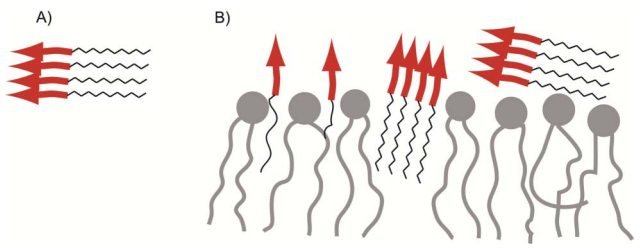

Fig. 8 The cartoon summarizes the major findings of the current study. In the absence of membranes, lipopeptides form highly ordered β-sheet like nanofibers with the lipid modifications in a rigid all *trans* conformation (A). When lipid membranes are present, larger peptide aggregates can bind to the membrane surface or insert into the membrane, but also dissociate and insert into the membrane as monomers, most likely with the lipid modification facing the membrane interior (B).

Conclusions

Overall, this study showed that the general molecular architecture of peptides comprising the building block -L-Phe-D-Oxd- is not altered by N-terminal lipid modifications if investigated in the crystalline state. However, in the presence of lipid membranes, the nanofibered structure only partially prevails; the molecules can also disaggregate and be immersed in the lipid membrane as many lipidated proteins and peptides do.

Experimental section

Materials and synthesis

The lipopeptides were synthesized as $C_n{}^2H_{(2n+1)}$CO-L-[U-^{13}C,^{15}N]-Phe-D-Oxd-OBn or $C_n{}^2H_{(2n+1)}$CO-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn to enable the ^{13}C and ^{15}N labeling for the Phe or Oxd, respectively. The lipopeptides differed in the length of their lipid modification: for lipopeptide **A** $n = 5$ and for **B** $n = 11$. For ^{2}H NMR experiments, the peptides were synthesized with a perdeuterated lipid modification, the perdeuterated precursors for the synthesis, hexanoic acid-d_{11} and dodecanoic acid-d_{23} as well as the isotopically labeled L-amino acids were purchased from Eurisotop GmbH, Saarbrücken, Germany. The molecular structure of the lipopeptides is shown in Fig. 1. It is worth mentioning that lipopeptides **A** and **B** have been synthesized starting from Boc-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn, while for lipopeptides **C** and **D** the enantiomer Boc-L-Phe-D-[U-^{13}C,^{15}N]-Oxd-OBn has been used, as isotopically labeled amino acids are available only in the L form.

The phospholipids 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC) and 1-palmitoyl-d_{31}-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC-d_{31}) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Al) and used without further purification.

The melting points of the compounds were determined in open capillaries and are uncorrected. High quality infrared spectra (64 scans) were obtained at 2 cm^{-1} resolution using a 1 mm NaCl solution cell and a Nicolet 210 FT-infrared spectrometer. All spectra were obtained in 3 mM solutions in dry CH_2Cl_2 at 297 K. All compounds were dried *in vacuo* and all the sample preparations were performed in a nitrogen atmosphere. Routine ^{1}H NMR spectra were recorded with spectrometers at 200 MHz. The measurements were carried out in $CDCl_3$. Chemical shifts are reported in δ values relative to the solvent ($CDCl_3$) peak.

Boc-L-[U-^{13}C,^{15}N]-Phe-D-Oxd-OBn. To a stirred solution of Boc-L-[U-^{13}C,^{15}N]-Phe-OH (0.67 g, 2.5 mmol) in dry acetonitrile (20 mL), HBTU (0.98 g, 2.6 mmol), then D-Oxd-OBn (0.59 g, 2.5 mmol) and lastly triethylamine (0.75 mL, 5 mmol) were added. The mixture was stirred over 1 h, then acetonitrile was removed under reduced pressure and replaced by ethyl acetate. The mixture

was washed with brine, 1 N aqueous HCl (1 × 15 mL), and 5% aqueous $NaHCO_3$ (1 × 15 mL), dried over sodium sulphate and concentrated *in vacuo*. The product was obtained pure in 88% yield (0.90 g) as a solid after silica gel chromatography (cyclohexane/ethyl acetate 8 : 2 → 1 : 1 as eluant). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.39 (s, 9H, *t*-Bu), 1.42 (d, 3H, J = 6.6 Hz, OCHCH_3), 2.50–2.89 and 3.15–3.65 (m, 2H, CHN-CH_2-Ph), 4.34 (d, 1H, J = 4.0 Hz, CHN), 4.53 (dq, 1H, J = 4.0, 6.6 Hz, CHO), 4.95 and 5.41 (bs, 1H, NH), 5.22 (s, 2H, OCH_2Ph), 5.42 and 6.19 (m, 1H, C*H*N-CH_2Ph), 6.72–7.00 and 7.43–7.78 (m, 5H, Ph), 7.10–7.37 (m, 5H, Ph).

L-[U-^{13}C,^{15}N]-Oxd-OBn. To a stirred solution of L-[U-^{13}C,^{15}N]-Thr-OH (1.21 mmol, 0.15 g) in 1 M NaOH (4 mL) a solution of triphosgene (1.21 mmol, 0.36 g) in dioxane (2.5 mL) was added dropwise at room temperature. The mixture was stirred for 3 h and then concentrated under reduced pressure. The residue was washed with warm acetonitrile to solubilize the product that was obtained pure after filtration and concentration of the liquid. The residue was dissolved in acetone (10 mL) and diisopropylethylamine (2.42 mmol, 0.42 mL), then benzyl bromide (1.21 mmol, 0.14 mL) was added dropwise at room temperature. The mixture was stirred overnight, then the acetone was removed under reduced pressure and replaced by ethyl acetate. The mixture was washed with water (3 × 10 mL), dried over sodium sulphate and concentrated *in vacuo*. The product was obtained pure in 88% yield (0.26 g) as a solid after silica gel chromatography (cyclohexane/ethyl acetate 8 : 2 as eluant). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.25 and 1.88 (q, 3H, J = 6.2 Hz, OCHCH_3), 3.66 and 4.37 (m, 1H, CHN), 4.42 and 5.16 (m, 1H, CHO), 5.22 (s, 2H, OCH_2Ph), 5.81 and 6.29 (dd, 1H, J = 3.8, 5.8 Hz, NH), 7.12–7.40 (m, 10H, Ph).

Boc-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn. For the preparation see above, starting from Boc-D-Phe-OH and L-[U-^{13}C,^{15}N]-Oxd-OBn. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.10 and 1.73 (q, 3H, J = 5.8 Hz, OCHCH_3), 1.39 (s, 9H, *t*-Bu), 2.98 (dd, 1H, J = 7.2, 13.2 Hz, CHN-CH*H*-Ph), 3.10 (dd, 1H, J = 4.4, 13.2 Hz, CHN-C*H*H-Ph), 3.95 and 4.73 (m, 1H, CHN), 4.12 and 4.92 (m, 1H, CHO), 5.15 (bs, 1H, NH), 5.21 (s, 2H, OCH_2Ph), 5.83 (m, 1H, C*H*N-CH_2Ph), 7.12–7.40 (m, 10H, Ph).

General method for the preparation of lipopetides A, B, C, or D

A solution of labeled Boc-L-Phe-D-Oxd-OBn (0.5 mmol) and TFA (9 mmol, 0.7 mL) in dry methylene chloride (10 mL) was stirred at room temperature for 4 h, then the volatiles were removed under reduced pressure and the corresponding amine salt was obtained pure in quantitative yield without further purification.

A solution of carboxylic acid [CD_3-$(CD_2)_4$-COOH or CD_3-$(CD_2)_{10}$-COOH] (0.5 mmol) and HBTU (0.2 g, 0.52 mmol) in dry acetonitrile (15 mL) was stirred under nitrogen atmosphere for 10 min at room temperature. Then, a mixture of the previously obtained amine salt (0.5 mmol) and Et_3N (1.6 mmol, 0.34 mL) in dry acetonitrile (10 mL) was added dropwise at room temperature. The solution was stirred for 40 min under nitrogen atmosphere, and then acetonitrile was removed under reduced pressure and replaced with ethyl acetate. The mixture was washed with brine, 1 N aqueous HCl (3 × 30 mL), and with 5% (w/v) aqueous $NaHCO_3$ (1 × 30 mL), dried over sodium sulphate and concentrated *in vacuo*. The product was obtained pure after silica gel chromatography (cyclohexane/ethyl acetate 9 : 1 → 7 : 3 as eluant).

$CD_3(CD_2)_4$CO-L-[U-^{13}C,^{15}N-Phe]-D-Oxd-OBn A. Yield 70%; m.p. = 145 °C; $[\alpha]_D$ −53.0 (*c* 0.8 in $CHCl_3$); IR (CH_2Cl_2, 3 mM): ν = 3428, 1793, 1703, 1671 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.44 (d, 3H, J = 6.4 Hz, OCHCH_3), 2.78 and 3.24 (m, 2H, CHN-CH_2-Ph), 4.35 (d, 1H, J = 4.0 Hz, CHN), 4.52 (dq, 1H, J = 4.0, 6.4 Hz, CHO), 5.21 (s, 2H, OCH_2Ph), 5.70 and 6.44 (m, 1H, C*H*N-CH_2Ph), 5.78 and 6.23 (d, 1H, J = 8.0 Hz, N*H*), 6.62–7.00 and 7.41–7.78 (m, 5H, Ph), 7.10–7.37 (m, 5H, Ph).

$CH_3(CH_2)_{10}$CO-L-[U-^{13}C,^{15}N-Phe]-D-Oxd-OBn B. Yield 68%; m.p. = 140 °C; $[\alpha]_D$ −50.0 (*c* 1.3 in $CHCl_3$); IR (CH_2Cl_2, 3 mM): ν = 3428, 1793, 1716, 1671 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.44 (d, 3H, J = 6.2 Hz, OCHCH_3), 2.71 and 3.42 (m, 2H, CHN–CH_2–Ph), 4.35 (d, 1H, J = 4.0 Hz, CHN), 4.54 (dq, 1H, J = 4.0, 6.2 Hz, CHO), 5.22 (s, 2H, OCH_2Ph), 5.71 and 6.45 (m, 1H, C*H*N-CH_2Ph), 5.79 and 6.23 (d, 1H, J = 8.0 Hz, N*H*), 6.65–7.00 and 7.38–7.79 (m, 5H, Ph), 7.18–7.37 (m, 5H, Ph).

$CD_3(CD_2)_4$CO-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn C. Yield 67%; m.p. = 145 °C; $[\alpha]_D$ −61.0 (*c* 0.8 in $CHCl_3$); IR (CH_2Cl_2, 3 mM): ν = 3424, 1785, 1703, 1667 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.13 and 1.78 (q, 3H, J = 5.0 Hz, OCHCH_3), 3.04 (dd, 1H, J = 7.0, 14.0 Hz, CHN-CH*H*-Ph), 3.20 (dd, 1H, J = 5.4, 13.4 Hz, CHN-C*H*H-Ph), 3.97 and 4.75 (m, 1H, CHN), 4.16 and 4.95 (m, 1H, CHO), 5.22 (s, 2H, OCH_2Ph), 6.07 (m, 2H, C*H*N–CH_2Ph + NH), 7.12–7.40 (m, 10H, Ph).

$CD_3(CD_2)_{10}$CO-D-Phe-L-[U-^{13}C,^{15}N]-Oxd-OBn D. Yield 66%; m.p. = 128 °C; $[\alpha]_D$ −39.0 (*c* 0.7 in $CHCl_3$); IR (CH_2Cl_2, 3 mM): ν = 3428, 1789, 1712, 1675 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.13 and 1.78 (q, 3H, J = 5.6 Hz, OCHCH_3), 3.05 (dd, 1H, J = 6.6, 13.8 Hz, CHN-CH*H*-Ph), 3.20 (dd, 1H, J = 5.6, 13.8 Hz, CHN-C*H*H-Ph), 3.97 and 4.75 (m, 1H, CHN), 4.16 and 4.93 (m, 1H, CHO), 5.22 (s, 2H, OCH_2Ph), 6.05 (m, 2H, C*H*N-CH_2Ph + NH), 7.08–7.42 (m, 10H, Ph).

Sample preparation

For the NMR investigations on the crystalline lipopeptides, the powdered sample as obtained after synthesis and lyophilisation was directly transferred into 4 mm MAS rotors without any further treatment.

For the NMR measurements, a mixture of the lipopeptide and the phospholipids (molar ratios of 1 : 5 or 1 : 50) were co-dissolved in acetonitrile/methanol. After the addition of cyclohexane the samples were lyophilized overnight at high vacuum. The obtained fluffy powder was suspended in 1 ml buffer (10 mM NaCl, 50 mM HEPES, pH = 7.4). This suspension was ultracentrifuged for 1 h at 79000 × *g*. The obtained pellets were equilibrated by freeze–thaw cycles and gentle centrifugation and finally transferred into 4 mm HR MAS rotors. We also prepared a peptide sample under exactly identical conditions but in the absence of POPC, which provided exactly identical ^{2}H NMR spectra.

Static solid-state NMR measurements

^{2}H NMR spectra were acquired on a Bruker Avance 750 MHz NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at a resonance frequency of 115.1 MHz for ^{2}H. A single-channel solid probe equipped with a 5 mm solenoid coil was used. The ^{2}H NMR spectra were accumulated with a spectral width of ±500 kHz using quadrature phase detection. A

phase-cycled quadrupolar echo sequence[51] was used. The typical length of a 90° pulse was between 3 and 6 μs, and a relaxation delay of 1 s was applied. All measurements were conducted at a temperature of 310 K.

The ^{2}H NMR powder spectra were dePaked[52] using the algorithm of McCabe and Wassall[53] and the order parameter profiles of the acyl chains were determined by a numerical spectral fitting procedure from the observed quadrupolar splitting $\Delta\nu_Q(n)$:

$$\Delta\nu_Q(n) = \frac{3}{4}\frac{e^2qQ}{h}S(n) \quad (1)$$

where e^2qQ/h is the quadrupolar-coupling constant (167 kHz for ^{2}H in a C-^{2}H bond) and $S(n)$ the chain order parameter for the n^{th} carbon position in the chain.

The static ^{31}P NMR spectra of membrane samples were acquired on a Bruker DRX 600 NMR spectrometer operating at a resonance frequency of 242.8 MHz for ^{31}P using a Hahn-echo pulse sequence. A ^{31}P 90° pulse length of 7 μs, a Hahn-echo delay of 50 μs, a spectral width of 100 kHz, and a recycle delay of 2 s were used. Continuous-wave proton decoupling was applied during signal acquisition.

^{13}C MAS NMR measurements

All ^{13}C CP MAS NMR experiments were carried out on a Bruker Avance 750 operating at a resonance frequency of 749.7 MHz for ^{1}H and 188.5 MHz for ^{13}C. A double-resonance MAS probe equipped with a 4 mm spinning module was used. ^{13}C chemical shifts were referenced externally relative to TMS. The length of the 90° pulse was 5 μs for ^{13}C and 4–5 μs for the ^{1}H. The CP contact time was 700 μs. For heteronuclear decoupling a ^{1}H radio-frequency field of 65 kHz was applied using the TPPM decoupling sequence.[54]

Spin diffusion experiments were carried out as described in literature.[32] After a 2 ms T_2 filter, magnetization was allowed to diffuse from the lipid moiety into the peptide. Spin diffusion was observed for time periods between 100 and 900 ms. Spin diffusion build up curves were corrected for T_1 relaxation and scaled to the intensity of the last data point.

The strength of the ^{13}C–^{1}H dipolar couplings for determination of the order parameters was measured using a constant time DIPSHIFT pulse sequence[55] with FSLG[56] as homonuclear decoupling sequence (80 kHz decoupling field). Experimental dipolar dephasing curves were simulated for one rotor period to extract the dipolar coupling of the respective signal.[57] Order parameters were calculated by dividing the motionally averaged dipolar coupling by its rigid limit value. Rigid limit values for the ^{1}H–^{13}C dipolar coupling were taken from literature.[58] All experiments were carried out at a temperature of 310 K and a MAS frequency of 7 kHz.

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