Biochimica et Biophysica Acta 1848 (2015) 833–841

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Control and role of pH in peptide–lipid interactions in oriented membrane samples

Julia Misiewicz [a], Sergii Afonin [a], Anne S. Ulrich [a,b,*]

[a] Karlsruhe Institute of Technology (KIT), Institute of Biological Interfaces (IBG-2), POB 3640, 76021 Karlsruhe, Germany
[b] Karlsruhe Institute of Technology (KIT), Institute of Organic Chemistry, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany

ARTICLE INFO

Article history:
Received 25 August 2014
Received in revised form 1 December 2014
Accepted 4 December 2014
Available online 12 December 2014

Keywords:
Membrane-active peptides
Buffering and pH control
Lipid phase transition
Oriented multilamellar membrane sample
Solid-state $^{2}H/^{31}P/^{19}F$ NMR spectroscopy
Differential scanning calorimetry

ABSTRACT

To understand the molecular mechanisms of amphiphilic membrane-active peptides, one needs to study their interactions with lipid bilayers under ambient conditions. However, it is difficult to control the pH of the sample in biophysical experiments that make use of mechanically aligned multilamellar membrane stacks on solid supports. HPLC-purified peptides tend to be acidic and can change the pH in the sample significantly. Here, we have systematically studied the influence of pH on the lipid interactions of the antimicrobial peptide PGLa embedded in oriented DMPC/DMPG bilayers. Using solid-state NMR (^{31}P, ^{2}H, ^{19}F), both the lipid and peptide components were characterized independently, though in the same oriented samples under typical conditions of maximum hydration. The observed changes in lipid polymorphism were supported by DSC on multilamellar liposome suspensions. On this basis, we can present an optimized sample preparation protocol and discuss the challenges of performing solid-state NMR experiments under controlled pH. DMPC/DMPG bilayers show a significant up-field shift and broadening of the main lipid phase transition temperature when lowering the pH from 10.0 to 2.6. Both, strongly acidic and basic pH, cause a significant degree of lipid hydrolysis, which is exacerbated by the presence of PGLa. The characteristic re-alignment of PGLa from a surface-bound to a tilted state is not affected between pH of 7 to 4 in fluid bilayers. On the other hand, in gel-phase bilayers the peptide remains isotropically mobile under acidic conditions, displays various co-existing orientational states at pH 7, and adopts an unknown structural state at basic pH.

1. Introduction

Membrane protein structure and the functional mechanisms of membrane-active peptides can only be fully understood in the presence of a proper lipid bilayer. Therefore – instead of employing detergent micelles, bicelles or organic solvent mixtures – many biophysical studies rely on the use of membrane samples, consisting of multiple stacked bilayers that contain the reconstituted peptide or protein in a liquid crystalline and well hydrated lipid environment [1–3]. Multilamellar vesicles are readily obtained in suspension using aqueous buffers, but a fundamental advantage can be gained by preparing them in a macroscopically oriented manner. This way, anisotropic information about the alignment of a whole protein, of a secondary structure element, or of an individual functional group becomes accessible. The most prominent anisotropic spectroscopic techniques include solid-state NMR (SSNMR) [1,4,5], oriented circular dichroism [6–8], attenuated total reflection infrared (ATR-IR) [9,10], electron paramagnetic resonance (EPR) [9,11,12], as well as scattering methods with X-rays and neutrons [13–16]. In all these methods it is very difficult to control the pH of a sample that has been macroscopically oriented on a solid support, so most of these studies tend to be pursued without pH control. The common use of distilled water together with synthetic peptides that are usually purified under acidic conditions by HPLC implies that the resulting data might suffer from unnaturally acidic conditions. Since the protonation state not only of the peptides/proteins but also of certain lipids can play a critical role in their structure and function, a critical assessment of pH effects and potential pitfalls is timely.

From our experience, SSNMR analysis of membrane-active peptides (MAPs) in oriented lipid bilayers is the most versatile and comprehensive approach to study peptide–lipid interactions. It can provide not

Abbreviations: MAP, membrane active peptides; PC, phosphatidylcholine; PG, phosphatidylglycerol; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (Na^{+} salt); PGLa, peptidyl-glycyl-leucine-carboxyamide peptide [GMASKAGAIAGKIAKVALKAL-NH2]; S-state, "surface-bound" state; T-state, "tilted" state; I-state, "inserted" state; Fmoc, 9-fluorenylmethoxycarbonyl; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; SSNMR, solid-state nuclear magnetic resonance spectroscopy; ATR-IR, attenuated total reflection infrared spectroscopy; EPR, electron paramagnetic resonance spectroscopy; HPLC, reversed-phase high-performance liquid chromatography; DSC, differential scanning calorimetry; Lα, fluid lamellar phase; L_{β}, lamellar gel phase; P_{β}, rippled gel phase; T_m, main transition temperature (P_{β}-L_{α}); T_p, pre-transition temperature (L_{β}-P_{β}); P/L, peptide-to-lipid (molar) ratio

* Corresponding author at: Karlsruhe Institute of Technology (KIT), Institute of Biological Interfaces (IBG-2), POB 3640, 76021 Karlsruhe, Germany. Tel.: +49 721 60843222; fax: +49 721 60844823.
E-mail address: Anne.Ulrich@kit.edu (A.S. Ulrich).

http://dx.doi.org/10.1016/j.bbamem.2014.12.006
0005-2736/

only a structural description (conformation, membrane alignment) [1,4,17,18] and dynamical information (molecular wobble, lateral diffusion, aggregation) [19–27] about the MAP in its functionally relevant membrane-bound state, but it can also monitor the response of the phospholipid matrix (lipid disorder, morphological transitions) [28–31]. The samples typically consist of stacks of several 1000 hydrated multibilayers that are spontaneously aligned on an inert solid support, like glass, quartz or silicon. They are very suitable as proper membrane models, because the lipid composition can be freely chosen [32–34], the peptide concentration can be varied over several orders of magnitude [17], the bilayers can be observed in their desired phase state, and it is easy to control the membrane charge [34,35], bilayer thickness [34,36], fluidity, spontaneous curvature [32,33]. Several reports on SSNMR applications have been concerned with optimizing the sample preparation, e.g. to achieve perfect alignment, but they were mainly focussed on adjusting the solvent composition for co-solubilizing peptide and lipids [38–41]. A commonly underestimated aspect, however, is the fact that many studies are carried out with synthetic peptides rather than recombinantly produced material. Synthetic peptides are routinely purified by commercial suppliers and in academic labs with RP-HPLC, which usually employs acidic eluents. If no post-purification treatment (e.g. neutralisation) is performed, any HPLC purified peptide carries with itself a stoichiometric excess of acid (commonly trifluoroacetic acid) [1,42–44]. When no sufficient buffer capacity is provided in the sample, this acid introduced by the peptide may significantly influence the system under study. A resulting effect can be, for instance, acid hydrolysis of the phospholipid molecules, leading to the generation of lyso-lipids, which will affect the membrane composition and properties [45,46]. Lipid hydrolysis as a factor modulating the behaviour of magnetically oriented "bicelles" has been intensively studied, and ether-linked phospholipids with reduced risk of hydrolysis are often used to circumvent this problem [47–49]. However, even this lipid choice is not a perfect solution, because the structure and alignment of MAPs in ether- vs. ester-linked phospholipid bilayers has been reported to differ [50,51].

Peptide-induced acidification or "spontaneous" hydrolysis in the peptide-containing samples is a practical problem that all experimental NMR spectroscopists must be aware of when using solid-supported oriented samples. In many cases the consequences are ignored, or precautions simply involve an acquisition of ^{31}P NMR spectra before and after the actual SSNMR measurement in order to assess the "damage" and to be alerted before interpreting the data. To the best of our knowledge, the problem of active pH control in oriented samples has not been explicitly discussed in the literature. Furthermore, it is well documented that lipid polymorphism depends not only on temperature, ionic strength and composition of the environment, but also on pH [52–55]. Since various ionizable groups are present in lipid membranes (Table 1), the pH of a sample can directly influence the membrane charge, spontaneous curvature and lateral pressure profile by protonation/deprotonation of the lipid headgroups. The protonation state of lipids and peptides can be further affected by the presence of anionic lipids, as they tend to lower the pH at the bilayer surface compared to the bulk [56].

Also the peptidic component in a membrane sample can be affected by pH. The HPLC derived acidity is not strong enough to cause hydrolysis of the amide backbone, but the ionization state of side chains can be easily affected, and thereby the peptide conformation and its interactions with the bilayer [56–59]. A prominent example is the pH-dependent re-orientation of amphiphilic helices triggered by the protonation/deprotonation of histidines [60]. Such pH-induced structural transitions have been explored to trigger the activity of viral fusion peptides and of ion channels, and in the design of pH-responsive materials [61–67]. Another pH-sensitive group on peptides is the N-terminus, which has a pKa value close to 7 and may accordingly interact in different ways with the amphiphilic bilayer.

Table 1
Ionizable groups in common phospholipids and their reported pKa values [54].

Phospholipid:	Ionizable group:	pKa
Phosphatidylcholines	phosphate	<2, 2–3
Phosphatidylglycerols	phosphate	3–3.5
Phosphatidyletanolamines	phosphate	<2, 2–3
	amine	8–11.25
Phosphatidylserines	phosphate	1.2
	carboxyl	3.6–5.5
	amine	9.8–11.5

Here, we address the fundamental question as to what extent typical SSNMR experiments can be influenced by pH over a broad range. Namely, whether/which pH would induce changes in the bilayer properties, and whether/how would the alignment of an α-helical peptide (without histidines) change upon varying the pH? To address these issues in a suitable test system, we chose a representative mixture of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) in a molar ratio of 3:1. Such anionic mixtures are often used as bacterial membrane mimics [1,4,34] and are therefore relevant for studies of antimicrobial MAPs. The polymorphism of both lipids has been well characterized, the two lipid species are almost ideally miscible [68], and their phase transitions occur in a convenient temperature regime. DMPC/DMPG mixtures are widely used to examine changes in other experimental conditions [69–71], both alone as well as in the presence of peptides or proteins [72,73]. This particular mixture of 3:1 has also been used extensively before in our earlier SSNMR studies of the present test peptide PGLa [17,34,35,37].

PGLa was selected as an exemplary peptide, being a typical cationic antimicrobial membrane-active peptide with 21 amino acids (GMASKAGAIAGKIAKVALKAL-NH_2) [74]. The peptide is unstructured in aqueous solution but adopts an amphiphilic α-helical structure in membrane-mimicking environments [75]. PGLa has been comprehensively characterized by SSNMR, revealing several different orientational states depending on the conditions. The helix undergoes a concentration dependent re-alignment in DMPC/DMPG bilayers at room temperature [17]. At low peptide-to-lipid ratios (e.g. 1:200) PGLa lies flat on the membrane in a surface-bound "S-state", while at higher concentration ($P/L \geq 1:50$) it adopts a tilted "T-state" that has been attributed to the formation of antiparallel dimers [17,76]. When mixed with its synergistic partner magainin 2, hetero-oligomers are formed in which the PGLa helix becomes aligned nearly parallel to the membrane normal [33]. This fully inserted "I-state" suggests that transmembrane pores have been formed, which can explain the antimicrobial mechanism. The I-state was also observed for PGLa alone at high peptide/lipid ratios (e.g. 1:50) in metastable gel-phase bilayers [37]. Changes between the three states (S, T, I) can be monitored by solid-state ^{19}F NMR, e.g. by observing a selectively ^{19}F-labelled peptide carrying a CF_3-containing amino acid, e.g. in the place of Ile13 [34]. This particular PGLa analogue is a good candidate to examine the influence of pH on the re-alignment behaviour of this well-known MAP.

Besides the ^{19}F NMR experiments on PGLa, we also used ^{2}H NMR and ^{31}P NMR to monitor the pH-dependent response of the DMPC/DMPG matrix. In addition, differential scanning calorimetry (DSC) measurements on isotropic lipid dispersions with different pH values were performed with multilamellar liposomes. As there is no ambiguity in pH or ionic strength in these samples, they provide an independent reference for the changes in lipid polymorphism and peptide–lipid interactions that are observed in the oriented NMR samples.

2. Materials and methods

2.1. Peptide, lipids, buffers

PGLa was synthesized using standard Fmoc-protocols, either as the native sequence or with a single CF_3-Bpg (2-amino-2-[3-(trifluoromethyl)bicyclo[1.1.1]pent-1-yl]ethanoic acid, obtained

from Enamine Ltd., Kyiv, Ukraine) in position of Ile13, as previously reported [77]. Crude material was purified by RP-HPLC (Jasco system with diode-array detector, Groß-Umstadt, Germany), with analytical (218TP54 Vydac®) and semipreparative (218TP1010, Vydac®) C18 columns, respectively (Grace, Deerfeld, USA). Linear water-acetonitrile gradients were used (both eluents supplemented with 5 mM HCl to avoid any ^{19}F NMR background from 2,2,2-trifluoroethanoic acid), as described before [42]. The collected RP-HPLC fractions were immediately freeze-dried from acidic water/acetonitrile solutions, without neutralising the pH at this stage. The identity and purity of the product were confirmed by analytical RP-HPLC and MALDI-TOF mass spectrometry (matrix-assisted laser induced desorption/ionization with time-of-fly detection, performed on Autoflex III mass spectrometer, Bruker Daltonics, Bremen, Germany).

The lipids DMPC, DMPC-d_{54} and DMPG were purchased from Avanti Polar Lipids (Alabaster, USA). To obtain the desired pH values in the oriented NMR samples, 100 mmol/L buffer solutions were prepared: glycine/HCl for pH 2.6; Na_2HPO_4/citric acid for pH 4.0; Na_2HPO_4/NaH_2PO_4 or HEPES for pH 7.0; Na_2CO_3/$NaHCO_3$ for pH 10. The ingredients were from Riedel-de-Haen (Seelze, Germany), AppliChem (Darmstadt, Germany), or Roth (Karlsruhe, Germany), and were dissolved in milliQ® water (Millipore, Billerica, USA). The pH was measured with a pH-meter (pH 315i, WTW, VWR International, Darmstadt, Germany) at room temperature, and adjusted with HCl (Roth) or NaOH (AppliChem).

2.2. DSC

DSC measurements were performed on a calorimeter VP-DSC (MicroCal, Freiburg, Germany). Original MicroCal software was used for data analysis. Multilamellar samples were prepared from 2 mg/mL stock solutions of the lipids in methanol/chloroform (1:2, vol:vol). They were mixed in the required ratio of DMPC/DMPG (3:1, mol:mol), such that each sample contained a total of 1.3 mg (= 2 mmol) of lipid. For the peptide containing samples, PGLa (unlabelled) was added from a 1 mg/mL stock solution in MeOH in the requested molar ratio of 1:200 or 1:50 peptide/lipid. The solvent was removed under a stream of nitrogen, and the remainder was suspended in milliQ® water and lyophilised overnight. The dried lipids were re-hydrated with 1 mL of a degassed buffer or milliQ® water and mixed carefully. Samples were incubated at 48 °C for 1 h, homogenized thoroughly by vortexing, and freeze-thawed six times. Samples were stored at −80 °C when not used. Before measurement, the samples were thawed at 48 °C for 15 min and degassed for 1 min under a stream of nitrogen. The initial heating and cooling scans of pure buffer were run before each sample to provide the individual baselines. Afterwards, the samples were measured with buffer solution as a reference, using ca. 0.5 mL of sample per measurement. The temperature range was from 5 to 40 or 80 °C, with a heating rate of 1 °C per minute. Samples were equilibrated at the starting temperature for 30 min before measuring. All experiments were performed at least twice with freshly prepared samples each time.

2.3. Solid-state NMR measurements

Solid-state NMR experiments were carried out with oriented samples of DMPC/DMPG 3:1. For ^{2}H NMR, 10% of DMPC was replaced by DMPC-d_{54}. For peptide containing NMR samples, the peptide-to-lipid ratio was 1:50 (mol:mol). The required amounts of peptide and lipid were co-dissolved in 340 μL $CHCl_3$:MeOH (3:1, vol:vol), namely 8.5 mg (0.12 mmol) lipid and 0.5 mg (2.4 μmol) of ^{19}F-labelled PGLa. Twice the amount of lipid and no peptide was used for the lipid-only samples. The solvent was evaporated under a stream of nitrogen, and the sample was freeze-dried overnight. The dried material was suspended in 170 μL of 100 mmol/L buffer (17 μmol) and 680 μL milliQ® water, and the pH of the fluid suspension was controlled with a portable pH meter (pH 315i, WTW, Weilheim, Germany). All samples had the desired pH value (+/− 0.1). The pH of the non-buffered sample was measured to be 6.1. Samples were then freeze-thawed 10 times in liquid nitrogen and a 48 °C water bath, with careful mixing after each cycle to form vesicles. Samples were then distributed onto 17 glass plates (50 μL resulting in 0.5 mg of lipid per glass plate) with dimensions of 18 mm × 7.5 mm × 0.08 mm (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). The glass plates were dried in vacuum (14–17 h), stacked and covered with an empty plate. The degree of hydration was adjusted by incubation in an atmosphere of saturated K_2SO_4 solution for 14–17 h at 48 °C. The stacks were wrapped in several layers of parafilm® and polyethylene foil to avoid dehydration. All samples were stored at −20 °C until measurement.

All solid-state NMR measurements were performed on a Bruker Avance 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany). ^{2}H NMR experiments were carried out at 76.77 MHz using a standard Bruker double resonance wideline probe equipped with a goniometer. A quadrupole echo sequence without ^{1}H decoupling was used with a 4.5 μs 90° pulse, an echo delay of 30 ms, and 80 ms recycle delay. Typically 25,600 scans were collected, starting the acquisition before the echo, and left-shifting the time domain data accordingly. The data were further processed by zero filling to 16,384 data points and a 400 Hz line broadening function, followed by Fourier transformation.

^{31}P NMR experiments were carried out at 202 MHz using a standard Bruker double-resonance wideline probe equipped with a goniometer. The ^{1}H-decoupled spectra were acquired with a Hahn echo sequence with a 90° pulse length of 7 μs, an echo delay time of 30 μs, and a recycle delay of 2 s. ^{31}P NMR spectra were referenced against a 85% solution (vol) of phosphoric acid as a primary reference (Roth). Usually 800 scans were collected, and line broadening of 300 Hz was used for processing.

^{19}F NMR was carried out at 470 MHz with a ^{19}F/^{1}H double-tuned flat-coil probe (Doty Scientific Inc., Columbia, USA). Simple single pulse experiments with a 90° pulse width of 1.8 μs, 10–15 kHz TPPM (two-pulse phase modulation) ^{1}H-decoupling, and a recycle delay of 1 s were used. ^{19}F NMR spectra were referenced by setting the fluoride resonance of 100 mmol/L NaF (Roth,) in aqueous solution (at 35 °C) to −119.5 ppm. Usually 6144 scans were collected, and line broadening of 0.5–1.5 kHz was used for processing.

3. Results

3.1. Preparation of oriented NMR samples with controlled pH

The biologically relevant pH range of interest lies between 5 and 8, reflecting the typical intra- and extracellular environments encountered by antimicrobial peptides and by other proteins in general. HPLC-purified peptides, however, can be rather acidic, down to pH 4 or worse. We therefore selected four representative pH values for this study: pH 2.6 (glycine/HCl) for highly acidic conditions, where the lipid phosphate groups start to become protonated; pH 4.0 (Na_2HPO_4/citric acid) to mimic typical conditions after HPLC; pH 7 (HEPES) to represent ambient conditions; and pH 10 (Na_2HPO_4/NaH_2PO_4 or Na_2CO_3/$NaHCO_3$) as a control for a basic environment in which the Lys groups on PGLa start to become deprotonated.

To optimize the preparation of mechanically aligned oriented samples for SSNMR on glass supports, we systematically varied the ratio of buffer to peptide and also tried different ways of adding and distributing the buffer in the multilamellar system. It was most critical to find a suitable concentration window in which the buffer capacity was strong enough to compensate the acid from the HPLC-purified peptide, but without introducing too much salt into the sample. High ionic strength was found to deteriorate the alignment quality of the oriented bilayers, and it is generally known to reduce the sensitivity of the NMR experiment, increase the pulse widths, etc. The most promising samples were obtained by the following protocol (see experimental section for details): (i) co-solubilization of the lipids (with/out peptide) in organic solvent, (ii) removal of solvent/drying, (iii) suspension of the peptide–

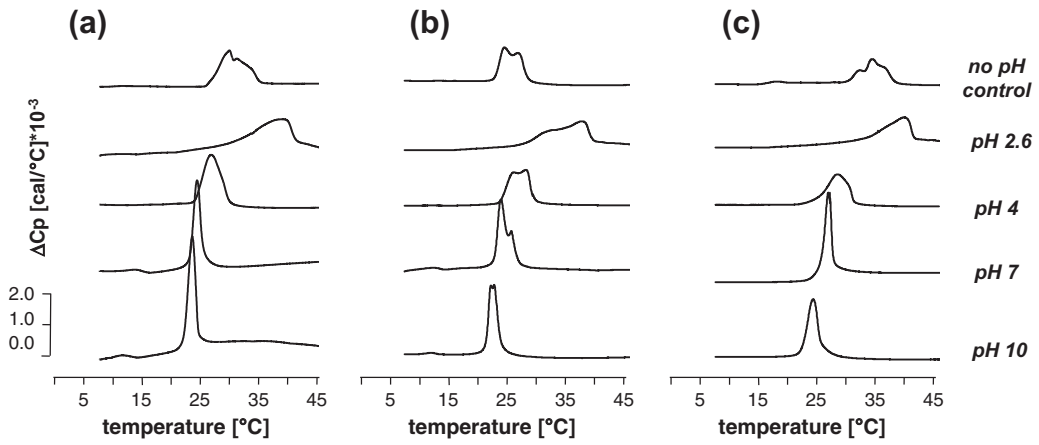

Fig. 1. DSC thermograms (heating scans) illustrating the lipid phase polymorphism of DMPC/DMPG multilamellar vesicles, without (a), and in the presence of externally added PGLa at a peptide-to-lipid (mol/mol) ratio of 1:200 (b), and of 1:50 (c).

lipid mixture (about 10 mg) in a generous amount of aqueous buffer (about 850 μl) with a suitable concentration (20 μmol buffer for 2.4 μmol peptide in 120 μmol lipid), (iv) pH control with a microelectrode and repeated freeze-thaw cycles, (v) distribution of the aqueous suspension onto about 17 glass supports (50 μL each, resulting in 0.5 mg of lipid per plate with an area of 135 mm^2 each), (vi) drying under vacuum, (vii) stacking the plates, (viii) hydration over saturated K_2SO_4 solution, and finally (ix) wrapping the stack with parafilm and polyethylene foil to prevent drying during the NMR experiment. Good spectra on the nuclei ^{31}P, ^{2}H and ^{19}F could be obtained by this method, as illustrated in the sections below. To ensure that the buffer capacity was sufficient, especially under the conditions of limiting hydration in the oriented samples, we first performed DSC experiments on a different set of non-oriented samples containing excess buffer. This way, the phase behaviour of the various sample conditions could be independently characterized and compared to the SSNMR results.

3.2. DSC

DSC is commonly used to examine the thermotropic polymorphism of lipids. Changes in the phase transitions can reveal interactions of various agents binding to and/or inserting into the lipid matrix. This thermodynamic technique is simple, essentially non-perturbing [78], and broadly applicable for studying peptide–lipid systems [79,80]. Here, we used DSC to complement the SSNMR analyses below, which were the main focus. First, as a control, suspensions of multilamellar vesicles of DMPC/DMPG (3:1, mol:mol) were characterized at defined pH values. Subsequently, the same experiments were carried out on samples containing different amounts of PGLa (Fig. 1).

In a standard DSC experiment with liposomes at neutral pH, two major endothermic events are expected for the DMPC/DMPG mixture used. First, a less cooperative low-enthalpy transition ("pre-transition", $T_p \approx 14$ °C) takes the lamellar gel phase (L_β) into a rippled gel phase (P_β). Upon further heating, the more enthalpic, highly cooperative chain-melting transition ("main transition", T_m, at ~24 °C) takes P_β into the lamellar fluid phase (L_α) [81]. These effects could be reproduced with two buffer systems for pH 7, namely phosphate and HEPES. The different buffers resulted only in slightly different absolute enthalpy values (data not shown), hence any changes in membrane polymorphism can be directly attributed to pH effects. Using further buffers to control the pH between 10 and 2.6, we observe a marked up-shift in T_m and a reduction in enthalpy (Fig. 1a) with decreasing pH. A similar effect is seen for T_p, which completely disappeared at pH 4 and below. At an extreme pH of 2.6, the main transition turned into a broad multi-component event ranging from 20 °C to 52 °C, and the vesicles started to precipitate visually upon further heating (see also footnote[1]). Notably, the T_m of the unbuffered sample is found to lie between that of pH 4 and pH 2.6, suggesting that this sample may be rather acidic, presumably due to the presence of DMPG. Its transition occurs over a broad temperature range (25 °C to 35 °C, Fig. 1a), which may be attributed to the known complex polymorphism of DMPG in aqueous solutions of low ionic strength [52,53] that may have affected the mixed DMPC/DMPG bilayers in our measurements.

When PGLa was added (Fig. 1b/c), the overall DSC behaviour changed only marginally. Both transitions, T_p and T_m, occurred at slightly higher temperatures than in the pure lipid mixture. At low peptide concentration (P/L = 1:200), the main transition showed two components at all pH values. One of them reproduced the transition in pure lipids, while the second component is attributed to a fraction of peptide-bound lipid molecules. This interpretation is supported by the fact that the latter component dominates the main transition at higher PGLa concentrations, as previously reported for PGLa and other peptides. The lipid pre-transition was visible at low peptide concentration at pH ≥ 7, but it disappeared completely at higher peptide concentration of P/L = 1:50. Without any pH control, the lipid chain melting transition occurred at temperatures between those of the pH 7 and pH 4 samples for P/L = 1:200, and between pH 4 and pH 2.6 for P/L = 1:50. These observations suggest that PGLa experiences and possibly exacerbates the acidity of an unbuffered sample, which can elevate the lipid phase transition temperature by up to 10 °C compared to pH 7.

3.3. Solid-state ^{2}H NMR analysis of the lipid chain melting transition

To further investigate the influence of pH on the phase state of DMPC/DMPG bilayers, we collected solid-state ^{2}H-NMR spectra with samples containing DMPC/DMPC-d_{54}/DMPG 2.7:0.3:1 (mol:mol:mol), with and without PGLa. ^{2}H NMR lineshapes of the deuterated acyl chains are very sensitive to molecular motions, and can thus be used to examine the phase state of the lipids [82]. Deuteration per se, though known to slightly shift the absolute phase transition temperatures, has only a marginal effect on the overall lipid polymorphism in the mixed systems, and we used a diluted isotope ratio of 1:9 ($^{2}H/^{1}H$). Given the low temperature resolution of 5 °C in the SSNMR experiments, the observed phase transitions should closely correspond to the DSC results,

[1] In repeated heating cooling cycles over ranges of either 5–40 °C or 5–80 °C, hysteresis effects were observed for the chain melting transition, as expected from the fast heating rate of 1 °C/min (data not shown). The pH influenced the overall lipid polymorphism in different ways. Samples with pH 7 and 10 showed reproducible behaviour in consecutive scans. Samples with pH 4 and below, however, were significantly changed already after a single exposure to high temperature (80 °C), presumably due to irreversible lipid hydrolysis.

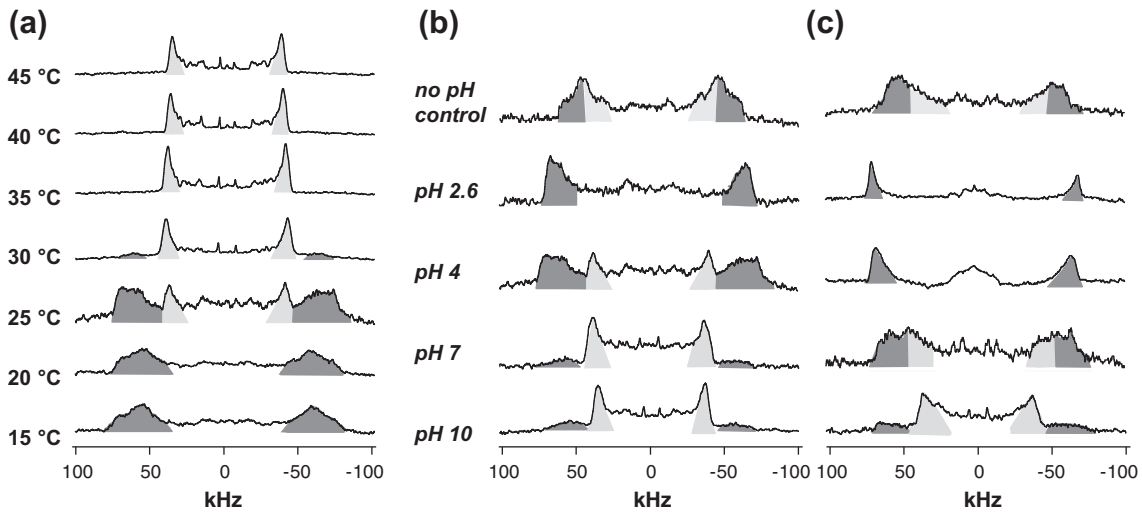

Fig. 2. Solid-state ^{2}H NMR was used to monitor the lipid chain melting transition in oriented DMPC(-d_{54})/DMPG samples at different pH. Spectra were acquired over a series of temperatures, equilibrated the sample at each temperature for 30 min before acquisition. (a) Representative spectra from a sample buffered at pH 4. The outermost broad signals are marked in dark grey and can be assigned to lipids in the gel-phase, while the light grey signals indicate lipids in the fluid phase. (b) Comparison of ^{2}H NMR spectra at 25 °C for the different lipid samples, showing the relative contributions of gel-phase (dark grey) and fluid phase (light grey). (c) Same conditions as in (b), but in the presence of PGLa (P/L = 1:50).

provided that the pH control in the oriented samples was successful, as was indeed the case.

^{2}H NMR spectra with oriented bilayers at different pH values were collected over a series of temperatures, as illustrated in Fig. 2a for a representative sample at pH 4 (the others are shown in Supporting Fig. S1a). At low temperature, lipids in the gel-state exhibit large ^{2}H quadrupolar splittings of about 100 kHz, reflecting the rigidified C^2H_2-segments near the glycerol moiety (individual signals are poorly resolved under these conditions). When increasing the temperature to 25 °C, additional signals emerge from lipids in the L_α phase that starts to co-exist at this temperature. At 35 °C, the outermost signals have completely disappeared, and the most prominent spectral component remains with a splitting below 50 kHz. This lineshape reflects a fluid bilayer with highly mobile acyl chains, indicating that the bilayer transition into the fluid phase is complete.

The ^{2}H NMR spectra obtained at neutral pH reflect the expected polymorphic behaviour. At 25 °C the peptide-free samples are right in the middle of the chain melting phase-transition, with gel-phase and fluid lipids co-existing. Throughout the series of pH values, the co-existence region is found to shift to higher temperature with decreasing pH (Fig. 2b), the same way as in DSC. At pH 10, the percentage of gel-phase lipids was quite small at 25 °C and had completely disappeared at 35 °C, whereas at pH 2.6 it was still dominant at 35 °C. Also the unbuffered sample showed an elevated chain melting transition (above 25 °C), just like in DSC, suggesting that this sample is indeed quite acidic. The same trends were observed for the samples containing PGLa (P/l = 1:50), namely an elevated chain melting temperature and signs of acidification by the presence of the peptide (see Supporting Fig. S1b).

3.4. Solid-state ^{31}P NMR analysis of lipid order and hydrolyis

^{31}P NMR was used to probe the response of the phospholipid headgroups, and to evaluate the quality of alignment in the solid-supported membrane samples, as it is routinely done for oriented bilayers [38–40]. Narrow signals around 20 to 30 ppm correspond to well oriented phospholipids in the lamellar phase, while a broader peak near -15 ppm indicates the presence of non-oriented domains in the sample. ^{31}P NMR spectra were acquired over a series of temperatures, as illustrated in Fig. 3a for a representative sample at pH 4 (the others are shown in Supporting Fig. S2a). The high quality of lipid orientation in this sample is confirmed. Even though the chemical shift of the phospholipid headgroup does not respond much to the lipid chain melting transition, we nevertheless compared the series of pH controlled spectra at 25 °C, as for ^{2}H NMR above. The pure lipid samples did not show much change, except for a slight shift of the DMPG signal relative to DMPC (Fig. 3b). However, in the presence of PGLa (P/L = 1:50), dramatic effects are seen that can be attributed to lipid hydrolysis at extreme pH values. At pH 2.6, additional signals emerge that can be assigned to membrane embedded lyso-lipids (15 ppm) and to isotropically mobile phosphorylated species (0 ppm). At the other extreme of pH 10, a large proportion of the samples has become disordered and lost its lamellar alignment. Overall, the extent of pH-dependent hydrolysis can be ranked as pH 7 ≈ unbuffered ≪ pH 4 ≪ pH 10 < pH 2.6, and it is exacerbated in the presence of high amounts of PGLa. Most importantly, however, there is no sign of lipid hydrolysis in the unbuffered samples, neither for the pure lipid nor in the presence of PGLa (P/L = 1:50). Even though they were found to be somewhat acidic according to DSC and ^{2}H NMR, the ^{31}P NMR data suggest that it may still be safe to investigate them without pH control, at least with regards to the risk of lipid hydrolysis.

3.5. Solid-state ^{19}F NMR analysis of the peptide alignment

^{19}F NMR of a selectively CF_3-labelled PGLa analogue was used to address any pH-dependent changes in its membrane-bound behaviour. The peptide conformation and orientation have been previously characterized in a comprehensive manner by means of ^{19}F NMR [17,37]. Therefore, the CF_3-splitting of a single label in the position of Ile13 can now be used as a fingerprint to deduce the alignment state of the entire helix [34]. We may thus expect to recognize the signals corresponding to the S-, T- or I-states of PGLa, which have been previously characterized in samples without pH control [8,17,33,35,37]. The surface-bound S-state is characterized by a large dipolar splitting of about +7 kHz to the left of the isotropic position, while the tilted T-state and the inserted I-state give splittings around −3 kHz and −4 kHz, respectively, on the right [17,37]. These latter signals may be obscured by "powder" contributions from immobilized molecules (−8 kHz), and from peptides in non-oriented domains of the bilayers (−3.5 kHz) [22].

Fig. 4 gives an overview of the ^{19}F NMR spectra for the peptide-containing samples with different pH, acquired over a series of different temperatures above and below the lipid phase transition. The previously described re-alignment of the peptide, which is known to occur around T_m, is well reproduced in the unbuffered sample. The S➔T(or I)➔S transition is readily discernible, as the NMR signals move from low-field (left of the isotropic position, positive splitting) to high-field (right of

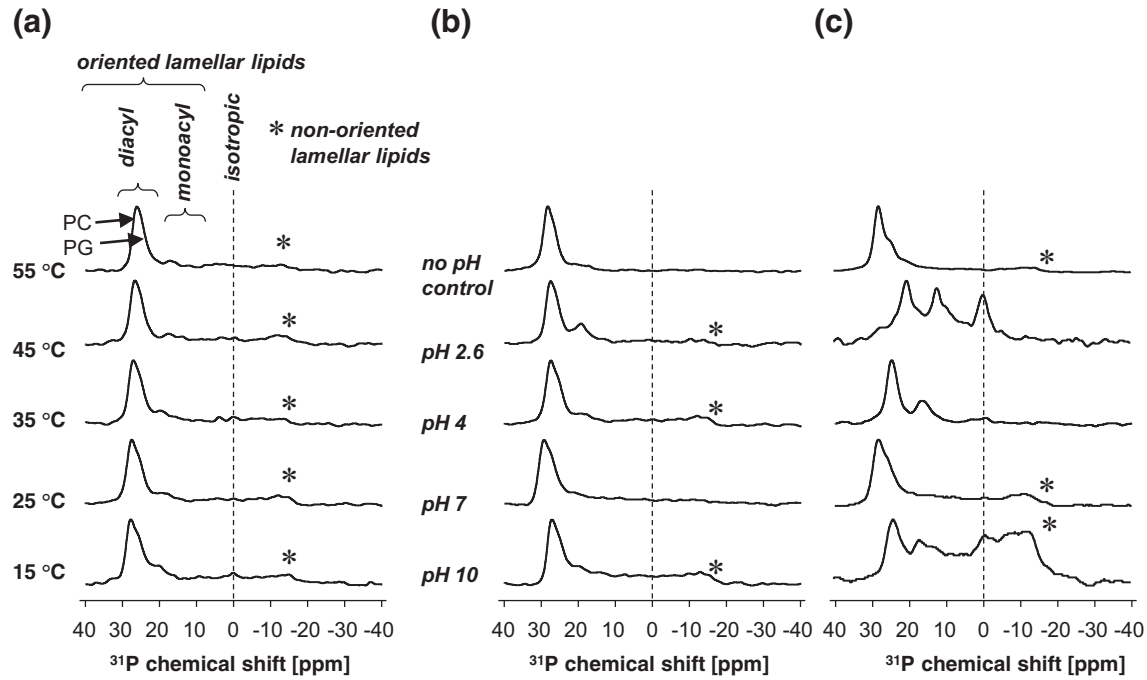

Fig. 3. ^{31}P NMR spectra from oriented samples of DMPC/DMPG. (a) The temperature series at pH 4 shows good alignment of the lipids, and little response to the chain melting transition. Signals are assigned to the well oriented diacyl- and monoacyl (i.e. lyso-) lipids, the isotropic frequency is indicated by a dashed line, and non-oriented lipid signals are marked with an asterisk. (b) Comparison of samples with different pH, measured at 25 °C. (c) Same as in (b), but with ^{19}F-labelled PGLa at a P/L ratio of 1:50. All spectra have been normalized to the same amplitude for convenience.

the isotropic position, small negative splitting) and back again. It is very reassuring to see that also the buffered sample at pH 7 displays the same behaviour as without pH control. This important result suggests that our earlier analyses have not been fraught with pH dependent problems, nor did they suffer from deleterious effects of acidification. In line with the DSC data and ^{2}H and ^{31}P NMR results above, the peptide-containing samples without pH control have a moderately acidic pH between 7 and 4, but this does not seem to perturb the biologically relevant peptide–lipid interactions of PGLa in DMPC/DMPG. In fact, the sample without pH control has a better quality than in the presence of pH 7 buffer, as the latter spectra show a significant proportion of "powder" contributions that are indicative of poorly oriented membrane domains.

As a matter of warning, however, PGLa behaved in unforeseeable and uncharacteristic ways in all pH controlled samples other than pH 7. Under acidic conditions of pH 4 and 2.6, it produced an isotropic signal when lowering the temperature towards the gel phase, suggesting that the peptide was excluded from the multibilayers or segregated in some isotropic lipid phase. The latter possibility can be excluded, because neither the ^{31}P or ^{2}H NMR spectra of the lipids show any

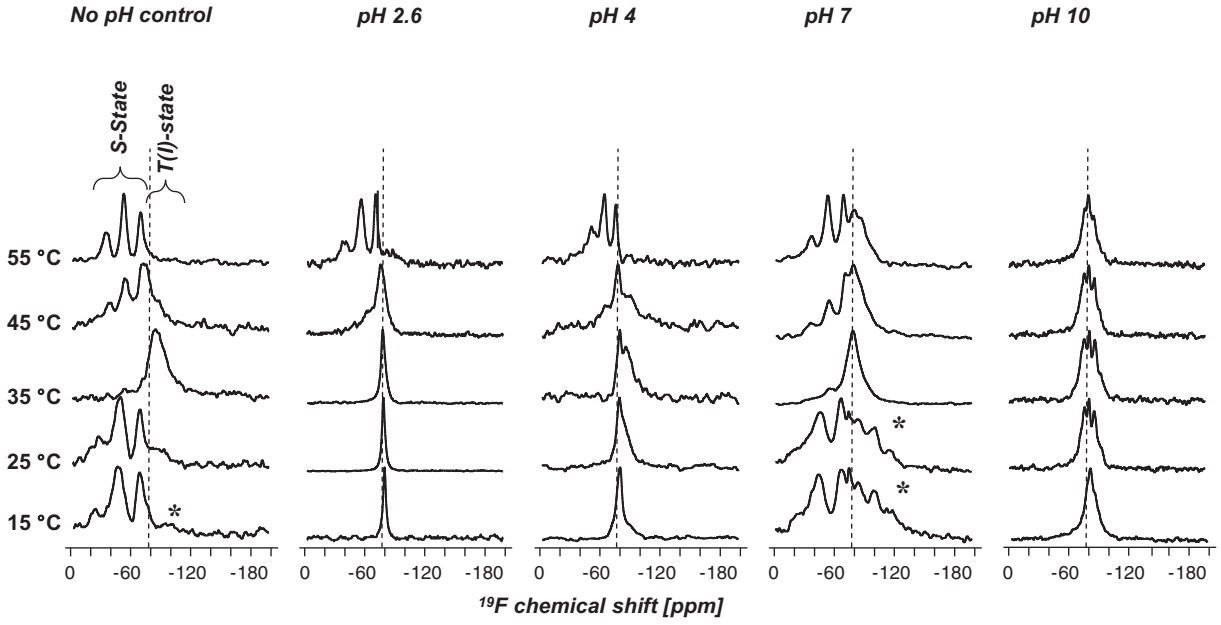

Fig. 4. ^{19}F NMR spectra of oriented DMPC/DMPG samples with different pH values, containing selectively CF_3-labelled PGLa with a P/L ratio of 1:50, acquired over a series of temperatures. The different alignment states (S, T, I) of the helical peptide have been previously assigned in samples with no pH control, so the characteristic spectra can be interpreted here like a fingerprint [17,37]. The isotropic frequency is marked with a dashed line, and powder-like signals with an asterisk.

corresponding isotropic component. Such unbinding/unfolding behaviour has not been documented before for PGLa in oriented samples, but may resemble that of some other peptides [83]. The ^{19}F NMR spectra at pH 10 with a persistent splitting of about −2 kHz differ profoundly from the other samples and from any previously reported data. This splitting is not compatible with any known alignment of PGLa and must reflect an entirely new structural state.

When passing through the lipid chain melting transition, most samples show a distinct change in spectral lineshape, so is possible to estimate the T_m of these peptide-containing oriented multibilayers. Compared to the phase transitions of the pure lipids, the presence of PGLa is found to elevate T_m by about 5–10 °C in the ^{19}F NMR series at ambient pH, in line with the DSC and ^{2}H NMR data above. Furthermore, it also shows a clear trend of increasing the transition temperature with decreasing pH, again in full agreement with DSC and ^{2}H NMR.

4. Discussion

Biophysical experiments on macroscopically oriented multilamellar membrane systems are typically carried out without any pH control, and without knowledge of the pH in the sample [1,4–8,11–22,24–29,31–42,51,77,83]. However, pH-induced effects can affect both, the lipids [52–56] and the peptides [57–67]. It is intrinsically difficult to control the pH in stacked multibilayers on a solid support, as these samples are usually hydrated via the vapour phase of a saturated salt solution with 96% relative humidity. They do not contain any excess water, unlike liposomal suspensions or bicelles, where buffering is a standard procedure. Here, we have demonstrated how oriented samples can be prepared with controlled pH on glass supports with a good quality of alignment, and we present an optimized protocol that was approved by solid-state NMR analysis and DSC. First, we had to verify that the buffer capacity was sufficient, and that the oriented membranes with limited hydration behaved the same way as in suspension with excess buffer. To this aim, the phase transitions of the two types of sample were compared, using SSNMR for the oriented bilayers and DSC for the multilamellar dispersions. Since the lipid phase behaviour was found to be fully consistent amongst these two types of experiments, we can conclude that the desired pH was indeed achieved and maintained in the NMR samples using the proposed protocol.

Yet, several technical challenges arise in an NMR experiment when buffer is used. Due to the high ionic strength not only hardware adjustments are problematic (e.g. tuning the probe), but also sample drying due to the enhanced radio-frequency induced heating is more pronounced than in non-buffered samples [84]. Drying can lead to marked changes in the sample, because dehydration of lipid bilayers generally causes an elevation of their main phase transition temperature [85]. This well-known effect is comparable in magnitude to what we observed here upon acidification of DMPC/DMPG, and both phenomena can lead to misinterpretation of the data. For example, we observed that a temperature series from 15 °C to 55 °C (by ^{31}P, ^{2}H, or ^{19}F NMR) can significantly decrease the level of hydration, largely due to the exposure to elevated temperatures. As a consequence, all T_m-associated events (e.g. the gel–fluid phase transition detected in ^{2}H NMR, or the peptide re-orientation seen in ^{19}F NMR) were shifted by about 5–10° when measured in a consecutive (2nd) temperature series. This means that a temperature series of only 6 h (e.g., 6 consecutive ^{31}P NMR spectra, each equilibrated for 30 min at the target temperature) is already enough to cause sample dehydration. Re-hydration of the sample in between two temperature series can attenuate this problem to a certain extent, but the stacked sample needs to be unwrapped and its quality of alignment can thereby deteriorate. The best precaution is to use only freshly prepared samples directly from the hydration chamber, as was done for all ^{2}H ^{31}P and ^{19}F NMR data presented here. Notably, the observation of peptides labelled with low-γ isotopes (^{15}N and ^{13}C) tends to require tens of hours or even several days per spectrum, and is expected to lead to more severe problems with drying.

Having found suitable buffer conditions, the effect of pH on the homogeneous DMPC/DMPG lipid mixture was compared systematically using calorimetry and ^{2}H NMR. Calorimetry revealed a steady increase in the lipid chain-melting temperature upon lowering the pH (Fig. 1a), and the same changes were seen by ^{2}H NMR (Figs. 2b, S1a). The non-buffered lipid samples behaved as if their pH was close to 4, but this intrinsic acidity could be successfully compensated by the buffer capacity in our pH-controlled samples. Notably, the DSC temperature maximum of the gel-to-fluid transition (P_β to L_α) in the non-buffered sample was found to be significantly higher (i.e. ~34 °C instead of ~24 °C), and the transition range was much broader (about 10 °C) than what is typically assumed and documented for DMPC and DMPG under ambient buffer conditions. The latter anionic lipid (as well as other phosphatidylglycerols) is known to exhibit rather complex phase transitions at low ionic strength. Full conversion of the unbuffered DMPC/DMPG mixture into the L_α phase was complete only above 37 °C, meaning that a sample measured around 25 to 35 °C may still be affected by the phase transition process.

^{31}P NMR was used to observe the phospholipid headgroups, in order to check for lipid hydrolysis, and to assess the quality of alignment in the oriented samples. In addition to the considerable impact of pH on the lipid phase transition, we observed pronounced hydrolysis under acidic and basic conditions. Not only did ^{31}P NMR detect large amounts of lyso-lipids at pH 2.6 and 10 (Figs. 3b, S2a), but also the ^{2}H NMR spectra contained more peaks than expected (Figs. 2b, S1a). The unbuffered sample, luckily, did not suffer from hydrolysis, just like the ones prepared freshly at pH 4.

When PGLa was added to the DMPC/DMPG matrix, the prevalent phase characteristics of the lipids remained, but the absolute phase transition temperatures and peak widths tended to increase even further, according to DSC and ^{2}H NMR. At low peptide-to-lipid ratio (P/L = 1:200), DSC showed a two-component main phase transition at pH 7. This effect had already been reported for PGLa and was attributed to the formation of peptide-poor and peptide-rich domains [86–88], or to lateral phase segregation of DMPC and DMPG based on preferential electrostatic interaction of the cationic PGLa with DMPG [35,42]. At high peptide concentration (P/L = 1:50), the DSC thermogram gave only a single peak, which corresponded to the higher temperature component of the peptide-poor samples. This observation supports the earlier interpretation of peptide-rich and peptide-free membrane domains.

The structural behaviour of PGLa itself in the membrane was monitored using ^{19}F NMR on a peptide analogue carrying a specifically CF_3-labelled side chain (Fig. 4). In earlier work, we had resolved three different alignment states of the amphiphilic helix in oriented DMPC/DMPG samples, but without pH control. PGLa was found to flip from a surface-bound S-state into a tilted T-state and an inserted I-state as a function of peptide concentration, temperature, partner peptides, etc. [17,33–35,37]. These structural changes were taken to explain its antimicrobial activity by the formation of dimers and transmembrane pores. An important result from the present study is the fact that the same re-alignment behaviour is observed now in the buffered sample at pH 7, which confirms that the earlier results without pH control are still valid, and that they are biologically relevant. This conclusion is reassuring, but the dramatic pH dependent effects seen in the other samples with controlled acidic or basic pH are highly precarious. At pH 4 and pH 2.6, PGLa was still properly aligned in the S-state in fluid membranes, but in the gel-phase it became excluded from the bilayers and produced an isotropic signal as an unbound and possibly unfolded molecule. This interpretation makes sense in the light of increased electrostatic repulsion of the cationic peptide from a protonated, charged lipid bilayer. At pH 10, on the other hand, the peptide assumed an entirely unknown structure that could not be assigned, and which did not respond to the lipid chain melting transition. Under these conditions, PGLa obviously lost its cationic character, since the lysine side chains have a pKa ≈ 10.3. Overall, we can thus conclude that our unbuffered PGLa samples were significantly acidic and must have been close to the

edge of the acceptable pH window within which an experiment and its interpretation are still meaningful. The same situation and note of caution probably also apply to most other cationic membrane-active peptides, which have been subject to an enormous number of biophysical analyses in oriented samples.

Extrapolating from the PGLa data, it is important to realize that an unbuffered system can only be considered as "safe" if the peptide does not possess too many acidic groups in its sequence. PGLa is an entirely cationic peptide (4 Lys, an ionisable N-terminus, and an amidated C-terminus), so it maintains a constant positive charge over the entire acidic pH range. It carries only anionic counter-ions from HPLC and does not act as a buffer itself, when dissolved in water without pH control. Carboxylic groups, however, such as Asp or Glu side chains or a free C-terminus, will be protonated after HPLC and can release these protons again in aqueous solution. Anionic peptides, therefore, can act themselves as buffers and will strongly acidify any membrane sample that is not subject to stringent pH control. It is a trivial but important conclusion that in unbuffered systems the risk of unwanted pH-induced effects is much higher for intrinsically acidic peptides than illustrated here for PGLa. Therefore, it can be recommended that those samples should be pH controlled.

The interaction of antimicrobial peptides with bacterial membranes usually occurs at neutral to low pH in a normal eukaryotic host, both at the level of cells and tissues, so the need for buffering of peptides with acidic groups should be generally realized. When investigating peptides that exert their activity in specific environments, pH control may become even more important. Peptides will encounter acidic conditions, e.g., after being endocytosed by eukaryotic cells and upon degradation in the lysosome. A pH-triggered endosomal escape mechanism has been described for viral proteins and can be utilized by cell penetrating peptides [89]. Other pH-exotic environmental niches are known, such as habitats for the enteric, skin or oral flora. In humans, basic pH > 8 is encountered on the skin and in the oral cavity upon exposure to certain cosmetic products or nutrients; in certain regions of the intestinal system; and in the ducts of the alkalinizing salivary glands and pancreas. Basic pH has also been described, e.g., to enhance the selective sterilization of Gram-negative bacteria [90], to boost transdermal drug delivery by cationic peptides [91], and to induce bacterial biofilms [92]. It is typically encountered in the manipulation of industrially relevant alkaliphiles [93], or in the environment of actively alkalinizing pathogenic microorganisms like *H. pylori* or *C. albicans* [94]. All these situations can be reliably studied in oriented samples when prepared with suitable buffers.

Acknowledgements

We thank Dr. Parvesh Wadhwani and Sezgin Kara for the peptide synthesis, and the KIT for a grant to J.M.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.12.006.

References

[1] K. Koch, S. Afonin, M. Ieronimo, M. Berditsch, A. Ulrich, Solid-state ^{19}F-NMR of peptides in native membranes, Top. Curr. Chem. 306 (2008) 89–118.
[2] D.E. Warschawski, A.A. Arnold, M. Beaugrand, A. Gravel, E. Chartrand, I. Marcotte, Choosing membrane mimetics for NMR structural studies of transmembrane proteins, Biochim. Biophys. Acta 1808 (2011) 1957–1974.
[3] T.A. Cross, D.T. Murray, A. Watts, Helical membrane protein conformations and their environment, Eur. Biophys. J. 42 (2013) 731–755.
[4] E. Strandberg, A.S. Ulrich, NMR methods for studying membrane-active antimicrobial peptides, Concepts Magn. Reson. A 23A (2004) 89–120.
[5] A. Naito, Structure elucidation of membrane-associated peptides and proteins in oriented bilayers by solid-state NMR spectroscopy, Solid State Nucl. Magn. Reson. 36 (2009) 67–76.
[6] G.A. Olah, H.W. Huang, Circular dichroism of oriented α-helices: I. Proof of the exciton theory, J. Chem. Phys. 89 (1988) 2531–2538.
[7] Y. Wu, H.W. Huang, G.A. Olah, Method of oriented circular dichroism, Biophys. J. 57 (1990) 797–806.
[8] J. Burck, S. Roth, P. Wadhwani, S. Afonin, N. Kanithasen, E. Strandberg, A.S. Ulrich, Conformation and membrane orientation of amphiphilic helical peptides by oriented circular dichroism, Biophys. J. 95 (2008) 3872–3881.
[9] D. Marsh, Orientation and peptide–lipid interactions of alamethicin incorporated in phospholipid membranes: polarized infrared and spin-label EPR spectroscopy, Biochemistry 48 (2009) 729–737.
[10] S.A. Tatulian, Attenuated total reflection Fourier transform infrared spectroscopy: a method of choice for studying membrane proteins and lipids, Biochemistry 42 (2003) 11898–11907.
[11] H. Ghimire, E.J. Hustedt, I.D. Sahu, J.J. Inbaraj, R. McCarrick, D.J. Mayo, M.R. Benedikt, R.T. Lee, S.M. Grosser, G.A. Lorigan, Distance measurements on a dual-labeled TOAC AChR M2δ peptide in mechanically aligned DMPC bilayers via dipolar broadening CW-EPR spectroscopy, J. Phys. Chem. B 116 (2012) 3866–3873.
[12] D. Marsh, M. Jost, C. Peggion, C. Toniolo, TOAC spin labels in the backbone of alamethicin: EPR studies in lipid membranes, Biophys. J. 92 (2007) 473–481.
[13] T. Salditt, Lipid–peptide interaction in oriented bilayers probed by interface-sensitive scattering methods, Curr. Opin. Struct. Biol. 13 (2003) 467–478.
[14] T. Salditt, C. Li, A. Spaar, Structure of antimicrobial peptides and lipid membranes probed by interface-sensitive X-ray scattering, Biochim. Biophys. Acta 1758 (2006) 1483–1498.
[15] K. He, S.J. Ludtke, H.W. Huang, D.L. Worcester, Antimicrobial peptide pores in membranes detected by neutron in-plane scattering, Biochemistry 34 (1995) 15614–15618.
[16] L. Yang, T.A. Harroun, W.T. Heller, T.M. Weiss, H.W. Huang, Neutron off-plane scattering of aligned membranes. I. Method of measurement, Biophys. J. 75 (1998) 641–645.
[17] R.W. Glaser, C. Sachse, U.H. Durr, P. Wadhwani, S. Afonin, E. Strandberg, A.S. Ulrich, Concentration-dependent realignment of the antimicrobial peptide PGLa in lipid membranes observed by solid-state ^{19}F-NMR, Biophys. J. 88 (2005) 3392–3397.
[18] S. Afonin, U.H.N. Dürr, P. Wadhwani, J. Salgado, A.S. Ulrich, Solid state NMR structure analysis of the antimicrobial peptide gramicidin s in lipid membranes: concentration-dependent re-alignment and self-assembly as a β-barrel, Top. Curr. Chem. 273 (2008) 139–154.
[19] P. Wadhwani, E. Strandberg, J. van den Berg, C. Mink, J. Bürck, R.A. Ciriello, A.S. Ulrich, Dynamical structure of the short multifunctional peptide BP100 in membranes, Biochim. Biophys. Acta 1838 (2014) 940–949.
[20] J. Salgado, S.L. Grage, L.H. Kondejewski, R.S. Hodges, R.N. McElhaney, A.S. Ulrich, Membrane-bound structure and alignment of the antimicrobial β-sheet peptide gramicidin S derived from angular and distance constraints by solid state ^{19}F-NMR, J. Biomol. NMR 21 (2001) 191–208 (2001).
[21] P. Wadhwani, J. Reichert, E. Strandberg, J. Bürck, J. Misiewicz, S. Afonin, N. Heidenreich, S. Fanghänel, P.K. Mykhailiuk, I.V. Komarov, A.S. Ulrich, Stereochemical effects on the aggregation and biological properties of the fibril-forming peptide $[KIGAKI]_3$ in membranes, Phys. Chem. Chem. Phys. 15 (2013) 8962–8971.
[22] P. Wadhwani, E. Strandberg, N. Heidenreich, J. Bürck, S. Fanghänel, A.S. Ulrich, Self-assembly of flexible β-strands into immobile amyloid-like β-sheets in membranes as revealed by solid-state ^{19}F NMR, J. Am. Chem. Soc. 134 (2012) 6512–6515.
[23] S.L. Grage, S. Afonin, A.S. Ulrich, Dynamic transitions of membrane-active peptides, Methods Mol. Biol. 618 (2010) 183–207.
[24] D. Grasnick, U. Sternberg, E. Strandberg, P. Wadhwani, A.S. Ulrich, Irregular structure of the HIV fusion peptide in membranes demonstrated by solid-state NMR and MD simulations, Eur. Biophys. J. 40 (2011) 529–543.
[25] P. Wadhwani, J. Bürck, E. Strandberg, C. Mink, S. Afonin, M. Ieronimo, A.S. Ulrich, Using a sterically restrictive amino acid as a ^{19}F NMR label to monitor and to control peptide aggregation in membranes, J. Am. Chem. Soc. 130 (2008) 16515–16517.
[26] O. Toke, R.D. O'Connor, T.K. Weldeghiorghis, W.L. Maloy, R.W. Glaser, A.S. Ulrich, J. Schaefer, Structure of $(KIAGKIA)_3$ aggregates in phospholipid bilayers by solid-state NMR, Biophys. J. 87 (2004) 675–687.
[27] S.L. Grage, A.V. Suleymanova, S. Afonin, P. Wadhwani, A.S. Ulrich, Solid state NMR analysis of the dipolar couplings within and between distant CF_3-groups in a membrane-bound peptide, J. Magn. Reson. 183 (2006) 77–86.
[28] S. Pujals, H. Miyamae, S. Afonin, T. Murayama, H. Hirose, I. Nakase, K. Taniuchi, M. Umeda, K. Sakamoto, A.S. Ulrich, S. Futaki, Curvature engineering: positive membrane curvature induced by epsin N-terminal peptide boosts internalization of octaarginine, ACS Chem. Biol. 8 (2013) 1894–1899.
[29] S.L. Grage, S. Afonin, M. Grüne, A.S. Ulrich, Interaction of the fusogenic peptide B18 in its amyloid-state with lipid membranes studied by solid state NMR, Chem. Phys. Lipids 132 (2004) 65–77.
[30] A. Filippov, A. Khakimov, S. Afonin, O. Antzutkin, Interaction of prostatic acid phosphatase fragments with a lipid bilayer as studied by NMR spectroscopy, Mendeleev Comm. 23 (2013) 313–315.
[31] S. Afonin, A. Frey, S. Bayerl, D. Fischer, P. Wadhwani, S. Weinkauf, A.S. Ulrich, The cell-penetrating peptide TAT(48-60) induces a non-lamellar phase in DMPC membranes, ChemPhysChem 7 (2006) 2134–2142.
[32] E. Strandberg, D. Tiltak, S. Ehni, P. Wadhwani, A.S. Ulrich, Lipid shape is a key factor for membrane interactions of amphipathic helical peptides, Biochim. Biophys. Acta 1818 (2012) 1764–1776.
[33] E. Strandberg, J. Zerweck, P. Wadhwani, A.S. Ulrich, Synergistic insertion of antimicrobial magainin-family peptides in membranes depends on the lipid spontaneous curvature, Biophys. J. 104 (2013) L9–L11.
[34] S. Afonin, R.W. Glaser, C. Sachse, J. Salgado, P. Wadhwani, A.S. Ulrich, ^{19}F NMR screening of unrelated antimicrobial peptides shows that membrane interactions are largely governed by lipids, Biochim. Biophys. Acta 1838 (2014) 2260–2268.
[35] P. Tremouilhac, E. Strandberg, P. Wadhwani, A.S. Ulrich, Conditions affecting the re-alignment of the antimicrobial peptide PGLa in membranes as monitored by solid state ^{2}H-NMR, Biochim. Biophys. Acta 1758 (2006) 1330–1342.

[36] C. Muhle-Goll, S. Hoffmann, S. Afonin, S.L. Grage, A.A. Polyansky, D. Windisch, M. Zeitler, J. Burck, A.S. Ulrich, Hydrophobic matching controls the tilt and stability of the dimeric platelet-derived growth factor receptor (PDGFR) β transmembrane segment, J. Biol. Chem. 287 (2012) 26178–26186.
[37] S. Afonin, S.L. Grage, M. Ieronimo, P. Wadhwani, A.S. Ulrich, Temperature-dependent transmembrane insertion of the amphiphilic peptide PGLa in lipid bilayers observed by solid state ^{19}F NMR spectroscopy, J. Am. Chem. Soc. 130 (2008) 16512–16514.
[38] K.J. Hallock, K. Henzler Wildman, D.K. Lee, A. Ramamoorthy, An innovative procedure using a sublimable solid to align lipid bilayers for solid-state NMR studies, Biophys. J. 82 (2002) 2499–2503.
[39] P.V. LoGrasso, F. Moll III, T.A. Cross, Solvent history dependence of gramicidin A conformations in hydrated lipid bilayers, Biophys. J. 54 (1988) 259–267.
[40] F.M. Marassi, K.J. Crowell, Hydration-optimized oriented phospholipid bilayer samples for solid-state NMR structural studies of membrane proteins, J. Magn. Reson. 161 (2003) 64–69.
[41] J.K. Rainey, B.D. Sykes, Optimizing oriented planar-supported lipid samples for solid-state protein NMR, Biophys. J. 89 (2005) 2792–2805.
[42] S. Afonin, R.W. Glaser, M. Berditchevskaia, P. Wadhwani, K.H. Gührs, U. Möllmann, A. Perner, A.S. Ulrich, 4-Fluorophenylglycine as a label for ^{19}F NMR structure analysis of membrane-associated peptides, ChemBioChem 4 (2003) 1151–1163.
[43] V.V. Andrushchenko, H.J. Vogel, E.J. Prenner, Optimization of the hydrochloric acid concentration used for trifluoroacetate removal from synthetic peptides, J. Pept. Sci. 13 (2007) 37–43.
[44] E. Kaiser, J. Rohrer, Determination of residual trifluoroacetate in protein purification buffers and peptide preparations by ion chromatography, J. Chromatogr. A 1039 (2004) 113–117.
[45] N. Fuller, R.P. Rand, The influence of lysolipids on the spontaneous curvature and bending elasticity of phospholipid membranes, Biophys. J. 81 (2001) 243–254.
[46] E.E. Kooijman, V. Chupin, N.L. Fuller, M.M. Kozlov, B. de Kruijff, K.N.J. Burger, P.R. Rand, Spontaneous curvature of phosphatidic acid and lysophosphatidic acid, Biochemistry 44 (2005) 2097–2102.
[47] F. Aussenac, B. Lavigne, E.J. Dufourc, Toward bicelle stability with ether-linked phospholipids: temperature, composition, and hydration diagrams by ^{2}H and ^{31}P solid-state NMR, Langmuir 21 (2005) 7129–7135.
[48] M. Ottiger, A. Bax, Bicelle-based liquid crystals for NMR-measurement of dipolar couplings at acidic and basic pH values, J. Biomol. NMR 13 (1999) 187–191.
[49] S. Cavagnero, H.J. Dyson, P.E. Wright, Improved low pH bicelle system for orienting macromolecules over a wide temperature range, J. Biomol. NMR 13 (1999) 387–391.
[50] K. Bertelsen, B. Vad, E.H. Nielsen, S.K. Hansen, T. Skrydstrup, D.E. Otzen, T. Vosegaard, N.C. Nielsen, Long-term stable ether–lipid vs conventional ester–lipid bicelles in oriented solid-state NMR: altered structural information in studies of antimicrobial peptides, J. Phys. Chem. B 115 (2011) 1767–1774.
[51] P.C. Dave, E. Billington, Y.-L. Pan, S.K. Straus, Interaction of alamethicin with ether-linked phospholipid bilayers: oriented circular dichroism, ^{31}P solidsState NMR, and differential scanning calorimetry studies, Biophys. J. 89 (2005) 2434–2442.
[52] K.A. Riske, L.Q. Amaral, M.T. Lamy-Freund, Thermal transitions of DMPG bilayers in aqueous solution: SAXS structural studies, Biochim. Biophys. Acta 1511 (2001) 297–308.
[53] M.T. Lamy-Freund, K.A. Riske, The peculiar thermo-structural behavior of the anionic lipid DMPG, Chem. Phys. Lipids 122 (2003) 19–32.
[54] J.M. Boggs, Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function, Biochim. Biophys. Acta 906 (1987) 353–404.
[55] H. Eibl, P. Woolley, Electrostatic interactions at charged lipid membranes. Hydrogen bonds in lipid membrane surfaces, Biophys. Chem. 10 (1979) 261–271.
[56] W.L. Vaz, A. Niksch, F. Jähnig, Electrostatic interactions at charged lipid membranes. Measurement of surface pH with fluorescent lipoid pH indicators, Eur. J. Biochem. 83 (1978) 299–305.
[57] J.B. Matthew, Electrostatic effects in proteins, Annu. Rev. Biophys. Biophys. Chem. 14 (1985) 387–417.
[58] K. Rosenheck, P. Doty, The far ultraviolet absorption spectra of polypeptide and protein solutions and their dependence on conformation, Proc. Natl. Acad. Sci. U. S. A. 47 (1961) 1775–1785.
[59] J.R. Whitaker, R.E. Feeney, Chemical and physical modification of proteins by the hydroxide ion, Crit. Rev. Food Sci. Nutr. 19 (1983) 173–212.
[60] T.C. Vogt, B. Bechinger, The interactions of histidine-containing amphipathic helical peptide antibiotics with lipid bilayers. The effects of charges and pH, J. Biol. Chem. 274 (1999) 29115–29121.
[61] W. Li, F. Nicol, F.C. Szoka Jr., GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery, Adv. Drug Deliv. Rev. 56 (2004) 967–985.
[62] F. Hu, K. Schmidt-Rohr, M. Hong, NMR detection of pH-dependent histidine–water proton exchange reveals the conduction mechanism of a transmembrane proton channel, J. Am. Chem. Soc. 134 (2011) 3703–3713.
[63] O.A. Andreev, D.M. Engelman, Y.K. Reshetnyak, pH-sensitive membrane peptides (pHLIPs) as a novel class of delivery agents, Mol. Membr. Biol. 27 (2010) 341–352.
[64] A. Aggeli, M. Bell, L.M. Carrick, C.W. Fishwick, R. Harding, P.J. Mawer, S.E. Radford, A.E. Strong, N. Boden, pH as a trigger of peptide beta-sheet self-assembly and reversible switching between nematic and isotropic phases, J. Am. Chem. Soc. 125 (2003) 9619–9628.
[65] J.L. Lorieau, J.M. Louis, C.D. Schwieters, A. Bax, pH-triggered, activated-state conformations of the influenza hemagglutinin fusion peptide revealed by NMR, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 19994–19999.
[66] D.W. Lowik, E.H. Leunissen, M. van den Heuvel, M.B. Hansen, J.C. van Hest, Stimulus responsive peptide based materials, Chem. Soc. Rev. 39 (2010) 3394–3412.
[67] N.K. Subbarao, R.A. Parente, F.C. Szoka Jr., L. Nadasdi, K. Pongracz, pH-dependent bilayer destabilization by an amphipathic peptide, Biochemistry 26 (1987) 2964–2972.
[68] R.N. Lewis, Y.P. Zhang, R.N. McElhaney, Calorimetric and spectroscopic studies of the phase behavior and organization of lipid bilayer model membranes composed of binary mixtures of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, Biochim. Biophys. Acta 1668 (2005) 203–214.
[69] D.L. Melchior, J.M. Steim, Thermotropic transitions in biomembranes, Annu. Rev. Biophys. Bioeng. 5 (1976) 205–238.
[70] V. Melchior, C.J. Hollingshead, D.L. Caspar, Divalent cations cooperatively stabilize close membrane contacts in myelin, Biochim. Biophys. Acta 554 (1979) 204–226.
[71] K.A. Riske, R.M. Fernandez, O.R. Nascimento, B.L. Bales, M.T. Lamy-Freund, DMPG gel–fluid thermal transition monitored by a phospholipid spin labeled at the acyl chain end, Chem. Phys. Lipids 124 (2003) 69–80.
[72] T. Silva, R. Adão, K. Nazmi, J.G. Bolscher, S.S. Funari, D. Uhríková, M. Bastos, Structural diversity and mode of action on lipid membranes of three lactoferrin candidacidal peptides, Biochim. Biophys. Acta 1828 (2013) 1329–1339.
[73] D. Jung, J.P. Powers, S.K. Straus, R.E.W. Hancock, Lipid-specific binding of the calcium-dependent antibiotic daptomycin leads to changes in lipid polymorphism of model membranes, Chem. Phys. Lipids 154 (2008) 120–128.
[74] K. Richter, H. Aschauer, G. Kreil, Biosynthesis of peptides in the skin of *Xenopus laevis*: isolation of novel peptides predicted from the sequence of cloned cDNAs, Peptides 6 (Suppl. 3) (1985) 17–21.
[75] A. Latal, G. Degovics, R.F. Epand, R.M. Epand, K. Lohner, Structural aspects of the interaction of peptidyl-glycylleucine-carboxyamide, a highly potent antimicrobial peptide from frog skin, with lipids, Eur. J. Biochem. 248 (1997) 938–946.
[76] J.P. Ulmschneider, J.C. Smith, M.B. Ulmschneider, A.S. Ulrich, E. Strandberg, Reorientation and dimerization of the membrane-bound antimicrobial peptide PGLa from microsecond all-atom MD simulations, Biophys. J. 103 (2012) 472–482.
[77] P.K. Mikhailiuk, S. Afonin, A.N. Chernega, E.B. Rusanov, M.O. Platonov, G.G. Dubinina, M. Berditsch, A.S. Ulrich, I.V. Komarov, Conformationally rigid trifluoromethyl-substituted α-amino acid designed for peptide structure analysis by solid-state ^{19}F NMR spectroscopy, Angew. Chem. Int. Ed. 45 (2006) 5659–5661.
[78] R.N. McElhaney, The use of differential scanning calorimetry and differential thermal analysis in studies of model and biological membranes, Chem. Phys. Lipids 30 (1982) 229–259.
[79] R.N. McElhaney, Differential scanning calorimetric studies of lipid–protein interactions in model membrane systems, Biochim. Biophys. Acta 864 (1986) 361–421.
[80] K. Lohner, E.J. Prenner, Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems, Biochim. Biophys. Acta 1462 (1999) 141–156.
[81] R.N. Lewis, N. Mak, R.N. McElhaney, A differential scanning calorimetric study of the thermotropic phase behavior of model membranes composed of phosphatidylcholines containing linear saturated fatty acyl chains, Biochemistry 26 (1987) 6118–6126.
[82] M.F. Brown, S. Lope-Piedrafita, G.V. Martinez, H.I. Petrache, Solid-state deuterium NMR spectroscopy of membranes, in: G.A. Webb (Ed.), Modern Magnetic Resonance, Springer, 2006, pp. 249–260.
[83] V.S. Kubyshkin, I.V. Komarov, S. Afonin, P.K. Mykhailiuk, S.L. Grage, A.S. Ulrich, Trifluoromethyl-substituted α-amino acids as solid state ^{19}F-NMR labels for structural studies of membrane-bound peptides, in: V. Gouverneur, K. Müller (Eds.), Fluorine in Pharmaceutical and Medicinal Chemistry: From Biophysical Aspects to Clinical Applications, Imperial College Press, 2012, pp. 91–138.
[84] J.-B. d'Espinose de Lacaillerie, B. Jarry, O. Pascui, D. Reichert, "Cooking the sample": radiofrequency induced heating during solid-state NMR experiments, Solid State Nucl. Magn. Reson. 28 (2005) 225–232.
[85] M. Trapp, T. Gutberlet, F. Juranyi, T. Unruh, B. Deme, M. Tehei, J. Peters, Hydration dependent studies of highly aligned multilayer lipid membranes by neutron scattering, J. Chem. Phys. 133 (2010) 164505.
[86] G.W. Seto, S. Marwaha, D.M. Kobewka, R.N. Lewis, F. Separovic, R.N. McElhaney, Interactions of the Australian tree frog antimicrobial peptides aurein 1.2, citropin 1.1 and maculatin 1.1 with lipid model membranes: differential scanning calorimetric and Fourier transform infrared spectroscopic studies, Biochim. Biophys. Acta 1768 (2007) 2787–2800.
[87] Y. Zhang, R.N. Lewis, R.N. McElhaney, R.O. Ryan, Calorimetric and spectroscopic studies of the interaction of *Manduca sexta* apolipophorin III with zwitterionic, anionic, and nonionic lipids, Biochemistry 32 (1993) 3942–3952.
[88] Y.P. Zhang, R.N. Lewis, R.S. Hodges, R.N. McElhaney, Peptide models of the helical hydrophobic transmembrane segments of membrane proteins: interactions of acetyl-K_2-$(LA)_{12}$-K_2-amide with phosphatidylethanolamine bilayer membranes, Biochemistry 40 (2001) 474–482.
[89] A. Erazo-Oliveras, N. Muthukrishnan, R. Baker, T.Y. Wang, J.P. Pellois, Improving the endosomal escape of cell-penetrating peptides and their cargos: strategies and challenges, Pharmaceuticals (Basel) 5 (2012) 1177–1209.
[90] W.F. Walkenhorst, J.W. Klein, P. Vo, W.C. Wimley, The pH dependence of microbe sterilization by cationic antimicrobial peptides: not just the usual suspects, Antimicrob. Agents Chemother. 57 (2013) 3312–3320.
[91] Y.C. Kim, S. Late, A.K. Banga, P.J. Ludovice, M.R. Prausnitz, Biochemical enhancement of transdermal delivery with magainin peptide: modification of electrostatic interactions by changing pH, Int. J. Pharm. 362 (2008) 20–28.
[92] J. Chew, P.S. Zilm, J.M. Fuss, N.J. Gully, A proteomic investigation of *Fusobacterium nucleatum* alkaline-induced biofilms, BMC Microbiol. 12 (2012) 189.
[93] K. Horikoshi, Alkaliphiles: some applications of their products for biotechnology, Microbiol. Mol. Biol. Rev. 63 (1999) 735–750.
[94] S. Vylkova, A.J. Carman, H.A. Danhof, J.R. Collette, H. Zhou, M.C. Lorenz, The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH, MBio 2 (2011) (e00055-00011).