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Aggregation and membrane permeabilizing properties of designed histidine-containing cationic linear peptide antibiotics‡

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Abstract: Members of the LAH4 family of cationic linear peptide antibiotics have been designed to form amphipathic helical structures in membrane environments and switch from alignments parallel to the bilayer surface to transmembrane orientations in a pH-dependent manner. Here the aggregation in aqueous buffer of two members of the family has been investigated by DLS. The peptides form monomers or small oligomers at pH = 5 but associate into nano-sized aggregates at physiological pH. The diameter of these latter complexes can be considerably reduced by sonication. Furthermore, the membrane interactions of the various supramolecular aggregates with POPC or mixed POPC/POPS vesicles have been investigated in calcein-release assays. In all the cases tested, the large preformed oligomeric peptide aggregates of 20–40 nm in size were more active than the structures with the smallest hydrodynamic radii in releasing the fluorescent dye from LUV. In contrast, the relative activity after sonication depends on the specific environment tested. The data suggest that these amphiphiles form micellar structures and support the notion that they can act in a manner comparable to detergents. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: polypeptide lipid interactions; peptide pore formation; dynamic light scattering; calcein release; bilayer; phospholipid membrane; peptide aggregates

INTRODUCTION

The discovery and analysis of bactericidal and fungicidal compounds has attracted considerable interest due to the necessity to keep up with the increasing resistance of pathogens against many of the commonly used antibiotics [1]. An interesting approach consists in searching for naturally occurring antimicrobial compounds produced by fungi, protista and eubacteria, and, in particular, plants and animals which are often rich sources as these organisms produce, store and secrete antibiotic peptides in exposed tissues, or synthesize such compounds upon induction [2,3]. The number of antimicrobial peptides from natural sources compiled in the Antimicrobial Sequences Database (http://www.bbcm.univ.trieste.it/~tossi/antimic.html) totals nearly 1000 and in addition, many more have been created by design [4–7].

By understanding the structure–function relationship of these peptides it is possible to design cheaper and/or more efficient analogues [7–9]. In particular linear cationic amphipathic peptides have attracted considerable interest as they can be prepared in large quantities by peptide synthetic methods or by bacterial overexpression [10,11]. Some of the best-studied members of this class are magainins, first found in amphibians, as well as cecropins, which were isolated from the pupae of the cecropia moths [2,3].

A reoccurring theme within this class of antimicrobials is the amphipathic distribution of polar and hydrophobic residues which can result in pronounced interactions of the peptides with phospholipid membranes [2,12]. Therefore, a common characteristic observed for membrane-active peptides is their capability to disturb bilayer integrity, either by creation of defects, disruption or pore formation. The resulting openings in lipid bilayers lead to the collapse of the transmembrane electrochemical gradients and, therefore, provide an explanation of the cell-killing activities of these peptides (reviewed, e.g. [12,13]). The formation of pores affects cellular respiration [14], deprives sensitive organisms of their source of energy by disrupting the electrochemical gradient across free-energy transducing membranes [15,16] and results in increased water and ion flow across the membrane concomitant with cell- swelling and osmolysis [17,18]. Alternatively, evidence has been found that for some peptides the membrane activities may not be the primary reason for cell-killing but merely serve as a means for the peptides to access the cell interior [19–22]. As a given peptide is often characterized by both cell-penetrating and

Abbreviations: DLS, dynamic light scattering; LUV, large unilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine.

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membrane-lytic activities, the ultimate killing mechanism probably depends on which of these activities is more pronounced in a given environment, and as a consequence which comes first, membrane lysis or attack of internal targets?

Interestingly, the chirality, sequence, composition and length of antimicrobial peptides can be modified to a large degree provided that the peptides retain the capacity to form amphipathic structures at the membrane interface and carry a substantial net positive charge [2,23]. It was therefore possible to extend the concept to peptides composed of β-amino acid oligomers [24,25], helical pseudo-peptides [26,27], peptoids [28], or amphipathic cationic polymers [29,30]. These observations indicate that the biological activities of such amphipathic compounds are not mediated through specific, chiral receptor interactions, but are mostly owing to direct interactions with the membranes. However, it should be noted that the possibilities of variation of charge and composition are limited as the interplay between overall charge, hydrophobicity, hydrophobic moment, hydrophobic surface and the shape of the molecule is important for selectivity as well as the degree of antibiotic activity [23,31–34].

By comparing the membrane interactions of antimicrobial peptides with those of detergents it becomes obvious that these compounds, albeit structurally different, share many properties with each other. As such molecules intercalate into the lipid bilayer interface, their charge and hydrophobic volume are important determinants of the polymorphic structures that are adopted by mixtures of amphiphiles and lipids. In particular, detergents and antimicrobial amphiphiles can have neutral or even stabilizing effects on the bilayer at low concentrations [35,36], cause transient openings when more peptides/detergents are added [37–40], and cause membrane disruption at high amphiphile-to-lipid ratios. The degree of membrane damage is determined by the peptide and lipid structure and the 'detergent-like' model has been discussed and reviewed in more detail recently [12,41].

A complete description of the peptide-membrane interactions and the resulting membrane morphologies would need to take into account a wide variety of parameters and conditions such as the peptide-to-lipid ratio, the detailed membrane lipid composition, temperature, hydration and buffer composition. In analogy to detergents this can be done by establishing phase diagrams (e.g. Figure 2 in Ref. 41). Notably, the detergent-like properties of amphipathic peptides are not in contradiction with previously suggested models which represent 'special areas' of the phase diagram where the conditions are such that this kind of supramolecular structures is observed. For example, in the 'wormhole model' the walls of the opening are lined with transmembrane peptides and lipids [42,43]. On the other hand the 'carpet model' applies when a high density of peptides assembles at the membrane surface and self-associates in a 'carpet-like' manner. When a threshold concentration of peptide is reached the membrane breaks into pieces [44]. By changing the lipid composition, temperature, or the peptide-to-lipid ratio different phases are obtained either by moving along one axis of the phase diagram or by shifting the phase boundaries. On the other hand, changing the peptide sequence causes shifts in the phase diagram which are correlated to the functional mechanisms.

In order to understand better the biological activities of linear cationic antimicrobial peptides, we have created amino acid sequences with a high propensity to form amphipathic α-helical structures in membrane environments (Table 1). The central core of these sequences is composed of leucines, alanines and four histidines, and consequently the peptides were named LAH4 [45]. The histidines allow one to manipulate the polarity and the hydrophobic moment of the helix by merely changing the pH. Indeed, a number of sequences has been created which are oriented along the membrane surface at pH <6, when the histidines carry positive charges, and adopt transmembrane alignments at neutral pH [45–47]. Interestingly, the peptides exhibit increased antimicrobial activities at low pH when compared to neutral environments [5,7]. The pH-dependent transition in charge and amphipathic properties play an important role also for the second known biological activity of this peptide family, namely, the transfection of DNA or RNA into eukaryotic cells [48–50].

During the design of the first generation of antimicrobial LAH4 peptides the primary goal was to equilibrate the hydrophobic, polar and charge interactions in such a manner to create an amphipathic helix with tunable membrane topology [45,51]. Although at this point the

Table 1 Sequences and nominal charges of LAH4 and LAH4-L1 helical peptides

Peptide	Sequence	Peptide length	Nominal charge at pH 7.4	Nominal charge at pH 5
LAH4	KKALLALALHHLAHLALHLALALKKA	26	+5	+9
LAH4-L1	KKALLAHALHLLALLALHLAHALKKA	26	+5	+9

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biological activities were of secondary consideration, the peptide turned out to exhibit antibacterial activities which were even more pronounced than those of magainin 2 at alignments of the LAH4 helix parallel to the membrane surface [5,7]. When located within the membrane interphase, amphipathic helical peptides exhibit effects on the lipid bilayer structure which are similar to those observed with cone- or wedge-shaped molecules [41]. Intercalation of peptides into the membrane interface therefore creates voids in the hydrophobic membrane region and, in addition, imposes curvature strain on the lipid bilayer.

The comparison of cationic amphipathic peptides with detergents implies the potential organization of the peptides into micelles in aqueous solution above a 'critical micelle concentration'. Here, we therefore investigated the aggregation characteristics of two different LAH4 peptides in a pH-dependent manner using DLS and correlated the membrane permeabilizing activities to their supramolecular structure formed under varying conditions. The peptides chosen as LAH4 is the parent peptide of the family and has been studied extensively and compared to the isomeric LAH4-L1 which has a slightly reduced hydrophilic angle and has similar antimicrobial and membrane disruptive properties in certain model membranes but is more active in transfection assays suggesting potential differences in properties depending on the membrane being challenged [7,52].

MATERIALS AND METHODS

Peptide and Lipids

LAH4 and LAH4-L1 peptides (Table 1) were synthesized using standard FMOC solid-state chemistry on a Millipore 9050 synthesizer. In crude peptide preparations a predominant peak was observed when analysed by HPLC with acetonitrile/water gradients. During HPLC purification the main peak was collected and the identity of the product confirmed by MALDI mass spectrometry. The lipids POPC and POPS were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification.

LUV Preparation

LUVs loaded with calcein were prepared by mechanical extrusion. Two lipid solutions, POPC and a mixture of POPC/POPS (3 : 1), were dissolved separately in chloroform/methanol. The solutions were dried and then hydrated in buffer (phosphate buffer : 50 mM, pH = 7.4, and acetate buffer : 50 mM, pH = 5) complemented with 50 mM of calcein disodium salt (Fluka, Switzerland) before undergoing several freeze-thaw cycles and then extrusion (11 times) through membranes with pores of 200 nm diameter (Avestin, Canada). The dye outside the calcein-loaded vesicles was removed by gel filtration through a Sephadex G-50 column (2.5 × 3.5 mm) (Sigma, USA) equilibrated with the adequate buffer (phosphate or acetate) that has been supplemented with 75 mM NaCl in order to compensate for the change in osmolarity induced by the presence of calcein and its sodium counter ions. During gel filtration the membranes were diluted by about seven-fold. However, the relative concentrations of the LUV suspensions eluting from the column could be compared by measuring the dye release using fluorescence spectroscopy.

DLS Measurement

DLS measurements were performed on a Zetasizer Nano-S system (Malvern Instruments, Worcestershire, UK). The samples were contained in 50 µl plastic cuvettes (trUView, Biorad, CA) and were placed in a thermally jacketed sample holder at 25 °C. All measurements were performed at a peptide concentration of 1 mg/ml and by using the standard automated measurement procedure proposed by Malvern. Prior to each measurement the calibration of the apparatus was verified by measuring the size of 60 nm polymer microspheres suspended in water (Duke Scientific, Palo Alto, CA). The intensity size distributions were obtained from the analysis of the correlation functions using a multiple narrow modes algorithm (Malvern DTS software) based upon a non-negative least squares fit [53]. The distribution of the number of particles was deduced and permitted to reject the bigger size intensity peaks that were considered as measurements or calculations artefacts.

Fluorescence Measurement

Calcein efflux measurements were performed on a Fluorolog 3–22 spectrometer (HORIBA Jobin-Yvon, Longjumeau, France). In a typical experiment, an aliquot of the LUV solution was added to 1.5 ml of buffer in a quartz cuvette and equilibrated for some minutes at 30 °C inside the spectrometer. To start calcein release 1–2.5 µl of peptide solution (1 mg/ml) was added to the cuvette while the sample was excited at $\lambda_{exc} = 480$ nm and the intensity of fluorescence (I) was recorded at $\lambda_{fluo.} = 515$ nm for about 10 min. A limited bandwidth ($\Delta\lambda < 1.2$ nm) was used for both excitation and emission. The percentage of calcein released from the vesicles ($I_{\%}$) was calculated according to the formula : $I_{\%} = 100 \cdot (I - I_0)/(I_{Max} - I_0)$, where I_0 represents the intensity of fluorescence before adding the peptide to the solution and I_{Max} is the maximum intensity observed after fully disrupting the vesicle with 10 µl of 10% Triton X-100. Care was taken to maintain constant I_{Max} in order to allow quantitative comparison between the multiple recordings.

RESULTS

In order to monitor the aggregation behaviour of the LAH4 peptides in aqueous buffers and to correlate the size of the oligomers to the membrane-disruptive properties of the sequences, their size was investigated in solution as a function of pH by DLS (Figure 1). The distribution of hydrodynamic diameters indicate a clear difference between the aggregation states in citrate buffer at pH 5 and phosphate buffer at pH 7.4. At pH 5 the peptides adopt a random coil conformation

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[5] and exhibit a distribution of apparent diameters of a few nanometers (Figure 1). This corresponds to the size of small oligomers, under the assumption of a globular shape, but when considering their random coil structure, [5] is more likely an extended monomer, in particular when considering the difficulties of detecting small structures using this technique. The size of the structures formed by LAH4 is, within experimental error, identical to that of LAH4-L1.

When the pH is increased to neutral the hydrodynamic radius of the aggregates formed by both peptides augments by an order of magnitude, which in the case of soft matter, such as polymers and vesicles, corresponds to about two orders of magnitude increase in number of subunits [54]. Notably, at pH = 7.4 the peptides have been shown to adopt predominantly helical conformations [5,50] and the assumption of a globular structure of the complexes seems an appropriate choice. Notably, even at this pH, the diameters of the peptide aggregates are considerably smaller than the wavelength of visible light, therefore, the samples appear transparent when inspected by eye. When the solutions, prepared at neutral pH, are further processed with a bath sonicator, structures of intermediate size are obtained.

When the LAH4 preparation at pH 7.4 was diluted in a stepwise manner and the scattering of light-monitored aggregates were detected even at a concentration as low as 8 μg/ml, thereby approaching the detection limit of the instrument (not shown).

When the peptide solutions prepared at pH = 5 were injected into the buffer at pH = 7.4 and vice versa the initial aggregate structures quickly adapted to the new environment. Unfortunately the time resolution of the DLS experiment is in the range of 5 min and therefore it was not possible to establish kinetic rate constants of these transitions.

In order to test how peptide aggregation influences the capacity of the LAH4 peptides to form membrane openings, fluorescence dye-release experiments were performed using members of the family of LAH4 peptides when prepared as various supramolecular structures, namely small oligomers at pH = 5, small sonicated aggregates at pH = 7.4 or 20–40 nm sized peptide aggregates at pH = 7.4. In a first series of experiments the peptides were injected into identical solutions of calcein loaded POPC/POPS 3 : 1 vesicles of 200 nm size at pH = 5. The same experiment was repeated five times and two sets of experiments where the calcein release is monitored as a function of time are shown in Figure 2. The dye release observed from one experiment to the other is highly reproducible when either large aggregates of LAH4-L1 or small mono- or oligomeric structures of LAH4-L1 are injected into the vesicle suspension. The differences reflect stochastic variations in the quantity of injected solutions carrying the peptides and/or the vesicles. When similar amounts of buffer without peptides (2.5 μl to 1.5 ml) were added to calcein-loaded vesicles, the intensity of fluorescence remained constant.

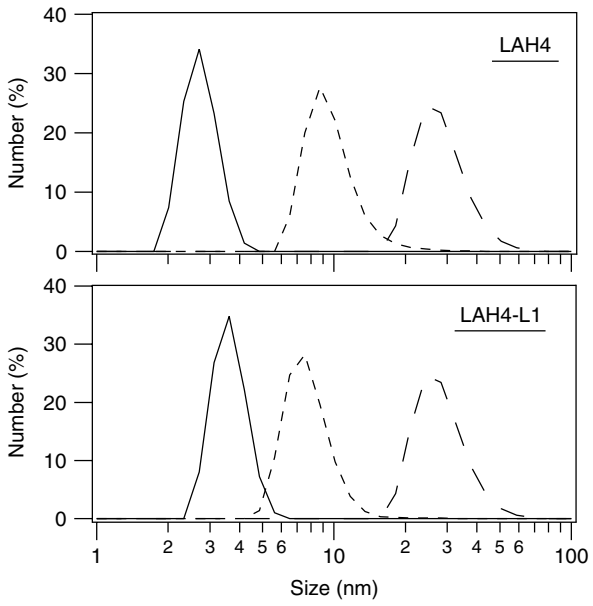

Figure 1 The hydrodynamic diameters of LAH4 (top) and LAH4-L1 (bottom) as measured by DLS. The average size distributions number displayed in each panel correspond to sample solutions where the peptides have been hydrated with either 50 mM citrate buffer, pH = 5 (solid line), with 10 mM phosphate buffer pH = 7.4 (long-dashed line), or with phosphate buffer followed by 40 s of sonication (short-dashed line). The width at half-height of the three size distribution functions displayed in each panel of the figure are 2, 4 and 15 nm, from left to right, respectively.

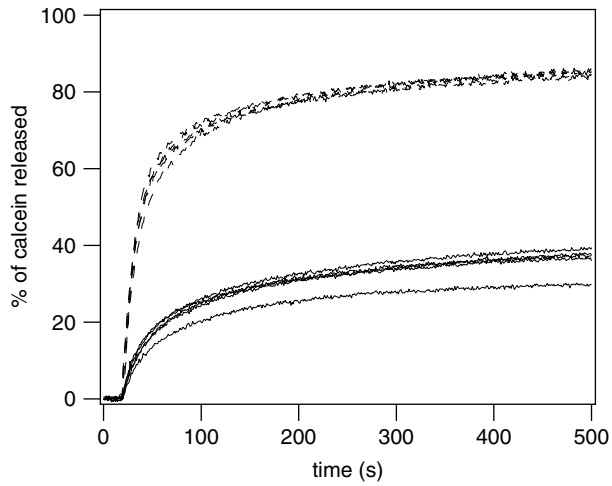

Figure 2 Calcein release from POPC/POPS (3 : 1) vesicles induced by LAH4-L1 peptide when added from citrate buffer at pH = 5 (solid lines) or phosphate buffer at pH = 7.4 (dashed lines). A series of ten recordings is displayed to show the good reproducibility of the measurements and to estimate the errors of the experiment. The lipid concentrations were 60 μM and the peptide-to-lipid ratios 1/100. The statistical error of these measurements is estimated <5%.

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In a next step LAH4 (Figure 3) or LAH4-L1 (Figure 4) in three different supramolecular assemblies were added to calcein-loaded LUVs which were either formed from POPC alone (panels *A*, *C*) or from mixtures of POPC/POPS 3 : 1 (panels *B*, *D*). Each of the experiments was performed in duplicate at pH = 5 (panels *A*, *B*) and at pH = 7.4 (panels *C*, *D*). In order to evaluate the membrane activity of the peptides the dye release kinetics or the amount of calcein released when the curves start to level off at about 5–10 min were considered. Clearly, under all conditions tested, the membrane-disruptive activities of both peptides are more pronounced when added in the form of large aggregates compared to the small structures formed at pH = 5.

Sonication of the LAH4 aggregates formed at pH = 7.4 results in an enhancement of pore-forming activity when injected to vesicles at neutral pH, but a decrease of activity even below that of the 'monomeric' peptides when injected to calcein loaded vesicles at pH = 5 (Figure 3). In contrast, sonication of the LAH4-L1 aggregates results in permeabilizing activities that are, at neutral pH, similar to or increased when compared to those of the large aggregates, and at acidic pH closely follow those observed for the large oligomers (Figure 4).

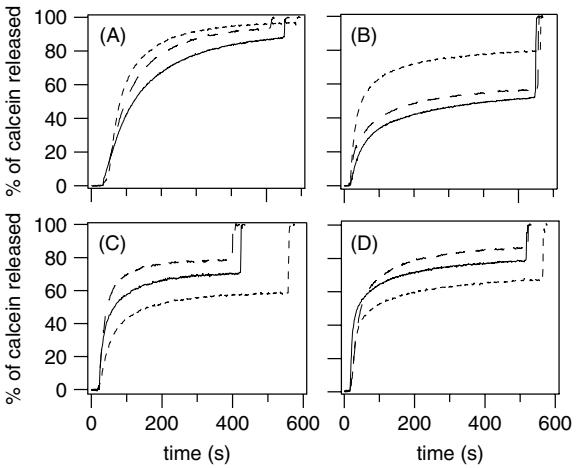

Figure 3 The release of calcein from large unilamellar vesicles (LUV) after addition of LAH4 is monitored as a function of pH and lipid bilayer composition. (A) and (B) are recorded at pH = 7.4, (C) and (D) at pH = 5.0. The vesicles are made from POPC (A, C) or POPC/POPS (B, D). In each frame the fluorescence intensity is shown as a function of time after addition of LAH4 in citrate buffer, pH = 5 (solid line), in phosphate buffer, pH = 7.4 (long-dashed line) or in phosphate buffer followed by 40 s of sonication (short-dashed line). The lipid concentration was 60 μM. The final peptide-to-lipid ratios were 1/100 except for panels (A) and (D) where a ratio of 1/250 was chosen to obtain similar fluorescence intensities.

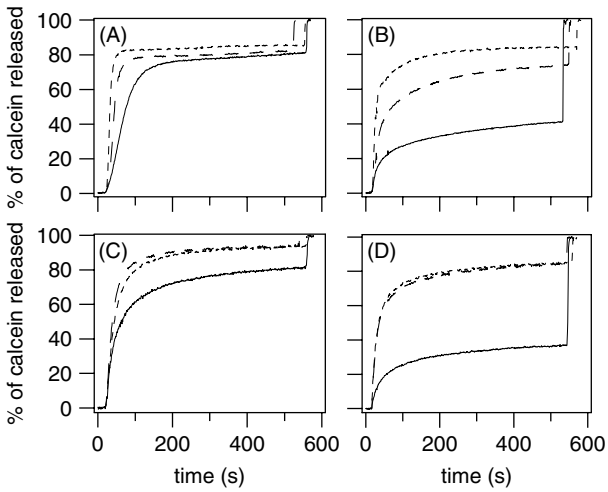

Figure 4 The release of calcein from large unilamellar vesicles (LUV) after addition of LAH4-L1 is monitored as a function of pH and of lipid bilayer composition. (A) and (B) are recorded at pH = 7.4, (C) and (D) at pH = 5.0. The vesicles are made from POPC (A, C) or POPC/POPS (B, D). In each frame the fluorescence intensity is shown as a function of time after addition of LAH4 in citrate buffer, pH = 5 (solid line), in phosphate buffer, pH = 7.4 (long-dashed line) or in phosphate buffer followed by 40 s of sonication (short-dashed line). The lipid concentration was 60 μM and the peptide-to-lipid ratio 1/100.

DISCUSSION

In the present study we show that not only the membrane alignment and interactions but also the organization state of the LAH4 peptides in aqueous solution are strongly dependent on pH. The peptides are monomeric or form small oligomers at acidic pH when the histidines of the central core of the sequence are protonated and the net overall charge of these sequences reaches +9. In contrast, they associate into aggregates of helical peptides 20–40 nm in size in aqueous buffer at neutral pH. In the following, we refer to these structures as 'peptide micelles', although clearly more work is needed to better define their properties. These peptide aggregates might either prevent or favour channel formation depending on how the membrane is permeabilized. On the one hand, they might encounter the bilayer and carry away a few lipid molecules thereby forming transient openings [12]. Furthermore, such 'peptide micelles' could insert into the membrane and form structures that resemble pores without, however, being well-defined in shape or size [12,55]. On the other hand, it remains possible that only mono- or small oligomers can insert efficiently in the membrane, where the peptides may form pores. Whereas in the former case pre-existing macroscopic structures would favour membrane permeabilization, aggregation in solution would reduce the number of active molecules in the latter case. The formation of aggregates has also been observed for melittin [56], *Staphylococcus* δ-toxin [57], dermaseptin S4 [58] and trichigonin GA VI [59] which suggest that similar considerations apply to the wider range of cationic linear peptides.

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When the LAH4 peptide 'monomers' or aggregates were added to calcein loaded vesicles they interact with these bilayers and permeabilize the membranes. Interestingly the calcein release is faster and more pronounced when preformed aggregates 20–40 nm in diameter are added when compared to monomers. This observation disfavours models, where membrane permeabilization has to follow a well-defined path with monomers interacting with the membrane, followed by membrane-partitioning and insertion, possible oligomerization and finally pore formation. In contrast, the preformed aggregates seem to directly interact with the bilayer and cause openings large enough for calcein to escape. Changing the size and the structure of the aggregates by sonication can shift the activity profile in either direction thereby suggesting that for each of the experimental conditions an optimal size of the aggregate exists which facilitates membrane interactions and the formation of openings.

In a related manner it has been demonstrated that membrane perturbation by the lipopeptaibol trichogin GA IV is due to the membrane interactions of peptide aggregates rather than monomers [59]. Furthermore, the ability to self-associate in aqueous medium may be important for target cell selectivity [58,60]. Whereas covalently linked pentameric bundles of cationic peptides expressed similarly potent antifungal, antimicrobial and haemolytic activities, regardless of the length of the peptide chains, the monomeric peptides showed length dependent antimicrobial activities and were devoid of haemolytic activity [61].

Previously we have shown that the LAH4 family of peptides exhibits a higher antimicrobial activity at acidic conditions when compared to neutral pH [5,7]. Notably, the peptides are aligned parallel to the membrane when most active and transmembrane at physiological pH [45,62]. It was therefore concluded that the membrane interaction and concomitantly antibiotic activity of the peptides could be described as 'detergent-like' behaviour rather than being due to formation of transmembrane helical bundles [5,12]. Furthermore, when interacting with membranes, the peptides induce fatty acyl chain disordering, packing defects and segregation of domains enriched in acidic phospholipids [52,63], the extent of which can be related to pore formation [64].

When the pH-dependence of the pore-forming activities of LAH4-L1 and LAH4 on model membranes (Figures 3 and 4) is compared to their antimicrobial activities, the differences due to pH are in general more pronounced when their antibiotic activities are investigated in either neutral or acidic environments [5,7]. This may be owing to the differences in the diffusion of complexes of different size and charge through the outer complex barriers of the cells and therefore the availability of these peptides at the level of the cell membrane and the cell cytoplasm [58,65]. Indeed, it has been shown that particle size is an important parameter since antimicrobial peptides that do not form oligomers in the presence of lipopolysaccharides have greater success in translocating across the outer membrane of the target bacterium [66,67]. In addition, mechanisms other than membrane-interactions are also important for the antibiotic activity of antimicrobial peptides [19–22]. It should also be taken into consideration that control experiments (not shown) have demonstrated that the life time of 'monomers' when injected into solutions at pH = 7.4 or of peptide oligomers when injected into acidic environments is shorter than the duration of the calcein release experiment and therefore, in these cases, the experimentally monitored differences between preparations are less apparent.

Nevertheless, we can conclude that the occurrence of cationic linear peptides in the form of aggregates and the high membrane permeabilizing activities of these 'micelles' provide a further parallel between the membrane-activities of detergents and cationic antimicrobial peptides. Notably, the description and analysis of the biological activities of these compounds is not only governed by the complex interactions with the membranes but also by their aggregation state in aqueous solution and that understanding and controlling the aggregation state of antimicrobial peptides may be an important consideration when evaluating and designing antimicrobial peptides with improved therapeutic capabilities.

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