



Investigating the lytic activity and structural properties of *Staphylococcus aureus* phenol soluble modulins (PSM) peptide toxins



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ABSTRACT

The ubiquitous bacterial pathogen, *Staphylococcus aureus*, expresses a large arsenal of virulence factors essential for pathogenesis. The phenol-soluble modulins (PSMs) are a family of cytolytic peptide toxins which have multiple roles in staphylococcal virulence. To gain an insight into which specific factors are important in PSM-mediated cell membrane disruption, the lytic activity of individual PSM peptides against phospholipid vesicles and T cells was investigated. Vesicles were most susceptible to lysis by the PSM α subclass of peptides (α 1–3 in particular), when containing between 10 and 30 mol% cholesterol, which for these vesicles is the mixed solid ordered (s_o)–liquid ordered (l_o) phase. Our results show that the PSM β class of peptides has little effect on vesicles at concentrations comparable to that of the PSM α class and exhibited no cytotoxicity. Furthermore, within the PSM α class, differences emerged with PSM α 4 showing decreased vesicle and cytotoxic activity in comparison to its counterparts, in contrast to previous studies. In order to understand this, peptides were studied using helical wheel projections and circular dichroism measurements. The degree of amphipathicity, alpha-helicity and properties such as charge and hydrophobicity were calculated, allowing a structure–function relationship to be inferred. The degree of alpha-helicity of the peptides was the single most important property of the seven peptides studied in predicting their lytic activity. These results help to redefine this class of peptide toxins and also highlight certain membrane parameters required for efficient lysis.

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1. Introduction

Staphylococcus aureus is an extremely versatile opportunistic pathogen, capable of causing a wide range of human and animal diseases [1,2]. This bacterium has a huge arsenal of virulence factors and has demonstrated high levels of multi-drug resistance. This has resulted in *S. aureus* becoming one of the most prominent causes of nosocomial infection [3,4], with previous studies suggesting that *S. aureus* contributes to a higher mortality than HIV/AIDS in the US [5]. *S. aureus* infections also cause a massive economic burden, resulting in approximately US\$570 million in additional hospital costs in the European Union [6]. Historically, methicillin-resistant *S. aureus* (MRSA) strains were restricted to the hospital and as such labeled health care-associated (HA-) MRSA, generally infecting immune-compromised patients. New MRSA strains have emerged over the last few decades that are able to infect healthy people; these community-associated (CA-) MRSA strains [7], have in their arsenal, both antibiotic resistance and also enhanced virulence and fitness [8].

The rationale for this study has arisen from previous work whereby phospholipid vesicles have been used as reporters for the presence of bacterial virulence factors, with the ultimate aim of developing an integrated sensor wound dressing [9,10]. Self-quenched fluorescein encapsulated within vesicles is released on contact with bilayer active toxins. Extensive work has been carried out using genetically engineered *S. aureus* strains to understand the interaction between cytolytic secreted components from this bacteria and the developed vesicle system [11].

Genomic and proteomic analysis of the toxins secreted by various CA-MRSA strains identified a class of peptides which are highly cytotoxic to host neutrophils [12]. These phenol-soluble modulins (PSMs) are generally classified as small, surfactant-like, alpha-helical peptides. They can be categorized into two groups; the alpha class consisting of four peptides of ~20 amino acids and the beta group comprising two longer ~40 amino acid peptides (Table 2). As well as being cytotoxic [12,13] these peptides are important in biofilm formation [14], exhibit antimicrobial properties [15] and have strong pro-inflammatory properties [16]. Interestingly, recent work has established the role of PSM α peptides in mediating phagosomal escape of both professional and non-professional phagocytes in clinically important *S. aureus* strains [17]. This survival within specific cells is an efficient method of immune evasion and, when in migrating immune cells, dissemination [17]. Importantly, deletion of the *psm* α operon leads to attenuation in a

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mouse infection model and a reduction in skin lesions, highlighting their central role in pathogenesis [12].

The PSM peptides are inter-related and share similar physicochemical and structural properties to the *S. aureus* delta toxin [12]. The mechanism of action of delta toxin has been investigated using 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) containing vesicles with specific ratios of cholesterol and sphingomyelin as a function of POPC [18]. The mechanism of membrane lysis by delta toxin involves binding and accumulation within liquid disordered lipid domains [18]. It is believed that delta toxin functions through association of the hydrophobic face of peptide molecules with the bilayer interior of cell membranes. Subsequent accumulation of the peptide in the membrane increases the bending modulus and allows for the formation of membrane discs which are bound by delta toxin to prevent interaction between the aqueous environment and the bilayer hydrophobic core. Concurrently, pores are generated in the membrane which leads to rapid osmotic destabilization of the cell [19]. A recent study investigating PSM interaction with POPC vesicles, demonstrated that all PSM peptides lyse such vesicles [20], importantly even those that show no cytolytic activities to eukaryotic cells, such as the PSM β 1 and β 2 peptides and the PSM α 4 [12]. It was also reported that all PSM peptides exhibited quite similar alpha-helical properties demonstrated by circular dichroism [12].

In the study presented here, PSM–lipid membrane interactions were studied, using phospholipid vesicles containing varying concentrations of cholesterol, encapsulating a self-quenched fluorescent dye, carboxy-fluorescein (CF). The effective concentration of peptide required to lyse 50% of vesicles, EC_{50} , was measured by quantifying fluorescence change as a function of PSM concentration using a dose response curve fitting to the Hill equation. Finally, experiments designed to investigate the structural properties of the PSMs were undertaken. Here helical wheel projections were generated to gain an understanding of differences in structural properties between these groups. While all PSMs displayed amphipathicity, which is vital for membrane lysis, we hypothesize that differences in charge, hydrophobicity and most importantly alpha-helical secondary structure explains the lytic differences between these related peptide toxins.

2. Materials and methods

2.1. Phospholipid vesicle formulation

The vesicles used in this study all utilize a photo-polymerized fatty acid, 10, 12-tricosadiynoic acid (TCDA) component, which is believed to create specific phase domains separate from the phospholipids. The vesicles can be thought of as hollow spheres consisting of a 'hard' shell of TCDA with pores containing 1, 2-dipalmitoyl-*sn*-glycero-phosphocholine (DPPC), cholesterol and a small fraction of 1, 2-dipalmitoyl-*sn*-glycero-phosphoethanolamine (DPPE) in a more fluid domain. Cholesterol concentration is expressed throughout as total mol% cholesterol, including the TCDA component, which will underestimate cholesterol concentration in the fluid lipid phase. Table 1 gives estimates of cholesterol in the fluid DPPC / DPPE phase.

Vesicles were formulated using 100 $\mu\text{mol dm}^{-3}$ stocks of DPPC, DPPE, TCDA, and cholesterol in chloroform. Different vesicle types with differing cholesterol compositions were synthesized using molar ratios shown in Table 1. Following solvent evaporation, lipid films were rehydrated using 5(6)-carboxyfluorescein (Sigma) buffer (50 $\mu\text{mol dm}^{-3}$), freeze thawed three times using liquid nitrogen and extruded at 60 °C through a 100 nm membrane (LiposoFast). Vesicles were further purified through a NAP-25 column (GE Healthcare) kept at 4 °C overnight and the incorporated TCDA was polymerized using a UV-crosslinker (UVP CL1000) at an energy of 1600 $\mu\text{J cm}^{-2}$ with the major component at 254 nm.

Vesicles remain stable in these conditions for a period of up to 20 days [21]. Size distribution and concentration of vesicle was measured via

Table 1

Vesicle types and composition.

Vesicle Composition (% molar ratio)	mol% cholesterol in fluid lipid domain
73% DPPC; 2% DPPE; 0% cholesterol; 25% TCDA	0
63% DPPC; 2% DPPE; 10% cholesterol; 25% TCDA	13.3%
53% DPPC; 2% DPPE; 20% cholesterol; 25% TCDA	26.7%
43% DPPC; 2% DPPE; 30% cholesterol; 25% TCDA	40%
33% DPPC; 2% DPPE; 40% cholesterol; 25% TCDA	53%
23% DPPC; 2% DPPE; 50% cholesterol; 25% TCDA	66.7%

Abbreviations: 1,2-dipalmitoyl-*sn*-glycero-phosphocholine, DPPC; 1,2-dipalmitoyl-*sn*-glycero-phosphoethanolamine, DPPE; 10, 12-tricosadiynoic acid, TCDA.

dynamic light scattering (Malvern) and nanosight tracking analysis (Nanosight Ltd) respectively, producing a size distribution of 90–110 nm and concentration of 1×10^8 particles μL^{-1} . Phospholipids and cholesterol were ordered from Avanti Polar Lipids.

2.2. Determining the lytic activity and EC_{50} of synthetic PSMs

The parameter for measuring the fluorescence intensity of lysed vesicles was set at excitation and emission wavelengths of 485–520 nm respectively and a gain of 650 using a FLUOstar fluorometer (BMG labtech). 50 μL of vesicle solution was incubated with 150 μL of individual synthetic PSMs (95% purity, Severn Biosciences) dissolved in PBS to a final concentration ranging from 10 $\mu\text{mol dm}^{-3}$ to 0.1 $\mu\text{mol dm}^{-3}$ (PSM α 1–4 and δ -toxin) and 50 $\mu\text{mol dm}^{-3}$ to 0.1 $\mu\text{mol dm}^{-3}$ (PSM β 1–2) for up to 1 h at 37 °C, with individual measurements taken in 1 min intervals. Each experiment was performed in triplicate, three times using different batches of vesicles in each experiment. Both positive (0.1% triton X-100) and negative (Hepes buffer) controls were used in fluorescence normalization. The effective concentration of specific synthetic peptides or bacterial supernatant required to generate 50% of the maximum possible vesicle lysis, (EC_{50}), within an individual experiment was determined by observing the fluorescence response of vesicles to each toxin concentration. Normalized fluorescence (NFI) values from 60 min were plotted against toxin concentration for each toxin. Each data set was fitted to the Hill Eq. (1):

$$NFI = \frac{[toxin]^n}{EC_{50}^n + [toxin]^n} \quad (1)$$

where EC_{50} = concentration at half max response and n = stoichiometric factor. Fitting was performed using Origin. The only fit parameter applied to the data was that the fit origin must not pass below 0.

2.3. Assessing the lytic response of vesicles to bacterial supernatant

S. aureus strain LAC (USA 300, CA-MRSA) [22] was routinely stored at –80 °C in 15% glycerol/broth stocks until required. This strain was maintained in tryptic soy (TS) media (Fluka). A single colony was incubated in 5 ml of TS broth (TSB) incubated for 18 h at 37 °C with shaking at 180 rpm. Bacterial supernatants were harvested from 1 ml of 18 h culture after centrifugation for 10 min at 14,000 g using a bench top centrifuge. The filtered supernatant was diluted with fresh TSB to gain a series of supernatant concentrations ranging from 100% to 1%. Experiments were done in triplicate, three times using the same protocol and parameters as above.

2.4. Cell toxicity assay

The immortalized T-cell line (T2 cells [23]) were grown in T75 tissue culture flasks (Corning) containing RPMI 1640 cell culture media (Gibco) containing 10% heat-inactivated fetal bovine serum, 1 $\mu\text{mol dm}^{-3}$ of L-glutamine, 200 units mL^{-1} of penicillin and 0.1 mg mL^{-1} of streptomycin, at 37 °C in a humidified incubator with

5% CO₂ in air. Cells were routinely viewed microscopically and split every 48–60 h. Cells were harvested by centrifugation at room temperature for 5 min at 500 g, gently washed and resuspended in tissue-culture grade phosphate buffered saline (PBS) (Invitrogen) to a final concentration of $1\text{--}1.5 \times 10^6$ cells mL⁻¹. This procedure typically yielded > 95% viability of cells, as determined by trypan blue exclusion using 0.4% trypan blue solution (Sigma). Individual PSMs were diluted to different concentrations in PBS and incubated at a 1:1 ratio with cells for 12 min at 37 °C. The number of cells lysed was determined by trypan blue exclusion and enumerated using a Fast-Read counting chamber (Immune System LTD) and the percentage viability calculated. All experiments were done in duplicate, three times and error represents the 95% confidence interval.

2.5. Circular dichroism measurement and helical wheel modeling

The structures of synthetic PSM peptides were analyzed by circular dichroism (CD) using a Chirascan spectrometer (Applied Photophysics) and a path length of 0.2 cm. Solutions of PSM peptides were prepared in 1 ml PBS at concentrations between 30 and 60 $\mu\text{mol dm}^{-3}$. Due to the hydrophobic nature of the PSM β 1 and 2 peptides, they were dissolved in 10 μL DMSO (final concentration 0.05%) initially and then to the required concentration with PBS. This buffer was also used in subsequent studies with these respective peptides. PSM beta peptides were also dissolved in 50% trifluoroethanol (TFE) (Sigma) to induce secondary structure. The experiments with the PSM β peptides in 50% TFE were conducted using a 0.5 mm cuvette. Measurements were converted to mean residual molar ellipticity (θ) and were performed in triplicate and the resulting scans were averaged, smoothed, and the buffer signal was subtracted. Helical wheel projections were constructed using helical wheel analysis software (<http://rslab.ucr.edu/>) and the primary amino acid sequence of the PSMs in Table 2.

3. Results and discussion

3.1. Investigation of the lytic activity of PSMs

The initial experiments reported here were designed to understand the concentration range required to lyse the 20 mol% cholesterol DPPC vesicles, quantified in terms of the respective PSM EC₅₀ values as shown in Fig. 1 (A–D). TCDA was added to stabilize the vesicles, as this molecule polymerizes within the membrane following UV exposure [10]. Fig. 1 (A–C) illustrates the normalized fluorescence (NFI) response to the PSMs over a concentration range of 0.1–10 $\mu\text{mol dm}^{-3}$ (or in the case of the β peptides 0.1–50 $\mu\text{mol dm}^{-3}$), following 60 min of incubation. The maximum fluorescence value for the PSM α 1, 2 and 3 peptides and delta toxin was between 0.8 and 0.95 NFI units, which was established with a toxin range of 2–4 $\mu\text{mol dm}^{-3}$, whereas PSM α 4 required 6 $\mu\text{mol dm}^{-3}$ to achieve 0.7 NFI units. The PSM β 1 and β 2 peptides were much less active (Fig. 1C), with an order of magnitude greater concentration (50 $\mu\text{mol dm}^{-3}$) of peptide required to reach a maximum normalized fluorescence of 0.95 (PSM β 1) and 0.8 (PSM β 2).

Fig. 1D shows that PSM α 2 and PSM α 3 have the greatest lytic activity to the vesicles, followed by PSM α 1 and delta toxin. The PSM β 1–2 toxins have an EC₅₀ almost an order of magnitude lower than the PSM α 1–3. Furthermore a significant increase between EC₅₀ values of these PSM α 1–3 peptides and PSM α 4 ($p = 0.014$) was observed. These results are in contrast with the work of Duong et al., where rapid lysis of POPC vesicles after exposure to 0.5 and 1 $\mu\text{mol dm}^{-3}$ PSM β 1, PSM β 2 and PSM α 4 was observed [20]. Our results are consistent with the PSM pattern in lytic activity observed with eukaryotic cells (Fig. 5), with PSM α 1–3 & delta toxin > PSM α 4 > PSM β 1–2.

3.2. Effect of cholesterol variation on PSM mediated vesicle lysis

The effect of membrane cholesterol concentration on the activity of the various peptide toxins are shown in Figs. 2 and 3. Fig. 2 shows the peptide concentration–response curves and fit from the Hill Eq. (1) for each peptide and cholesterol concentrations between 0 and 50 mol%; Fig. 3 is a histogram of the EC₅₀ values (where able to be reasonably estimated) for obtained from the Hill fit of data in Fig. 2. Certain trends become clear: The general peptide activity profile seen for 20 mol% cholesterol case discussed in Fig. 1 is seen for virtually all cholesterol concentrations i.e. PSM α 1–3 & δ toxin > PSM α 4 > PSM β 1–2. Trends in the effect of variations of lower concentrations of cholesterol are less clear, but by viewing the EC₅₀ values (Fig. 3) estimated from a Hill fit of data in Fig. 2, it can be seen that having some cholesterol in the membrane (between 10 and 30 mol%) increases the lytic activity of all the peptides except δ toxin, which is insensitive to membrane cholesterol concentration between 0 and 40% but totally inhibited at 50% cholesterol.

The dose response curves for both the PSM β peptides in Fig. 2 (F–G) show that the lytic function of the peptide is effectively inhibited by membrane cholesterol concentrations of 40 and 50 mol%. An EC₅₀ value was not determined for the activity of the PSM β 1 and PSM β 2 against 40 and 50 mol% cholesterol, due to a low response and poor fit to the Hill equation.

Studies by other groups of PSM mediated POPC vesicle lysis showed a reverse lytic pattern to that observed using the DPPC vesicles in this study, with PSM α 4 and PSM β 1 and β 2 highlighted as the most efficient at vesicle lysis [20]. We hypothesize that the effect of cholesterol in the membrane and the high phase transition temperature of vesicles without, or with low concentrations of cholesterol mean that the DPPC vesicles respond quite differently to POPC vesicles, which contain unsaturated fatty acid tails and much lower phase transition temperatures.

The variation in the response of vesicles to PSMs with change in cholesterol concentration may have a biological context. As previously demonstrated, PSMs are active against numerous cell types in numerous organisms, which will have varying and dynamic bilayer compositions. For example, neutrophils of adult rats have been demonstrated to have a cholesterol to phospholipid ratio of 0.011 [24], while erythrocytes from adult humans give a ratio of 0.59 [25]. This variation in the PSMs capacity to lyse membranes of varying cholesterol concentration may indicate a mechanism employed by *S. aureus* to selectively lyse a variety of cell types dependent on the membrane composition,

Table 2

Amino acid sequence of peptide toxins and respective hydropathicity and charge values.

PSM peptide	Amino acid sequence	Hydropathicity	Charge
Delta	fMAQDIISTISDLVKWIIDTVNKFTHK (26)	0.135	0
PSM α 1	fMGIIAGIIKVIKSLIEQFTGK (21)	0.957	+2
PSM α 2	fMGIIAGIIKFIKGLIEKFTGK (21)	0.890	+3
PSM α 3	fMEFVAKLFKFFKDLLGKFLGNN (22)	0.305	+2
PSM α 4	fMAIVGTIIKIIKAIIDIFAK (20)	1.700	+2
PSM β 1	fMEGLFNAIKDVTVAIINNDGAKLGTISIVSIVENGVLGKLGFG (44)	0.570	–1
PSM β 2	fMTGLAEAIANTVQAQQHDSVKLGTSIVDIVANGVLLGKLGFG (44)	0.607	0

Primary sequence of delta and PSMs toxins, all of which were N-formylated.

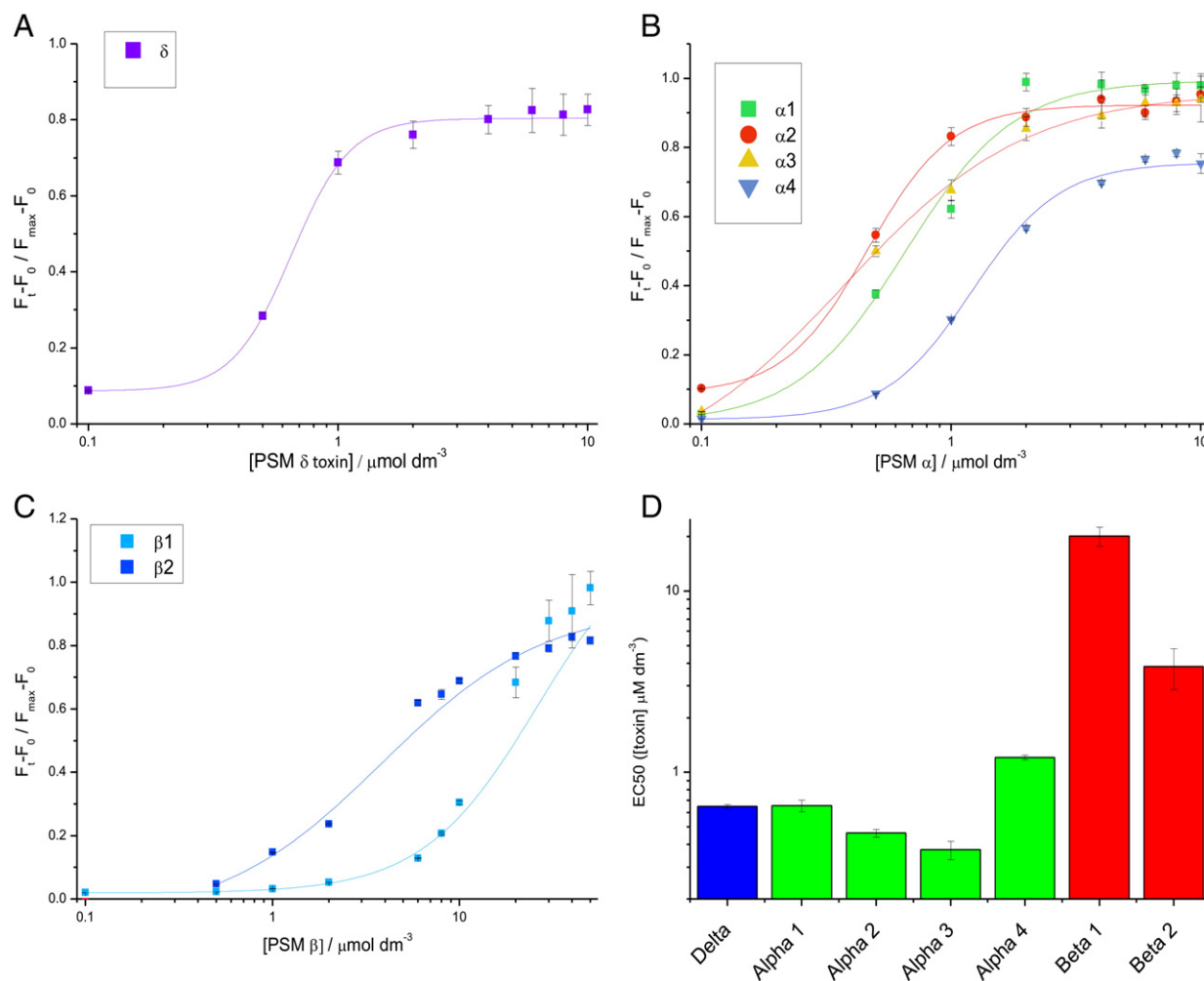


Fig. 1. Study into the effect of delta, PSM alpha and beta toxins against DPPC53 vesicles. Panels A), B) and C) show the response of DPPC (20 mol% cholesterol) vesicles to δ toxin, the PSM α 1–4 and the PSM β 1–2 toxins respectively. Fluorescence values were taken after 60 min of exposure of the vesicles to the indicated peptide concentration. Peptide concentrations from 0.1 $\mu\text{mol dm}^{-3}$ to 10 $\mu\text{mol dm}^{-3}$ were used for delta toxin and the PSM α 1–4 toxins. For the PSM β 1–2 a concentration range from 0.1 $\mu\text{mol dm}^{-3}$ to 50 $\mu\text{mol dm}^{-3}$ was used. D) EC_{50} values (effective concentration of toxins required to reach a fluorescence value half that of the maximum), of each of the peptides tested against the vesicles. EC_{50} values were determined through fitting of the normalized fluorescence vs toxin concentration data to a dose response curve.

particularly cholesterol concentration. It has been shown previously that PMNs from different mammals have different susceptibilities to PSMs, with mouse neutrophils (BALB/c and C57/BL6) being more resistant to PSMs than rabbit or human neutrophils [26]. *S. aureus* may have evolved to secrete small peptides which have preferences to cell types based purely on membrane composition, adding to the hugely effective arsenal of toxins in which this bacterium possess [27]. This preference for membrane structure, dictated by cholesterol gains credibility considering that there are no known protein receptors for PSMs.

Cholesterol can impart liquid ordered like character to localized areas of the bilayer by influencing chain packing behavior of lipids in its immediate vicinity as previously demonstrated by Pokorny et al., with delta toxin partitioning primarily to the liquid-ordered phase of a bilayer [28]. Measurements here were all made at 37 °C which makes the vesicles quite sensitive to cholesterol composition: pure DPPC has a phase transition temperature of 41 °C so the effect of adding cholesterol takes the membrane from the solid ordered phase s_o at <7 mol% cholesterol; mixed s_o –liquid ordered l_o phases up to ca. 30 mol% cholesterol; and liquid ordered above 30 mol% cholesterol [29].

3.3. Lysis of vesicles by *S. aureus* supernatant

Bacterial supernatant is a mixture of proteins, peptides and small molecules secreted by bacteria as they grow, as well as components of

growth media. Previous work has identified PSMs as the primary secreted *S. aureus* virulence factors that lyse DPPC vesicles [11]. The clinically important *S. aureus* strain, LAC (USA300), secretes a cocktail of both delta toxin and PSMs. Experiments were carried out to study the lytic effect of dilutions of supernatant from this strain as a function of vesicle cholesterol concentration (Fig. 4).

The attenuation of vesicle response at high (50 mol%) cholesterol concentration is observed in a similar fashion to the pure PSMs (Fig. 2). The undiluted LAC supernatant was highly lytic, the interesting difference in response is at higher dilutions, where the response of the vesicles is inversely proportional to membrane cholesterol concentration (see for example 6% LAC supernatant) not fully observed for most of the pure toxins, where maximum fluorescence response was generally seen in vesicles with between 10 and 30 mol% cholesterol. These results suggest that when PSMs are acting synergistically the rigidity in membrane fluidity imposed by cholesterol negatively affects PSM-mediated permeabilization.

3.4. Lysis of T-cell mediated by PSM peptides

The results from the study of vesicles with 20 mol% cholesterol shown in Fig. 1 suggest three distinct levels of lytic activity depending on the specific peptide. Experiments were carried out to see whether

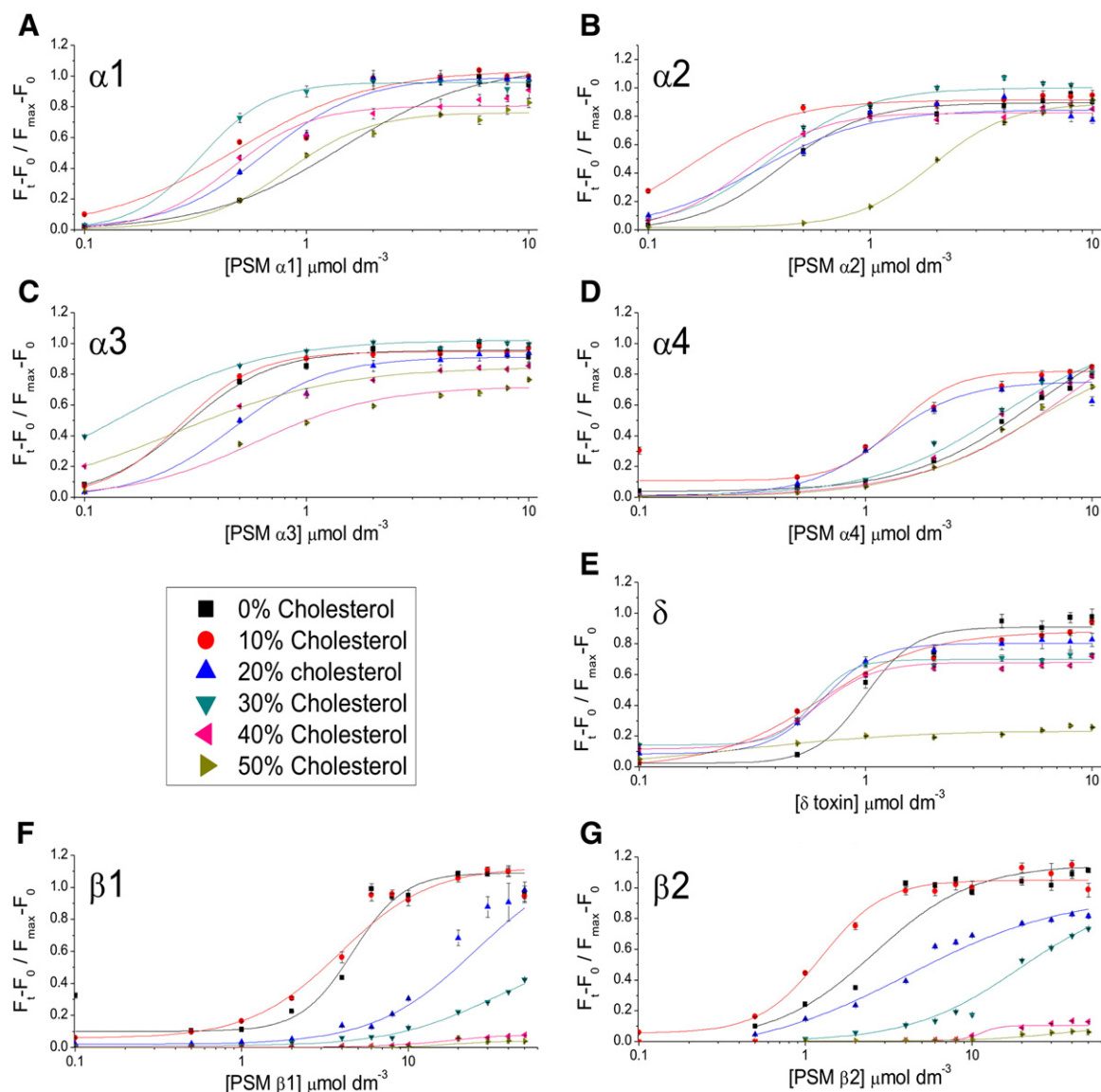


Fig. 2. The effect of cholesterol variation on the lysis of vesicles by PSMs. Vesicles with differing concentrations of cholesterol, 0% to 50% were incubated with A) PSM α 1, B) PSM α 2, C) PSM α 3, D) PSM α 4, E) δ toxin, in the concentration range of 0.1 to 10 $\mu\text{mol dm}^{-3}$ and F) PSM β 1 and G) PSM β 2, in the concentration range of 0.1 to 50 $\mu\text{mol dm}^{-3}$ for 1 h and fluorescence values measured and normalized.

this trend would correlate with cytotoxicity of the peptides to T cells (Fig. 5).

The range of concentrations used in the T cell experiments were higher than those used in the vesicle studies due to poor or nominal killing seen over the 0.1–10 $\mu\text{mol dm}^{-3}$ concentration range for the majority of toxins. The general pattern of lysis in terms of the most, and least, lytic species of PSMs correlates well with the pattern seen for the vesicles. As shown in Fig. 5, PSM α 2 and PSM α 3 show the highest levels of cytotoxicity, closely followed by PSM α 1. The EC_{50} for PSM α 1 and delta toxin are equivalent in the vesicle assay, but PSM α 1 shows significantly greater killing in the T cell assay. PSM α 4 is again seen to be less lytic than delta toxin or the rest of the PSM α s. As with the vesicle assay, the PSM β class are significantly less lytic than the PSM α class or delta toxin, however the difference between PSM β 1 and PSM β 2 observed in the vesicle assay is not seen in the cell toxicity assay as no killing was observed despite the relatively high toxin concentrations used. Overall, the results generated in the vesicle-PSM studies are consistent with the effect of PSM on important immune cells. This pattern of cytotoxicity is similar to that measured by other researchers on PMNs [12] and erythrocytes, with the exception that PSM β 1 showed greater hemolytic

activity than β 2 [13] which is the opposite of results obtained using the DPPC vesicles (Fig. 1) and is not seen in the T cell assay (Fig. 5) or PMN assay [12].

3.5. Secondary structure analysis

Studies of antimicrobial peptides (AMPs) suggest that there are two functional requirements needed for active membrane destabilization: a net positive charge to facilitate interaction with the negatively charged phospholipid membrane; and the potential to form amphipathic structures which allow incorporation into the membrane [30,31]. Other parameters which can strengthen the incorporation of peptides into lipid membranes include the overall hydrophobicity of the peptide, the ratio of hydrophobic to charged residues (h:c) and the degree of structuring [32–34]. In light of these parameters and using the hypothesis that PSMs act in a similar fashion to AMPs and delta toxin, we investigated these properties in an attempt to understand the different lytic activities of these peptides.

The delta and PSM α 1–3 peptides consist of between 46 and 54% hydrophobic residues, which is consistent with the requirement for an

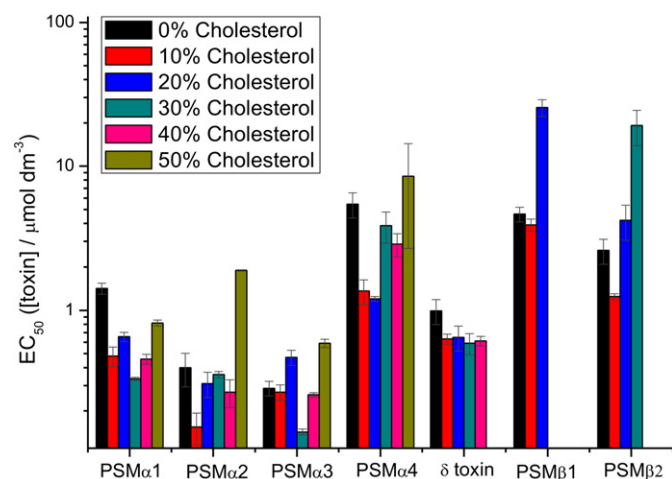


Fig. 3. EC_{50} values of PSMs when incubated with phospholipid vesicles of different cholesterol concentrations. EC_{50} values were determined from the dose response fit of the normalized fluorescence vs concentration data. Where fluorescence maxima did not reach the required 0.5 normalized fluorescence units value, they were excluded.

amphipathic helical structure [30]. However, the PSM α 4 peptide contained 70% hydrophobic residues, giving the highest hydropathicity score using the Kyte and Doolittle algorithm, where a high hydropathicity value indicates high hydrophobicity (Table 2) [35]. Previous studies using AMPs observed that increased hydrophobicity lead to a decrease in interaction with negatively charged membranes and under certain conditions the more hydrophobic a peptide, the lower its membrane lytic capability [30,36]. PSM α 4 also has the highest hydrophobicity to charge (h:c) ratio among the PSM peptides, which may further explain its lower lytic capacity. Overall charge on the delta and PSM α 1–4 was positive (Table 2) with PSM α 2 > PSM α 1 = PSM α 3 = PSM α 4 > delta, within the limits of charge typical for lytic peptides, as excessive charge is potentially damaging towards lytic activity by preventing suitable structuring [37]. The PSM β 1 peptide has an overall negative charge whereas PSM β 2 displays a neutral charge, both peptides have a high h:c ratio which may in part explain the low lytic phenotype.

Helical wheel projections of these peptides (Fig. 6) illustrate that like delta toxin, all the PSM α peptides have a predicted helical structure

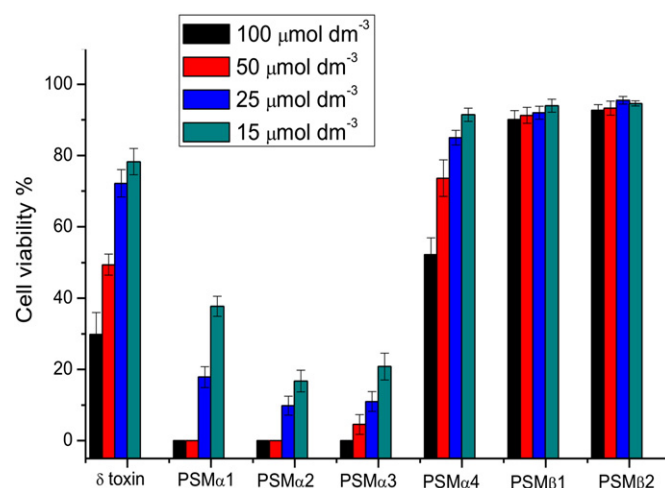


Fig. 5. PSM mediated lysis of T cells. T cells were incubated with increasing concentrations of synthetic PSMs in the range of 15–100 $\mu\text{mol dm}^{-3}$, and resulting T cell viability was assessed. The PSM α 1–3 shows the highest toxicity, giving the lowest T cell survival. The PSM β 1–2 showed no toxicity against T cells whereas PSM α 4 was only toxic at high concentrations. Delta (δ) toxin illustrated an intermediate level of toxicity.

with a high degree of amphiphilicity, with PSM α 3 showing the largest hydrophobic moment (PSM α 3 > delta > PSM α 4 > PSM α 2 > PSM α 1, ranging from 9.16 to 5.96) and PSM β 1 and β 2 relatively lower (6.23 and 4.29, respectively). PSM α 3 is the most toxic towards polymorphonuclear leukocytes (PMNs) [12] and in this study, is most lytic to lipid vesicles and T cells. It has been observed that ionic or salt bridges may form when negatively charged residues are spaced 3 to 4 positions from positively charged residues, which may promote helix formation [38]. PSM α 3 shows the largest number of charged residues (positive and negative) on the hydrophilic phase of the predicted helix, indicating the potential formation of helix stabilizing ionic bonds. This coupled with the large hydrophobic moment for PSM α 3 will likely increase both peptide agglomeration in solution and partitioning of the peptide into the lipid bilayer [39].

Circular dichroism spectroscopy analyses (Fig. 7A–B) illustrate those PSM peptides that were highly lytic (PSM α 1–3) displayed a higher degree of alpha-helical secondary structure whereas PSM α 4 and particularly PSM β 1–2 show reduced helical characteristics (based on CD peak intensity at 190 and 210 nm). Alpha-helicity is important in the ability of small peptides to integrate within the lipid membrane and cause disruption as this type of structure allows for the formation of both hydrophilic and hydrophobic faces [40–42]. The structuring is influenced by both the overall hydrophobicity of the peptide, which will govern initial interaction with the bilayer surface and subsequent interactions between individual residues on the hydrophobic face of the peptide and the bilayer interior. It is worth noting that the PSM α 1–3 peptides possess helix stabilizing alanine residues at position 5 whereas PSM α 4 contains a helix destabilizing glycine in this position which may reflect the observed difference in alpha-helicity.

Delta toxin exhibited a high degree of both amphipathicity and alpha-helicity, with well-defined hydrophobic and hydrophilic phases, but had lower lytic and cytolytic activity than PSM α 2–3 (Figs. 1D and 5). The lower lysis activity is intriguing and may be due to the low hydropathicity value and a neutral charge affecting efficient initial interaction and insertion within the membrane. Previous studies have demonstrated that aliphatic residues decreasingly promote alpha-helix stability in the order Leu > Ile > Ala > Val > in a lipid environment [43, 44]. The combined number of all aliphatic residues within each peptide (discounting glycine), varies in the order 6 (PSM α 3), 8 (PSM α 2), 9 (PSM α 1), 9 (delta toxin) and 12 (PSM α 4). Of those residues PSM α 3 has proportionately the highest leucine content while PSM α 4 has the

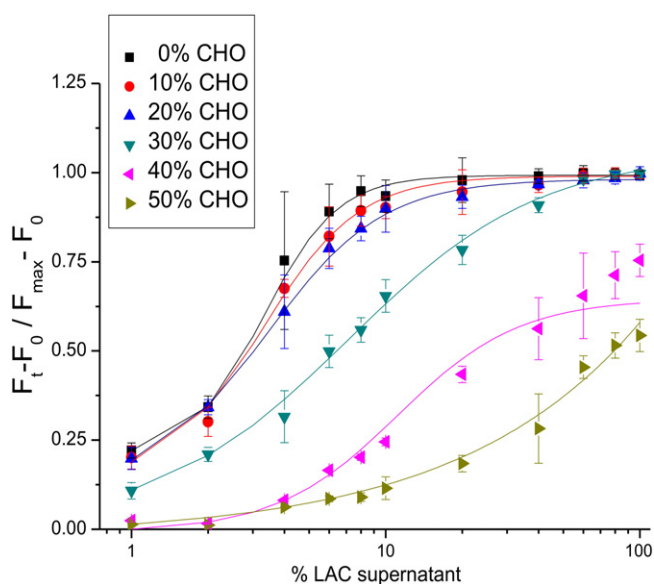


Fig. 4. Effect of cholesterol on vesicle lysis mediated by *Staphylococcus aureus* strain LAC supernatant. Different dilutions of cell-free supernatant (0–100%) were incubated with vesicles with varying cholesterol concentrations (0–50%).

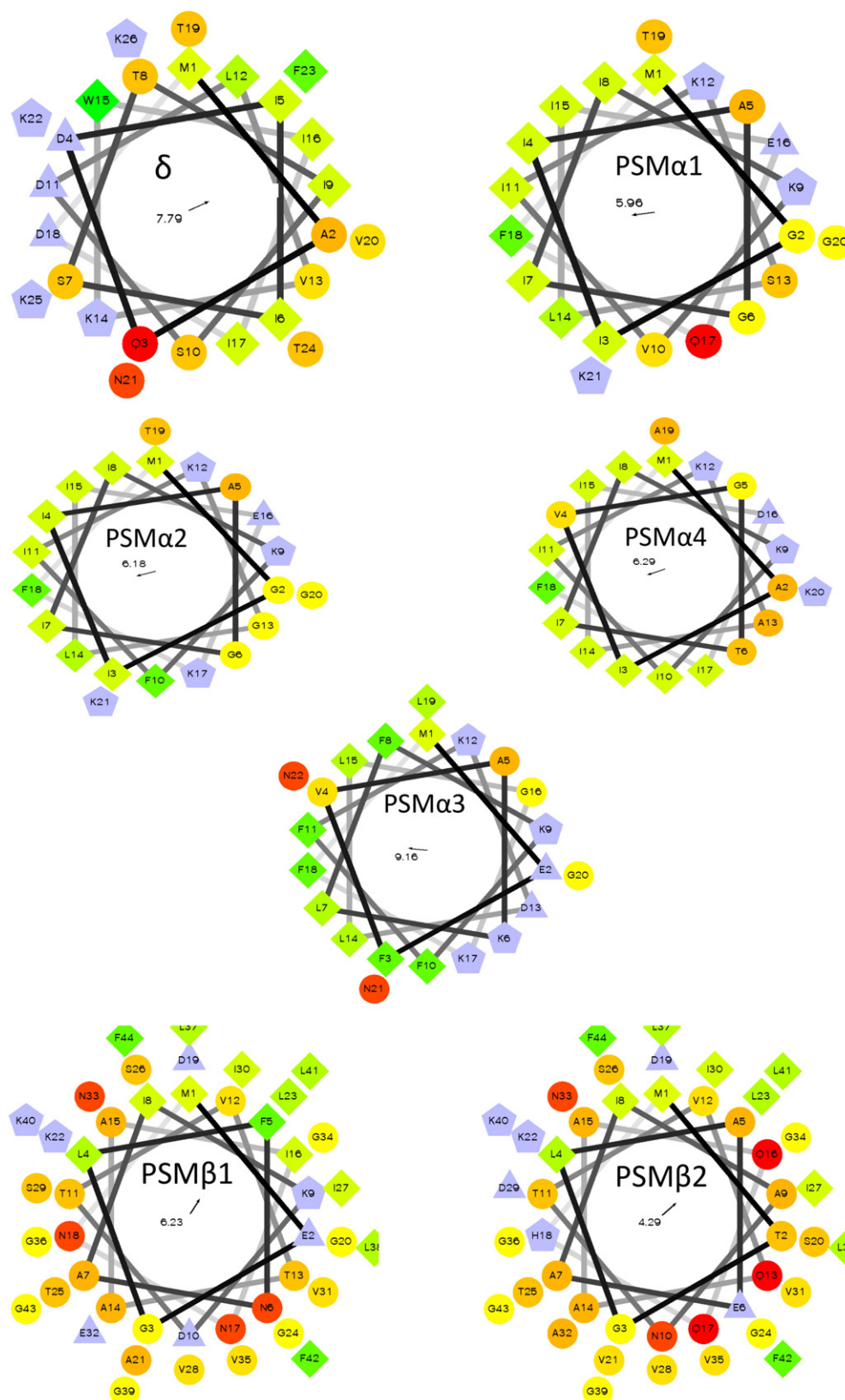


Fig. 6. Helical wheel projections of delta toxin, PSMα1, α2, α3, α4, PSMβ1 and β2. Hydrophobicity is color coded from dark green (most hydrophobic) to yellow (zero hydrophobicity). Hydrophilic amino acids are shaded from red (most hydrophilic) to yellow, charged residues are shown in light blue (reference; <http://r3lab.ucr.edu/>). The arrows indicate the directions of the hydrophobic moments.

lowest. The number of aliphatic residues a peptide contains therefore shows an approximate inverse correlation with EC_{50} values obtained for each when challenging 20 mol% cholesterol vesicles Fig. 1D.

It is important to note that delta and PSMα1–3 illustrated good alpha-helical spectra in the absence of lipid membranes and alpha-helical inducing solvents such as TFE. The PSMβ1–2 peptides, which

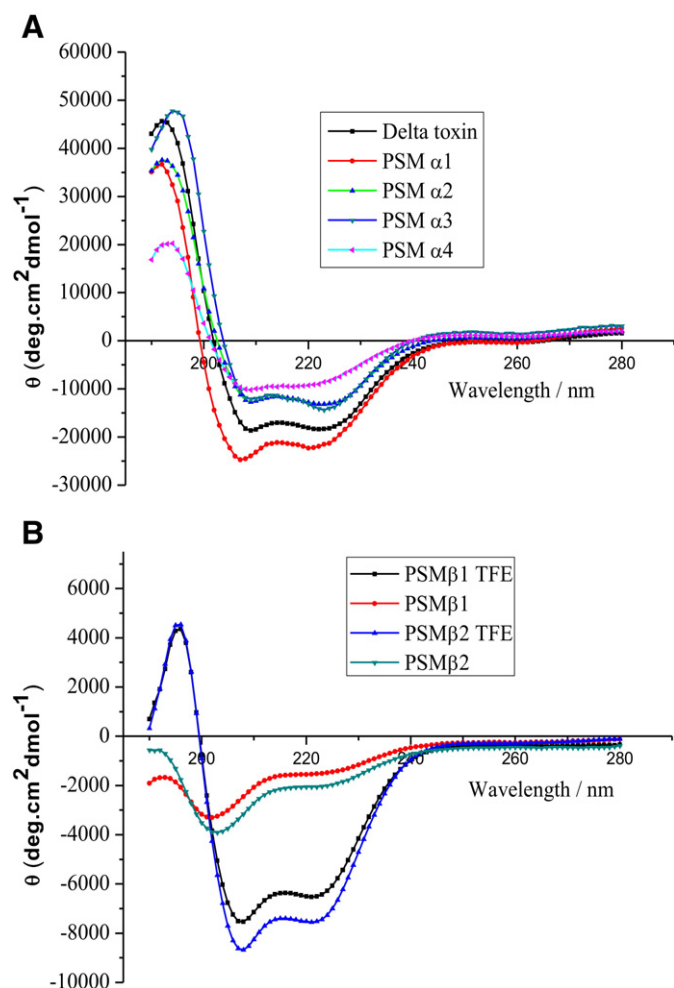


Fig. 7. Circular dichroism (CD) spectra of synthetic PSMs. A) CD spectra of PSM α 1–4 and delta toxin prepared in PBS. B) CD spectra of PSM β 1 peptide in 50% TFE (trifluoroethanol), PSM β 1 peptide in PBS buffer, PSM β 2 peptide in 50% TFE and PSM β 2 peptide in PBS buffer. The peaks at 190 and 210 nm are indicative of alpha-helicity. The negative peaks at 202 nm and minimal negative peaks at 220 nm indicate random coil secondary structure.

show the lowest lytic potential, are relatively unstructured in the absence of TFE. These peptides instead possess a random coil structure. However, under our conditions, PSM β 1–2 exhibited increasing alpha-helicity when dissolved in 50% TFE as shown in Fig. 7B. Previous results have shown that all PSM possess alpha-helicity, however these peptides were dissolved in 50% TFE [12] which is known, and observed by us, to induce alpha-helicity. Our results suggest that the ability to form alpha-helical structure is extremely important in lysing cholesterol containing lipid vesicles, as those PSM which have poor alpha-helical folding also have poor lytic activity.

4. Conclusions

The lytic activity of PSM peptides from virulent strains of *S. aureus* has been studied here. Physico-chemical properties of peptides affect their ability to both penetrate and lyse phospholipid membranes. On the basis of the results presented here, the degree of alpha-helicity is the most important property for correct insertion and lysis of membranes for PSM peptides.

The PSM β 1–2 peptides, which show the lowest lytic potential, are relatively unstructured in the absence of TFE, instead possessing a random coil structure which is not conducive to high lytic activity. Why the PSM α 4 or PSM β 1–2 peptides have lower alpha-helicity is not completely understood as yet but maybe due to the presence of helix breaking residues. Therefore, we suggest refining this class of peptides,

as not all of the members have alpha-helicity, which is an important factor in membrane lysis.

The ability of these PSM to act synergistically with other toxins has been recently reported and shown to be involved in *S. aureus* escape from epithelial and endothelial phago-endosomes [45]. This study reported the synergistic effect of delta toxin or PSM β 1–2 with the sphingomyelinase β -toxin in disrupting the endosomal membrane when these toxins were overexpressed in non-virulent strains. Interestingly, no synergistic effect was observed with the PSM α peptides, which are more related to delta toxin than the PSM β peptides and recently shown to be important in phagosomal escape [17]. Currently, it is unknown if PSMs can act synergistically with each other, and what molecular interaction may dictate such effects. Experiments designed to investigate how PSMs acting in synergy affect different phospholipid vesicle membranes and how they influence the structure of one another are ongoing and will hopefully shed light onto how these interesting peptides act during pathogenesis.

Recent work has shown that serum lipoproteins inactivate the lytic activity of PSMs [46]. This result, in partnership with studies showing the importance of PSM α peptides in the lysis of intracellular compartments, suggests that the main mode of action of PSMs is lysis of the phagosomal membrane. Interestingly, cholesterol is a major component of lipid rafts which is required for initiating signals for phagocytosis [47]. Cholesterol containing lipid rafts are also evident in the phagosomal membrane [47]. Our results indicate that the expression of delta toxin, PSM α 1, -2 or -3 will lead to the lysis of cholesterol-containing vesicles and that the progression from a liquid-disordered to liquid ordered state following the addition of cholesterol to the membrane results in decreased lytic activity. Differences in cholesterol concentration in phagosomes from different cell types/species may impact of PSM-mediated phagosome escape and subsequent survival of *S. aureus*.

Finally, it appears that there is a good correlation between the results obtained from the vesicle assay (Fig. 1) and the cytotoxicity measurements of PSM against T cells (Fig. 5), which suggests that using lipid vesicles of the type and composition studied here could provide a way of screening peptides with high lytic activity and identifying bacteria which secrete highly cytolytic proteins.

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