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Membrane binding and permeation by indolicidin analogs studied by a biomimetic lipid/polydiacetylene vesicle assay

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Abstract

Membrane binding and relative penetration of indolicidin analogs were studied using lipid/polydiacetylene (PDA) chromatic biomimetic membranes. Colorimetric and fluorescence analyses determined that an indolicidin analog with a proline and tryptophan residue substituted with lysines showed more pronounced bilayer surface interactions, while indolicidin and particularly an indolicidin analog in which all prolines were replaced with alanine residues exhibited deeper insertion into the lipid bilayer. The colorimetric data demonstrated that more pronounced blue-red transitions were observed when the chromatic vesicles incorporated lipopolysaccharide (LPS) within the lipid bilayer, indicating that LPS promoted preferred binding and incorporation of the peptides at the lipid/water interface. The fluorescence quenching experiments further confirmed this outcome. The results indicate that the antibacterial activity of indolicidin most likely requires initial binding to the LPS moieties within bacterial membranes, as well as disruption of the bilayer interface. The degree of hemolysis induced by the analogs, on the other hand, correlated to the extent of penetration into the hydrophobic core of the lipid assembly.

Keywords: Indolicidin; Antimicrobial peptides; Membranes; Colorimetric assays; Polydiacetylene; Fluorescence quenching; NBD; Dansyl–PMBN

1. Introduction

Indolicidin is a 13-residue cationic antimicrobial peptide (sequence carboxy-terminal amidated ILPWKWPWWPWRR, single letter code) that was purified from the cytoplasmic granules of bovine neutrophils [40]. Among the unique properties of indolicidin is the high content of proline (23%) and the highest percentage of tryptophan found in antimicrobial peptide sequences. Indolicidin was shown to be highly effective in killing both Gram-negative and -positive bacteria, fungi [1], protozoa [2], HIV-1 [35], and rat and human T lymphocytes [38]. The mechanism of indolicidin action, however, has not been fully elucidated. Various investigations have pointed to cellular membrane permeation by indolicidin as a significant factor for its bactericidal activity. Falla et al. outlined a model in which the peptide penetrates through the cytoplasmic membrane via a "barrel-stave" mechanism [8] resulting in formation of small transient pores, rather than well-defined channels [41]. Other models propose that the lytic activity of indolicidin is related to permeation of the outer cell wall and micellization of the cytoplasmic membrane [41] or to transit across the cytoplasmic membrane via an aggregate intermediate and interaction with cytoplasmic targets [45].

Several indolicidin analogs were synthesized with the goal of producing less toxic effects compared to the parent peptide yet having similar or higher antimicrobial activities. CP11 [ILKKWPWWPWRRK-NH_2] has two extra positive charges resulting from substitution of Pro-3 and Trp-4 with a single lysine residue and another Lys added at the C-terminus [45]. CP11 demonstrated better permeability of both outer and inner membranes of *Escherichia coli* compared to native indolicidin, with less hemolytic activity and improved activity against Gram-negative bacteria [1,45]. Like indolicidin [37], CP11 adopts a boat-shaped structure as determined by NMR spectroscopy in lipidic environments (Rozek and Hancock, unpublished results).

Another indolicidin analog denoted CP10A, in which the three proline residues were replaced with alanine, demonstrated similar activity as indolicidin towards Gram-negative bacteria but significantly higher lytic action against Gram-positive bacteria [10]. CP10A, unlike the other peptides, adopts an α-helical structure in lipid environments

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[11]. It was demonstrated that small concentrations of CP10A almost completely depolarized the cytoplasmic membrane of *E. coli and Staphylococcus aureus* [11], phenomena that were not observed for indolicidin and CP11 [7,10].

The goal of this study was to probe membrane interactions of indolicidin and its two analogs CP10A and CP11, and to relate this information to the mechanisms of their biological action. We specifically evaluated the significance of membrane composition and the effects of membrane constituents such as lipopolysaccharide, upon binding and permeation. The biophysical investigation presented here was carried out using the newly-developed lipid/polydiacetylene (PDA) biomimetic membrane assay [15]. Lipid/PDA vesicle assemblies consist of mixed domains of lipid bilayers and conjugated PDA that together mimic membrane environments as well as undergo visible blue-red transformation induced by varied membrane processes [18–21]. Previous studies of the biophysical properties of lipid/PDA vesicles indicate that the lipids and polydiacteylene form interspersed, interacting microscopic phases [6,12,22,44]. Importantly, the phospholipids incorporated within the PDA matrix adopt a bilayer structure, the dominant lipid organization within cellular membranes [22].

The PDA assemblies exhibit dramatic blue-red transitions directly induced by biological interactions occurring within the embedded lipid domains [18–21]. Published data point to the contribution of changes in fluidity within the lipid domains for induction of the colorimetric transformations [18,22]. We have previously shown that the colorimetric lipid/PDA assay could be used for studying lipid association and membrane penetration of antimicrobial peptides. Here we apply colorimetric, fluorescence, and circular dichroism analyses to investigate lipid–peptide interactions of indolicidin and its analogues.

2. Materials and methods

2.1. Materials

Phospholipids, including dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), and lipopolysacharides (LPS) (*E. coli* 055:B5) were purchased from Sigma. LPS was dialyzed in EDTA (Sigma) (1 mM, 24 h) solution followed by distilled water in order to remove excess divalent cations (which generally cause precipitation of lipid/PDA vesicles). The diacetylenic monomer 10,12-tricosadiynoic acid was purchased from GFS Chemicals (Powell, OH), washed in chloroform, and filtrated through 0.45 μm filters prior to use Tris[hydroxymethyl]aminomethane (Tris buffer) was purchased from Sigma.

Indolicidin (ILPWKWPWWPWRR-NH_2), CP10A (ILAWKWAWWAWRR-NH_2), and CP11 (ILKKWPWWPWRRK-NH_2) were synthesized by solid-phase F-moc peptide synthesis at the Nucleic Acid/Peptide Synthesis (NAPS) facility at the University of British Columbia. Purity of the peptides was verified by high performance liquid chromatography and mass spectrometry.

2.2. Hemolytic activity

Human erythrocytes were isolated from heparinized blood by centrifugation (3000 rpm for 10 min) after washing three times with Tris–HCl buffered saline (5 mM Tris–HCl pH = 7.4, 150 mM NaCl). Erythrocytes were diluted with saline to a 1/50 packed volume. Test tubes containing 1 ml of erythrocyte solutions were incubated with different peptide concentrations for 60 min at 37 °C. The tubes were then centrifuged and the absorbance of the supernatants was measured at 540 nm. Lysis induced by 1% Triton X-100 was taken as 100%.

2.3. Vesicle preparation

Lipid components and the monomeric unit 10,12-tricosadiynoic acid at the appropriate ratios (DMPG/DMPC/PDA—1:1:3 mole ratio, LPS/DMPC/PDA—0.1:2:6 ratio) were dissolved in chloroform/ethanol (1:1) and dried together in vacuo, followed by addition of deionized water and probe-sonication at 70 °C for 2–3 min. The vesicle solution was then cooled at room temperature and was kept at 4 °C overnight. The vesicles were then polymerized using irradiation at 254 nm for 10–20 s, with the resulting solutions exhibiting an intense blue appearance.

2.4. Dansyl–PMBN binding assay

The fluorescence of dansyl–PMBN bound to vesicles was measured at 27 °C using a Spectra Mac Gemini spectrofluorimeter (Molecular devices) with excitation at 340 nm and emission acquired at 485 nm. Increasing quantities of dansyl–PMBN (1×10^{-5} to 1×10^{-3} M) were added to microplate 96-well containing vesicle solution (0.4 mM) and buffer (Tris-base 25 mM, pH 8). The solution was diluted to 0.2 ml with distilled water and fluorescence was recorded. The fluorescence of vesicle-bound dansyl–PMBN (1×10^{-4} M) was determined at 85% of maximum fluorescence, which served as the control sample. The decrease in dansyl–PMBN fluorescence (percent inhibition from control) as a result of progressive addition of peptides was then calculated.

2.5. Colorimetric measurements

Samples were prepared by adding peptides (at final concentrations of between 20 and 150 μM) to 0.06 ml vesicle solutions at 0.2 mM total lipid concentration and 25 mM Tris (pH 8). The peptides and derivatives were dissolved in water. Following addition of the peptides, the solutions were

diluted to 1 ml and UV-Vis spectra were acquired. UV-Vis spectroscopy measurements were carried out at 28 °C on a Jasco V-550 spectrophotometer, using a 1 cm optical path cell.

A quantitative value for the extent of the blue-to-red color transitions within the vesicle solutions is given by the colorimetric response (%CR), which is defined as follows [15]:

$$\%\mathrm{CR} = \left(\frac{\mathrm{PB}_0 - \mathrm{PB}_\mathrm{I}}{\mathrm{PB}_0}\right) \times 100$$

where

$$\mathrm{PB} = \frac{A_{\mathrm{blue}}}{A_{\mathrm{blue}} + A_{\mathrm{red}}}$$

A is the absorbance either at the "blue" component in the UV-Vis spectrum (640 nm) or at the "red" component (500 nm). (Note: "blue" and "red" refer to the visual appearance of the material, not its actual absorbance.) PB_0 is the blue/red ratio of the control sample (before induction of a color change), and PB_I is the value obtained for the vesicle solution after colorimetric transition occurs.

Graphs correlating %CR and *vesicle-bound* peptide rather than *total* peptide concentrations were calculated [16]. Vesicle-bound peptide concentrations were determined through calculation of partition coefficients of the peptides employing an ultracentrifugation technique described elsewhere [16].

2.6. Tryptophan fluorescence measurements

Reduction of the Trp fluorescence by lipid-embedded spin-probe was measured as the following. The spin label 16-doxyl-stearic acid (16-DS, Sigma) was added to the vesicles after the polymerization step in a molar ratio of 1:100 (spin label:lipids). Spin-labels quench tryptophan fluorescence when in close proximity. Samples were prepared by adding CP10A/indolicidin or CP11 peptides (at concentrations of 110 and 75 μM, respectively) to 0.06 ml vesicle solutions at 0.2 mM total lipid concentration and 25 mM Tris base (pH 8). The decrease in tryptophan fluorescence emission was recorded at 28 °C using 284 nm excitation and 345 nm emission on a SLM Aminco Bowman spectrofluorimeter. The excitation and emission slit widths set to 4 and 8 nm, respectively.

2.7. NBD-PE fluorescence quenching

N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) was purchased from Molecular Probes, Inc. (Eugene, OR). NBD-PE was dissolved in chloroform, added to lipids in a 1 mol% and dried together in vaccuo before sonication (see Section 2.3). Addition of NBD-PE did not affect the colorimetric properties of the vesicles. Samples were prepared by adding peptides at concentration of 70 μM (vesicle-bound concentrations) to 0.06 ml vesicle solutions containing 0.2 mM total lipid concentration at 25 mM Tris buffer (pH 8). The quenching reaction was initiated by adding sodium dithionite from a stock solution of 0.6 M, prepared in 50 mM Tris base buffer (pH 11) to a final concentration of 10 mM. The decrease in fluorescence (100% corresponds to fluorescence value prior to addition of dithionite) was recorded for 300 s at 28 °C using 467 nm excitation and 535 nm emission on a Jobin Horiba FL3-22 spectrofluorimeter.

2.8. Circular dichroism (CD)

CD spectra were acquired on an Aviv 62A-DS Circular Dichroism Spectrometer (Aviv Inc., NJ). Three scans were recorded between 195 and 250 nm with 1 nm-acquisition steps. A 2 mm optical path length was used. Samples were prepared by adding 40 μM of peptides to 0.2 ml vesicle solutions at 0.3 mM total lipid concentration and 10 mM Tris-base buffer pH = 8. To compensate for scattering due to the vesicles, the CD spectrum of a vesicle solution alone was subtracted from that of the peptide in the presence of the vesicles.

3. Results

3.1. Hemolysis data

To evaluate one of the biological activities of the peptides we compared their hemolytic properties against human erythrocytes (Fig. 1). Fig. 1 depicts the percent hemolysis of erythrocytes as a function of peptide concentrations. CP10A induced the highest lytic activity among the three peptides, with almost 70% lysis observed at a concentration of 20 μM compared to less than 10% hemolysis at that concentration for the two other peptides (Fig. 1). Fig. 1 also indicates that CP11 exhibited lesser hemolytic activity than indolicidin.

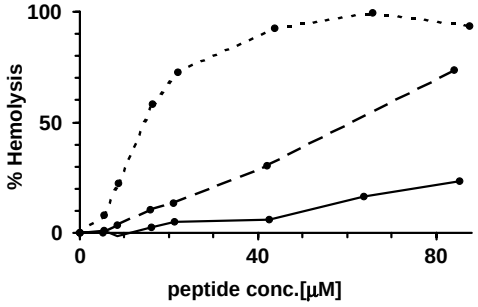

Fig. 1. Hemolysis of human erythrocytes as a function of peptide concentration. Erythrocytes were incubated in Tris–HCl 5 mM saline buffer containing varying concentrations of the peptides for 1 h. Indolicidin analogs examined were: CP11 (bold line); CP10A (short dash); native indolicidin (long dash).

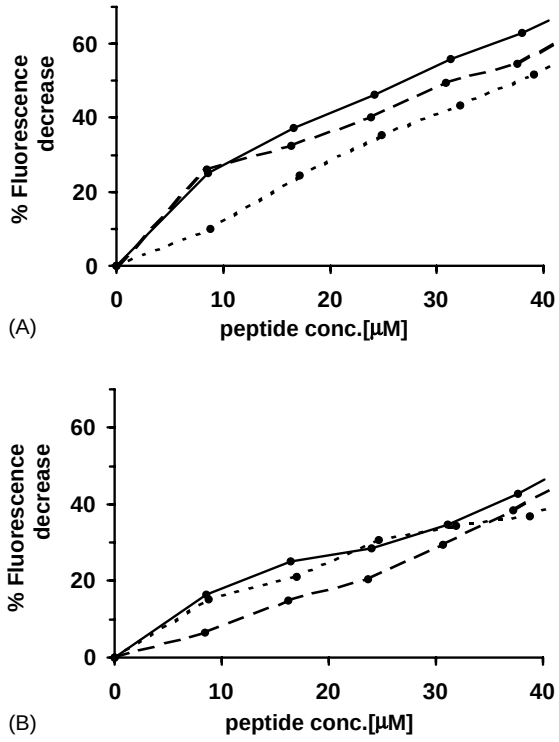

Fig. 2. Dansyl–PMBN displacement assay. Indolicidin analogs examined were: CP11 (bold line); CP10A (short dash); native indolicidin (long dash). Vesicles examined were (A) DMPG/DMPC/PDA vesicles (1:1:3 ratio); (B) LPS/DMPC/PDA vesicles (0.1:2:6 ratio).

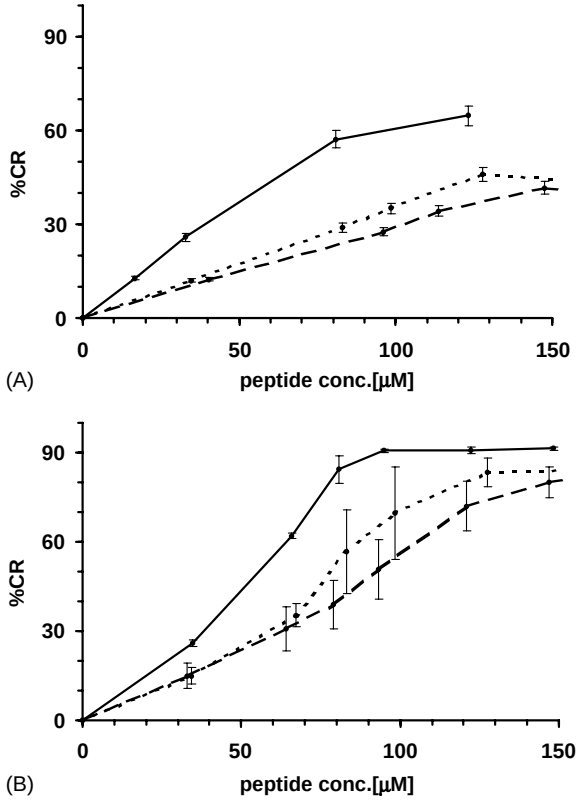

Fig. 3. Colorimetric transitions induced in lipid/PDA vesicles by the peptides. Relationships between peptide concentrations and colorimetric response (%CR, Section 2) induced in (A) DMPG/DMPC/PDA vesicles (1:1:3 ratio); (B) LPS/DMPC/PDA vesicles (0.1:2:6 ratio). Indolicidin analogs examined were: CP11 (bold line); CP10A (short dash); native indolicidin (long dash).

Previous studies reported differences in hemolytic activity between indolicidin and CP10A against rat erythrocytes, albeit to lesser degrees than observed here (Fig. 1) [42]. These observations might be related to differences in membrane lipid composition of erythrocytes from different organisms [27]. Previous studies have shown, for example, that the extent of erythrocyte permeability is strongly related to the relative abundance of lecithin within the membrane [5]. This factor might account for the differential hemolysis data we recorded here for human erythrocytes (Fig. 1), which contain around 19% lecithin, compared to 43% in rat erythrocytes [3,27], which might give rise to different hemolytic properties.

3.2. Dansyl–PMBN displacement assay

The apparent sensitivity of the biological activities of indolicidin and its analogs to lipid composition was further examined in biomimetic lipid/PDA vesicles using several analytical and spectroscopic techniques (Figs. 2–6). Two vesicle models were examined: DMPG/DMPC/PDA (1:1:3 mole ratio) in order to evaluate the contribution of negatively-charged phospholipids, relatively abundant in the cytoplasmic membranes of bacterial cells [13], and LPS/DMPC/PDA (0.1:2:6 ratio) for elucidating the role of LPS in promoting surface binding and outer membrane permeation of the peptides [32].

We first measured the relative peptide association with the biomimetic vesicles through application of the dansyl–PMBN displacement assay (Fig. 2) [9,43]. The dansyl group fluoresces when located within a hydrophobic lipid environment, while it is quenched in the aqueous solution following displacement by membrane-bound species [9]. Accordingly, application of the dansyl–PMBN assay in this work provided information upon the relative affinities of the indolicidin analogs to the lipid/PDA vesicles by measuring the decrease in fluorescence of dansyl–PMBN residues displaced by the bound peptides [9,43].

Fig. 2 shows that, as expected, increasing peptide concentrations induced higher inhibition (quenching) of the dansyl fluorescence due to displacement of the dansyl–PMBN molecules into the aqueous solution [24,39]. Differences were observed however, albeit small, in the binding properties among the peptides, and depending upon lipid composition (Fig. 2). CP11 seemed to exhibit the most pronounced displacement capabilities in DMPG/DMPC/PDA vesicles (Fig. 2A) and LPS/DMPC/PDA (Fig. 2B); for the latter similar displacement was recorded with CP10A. This result suggests that CP11 attaches predominantly to the surface of the lipid bilayer, since previous work has indicated that

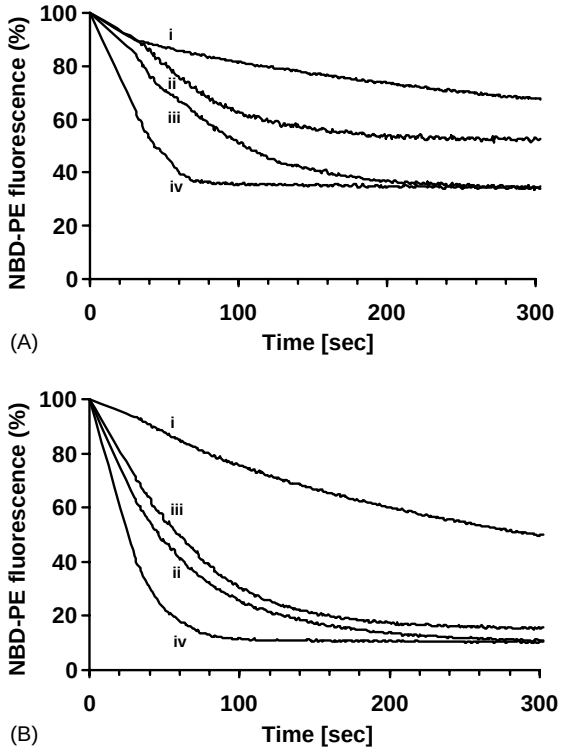

Fig. 4. Fluorescence quenching of NBD-PE. The percentage of fluorescence recorded from NBD-PE/lipid/PDA vesicles after peptide addition and quenching by soluble sodium dithionite. Sodium dithionite was added at a concentration of 10 mM to vesicle solutions (0.2 mM) at 70 μM peptide concentrations. (A) NBD-PE/DMPG/DMPC/PDA vesicles (0.02:1:1:3 mole ratio); (B) NBD-PE/LPS/DMPC/PDA vesicles (0.02:0.1:2:6). Curves corresponding to: (i) control (no peptide added); (ii) CP10A; (iii) native indolicidin; (iv) CP11.

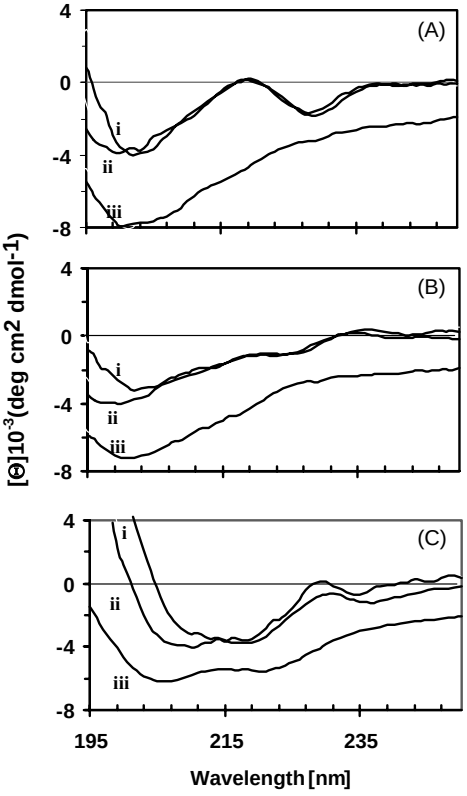

Fig. 6. CD spectra of peptides: (A) CP11; (B) native indolicidin; (C) CP10A. Traces corresponding to: (i) DMPG/DMPC/PDA (1:1:3 molar ratio); (ii) LPS/DMPC/PDA (0.1:2:6); (iii) no vesicles (aqueous solution). Peptide concentrations in all measurements were 40 μM and vesicle concentrations in Tris buffer (pH = 8) were 0.3 mM.

this is also the preferred location of dansyl–PMBN binding sites [25].

Fig. 2B demonstrates that native indolicidin gave rise to relatively lower displacement of dansyl–PMBN compared to the two analogues in the LPS/DMPC/PDA vesicle assembly. Various studies have suggested that initial interactions of antimicrobial peptides with Gram-negative bacteria involve binding to LPS [26,32]. Thus, the higher affinity of CP10A and CP11 for the LPS/DMPC/PDA system (Fig. 2B), compared to native indolicidin, might correspond to the increased interaction with and self-promoted uptake across the outer membrane leading to increased bactericidal activity against Gram-negative bacteria of these analogues [26,32].

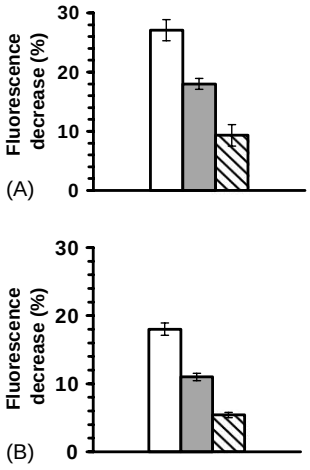

Fig. 5. Quenching of Trp fluorescence by 16-DS spin probe after peptide addition: CP10A (white column); native indolicidin (grey column); CP11 (wide downward diagonal column). (A) DMPG/DMPC/PDA (1:1:3 molar ratio); (B) LPS/DMPC/PDA (0.1:2:6).

3.3. Colorimetric analysis

The fluorescence inhibition data in Fig. 2 showed differences in peptide binding to the lipid assemblies. The biological activities of antimicrobial peptides are determined, however, not only by their binding properties but also to a large extent by their effects on membrane structure and organization [8]. Penetration and lipid bilayer disruption by the indolicidin derivatives were compared here through analysis of the colorimetric transitions induced within DMPG/DMPC/PDA vesicles and LPS/DMPC/PDA vesicles (Fig. 3).

Previous studies have indicated that lipid/PDA vesicles mimic cellular membrane environments and provide infor-

mation on membrane processes [18,19]. We have shown that the lipid moieties in the mixed vesicles form organized bilayer domains within the polymer matrix [22,44]. In particular, the blue-red transformations in lipid/PDA vesicles depend upon disruption of the lipid interface by membrane-interacting compounds and by their depth of penetration into the lipid layer [19,21]. A correlation was shown between the surface perturbation induced by membrane-active peptides and the degree of color changes (recorded as percent colorimetric change, %CR) [6,19,21,22].

The data depicted in Fig. 3 demonstrate that the colorimetric transitions exhibit different dependencies on peptide concentrations among the three analogs. In particular, in the two vesicle models a steeper %CR/concentration curve was observed for CP11, compared to indolicidin and CP10A, which induced similar lower chromatic changes (Fig. 3). At a peptide concentration of 50 μM, for example, CP11 induced a %CR of 30% in the DMPG/DMPC/PDA vesicle solution compared to the value of approximately 15% induced by the other two peptides (Fig. 3A). Similar values were obtained in the LPS/DMPC/PDA system (Fig. 3B).

The colorimetric data in Fig. 3 suggest that CP11 exhibited much more significant surface interactions compared to the other two peptides. Previous studies have demonstrated that peptides that preferably disrupt the lipid head-group region were able to induce more pronounced color transitions, while deeper penetration into the hydrophobic lipid core generally gave rise to more moderate blue-red transformations [18]. This correlation has been ascribed to the perturbation of the pendant side-chains of the PDA framework induced by the interfacial effect within the lipid domain's transitions [29,34]. Thus, greater lipid surface interactions would result in more pronounced perturbations of the adjacent polymer domains, giving rise to higher colorimetric response (%CR). This may contribute to the apparent better interaction of CP11 with the surface (outer) membrane of Gram-negative bacteria, leading to improved self-promoted uptake and consequent antibiotic activity [10].

Surface localization of CP11 was also apparent in the dansyl–PMBN displacement results shown in Fig. 2. Lower penetration of CP11 into the DMPG/DMPC bilayers in DMPG/DMPC/PDA vesicles could be explained by the higher positive charge of the peptide, resulting in more pronounced electrostatic surface interactions. This interpretation is consistent with studies employing Langmuir monolayers of zwitterionic and negatively charged phospholipids indicating deeper penetration of indolicidin and CP10A compared to CP11 [37]. Similarly, calcein release experiments showed a significantly smaller leakage induced by CP11 compared to indolicidin and CP10A [46].

The resemblance of the %CR graphs of indolicidin and CP10A (Fig. 3) indicates that these two peptides undergo a similar degree of penetration into the bilayer in the two vesicle models, despite the rather different structures they adopt. Importantly, greater %CR was recorded for all peptides at higher peptide concentrations in the LPS/DMPC/PDA vesicle assembly (Fig. 3B) compared to the DMPG/DMPC/PDA vesicles (Fig. 3A). This result indicates that more pronounced surface interactions occurred in the LPS-containing vesicles, consistent with the self-promoted uptake model [26,32].

3.4. Fluorescence quenching of NBD-PE

Further insight into the surface interactions of the indolicidin analogs was provided by experiments recording the fluorescence quenching of NBD-PE incorporated within the chromatic vesicles by sodium dithionite dissolved in the aqueous solution [23] (Fig. 4). The dithionite quenching assay had been previously used to measure the lipid "flip-flop" processes and transport of NBD-phospholipids from the inner leaflet to the outer leaflet in lipid bilayers [14,17]. Higher accessibility of the dithionite quencher to the NBD moieties was also related to bilayer disruption by membrane-active compounds [23].

Fig. 4 depicts dithionite-induced fluorescence quenching of NBD-PE incorporated within DMPG/DMPC/PDA and LPS/DMPC/PDA vesicles, respectively, after peptide association. The control kinetic data were measured by incubation of the vesicles with sodium dithionite in the absence of peptides. In both vesicle models, the added peptides generally induced faster fluorescence quenching, however, differences in the rates of fluorescence decrease were clearly apparent among the indolicidin analogs. CP11 induced the most rapid fluorescence quenching (Fig. 4A and B). This observation most likely corresponded to the preferred localization of the peptide at the lipid-water interface thus strongly affecting the NBD probe, which is also displayed at the hydrophilic phospholipid headgroup. In particular, the higher positive charge on CP11 likely modulated the electrostatic interactions occurring at the bilayer headgroup region, thus allowing more access of the dithionite moieties to quench the NBD fluorescence.

Indolicidin and CP10A gave rise to similar quenching rates, slower than CP11, in both vesicle models (Fig. 4). The lesser effects of indolicidin and CP10A on the fluorescence quenching of surface-displayed NBD-PE are most likely due to the relatively deeper bilayer insertion of the two peptides. This interpretation is consistent with the colorimetric data (Fig. 3) in that the "grouping" of the indolicidin and CP10A curves observed in Fig. 4 was similarly to that recorded in the colorimetric experiment (Fig. 3).

The fluorescence quenching data further confirmed the significance of LPS in promoting surface interactions of the peptides. The quenching rates associated with each peptide in the NBD-PE/LPS/DMPC/PDA system were faster than the corresponding rates recorded in the NBD-PE/DMPG/DMPC/PDA assembly indicating a preferential interaction with polyanionic LPS.

3.5. Quenching of tryptophan fluorescence by lipid-embedded radical probe

We further evaluated the degree of peptide insertion into the lipid bilayer through recording the decrease in tryptophan (Trp) fluorescence induced by proximity to a spin probe embedded within the lipid/PDA vesicles. These experiments employed a doxyl spin probe covalently attached to a stearic acid moiety at carbon position 16 (16-DS) [28] (Fig. 5). 16-DS has been used as an ESR probe for investigating the environment and dynamics at the hydrophobic cores of lipid bilayers [28,33]. In the experiments depicted in Fig. 5, the percentage quenching of Trp fluorescence emission of the indolicidin analogs was measured in 16-DS/DMPG/DMPC/PDA (0.01:1:1:3 mole ratio) vesicles and 16-DS/LPS/DMPC/PDA (0.01:0.1:2:6) vesicles, respectively.

Fig. 5 demonstrates variations in the percentage of Trp quenching by the spin-probe co-incorporated within the vesicles. The control samples, used were lipid/PDA vesicles *not* containing 16-DS (Section 2). The highest quenching in the two vesicle models observed for CP10A was around 28% in the 16-DS/DMPG/DMPC/PDA vesicles (Fig. 5A) and 18% when the peptide was added to 16-DS/LPS/DMPC/PDA (Fig. 5B). This result indicates that the distances between the spin label of 16-DS and the Trp residues in CP10A were relatively short compared to those measured for CP11 and the parent indolicidin peptide. This observation again indicates deeper bilayer insertion of CP10A consistent with the colorimetric analysis (cf. Fig. 3), and most likely reflects the higher hydrophobicity of the CP10A sequence due to the triple Pro → Ala substitution.

The Trp fluorescence of indolicidin and CP11 decreased less compared to that of CP10A in the vesicles containing 16-DS (Fig. 5). CP11 in particular showed only 10% fluorescence decrease in the 16-DS/DMPG/DMPC/PDA vesicles (Fig. 5A) and only ∼6% quenching in 16-DS/LPS/DMPC/PDA vesicles (Fig. 5B). These data indicate very limited contact between the peptide and vesicle-embedded spin-probe, confirming that CP11 has a preferred surface localization in the two bilayers. Indolicidin fluorescence underwent quenching of approximately 18 and 10% in the 16-DS/DMPG/DMPC/PDA and 16-DS/LPS/DMPC/PDA vesicle assemblies, respectively (Fig. 5), indicating intermediate penetration into the bilayer cores.

The fluorescence quenching data depicted in Fig. 5 emphasizes the effect of LPS in promoting surface association of the peptides. Specifically, the percent fluorescence reductions recorded in the 16-DS/DMPG/DMPC/PDA-vesicle system were consistently higher for all three analogs compared to 16-DS/LPS/DMPC/PDA, indicating deeper peptide incorporation and thus closer proximity to the spin label in the former vesicles. This result is consistent with the dansyl–PMBN displacement assay (Fig. 2) and colorimetric data (Fig. 3) discussed above. It is also consistent with the concept that self-promoted uptake across the outer membrane is triggered by disruption of the electrostatic outer layer of the outer membrane through binding to anionic LPS.

3.6. Circular dichroism (CD)

Circular dichroism (CD) experiments were carried out in order to analyze the structural properties of the peptides in the lipid/PDA environments (Fig. 6). Previous studies have detected distinct conformational changes of the indolicidin analogs induced upon their interactions with model lipid bilayers [8,37]. The CD traces shown in Fig. 6 are similar to published studies using other vesicle models and indicate that all peptides underwent structural modifications to some extent upon their incorporation with the chromatic vesicles.

The CD spectra of CP11 (Fig. 6A) and indolicidin (Fig. 6B) in an aqueous buffer were characterized by a minimum at around 200 nm most likely corresponding to unordered structures [30,31]. The observed ellipticities, however, were smaller than values reported for random-coil conformations and suggest that sub-populations of ordered conformations such as β-turns or poly-L-proline helices were present in the solution [8,36]. The spectra shown in Fig. 6A and B further demonstrate that interactions of CP11 and indolicidin with both DMPG/DMPC/PDA and LPS/DMPC/PDA vesicles affected their CD traces, clearly reducing the ellipticity of the peptide. The lower ellipticities, previously observed for CP11 and indolicidin in other vesicular and micellar environments, might correspond to more pronounced secondary structures. The positive shift at around 218 nm and the minimum at 228 nm apparent in the CD spectra of CP11 in the presence of the lipid/PDA vesicles (Fig. 6A) were further consistent with higher Trp side-chain ordering of the peptide, a phenomenon detected in other vesicle systems [8,37].

The CD spectra of CP10A (Fig. 6C) were significantly different than those acquired for CP11 and indolicidin, both in the aqueous solution without vesicles as well as in the lipid/PDA environments. The CD trace of CP10A in a vesicle-free solution exhibited two minima at around 205 and 222 nm, respectively, which indicate a residual helical conformation [4]. In the presence of DMPG/DMPC/PDA vesicles or LPS/DMPC/PDA vesicles the two-minima appearance of the CD traces is retained, however, the ellipticity was again attenuated (as observed for the two other peptides, Fig. 6A and B) and shifts in the spectral positions of the minima were observed (Fig. 6C). Similar to the CD results obtained for CP11 (Fig. 6A), the positive–negative appearance of the CD trace between 215 and 230 nm is most likely indicative of an effect of the lipid moiety on Trp residues in the bound peptide [37].

Overall, the CD data depicted in Fig. 6 indicate that the structures of the indolicidin analogs are clearly affected by their binding and association with the lipid/PDA vesicles. Interestingly, similar CD traces were observed in solutions containing DMPG/DMPC/PDA vesicles or LPS/DMPC/PDA vesicles, suggesting that the differences in permeation properties between the two models was not

related to distinct structural features, but rather to specific interactions (electrostatic, hydrophobic, amphipathic) between the peptides and membrane components. Importantly, similar CD results as presented in Fig. 6 were obtained in varied published studies utilizing different vesicle systems [8,37], which additionally confirm the biological applicability of the lipid/PDA platform.

4. Discussion

Colorimetric, fluorescence and CD experiments employing biomimetic lipid/PDA vesicles provided insight into the mechanisms of membrane interactions of indolicidin analogs. The data confirmed that membrane binding and permeation depend upon the molecular composition of the bilayer. In particular, the incorporation of LPS in the bilayer clearly promoted surface interactions and localization of the peptides, an observation which supports the promoted self-uptake model pertaining to permeation of indolicidin through the outer bacterial wall.

The spectroscopic analysis points to differences in bilayer insertion among the indolicidin analogs. CP11 exhibited more pronounced interactions at the lipid/water interface, most likely accounted for by the higher positive charge on the peptide. CP10A inserted deeper into the lipid assembly compared to CP11, which is probably due to the higher propensity of the peptide towards helical structure. The experimental data highlight the significance of surface interactions for the antibacterial action of indolicidin, but also suggest that hemolytic activity requires deeper peptide insertion into the erythrocyte membrane.

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