Monovalent Salt Effects on the Membrane Activity of Antimicrobial Polymers

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Summary: Synthetic mimics of antimicrobial peptides (SMAMPs) are known to disrupt cellular membranes in aqueous media. The impact of ionic strength, or the salt concentration, on membrane activity of these SMAMPs is an important issue since some AMPs are known to lose their activity at higher salt concentrations. In this report, the effect of salt concentration on membrane activity was evaluated using fluorescence dye leakage assays. Salt concentration did not affect the membrane activity of these SMAMPs significantly except for the 100% anionic vesicles (phosphatidylglycerol (PG) or phosphatidylserine (PS) only), where membrane activity decreased with increasing salt concentration. The results also indicated that the membrane activity of SMAMPs with monoamine side chains is independent on ionic strength against cardiolipin (CL) vesicles; however, SMAMPs containing bis- or tris-amines exhibited salt concentration dependent membrane activity for CL liposomes.

Keywords: antimicrobial polymer; magainin; norbornene; phospholipids; polyelectrolyte; ROMP; salt concentration

Introduction

Natural antimicrobial peptides (AMPs), which are extracted from multicellular organisms, cover a broad range of antimicrobial activities and selectivities.[1,2] AMPs are characterized by great diversity with regard to their lengths (12–50 amino acid residues), primary amino acid sequences, and secondary structures. However, despite this significant diversity, most AMPs are cationic and generally adopt amphiphilic architectures in which the hydrophilic and hydrophobic side chains are segregated into distinct regions or faces of the molecules.[2,3] With the elucidation of this common facially amphiphilic feature of AMPs, there has been increasing research interest in the past few years toward preparing synthetic mimics of antimicrobial peptides (SMAMPs).[4–6] These include SMAMPs that are made of α- and β-amino acids,[7–9] peptoids,[10] aromatic oligomers,[11–14] and synthetic polymers.[15–24] We previously reported the synthesis of functional polynorbornenes, based on ring-opening metathesis polymerization (ROMP), that captured the biological function of AMPs.[18,20,21,25,26]

These SMAMPs are designed to be membrane active and demonstrate selectivity between prokaryotic pathogens and eukaryotic host cells. Recently, AMPs and many of their structural derivatives have been shown to differentiate between bacteria cells and mammalian red blood cells (RBCs). The origin of this selectivity is thought to arise from interactions between antimicrobial molecules and molecules present in bacterial cell membranes.[1,27] A plausible mechanism for the activity of AMPs against bacteria is based on their favorable interactions with the negatively charged and hydrophobic components of the membranes.[3,27,28] Interactions of AMPs/SMAMPs with multiple targets inside the cells and with other membrane components (e.g., Toll like membrane receptor proteins, lipopolysaccharide) are

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also possible.[2,28–30] Apart from different constituents such as membrane proteins, bacteria cell walls and RBC membranes are very dissimilar with respect to their phospholipid types, charges, compositions, and ratios. Most bacterial membranes (e.g., in *E. coli*) have 70–80% phosphatidylethanolamine (PE) as their most common zwitterionic lipid, and also contain 20–25% of negatively charged lipids such as phosphatidylglycerol (PG) and cardiolipin (CL).[4] In contrast, the outer leaflets of RBC membranes are neutral at physiological pH, and mainly composed of zwitterionic lipids such as phosphatidylcholine (PC), and sphingomyelin (SM). Human RBCs contain a relatively small amount (10%) of the negatively charged phosphatidylserine (PS) and this is usually confined to the inner leaflet of the membrane. In addition to lipid composition, lipid ordering within the membrane is also known to influence how molecules interact with the bilayer.[4,5,31,32] Because of these differences in lipid compositions between prokaryotic and eukaryotic cells, understanding the interaction of SMAMPs with specific lipid membranes is of significant and major interest.

One plausible mechanism for the activity of AMPs/SMAMPs against bacteria is based on their favorable electrostatic interactions with the negatively charged lipid head groups of the bacteria membranes.[3,27] Therefore, understanding the effects ionic strength play on SMAMP-membrane activity is worthwhile. Cell or vesicle disruption requires that the AMP/SMAMP be attracted to the membrane surface. This attraction can have a substantial electrostatic component and should therefore be attenuated with increasing salt concentrations.[33] In this report, we study the membrane leakage of a series of polymeric SMAMPs with different liposomes having the lipid compositions of bacterial cell walls or the mammalian cell membranes. Different large unilamellar vesicles (LUVs) loaded with self-quenching calcein dye, having either single or mixed lipid compositions, were prepared. The interactions of four different SMAMPs with these LUVs were characterized at different salt concentration. The results indicated that the membrane activity of SMAMPs with monoamine side chains is independent of ionic strength against mixed lipid systems; however, SMAMPs containing bis- or tris- amines exhibited salt concentration dependent membrane activity for certain lipid vesicles. In 100% anionic vesicles, SMAMP activity decreased upon increasing the salt concentration of the assay media.

Experimental Part

Materials

All chemicals were of the highest grade available from Sigma-Aldrich and were used without further purification. Lipids were obtained from Avanti Polar Lipids, Inc. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE); 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC); 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG); 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS); 1,1′,2,2′-tetraoleoyl cardiolipin (TOCL). All water used was Millipore water. Fluorescence measurements were performed on a PerkinElmer-LS55 luminescence spectrometer.

Vesicle Preparation

A 10 mL round-bottomed flask was charged with a single or mixed lipid (chloroform stock solution) composition totaling 10 mM and diluted with approximately 2 mL chloroform. The solvent was evaporated slowly at reduced pressure on a rotary evaporator and then placed under vacuum > 2 h to prepare a thin lipid film. The thin film was then hydrated for 1 h with 1 mL buffer A (40 mM calcein, 1 mM Na_2HPO_4, pH 7.0). The resulting suspension was subjected to five freeze-thaw cycles (using liquid nitrogen to freeze and warm water bath to thaw) and extruded 11 times using a mini-extruder through a polycarbonate membrane (Whatman, pore size 400 nm), stacked between two pairs of

membrane supports (Avanti). Excess calcein was removed by gel filtration through a Sephadex G-50 column equilibrated with buffer B (1 mM Na_2HPO_4, 10 mM or 100 mM NaCl, pH 7.0). The resulting 1.0 mL vesicle solution was diluted with buffer B to 5 mL to give calcein loaded LUVs stock solution having final lipid concentration of ~2 mM. Calcein-dye loaded vesicles with the following lipid compositions (molar ratio) were made: DOPE:DOPG (3:1); DOPC:DOPG (3:1); DOPS; DOPG; DOPE:TOCL (6:1).

Dye Leakage Assays

Calcein loaded LUVs (20 μL) were added to 1.98 mL buffer B (1 mM Na_2HPO_4 buffer at pH 7.0 that was varied in salt concentration from 0, 10, 50, 75, 100, and 150 mM NaCl) in a fluorescence cuvette (final lipid concentration in the cuvette 20 μM). Fluorescence emission intensity I_t ($\lambda_{em} = 510$ nm, $\lambda_{ex} = 450$ nm) was monitored as a function of time (t) over 1,000 seconds during addition of 20 μL DMSO solution of SMAMP. The maximum intensity was measured by inducing lysis of the vesicles by the addition of 20 μL 20% Triton X-100. Flux curves were normalized to percentage leakage activity Y, where $Y = [(I_t - I_0)/(I_\infty - I_0)]^*100$. I_0, is I_t before addition of SMAMP, and I_∞ is I_t after addition of 20% Triton X-100.

This procedure was repeated for each vesicle composition and SMAMPs. The antimicrobial compounds and their concentrations (μg/mL) used were the following: **Poly A1** (0.5 μg/mL); **Poly A2** (0.5 μg/mL); **Poly A3** (0.5 μg/mL); **Poly A4** (0.5 μg/mL).

Results and Discussions

The syntheses and characterization of **Poly A1**, **Poly A2**, **Poly A3** and **Poly A4** were previously reported in the literature using ROMP chemistry (see Figure 1).[20,25] The number-average molecular weights (M_n) of all polymers were approximately 11.2–12.9 kDa, and the PDIs were 1.05–1.11, as measured by GPC for the Boc-protected polymers. All these polymers are highly active against bacteria cells.[20,25] However, **Poly A2**, **Poly A3** and **Poly A4** were less hemolytic compared to **Poly A1**. To understand the role of charge density and its influence on membrane activity, we examined the polymer-induced dye leakage from different calcein dye loaded vesicles at various salt concentrations (0–150 mM). In a typical experiment, calcein loaded LUVs were added to phosphate buffer of a specified ionic strength (final lipid concentration 20 μM) and fluorescence emission intensity was monitored as a function of time after the addition of 0.5 μg/mL SMAMPs. 100% lysis was achieved by Triton X-100 at the end of each experiment, and %leakage was determined (see Figure 2A). These measurements were performed for different vesicle compositions at each salt concentration for all four SMAMPs **Poly A1-4**. Six different NaCl concentrations (0 mM, 10 mM, 50 mM, 75 mM, 100 mM, and 150 mM) were used. The % leakage at 800s was plotted against the salt concentration, which is exemplified in Figure 2B.

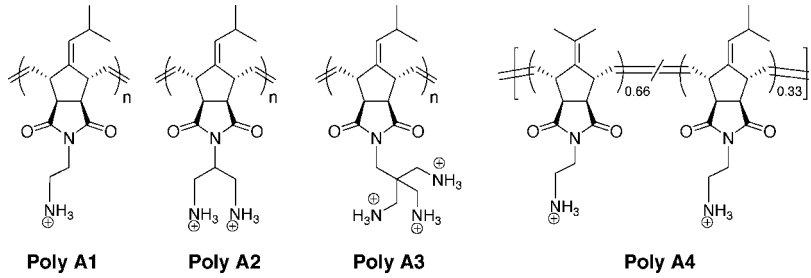

Figure 1.
The homopolymers and copolymers studied.

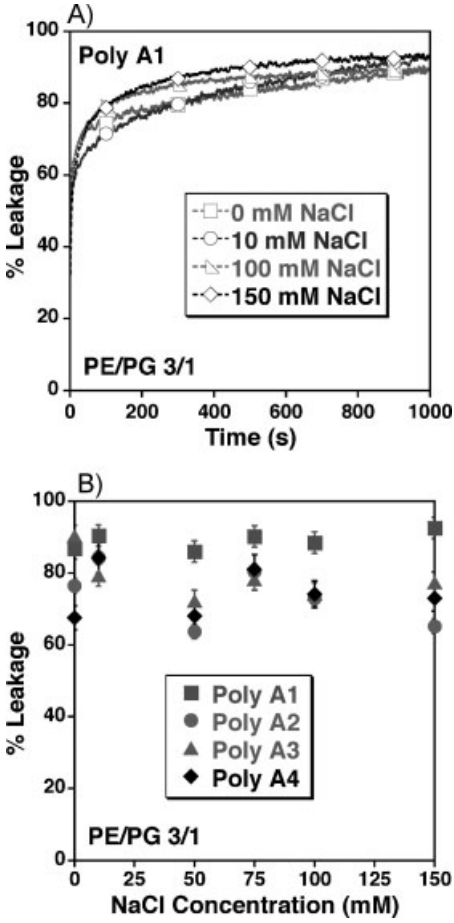

Figure 2.
(A) Percentage (%) of calcein dye leakage from 3:1 PE/PG liposomes; final lipid concentration is 20 μM. The dye leakage experiments were performed in 10 mM phosphate and 0–150 mM NaCl buffer (pH 7.0). At $t = 0$ s, 0.5 μg/mL polymeric SMAMP (in DMSO) was added, and at $t = 1000$ s, 20% Triton X-100 was added to provide 100% leakage. (for simplicity the raw data for only four salt concentrations has been plotted). (B) Effect of NaCl concentration on the PE/PG membrane activity of SMAMPs. The value of % leakage at 800 s was plotted against different salt concentrations.

To understand the interaction of these SMAMPs with bacteria membrane at different ionic strength, dye leakage experiments were performed in 3:1 PE/PG vesicles (Figure 2A and 2B). 3:1 PE/PG vesicles are close mimics of Gram-negative bacterial membranes.[4] No significant difference in membrane activity was observed with increasing salt concentration for any of the SMAMPs. If electrostatic interactions are central to the membrane activity of the SMAMPs, the membrane activity would be expected to attenuate with increasing salt concentration as at higher salt concentration SMAMP (cationic)-membrane surface (anionic) interactions would be masked.

The outer surfaces of red blood cell membranes are rich with PC which is zwitterionic lipid. In order to study the effect of salt concentration on PC containing membranes, dye leakage experiments were performed in 3:1 PC/PG vesicles (Figure 3). The fixed PG content (25%) in this vesicle and the previous vesicle was maintained to keep the overall charge constant for both the vesicles, which is important to compare the effect of ionic strength on membrane activity, although the reader should be reminded that RBCs do not contain PG or 25% anionic lipid (PS). The result again showed no significant change in activity with changing salt concentration for any of the SMAMPs. This also supports the idea that simple electrostatic interactions (between cationic SMAMP and anionic membrane surface) may not be crucial for the PE/PG or PC/PG membrane activity of these SMAMPs.[31,32]

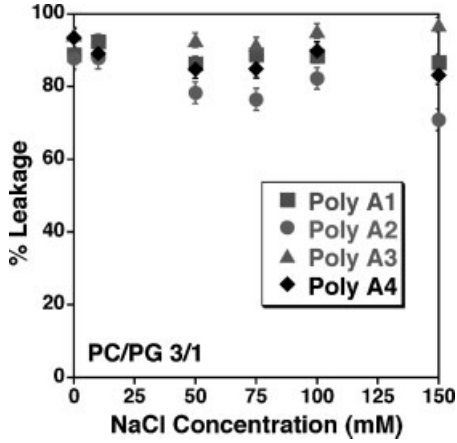

Figure 3.
Effect of NaCl concentration on the SMAMPs activity against 3:1 PC/PG membrane. The value of % leakage at 800 s was plotted against different NaCl concentrations.

However, 3:1 PE/PG and 3:1 PC/PG vesicles are mostly (75%) composed of neutral lipid (PE or PC) with only a minor amount (25%) of negatively charged PG lipid. In order to evaluate the effect of salt concentration on the electrostatic interaction between SMAMP and the membrane, liposomes were prepared composed of only negatively charged lipid (PG or PS). PG is the major negatively charged lipid found in Gram-negative bacteria membrane and PS is the major negatively charged lipid found in RBCs.[4,5] SMAMPs mediated dye leakage experiments were performed against 100% PG and 100% PS vesicles at different NaCl concentrations (Figure 4A, and 4B). Interestingly, salt concentration influenced the membrane activity of the SMAMPs for these negatively charged vesicles PG and PS. For both the vesicles, PG and PS, all four SMAMPs exhibited decreased activity with increasing salt concentration. This observation is in agreement with the importance of electrostatic interactions required for the SMAMPs activity with these vesicles. It also shows that there is little difference between PG and PS, supporting the use of PG in the previous studies with PC. At high salt concentration, the ionic interactions between the cationic SMAMPs and anionic membranes diminish resulting in less membrane leakage. Similar effects of salt concentration on the membrane activity of the SMAMPs was not observed for PE/PG and PC/PG vesicles, which contains only 25% negatively charged lipid. Most likely for PE/PG and PC/PG vesicles electrostatic interactions are suppressed by the other lipid properties (e.g., lipid intrinsic curvature) of the zwitterionic lipid (PE or PC) components. The specific role of PE in SMAMP-induced pore formation was recently reported.[14,31,32]

Gram-positive bacterial (e.g., *S. aureus*) membranes are mainly composed of anionic phospholipids cardiolipin (CL). These

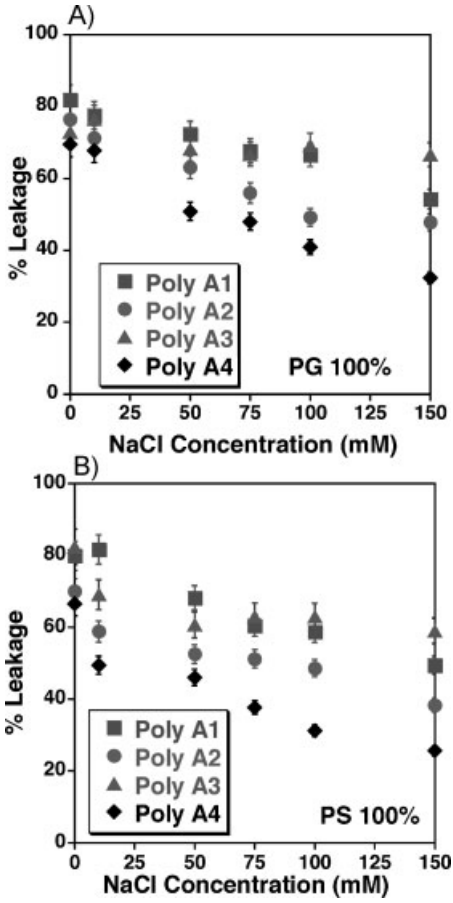

Figure 4.
Effect of NaCl concentration on the SMAMPs activity against (A) only PG membrane, and (B) only PS membrane. The value of % leakage at 800 s was plotted against different salt concentrations.

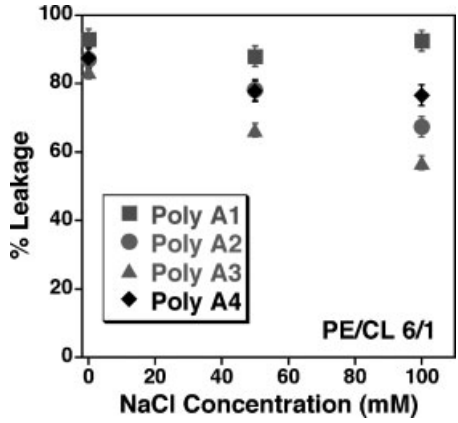

Figure 5.
Effect of NaCl concentration on the SMAMP's activity against PE/CL (6:1) vesicles. The value of % leakage at 800 s was plotted against different salt concentrations.

SMAMPs were tested against 6:1 PE/CL vesicles at different salt concentrations (Figure 5). As the dianionic lipid CL is a dimer of monoanionic lipid PG, 6:1 PE/CL lipid was used for the direct comparison with 3:1 PE/PG lipid vesicles. **Poly A1** and **Poly A4** hardly showed any change in membrane activity at varied salt concentration, similar to PE/PG system. However, the activity of **Poly A2** and **Poly A3** decreased with increasing NaCl concentrations, unlike PE/PG system. It is worth noting that **Poly A1** and **Poly A4** have only one amine group per monomer, while **Poly A2** and **Poly A3** have two and three amine groups, respectively per monomer. In addition, the decrease is larger for **Poly A3** than **Poly A2**. This observation suggests that higher amine (charge) density on the polymer repeat units exhibits electrostatic interaction with dianionic CL membrane even in the presence of other lipids like PE.

Conclusions

This study demonstrated the effect of salt concentration on the membrane activity of polymeric SMAMPs. Unless the liposomes contained only anionic lipids, the salt concentration did not affect the membrane activity of the SMAMPs significantly. This suggests that the membrane interaction is not crucially dominated by the Coulombic interactions when these polymers are exposed to mixed lipid system. Coulombic interactions between these SMAMPs and the membrane become visible for 100% anionic liposomes, PG and PS. Contribution of the Coulombic interactions also shows up for PE/CL vesicles when there are more than one amine group is present in the repeat units of the SMAMPs (**Poly A2** and **Poly A3**).

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