Revealing the Hidden Relationship Between Pore-Forming Proteins and Biomembranes

Michael H.L. Nguyen, Mitchell DiPasquale, Brett Rickeard, Drew Marquardt *Department of Chemistry & Biochemistry, University of Windsor, Canada*

The plasma membrane is a cell's first line of defense, protecting and separating important cellular content from harmful external agents. Evolutionarily, a variety of proteins have developed to destroy this barrier. For instance, amphipathic lytic peptides can embed themselves into the plasma membrane, and induce pores which can disrupt the electrochemical gradient, cell motility and shape[1], eventually leading to cell death. This property makes them viable pharmaceutical options as they display antibiotic, fungicidal and anti-tumor properties[2], and selective toxicity[3]. As strains of antibiotic- resistant bacteria continue to arise, synthetic antimicrobial peptides can provide a plethora of diverse curative agents. Thus, uncovering new mechanisms of antimicrobial peptide activity is required so that novel therapeutic peptides can be created.

A postulated mechanism these pore-inducing proteins can operate is the loss of phospholipid asymmetry in bilayers. The cell expends an enormous amount of energy establishing and maintaining this asymmetry[4]; rightly so, as it is involved in physiological functions such as blood clotting and apoptosis[5-6]. Therefore, a loss of asymmetry can act as a significant source of cell death. There are studies that claim the presence of flip-flop (where phospholipids translocate to the opposing monolayer) after the introduction of lytic peptides suggests certain pore formation[7-9]. However, research demonstrating that these pores are the direct cause of phospholipid flip-flop lack reliability because of the membrane-disruptive and interpretative techniques used. For example, fluorescence and electron spin resonance studies rely on labelled lipid to monitor transverse lipid diffusion. The issue is two-folds: the bulkiness of these probes perturbs membrane features; and the flip-flop rate measured is not that of native lipids but chemically and structurally altered lipids.

To circumvent these issues, we applied probe-free small angle neutron scattering (SANS) to monitor the rate of transverse lipid diffusion in asymmetric large unilamellar vesicles[10]. This is possible as a large scattering contrast can be generated using selective deuteration of one leaflet, exploiting the scattering difference between hydrogen and deuterium. Our results showed an "uplift" in intensity that appeared in the high q range of asymmetric vesicle scattering curves. With the addition of the pore- forming peptide, gramicidin, a contrast decay (in other words, intensity decrease) was observed, indicating a loss of phospholipid asymmetry. The gradual loss of intensity over a period of 3 days can be converted into a contrast decay curve and used to determine the flip-flop rate constant and thermodynamic properties.

Our preliminary data also showed that the incorporation method for each peptide is critically important for its insertion into the membrane, and in turn the ability to visualize flip-flop using SANS. While pre-incorporated (during the asymmetric vesicle prep[10]) gramicidin demonstrated flip- flop, other peptides, such as melittin, alamethicin, and pHLIP, when pre-incorporated into vesicle systems would precipitate out, even with low peptide-to-lipid ratios and in different solvents. Following, an alternative method of addition was conducted; peptides were added to complete aLUVs and showed almost instantaneous scrambling or occurred on a time-scale the SANS instrument was unable to detect. A new method of peptide addition must be devised to accurately determine the presence of peptide-induced flip-flop. Our work demonstrates a novel use of SANS to biologically relevant systems and is helping uncover new insights into proteolipidic research.

References

[1] Beven L. *et al.* Effect of natural amphipathic peptides on viability, membrane potential, cell shape and motility of mollicutes. *Research in Microb*. Volume 148, 1997, Issue 2, pp. 163-175.

[2] Leuschner C. *et al.* Membrane Disrupting Lytic Peptides for Cancer Treatments. *Curr. Pharm. Design*. Volume 10, 2004, Number 19, pp. 2299-2310(12).

[3] Schweizer F. Cationic amphiphilic peptides with cancer-selective toxicity. *Eur. J. of Pharm*. Volume 625(1-3) 2009, pp. 190-194.

[4] Seigneuret M. *et al.* ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc Natl Acad Sci* USA 1984;81:3751.

[5] Zwaal RFA. Membrane and lipid involvement in blood coagulation. *Biochim Biophys Acta* 1978;515:163.

[6] Martin SJ. *et al.* Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995;182:1545.

[7] Sobko, AA., *et al*. Lipid Dependence of the Channel Properties of a Colicin E1-Lipid Toroidal Pore. *Journal of Biological Chemistry, 281*(20), 2006,14408-14416.

[8] Yoneyama, F., *et al*. Peptide-Lipid Huge Toroidal Pore, a New Antimicrobial Mechanism Mediated by a Lactococcal Bacteriocin, Lacticin Q. *Antimicrobial Agents and Chemotherapy,53*(8), 2009, 3211-3217.

[9] Raghava, S., *et al.* SV40 Late Protein VP4 Forms Toroidal Pores To Disrupt Membranes for Viral Release. *Biochemistry,52*(22), 2013, 3939-3948.

[10] Heberle F. *et al.* Subnanometer Structure of an Asymmetric Model Membrane: Interleaflet Coupling Influences Domain Properties. *Langmuir*, *32* (20), 2016, pp 5195–5200.