Published in final edited form as:

J Phys Chem B. 2008 March 20; 112(11): 3495–3502. doi:10.1021/jp077487j.

Influence of Lipid Composition on Membrane Activity of Antimicrobial Phenylene Ethynylene Oligomers

Abhigyan Som and Gregory N. Tew*

Department of Polymer Science and Engineering, University of Massachusetts, Amherst, 120 Governors Drive, Amherst, Massachusetts 01003

Abstract

Host defense peptides (HDPs), part of the innate immune system, selectively target the membranes of bacterial cells over that of host cells. As a result, their antimicrobial properties have been under intense study. Their selectivity strongly depends on the chemical and mostly structural properties of the lipids that make up different cell membranes. The ability to synthesize HDP mimics has recently been demonstrated. To better understand how these HDP mimics interact with bilayer membranes, three homologous antimicrobial oligomers (AMOs) **1–3** with an *m*-phenylene ethynylene backbone and alkyl amine side chains were studied. Among them, AMO **1** is nonactive, AMO **2** is specifically active, and AMO **3** is nonspecifically active against bacteria over human red blood cells, a standard model for mammalian cells. The interactions of these three AMOs with liposomes having different lipid compositions are characterized in detail using a fluorescent dye leakage assay. AMO **2** and AMO **3** caused more leakage than AMO **1** from bacteria membrane mimic liposomes composed of PE/PG lipids. The use of *E. coli* lipid vesicles gave the same results. Further changes of the lipid compositions revealed that AMO **2** has selectively higher affinity toward PE/PG and *E. coli* lipids than PC, PC/PG or PC/PS lipids, the major components of mammalian cell membranes. In contrast, AMO **3** is devoid of this lipid selectivity and interacts with all liposomes with equal ease; AMO **1** remains inactive. These observations suggest that lipid type and structure are more important in determining membrane selectivity than lipid headgroup charges for this series of HDP mimics.

Introduction

Antibacterial molecules, a main component of innate immunity, are of immense importance to resist contagious diseases. By definition, host defense peptides (HDPs) are a class of membrane active peptides that exhibit a broad spectrum of antimicrobial activity and are nontoxic to mammalian cells.¹ Animals defend themselves against invading pathogenic microorganisms by utilizing cationic HDPs, which rapidly kill various microorganisms without exerting toxicity against the host cells. The ability of amphiphilic molecules to insert into membranes is an active area of research, and recently, the development of HDP mimics has been reviewed.^{2,3} “Why and how are peptide lipid interactions utilized for self-defense” was studied by Matsuzaki for α -helical HDPs such as Magainin-2, PGLa, Ceropin-P1, and β -sheet peptide Tachyplesin-1.^{4,5} Different modes of membrane interactions by HDPs, such as *toroidal pore*, *barrel stave*, and *carpet* models have been extensively studied and reviewed.^{6–8} In recent years, research in the area of biomimetic facially amphiphilic molecules have proven that they are good candidates for the development of a new class of antibiotics.^{9–12}

These HDP mimics are designed to be membrane active and demonstrate selectivity between prokaryotic pathogens and eukaryotic host cells. Recently, HDPs and many of their structural derivatives, such as α -peptides,^{13–17} peptoids,¹⁸ cyclic and noncyclic peptides containing D-, L-amino acids,^{19,20} arylamides,⁹ and phenylene ethynylenes,²¹ 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 lipids that are present in cell membranes. Interactions of AMOs/HDPs with multiple targets inside the cell are also possible.⁶ Apart from different constituents such as membrane proteins, bacteria cell walls and RBC membranes are very dissimilar with respect to their phospholipid types, compositions, and ratios.²² 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).^{23–27} In contrast, the outer leaflets of RBC membranes are neutral at physiological pH, and mainly composed of zwitterionic lipid such as phosphatidylcholine (PC), and sphingomyelin (SM). Human RBCs contain a relatively small amount (~10%) of the negatively charged phosphatidylserine (PS), usually confined to the inner leaflet of the membrane. In addition to lipid composition, lipid ordering within the membrane can have a profound influence on how molecules interact with the bilayer.²² The ability of lipids to organize into the fundamental bilayer membrane is dictated by their amphiphilic character and intrinsic curvature (C_0). This intrinsic curvature creates membrane domains and organizes various membrane activities associated with cell survival. The membrane curvature is controlled by the size ratio of the hydrophilic head group to the hydrophobic acyl tails of the lipid molecules.^{10,28} Because of these factors and the differences in lipid compositions between prokaryotic and eukaryotic cells, understanding the interaction of antimicrobial molecules with lipid membranes is of significant and major interest. The general structure and volume ratio of head group to acyl tails of some important lipids (PE, PC, SM, PG, PS, and CL), along with their intrinsic curvature, are shown in Figure 1.

The mode of action of synthetic antibacterial molecules with bilayer membranes is still unclear for many systems. It is believed that the amphiphilicity of AMOs/HDPs is likely responsible for their interactions with membranes. One possible scenario involves electrostatic interaction of the cationic part of AMOs/HDPs with the negatively charged outer leaflet of the bacterial membrane. However, recent studies showed that lipid type and the intrinsic lipid curvature (C_0) are critically important factors for the activity of AMOs/HDPs at the lipid membrane interface.^{26,29–31} Lohner and co-worker demonstrated that protegrin-1 can discriminate between liposomes of the different anionic phospholipids having the same net charge.³² A variety of biophysical techniques have been employed to probe the interactions between antimicrobials (HDPs, antimicrobial polymers, and oligomers) and model bilayer membranes, including X-ray scattering, solid-state NMR, differential scanning calorimetry, vibrational spectroscopy, calorimetry, microscopy studies, and so forth.¹⁰ The fluorescence dye leakage assay is one of the earliest and most widely used techniques to detect HDP-membrane interactions. Many HDPs and their synthetic mimics are able to bind with and cause leakage from lipid vesicles thereby releasing entrapped fluorescence dyes such as dextran, calcein, carboxyfluorescein, and other probes.^{33–37} However, it is surprising that the literature contains many fewer examples of dye leakage experiments designed to examine the effect of lipid composition on the activity of antimicrobial molecules. The differences in bacteria cells and eukaryotic cells are often not captured carefully (or even closely sometimes) in the dye leakage experiments. The approach used here involves many-component lipid mixtures in an effort to more closely mimic natural cell membranes. Although these models do not capture the protein content of natural membranes, they are already more scientifically interesting, yet complex.

In this report, we describe the interaction of a series of AMOs, by varying only the alkane linker, (**1–3**, Figure 2) with different liposomes, aimed to mimic either bacterial cell walls or the mammalian cell membranes. The use of lipopolysaccharide (LPS), although an extremely important component, is not discussed here since the focus is on AMO-lipid interactions of the core bilayer membrane. AMO **2** is known to bind LPS.³⁸ Different large unilamellar vesicles (LUVs) loaded with self-quenching calcein dye, having either single or mixed lipid compositions, were prepared. The interactions of three different AMOs with these LUVs were characterized. These studies explicitly show that the lipid composition, and not lipid charge, of the membrane significantly influences the level of AMO's interactions. The direct implication that PE concentration is critical to AMO-membrane interaction implies negative intrinsic curvature ($C_0 < 0$) may be an important feature of AMO activity. In vitro biological activity studies revealed that AMO **2** had low MIC (0.8 $\mu\text{g/mL}$) and high selectivity between bacterial cells and human RBCs ($\text{HC}_{50}/\text{MIC} = 93$), AMO **1** showed no activity toward either bacterial cells or RBCs, whereas, AMO **3** was found to be active against both bacterial cells and RBCs ($\text{MIC} = 1.6 \mu\text{g/mL}$, $\text{HC}_{50}/\text{MIC} = 2.0$).³⁹ Dye leakage experiments from different artificial liposomes were conducted in order to gain an insight on the interactions of AMO **1**, **2**, and **3** with lipid membranes.

Experimental Section

General reagents for syntheses were purchased from Sigma-Aldrich. Column chromatography was carried out on silica gel 60 (Fluka). Analytical TLC was performed with a silica gel 60 coated aluminum plate (Fluka). ^1H NMR spectra were obtained on a 300 MHz Bruker DPX-300 NMR spectrometer. Fluorescence measurements were performed on a Perkin-Elmer-LS55 luminescence spectrometer and Jobin Yvon Fluorolog-3. Phospholipids, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl ethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG); 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS), *E. coli* total lipid extract, and egg-L- α -lysophosphatidylcholine (EYPC) were purchased from Avanti Polar Lipids Inc. Calcein was purchased from Sigma, all salts and buffers of the best grade were available from Sigma-Aldrich, and used as received.

Synthesis and Characterization of AMOs

AMO **2** was synthesized using a technique similar to that reported for *m*-phenylene ethynylene molecules, and characterization of AMO **2** has been reported elsewhere.^{40–43} AMO **1** and **3** were synthesized using a similar procedure.⁴² In brief, the dibromoethylamine monomer was coupled with diethynylenebenzene using Sonogashira coupling to obtain Boc-protected AMO (General procedure A). An air-free Schlenk flask equipped with a magnetic stir bar was dried in an oven and then cooled under N_2 in a vacuum desiccator. *m*-Diethynylbenzene (1.34 mmol, 0.25 equiv) was weighed directly into the Schlenk flask. The monomer (5.38 mmol, 1 equiv.), $\text{Pd}(\text{PPh}_3)_4$ (0.16 mmol, 0.03 equiv.), and CuI (0.32 mmol, 0.06 equiv.) were added to the flask. The flask was then returned to positive nitrogen pressure. Distilled diisopropylamine (DIPA) (17.3 mL, 123.6 mmol, 23.0 equiv) and dry toluene (60 mL) were added via a syringe. The reaction was then placed in an oil bath at 65 $^\circ\text{C}$ and stirred overnight. The reaction mixture was evaporated to dryness and the product was purified by column chromatography, first eluting with dichloromethane, followed by 1% acetone/dichloromethane to yield a white solid (55–70%).

The Boc-protected AMO was treated with 4M HCl in dioxane at 0 $^\circ\text{C}$ to obtain free amine (general procedure B). The reaction mixture was allowed to warm up to room temperature and stirred overnight. The solvent was evaporated under reduced pressure and the solid was titrated with ether three times and dried under vacuum to produce a white solid AMO (quantitative yield). Purity of the product was 98% as checked by HPLC.

Boc-protected AMO **1**: ^1H NMR (CDCl_3): 7.70 (t, 1H, phenyl H), 7.65 (t, 2H, phenyl H), 7.52 (m, 3H, phenyl H), 7.49 (m, 3H, phenyl H), 7.40–7.37 (m, 1H, phenyl H), 5.45 (s, 2H, NH), 5.07 (s, 4H, 2CH_2), 1.52 (s, 18H, 6CH_3); MS (m/z) [$\text{M}^+ + \text{Na}$] 719.2, [$\text{M}^+ + \text{Na} - t\text{-butyl}$] 663.2, [$\text{M}^+ + \text{Na} - 2t\text{-butyl}$] 607.1. Boc-protected AMO **1** was deprotected following general procedure B to yield **1**. ^1H NMR ($\text{DMSO}-d_6$): 7.94 (s, 4H, 2NH_2), 7.79 (m, 1H, phenyl H), 7.70–7.62 (m, 3H, phenyl H), 7.61 (d, 1H, phenyl H), 7.57 (m, 2H, phenyl H), 7.51 (s, 3H, phenyl H), 4.52 (s, 4H, 2CH_2).

Boc-protected AMO **3**: ^1H NMR (CDCl_3): 7.69 (t, 1H, phenyl H), 7.53 (m, 2H, phenyl H), 7.49 (m, 2H, phenyl H), 7.37 (d, 1H, phenyl H), 7.33 (m, 1H, phenyl H), 7.30 (m, 2H, phenyl H), 7.28 (s, 1H, phenyl H), 4.57 (s, 2H, 2NH), 3.19 (m, 4H, 2CH_2), 2.64 (t, 4H, 2CH_2), 1.84 (m, 4H, 2CH_2), 1.47 (s, 18H, 6CH_3); MS (m/z): [$\text{M}^+ + \text{Na}$] 773.3, [$\text{M}^+ + \text{Na} - t\text{-butyl}$] 717.2, [$\text{M}^+ - \text{Boc}$] 649.1, [$\text{M}^+ + \text{Na} - 2\text{Boc}$] 572.2. Boc-protected AMO **3** was deprotected following general procedure B to yield AMO **3**. ^1H NMR ($\text{DMSO}-d_6$): 7.89 (s, 4H, 2NH_2), 7.77 (m, 1H, phenyl H), 7.65–7.63 (m, 3H, phenyl H), 7.61 (d, 1H, phenyl H), 7.56 (m, 2H, phenyl H), 7.48 (m, 3H, phenyl H), 2.77–2.67 (m, 8H, 4CH_2), 1.87 (m, 4H, 2CH_2).

Antimicrobial Studies

The antimicrobial activities of these compounds were evaluated using standard methods.⁴⁴ Stock solutions were made in DMSO and then diluted into 96-well plates with Mueller Hinton (MH) medium to a constant volume. All bacteria (used cell lines: *E. coli* D31, *S. aureus*, ATCC 25923) were either taken from stock glycerol solutions or from a frozen stock, diluted into MH medium, and grown overnight at 37 °C. Sub-samples of these cultures were grown for 3 h, the OD_{600} was measured and then the cells were diluted to 0.001 OD_{600} . The diluted cell solutions (approximately 105 cells/mL) were then added to the 96-well plate and incubated at 37 °C for 6 h. The MIC values reported are the minimum concentration necessary to inhibit 90% of the cell growth. Experiments were run in duplicate.

Hemolysis Studies

Fresh human erythrocytes were obtained by centrifuging whole blood (3000 rpm, 10 min) to remove plasma and white blood cells. The RBCs (1 mL) were diluted with 9 mL of TBS buffer (10 mM Tris buffer, pH = 7.0, 150 mM NaCl), and this suspension was further diluted by a factor of 40 to give a RBC stock suspension (0.25% blood cells). This RBC stock (120 μL), TBS buffer (15 μL), and the AMO stock solutions (15 μL) (or control solutions) were added to a 200 μL centrifugation tube and incubated at 37 °C for 1 h. The tube was centrifuged at 4000 rpm for 5 min. Supernatant (30 μL) was diluted with TBS buffer (100 μL), and OD_{414} of the solution was measured as hemoglobin concentration. A control solution containing only DMSO was used as a reference for 0% hemolysis. Complete hemolysis was measured by adding 1% Triton X-100 to the RBCs and measuring OD_{414} . Nonlinear exponential curve-fitting plots of OD_{414} vs AMO concentration determined the HC_{50} , the hemolytic dose required to lyse 50% of the RBCs.

Liposome Preparation

The following buffers were used: Buffer A, 40 mM calcein, 10 mM Na_2HPO_4 pH 7.0; buffer B, 10 mM Na_2HPO_4 , 90 mM NaCl, pH 7.0. Homogeneous LUVs were prepared by the extrusion method, as described in the Avanti Polar Lipid Inc. website.⁴⁵ Vesicle abbreviations with the corresponding lipid compositions, inside and outside buffers, and overall net lipid charge are listed in Table 1.

Dye loaded vesicle V_1 preparation: (general procedure C) 560 μL of DOPE solution (10 mg/mL CHCl_3) and 200 μL of DOPG solution (10 mg/mL CHCl_3) were mixed and evaporated slowly at reduced pressure on a rotary evaporator and then placed under vacuum >2 h to prepare a thin film. The thin film was then hydrated for 1 h with 1 mL buffer A. The resulting suspension was subjected to five freeze–thaw cycles (using liquid nitrogen to freeze and warm water bath to thaw) and extruded >10 times using a mini-extruder through a polycarbonate membrane (Whatman, pore size 400 nm), stacked between two pairs of membrane supports. The external calcein was removed by gel filtration (Sephadex G-50) using buffer B and the resulting 1.0 mL vesicle solution was diluted with buffer B to 5 mL to give calcein loaded 75/25 DOPE/DOPG LUVs stock solution (V_1), having final lipid concentration of ~2 mM. Following the general vesicle preparation procedure C, other vesicles V_2 – V_{14} were prepared using appropriate lipid ratios. Empty vesicles V_{15} – V_{17} , containing no dyes inside, were prepared only by slight modification of general procedure C, the thin film was hydrated with 1 mL buffer B instead of buffer A.

Dye Leakage Assays

Calcein loaded LUVs (20 μL) were added to 1.98 mL buffer B in a fluorescence cuvette (final lipid concentration in the cuvette 20 μM). Fluorescence emission intensity I_t ($\lambda_{\text{em}} = 510 \text{ nm}$, $\lambda_{\text{ex}} = 450 \text{ nm}$) was monitored as a function of time (t) during addition of 20 μL DMSO solution of AMO 1, 2, or 3 and 50 μL of 20% Triton X-100. Control experiments were performed adding only 20 μL DMSO. Flux curves were normalized to percentage leakage activity Y , where $Y = [(I_t - I_0)/(I_{\infty} - I_0)] \times 100$. I_0 is I_t before addition of samples (AMOs or DMSO), and I_{∞} is I_t after addition of 20% Triton-X.

For the c_M profile (concentration vs activity), the plot of leakage activities (Y) versus AMO 2 concentration (c_{AMO}) was fitted to the Hill equation, $Y = Y_{\infty} + (Y_0 - Y_{\infty}) / \{1 + (c_{\text{AMO}} / EC_{50})^n\}$. Where Y_0 is Y without AMO, Y_{∞} is Y with excess AMO to give the Hill coefficient n and the effective concentration EC_{50} .⁴⁶

Membrane-Potential Sensitive diSC3–5 Dye Release

In vitro, diSC3–5 dye release experiments from *S. aureus* were carried out at varied AMO 2 concentrations as described in a previous report.²¹ *S. aureus* ATCC 25923 was grown at 37 °C and the cells were collected by centrifugation, washed once with buffer (5 mM HEPES, pH 7.0, 5 mM glucose), and re-suspended to an optical density (OD_{600}) of 0.05. The cells were incubated with 0.4 μM diSC3–5 for an hour, 100 mM KCl was added to equilibrate the cytoplasmic and external potassium ion concentration. The cells were mixed with the desired concentration of AMO, and the fluorescence was monitored with an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The plot of diSC3–5 dye release activities (Y) versus AMO 2 concentration (c_{AMO}) was fitted to the Hill equation, $Y = Y_{\infty} + (Y_0 - Y_{\infty}) / \{1 + (c_{\text{AMO}} / EC_{50})^n\}$.

Results and Discussion

Antibacterial Activity of 1–3

Small amphiphilic oligomers are known to have antimicrobial activity, yet be nontoxic to human RBCs.^{42,47} AMO 2 displayed excellent antimicrobial activity against *E. coli* and *B. subtilis* with very low MIC values of 0.8 $\mu\text{g/mL}$ and 1.7 $\mu\text{g/mL}$, respectively. This highly active AMO 2 exhibited a broad spectrum of antimicrobial activity against a wide range of bacteria, yeast and fungi.²¹ *E. coli* and *B. subtilis* were used for initial screenings, since they have been shown to be reliable markers for widespread Gram-negative and Gram-positive bacteria.^{9,12,21} The corresponding MIC and HC_{50} values ($HC_{50} = 75 \mu\text{g/mL}$) of the AMOs clearly indicate that AMO 2 has very high selectivity (44 against *B. subtilis* and 93

against *E. coli*) between bacteria and RBCs. When the ethylamine linker of the specifically active AMO **2** was shortened to a single methylene (AMO **1**), the AMO lost all activity against both bacteria and RBCs. In contrast, when the ethylamine linker was lengthened to propylamine (AMO **3**), the AMO became active against both bacteria and RBCs (MIC, 1.6 $\mu\text{g/mL}$ against *E. coli*, and 3.2 $\mu\text{g/mL}$ against *B. subtilis*; $\text{HC}_{50} = 3.2 \mu\text{g/mL}$), showing no selectivity.

Interaction with PE/PG Vesicles (bacteria membrane mimic)

To better understand the basic interactions of these AMOs with bacterial membranes, artificial vesicles V_1 containing 75/25 PE/PG lipids were prepared (see Table 1 for vesicle details). Dye leakage from these vesicles showed that AMO **2** and AMO **3** caused ~80% leakage, whereas, only 10% leakage was observed for AMO **1** (Figure 3A) when all three AMOs were studied at identical concentrations (1.0 $\mu\text{g/mL}$). No leakage was observed when 20 μL of DMSO was added instead of the AMOs, indicating membrane disruption is not mediated by 1% DMSO. Likewise, a Boc-protected AMO that did not have the amine functionality exposed showed no membrane activity, which implies the amine groups present in the AMOs are necessary for the membrane activity.

Leakage activity of AMO **2** against vesicle V_1 (PE/PG 75/25) was studied at different AMO concentrations and the c_M profile (AMO concentration vs leakage activity) was generated (Figure 3B). In general, thermodynamics of pore formation are reflected in the dependence of fractional activity Y on the AMO concentration c_M as described in the logarithmic form of the Hill equation, $\log Y = n \log c_M - n \log (EC_{50})$. The Hill coefficient (n) for AMO **2** was calculated by fitting the nonlinear c_M profile into the Hill equation. For AMO **2** in PE/PG vesicles V_1 , at pH 7.0, the value of the Hill coefficient was found to be ~3. Therefore, it requires, on average, three molecules of AMO **2** to form an active structure in the bacteria mimic membrane. This is also supported by the previously reported electron density profile model, computed from small-angle X-ray scattering (SAXS) data, which showed the horizontal arrangement of ~3 AMO's per pore.²⁹ The effective AMO concentration required for 50% dye release (EC_{50}) was also obtained from the Hill equation curve fit. The calculated EC_{50} value for AMO **2** against PE/PG vesicles V_1 was found to be 1 μM , which is comparable to the EC_{50} values of natural HDPs.⁴⁸ Moreover, the c_M profile for the release of membrane potential cyanine dye diSC3-5 from *S. aureus* provided similar n and EC_{50} values (Figure 3C).

In another experiment to understand the dynamics of AMO-liposome interactions, 1.0 $\mu\text{g/mL}$ of AMO **2** was added to liposome V_1 in two different ways: (a) all at once or (b) in two 0.5 $\mu\text{g/mL}$ doses. Figure 3D shows that both experiments caused ~80% dye leakage from the liposomes. However, the sequential addition of two 0.5 $\mu\text{g/mL}$ doses showed only slightly higher leakage suggesting the type and order of addition makes little difference. This is important to understanding the dynamics of the system and here suggests at a minimum that the systems are well mixed and the process is not diffusion limited.

Interaction with PC Vesicles and PC/PS Vesicles (RBC mimic)

The outer surface of RBC membranes is rich with PC that is zwitterionic and thus overall charge neutral. Therefore, a calcein dye loaded liposome V_2 , composed of PC lipids was prepared, and AMO induced leakage activities were monitored. AMO **1** and AMO **2** showed only 9–18% activity, whereas the activity of AMO **3** against V_2 (~80%) was comparable to vesicles V_1 (Figure 4). These dye leakage studies are consistent with the biological activities (MIC and HC_{50}) of the AMOs. Biologically inactive AMO **1**, and selectively active AMO **2** did not show significant leakage activity against V_2 ; however, in contrast, nonselectively active AMO **3** showed similar affinity toward V_2 and V_1 . Since all three AMOs have two

positive charges and the fact that AMO 3 remains potentially active against neutral PC vesicles, it appears that a simple electrostatic-dominated driving force for AMO–membrane interactions is not applicable here.

To further explore the interactions of AMO 2 with PC based liposomes, calcein loaded vesicles V₃, V₄, and V₅ were prepared using different ratios of PC and PS lipids.⁴⁹ As shown in Table 2, there is little difference in leakage activity between liposomes containing only PC (PC/PS 100/00) and those containing 10 and 25 mol % PS. All of these liposomes showed 25 ± 5% dye leakage. A comparison of the three PS concentrations does show that increasing PS concentration leads to increased dye leakage but the total dye leakage is still much less than that observed for PE/PG (75/25) liposomes. These studies with PS concentrations indicate some Coulombic interactions may exist between the positively charged AMOs and negatively charged PC/PS liposomes although other interactions may be involved. Either way, to induce significantly more leakage (but still only about double) from pure, neutral PC liposomes, 50% PS must be added which is 40% more than naturally occurs in mammalian cells (RBCs contain only 10% PS).

Comparison of AMO 2 mediated leakage from liposomes V₁ (75/25 PE/PG) and V₄ (75/25 PC/PS) revealed interesting information about the mode of interactions of these AMOs with specific lipids. Vesicles with the same molar ratio of neutral and anionic lipids showed significantly different % leakage values. Surprisingly, AMO 2 is >50% more active against V₁ compared to V₄ (Table 2). The biggest differences are that zwitterionic PE is negative intrinsic curvature and zwitterionic PC is zero intrinsic curvature. Among the lipids in V₁ and V₄, only PE is capable of forming negative curvature ($C_0 < 0$), whereas all other lipids (PG, PC, PS) have zero intrinsic curvature ($C_0 = 0$) (Figure 1). In a previous report, SAXS experiments indicated that the PE/PC ratio of the target membrane directly influenced the ability of these AMOs to induce inverted hexagonal H_{II} phase pores, and AMO 2 requires a certain mole % of PE lipids for its membrane activity.²⁹ Thus, along with these dye leakage data, membrane composition rather than the net surface charge of the membrane seems like the dominating contributor to membrane activity.

Interaction with *E. coli* Vesicles

Dye leakage from the model membrane V₆, composed of pure *E. coli* lipids, was studied to evaluate the bioactivity of the AMOs. *E. coli* extract was used as a closer comparison to real bacteria membranes. The leakage activity of the AMOs against V₆ was comparable with the leakage activity against the bacteria mimic V₁ (Figures 3A and 5A). AMO 1 exhibited minimal activity, less than 20% leakage, whereas AMO 2 and AMO 3 caused ~80% leakage. These leakage data follow the biological activity trends of the AMOs as expected and indicate that PE/PG model membranes are reasonable mimics.

Interaction with *E. coli*/PC Vesicles

Previous SAXS experiments hypothesized intrinsic membrane curvature as a necessary component for membrane specificity of the AMOs.²⁹ In order to explore this outcome, AMO induced dye leakage from vesicles (V₆, V₇, V₈, V₉) composed of *E. coli* extract diluted with an increasing concentration of zero-curvature ($C_0 \approx 0$) PC lipid was studied. Change in percent leakage for each AMO was observed as the concentration of *E. coli* extract was reduced and replaced by PC (Figure 5 and Table 2). These changes are due to the increasing concentration of PC lipids in the vesicles. AMO 2 showed the largest dependence on *E. coli* volume fraction. This was substantiated by the decrease in leakage from 80% for V₆ to 37% for V₉. In contrast, AMO 3 showed approximately 72% leakage for all of the vesicles, indicating that AMO 3 has no dependence on the *E. coli* lipid volume fractions. Similarly, AMO 1 showed only a 5% decrease in leakage from V₆ to V₉ and

always exhibited considerably less dye leakage than AMO 2 or AMO 3 for all liposomes (Figure 5 and Table 2).

Interaction with PE/PG/PC Vesicles

To better comprehend the influence of lipid type in membrane selectivity, we studied all three AMOs induced dye leakage from four different vesicles (V_{10} , V_{11} , V_{12} , and V_{13}) having ternary lipid compositions with constant anionic lipid content and tunable intrinsic curvature, C_0 (see Figure 1 and Table 2). In these sets of experiments, the ratio of PE ($C_0 < 0$) to PC ($C_0 \approx 0$) lipids were varied at a fixed PG concentrations with variable PE/PG/PC ratios (80/20/00, 60/20/20, 40/20/40, 00/20/80). AMO 1 remained inactive for the entire range of PE concentrations (Figure 6A). Membrane activity of AMO 2 diminished significantly below a certain PE concentration (60%) (Figure 6B). In contrast, AMO 3 remained sufficiently active, with only a slight decrease in activity with increasing PC concentration against all four vesicles (Figure 6C). These observations further support membrane activity of these AMOs does not depend on the presence of anionic lipid in the membrane but instead on the lipid composition. These results clearly show lipid type and specifically the concentration of negative intrinsic curvature lipid (PE, $C_0 < 0$) seems to dominate membrane activity of AMO 2 and therefore selectivity of this AMO.

Competitive Binding Study and Lipid Affinity

To demonstrate the strong affinity of AMO 2 toward bacterial lipids, empty vesicles V_{15} , V_{16} , and V_{17} were prepared containing no dye inside (Table 1). In three individual sets of experiments, AMO 2 was pretreated with these three empty vesicles for an hour. The incubated AMO/vesicle mixture was added to the dye loaded V_1 (75/25 PE/PG) to observe the leakage activity (Figure 7). Once AMO 2 interacts with PE/PG or *E. coli* lipid (V_{15} or V_{16}), the AMO is no longer available for the newly added V_1 vesicles and induced no leakage. This result indicates irreversible interaction of AMO 2 with both PE/PG vesicles (V_{15}) and *E. coli* vesicles (V_{16}). However, when AMO 2 is pretreated with PC/PS vesicles (V_{17}), it interacted with newly added PE/PG vesicles (V_1) (Figure 7). This suggests AMO 2 either does not bind appreciably or binds reversibly to PC/PS liposomes. This set of data supports the strong affinity of AMO 2 toward bacterial lipid (PE/PG or *E. coli*), even if other lipids are present in the medium and is consistent with previous in vitro experiments in which the MIC was not adversely impacted by the presence of RBCs.⁴²

AMO to Lipid Ratios (P/L)

Most biophysical techniques observed a threshold P/L , ranging from 1/200 to 1/10, which causes a major change in HDP/AMO-membrane interactions.⁵⁰ Taking into consideration that in a typical MIC experiment there are about 10^5 cells in 1 mL and each cell comprises 2.2×10^7 lipids; the calculated P/L at the MIC (0.8 $\mu\text{g/mL}$) for AMO 2 is 1/0.003 (or 333/1). Although the dye leakage experiments were conducted at $P/L \approx 1/10$ (or 0.1/1), this is still 3000 $\times$ lower than the MIC experiments. Therefore, it is likely that the observed membrane perturbation occurring at these low P/L ratios in the dye leakage experiments will also occur at the MIC. It is also consistent with the known ability of AMO 2 to bind LPS which would require a higher concentration of AMO to kill bacteria.³⁸

Summary of Lipid Selectivity

A summary of dye leakage from different phospholipid vesicles induced by AMOs is presented in Table 2. The study shows that subtle changes in the chemical structure of three AMOs give different behaviors associated with the lipid bilayer interaction. Selectively active AMO 2 induced the highest activity against both PE/PG and *E. coli* vesicles, which have overall negatively charged surfaces but also showed much lower activity against

neutral PC and anionic PC/PS vesicles. PC/PS vesicles have similar surface charge compared to PE/PG vesicles yet AMO 2 was at least three times as active against PE/PG. Interestingly, this high activity of AMO 2 was also observed against PE/PS vesicles, V_{14} demonstrating the role of PG is not critical. Furthermore, the dye leakage activity from different *E. coli*/PC vesicles proved that a certain concentration of *E. coli* lipid is necessary for the activity of the AMOs. Conversely, AMO 3 is active against both anionic vesicles (PE/PG or *E. coli*) and neutral vesicles (PC). Dye leakage studies from the liposomes having ternary lipid compositions (PE/PG/PC) revealed that a threshold concentration of PE lipids, not the net charge of the membrane, drives the membrane activity of AMO 2. AMO 1 remains inactive, and AMO 3 is able to show activity even at low PE concentrations. The lipid affinity study demonstrated that AMO 2 interacts strongly with PE/PG or *E. coli* liposomes even in the presence of PC/PS liposomes. All of these findings strongly support the idea that specific lipid types influence AMO-membrane interactions, and negative intrinsic curvature lipids (PE) play an important role in membrane selectivity. It appears that binding of AMO 2 induces a local change in the concentration of negative intrinsic curvature lipids which in turn destabilize the membrane leading to bacterial death.

Conclusion

The dye leakage experiments conclude that AMOs selectively interact with particular types of membrane lipids. The nature of this membrane selectivity is dominated by the intrinsic lipid curvature although some Coulombic AMO–lipid interaction or other specific interactions with PE cannot be completely ruled out. While this study has focused almost exclusively on PE (i.e., Gram-negative bacteria models), recent work within the group studying CL (i.e., Gram-positive bacteria models) also appears to implicate negative intrinsic curvature. The unique membrane activity of these homologous AMOs is likely to provide insight into other membrane phenomena such as fusion, translocation, drug delivery, endocytosis, and so forth.

Acknowledgments

We thank the NIH (RO1-GM-65803), the ONR (N00014-03-1-0503), and PolyMedix Inc. for financial support.

Abbreviations

AMO	antimicrobial oligomers
C_0	Intrinsic lipid curvature
CL	cardiolipin
DO	dioleoyl
EC	effective concentration
HC₅₀	hemolytic concentration
HDP	Host defense peptides
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
OD	optical density
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PG	phosphatidylglycerol
PS	phosphatidylserine
RBC	red blood cell
SAXS	small angle X-ray scattering
SM	sphingomyelin

References and Notes

1. Zasloff M. *Nature*. 2002; 415:389. [PubMed: 11807545]
2. Cheng RP, Gellman SH, DeGrado WF. *Chem Rev*. 2001; 101:3219. [PubMed: 11710070]
3. Patch JA, Barron AE. *Curr Opin Chem Biol*. 2002; 6:872. [PubMed: 12470744]
4. Matsuzaki K. *Biochim Biophys Acta*. 1999; 1462:1.
5. Matsuzaki K. *Biochem Soc Trans*. 2001; 29:598. [PubMed: 11498035]
6. Brogden KA. *Nature*. 2005; 3:238.
7. Yount NY, Bayer AS, Xiong YQ, Yeaman MR. *Biopolymers*. 2006; 84:435. [PubMed: 16736494]
8. Oren Z, Shai Y. *Biopolymers*. 1999; 47:451. [PubMed: 10333737]
9. Tew GN, Liu DH, Chen B, Doerksen RJ, Kaplan J, Carroll PJ, Klein ML, DeGrado WF. *Proc Natl Acad Sci USA*. 2002; 99:5110. [PubMed: 11959961]
10. Gabriel GJ, Som A, Madkour AE, Eren T, Tew GN. *Mater Sci Eng R*. 2007; R57:28.
11. Liu DH, Choi S, Chen B, Doerksen RJ, Clements DJ, Winkler JD, Klein ML, DeGrado WF. *Angew Chem, Int Ed*. 2004; 43:1158.
12. Tang H, Doerksen RJ, Jones TV, Klein ML, Tew GN. *Chem Biol*. 2006; 13:427. [PubMed: 16632255]
13. Porter EA, Wang XF, Lee HS, Weisblum B, Gellman SH. *Nature*. 2000; 404:565. [PubMed: 10766230]
14. Raguse TL, Porter EA, Weisblum B, Gellman SH. *J Am Chem Soc*. 2002; 124:12774. [PubMed: 12392424]
15. Liu DH, DeGrado WF. *J Am Chem Soc*. 2001; 123:7553. [PubMed: 11480975]
16. Hamuro Y, Schneider JP, DeGrado WF. *J Am Chem Soc*. 1999; 121:12200.
17. Porter EA, Weisblum B, Gellman SH. *J Am Chem Soc*. 2002; 124:7324. [PubMed: 12071741]
18. Patch JA, Barron AE. *J Am Chem Soc*. 2003; 125:12092. [PubMed: 14518985]
19. Fernandez-Lopez S, Kim HS, Choi EC, Delgado M, Granja JR, Khasanov A, Kraehenbuehl K, Long G, Weinberger DA, Wilcoxon KM, Ghadiri MR. *Nature*. 2001; 412:452. [PubMed: 11473322]
20. Oren Z, Shai Y. *Biochemistry*. 1997; 36:1826. [PubMed: 9048567]
21. Arnt L, Rennie JR, Linser S, Willumeit R, Tew GN. *J Phys Chem B*. 2006; 110:3527. [PubMed: 16494408]
22. Vance, DE.; Vance, J. *Biochemistry of Lipids, Lipoproteins and Membranes*. 2. Vol. 20. Elsevier Science; Amsterdam: 1991.
23. Glukhov E, Stark M, Burrows LL, Deber CM. *J Biol Chem*. 2005; 280:33960. [PubMed: 16043484]
24. Dathe M, Nikolenko H, Meyer J, Beyermann M, Bienert M. *FEBS Lett*. 2001; 501:146. [PubMed: 11470274]
25. Kol MA, van Laak ANC, Rijkers DTS, Killian JA, de Kroon AIPM, de Kruijff B. *Biochemistry*. 2003; 42:231. [PubMed: 12515559]
26. Epand RF, Schmitt MA, Gellman SH, Epand RM. *Biochim Biophys Acta*. 2006; 1758:1343.
27. Clejan S, Krulwich TA, Mondrus KR, Seto-Young D. *J Bacteriol*. 1986; 168:334. [PubMed: 3093462]
28. McMahon HT, Gallop JL. *Nature*. 2005; 438:590. [PubMed: 16319878]

29. Yang L, Mishra A, Purdy K, Som A, Tew GN, Wong GCL. *J Am Chem Soc.* 2007; 129:12141. [PubMed: 17880067]
30. Matsuzaki K, Sugishita K, Ishibe N, Ueha M, Nakata S, Miyajima K, Epand RM. *Biochemistry.* 1998; 37:11856. [PubMed: 9718308]
31. Epand RF, Martinou JC, Fornallaz-Mulhauser M, Hughes DW, Epand RM. *J Biol Chem.* 2002; 277:32632. [PubMed: 12082098]
32. Jing WG, Prenner EJ, Vogel HJ, Waring AJ, Lehrer RI, Lohner K. 2005; 11:735.
33. Zhao HX, Mattila JP, Holopainen JM, Kinnunen PKJ. *Biophys J.* 2001; 81:2979. [PubMed: 11606308]
34. Matsuzaki K, Yoneyama S, Miyajima K. *Biophys J.* 1997; 73:831. [PubMed: 9251799]
35. Ladokhin AS, Selsted ME, White SH. *Biophys J.* 1997; 72:1762. [PubMed: 9083680]
36. Kang JH, Shin SY, Jang SY, Lee MK, Hahm KS. *J Pep Res.* 1998; 52:45.
37. Hristova K, Selsted ME, White SH. *J Biol Chem.* 1997; 272:24224. [PubMed: 9305875]
38. Beckloff N, Laube D, Castro T, Furgang D, Park S, Perlin D, Liu DCD, Tang H, Scott RW, Tew GN, Diamond G. *Antimicrob Agents Chemother.* 2007; 51:4125. [PubMed: 17785509]
39. MIC is the minimum concentration (in $\mu\text{g/mL}$) of the compound required to inhibit bacteria growth by 90%. The hemolytic concentration, HC_{50} , is the concentration (in $\mu\text{g/mL}$) at which 50% of the red blood cells (RBCs) lyse. The ratio between HC_{50} and MIC is termed as selectivity.
40. Arnt L, Nusslein K, Tew GN. *J Polym Sci Part A: Polym Chem.* 2004; 42:3860.
41. Arnt L, Tew GN. *J Am Chem Soc.* 2002; 124:7664. [PubMed: 12083913]
42. Nusslein K, Arnt L, Rennie J, Owens C, Tew GN. *Microbiol.* 2006; 152:1913.
43. The synthesis and commercial use of AMO-2 are protected by US Provincial Patent WO-02/072007-A2.
44. Rennie J, Arnt L, Tang HZ, Nusslein K, Tew GN. *J Ind Microbiol Biotechnol.* 2005; 32:296. [PubMed: 15959729]
45. <http://www.avantilipids.com/ExtrusionTechnique.html>.
46. Litvinchuk S, Bollot G, Mareda J, Som A, Ronan D, Shah MR, Perrottet P, Sakai N, Matile S. *J Am Chem Soc.* 2004; 126:10067. [PubMed: 15303883]
47. Liu C, Xiao C. *J Appl Polym Sci.* 2004; 93:1868.
48. Wieprecht T, Apostolov O, Beyermann M, Seelig J. *Biochemistry.* 2000; 39:442. [PubMed: 10631006]
49. In RBC membranes, PS lipids are found only in the inner leaflet.
50. Huang HW. *Biochim Biophys Acta.* 2006; 1758:1292q.

Figure 1.

Zwitterionic lipids PE, PC, SM, and anionic lipids PG, PS, CL. The lipid molecules contain a polar phosphate head group and hydrophobic fatty acyl tails R_1 and R_2 (e.g., in *DOPG* lipids, $R_1 = R_2 =$ oleoyl). The head group of PE is smaller compared to its acyl tails, thereby making PE a negative intrinsic curvature lipid ($C_0 < 0$) having an inverted cone shaped conformation. PC, PG, and PS are zero intrinsic curvature lipids ($C_0 \approx 0$) with cylindrical conformation because of their similar width of polar head group and hydrophobic tails. SM is structurally similar to PC and CL is essentially a dimer of PG. Therefore, these two lipids were not included in this study but are under investigation in our laboratory.

Figure 2. Amphiphilic phenylene ethynylene based antimicrobial oligomers (AMOs). AMO **1** is nonactive, AMO **2** is specifically active (low MIC, high HC_{50}), and AMO **3** is nonspecifically active (low MIC, low HC_{50}) against bacteria over human red blood cells (RBCs).

Figure 3.

(A) Percentage of calcein dye leakage from 75/25 PE/PG vesicles (V_1) (lipid concentration 20 μM) upon addition of 1.0 $\mu\text{g/mL}$ AMO 1 (green), AMO 2 (red), and AMO 3 (blue). (B) Concentration dependent leakage curve (data taken from the leakage curves at 800 s) for AMO 2 against V_1 , the curve was fitted to the Hill equation. (C) AMO 2 concentration dependent release of membrane potential cyanine dye diSC3-5 from *S. aureus* (data taken from the leakage curves at 300 s), this curve was also fitted to the Hill equation. (D) Stepwise addition of AMO 2 to V_1 . Step 1: leakage caused by the addition of 0.5 $\mu\text{g/mL}$ AMO 2 (blue), step 2: increase of leakage activity upon addition of extra 0.5 $\mu\text{g/mL}$ AMO 2, in the same experiment (blue). One time addition (without any steps) of 1.0 $\mu\text{g/mL}$ AMO 2 provided 80% leakage from V_1 (red).

Figure 4. The percent leakage of calcein dye from PC vesicles (V_2) upon addition of $1.0 \mu\text{g/mL}$ AMO 1 (green), AMO 2 (red), AMO 3 (blue). Final lipid concentration is $20 \mu\text{M}$ for all the liposomes.

Figure 5. Calcein dye leakage from *E. coli*/PC 100/0 (A), 50/50 (B), 33/66 (C) vesicles promoted by 1.0 $\mu\text{g/mL}$ AMO 1 (green), 2 (red), and 3 (blue). Only AMO 2's (red) activity changes significantly as the concentration of PE lipids changes. Final lipid concentration is 20 μM for all the liposomes.

Figure 6. Calcein dye leakage from PE/PG/PC 80/20/00 (black), 60/20/20 (red), 40/20/40 (green), 00/20/80 (blue) vesicles (final lipid concentration is 20 μM) promoted by 1.0 $\mu\text{g/mL}$ AMO 1 (A), AMO 2 (B), and AMO 3 (C). AMOs with different biological activities require different concentration of PE lipids in the target membrane to induce sufficient leakage. Final lipid concentration is 20 μM for all the liposomes.

Figure 7.

Calcein dye leakage from 75/25 PE/PG vesicles V_1 promoted by $1.0 \mu\text{g/mL}$ AMO 2 after 1 h preincubation with PE/PG vesicle V_{15} (blue), after 1 h preincubation with *E. coli* vesicle V_{16} (magenta), after 1 h preincubation with PC/PS vesicle V_{17} (green), and without any preincubation with empty vesicles (red). The graph indicates that AMO 2 has strong affinity toward bacteria membrane mimic PE/PG vesicles and *E. coli* vesicles, and poor affinity toward mammalian mimic PC/PS vesicles.

TABLE 1

Vesicle Abbreviations with the Corresponding Lipid Compositions^a

vesicles	lipid composition	buffer inside ^b	buffer outside ^b	net charge
V ₁	DOPE/DOPG 75/25	A	B	negative
V ₂	DOPC 100%	A	B	neutral
V ₃	DOPC/DOPS 50/50	A	B	negative
V ₄	DOPC/DOPS 75/25	A	B	negative
V ₅	DOPC/DOPS 90/10	A	B	negative
V ₆	<i>E. coli</i> extract 100%	A	B	negative
V ₇	<i>E. coli</i> /EYPC 66/33	A	B	negative
V ₈	<i>E. coli</i> /EYPC 50/50	A	B	negative
V ₉	<i>E. coli</i> /EYPC 33/66	A	B	negative
V ₁₀	DOPE/DOPG/DOPC 80/20/00	A	B	negative
V ₁₁	DOPE/DOPG/DOPC 60/20/20	A	B	negative
V ₁₂	DOPE/DOPG/DOPC 40/20/40	A	B	negative
V ₁₃	DOPE/DOPG/DOPC 00/20/80	A	B	negative
V ₁₄	DOPE/DOPS/DOPC 80/20/00	A	B	negative
V ₁₅	DOPE/DOPG 75/25	B	B	negative
V ₁₆	<i>E. coli</i> extract 100%	B	B	negative
V ₁₇	DOPC/DOPS 75/25	B	B	negative

^a All of the lipid ratios are defined as molar % of lipids.^b Buffer A, 40 mM calcein, 10 mM Na₂HPO₄ pH 7.0; buffer B, 10 mM Na₂HPO₄, 90 mM NaCl, pH 7.0.

TABLE 2Percentage of Dye Leakage from Different Liposomes Induced by AMOs^a

lipid ratios	AMO 1	AMO 2	AMO 3
PE/PG (75/25) V ₁	9	80	84
PC/PS (50/50) V ₃	--	43	--
PC/PS (75/25) V ₄	--	32	--
PC/PS (90/10) V ₅	--	27	--
<i>E.coli</i> /PC (100/0) V ₆	15	80	71
<i>E.coli</i> /PC (66/33) V ₇	18	81	73
<i>E.coli</i> /PC (50/50) V ₈	12	57	72
<i>E.coli</i> /PC (33/66) V ₉	11	37	73
<i>E.coli</i> /PC (0/100) V ₂	9	18	80
PE/PG/PC (80/20/00) V ₁₀	3	75	98
PE/PG/PC (60/20/20) V ₁₁	2	60	96
PE/PG/PC (40/20/40) V ₁₂	1	22	78
PE/PG/PC (00/20/80) V ₁₃	0.5	9	71
PE/PS/PC (80/20/00) V ₁₄	3	87	82

^aData taken from the leakage curves, at 800s.