13

Interaction of Cationic Peptides with Bacterial Membranes

Shafique Fidai, Susan W. Farmer, and Robert E. W. Hancock

1. Introduction

A common feature of cationic peptides is that their site of action is at the membrane due to channel formation, and that they tend to possess strong selectivity towards their target membrane. For example, although moth cecropin and bee melittin are members of the same family of peptides that adopt amphipathic α-helical structures, the cecropins are strongly antibacterial and demonstrate minimal eukaryotic selectivity (i.e., toxicity), whereas melittin is a weak antibacterial compound but a potent toxin. Whereas the basis for selectivity is not completely understood, it has been shown to be due to the size of the transmembrane electrical potential gradient (up to −140 mV in bacterial cytoplasmic membranes compared with about −20 mV or less in eukaryotic membranes) and the lipid composition (bacterial membranes contain a large number of anionic lipids such as phosphatidyl glycerol and cardiolipin and lack cholesterol in their membranes). Gram-negative bacteria have an additional, outer membrane, and our data suggests that a further level of selectivity is expressed there in that there are Gram-positive bacteria-selective peptides that interact poorly with the outer membrane but (presumably) well with cytoplasmic membranes, whereas we have identified peptides that interact with the outer membrane, but are not bactericidal and thus do not interact with cytoplasmic membranes.

Although specific details may vary depending on the peptide, enough data exist to present a general model for the mechanism of action of cationic peptides against Gram-negative bacteria. This process is described below, and can be summarized as a sequence of events involving interaction with lipopolysac-

From *Methods in Molecular Biology, Vol 78 Antibacterial Peptide Protocols*
Edited by W M Shafer, Humana Press Inc , Totowa, NJ

charide (LPS) and self-promoted uptake of the peptides across the outer membrane, electrostatic interaction with the negatively charged head groups of lipids in the cytoplasmic membrane, and insertion into the membrane and channel formation leading to leakage of essential nutrients from the cell.

1.1. Dansyl Polymyxin B Displacement Assay

Cationic peptides *(1,2)*, like other polycationic antibiotics *(3)*, traverse the outer membrane using a process termed self-promoted uptake; in contrast small hydrophilic antibiotics such as β-lactams diffuse through the water-filled channels of porin proteins *(4)*. According to the self-promoted uptake model, compounds that access this pathway initially bind to the divalent-cation-binding sites of LPS To study this, dansyl polymyxin B displacement assays can be performed using purified LPS or whole cells. Dansyl polymyxin B has been shown to bind to the divalent-cation-binding sites of LPS, resulting in greatly enhanced fluorescence of the dansyl group *(5)*. This property led to the development of the above assay for determining the relative LPS-binding affinities of antibiotics based on their ability to competitively displace dansyl polymyxin B from LPS *(5)*.

1.2. Antiendotoxin Activity

As mentioned above, the initial step in the uptake of cationic peptides across the outer membrane is binding to LPS This binding, which is specific to the lipid A portion of LPS (i.e., endotoxin), can neutralize the ability of LPS to induce tumor neurosis factor (TNF) in macrophage cell lines.

1.3. Lysozyme Lysis Assay

Since the cationic peptides have an affinity for LPS that is three orders of magnitude higher than the native divalent cations, Mg^{2+} or Ca^{2+} *(1)*, they competitively displace these cations. This causes a distortion of outer membrane structure, that has been visualized in the electron microscope as induction of outer membrane blebs *(1)*, and a consequent permeabilization of the membrane to various probe molecules. One of them, lysozyme, is a 14-kDa basic protein that is unable to penetrate intact outer membranes, but can diffuse across disrupted membranes to exert its ability to enzymatically cleave peptidoglycan leading to cell lysis *(6)*. Because of its large size, one would expect that a significant destabilization of the outer membrane would be required for it to penetrate to its peptidoglycan substrate. Cationic peptides can therefore be tested in a lysozyme lysis assay for their ability to permeabilize the outer membrane of the test bacterium and facilitate the uptake of lysozyme.

1.4. NPN Uptake Assay

A second probe molecule, 1-*N*-phenyl-napthylamine (NPN), is an uncharged, hydrophobic fluorescent probe that has been used to study membrane permeabilization *(1,7)*. NPN fluoresces weakly in an aqueous environment, but strongly in the hydrophobic interior of a membrane When NPN is mixed with cells, it fluoresces weakly since it is unable to breach the outer membrane permeability barrier (or perhaps is rapidly effluxed from such bacteria; *see* **ref. 7**). Upon outer membrane destabilization in the presence of an energy inhibitor, however, it can partition into the hydrophobic environment of the membrane, where it emits a bright fluorescence. NPN is both smaller and more hydrophobic than lysozyme, which enables it to insert into membranes more easily than lysozyme *(7)*, although many cationic peptides cause major disruptions of the outer membrane even at low concentrations, leading to permeabilization to both probes.

1.5. Synergy with Antibiotics

The ability of cationic peptides to act in synergy with certain classical antibiotics *(8)* can be explained in part by their ability to disrupt outer membrane integrity, promoting the uptake of antibiotics across this barrier. Interestingly, the most potent cationic peptides do not have this "enhancer" activity for most antibiotics presumably since they kill cells at concentrations equal to their permeabilizing concentrations *(2)*. This is analogous to the situation for the polycationic antibiotic polymyxin B, which is not an enhancer, whereas its deacylated derivative PMBN (which interacts weakly with cytoplasmic membranes but strongly with outer membranes) is a potent enhancer of antibiotic activity *(9)*. To measure possible synergy, fractional inhibitory concentration (checkerboard) assays can be performed using the test antibiotics in the presence of sub-MIC concentrations (e.g., one-half or one-fourth MIC values) of the peptide *(10)*.

1.6. Planar Lipid Bilayer

Following uptake across the outer membrane, the peptides rapidly associate with the negatively charged head groups of lipids on the cytoplasmic membrane in a cooperative process. The extent of binding corresponds to the zeta potential of the lipids involved, strongly suggesting that binding is governed by electrostatic interactions *(11,12)*. It is uncertain as to whether at this stage the permeability of the target lipid membrane changes, although it is probable that the cationic peptide undergoes a change in conformation and aggregation state as a result of this interaction. At a threshold concentration of peptides bound to the membrane surface, the peptides are able to insert into the mem-

brane and form channels. Although some peptides can insert spontaneously into membranes with little or no transmembrane potential, it seems likely that the membrane potential of living cells (oriented interior negative) is an important factor in peptide insertion. The process of insertion can also cause a conformational change in the cationic peptide (e.g., from unstructured to α-helical), as for example with melittin *(13)* and magainins *(14,15)*. In many cases the peptides are thought to end up spanning the membrane bilayer *(13,16,17)* in multimeric complexes. Other peptides are too short to span the bilayer and presumably must stack to form aggregates to permit transmembrane channel formation *(15,17,18)*. The net effect of channel formation is to disrupt the integrity of bacterial cytoplasmic membranes. This would have the effect of permitting leakage of ions and small metabolites and destroying the ability of bacteria to maintain a transmembrane proton gradient (proton-motive force) with consequent loss of ability to generate ATP and to transport substrates.

The strongest evidence for channel formation has been in model membrane experiments using the planar lipid bilayer technique. In these experiments, the membrane potential, which is provided as an applied voltage, must be oriented positive on the *cis* side (where the cationic peptides are added) and negative on the *trans* side of the membrane (toward which the cationic peptides tend to move as they enter the membrane). This results in an observable increase in conductance as the peptides enter the membrane and forms channels *(19–23)*. Reversal of the voltage actually causes peptides to leave the membrane *(19)*. It should be noted, however, that channel formation may not be the only mechanism for cell lysis. Several studies using model liposomes have suggested that lysis can occur by a nonpore mechanism *(24,25)* in which cationic peptides form a "carpet-like" layer on the membrane surface that leads to a severe disruption in the lipid bilayer packing and eventual membrane disintegration. In addition it is known that cationic peptides can stimulate the intrinsic autolytic mechanisms of bacteria.

2. Materials

2.1. Dansyl Polymyxin Displacement Assay

2.1 1. Dansyl Polymyxin Synthesis

1 Polymyxin B sulphate (PxB).
2 0.1*M* $NaHCO_3$.
3. Dansyl chloride (Sigma D2625)
4 Acetone.
5 10 m*M* Na_2HPO_4, 0 145*M* NaCl
6. 10 m*M* NaH_2PO_4, 0 145*M* NaCl

7 Column, 50 × 2 5 cm packed with Sephadex G25 or G50
8 *n*-Butanol.
9 5 m*M* HEPES buffer, pH 7 2 Filter sterilize; do not autoclave
10 UV lamp or UV lightbox

2.1.2. Dansyl Polymyxin Quantitation (Dinitrophenylation Assay)

1. Polymyxin B sulfate (PxB).
2. 1% (w/v) $Na_2B_4O_7 \cdot 10\ H_2O$.
3. 100 m*M* 2,4 -dinitro-1-fluorobenzene (=1-fluoro-2,4-dinitrobenzene) in ethanol.
4. 2*N* HCl
5 *n*-Butanol.

2.1.3. Dansyl Polymyxin Displacement Assay

1 100 µ*M* dansylated polymyxin B (DPX).
2 5 m*M* HEPES buffer, pH 7.2 (as in **Subheading 2.1.1.**)
3 LPS 3 µg/mL in above HEPES buffer. Store at 4°C Solutions are stable for several months (*see* **Notes 1, 2,** and **8**)
4 Displacement compounds for testing stock solutions of polymyxin B at 1 0 mg/mL, gentamicin at 10 mg/mL, and magnesium chloride at 100 m*M*
5. 3% (w/v) Triton X-100.
6 Fluorescence spectrophotometer (we use a Perkin Elmer [Norwalk, CT] 650-10S machine with a strip chart recorder attached)

2.2. LPS/Endotoxin Neutralization Assay

1 Dulbecco's modified Eagle medium (DMEM). Filter sterilize through a 0 22-µm filter
2. Hanks' balanced salt solution: Filter sterilize through a 0.22-µm filter
3. 1*M* HEPES: Sterilize by autoclaving.
4 Hanks'-HEPES buffer: To 500 mL of Hanks' balanced salt solution, aseptically add 12 5 mL sterile HEPES buffer; store at 4°C
5. L-glutamine. 29.2 mg/mL dissolved in ddH_2O Filter sterilize through a 0 22-µm filter and store in 6-mL aliquots in 15-mL sterile tubes
6 Trypan blue 4 mg/mL dissolved in sterile PBS. Filter sterilize through a 0.44-µm filter and store in 0.4-mL aliquots at room temperature
7. MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; thiazolyl blue) 5 mg/mL dissolved in DMEM (without phenol red) and stored at 4°C in dark or foil covered bottles (this compound is light-sensitive).
8 Actinomycin D: 40 µg/mL dissolved in absolute ethanol and stored at 4°C (this compound is light-sensitive). For use in the TNF assay, dilute 1:10 into RAW cell media.
9 Penicillin: 10^4 U/mL sterile solution
10 Streptomycin· 1 mg/mL sterile solution
11 β-mercaptoethanol· 0 1*M* sterile solution

12. Heat-inactivated fetal bovine serum
13 RAW cell media: DMEM containing 2 4 m*M* L-glutamine, 60 U/mL penicillin, 6 µg/mL streptomycin, 1 2m*M* β-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS).
14 L929 Growth media same as RAW cell media, but can substitute heat inactivated horse serum for FBS
15 TNF assay media: RAW cell media containing 4 µg/mL Actinomycin D.
16. Cell dissociation solution, non enzymatic (Sigma, St. Louis, MO). Store at 4–6°C. Do not freeze
17 Trypsin-EDTA solution (1X).
18 Test peptides

2.3. Lysozyme Lysis Assay

1 Luria broth (LB): 10 g tryptone, 5 g yeast extract/ L dH_2O, no added NaCl
2 5 m*M* HEPES buffer, pH 7.2 with 5 m*M* sodium azide or 5 m*M* potassium cyanide (to inhibit respiration and prevent active excretion). NaN_3 and KCN are both poisonous Do not autoclave. Filter sterilize if desired for longer shelf life, sterile buffer is not required for assay
3. Lysozyme: 5 mg/mL dH_2O.
4 Permeabilizing compounds Test compounds and positive controls stock solutions of gentamicin at 10 mg/mL, polymyxin B at 1 mg/mL, and EDTA at 500 m*M*.
5 Spectrophotometer (We use a Perkin Elmer Lambda 3 dual beam spectrophotometer with a strip chart recorder attached).

2.4. NPN Uptake

1 LB broth (*see* **Subheading 2.3.**)
2 For *P aeruginosa*: 5 m*M* HEPES buffer, pH 7 2, containing 5 m*M* NaN_3 or 5 m*M* KCN (*see* **Subheading 2.3.**) For *E coli*: 5 m*M* HEPES buffer, pH 7.2, containing 5 m*M* glucose, 5 m*M* NaN_3 or 5 m*M* KCN or 5 µM CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) CCCP works the best but is unstable and light sensitive Make stock solutions in ethanol and store at –20°C in the dark (solutions are stable for several years). Add to buffer to make enough working solution for that day
3 1-*N*-phenylnapthylamine (NPN) Prepare a 5-m*M* stock and 0 5 m*M* working solutions in acetone Store at –20°C in foil covered bottles (solutions are stable for several months/1–2 yr)
4. Permeabilizing compounds prepared in serial dilutions at 100X the desired final concentration Concentrations needed will vary with the compound and the bacteria used, e g , polymyxin B at 0 2–0 64 mg/mL, gentamicin 1 5–10 0 mg/mL
5 Fluorescence spectrophotometer with a strip chart recorder attached.

2.5. Fractional Inhibitory Concentration Assay for Synergy

1 Untreated, polypropylene, 96-well, round-bottomed, sterile microtiter trays (Costar 3790, Cambridge, MA) (*see* **Note 23**)

2. Growth media, e.g., Mueller Hinton broth. (*see* **Note 18**)
3. Antibiotic/compound "A," at four times the desired final concentration, in media (*see* **Note 19**)
4. Antibiotic/compound "B," at two times the desired final concentration, in media (*see* **Note 19**).
5. Test strains of bacteria, log-phase or overnight cultures (*see* **Note 20**)
6. Multipipettor(s)

3. Methods

3.1. Dansyl Polymyxin (DPX) Displacement Assay

3.1.1. Dansyl Polymyxin Synthesis (26)

1. Prepare column buffer by adding Na_2HPO_4 and NaH_2PO_4 solutions together in an approx 2 1 ratio until the pH is 7 1. Equilibrate a packed column (50 × 25 cm) with at least 100 mL of buffer.
2. Dissolve 40 mg of polymyxin B in 1.2 mL of 0.1*M* $NaHCO_3$.
3. Dissolve 10 mg dansyl chloride in 0.8 mL acetone Add to the polymyxin B mixture from above and then incubate in the dark at 23°C for 90 min
4. Load the mixture on to the column, collect 5- to 6-mL fractions. Monitor fractions using a UV lamp. The dansyl-polymyxin (DPX) fluoresces yellow-orange and comes out as a broad peak (at approx 50–100 mL) before the dansyl-chloride which fluoresces blue-green (at approx 100–130 mL)
5. Pool the DPX fractions Add one-half the volume of butanol and mix well. Allow the butanol phase to partition or spin gently (100 rpm for 3 min) to separate the phases. Save the butanol phase and place it in shallow glass dish in desiccator. Evacuate the desiccator and place at 37°C until dry, approx 24 h
6. Dissolve the dried DPX in 3 mL HEPES buffer, and store in aliquots at –20°C, in the dark (lasts for several years)
7. The concentration of the DPX is determined by the dinitrophenylation assay.

3.1.2. Dansyl Polymyxin Quantitation (Dinitrophenylation Assay) (27)

1. Prepare a standard curve using a 1 0 mg/mL stock solution of polymyxin B to give 0, 5, 10, 20, 30, 40, and 50 µg/mL Use 5 and 50 µL of the DPX solution to be tested. Bring the volume of all samples to 50 µLwith dH_2O.
2. Add 25 µL of 2,4-dinitro-1-fluorobenzene to each sample, mix, and incubate at 37°C for 1 h
3. Add 1 mL of 2*N* HCl
4. Add 1 mL of *n*-butanol and vortex
5. Centrifuge in a clinical centrifuge at 100 rpm for 3 min
6. Remove the top butanol phase and measure the OD_{420} in a spectrophotometer.
7. Polymyxin B has five free amino groups; DPX has four. Therefore read the corresponding value from the unknown samples off the standard curve and multiply by 1.25 to obtain the quantity of DPX in the sample tube Calculate the dilution factor to obtain the final concentration of DPX solution.

3.1.3. Dansyl Polymyxin Displacement Assay

1 Set the excitation and emission wavelengths of a fluorescence spectrophotometer to 340 nm and 485 nm respectively, using narrow slit widths of 5 nm
2 Add 50 µL of 100 µ*M* DPX to 1 mL of 3% Triton X-100 Measure the fluorescence level Adjust the spectrophotometer sensitivity to give a reading of 90–100% of maximum deflection on the chart recorder

3.1 3 1 Determining Background Fluorescence of DPX solution

1 Add 5 µL of 100 µ*M* of DPX solution (final amount equals 500 pmol) to 1 mL of HEPES buffer Measure the fluorescence level
2. Repeat addition of 5 µL of 100 µ*M* DPX 5–10 times, adding DPX to the same cuvet and measuring the fluorescence for approx 10–20 s after each addition Each addition should result in a small, equal increase in fluorescence

3.1 3 2. Determining Saturation of LPS with DPX

1. Add 5 µL of DPX to 1 mL of LPS 3 µg/mL. Measure the fluorescence level
2 Repeat, adding 5-µL amounts of DPX to the same cuvette and measuring the fluorescence until the level of fluorescence plateaus off and the increase is only a result of the change in background fluorescence
3 Using the data from the above two steps, determine the amount of DPX that must be added to the 3 µg/mL LPS to give 85–90% of saturation. Call this concentration/amount "Z"
4 Add "Z" amount of DPX to 1 mL of 3 µg/mL LPS Reset the spectrophotometer so this value gives 90% of maximum scale deflection of the chart recorder (*see* **Note 7**)

3.1.3.3. Determining Inhibition/Displacement of DPX.

1 Add "Z" amount of DPX to 1 mL of 3 µg/mL LPS. Measure the fluorescence level (should be 90%).
2 Add a small aliquot (5–10 µL) of the potential displacer Measure the fluorescence level for 30–60 s If the test compound displaces DPX there will be a decrease in fluorescence as the DPX is removed from the LPS and goes into the aqueous solution
3 Repeat addition of aliquots 5–10 times or until the maximum displacement is reached (i e , additional compound no longer results in decreased fluorescence) (*see* **Notes 3–6**)
4 Plot the data as percent inhibition versus the concentration of inhibitor (µ*M*) If enough inhibitor is used the maximal % inhibition (I_{max}) can be seen when the fluorescence decrease levels off. The I_{50} is the concentration of compound resulting in half maximal displacement of DPX from LPS The data can also be plotted as a Lineweaver-Burke plot, plotting reciprocals of each axis of the first plot (i.e , 1/%inhibition vs 1/µ*M* concentration). Extrapolate to determine X and Y intercepts if necessary I_{max} is then calculated as 100/Y intercept and I_{50} is calculated from –1/X intercept.

3.2. LPS/Endotoxin Neutralization (28) (see Note 9)

3.2.1. Preparation of RAW Cells

1. Grow the murine RAW 264 7 macrophage cell line by seeding 10^6 cells into a 162-cm^2 cell culture flask and incubating at 37°C, 5% CO_2 for 1 wk.
2. Completely remove the RAW cell media and incubate with 10 mL of cell dissociation solution for 10 min at 37°C, 5% CO_2.
3. Remove cells from the flask and dilute with 10 mL RAW cell media
4. Centrifuge cells at 500*g* for 5 min and resuspend the cell pellet in 5 mL of media
5. Count the number of cells using a hemocytometer by removing a 0 1-mL aliquot and mixing with 0.4 mL of trypan blue.
6. Dilute the cell suspension to 10^6 cells/mL and add 1 mL of suspension to each well of a 24-well plate
7. Incubate the plate at 37°C in 5% CO_2 overnight for use in the assay
8. Save several milliliters of cell suspension from **step 6** to seed a new flask

3.2.2. Induction of TNF

1. After overnight incubation, the medium is aspirated from each of the wells in the 24-well plate
2. Wash each well with 1 mL of Hanks' balanced salt solution and aspirate off
3. Either synthetic LPS or whole bacteria can be used to induce TNF production If synthetic LPS is used, add 0.1 mL to each well for a final concentration of 100 ng/mL, and continue with **step 6** (*see* **Note 10**)
4. For experiments using whole bacteria, overnight cultures are diluted to an OD_{600} of 0 3 (10^8 cells/mL)
5. Further dilute the bacteria 1.10, and perform a viable count measurement to determine the exact number of bacteria used.
6. Add 0.1 mL of the diluted bacteria (approx 10^6 cells) to a Millipore (Bedford, MA) transwell filter insert (<0 2 μm) and place in the wells
7. Add peptide to specific wells at the desired concentration.
8. Add RAW cell media (without penicillin/streptomycin) to all of the wells to give a final volume of 1 0 mL, and incubate the plate for 6 h at 37°C and 5% CO_2
9. Remove supernatants from the wells and store at 4°C overnight.

3.2.3. Preparation of L929 Cells

1. Grow the TNF-sensitive mouse fibroblast cell line by seeding 10^6 cells into a 162-cm^2 cell culture flask and incubating at 37°C, 5% CO_2 for 1 wk
2. Completely remove the L929 cell media and incubate with 10 mL of trypsin EDTA solution for 5–10 min at 37°C, 5% CO_2
3. Remove cells from the flask and dilute with 10 mL L929 cell media.
4. Centrifuge cells at 500*g* for 5 min and resuspend the cell pellet in 5 mL of media.
5. Count the number of cells using a hemocytometer by removing a 0 1-mL aliquot and mixing with 0 4 mL of trypan blue.

6. Dilute the cell suspension to 10^6 cells/mL for use in L929 cell toxicity assay.
7. Save several milliliters of cell suspension from **step 6** to seed a new flask

3.2.4. Cell Cytotoxicity Assay

The levels of TNF released into the supernatants can be measured using the following L929 cell cytotoxicity assay

1 Add 50 µL of TNF media to all the wells of a 96-well microtiter plate except to those in the first row
2. Add 10 µL of murine TNF standard (20 ng/mL) and 90 µL of TNF medium in duplicate to wells A1 and A2 in the first row and dilute 1·2 (i e., –50 µL from A1 into B1, 50 µL from B1 into C1, and so on) down the plate until the second to last row Discard the extra 50 µL from row G; do not add to row H (*see* **Note 11**)
3. 75 µL of the test samples comprising the supernatants from the RAW cell assay are then added in duplicate to the next columns in the first row (i.e , first sample added to A3 and A4, second sample to A5 and A6, and so on) The samples are diluted 1:3 down the plate until the second to last row (i e , 25 µL from A3 into B3, 25 µL from B3 into C3, and so on). Discard the extra 25 µL from row G; do not add to row H
4 All of the wells should now contain a volume of 50 µL
5 Add 100 µL of the previously prepared L929 cell suspension (10^6 cells/mL) to each of the wells except H11 and H12
6 The last row (H1–H10) contains only TNF media and cells and is used as a negative control, wells H11 and H12 contain only TNF media and are used as blank control
7 Incubate the plate at 37°C, 5% CO_2 for 2 d

3.2 5. TNF Measurement

1 Aspirate the medium from the plate and replace with 0 1 mL of the dye MTT (0 5 mg/mL) in DMEM without phenol red
2 Incubate the plate at 37°C and 5% CO_2 for 3 h.
3 Remove the dye and replace with 0.1 mL absolute ethanol
4 Incubate the plate at room temperature for 10–15 min to dissolve the formazan dye crystals
5 Read plate at 570 nm in an ELISA plate reader with a 690-nm reference filter One unit of TNF activity is defined as the amount of TNF required to kill 50% of the L929 cells

3.3. Lysozyme Lysis Assay

1. Use 1 mL overnight culture to inoculate 50 mL LB. Grow cells to mid-log phase OD_{600} 0.4–0.6 (*see* **Note 12**)
2. Centrifuge at 3000*g* for 10 min at 23°C Resuspend in HEPES buffer, wash once, and then resuspend to a final OD_{600} of 0.5 (*see* **Note 13**)

3. Add 1 mL of cells to the cuvette. Measure OD_{600} for approx 10 s.
4. Add lysozyme to a final concentration of 50 µg/mL Measure OD for approx 10 s There should be no lysis
5. Add permeabilizer to the desired final concentration. Add small volumes (5–10 µL) of stock solutions so cells/lysozyme are not heavily diluted. Measure OD for at least 1 min.
6. Measure percent cell lysis as the percent decrease in OD_{600} at a set time point after addition of the permeabilizer, i.e 3 min. If a chart recorder is attached, compare lysis curves. The result depends on the concentration of permeabilizer and a plot of percent lysis vs permeabilizer concentration can be developed.
7. Measure negative controls: cells and lysozyme (no permeabilizer), buffer and lysozyme and permeabilizer (no cells), cells and permeabilizer (no lysozyme) Note. some peptides stimulate autolysis (*see* **Notes 14** and **15**).

3.4. NPN Uptake Assay

1. Use 1 mL overnight culture to inoculate 50 mLs of LB. Grow cells to mid-log phase (OD_{600} = 0.4–0.6) (*see* **Note 16**)
2. Centrifuge at 3000*g* for 10 min at 23°C Resuspend in HEPES buffer, wash once, and then resuspend to a final OD_{600} of 0.5. Maintain cells at 23°C.
3. Set the excitation and the emission wavelengths of a fluorescence spectrophotometer to 350 nm and 420 nm, respectively, using narrow slit widths of 5 nm Fluorescence is measured in arbitrary units. To standardize experiments, choose one set of conditions and adjust the sensitivity of the spectrophotometer and the chart recorder to a set value each time. For example to 1 mL of cells (OD_{600} = 0 5) add 10 µm NPN and 6.4 µg/mL polymyxin B and adjust sensitivity to give 90% of maximum scale deflection of the chart recorder. Under these conditions the NPN background level (without polymyxin B) is usually 5–15%.
4. Add 1 mL of cells to the cuvet and measure the fluorescence level after 5 s.
5. Add 10 µ*M* NPN to the same cuvet Mix Measure the new fluorescence level after 5 s.
6. Add 5–10 µL of permeabilizer to the same cuvet. Mix. Measure the fluorescence level until there is no further increase (*see* **Note 17**).
7. Using a fresh cuvet of cells with 10 µ*M* NPN each time, repeat steps 4–6 over a wide range of concentrations for each compound to give the full range of levels of fluorescence. Dilute the compounds so that equal volumes are added to each cuvette of cells, rather than having one concentration of compound and adding different volumes.
8. Measure as negative controls cells + NPN + antibiotic "solvent," NPN in buffer only (no cells, no antibiotics control), NPN in buffer only + antibiotic (no cells control).
9. To plot data, subtract the background level of fluorescence of the NPN only from the total fluorescence and plot versus the concentration of the compound

3.5. Fractional Inhibitory Concentration Assay for Synergy (29)

1. Pipet 100 µL of media only into each well of the 96-well microtiter tray in which columns are numbered 1–12 and rows A–H
2. Add 100 µL of compound A (4X) to each well in column 1.
3. Do doubling dilutions *across* the plate from columns 1–11 using a multipipettor, carefully mix all the wells in column 1 , then transfer 100 µL from that column to the next one Repeat until column 11, then discard the last 100 µL, do not add it to column 12
4. Add 100 µL of compound B (2X) to each well in the top row A of the plate
5. Do doubling dilutions *down* the plate from rows A–G using a multipipetor, carefully mix all the wells in row A , then transfer 100 µL from that row to the next one Repeat until row G, then discard the last 100 µL, do not add it to row H
6. Add 100 µL of media only to row H Mix, then discard 100 µL This will bring the concentration of compound A in each column of row H to the same concentration as the other rows for each respective column.
7. Add 5–10 µL of bacteria to each well to give a final inocculum of 5×10^5CFU/mL
8. Incubate at 37°C and read visually after 18–24 h Well H12 serves as a positive control as it contains only the growth medium and the bacteria.
9. Calculate the MIC for each compound from row H (for compound A) and column 12 (for compound B) The MIC is read as the minimal concentration necessary to inhibit growth by at least 50% Calculate the FIC index

3.5.1. Calculation of the FIC Index

The FIC index is calculated from the formula:

$$\text{FIC index} = \text{FIC A} + \text{FIC B} = (\text{A})/(\text{MIC A}) + (\text{B})/(\text{MIC B})$$

where (A) is the concentration of compound A in the microtiter well that is the lowest inhibitory concentration of compound A in its row. (MIC A) is the MIC of drug A alone. FIC A is the fractional inhibitory concentration of compound A. (B), (MIC B), and FIC B are defined for compound B in the same way as for compound A.

An FIC index of ≤ 0.5 indicates synergy, when the results with two drugs are significantly greater than the additive response. An FIC index of >1.0 indicates antagonism, when the results with two drugs are significantly less than the additive response. An FIC index = 1.0 indicates additivity; when the results with two drugs are equal to the sum of the results for each of the drugs used separately. An FIC index = FIC A or FIC B alone indicates autonomy, when the results with two drugs do not significantly differ from the result with the most effective drug alone.

In the example in **Fig. 1**, at combination * (well E6), (A) = 2, (B) = 1.25; therefore, the calculation is as follows:

	row	1	2	3	4	5	6	7	8	9	10	11	12
column	conc	64	32	16	8	4	2	1	.5	.25	.12	.06	.03
A	20												
B	10												
C	5											+	+
D	2.5											+	+
E	1.25						*	+	+	+	+	+	+
F	0.63						+	+	+	+	+	+	+
G	0.32					+	+	+	+	+	+	+	+
H	0.15				+	+	+	+	+	+	+	+	+

Fig. 1. Example to calculate FIC index. Growth is indicated by +, the MIC of compound A = 16 (well H3), the MIC of compound B = 10 (well B12) *Indicates well used to illustrate calculation of FIC index

$$\text{FIC index} = (A)/(\text{MIC A}) + (B)/(\text{MIC B})$$

$$\text{FIC index} = 2/16 + 1.25/10 = 0.125 + 0.125 = 0\ 25$$

Since 0.25 is ≤0.5, this therefore indicates synergy between compounds A and B. Please note that the FIC index for well F5 is equal to 0 31 and also indicates synergy Usually the lowest calculation of FIC index is used (*see* **Notes 21** and **22**).

3.6. Planar Lipid Bilayer

The planar lipid bilayer method offers a rapid way to experimentally measure the ability of peptides to form transbilayer channels. In general, cationic peptides form multistate channels, and planar bilayer experiments demonstrate a substantial range of channel sizes, with single channel conductances (which reflect size) varying from 10–2000 pS (**refs. *19–23***) and lifetimes ranging from milliseconds to seconds. The planar lipid bilayer method, described briefly below, utilizes specialized apparatus that usually requires some training in its use.

The central part of the apparatus comprises a chamber that is machined from a 5 × 2.5 × 3-cm block to create two equal compartments, separated by a 1-mm Teflon divider. One of these compartments contains a viewing window, and the Teflon divider is perforated by a 0 1- to 2-mm^2 hole. The hole is anointed at its edges with a lipid solution and dried under a jet of hot air to provide a surface to which a membrane can adhere. The compartments are then each filled with 6 mL of a salt solution (e.g., 1*M* KCl). The hole is then covered with lipid by wiping with a Teflon rod onto which 5 µL of the lipid solution has been pipetted. Successful "painting" of the lipid over the hole is assessed by measuring a high resistance when a voltage is passed across two electrodes dipping into the two compartments. Within a short time, the lipid thins out

until it forms a bilayer. This can be observed using a short focal length telescope in incident light coming from a suitable focused light source such as a microscope light, since the observed lipid changes from multicolored to black, due to the optical properties of lipid globules and lipid bilayers respectively. This gives the methodology its alternate name black lipid bilayers.

To the electrode dipping into the solution in one compartment, a direct current voltage source is attached. To the other electrode is attached a current amplifier (Keithly 427 [Cleveland, OH]), an oscilloscope to monitor the amplified signal (Tektronix 511A [Beaverton, OR]) and a rapid response chart recorder (Houston Instruments 4512). With the naked membrane, application of a voltage (typically 30–100 mV) results in a very small current (approx 2 pA) since bilayers have little permeability to ions. When peptide is added to the compartment containing the cathode (*cis* side) on one side of the membrane, depending on its affinity, it will incorporate into the membrane at a threshold voltage and form a conduit for the movement of ions through the channel This can be observed, after signal amplification, as a stepwise increase in conductance in both real time on the chart recorder and at a faster resolution on the oscilloscope.

Some of the channel properties that can be easily checked by varying experimental parameters are whether the channel is water-filled, whether there is a strong selectivity for cations over anions, or vice versa, the influence of lipid composition, whether the channel aggregates in the membrane, the effect of voltage on channel properties and on the ability of channels to insert into the membrane (voltage induction) or open under a voltage (voltage gating), whether the channel permits only unidirectional flux of ions, and the variability in the sizes of individual channels. The major readout, however, is average single channel conductance of the channel in given salts, which is itself proportional to the volume of the channel and its geometry.

With a slightly different setup in which only a voltage source and a multimeter (Keithly 610) are connected to the electrodes, one can measure macroscopic conductance, and determine such properties as voltage dependence and selectivity for one ion over another.

4. Notes

1 LPS may be purchased or prepared from bacterial cells *(8)*
2 LPS is more stable and gives more consistent results after further purification by chloroform/methanol extraction to remove contaminating lipids, and conversion to the sodium salt form which exchanges sodium for magnesium at cross-bridging sites (***30***, but omit $MgCl_2$ dialysis step)
3 Try to use a concentration of displacer that will give several relatively even step decreases in fluorescence of 5–15% before reaching maximum displacement.

4. Usually the decrease in fluorescence is immediate and fairly stable. If necessary measure the fluorescence decrease for a longer time until the decrease levels off.
5. If the cuvet containing LPS and DPX alone gives erratic or drifting fluorescence recordings, try briefly sonicating the LPS solution.
6. Many common antibiotic solvents such as alcohols, and acidic or basic solutions will cause decreases in fluorescence (i e , false positive results) Similarly, detergents can cause increases in fluorescence. Be sure to include a negative control using your "solvent" only
7 Once the "Z" concentration of DPX is determined, the background and saturation points do not have to be retested if using the same conditions (DPX concentration and LPS stock). For further displacement experiments, just begin at **Subheading 3.1.3.3.**
8 Lipid A at 4 µg/mL or whole cells may be used instead of LPS. For whole cells, grow up cells to mid-log phase in LB Pellet, wash in HEPES buffer containing 5 m*M* sodium azide (to inhibit respiration) Resuspend to an OD_{600} of 0 5.
9 Plan the experiment properly so that everything will be ready at the necessary time. On d 1, seed the RAW cells (**Subheading 3.2.1., step 1**), on d 3, seed the L929 cells (**Subheading 3.2.3., step 1**). On d 8 set up the RAW cells into the 24-well plates (**Subheading 3.2.1., steps 2–7**), then induce them with TNF on d 9 and save the supernatants (**Subheading 3.2.2.**). On d 10, use the supernatants to set up the microtiter plates for the L929 cell toxicity assay (**Subheading 3.2.4.**), do all the dilutions before getting the L929 cell ready (**Subheading 3.2.3., steps 2–8**). Incubate the plates and then read on d 12 (**Subheading 3.2.5.**).
10 If whole bacteria are used in the induction of TNF (**Subheading 3.2.2., steps 2–5**), they can be added without using the Millipore transwell filters, but after removing supernatants centrifuge them in 0 2-µm filter Eppendorf tubes and store the clarified supernatants at 4°C overnight.
11. The TNF standard (positive control) is only required on one plate for the L929 cell toxicity assay (**Subheading 3.2.4., step 2**)
12 Other growth media may be used, but high levels of cationic compounds such as Mg^{2+} and Ca^{2+} may interfere by binding to the same binding sites that the permeabilizer would access
13 Spin and keep cells at 23°C to avoid "leaky" cells Do not keep on ice! *Pseudomonas aeruginosa* is especially affected, and can become very leaky even if cells are only refrigerated during centrifugation. Resuspend cell pellets gently with a pipet; do not vortex.
14 If the test compound causes cell lysis without lysozyme addition, try to stabilize cell osmolarity by using 80 m*M* (or higher) NaCl in the buffer Lysis by the test compound alone can also be subtracted as background
15. Watch the cuvets for any signs of cell clumping, which will also cause changes in OD. If in doubt, check the cells using a microscope.
16. Cell stability is very important in this assay. See notes in **Notes 12** and **13** regarding the growth of cells Measure the background level of fluorescence of the cells alone plus NPN for at least 1 min at the beginning of the assays. Keep an eye on

the background levels throughout successive experiments and again measure the value with the cells alone plus NPN if the background seems to have increased substantially, or at least every hour to ensure cells are stable Do not continue the experiment once the cells have become unstable (after 1–3 h) If the cells seem unstable from the very beginning, it is better to discard them and grow another batch.

17. Add the permeabilizer soon after adding the NPN, since the level of fluorescence with the permeabilizer is usually somewhat lower if it is, for example, added 1 min as compared to adding it 10 s after NPN addition
18 Different growth media may be used but several factors can influence MIC results. Mueller Hinton is the most commonly used and standardized media for MIC measurements
19. Use concentrations of antibiotic ranging from at least twofold above the MIC to at least fourfold below the MIC
20 Generally, overnight cultures are around 10^9–10^{10} CFU/mL, therefore dilute 1/10,000 and use 5–10 µL to inoculate, giving a final inoculum of approx 10^5 CFU/mL Check inoculum by plate counts Adjust dilutions for further experiments as necessary for your strains Try to ensure final inoculum is between 10^4–10^6 CFU/mL for accurate MIC values The MIC results for peptides are not usually different using log-phase cells or overnight cultures, but many antibiotics target growing cells and require log-phase cells for accurate MIC values.
21 Some compounds do not inhibit growth (i.e., have no MIC themselves), but will be synergistic with other compounds These compounds usually permeabilize the cells, allowing more of the other compound (that inhibits the bacteria) into the cell. A true FIC index cannot be calculated, since there is no MIC, but the pattern of synergy can be easily seen.
22 When calculating FIC indexes, each well will not usually have the same result Two compounds may be synergistic at one particular combination but not at others
23. Do not use tissue culture or ELISA plates; they are treated and tend to bind cationic peptides. Polypropylene also binds peptides less than standard polystyrene plates

Acknowledgments

The financial assistance of the Canadian Cystic Fibrosis Foundation, the Medical Research Council of Canada, the Canadian Bacterial Diseases Network, and Micrologix Biotech is gratefully acknowledged.

References

1. Sawyer, J. G , Martin, N L , and Hancock, R E W (1988) Interaction of macrophage cationic proteins with the outer membrane of *Pseudomonas aeruginosa. Infec. Immun* **56,** 693–698
2 Piers, K. L. and Hancock, R E W (1994) The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa Molec Microbiol* **12,** 951–958

3 Hancock, R. E. W. (1981) Aminoglycoside uptake and mode of action—with special reference to streptomycin and gentamicin. II. Effects of aminoglycosides on cells. *Antimicrob Chemother.* **8,** 429–445.
4. Hancock, R E W (1991) Bacterial outer membranes evolving concepts *ASM News* **57,** 175–182
5 Moore, R. A , Bates, N C , and Hancock, R E. W (1986) Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin *Antimicrob. Agents Chemother* **29,** 496–500
6. Hancock, R. E. W and Wong, P. G. W. (1984) Compounds which increase the permeability of the *Pseudomonas aeruginosa* outer membrane *Antimicrob. Agents Chemother.* **26,** 48–52.
7 Loh, B , Grant, C , and Hancock, R E W (1984) Use of the fluorescent probe 1-N-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa. Antimicrob. Agents Chemother.* **26,** 546–551
8 Darveau, R. and Hancock, R E.W. (1983) Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* **155,** 831–838
9. Vaara, M (1992) Agents that increase the permeability of the outer membrane *Microbiol Rev.* **56,** 395–411.
10 Piers, K. L., Brown, M H , and Hancock, R E W. (1994) Improvement of outer membrane-permeabilizing and lipopolysaccharide-binding activities of an antimicrobial cationic peptide by C-terminal modification *Antimicrob Agents Chemother.* **38,** 2311–2316
11 Matsuzaki, K , Harada M , Funakoshi, S , Fujii, N., and Miyajima, K (1991) Physiochemical determinants for the interactions of magainins 1 and 2 with acidic lipid bilayers. *Biochem Biophys Acta* **1063,** 162–170
12 Sekharam, K M , Bradrick, T D , and Georghiou, S (1991) Kinetics of melittin binding to phospholipid small unilamellar vesicles. *Biochem Biophys Acta* **1063,** 171–174.
13. Vogel, H. and Jahnig, F (1986) The structure of melittin in membranes *Biophys J.* **50,** 573–582
14. Bechinger, B , Zasloff, M., and Opella, S. J. (1992) Structure and interactions of magainin antibiotic peptides in lipid bilayers· a solid-state nuclear magnetic resonance investigation *Biophys J.* **62,** 12–14
15 Williams, R. W., Starman, R , Taylor, K M P., Gable, K , Beeler, T , Zasloff, M., and Covell, D (1990) Raman spectroscopy of synthetic antimicrobial frog peptides magainin 2a and PGLa *Biochemistry* **29,** 4490–4496.
16 Sipos, D., Andersson, M , and Ehrenberg, A (1992) The structure of the mammalian antibacterial peptide cecropin P1 in solution, determined by proton-NMR. *Eur J Biochem.* **209,** 163–169
17. Andreu, D., Ubach, J , Boman, A., Wahlur, B., Wade, D , Merrifield, R. B., and Boman, H. G. (1992) Shortened cecropin A-melittin hybrids. Significant size reductions retains potent antibiotic activity *FEBS Letts* **296,** 190–194

18. Agawa, Y , Lee, S., Ono, S , Aoyagı, H., Ohno, M , Tamguchı, T , Anzaı, K , and Kırıno, Y. (1991) Interaction with phospholipid bılayers, ion channel formation, and antimicrobial activity of basic amphiphilic alpha-helical model peptides of various chain lengths *J Biol. Chem.* **266,** 20,218–20,222
19 Christensen, B , Fink, J., Merrifield, R B., and Mauzerall, D. (1988) Channel forming properties of cecropins and related model compounds incorporated into planar lipid membranes *PNAS.* **85,** 5072–5076
20 Kagan, B. L., Selsted, M E , Ganz, T., and Lehrer, R. I (1990) Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes *PNAS* **87,** 210–214
21 Hanke, W., Methfessel, C., Wilmsen, H U , Katz, E., Jung, G , and Bohem, G (1983) Melittin and a chemically modified trichotoxin form alamethicin-type multistate pores. *Biochem. Biophys. Acta* **727,** 108–114.
22. Kordel, M., Benz, R , and Sahl, H. G. (1988) Mode of action of the staphylococcin like peptide Pep5 voltage dependent depolarization of bacterial and artificial membranes *J Bacteriol.* **170,** 84–88
23. Cociancich, S , Ghazi, A , Hetru, C , Hoffman, J A , and Letellier, L. J (1993) Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus. Biol Chem* **268,** 19,239–19,245
24 Pouny, Y., Rapaport, D., Mor, A , Nicolas, P., and Shai, Y (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes *Biochemistry* **31,** 12,416–12,423
25. Gazit, E , Boman, A., Boman, H G , and Shai, Y (1995) Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles *Biochemistry* **34,** 11,479–11,488
26. Schindler, P. R. G. and Tueber, M (1975) Action of Polymyxin B on bacterial membranes. morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. *Antimicrob Agents Chem* **8,** 95–104.
27 Bader, J. and Teuber, M. (1973) Binding to the O-antigenic lipopolysaccharide of *Salmonella typhimurium. Z. Naturforsch.* **28c,** 422–430.
28 Kelly, N M , Young, Y , and Cross, A S (1991) Differential induction of tumor necrosis factor by bacteria expressing rough and smooth lipopolysaccharide phenotypes. *Infect Immun* **59,** 4491–4496
29 Amsterdam, D (1991) Antimicrobial combinations, in *Antibiotics in Laboratory Medicine.* (Lorian, V , ed) Williams and Wilkins, Baltimore, pp 432–492
30. Peterson, A. A , Hancock, R. E. W , and McGroarty, J (1985) Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa J Bacteriol* **164,** 1256–1261