

Quantifying Lipid Mobility and Peptide Binding for Gram-Negative and Gram-Positive Model Supported Lipid Bilayers

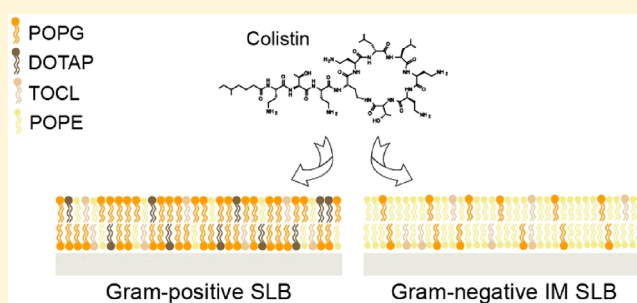
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Supporting Information

ABSTRACT: Model membranes are a valuable tool to investigate the mechanism of interaction between antibiotic compounds and bacterial membranes. However, the development of supported lipid bilayer (SLB) models for Gram-negative and Gram-positive bacteria has been challenging because of the high charge and spontaneous curvature of the lipids that make up these membranes. Here we describe a method for preparing mimetic Gram-negative inner membrane and Gram-positive membrane SLBs, including asymmetric SLBs (asy-SLBs) that contain a fluorescent tracer only in the upper leaflet of the membrane. We quantified the dynamics of the lipids in these membranes with fluorescence correlation spectroscopy (FCS) and found that lipid diffusion is slower in Gram-negative SLBs/asySLBs than in Gram-positive SLBs/asySLBs. Peptide binding to these membranes was also characterized using colistin, a Gram-negative specific antibiotic. Interactions between colistin and membrane lipids phosphatidylethanolamine (PE) or cardiolipin (TOCL) were probed with pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS). Overall, our data provide unique insight into the diffusion dynamics of lipids in Gram-negative and Gram-positive membranes as well as a novel platform for investigating the mechanism of interaction between antibiotic peptides and bacterial membrane lipids.



INTRODUCTION

The membranes of Gram-negative and Gram-positive bacteria are complex lipid mixtures with lipopolysaccharides, anionic phospholipids such as cardiolipin and PG and cationic phospholipids like lysyl-PG.^{1–3} Model membrane supported lipid bilayers (SLBs) are an ideal system to study the physical properties of Gram-negative and Gram-positive bacterial membranes and investigate lipid interactions with antibiotic peptides.^{1,4–6} However, the formation of a fluid and stable SLB with physiologically relevant concentrations of lipids that mimic Gram-negative and Gram-positive bacterial membrane is very challenging because they contain a high percentage of charge lipids. For example, the inner membrane (IM) of Gram-negative bacteria has around 30% charged lipids and Gram-positive membrane has around 80% charge lipids.^{7,8} The presence of charged lipids leads to strong Coulombic interaction between lipids and the solid support surface and affects the motion of lipids in the model membrane.^{9,10} In addition, one important component of Gram-negative and Gram-positive membranes is PE, which has a negative curvature and also limits its ability to incorporate into the SLB.^{11,12} Thus, fabrication of a stable model membrane with a high concentration of PE lipid, especially for Gram-negative membranes (~70 mol %), is very difficult.¹³ Recently, the

formation of SLBs using 1:3 lipid mixture of PE:PG in CaCl₂ solutions has been reported to mimic the Gram-negative membrane.¹⁴ As an alternative to overcome these issues, we used high salt concentrations to prepare the lipid vesicle suspensions and changed the glass surface treatment to isopropanol instead of sodium hydroxide or Piranha solution.⁹ These changes allowed us to make fluid SLBs using mixtures of PE, PG, TOCL, and DOTAP lipids to mimic Gram-negative and Gram-positive membranes (Figure 1) (DOTAP is a positively charged lipid used to replace lysyl-PG).^{3,8} To more directly probe the interaction between bacterial membrane lipids and antimicrobial peptides, we prepared asymmetric supported lipid bilayers (asy-SLBs) that have the fluorescent tracer lipid only in the top leaflet of the bilayer.¹⁵ This configuration avoids interactions between the fluorescent lipids and the substrate and increases the proportion of fluorescent lipid available for peptide binding in the upper leaflet.¹⁵

One advantage of model SLBs is the ability to perform high resolution imaging and spectroscopy methods to study various membrane localized biological processes. Here, we studied the

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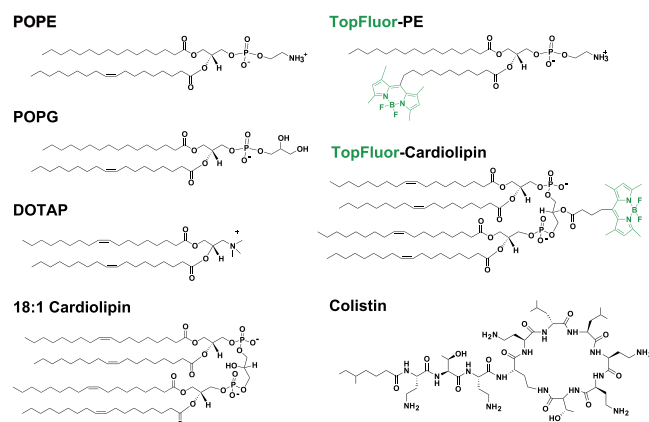


Figure 1. Molecular structures are shown for the lipids used in this study: POPE, POPG, DOTAP, 18:1 Cardiolipin (TOCL), TopFluor-PE, TopFluor-TOCL lipids, and Colistin.

interactions between Gram-negative and Gram-positive SLBs/asy-SLBs membrane and an antimicrobial peptide, colistin. Colistin is a cationic peptide, derived from polymyxin, which permeabilizes the bacterial membrane, leading to cell death.^{16–19} Colistin has been reported to inhibit about 63% of the Gram-negative and 13% of Gram-positive bacteria.²⁰ The mechanism of colistin antibacterial activity in Gram-negative bacteria involves two steps: translocation across the outer membrane and then insertion to the inner membrane, which is constituted of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (TOCL).^{2,21} Recent work with diffuse X-ray scattering and neutron reflectometry in multilamellar model membranes measured the mechanical properties of the membranes at various concentrations of colistin.⁸ The authors found that membrane rigidity increased then decreased around a critical lipid:peptide ratio for Gram-negative inner membranes. They also determined that the insertion depth of colistin into Gram-negative membranes is larger than that for Gram-positive membranes and that the secondary structure of colistin is unchanged. These results provide substantial molecular insight into the antibiotic activity of colistin. In this work we sought to probe the diffusion and dynamic association of single lipid and peptide species in SLBs. This approach adds molecular-level detail to how colistin disrupts Gram-negative membranes and provides a new methodology for investigating novel antibiotic interactions with bacterial membranes.

Two fluorescence-based methods were used to quantify the lipid dynamics of Gram-negative and Gram-positive model membranes and their interaction with colistin. We first characterized the fluidity of the bilayers by fluorescence recovery after photobleaching (FRAP).²² Then, we analyzed molecular diffusion and interactions with pulsed interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS), which has been successfully used to study peptide-lipid interactions in our previous work.²³ By labeling the lipids and colistin with fluorescence dyes, PIE-FCCS measured the concentration, mobility, and correlated diffusion of lipids and peptides.^{24,25} Acyl chain labeled TopFluor-PE and headgroup labeled TopFluor-TOCL, were used to monitor lipid diffusion. We found that lipid diffusion was faster in Gram-positive membranes than in Gram-negative membranes. In addition, we analyzed the effectiveness and applicability of the Gram-positive and Gram-negative membranes by investigating the

interaction between fluorescence lipids and colistin. The mobility of both TopFluor-PE and TopFluor-TOCL was altered in the presence of colistin, especially for TopFluor-PE. The different mobilities of TopFluor-PE and TopFluor-TOCL with colistin suggest that PE display a stronger colistin binding affinity than TOCL. We also investigated the degree of colistin-lipid binding via fraction correlated, f_c . No cross-correlation was found between AF-colistin and TopFluor-TOCL, but a measurable cross-correlation was obtained between AF-colistin and TopFluor-PE, consistent with the mobility result.

METHODS

Lipids and Reagents. The following lipids were used in the model membrane systems described below: 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol)) (sodium salt) (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-3-trimethylammonium propane (chloride salt) (DOTAP), 1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-glycerol (sodium salt) (18:1 Cardiolipin), 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-*sn*-glycero-3-phosphoethanolamine (TopFluor-PE) and 1,1',2,2'-tetraoleoyl cardiolipin[4-(dipyrrometheneboron difluoride)-butanoyl] (ammonium salt) (TopFluor-Cardiolipin). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Colistin sulfate salt was obtained from Sigma-Aldrich. Molecular probe Alexa Fluor 568 C5-maleimide was purchased from ThermoFisher Scientific and was used to label colistin salt and form a stable protein-dye conjugation according to the manufacturer's protocol. Molecular structures of POPE, POPG, DOTAP, Cardiolipin (TOCL), TopFluor-PE, TopFluor-Cardiolipin (TopFluor-TOCL), and colistin are shown in Figure 1.

Preparation of Gram-Positive and Gram-Negative Supported Lipid Bilayers. The Gram-negative membranes were prepared with POPE 69.99%, POPG 20%, TOCL 18:1 10% and TopFluor-PE 0.01% or TopFluor-TOCL 0.01% (Table 1). Gram-positive membranes were prepared with

Table 1. Mole Percent of Each Lipid Component in the Lipid Mixture for Gram-Negative and Gram-Positive SLBs

	Gram-negative	Gram-positive
POPE	69.99	14.99
POPG	20.00	60.00
TOCL	10.00	10.00
DOTAP	—	15.00
TopFluor-PE/TOCL	0.01	0.01

POPG 60%, TOCL 18:1 10%, DOTAP 15%, POPE 14.99%, and TopFluor-PE 0.01% or TopFluor-TOCL 0.01% (Table 1). SLBs were prepared using a modified vesicle fusion method.¹⁵ First, lipid mixtures were dissolved in chloroform and dried by rotational evaporation under vacuum. After drying, 1 mL of 2 M NaCl solution was added to the lipid films to produce a lipid vesicle suspension with a lipid concentration of 1 mg/mL. The lipid vesicle suspensions were heated to 38 °C for 3 min before the formation of SUVs to reach the melting temperature of POPE. Next, to prepare the small unilamellar vesicles (SUVs), lipid vesicle suspension was extruded by a 100 nm diameter porous membrane 15 times and then centrifuged at 13000 rpm for 10 min, 50% of top SUVs was collected. Vesicles were stored at room temperature for up to 2 h before being used to

prepare SLBs. SLBs were formed in 96-well glass bottom plate. It should be noted that the glass surface was treated only with isopropanol, instead of using common chemical etchant (NaOH or piranha). The plate was first incubated with isopropanol for 45 min to remove the contaminants from the glass surface, rinsed with 300 μL deionized water 3 times, and then dried with nitrogen gas. After the cleaning steps, 100 μL of SUV suspension was deposited on the microwell surface to form an SLB. In order to rupture the vesicles, 150 μL deionized water was added to the well. The supported bilayer formed in the well and, after 1-h incubation, was rinsed with 200 μL deionized water three times to remove the unfused vesicles.

Colistin was added to the SLBs to mimic the interactions with Gram-negative and Gram-positive bacterial membranes. The SLBs were incubated with colistin at room temperature for 20 min to allow the peptide binding to the supported lipid bilayer. After incubation, the SLBs were washed with 200 μL of deionized water three times before measurement.

An alternative procedure to prepare the Gram-negative and Gram-positive SLBs with colistin was also used in the FCS study. The glass was first cleaned in isopropanol, rinsed with deionized water, dried with nitrogen gas. Then, colistin was directly added to lipid vesicle suspension and incubated for 30 min to allow peptide binding before extrusion. The final vesicle suspensions containing colistin were stored at room temperature and used within 2 h. The formation of SLBs from vesicles containing colistin was the same as described above.

Preparation of Gram-Positive and Gram-Negative Asymmetric Supported Lipid Bilayers. Asy-SLB was prepared by incorporating the fluorescence lipid into the upper leaflet of the host SLB.²⁵ Host SLBs were prepared with Gram-negative lipid mixtures containing POPE, POPG and 18:1 Cardiolipin with a 70/20/10 molar ratio and Gram-positive lipid mixtures containing POPG, 18:1 Cardiolipin, DOTAP, and POPE with a 60/10/15/15 molar ratio (Table 2). The lipid mixtures were dried by rotational evaporation

Table 2. Lipid Composition of SUVs Used To Make Gram-Negative and Gram-Positive SLBs Prior to Adding the Fluorescent Lipids To Make asy-SLBs

	Gram-negative	Gram-positive
POPE	70	15
POPG	20	60
TOCL	10	10
DOTAP	—	15

under vacuum, and then the lipid films were hydrated in 1 mL of 2 M NaCl solution for 1 h to produce a lipid vesicle suspension of 1 mg/mL. Next, lipid vesicle suspensions were heated to 38 °C for 3 min to reach the T_m of POPE. The Gram-negative and Gram-positive lipid suspensions were then passed through an extruder 15 times with a 100 nm diameter porous membrane to yield small unilamellar vesicles (SUVs). In addition, before the formation of host SLBs, SUVs were centrifuged at 13000 rpm for 10 min and collected 50% of top solution. The 96-well glass plate used for host SLBs was also cleaned by incubating in isopropanol for 45 min and rinsed with deionized water before drying with nitrogen gas. A 100 μL aliquot of SUV was deposited in the well, and then 150 μL of deionized water was added and the mixture incubated for 1 h.

After incubation, the excess vesicles were raised with deionized water 3 times.

For the fluorescent lipids, TopFluor-PE or TopFluor-TOCL lipid film was hydrated by adding 1 mL of deionized water. A small amount of the fluorescence lipid suspension was added to the water above the host SLBs and incubated for 20 min while the fluorescence lipid incorporated into the host SLBs. After the incubation step, the asy-SLBs were washed with 150 μL of deionized water three times to remove the excess fluorescence lipid. Fluorescence labeled colistin was added to asy-SLBs and incubated in deionized water for 20 min. Deionized water was used again to remove the free colistin before measurement.

PIE-FCCS Data Collection and Analysis. The PIE-FCCS experiments were performed on a customized microscope described in several publications from our group.^{23,26} The excitation laser powers were set to 800 nW (488 nm, blue) and 600 nW (561 nm, green). TCSPC data were collected with 15-s acquisition times from five areas of interest in each individual SLB or asy-SLB. The samples were kept at room temperature (20 °C) on the microscope stage during data processing.

The complex composition of the model membrane can give rise to anomalous diffusion behavior of both the lipids and colistin under certain conditions. Hence, the autocorrelation functions were fitted with a 2D anomalous diffusion model as follows:

$$G(\tau) = \frac{1}{\langle N \rangle} \left[1 + \frac{\tau}{\tau_D} \right]^{-\alpha}$$

Here α is the anomalous factor. If α is equal to 1, the fluorescent particles are undergoing pure Brownian diffusion, while α values below 1 indicate anomalous diffusion. The dwell (τ_D) of the fluorescence molecules is also extracted from the 2D anomalous diffusion model and used to calculate the diffusion coefficient by the equation:

$$D = \frac{\omega^2}{4\tau_D}$$

To characterize the degree of the colistin-lipid binding, we use fraction correlated, f_c for the data analysis, given by

$$f_c = \frac{N_{rg}}{\min[(N_r + N_{rg}), (N_g + N_{rg})]}$$

In our experiment, N_r is the average number of free Alexa Fluor 568-labeled colistin, N_g is the average number of free TopFluor-labeled lipids and N_{rg} is the average number of bound molecules. In principle, $0 < f_c < 1$, where an f_c value equal to 0 indicates no codiffusion and an f_c value equal to 1 indicates complete codiffusion.

RESULTS

Formation and Characterization of Gram-Negative and Gram-Positive Symmetric SLBs. We first set out to create supported lipid bilayers that mimic the inner membrane of Gram-negative bacteria and the plasma membrane of Gram-positive bacteria. We chose lipid compositions to match those of a previous publication for direct comparison to those and other previous results.⁸ The Gram-negative inner membrane SLBs consisted of POPE/POPG/TOCL18:1/TopFluor-PE mixture at the 69.99/20/10/0.01 molar ratio, and the composition of Gram-positive SLBs was POPG/TOCL18:1/

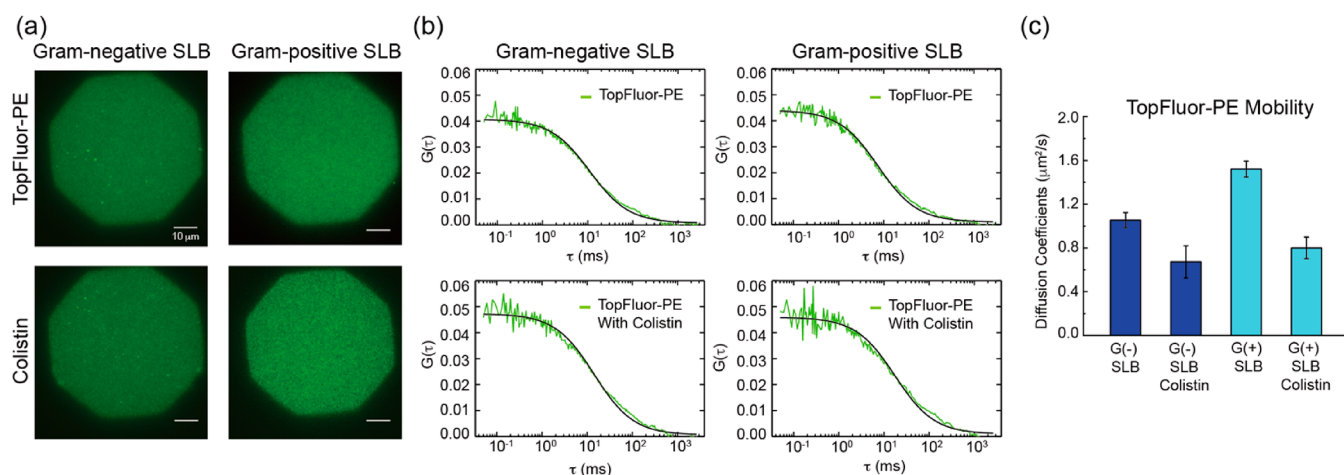


Figure 2. (a) Epifluorescence images of Gram-negative and Gram-positive SLBs containing TopFluor-PE (top images) and after nonlabeled colistin adsorption for the same bilayer (bottom images). The scale bars are 10 μm . (b) FCS data shown for Gram-negative and Gram-positive SLBs containing TopFluor-PE and bound with nonlabeled colistin. (c) Diffusion coefficients of TopFluor-PE lipids on Gram-negative and Gram-positive SLBs. Error bars are the standard deviation.

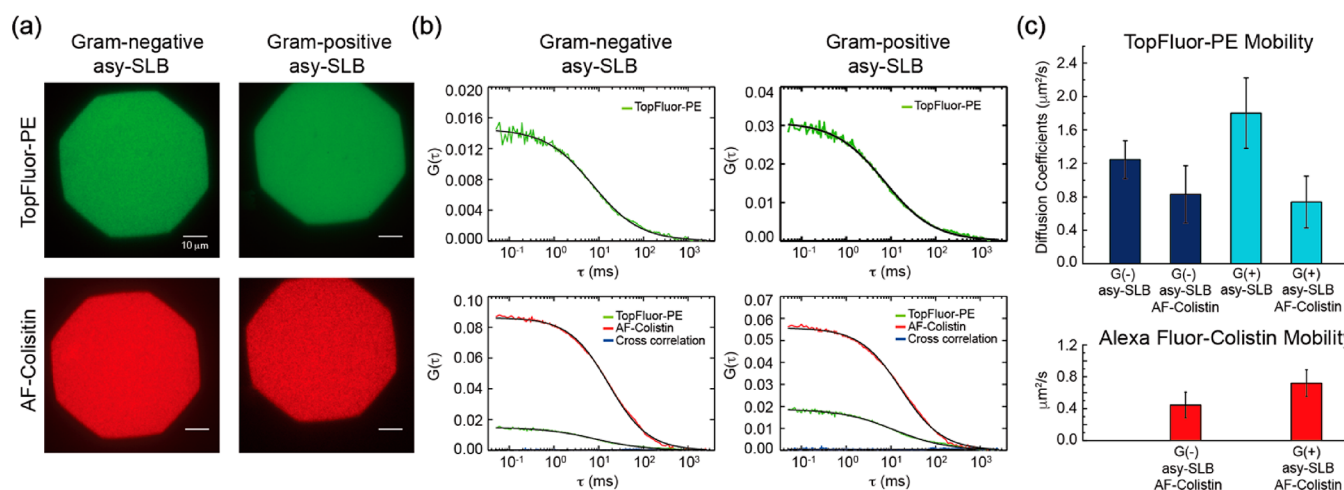


Figure 3. (a) Epifluorescence images of Gram-negative and Gram-positive asy-SLBs containing TopFluor-PE (green, top images) and adsorbed AF-colistin (red, bottom images). The scale bars are 10 μm . (b) Representative PIE-FCCS data is shown for the bilayers. The FCS data corresponding to TopFluor-PE are shown in green before (top) and after (bottom) addition of AF-colistin. The AF-colistin FCS data is shown in red and the cross-correlation functions (very low near baseline) are shown in blue. Black lines represent fits to the data. (c) Diffusion coefficients of TopFluor-PE and AF-colistin in Gram-negative and Gram-positive asy-SLBs obtained from PIE-FCCS data. Error bars represent the mean $\pm$ s.d.

DOTAP/POPE/TopFluor-PE with a molar ratio 60:10:15:14.99:0.01. TopFluor-PE, is a fluorescent PE analogue based on a BODIPY fluorophore attached to the acyl chain of lipid, and is amenable to single molecule fluorescence imaging and FCS.^{25,27} Routine approaches to rupture these SUVs following standard protocols failed to produce a continuous SLB. Two changes were made to the protocol that enabled successful creation of SLBs. First, the salt concentration was raised significantly to 2.0 M NaCl. Second, the glass surface was cleaned and sonicated in water and IPA, but not treated with solvents like NaOH or piranha etched ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$). Epifluorescence images of SLBs formed with this protocol are shown in Figure 2a and demonstrate that the distribution of TopFluor-PE lipid in both Gram-negative and Gram-positive SLBs is homogeneous and uniform.

To measure the two-dimensional mobility of the membrane, we conducted FCS measurements of TopFluor-PE in Gram-negative and Gram-positive SLBs (Figure 2b). All the data fit

well to a 2D anomalous diffusion model, indicating that TopFluor-PE is fully mobile in both Gram-negative and Gram-positive SLBs. The anomaly factor, α , was used to characterize the deviation from ideal 2D diffusion, where $\alpha < 1$ indicates anomalous diffusion and $\alpha = 1$ for normal Fickian diffusion.²⁸ The average α values for the TopFluor-PE FCS data were 0.81 ± 0.03 for the Gram-negative SLBs and 0.76 ± 0.1 for the Gram-positive SLBs (Figure S1). This anomaly is consistent with a slow-moving component that may result from lipid clustering in the SLBs.²⁹ To determine if the substrate interferes with the mobility of the lipids in the lower leaflet of the bilayer we made asymmetric lipid bilayers described in a later section. By measuring the dwell time (τ_D) from the FCS data we calculated the diffusion coefficient through the relationship $D = \omega^2/4\tau_D$ (Figure 2c). The average diffusion coefficient of TopFluor-PE in Gram-negative SLBs was $1.05 \mu\text{m}^2/\text{s}$, which was substantially lower than the diffusion

coefficient of TopFluor-PE in Gram-positive SLBs ($1.52 \mu\text{m}^2/\text{s}$).

Effects of Colistin Binding on PE Lipid Mobility. We next applied these model membranes to study the interaction between Gram-negative and Gram-positive SLBs and an antibiotic peptide, colistin, which is active against Gram-negative bacteria. The samples were prepared by incubating nonfluorescently labeled colistin with lipid mixtures before the start of SLB formation. As shown in Figure 2a, the epifluorescence images of Gram-negative and Gram-positive SLBs associated with unlabeled colistin showed a homogeneous distribution of TopFluor-PE intensity, suggesting that colistin association does not have a significant effect on the lateral distribution of the fluorescence lipids. To test whether colistin influenced the mobility of the lipids, we quantified the diffusion of TopFluor-PE after the addition of colistin using FCS (Figure 2b). In the presence of colistin, the decay time, τ_D , is lower than without colistin for both Gram-positive and Gram-negative SLBs. The Gram-negative SLBs without peptide binding yielded a diffusion coefficient value of $D = 1.05 \mu\text{m}^2/\text{s}$, compared to $D = 0.67 \mu\text{m}^2/\text{s}$ in the presence of colistin. This decrease in lipid mobility after peptide binding is likely due to a hindered diffusion that has been seen for other lipid binding systems.^{25,30} Similarly, the diffusion coefficient for Gram-positive SLBs decreased from 1.52 to $0.80 \mu\text{m}^2/\text{s}$ in the presence of colistin.

Formation and Characterization of Asymmetric SLBs with Alexa Fluor-Labeled Colistin. To more directly assess the correlation between lipid and peptide diffusion, we next conducted experiments with fluorescently labeled colistin on the model membranes discussed in the previous sections. In order to avoid interactions between the substrate and fluorescent lipids, we created an asymmetric SLB (asy-SLB), for which the fluorescent marker lipid was only in the upper leaflet (Figure 3a). The asy-SLBs also allowed us to isolate the mobility of lipids in the upper leaflet of the bilayer as they encounter peptides added to the buffer above the membrane. In the first set of experiments, Gram-negative and Gram-positive asy-SLBs were created with TopFluor-PE as the fluorescent lipid. To monitor peptide binding and mobility we labeled colistin with Alexa Fluor 568 (AF-colistin), which was added to the system after formation of the asy-SLB. We verified the continuity and fluidity of the asy-SLBs with FRAP as shown in Figure S2. We found that the intensity of the bleached area was fully recovered within 210 s. The FRAP data for both Gram-negative and Gram-positive asy-SLBs showed that the recovery of TopFluor-PE is essentially 100%. Lipid and peptide mobilities were quantified with PIE-FCCS as discussed below.

From the PIE-FCCS data (Figure 3b), we first quantified the effect of colistin binding on lipid mobility in the asy-SLBs. For the Gram-negative membranes, TopFluor-PE had a diffusion coefficient of $1.24 \pm 0.23 \mu\text{m}^2/\text{s}$ before peptide binding (Figure 3c). This value is about 20% higher than the diffusion coefficient of the same lipid in the symmetric SLB and is consistent with previous observations that substrate interactions have a small but measurable effect on lipids in the lower leaflet of the SLB.^{8,25} After incubating with AF-colistin, the diffusion coefficient of TopFluor-PE decreased to $0.83 \pm 0.34 \mu\text{m}^2/\text{s}$ (Figure 3c), consistent with the changes seen in the symmetric bilayer results in the previous section. For Gram-positive membranes, TopFluor-PE had a diffusion coefficient of $1.80 \pm 0.42 \mu\text{m}^2/\text{s}$ before peptide binding and

$0.74 \pm 0.31 \mu\text{m}^2/\text{s}$ after colistin binding (Figure 3c). As expected, these values are higher than was observed in the symmetric SLBs but show a similar decrease in mobility when colistin is bound to the membrane. The anomaly factors for TopFluor-PE before peptide binding are 0.81 and 0.79 in Gram-negative and Gram-positive asy-SLB respectively (Figure S3). These values are essentially identical to those in the symmetric SLBs above and suggests that the anomaly is not assignable of interactions between lipids in the lower leaflet and the substrate. With asy-SLBs we were able to suggest that the anomaly instead is likely due to lipid clustering. Upon peptide binding, the anomaly factor decreases to 0.66 and 0.65 for Gram-negative and Gram-positive bilayers, respectively (Figure S3). This change indicates that the peptide causes significant reorganization in the membrane consistent with previous studies.⁸

Next, we quantified the mobility of AF-colistin on the model membranes. The FCS data corresponding to AF-colistin fit well to the 2D diffusion model used for lipid diffusion. From the fit, we calculated an average diffusion coefficient of $0.44 \pm 0.12 \mu\text{m}^2/\text{s}$ for AF-colistin in Gram-negative asy-SLBs. This value is approximately half the value of the TopFluor-PE diffusion coefficient in the same membranes ($0.83 \pm 0.34 \mu\text{m}^2/\text{s}$) (Figure 3c). The lipids and peptides experience the same bulk viscosity, however, differences in their size, shape and chemical interactions influence how they diffuse in the membrane. The lower mobility of the peptide likely reflects multivalent lipid interactions or a larger effective area in the membrane. The diffusion coefficient of AF-colistin in the Gram-positive asy-SLBs is $0.80 \pm 0.15 \mu\text{m}^2/\text{s}$, which is much closer to the diffusion coefficient of TopFluor-PE in the same membranes (Figure 3c). The higher mobility of AF-colistin in Gram-positive vs Gram-negative membranes is consistent with the difference in TopFluor-PE diffusion before peptide binding suggesting that it can be assigned to differences in the bulk fluidity of the two membranes. The fact that the lipid and peptide diffusion coefficients are similar in Gram-positive bilayers is evidence that there are stronger lipid-peptide associations in those membranes. The AF-colistin FCS data was also fitted with an anomalous factor, α , but for both membranes the α value was greater than 0.9, suggesting that there is no substantial clustering or other anomalous diffusion process for peptides bound to membranes at these low concentrations (Figure S4).

Mobility and Peptide Interactions of Cardiolipin in Gram-Negative and Gram-Positive asy-SLBs. Having characterized the behavior of a fluorescence PE lipid analogue in model SLBs, we next sought to quantify the mobility and peptide interactions of another major component of bacterial membranes: cardiolipin. For these experiments we utilized a headgroup-labeled cardiolipin analogue, TopFluor-TOCL (Figure 1). The TopFluor-TOCL lipids inserted into the top leaflet of the Gram-negative and Gram-positive SLBs to make asy-SLBs, and representative images are shown in Figure 4a. After binding AF-colistin, both Gram-negative and Gram-positive SLBs displayed a homogeneous distribution of lipid and peptide with no observable clusters. The fluidity of TopFluor-TOCL in Gram-negative and Gram-positive asy-SLB was also determined by FRAP. These data show that the bilayer is continuous and mobile, exhibiting 100% recovery after about 3 min (Figure 4b). PIE-FCCS data were collected before and after binding of AF-colistin (Figure S5), and the diffusion coefficients of TopFluor-TOCL are summarized in

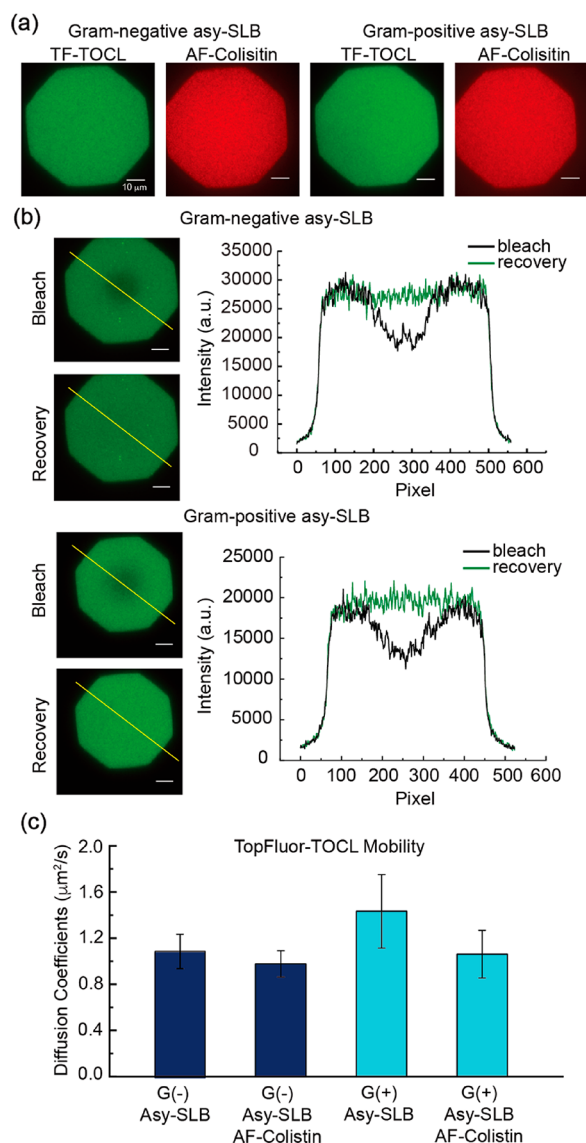


Figure 4. Epifluorescence images of (a) Gram-negative and Gram-positive asy-SLBs containing TopFluor-TOCL and adsorbed AF-colistitin. Green channel represents the TopFluor-labeled PE and TOCL lipid; Red channel represents the Alexa Fluor 568-labeled colistitin. The scale bars are 10 μm. (b) Fluorescence recovery after photobleaching (FRAP) experiments for TopFluor-TOCL are shown for a Gram-negative and Gram-positive asy-SLB. Epifluorescence images of Gram-negative and Gram-positive asy-SLB immediately after photo bleaching (top) and following 210 s after recovery (bottom). The image intensity along the yellow lines in the images are plotted on the right to show full recovery. (c) Diffusion coefficients of TopFluor-TOCL lipids on Gram-negative and Gram-positive asymmetric SLBs and adsorbed nonlabeled colistitin (left, dark blue).

Figure 4c. The peptide diffusion coefficients were essentially identical to those observed in the TopFluor-PE membranes as seen in Figure S6.

As shown in Figure 4c, the average diffusion coefficient of TopFluor-TOCL in Gram-negative asy-SLB is $1.08 \pm 0.15 \mu\text{m}^2/\text{s}$. This value is slightly smaller than that measured for TopFluor-PE in a similar membrane. In the Gram-positive membranes, the TopFluor-TOCL diffusion coefficient is $1.43 \pm 0.32 \mu\text{m}^2/\text{s}$, which is higher than in the Gram-negative asy-SLB but again slightly lower than in TopFluor-PE in similar

membranes. The difference between diffusion in the two membranes is consistent with the TopFluor-PE and AF-colistitin data, making it clear that all of the components in the Gram-negative membranes diffuse more slowly than in the Gram-positive membranes. The difference between TopFluor-PE and TopFluor-TOCL in similar membranes is likely because individual TOCL lipids take up a larger effective area than TopFluor-PE lipids, which will lead to lower diffusion in the same membrane environment. With the addition of fluorescently labeled colistitin, the average diffusion coefficient of TopFluor-TOCL in Gram-negative asy-SLBs is $0.98 \pm 0.11 \mu\text{m}^2/\text{s}$, which is statistically identical to the diffusion coefficient before the peptide is added. For Gram-positive membranes, there is a significant decrease in the diffusion coefficient of TopFluor-TOCL when peptide is added ($1.06 \pm 0.21 \mu\text{m}^2/\text{s}$).

With both the lipid and peptide labeled with spectrally distinct fluorescent probes, we also calculated the relative cross-correlation, f_c , to assess the fraction of TopFluor-lipid that codiffuses with AF-colistitin. Cross-correlation is a direct measure of stable interactions and is related to the binding affinity between the lipids and peptide. As shown in Figure 5,

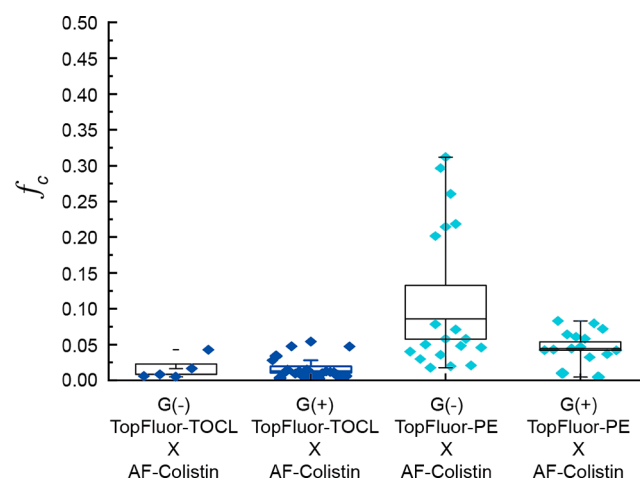


Figure 5. f_c values measured for TopFluor-PE and TopFluor-TOCL Gram-negative and Gram-positive asy-SLBs. TopFluor-PE asy-SLB is plotted in dark blue and TopFluor-TOCL asy-SLB is in light blue. The line in the center of each box is the median of the distribution.

AF-colistitin with TopFluor-TOCL Gram-negative and Gram-positive asy-SLB data showed f_c values were near zero, with a value of 0.016 and 0.019, respectively, indicating that no colistitin–lipid complexes formed above our limit of detection. The f_c data for colistitin with TopFluor-PE Gram-negative and Gram-positive asy-SLB were also recorded (Figure 5). The average f_c value for colistitin with Gram-positive asy-SLB is 0.048, which is also near zero. However, it is interesting to note that the average f_c value for colistitin with TopFluor-PE Gram-negative asy-SLB is 0.083. This non-near zero f_c value ($f_c > 0.05$), suggesting that colistitin may interact with TopFluor-PE lipid and form colistitin–lipid complexes in Gram-negative bilayer. One limitation of this data is that concentration of the TopFluor-PE lipid is about 3 orders of magnitude lower than that of the bulk unlabeled PE lipid. Therefore, the interaction is likely being severely outcompeted by unlabeled lipid and the effective cross-correlation is severely under-representing the degree of association. While the data cannot be used to quantify the binding affinity, the presence of

nonzero cross-correlation is strong evidence for a PE/colistin complex.

CONCLUSIONS

In this work we have reported a modified vesicle fusion method to make stable, fluid Gram-negative and Gram-positive model membranes containing a high percentage of charged lipid and PE lipid. By using fluorescence imaging and PIE-FCCS, we have characterized the lipid dynamics of Gram-negative and Gram-positive bacterial membranes. The fluorescence images and FRAP data provided evidence for the formation of a fluid SLB and asy-SLB. The FCS data were used to experimentally measure lipid diffusion coefficients showing that TopFluor-TOCL and TopFluor-PE mobility was lower in Gram-negative bilayers compare with Gram-positive bilayers. Moreover, the diffusion coefficient of TopFluor-PE in Gram-negative and Gram-positive SLBs were found to be slower than those in asymmetric SLBs. The higher lipid mobility in asy-SLBs suggests that they are an ideal model system for measuring peptide interactions with Gram-negative and Gram-positive membranes.

In order to understand the interactions of colistin with Gram-negative and Gram-positive bacterial membranes, we also performed PIE-FCCS measurements on asy-SLBs with AF-colistin. Colistin binding reduced the mobility of TopFluor-PE and TopFluor-TOCL in both Gram-negative and Gram-positive asy-SLBs. However, we found that the decrease of diffusion coefficient in TopFluor-PE asy-SLBs to be approximately 2-fold higher than those for TopFluor-TOCL asy-SLBs, indicating stronger binding of TopFluor-PE with colistin compare to TopFluor-TOCL. Moreover, based on the diffusion coefficient of colistin, we found that Gram-positive bilayer exhibits the fastest diffusion in all types of model membrane, suggesting that colistin has a stronger interaction with Gram-negative membrane. Future work should be done to resolve the affinity between colistin and other lipids, especially PG.

The approach outlined here for preparation Gram-negative and Gram-positive model membrane should create new opportunities to investigate biophysical phenomena in bacterial membranes. Our bilayer preparation protocol differs from a recent report in which mM concentrations of Ca^{2+} were used to make SLBs with high PE and PG content.¹⁴ These studies indicate that multiple factors affect the formation of bacterial membrane mimetics and suggest that more work needs to be done to resolve the mechanism of formation and the limits of these techniques.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.9b09709>.

Additional data including Figures S1–S8 and Table S1 (PDF)

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Notes

The authors declare no competing financial interest.

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