Interaction of Daptomycin with Lipid Bilayers: A Lipid Extracting Effect

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Supporting Information

ABSTRACT: Daptomycin is the first approved member of a new structural class of antibiotics, the cyclic lipopeptides. The peptide interacts with the lipid matrix of cell membranes, inducing permeability of the membrane to ions, but its molecular mechanism has been a puzzle. Unlike the ubiquitous membrane-acting host-defense antimicrobial peptides, daptomycin does not induce pores in the cell membranes. Thus, how it affects the permeability of a membrane to ions is not clear. We studied its interaction with giant unilamellar vesicles (GUVs) and discovered a lipid-extracting phenomenon that correlates with the direct action of daptomycin on bacterial membranes observed in a recent fluorescence microscopy study. Lipid extraction occurred only when the GUV lipid composition included phosphatidylglycerol and in the presence of Ca²⁺ ions, the same condition found to be necessary for daptomycin to be effective against bacteria. Furthermore, it occurred only when the peptide/lipid ratio exceeded a threshold value, which could be the basis of the minimal inhibitory concentration of daptomycin. In this first publication on the lipid extracting effect, we characterize its dependence on ions and lipid compositions. We also discuss possibilities for connecting the lipid extracting effect to the antibacterial activity of daptomycin.

Daptomycin (Figure 1) is an FDA (the U.S. Food and Drug Administration)-approved lipopeptide antibiotic, notably active against multidrug-resistant, Gram-positive pathogens, including methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE). In recent reviews,¹⁻³ it was concluded that despite nearly 25 years of study and 9 years of clinical use, aspects of the mechanism of action of daptomycin remained poorly understood. The evidence so far suggests that its main target is the cytoplasmic membrane, where daptomycin causes leakage of cations leading to loss of membrane potential and cell death.¹²⁻⁴⁻⁶ The membrane function is compromised in the absence of cell lysis or leakage of molecules other than atomic cations.⁶⁻⁷ What is not yet clear is what molecular process underlies the action of daptomycin. One major concern about the clinical use of daptomycin is the development of resistance during therapy. Many of the mutations that alter susceptibility to daptomycin have been shown to directly affect the membrane lipid composition.⁸⁻¹² This further supports the role of the membrane being the central target for the action of daptomycin. Thus, the interaction of daptomycin with lipid bilayers is fundamental to its antibiotic activity.

The antibacterial activity of daptomycin is calcium-dependent¹³ and correlates with the target membrane’s content of phosphatidylglycerol (PG).¹⁰ In the presence of Ca²⁺ ions, daptomycin spontaneously binds to artificial membranes,¹⁴ but only minor effects of daptomycin on lipid vesicles have been detected in past experiments.¹⁴⁻¹⁶ Fluorescence resonance energy transfer (FRET) experiments have shown daptomycin aggregation in membranes,¹⁷⁻¹⁹ but no leakage of calcine from lipid vesicles was detected.¹⁵ The only noticeable effect of daptomycin on the membrane properties was causing leakage of cations from lipid vesicles, but the effect was weak compared with that of an ionophore valinomycin at comparable concentrations.²⁰ These earlier studies used artificial membranes in the form of small liposomes (<100 nm).¹⁴⁻¹⁶,¹⁸,²¹ Here we used giant unilamellar vesicles (GUVs) (>10 μm) that allowed us to observe the action of daptomycin under a microscope. Our results are consistent with all previous measurements, but in addition, we discovered an effect of lipid extraction that appears to be similar to the direct action of daptomycin on bacterial membranes.¹

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Figure 1. Structural formula of daptomycin. The molecule has a net charge of approximately −3 at pH 7.
Lipid extraction occurred only when the GUV lipid composition included phosphatidylglycerol (PG) and in the presence of Ca\(^{2+}\) ions, exactly the same condition found to be necessary for daptomycin to be effective against bacteria. The greatest advantage of the GUV method over the methods of smaller liposomes is that we can measure the membrane area change as daptomycin binds to it. Thus, we obtained a quantitative measure of the lipid extracting effect and showed that it has a daptomycin concentration dependence. We found that lipid extraction by daptomycin is a cooperative phenomenon with a threshold concentration. The effect was detected only for daptomycin concentrations above this threshold.

Because daptomycin targets bacterial cell membranes, the resistance mechanisms have been suspected to involve changes in the lipid composition of the bacterial cell membrane.\(^9\) One possibility is decreasing the extent of the synthesis of PG or increasing the extent of the conversion of PG to lysylphosphatidylglycerol (LPG).\(^9\) These were found in the resistant mutants of \textit{Staphylococcus aureus}\(^22\) and \textit{Bacillus subtilis}.\(^23\) Our experiment shows that replacing PG with LPG has the effect of diminishing the lipid extracting effect. Another possibility involves cardiolipin.\(^12\) Recent reports suggested interference of cardiolipin with the action of daptomycin on membranes.\(^24,25\)

Our experimental results described here are inadequate to address the reported effects of cardiolipin.

**MATERIALS AND METHODS**

**Materials.** Daptomycin was purchased from Selleckchem (Munich, Germany) and from Haorui-Pharma Chem Inc. (Irvine, CA); 18:1 (Δ9-Cis) PC (DOPC), 18:1 (Δ9-Cis) PG (DOPG), 18:1 Liss Rhod PE (Rh-PE), 18:1 cardiolipin, and 18:1 Lysyl PG (lysyl PG) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red sulfonyl chloride (MW 625) was purchased from Invitrogen Probe (Grand Island, NY). Fluorescence-labeled BODIPY-daptomycin was a gift from Cubist Pharmaceuticals, Inc. (Lexington, MA).

**Aspirated GUV Experiment.** This experiment was a modification of the original method of Kwok and Evans\(^26\) and of Longo et al.\(^27\) as described by Sun et al.\(^28\) Giant unilamellar vesicles (GUVs) of a chosen lipid composition were produced by the electroformation method\(^29\) in a solution containing 199 mM sucrose for the purpose of controlling the osmolality and 1 mM Tris (pH 7). Ten microliters of a GUV suspension was injected into a control chamber that contained 190 mM glucose and 10 mM Tris (pH 7). A selected GUV (diameter of ∼20–30 μm) was aspirated at a low constant negative pressure (∼100 Pa producing a membrane tension of ∼0.4 mN/m) with a micropipette, which was connected to an oil-filled U tube in which a negative pressure was produced and controlled by reference to the atmospheric pressure.\(^28\) A separate observation chamber contained ∼190 mM glucose, 10 mM Tris (pH 7), Ca\(^{2+}\) ions, and daptomycin. The aspirated GUV was then transferred through a movable pipe\(^28\) from the control chamber to the observation chamber. We used sucrose inside and glucose outside of GUV, so that GUVs sank to the bottom for ease of aspiration manipulation. Also the phase contrast between sucrose and glucose could be monitored by a phase condenser to detect possible leakage of sugar through the membrane. We included a small amount of dye lipid (1 mol % Rh-PE) to make the boundary of the GUV clear, except in the case of the dye leakage experiment in which 10 μM Texas Red sulfonyl chloride was encapsulated in the GUV. The osmolality of each solution used in the experiment was measured by the Wescor (Logan, UT) model 5520 vapor pressure osmometer. Equi-osmolality between the inside and outside of the GUV was the initial condition in all GUV experiments. The response of the GUV to daptomycin binding was observed and recorded by a camera-equipped microscope. All GUV experiments were performed at room temperature (∼25 °C).

The binding of daptomycin to a GUV can be monitored by the expansion of the membrane area. This expansion is too small to be measured accurately by the change in the diameter of GUV. We used a technique developed by Kwok and Evan,\(^26\) in which a spherical GUV was aspirated partially into a micropipette by a constant, low membrane tension (∼0.4 mN/m) (Figure 2A). The shape of the GUV then consisted of two parts, a spherical part and a cylindrical part, with different surface area/volume (A/V) ratios. An overall A/V change due to peptide binding would cause a repartition between these two parts, a spherical part and a cylindrical part, with different surface area/volume (A/V) ratios.

![Figure 2. Effect of daptomycin on 7/3 DOPC/DOPG GUVs (including 1% Rh-PE) in the presence of Ca\(^{2+}\) ions, in widefield images. (A) Rh-PE fluorescence widefield images of GUVs at different daptomycin concentrations (0.3, 1.0, and 5.0 μM from top to bottom, respectively). Note that the length of protrusion into the micropipette increased to a maximal value at 0.3 μM and increased and then decreased at 1.0 and 5.0 μM. When the protrusion length decreased, aggregates appear on the GUV surface. (B) Fractional area change ΔA/A of the GUVs over time. For the sake of clarity, only two or three representative runs are shown for each daptomycin concentration. The red data points and blue triangles ended when the GUV ruptured. (C) As ΔA/A decreased, substances including Rh-PE came off or appeared on the GUV surface, as indicated by arrows (see Movies S1 and S2 of the Supporting Information). The scale bar is 10 μm.](image-url)
parts, from which the change in area could be accurately measured. From the microscopic images, the length of the protrusion into the micropipette ($L_p$), the radius of the micropipette ($R_p$), and the radius of the GUV ($R_v$) were carefully measured. Then it is straightforward to show $\Delta A = 2\pi R_v L_p + 8\pi R_v R_p$, and $\Delta V = \pi R_v^2 L_p + 4\pi R_v^2 R_p$. If there were no molecular leakage (see below) and as long as the inside and outside of the GUV had the same osmolality, there would be no change in volume. When $\Delta V = 0$, $\Delta A$ is directly proportional to $\Delta L_p$: $\Delta A = 2\pi R_v (1 - R_p/R_v) \Delta L_p$. The fractional area change ($\Delta A/A$) is calculated from the change in protrusion length $\Delta L_p$. When the protrusion length decreased, the data analysis was limited to the point at which the protrusion length vanished. The maximal negative $\Delta A/A$ value depended on the initial protrusion length (before the exposure to daptomycin) that varied from vesicle to vesicle. All experiments reported here were highly reproducible, although small quantitative variations were expected of GUV experiments. For the sake of clarity, only two or three representative results are presented in the figures for each type of experiment, approximately 20–30% of actual measurements. No error bars are shown because the results are interpreted qualitatively, except for the measurement of the threshold concentration.

**RESULTS**

**Lipid Extracting Effect.** The antibacterial activity of daptomycin requires Ca$^{2+}$ ions.$^{13}$ The standard media for in vitro susceptibility testing of daptomycin in clinical laboratories are set to a calcium content of 50 mg/L ($\sim$1.25 mM),$^{30}$ which is close to the calcium concentration in human serum.$^2$ The reported minimal inhibitory concentration (MIC) values of daptomycin are in the micromolar range.$^{2,13}$ Thus, we chose 1 $\mu$M daptomycin and 1 mM Ca$^{2+}$ as our reference condition (we found our results to be insensitive to Ca$^{2+}$ concentration from $\sim$0.5 to $\sim$2 mM). Also, the susceptibility of bacteria to daptomycin is correlated to the target membrane's content of phosphatidylylycerol (PG).$^{9,23}$ To simulate the PG content of bacterial membranes, we used lipid bilayers of a DOPC/DOPG mixture at a 7/3 ratio. PC/PG mixtures were commonly used in previous model membrane experiments$^{15,17,18}$ for daptomycin.

First, for the control experiments, in the absence of daptomycin, we detected no membrane expansion of a 7/3 DOPC/DOPG GUV with Ca$^{2+}$ (Figure S1 of the Supporting Information). Figure 2A shows the responses of the GUVs to the binding of daptomycin. The GUVs were made visible by including a small fraction (1 mol %) of 18:1 Liss Rhod PE (Rh-PE). They were transferred at zero time into an observation chamber containing a solution of daptomycin and Ca$^{2+}$ ions ($\sim$1.25 mM),30 which would change the osmolality (see Movies S1 and S2 of the Supporting Information). Figure 3 shows that the exuded aggregates contained both Rh-PE and BODIPY-daptomycin.

To show that the aggregates are those of both lipid and daptomycin, we performed the same experiment with daptomycin, including 30% fluorescence-labeled BODIPY-daptomycin. (Note that BODIPY-daptomycin is 2–4 times less active than the unmodified molecule.3) Figure 3 shows that the exuded aggregates contained both Rh-PE and BODIPY-daptomycin.

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Molecular Leakage Experiment. To test if the lipid extracting effect causes any molecular leakage, we included 10 μM Texas Red sulfonyl chloride (TRsc, MW 625) in the GUV. In this case, no dye lipid (Rh-PE) was used, because Lissamine Rhodamine B (Rh) and Texas Red are of the same color; consequently, the aggregates were not directly visible (Figure 5). The change in the slope of ΔA/A signaled the occurrence of lipid extraction, but there was no leakage of the content dye. The fluorescence intensity of the encapsulated TRsc decreased slightly over time at a rate comparable to that of photo-bleaching of the background fluorescence. For comparison, the TRsc leakage caused by melittin pores is shown in Figure S2 of the Supporting Information.

Ion Dependence. What is the role of Ca²⁺ ions in the action of daptomycin on membranes? In the absence of Ca²⁺ ions, no binding of daptomycin was detected at a concentration of 1 μM (not shown). Even when Ca²⁺ was replaced with Mg²⁺ (at 1 mM), we saw little binding of daptomycin to the PG-containing membranes (Figure 6). This is consistent with the reports that the antibacterial activity of daptomycin requires Ca²⁺ ions and that the MICs increased at least 32-fold if Ca²⁺ ions were replaced with Mg²⁺ ions.²¹

However, if the daptomycin concentration was increased to 5 μM, there was a membrane area expansion indicating daptomycin binding at higher concentrations in the absence of Ca²⁺ ions. At 50 μM daptomycin without Ca²⁺ ions, the response of the GUV was similar to that with 1 μM daptomycin with Ca²⁺ (Figure 2). This result indicates that the role of Ca²⁺ is to facilitate the binding of daptomycin to lipid bilayers, as suggested in an earlier study, but the binding affinity of daptomycin for membranes is not zero in the absence of Ca²⁺. As long as there is a sufficient amount of daptomycin bound to a PG-containing membrane, there is a lipid extracting effect. Again, this is consistent with the MICs increasing 32-fold in the absence of Ca²⁺ ions.²¹

Lipid Dependence. The susceptibility of bacteria to daptomycin is correlated with the membrane content of PG.⁹ Indeed, we found that the lipid extracting effect occurred only when GUVs contained PG. Interestingly, daptomycin binds to the electrically neutral pure DOPC membrane in the presence of Ca²⁺ but exhibited no lipid extraction (Figure 7). The peptide moiety of daptomycin is anionic [net charge of approximately -3 at pH 7 (Figure 1)], yet it still has a low binding affinity for the DOPC/PG anionic lipid bilayer in the absence of Ca²⁺ (Figure 6). With Ca²⁺, daptomycin has a stronger affinity for DOPC than for DOPC mixed with anionic CL or LPG (Figure 7). Thus, the binding affinities of daptomycin for lipid bilayers do not follow a naive electrostatic argument based on the net charge.

Some studies of the resistance to daptomycin implied the possibilities of shifting the phospholipid composition from PG to LPG in one case, and to CL in another. We found that replacing PG with either LPG or CL eliminated the lipid extracting effect at a daptomycin concentration of 1 μM (Figure 7). However, the most recent studies implied possible interference of CL with the action of daptomycin. This clearly calls for much more extensive studies of daptomycin with the lipid mixtures of CL and PG.

Existence of a Threshold Concentration. The lipid extracting effect has a strong daptomycin concentration dependence. Below a threshold concentration, the effect was absent. Above the threshold, the effect occurred in a shorter and shorter time as the daptomycin concentration increased. To quantify this dependence, we plotted the time τ for reaching the maximum of ΔA/A (ΔA/A) as a function of daptomycin concentration (Figure 8). As the daptomycin concentration decreased from 4 to 0.4 μM, the time to reach (ΔA/A) exponentially increased. When the concentration decreased to 0.3 μM, the membrane area increased to ~3% and...
stayed constant without a decrease (Figure 2); there was no lipid extracting effect, equivalent to \( \tau \rightarrow \infty \) Thus, the threshold concentration for the lipid extracting effect is \( \sim 0.3 \mu M \) or, more exactly, between 0.3 and 0.4 \( \mu M \) These measurements were first performed at a constant \( \mathrm{Ca}^{2+} \) concentration of 1.25 mM The same result was reproduced at 0.5 and 1 mM \( \mathrm{Ca}^{2+} \) (Figure 8).

We examined all GUV experiments with a 7/3 DOPC/DOPG GUV and \( \mathrm{Ca}^{2+} \) ions and concluded that there is a critical value of \( (\Delta A/A)_{\text{max}} \) of \( \sim 3\% \) [This is the average of \( (\Delta A/A)_{\text{max}} \) values for daptomycin at 0.4–0.5 \( \mu M \)] At <0.4 \( \mu M \) daptomycin, \( \Delta A/A \) reaches a constant value of \( \leq 3\% \) without a lipid extracting effect At >0.4 \( \mu M \) daptomycin, \( \Delta A/A \) increases to a \( (\Delta A/A)_{\text{max}} \) of >3\% and then decreases, concurrent with a lipid extracting effect The lipid extracting effect started when the membrane expansion \( \Delta A/A \) reached the critical value of \( \sim 3\% \) However, at high daptomycin concentrations, the membrane area continued to increase even after the lipid extracting effect had started, because daptomycin continued to bind to the lipid bilayer from the solution Consequently, the \( (\Delta A/A)_{\text{max}} \) value could reach \( \sim 6\% \) before the membrane area started to decrease showing the effect of lipid extraction (Figure 2B).

**DISCUSSION**

Daptomycin is the first approved member of a new structural class of antibiotics, the cyclic lipopeptides (Figure 1) It is a water-soluble membrane-acting antibiotic1,2,4–8 The apparent membrane-acting feature of the molecule is its hydrophobic N-terminal decanoyl chain It is worth noting that the decanoyl chain can be replaced by varieties of hydrophobic substitutes without losing antibacterial activity10,32 The hydrophobic moiety is expected to insert into the hydrocarbon region of a membrane However, the research so far has not yielded the molecular configuration of membrane-bound daptomycin There is a change in the circular dichroism (CD)15 of daptomycin when it binds to PG-containing lipid bilayers in the presence of \( \mathrm{Ca}^{2+} \), indicating a possible structural change upon membrane binding, but there has been no theoretical interpretation of the CD spectra of daptomycin Nuclear magnetic resonance studies were limited to daptomycin in solutions without lipid bilayers15,33,34 The lack of structural information about membrane-bound daptomycin is the main obstacle for understanding its molecular mechanism.

Over the past two and a half decades, the field of membrane-active peptides has grown from the studies of host-defense antimicrobial peptides (AMPs).35–41 AMPs are characterized by their abilities to form pores in membranes and cause leakage of dye molecules from lipid vesicles35–41 Thus, very active research of AMPs has focused on the molecular mechanisms of AMPs that cause molecular leakage35–41 In contrast, daptomycin does not induce molecular leakage across a membrane67, the proof is shown here in Figure 5 (and Figure S2 of the Supporting Information; see Note S2 of the Supporting Information) Compared with the research publications for AMPs, there are fewer studies of daptomycin–membrane interactions because of the lack of obvious effects on the membrane Until now, the only detected reaction...
in membranes is oligomerization or aggregation of daptomycin by the FRET technique.\textsuperscript{17,18} A hypothesis for transmembrane octameric daptomycin pores has been postulated from the FRET and ion leakage experiments.\textsuperscript{19,20,25} This hypothesis required many assumptions\textsuperscript{19,20,25} that have not been critically evaluated. There is no structural evidence of such pores, and there are no free energy arguments about why daptomycin, given its molecular structure, would form such an octameric complex and why such a complex would be stable inside a lipid bilayer. In contrast, pores induced by AMPs have been detected by X-ray diffraction in lipid crystallines\textsuperscript{41,42} and by neutron scattering in fluid membranes,\textsuperscript{43,44} and they are free energy minimum states (see references in refs 40 and 41).

Here we discovered an effect of daptomycin on lipid bilayers that appeared to be similar to the direct action of daptomycin on bacterial membranes observed in a recent fluorescence microscopy study,\textsuperscript{1} i.e., inducing lipid aggregates on the surface of a bacterial membrane. We emphasize that the observed aggregates are the end product of the daptomycin interaction. We imagine that the aggregates must have started from a microscopic size and became visible only after they grew or combine to micrometer size. With a microscope, we cannot see the aggregation process. Most of the macroscopic aggregates appeared to stay on the GUV surface. They moved away from the GUV surface if there was a flow in the solution (see Movies S1 and S2 of the Supporting Information). As far as we could detect, the aggregates staying on the surface of GUV had no effect on the property of the GUV bilayer. Therefore, we believe that the main effect of daptomycin on the membrane is removing lipid molecules from the bilayers as a result of the interaction.

The lipid extracting effect is a predictable reaction of daptomycin with lipid bilayers under specific conditions. It occurred only when the GUV lipid composition included PG and in the presence of Ca\textsuperscript{2+} ions, the same condition found to be necessary for daptomycin to be effective against bacteria.\textsuperscript{10,13} Most surprisingly, the lipid extracting effect has an all-or-none cooperative concentration dependence that is similarly to a MIC. This suggests that the lipid extracting effect and the antibacterial activity of daptomycin are correlated. The current consensus view of daptomycin’s bactericidal activity is that it causes leakage of atomic cations through the cytoplasmic membrane of bacteria, leading to loss of membrane potential and cell death.\textsuperscript{1,2,4,6-8} It compromises the membrane function without cell lysis or molecular leakage.\textsuperscript{6,7} Can the lipid extracting effect be the underlying mechanism for the bactericidal activity? We discuss this possibility by offering the following observations.

**Lipid Extraction and Ion Leakage.** The permeation of a membrane by ions is one of the subjects most studied by molecular simulations.\textsuperscript{45-49} The consensus results can be summarized as follows. (i) Ion permeation is an activated process; spontaneous ion permeation is rare.\textsuperscript{48,49} (ii) Ions enter the bilayer with a trail of water molecules called water fingers\textsuperscript{45} (the potential energy for an isolated ion in membrane is too high). The water finger is lined by lipid headgroups. This results in a major rearrangement of neighboring lipids, commonly called water pore defects.\textsuperscript{45,49} (iii) Simulations by displacing a pair of chain-to-chain lipid molecules from a bilayer create a water pore defect, allowing ion permeation.\textsuperscript{47} (iv) The transient water pores created by activated ion permeation by or lipid molecule displacement have radii of <1 nm\textsuperscript{46} and lifetimes of 10-100 ns.\textsuperscript{46,47}

Thus, it is reasonable to expect that daptomycin-induced lipid extraction can lead to ion permeation. So far, daptomycin-induced K\textsuperscript{+} and Mg\textsuperscript{2+} leakage has been observed in bacteria. The negative counterions were most likely blocked from permeation by the presence of anionic lipid PG. The water pore defects induced by the lipid extracting effect would be too transient and too small to allow molecular leakage. It is well-known that very small amounts of net charge translocation can cause significant changes in membrane potential; e.g., it has been demonstrated that the translocation of only 2.9 \times 10\textsuperscript{3} H\textsuperscript{+} ions per Escherichia coli cell was sufficient to change the membrane potential by 16 mV.\textsuperscript{50,51}

**Molecular Process That Causes Lipid Extraction.** The lipid extraction effect is not unique to daptomycin. Similar effects have been observed previously in the studies of \(\beta\)-amyloid peptides human islet amyloid polypeptide (hIAPP)\textsuperscript{52} and amyloidogenic peptide penetratin.\textsuperscript{53,54} Despite having different molecular structures, daptomycin and \(\beta\)-amyloid peptides exhibit similar behaviors in solution. \(\beta\)-Amyloid peptides are monomeric at low concentrations but aggregate at high concentrations.\textsuperscript{55,56} Similarly, daptomycin exists as soluble monomers at micromolar concentrations\textsuperscript{57} but oligomerizes at millimolar concentrations.\textsuperscript{15,21,33}

In the presence of lipid bilayers, \(\beta\)-amyloid peptides first bind to bilayers as \(\alpha\)-helical monomers, but when the bound peptide/lipid ratio exceeds a threshold, peptides transform from \(\alpha\)-monomers to \(\beta\)-sheet aggregates (can be detected by CD).\textsuperscript{52-54} The latter appeared as peptide–lipid aggregates on the membrane surface,\textsuperscript{52,53} exactly like the aggregates induced

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![Figure 8](https://example.com/figure8.png)

**Figure 8.** Time \(\tau\) to the maximal \(\Delta A/A\) \((\Delta A/A)_{\text{max}}\) vs daptomycin concentration. The Ca\textsuperscript{2+} concentration was constant at 1.25, 1.0, and 0.5 mM (from top to bottom, respectively). The inset shows examples of how \(\tau\) was measured for 1 and 2 \(\mu\)M daptomycin (see arrows). The curves are a guide to the eye. The vertical dashed lines are at 0.3 \(\mu\)M. The error bars represent the ranges of reproducibility by several runs. The results measured at three different Ca\textsuperscript{2+} concentrations are essentially the same.
by daptomycin. Normally, a molecule bound to a lipid bilayer either adsorbs on the interface between the headgroups and the chains or embeds inside the chain region. Both the β-peptide aggregates and the daptomycin aggregates unfold from the lipid bilayer, because, we believe, they are not compatible with being a bound state in lipid bilayers. It is not clear in either case whether lipid is intrinsically part of the molecular aggregation or lipid molecules attach to the peptide aggregates when the latter unfold from the bilayer. In either case, the actions of molecular aggregation and unfolding from the bilayer have an effect of lipid extraction, i.e., removing lipid molecules from the bilayer.

The connection of the lipid extracting effect to daptomycin’s bactericidal activity is reinforced by the findings that hIAPP and penetratin are cytotoxic56,57 and antibacterial,58,59 respectively. They were also found to induce transmembrane ion conduction without daptomycin (Figure S1), additional runs of the GUV are almost the same (Movie S3), control experiments without daptomycin. Normally, a molecule bound to a lipid bilayer by daptomycin. Normally, a molecule bound to a lipid bilayer

![Image]

**ASSOCIATED CONTENT**

**Supporting Information**

Movies of Figure 2 showing the effect of daptomycin on 7/3 DOPC/DOPG GUVs (including 1% Rh-PE) in the presence of Ca²⁺ ions (Movies 1 and 2), movie of Figure 4 showing the lipid extracting effect when the inside and outside solutions of the GUV are almost the same (Movie S3), control experiments without daptomycin (Figure S1), additional runs of the molecular leakage experiment as shown in Figure 5 (Figure S2), and additional notes and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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