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Interaction of Daptomycin with Lipid Bilayers Correlated to Its Action on Cell Membranes

by

Yen-Fei Chen

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APPROVED, THESIS COMMITTEE

Huey W. Huang, Chair
Professor of Physics and Astronomy

Frank R. Toffoletto
Professor of Physics and Astronomy

Thomas C. Killian
Professor of Physics and Astronomy, Department Chair

HOUSTON, TEXAS
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ABSTRACT

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Daptomycin is a lipopeptide antibiotic notably against multidrug-resistant, gram-positive pathogens. Evidence shows that the antibiotic acts neither on DNA nor on the proteins. It has been shown to insert and aggregate in the bacteria membrane; nevertheless, how the molecular interaction leads to cell death is unknown. In this work, the physical properties of interactions between daptomycin and model membranes are studied in order to understand the underlying mechanism of daptomycin. First, daptomycin’s binding affinity to membranes was found to be proportional to Ca\(^{++}\) concentration. The effect of Ca\(^{++}\) cannot be replaced by other divalent ions such as Mg\(^{++}\). After binding, daptomycin was found to form lipid-peptide aggregations on phosphatidylglycerol(PG)-containing vesicles. PG is required for the formation of lipid-peptide aggregates, which eventually lead to membrane ruptures. Cardiolipin, another main component in bacterial membrane, cannot substitute PG to induce the same membrane defect. In addition, with a fixed concentration of Ca\(^{++}\), it requires a minimum concentration of daptomycin to trigger the membrane defect process. The membrane defect results mainly from lipid-peptide aggregations rather than from pores forming in the membrane. Finally, x-ray data imply that daptomycin binds to the headgroup region of the bilayer, which
causes membrane thinning. The elastic energy of membrane thinning elevates the energy level of the daptomycin binding state, which explains the transition to the aggregation state.
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Daytomycin is a lipopeptide antibiotic notably against multidrug-resistant, gram-positive pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE). Despite nearly 25 years of study and 9 years of clinical use, aspects of the mechanism of action of daptomycin remain poorly understood [1-3]. It is known that its main target is the bacterial plasma membrane, where daptomycin causes leakage of potassium (and possibly other) ions leading to loss of membrane potential and cell death [2, 4]. It is also known that the membrane function is compromised in the absence of cell lysis or creation of large pores [5]. One major concern of the use of daptomycin for the treatment of infection 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 [6-9]. This further supports the role of the membrane as the central target for the action of daptomycin. To understand the
underlying mechanism of resistance to daptomycin, we must first understand the mode of action of daptomycin. From the data available so far, it is already clear that the action of daptomycin is entirely different from that of better known pore-forming antimicrobial peptides [10-12]. Diverse ways of killing bacteria via membrane interaction are not only interesting from the viewpoint of membrane biophysics, the insights gained from such studies could also be useful for combating the growing threat from antibiotic-resistant infections.

Our ultimate goal is to gain the structural information about how daptomycin interacts with lipid bilayers and understand the energetics of the interacting process. But the first step is to identify the direct effect of daptomycin and separate it from the cellular reaction to the direct effect. Confusing the secondary cellular effects with the direct effect is often the source of controversies. For instance, despite significant evidence pointing to action against the cell membrane in susceptible bacteria, the cell wall has also been suspected to be an important target of daptomycin [13]. Using time lapse fluorescence microscopy to visualize the interaction of daptomycin with the model Gram-positive bacterium *Bacillus subtilis*, Pogliano et al. [13] found that the first, direct effect of daptomycin binding to cells was creating membrane defects, which appeared as aggregates on membranes. The defects were distributed at random positions throughout the cell while the membranes were intact. In time, the daptomycin-induced membrane defects appeared to recruit essential cell envelope proteins which then disrupted cell wall biogenesis and contributed directly to the mechanism of lethality.
The second crucial information from the cellular studies is that the minimum inhibitory concentration (MIC) of daptomycin is in the range of micromoles. At this range of concentration (1 M=1.6 g/ml), the amount of peptide bound to a lipid bilayer, measured by peptide-to-lipid molar ratio (P/L), is in the order of 1/100 or higher. This is the same range of MIC values for host-defense antimicrobial peptides which are known to form pores in the membranes [11, 12]. This means the action of daptomycin is a many-body effect, rather than the action by a small number of peptides. As far as we know, the question of why its MIC is at this level has never been discussed.

In this report, I will show that I have successfully identified an interaction phenomenon between daptomycin and membranes of specific lipid composition that correlates with what was observed in cell studies. I will also show that this effect appears only when the daptomycin concentration is above a certain critical value corresponding to the MIC values of daptomycin. Following this discovery, I plan to use spectroscopic and X-ray methods to gain molecular and structural information about this daptomycin effect.

**Molecular property of daptomycin**

A structural class of antibiotics is called the cyclic lipopeptides, each consisting of a cyclopeptide ring and an exocyclic N-terminal acylated to a fatty acid. Daptomycin has 13 amino acid residues, of them the cyclic decapeptide core is formed by an ester bond between threonine-4 and kynurenine-13; the 3-residue N-terminus is acylated with an n-decanoyl fatty acid side chain. Four of the residues
are acidic and one basic, resulting in a total molecular charge of -3 at neutral pH [14], and a high water solubility. The antibacterial activity of daptomycin is highly calcium dependent. The efficacy is optimal in the presence of a Ca\(^{2+}\) concentration around 1.25 mM, which corresponds to levels usually measured in human serum [15], and negligible in the absence of Ca\(^{2+}\) [16]. Susceptibility of bacteria to daptomycin is correlated with the membrane content of phosphatidylglycerol (PG); it is decreased by mutations that promote the conversion of PG to lysylphosphatidylglycerol [7]. On bacterial cell membranes, fluorescently labeled daptomycin localizes to phosphatidylglycerol-rich membrane areas [17].

![Chemical structure of daptomycin.](image)

**Figure 1.1.1** Chemical structure of daptomycin.

At least three independent groups [18-20] studied the NMR solution structures of daptomycin, including the effect of Ca\(^{2+}\), but did not yield a consensus result. The disagreements include whether daptomycin in solution had a definitive structure or had a change of conformation after binding Ca\(^{2+}\). This seems to indicate the variability of daptomycin structure in solution. However fluorescence studies
clearly showed Ca$^{2+}$-facilitated binding to lipids [19, 21]. Oligomerization of daptomycin on lipid membranes was recently demonstrated by fluorescence resonance energy transfer (FRET) [22] and by perylene excimer fluorescence [23]. Oligomerization was detected on liposome membranes containing PG combined with phosphatidylcholine (PC) or phosphatidylethanolamine (PE) plus cardiolipin. In contrast, no oligomerization was observed with liposomes containing PC alone or in solution at daptomycin concentrations similar to those required for antibacterial activity. The results strongly suggest PG mediated oligomerization of daptomycin, but the degree of oligomerization, whether it is dimers or higher orders, is unknown.

In this study we use giant unilamellar vesicles (GUVs) to study the interaction of daptomycin with lipid bilayers. GUVs are aspirated by a micropipette to monitor its membrane area change. Fluorescent lipids and solution dyes are used to observe the membrane surface and molecular leakage through the bilayer. First we confirm the ion and lipid dependence of daptomycin by this technique. Unique peptide-lipid aggregates were observed only in the combination of Ca$^{2+}$ and PG. The aggregates are similar in appearance to what was detected on the membrane of \textit{B. subtilis}. Such peptide-lipid aggregates appear only if the daptomycin concentration exceeds a level similar to the MIC values reported in the bacterial studies.
Chapter 2

Materials and Methods

2.1. Materials

Daptomycin was purchased from Selleckchem (Munich, Germany) and from Haorui-Pharma Chem Inc (Irvine, CA). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)(DOPG), 1',3'-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol (sodium salt)(Cardiolipin), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)(Rh-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red sulfonyl chloride (TRsc) (MW 625) was purchased from Invitrogen (Grand Island, NY). Calcium chloride and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. All experiments were performed at room temperature ~25°C unless specified otherwise.
2.2. Giant Unilamellar Vesicle

2.2.1. Sample Preparation

Giant unilamellar vesicles (GUVs) were produced by an electroformation method [24]. To produce DOPC GUVs, for example, 50 µl DOPC (25 mg/ml) and 0.5% molar ratio of Rh-DOPE (for clear imaging) were dissolved in 1:1 (v/v) tetrafluoroethylene and chloroform. The lipid solution (~0.05 mg lipid) was deposited onto two indium tin oxide (ITO)-coated glass coverslips. After vacuuming to remove the organic solvent, an o-ring was sandwiched in between the two ITO coverslips, and the space inside of the o-ring was filled with the interior solution of GUVs. Next 3 V ac at 10 Hz was applied between the two ITO electrodes for 2 hours. Subsequently, the frequency was adjusted to 5 Hz for 10 minutes, and then 1 Hz for 10 minutes. Finally the GUV suspension was gently collected into a vial (reservoir) saved for use within 24 hours.

The GUV experiment was performed with vesicles containing ~200 mM sucrose solution inside and with same osmolality of glucose outside. The osmolality of each solution was measured by a Wescor Model 5520 dew point Osmometer (Wescor, Logan, UT). The refractive index contrast between sucrose and glucose makes the vesicles visible. The density contrast makes the vesicles sink to the bottom of the solution for the ease of manipulation.
2.2.2. GUV Aspiration Experiment

To perform a GUV aspiration experiment, the vesicles were first transferred to a control chamber containing a solution of 200 mM glucose in 10 mM Tris buffer at pH 7.5, which is the physiological pH of most living organisms. A micropipette of inner radius 8–10 µm was coated with 0.5% bovine serum albumin (BSA) overnight to neutralize the charge on the bare glass surface [25] and washed extensively with water. The tail end of the aspiration pipette was connected to an oil-filled U tube, where a negative pressure was generated by reference to the atmosphere. The controlled negative pressure of the micropipette can aspirate a GUV with 25-35 µm in radius, and created a membrane tension of 0.5 dyn/cm [26].

![Figure 2.1 Scheme of the GUV experiment. (1) An aspirated GUV was inserted ~0.7 mm into the transfer pipette in the control chamber. (2) The aspirated GUV in the transfer pipette was moved from the control chamber to the observation chamber. (3) Then the transfer pipette was swiftly moved away so that the GUV was exposed to the peptide/glucose/tris solution. Time was marked as $t=0$.](image-url)
Next, the aspirated GUV was transferred to an observation chamber, which contained peptide/glucose/Tris solution with pH and osmolality controlled (see schematic in Fig. 2.3.1). The observation chamber was set side-by-side with the control chamber, separated by 0.5 cm. A transfer pipette with inner diameter 0.75 mm was filled with the control solution, and inserted from the opposite side of the aspiration pipette through the observation chamber into the control chamber. The aspiration pipette and the transfer pipette were held separately by motor-driven micromanipulators Narishige MM-188NE (East Meadow, NY). The aspirated GUV was inserted ~0.7 mm into the transfer pipette in the control chamber. By moving the microscope stage, the aspirated GUV in the transfer pipette was moved from the control chamber to the observation chamber. Then the transfer pipette was swiftly moved away so that the GUV was exposed to the peptide/glucose/Tris solution. As soon as transfer pipette was removed, time was set as zero. The GUV status was observed by a conventional inverted wide-field microscope (IX81, Olympus, Tokyo, Japan) and recorded by a Hamamatsu Photonics digital CCD Camera (model C10600-10B) (Hamamatsu City, Japan).

### 2.2.3. Data Analysis

In response to peptide binding, a GUV can potentially change its membrane area $A$ and volume $V$. The GUV protrusion inside the micropipette serves as an amplifier for the measurement of such changes. From the microscopic images, $L_p$ the length of the protrusion, $R_p$ the radius of the micropipette, and $R_v$ the radius of the GUV were carefully measured. It is then straightforward to show $\Delta A = 2\pi R_p \Delta L_p +$
$8\pi R_v \Delta R_v$ and $\Delta V = \pi R_p^2 \Delta L_p + 4\pi R_v^2 \Delta R_v$ [26]. If there is no molecular leakage and as long as the inside and outside of the GUV have the same osmolality, there should be no change of volume. Under the condition $\Delta V=0$, $\Delta L_p$ directly proportional to $\Delta A$: $\Delta A = 2\pi R_p(1-R_p/R_v)\Delta L_p$.

![Figure 2.2 The protrusion length analysis of a GUV. $L_p$, $R_p$, and $R_v$ are as indicated.](image)

### 2.3. Circular Dichroism (CD)

#### 2.3.1. Background

Circular Dichroism (CD) is widely used to investigate the secondary structures of proteins and peptides. Usually the molecules of interest are measured in solution within a far-UV region from 190 to 250 nm. The structural asymmetry of proteins and peptides causes an absorption disparity between left-handed and
right-handed circularly polarized light. The absence of regular structure in proteins and peptides results in zero CD intensity while an ordered structure results in a spectrum containing both positive and negative signals. These ordered structures could be roughly classified into three groups: α-helix, β-sheet, and random coil. Each has its own characteristic CD spectrum shown in figure 2.3.

![Characteristic CD spectra of secondary structures](image)

**Figure 2.3** Characteristic CD spectra of secondary structures: α-helix, β-sheet, and random coil.
2.3.2. Sample preparation

To explore the daptomycin structure in a bacteria membrane-like environment, we prepared large unilamellar vesicles (LUVs) by extrusion. Their sizes are < 0.2 \( \mu \text{m} \). First, lipid mixture was dried in a vial by evaporation, and later hydrated by Tris buffer at pH 7.4 for 30 minutes. The lipid suspension should be kept above the phase transition temperature of the lipid during hydration and extrusion. To increase the efficiency of entrapment of water-soluble compounds, the hydrated lipid suspension is subjected to 7-10 freeze/thaw cycles by alternately placing the sample vial in a liquid nitrogen bath and warm water bath. Once the sample is fully hydrated, load the sample into one of the gas-tight syringes, and gently push the plunger of the filled syringe until the lipid solution is completely transferred to the alternate syringe. This step was repeated 15 times to let the lipid solution become more homogenous. The final extrusion should fill the alternate syringe to reduce the chances of contamination with larger particles or foreign materials. The lipid suspension should begin to clear to yield a slightly hazy transparent solution. The haze is due to light scattering induced by residual large particles remaining in the suspension. All vesicles are used on the same day they were prepared.

2.3.3. Experimental Setup

CD spectra were measured by a Spectropolarimeter (Jasco J-810, Tokyo, Japan). The equipment layout is illustrated in figure 2.4. The linearly polarized monochromatic
light comes out from a xenon lamp. A photoelastic modulator is used to modulate the polarization of the incoming linearly polarized light source. Modulated right-handed and left-handed components go through the optically active sample and result in preferential absorption of right-handed or left-handed polarization. The photomultiplier is then used to accumulate signals for a specific wavelength in the far-UV region. The prepared sample solution is placed in a quartz cuvette, which is transparent to far-UV lights. Each sample solution is measured 5 times and then averaged for further data analysis.

Figure 2.4  Spectropolarimeter layout.
2.4. X-ray Lamellar Diffraction (XRD)

Lamellar X-ray diffraction is a technique to study the structure of the lipid membrane because of the one-dimensional regular stacking of aligned multilayers. Of particular interest here is the change of the lipid bilayer structure upon the addition of other molecules, such as membrane acting peptides. To clearly delineate XRD with our research interest, I will discuss the XRD technique separately in the following sections: (1) capsule of the sample preparation, (2) derivation of the basic equations of X-ray diffraction as applied to lamellar scattering, (3) discussion of the problem of phasing and its solution with the help of swelling method, (4) description of the experimental setup, and (5) introduction of the corrections necessary to connect the measured diffraction intensity with the theoretical calculation.

2.4.1. Sample Preparation

Same amount of Daptomycin and CaCl$_2$ were first dissolved in methanol. The methanol solution and lipids were then mixed into 1:1 (v/v) chloroform and tetrafluoroethylene at a chosen peptide/lipid (P/L) molar ratio. Next, the mixture was deposited on a cleaned, 1 cm$^2$ silicon wafer. The amount of lipid on each substrate was fixed to 0.3 mg as a reference for comparing different P/L ratio. After the solvent was removed in vacuum, the samples were hydrated by saturated water vapor at 35°C overnight. The results were well aligned, parallel, hydrated bilayers as
proven by x-ray diffraction. The samples were kept in a temperature humidity chamber during the measurement. All experiments were performed at 25°C.

2.4.2. Basic Theory

X-rays interact with the electrons of the sample. The scattered waves from the sample interfere to result in an observable pattern, which is a function of the electron density distribution. I apply the principles of X-ray diffraction to a special case of lamellar samples.

First, we define the momentum transfer vector \( \mathbf{q} \) as the difference between the wave vectors of the incoming beam \( \mathbf{k}_i \) and the diffracted beam \( \mathbf{k}_f \), i.e.

\[
\mathbf{q} = \mathbf{k}_f - \mathbf{k}_i
\]  

(2-1)

For elastic scattering, energy remains constant, hence only the direction of incoming radiation is changed. Based on \(|\mathbf{k}_i| = |\mathbf{k}_f| = 2\pi/\lambda\) and figure 2.5, we can have a relation of the momentum transfer vector

\[
|\mathbf{q}| = \frac{4\pi}{\lambda} \sin \theta.
\]  

(2-2)
Next, the intensity of X-ray scattering is given by B. Warren [27] as

$$I(q) = I_e \left| \int_V \rho(r) e^{-i\mathbf{q} \cdot \mathbf{r}} d^3r \right|^2,$$  \hspace{1cm} (2-3)

where $I_e$ is the intensity of scattering by a single free electron, and $\rho(r)$ is the electron density of the sample of volume $V$. We assumed a multilayer sample composed of $N$ bilayers that are separated by a distance $D$ in the $z$-direction. $\rho(x, y, z)$ is the electron density of a single membrane in this stack. Since the membranes in our sample are fluid, and lipids are evenly distributed within the $x$-$y$ plane, i.e. $\rho(x, y, z) = \rho(z)$. The membranes are assumed to have uniform lateral structure. Therefore, the electron density of the complete sample $\rho_{tot}$ can then be written as

$$\rho_{tot}(x) = \rho(x + nD)$$
with \(-\frac{D}{2} \leq z \leq \frac{D}{2}\), \(n = 0, 1, 2, \ldots, N - 1\). (2-4)

Accordingly, the integral in equation (2-3) can be written as

\[
\int_{\mathcal{V}} \rho_{\text{tot}}(r)e^{-iqzD^3}r\,d^3r = \int_{-D/2}^{(N-1)D/2} \rho(z) e^{-iqz^2}dz \\
= \sum_{n=0}^{N-1} \int_{-D/2}^{D/2} \rho(z)e^{-iqz^2}e^{-inqzD}dz \\
= \int_{-D/2}^{D/2} \rho(z)e^{-iqz^2}dz \sum_{n=0}^{N-1} e^{-inqzD} 
\]

(2-5)

Applying the relation

\[
\sum_{n=0}^{N-1} e^{in\pi x} = \frac{1-e^{iN\pi x}}{1-e^{i\pi x}},
\]

(2-6)

we can rewrite the last summation in equation (2-5) as

\[
\sum_{n=0}^{N-1} e^{in\pi x} = \frac{\sin(NqD/2)}{\sin(qD/2)} e^{i\pi(N-1)qD} 
\]

(2-7)

The exponential factor at the end is a phase factor, which becomes unity after absolute squaring. As a result, we have the diffraction intensity from a stack of aligned membranes from equation (2-3):

\[
I(q_z) = I_0 \left| \frac{\sin^2(Nq_zD/2)}{\sin^2(q_zD/2)} \int_{-D/2}^{D/2} \rho(z)e^{-iq_z^2}dz \right|^2 \\
= I_0 S(q_z)|F(q_z)|^2
\]

(2-8)

with the definition of
\[ F(q) \equiv \int_{-D/2}^{D/2} \rho(z) e^{-iqz} dz \] (2-9)

and

\[ S(q) \equiv \frac{\sin^2(NqzD/2)}{\sin^2(qzD/2)} \] (2-10)

\(F(q)\) is the structure factor of the bilayer, which is the Fourier transform of the electron density of each lipid bilayer plus the surrounding water. While \(S(q)\) is the interference function of the sample, which indicates the arrangement of membrane stacking in the multilayer sample.

Figure 2.6 gives an example of the interference function \(S(q)\) of different number of lipid bilayer, which is indicated as \(N\). When \(N\) is increasing, the peaks of \(S(q)\) become sharper while the oscillations between each peak get smaller.

Accordingly, with a large number of \(N\), the interference function \(S(q)\) only has perceivable values at

\[ q \equiv q_h = \frac{2\pi h}{D} \quad \text{with } h = 0, \pm 1, \pm 2, \ldots \] (2-11)

The value at the peak position is \(N^2\) while the peaks have a width of \(2\pi/(ND)\).
Figure 2.6  The interference function $S(q)$ for several values of $N$. The function peaks at $q = 2\pi h/D$ with a peak value of $N^2$.

Because we neglected that we can only measure the absolute value of diffraction patterns in the experiment. In order to reconstruct the profiles of electron density profiles of the sample, we need the phases associated with the diffracted waves. In other words, as long as we apply the phase angles to the observed amplitudes, we can invert the Fourier transformation of equation (2-3) to calculate the electron density and determine the bilayer structure. This is referred to as phasing problem. It is the major challenge of structure determination in X-ray diffraction.

Since the membrane are centrosymmetric, i.e. $\rho(x) = \rho(-x)$, the phase problem can be simplified by the fact that the structure factor $F(q)$ is a pure real function. Therefore, $F(q)$ can be considered as a linear combination of its amplitude $|F(q)|$ and phase $\alpha(q)$ as
\[ F(q) = |F(q)|e^{i\alpha} = |F(q)|\cos \alpha + i |F(q)|\sin \alpha \]  \hspace{1cm} (2.12)

Besides, \( F(q) \) is purely real suggesting \( \alpha = n\pi \), and \( n = 0, \pm 1, \pm 2 \ldots \). Since the structure factor \( F(q) \) can be only integral when phase equals to multiples of \( \pi \), the phase problem in this case is reduced to the determination of the signs of each observed amplitude \( |F(q)| \). In experiment, this task can be achieved by the swelling method.

In the swelling method, we change the hydration state of the membrane, which is equivalent to changing the repeat distance \( D \). From equation (2.11), the peak position, \( q \), of the interference function changes accordingly (figure 2.7). By Shannon’s theorem [28], we are able to reconstruct the continuous Fourier transform \( F(q) \) from the discrete Fourier series of the XRD measurement. The equation can be written as

\[ F(q) = \sum_{h=-\infty}^{h=\infty} F \left( \frac{2\pi h}{D} \right) \text{sinc} \left( \frac{qD}{2} - \pi h \right). \]  \hspace{1cm} (2.13)

Thus the total scattering intensity becomes

\[ \int_{-\infty}^{\infty} |F(q)|^2 dq = \frac{2\pi}{D} \sum_{h=-\infty}^{h=\infty} \left| F \left( \frac{2\pi h}{D} \right) \right|^2. \]  \hspace{1cm} (2.14)

In addition, Blaurock [29] has demonstrated that the total diffracted intensities for two different D-spacings must be equal, which leads to a scaling law

\[ \frac{\sum_{h=1}^{h=\infty} |F(2\pi h/D_1)|^2}{\sum_{h=1}^{h=\infty} |F(2\pi h/D_2)|^2} = \frac{D_1}{D_2}. \]  \hspace{1cm} (2.15)
By applying this relation to rescale the measured intensities for different swelling states and plotting them versus $q$, we are able to decide the right phases associated with the measured amplitudes by connecting them with the smoothest possible curve.

**Figure 2.7** An example of diffraction patterns of pure DOPC. The peak position shifts and the peak amplitude changes at different relative humidities (RH). The discontinuity at 1.3° is due to the attenuator used (details explained in section 2.4.3).
Figure 2.8  An example of scaling diagram for determining the phases of pure DOPC at different relative humidities (RH).

Once the phase factor for each measured intensity is decided, we can calculate the relative electron density profile of the lipid bilayers at different relative humidity (RH) as follow.

\[
\rho(z) - \rho_0 = \frac{1}{d} \sum_n F(2\pi h/D) \cos(2\pi h z/D)
\]  

(2-16)

The correspondence of the obtained electron density profile and the membrane model is shown in figure 2.9. The lipid head groups have the highest electron density.
density in the membrane. Hence the peak-to-peak distance (PtP) indicates the membrane thickness. The point of the lowest electron density corresponds to the center of the bilayer.

Figure 2.9 The correspondence of the electron density profile to the lipid bilayer. The bottom is an example of electron density profile of pure DOPC at different relative humidities (RH).
2.4.3. Experimental Setup

Multilamellar samples were measured in a temperature-humidity chamber. ω-2θ diffraction was collected on a four-circle Huber goniometer (Huber Diffraktionstechnik, Rimsting, Germany), with a vertical line-focused Cu Ka source (\(\lambda = 1.542 \, \text{Å}\)) operating at 30 kV and 15 mA. The incident beam was collimated by a horizontal soller slit and two vertical slits on the front and the back sides of the soller slit. The horizontal and vertical divergences of the incident beam were 0.23° and 0.3°, respectively. The diffracted beam first passed through a vertical slit and then was discriminated by a bent graphite monochromator (selected for 1.542 Å) before entering a scintillation detector. Thus, the detector excluded Compton scattering, fluorescence, and most of the x-rays from air scattering. This diffractometer was designed to minimize the background signal, thus allowing the measurement of high diffraction orders. A schematic view of the experimental setup is given in figure 2.10.
Before the measurement, the aligned multilayer sample was carefully positioned at the center of the x-ray beam and was oriented so that $\omega = 0^\circ$ and $\theta = 0^\circ$ coincided. We have established an elaborate routine for positioning and orienting the sample as described in Wu et al. [30]. A two-dimensional $\omega$-$\theta$ scan around the second or the third Bragg order was used to check the alignment of the $\omega$-angle and the mosaic of the multilayers alignment. Typically, the full width at half-maximum of the peak on the $\omega$ axis (of the $\omega$-$\theta$ scan) was $0.2–0.3^\circ$. Once the sample was properly positioned and aligned on the diffractometer, each $\omega$-$2\theta$ scan was performed from $\omega = 0.5^\circ$ to $\omega = 7.5^\circ$ with a step size of $\Delta \omega = 0.01^\circ$. An attenuator
was used to prevent the first-order Bragg peak from saturating the detector. The scan was repeated 2–3 times at each of several hydration levels and averaged at each hydration level for data analysis. Measurements at several hydration levels were for the purpose of phase determination by the swelling method. Note that the readings of commercial humidity sensors are not accurate above 98% RH; we used the maximum RH above which the membrane samples were washed out.

2.4.4. Data Analysis

The measured diffraction intensity $I_m$ was corrected for artifacts and distortions caused by the experimental setup and geometry. After considering all the correction terms, the corrected intensity $I_{cor}$ can be written as

$$I_{cor} = (I_m C_{det} C_{att} - B \gamma) C_{sam} C_{abs} C_{pol} \int \int \int C_{Lor}$$  \hspace{1cm} (2-17)

where $C_{det}$ was the correction for the detector dead time, and $C_{att}$ was the correction for the attenuator. After applying these two corrections, the background signals $B \gamma$ was determined and subtracted from the data. $C_{sam}$ corrected for the angular dependence of apparent sample size in the direction of the beam. While $C_{abs}$ adjusted the effects due to the absorption of the radiation in the sample. After multiplying by the polarization correction factor $C_{pol}$, the diffraction peaks were integrated before the Lorentz correction factor $C_{Lor}$ was applied.

The relative magnitude of the diffraction amplitude was the square root of the integrated intensity. With their phases determined by the swelling method, the diffraction amplitudes were used to reconstruct the electron density profile of the
bilayer. According to the bilayer profile, the phosphate peak to phosphate peak distance (PtP) was measured for the bilayer thickness.
Chapter 3

Results

Pogliano et al. [31] found the bacteria, *Bacillus subtilis*, suffered membrane deformations and ultimately lost their membrane integrity when cells were grown on an agarose pad containing daptomycin. They identified daptomycin-induced lipid-daptomycin aggregates on bacterial membrane (figure 3.1, A,B,C). The lipid-daptomycin aggregates recruit the essential proteins of cell wall biosynthesis and halt activates of proteins. The interruption of cell wall production results in the bent structure of cell wall (figure 3.1, D,E). These striking changes in cell morphology consequently lead to cell death.
In addition to the fact that the main target of daptomycin is the bacteria plasma membrane, previous studies suggest that daptomycin causes leakage of potassium and possibly other ions to depolarize cell membranes and results in cell death. Some other studies on bacteria also indicate that the antibiotic activity of daptomycin strictly depends on both the presence of calcium and phosphatidylglycerol-containing (PG) membrane.

Here my work provides a new biophysical view from the traditional biological studies to understand the daptomycin-membrane interaction. To investigate how calcium and PG affect daptomycin activity, I tested a series of combinations of calcium and PG composition by the techniques of GUV aspiration, CD, and x-ray. Through these different methods, we could obtain more information from different point of views to depict the membrane defect process induced by

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**Figure 3.1** Daptomycin-induced membrane deformations on *Bacillus subtilis*. A vital membrane stain FM 4-64 were used for visualization. Figure adopted from *Pogliano et al., 2012*. 

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In this chapter, I will first present the results of GUV aspiration as follows: (1) behaviors of daptomycin with the presence of calcium and PG, (2) calcium effects on antibiotic activity, (3) PG effects on antibiotic activity, and (4) minimum inhibitory concentration of daptomycin. Next, we will show the CD spectra of daptomycin with LUVs composed of (1) DOPC/DOPG (7:3) and (2) pure DOPC. Finally, the membrane thickness measured by x-ray diffraction will be discussed.

3.1. GUV Aspiration Experiment

Artificial GUVs with designed lipid compositions provide a simplified model to focus on specific lipid components. They also eliminate the complexity of living cell membrane due to embedded proteins and enzymes. GUV aspiration is a useful technique for studying the interaction between membrane-acting molecules and lipid bilayers. The change of GUV protrusion length in the micropipette reflects the change of the area/volume ratio. The ratio can be further referred to molecule binding rate to membrane, which depicts a dynamic picture between molecules and the lipid bilayer.

3.1.1. Basic Properties of Daptomycin

Responses of a DOPC/DOPG (7:3) GUV in a solution containing 2.5 µM daptomycin and 5 mM CaCl₂ are shown in figure 3.2, A and B. After exposing to the solution, the GUV protrusion initially stretched without any aggregates appeared.
Subsequently, the protrusion shrank back while aggregates formed on the GUV surface. Finally, the GUV ruptured. To compare the amount of surface area stretched, the protrusion length change, $\Delta L_p$, was converted into a fractional area change, $\Delta A/A$, by the relation $\Delta A = 2\pi R_p \Delta L(1 - R_p/R_o)$. The increase of $\Delta A/A$ indicates daptomycin binding while the increasing rate is positively correlated with the concentration of daptomycin and calcium. The process of GUV protrusion change and aggregate forming will be referred as daptomycin-induced membrane defect in the following context.

Pore forming on bacterial membranes is commonly found during the treatment of antimicrobial peptides. To confirm whether daptomycin formed pores on a GUV membrane in the presence of calcium, the content dye experiment was performed. GUVs were produced in a sucrose solution with 20 $\mu$M content dye (Texas Red), and later were introduced into a glucose solution with 1 $\mu$M daptomycin and 1.25 mM CaCl$_2$. Once pores are formed, outside and inside solutions of the GUV will exchange through the pores. Owing to the molecular weight difference of glucose (MW 180) and sucrose (MW 342), the sucrose inside the GUV diffuses slower than the outside glucose does. Hence there is an influx of glucose that leads to the increase of GUV volume and the decrease of protrusion length. Moreover, the content dye will effuse from the inside of the GUV, and cause the dye intensity to suddenly drop. On the other hand, if no pores are formed, the content dye intensity will either remain the same or decrease slightly due to photobleaching.
The results of content dye leakage experiment are shown in figure 3.2, C and D. In figure 3.2 C, the behaviors of the protrusion length change verify the daptomycin-induced membrane defect process. The GUVs in both trials ruptured within 80 seconds. The content dye intensity remained similar during the period. The 0.1% decreases of the fluorescence intensity are consistent with the decrease in a control experiment, which is performed by introducing a GUV into a glucose solution in the absence of daptomycin and calcium. These results suggest that the daptomycin does not form pores on the membrane.

![Figure 3.2](image)

**Figure 3.2** Properties of an aspirated DOPC/DOPG (7:3) GUV in a solution of 2.5 µM daptomycin and 5 mM CaCl$_2$. (A) Images of a GUV with surface dye show the protrusion first increased due to daptomycin binding. After the protrusion reached a maximum, lipid aggregated on the GUV surface while the protrusion decreased, and finally ruptured. (B) The length of the protrusion, $L_p$, was converted into fractional area changes, $\Delta A/A$, by the relation $\Delta A = 2\pi R_p(1-R_p/R_v)\Delta L_p$. Daptomycin with calcium does not form pore on the GUV membrane. (C) and (D) show Texas red (contain dye) intensities in time sequence after a GUV was introduced to a 1 µM daptomycin and 1.25 mM CaCl$_2$.
solution. A control trial of a GUV in a glucose solution is shown for comparison (solid curve). For all trials, the 0.1% decrease within 70 sec was due to photobleaching.

3.1.2. Calcium Effect on Daptomycin Antibiotic Activity

To understand what role calcium is playing in the daptomycin-induced membrane defect, we compared the responses of DOPC/DOPG GUVs in three different experimental settings: (1) in the presence of calcium, (2) in the absence of calcium, and (3) in the presence of magnesium, a cation with two positive charges as calcium.

The GUV images in figure 3.3 A show that the length of the GUV protrusion steadily increased but did not decrease in 5 μM daptomycin solutions without calcium present. Increasing the daptomycin concentration to 50 μM, the protrusion length increased faster and then decreased (figure 3.3, B). Because NBD surface dye was utilized in the experiment of figure 3.3 B, the image looked dimmer than figure 3.3 A. NBD surface dye is commonly used to distinguish the red-colored contain dye, such as Texas red. Besides this trial, GUVs of all other experiments were prepared with surface dye, Rhodamine B, to obtain better contrast in images. In both figure 3.3A and B, no obvious membrane aggregates had been found.

The changes of the protrusion length in both cases were converted into the fractional area changes, and were compared with the case of GUV in 5 μM daptomycin and 5 mM CaCl₂ shown in figure 3.2 B. A low concentration of
daptomycin without the assistance of calcium can barely bind to the membrane. Despite daptomycin being able to bind to the membrane when the concentration was risen to 10-fold higher, compared to 5 µM daptomycin/5 mM CaCl$_2$-treated cases, the maximum of ΔA/A is smaller (2% vs 6.5%) and the time of process completion is longer (50 seconds vs 10 seconds). These results suggest that calcium can increase the efficiency of antibiotic activity of daptomycin.
Figure 3.3 The effect of calcium on daptomycin binding to the membrane. Top images are DOPC/DOPG (7:3) GUVs in time sequence after introduced into solutions containing (A) 5 μM daptomycin, (B) 50 μM daptomycin. When the calcium was absent, the protrusion length increased slowly during 5 minutes. No aggregations were observed, and GUVs did not rupture. While when daptomycin concentration was risen up to 10 times, the protrusion length increased and decreased without the help of calcium. (C) A comparison of ΔA/A among (A), (B), and the condition of 5 μM daptomycin and 5 mM CaCl$_2$. With the assistance of calcium, daptomycin performed a better binding ability to the membrane.
Since calcium is essential for membrane binding of daptomycin, this raised a critical question that if other divalent ions have the same effect on the daptomycin-lipid binding process. Thus, the responses of GUV protrusion length were compared when calcium was replaced by magnesium. Here, daptomycin was kept the same but magnesium was substituted for calcium. In the presence of magnesium, the protrusion of the GUV initially lengthened. However, the protrusion length subsequently became stable and the GUV did not rupture (figure 3.4). The results indicate that magnesium cannot replace the role of calcium in assisting daptomycin-membrane binding. Furthermore, the results may also imply that other cations with two positive charges are not able to improve daptomycin binding to the membrane.
3.1.3. PG Effect on Daptomycin Antibiotic Activity

Because peptide-lipid aggregates were observed only in the combination of calcium and PG, it indicated that PG is also a crucial factor in daptomycin antibiotic activity. Here GUVs were compared under different lipid compositions, including DOPC/DOPG (7:3), pure DOPC, and DOPC/cardiolipin (7:3). Two important reasons
raise our interest in comparing GUV reactions when DOPG is replaced by cardiolipin. First, both PG and cardiolipin are the main components of bacteria membrane. Second, in bacteria, cardiolipin is synthesized from two PGs by cardiolipin synthase (figure 3.6). Therefore, cardiolipin has a structural similarity with PG. All GUVs were introduced to the same solution of 5 µM daptomycin and 5 mM CaCl₂.

The fractional area changes of DOPC and DOPC/cardiolipin (7:3) GUVs both increase up to 3~4% in the initial 80 seconds. The results indicate that daptomycin binds to the membrane (figure 3.5). However, after the protrusions reach a maximum, they do not shrink back even though little aggregates appeared on the GUV surface. Meanwhile, GUVs in both cases stay intact for more than 5 minutes. The data show that PG accelerates the formation of daptomycin-lipid aggregate and eventually lead to GUV rupture. Cardiolipin cannot replace the role of PG in the precipitation of both lipid aggregate and GUV rupture. On the other hand, the effect of cardiolipin can be related to the daptomycin resistance of bacteria.
Figure 3.5  The effect of PG on daptomycin binding to the membrane. Top images show GUVs composed of (A) pure DOPC and (B) DOPC/cardiolipin (7:3) in a solution with 5 μM daptomycin and 5 mM CaCl₂. In both case, the GUV protrusion gradually increased with time. Although a small amount of aggregates was observed, the GUVs had neither protrusion decrease nor rupture during 5 minutes. The bottom figure presents ΔA/A corresponding to (A) and (B). A case of DOPC/DOPG (7:3) GUV is shown for comparison. The curves demonstrate that PG is required for daptomycin-induced membrane defect.
3.1.4. Minimum inhibitory concentration

To determine whether daptomycin-induced membrane defect results from the individual contribution or the corporate effort of molecules, I tested the time for DOPC/DOPG (7:3) GUVs to have a maximum protrusion length at various concentrations of daptomycin with 0.5, 1, and 1.25 mM calcium. Here 1.25 mM calcium is the physiological concentration in human serum. The time required for each GUV to reach a maximum protrusion length was recorded and plotted versus daptomycin concentration in figure 3.7. The plot shows a critical concentration around 0.4 µM. When daptomycin concentration is above 0.4 µM, the time required for the protrusion to reach a maximum is consistent. However, when daptomycin
concentration is close to 0.4 \( \mu \text{M} \), the time required for reaching a maximum protrusion length varies. Moreover, when daptomycin concentration is below 0.4 \( \mu \text{M} \), the protrusion length increases steadily and never decreases back. These results suggest that it requires a minimum concentration to trigger the daptomycin-induced membrane defect. Hence the antimicrobial activity of daptomycin relies on the corporate effort of molecules.

![Image](image.png)

**Figure 3.7** The minimum inhibitory concentration (MIC) of daptomycin with 0.5, 1, and 1.25 mM calcium. Each symbol represents a trial with different concentration of daptomycin. The time durations for protrusion length to reach a maximum are plotted versus daptomycin concentration.

By applying GUV aspiration technique, I identified the essential factors for daptomycin antibiotic activity. First, calcium is indispensable for daptomycin-lipid binding process and calcium cannot be replaced by magnesium. Second, PG enhances lipid aggregation and is required for daptomycin-induced membrane
defects. Third, with sufficient amount of calcium, daptomycin requires a minimum concentration to trigger the antibiotic activity. In the remaining section, I applied circular dichroism and x-ray diffraction to characterize the interactions between daptomycin and membrane in a structural level.

### 3.2. Circular Dichroism

Although circular dichroism (CD) cannot detect the orientation or evaluate the quantity of molecules of interest, it is still valuable for showing the conformation changes of proteins or peptides. The conformation changes of daptomycin implied by CD spectra can be related to the phenomena observed in GUV experiments. The connections between CD and GUV data might give more details about the mechanism of daptomycin antibiotic activity.

As mentioned in the method section, by measuring the differential absorption of left and right circularly polarized light of peptide in far UV region, CD can discriminate secondary structures of peptides, including α-helix, β-sheet, or random coil. However, none of the secondary structures can be used to describe daptomycin because of its cyclic structure. Based on the study of Grishina and Woody [32], several evidences had been shown that aromatic side chains can make significant contributions to the CD of proteins. Tryptophan and kynurenine, the amino acids of daptomycin side chains, were measured and compared with the CD spectra of daptomycin in the presence of calcium and LUVs that consist of different lipid composition. Here the usage of LUVs is because of its easy and large production.
In figure 3.9, the CD spectra demonstrated following conditions: (1) 10 µM daptomycin (blue), (2) 10 µM daptomycin with 5 µM CaCl₂ (red), (3) 10 µM daptomycin with DOPC/DOPG (7:3) LUVs (green), (4) 10 µM daptomycin and 5 µM CaCl₂ with DOPC/DOPG (7:3) LUVs (purple). All samples were controlled at pH7.2 in 10 mM Tris buffer. For conditions of daptomycin without adding either PG or calcium, the spectra are similar and have a peak around 230 nm. When daptomycin is measured in the presence of PG and calcium together, the spectrum flips with a trough at around 233 nm. We will refer this condition of the flipped curve as the working condition of daptomycin in the following context.
Figure 3.9 CD spectra of 10 µM daptomycin in 10 mM Tris pH7.2 with (1) none (blue), (2) 5 mM CaCl₂ (red), (3) 0.2 mg/ml DOPC/DOPG (7:3) LUVs (green), and (4) 5 mM CaCl₂ and 0.2 mg/ml DOPC/DOPG (7:3) LUVs (purple).

Since the CD spectrum of daptomycin does not result from the secondary structure but from the aromatic side chains, tryptophan and kynurenine (green and red in figure 3.10), I measured either Tryptophan or kynurenine independently and compare them with daptomycin. Each amino acid was dissolved in pH 7.2, 10 mM Tris buffer at a concentration of 800 µM. The spectrum of each amino acid has a peak around 227 nm, which does not meet the features of daptomycin spectra under either working or non-working conditions.
Figure 3.10  CD spectra of (1) 10 µM daptomycin in 10 mM Tris pH7.2 (blue), (2) 10 µM daptomycin and 5 mM CaCl$_2$ with DOPC/DOPG (7:3) LUVs (purple), (3) 800 µM Tryptophan in 10 mM Tris pH7.2 (green), (4) 800 µM Kynurenine in 10 mM Tris pH7.2 (red).

Figure 3.11 presents a comparison of the PG effect on daptomycin CD spectrum. First the zero amplitude of pure DOPC LUVs suggests lipids do not contribute to CD signal (green). The spectrum of daptomycin with pure DOPC LUVs (blue) is similar to the spectrum of daptomycin without any lipid and ion. When DOPC LUVs and calcium are added into daptomycin solution, the spectrum becomes flatter (purple). This spectrum has larger amplitudes than pure DOPC does. Here, a spectrum of daptomycin under working condition (red) is shown for reference.
3.3. X-ray Lamellar Diffraction

In this study, daptomycin, calcium, and lipid mixtures were prepared as parallel multiple bilayers on a flat substrate. Each sample was measured by X-ray diffraction to produce the electron density profile of the bilayer. To have the lipid bilayers in an environment as hydrated as possible, diffraction data were collected with increasing relative humidity (RH) until the sample was washed out by the condensed water.

The electron density measured by lamellar diffraction is the density per unit length in the direction normal to the bilayer, averaged over the plane of the bilayer.
This density profile is mainly from the lipid hydrophilic headgroup because of the in-plane orientation of DOPC and DOPG, and the high correlation of the headgroup position from layer to layer. Interfacial bound peptides do not produce additional density to the profile because of two reasons. First, the normal density of peptides is lower than the density of in-plane lipids. Second, the peptides have poor correlation in positions from layer to layer.
Figure 3.12  Diffraction patterns of daptomycin with CaCl$_2$ and DOPC/DOPG 7:3 at different peptide-lipid (P/L) ratio.

Figure 3.12 shows the diffraction patterns of daptomycin with CaCl$_2$ and DOPC/DOPG 7:3 at different peptide-lipid (P/L) ratio. The discontinuity at 1.3° is caused by an attenuator, which is used for the first-order Bragg peak to prevent from saturating the counter. The patterns are displaced for clarity. The analyzed membrane thicknesses are plotted in figure 3.13. When increasing the
daptomycin/lipid (P/L) ratio in the presence of calcium, daptomycin tends to thin the membrane. However, the consistency of ptp values at each P/L ratio needs to be improved. Two causes possibly lead to these large variations. First, in general, samples of high peptide-lipid ratio (P/L) are more difficult to achieve uniform alignment. Second, owing to the small amount of solvent contained by each sample, calcium chloride is inclined to crystallize. The crystallization may randomly disarrange the lipid bilayers.

Figure 3.13  Membrane thickness at different P/L ratio. The thicknesses are calculated from the distances between two peaks of electron density profiles.
Chapter 4

Discussion

4.1. GUV Responses to Daptomycin

Each membrane acting peptide has its unique interaction to lipid bilayers. Thus distinct phenomena have been explored by applying GUV aspiration technique. Previously, our group had discovered several molecules that caused distinct reactions of GUVs. Curcumin could simply incorporate into the lipid bilayer [33]. Melittin could form pores in the membrane [34]. Penetratin could turn into aggregates [35]. Tea catechin could extract lipid molecules from the membrane [36]. These characteristics of peptide-membrane interaction have been shown with a great variety of lipid compositions and different peptide-lipid (P/L) ratio [37].

According to my experimental results, I prove that the antibiotic activity of daptomycin strongly depends on calcium. Details will be illustrated in the following paragraphs. Calcium enhances daptomycin binding to membranes and following
with lipid aggregates forming on the membrane surface. In the absence of calcium, even though daptomycin can bind to membranes in a relative high concentration, the fractional area changes are smaller and the process of membrane defect is less efficient. After the protrusion length decreases from a maximum, no obvious aggregations are observed on the membrane surface.

A possible explanation for the difference in aggregation formations mentioned above is the charge effect of calcium. When molecules are close, aggregations form because of hydrophobic effect. The distances between molecules are mainly depends on the probability of collision. Therefore, with calcium, the charge attraction raises the possibility of collision between molecules, and thus precipitates in the aggregation process.

Another important factor, PG, is also carefully examined in this work. For a pure DOPC GUV, daptomycin binds to the membrane, but does not form aggregates. The maximum of the fractional area change is consistent with the maximum value of a DOPC/DOPG (7:3) GUV. This result strongly supports the idea that calcium improves daptomycin binding due to charge attraction-induced hydrophobic effect.

4.2. The Potential Role of Cardiolipin in Daptomycin-resistance Bacteria

An interesting finding in my work is replacing PG with cardiolipin results in the loss of antibiotic activity of daptomycin. PG and cardiolipin are both main
components of bacteria membrane. Cardiolipin are composed of two PGs. Thus, cardiolipin has two headgroups as same as the PG headgroup. The results indicate that daptomycin binds to DOPC/cardiolipin (7:3) GUV in the presence of calcium. However, despite small amount of lipid aggregates form, the protrusion length does not decrease after reaching a maximum and the GUV status remains stable. These different aggregation responses lead to an important question: what is the mechanism of daptomycin-induced lipid aggregation? To address this important question, I will perform more studies to understand the composition of aggregates and the forming mechanism. For example, explore the lipid-peptide aggregates by electron microscopy, and investigate the difference of aggregation formation between DOPG and cardiolipin. In addition, according to the finding on daptomycin and cardiolipin, it raises another important question whether bacteria can increase the ratio of cardiolipin to develop daptomycin resistance. Interestingly, previous studies reported that many daptomycin-resistance gram-positive bacteria have genetic mutations on cardiolipin synthase and Davlieva et al. [38] recently shown these mutations increase the enzyme activity of cardiolipin synthase and also highly associated with daptomycin resistance. However, it still remains unknown why increase the activity of cardiolipin synthase directly benefits gram-positive to develop daptomycin resistance. As mentioned in section 3.1.3 and figure 3.6, cardiolipin synthase catalyzes two PGs become one cardiolipin. When cardiolipin synthase activity increases, the ratio of cardiolipin in bacteria membrane may also increase. Therefore, my GUV aspiration experiment on daptomycin and cardiolipin
is the first direct evidence to answer why bacteria tend to increase ratio of cardioli

cardiolipin because daptomycin fails to induce membrane defect at cardioli

membrane.

4.3. Exciton Theory

Daptomycin does not have typical secondary structures of peptides, including $\alpha$ helix, $\beta$ sheet or random coil. Therefore, the CD spectra that are widely used to characterize the structures of peptides cannot be applied to daptomycin. However, due to the disruption of left and right circularly polarized light by aromatic side chains of daptomycin, the CD spectra can still reveal information of daptomycin-daptomycin interactions. Based on my experimental results, the side chains of daptomycin, tryptophan and kynurenine, give rise to distinct spectra from the spectra of daptomycin alone (figure 3.10). A possible explanation for this diversity of spectra is exciton theory [32]. According to the theory, if two identical chromophores are sufficiently close, their excitation states will interact. The interaction causes the excited state split into two components, which are called exciton components. One component arises from an in-phase combination of two monomeric excitations and the other component from the out-of-phase combination. These two exciton components are separated by twice of the energy owing to the interaction between the two transition dipole moments. The rotational strengths for the two components have the same magnitude but are opposite in
sign. Therefore, the couplet of the two states might give rise to the CD spectrum of daptomycin.

Figure 4.1 An exciton couplet in CD. The positive (...) and negative (...) bands are the two exciton components, separated by the exciton splitting, $\Delta \lambda$. The solid curve is the resultant of the two components. Figure is adopted from *Grishina and Woody, 1994*.

4.4. CD Spectra and GUV Aspiration

In this section, I will discuss three characteristic CD spectra of daptomycin in figure 4.2. First, the blue spectrum suggests the condition when daptomycin in the absence of calcium. Second, the green spectrum presents the state of daptomycin with calcium but without PG. Third, the red spectrum shows daptomycin under working condition, which is a condition in the presence of calcium and PG. Since CD signals of daptomycin are mainly contributed from the interaction of its side chains,
Tryptophan and Kynurenine, the changes of spectra suggest certain conformation changes between these two aromatic groups, for example, distance and orientation.

My hypothesis to explain all three spectra is to consider two steps in the membrane defect process. Before interacting with calcium and PG, daptomycin is randomly distributed in a free solution (figure 4.2, blue). Next, binding with calcium makes daptomycin have a conformation change, which promotes the binding ability of daptomycin to the GUV membrane. The alignments of these binding daptomycins may alter the interaction of the aromatic groups of the side chains. Thus the CD spectrum changes accordingly into the green spectrum in figure 4.2, which is identified as the intermediary state of the membrane defect process. Finally, when daptomycin and calcium form aggregates with PG, the orientation of aromatic groups change again. The interactions among the aromatic groups give rise to the red spectrum in figure 4.2. In order to integral CD and GUV results and to support the hypothesis, detailed investigation will be performed.
Figure 4.2  Characteristic solution CD spectra of daptomycin in three conditions: (blue) daptomycin alone, (green) daptomycin and calcium bind to DOPC membrane, (red) daptomycin and calcium bind to DOPC/DOPG membrane (working condition of daptomycin).

4.5. Membrane thinning

From GUV aspiration experiment, I can estimate how many daptomycin binding to the membrane from the fractional area changes. However, GUV aspiration technique is not able to provide enough information to address an important question: how depth is the binding site on the membrane? In other words, whether daptomycin can incorporate into the hydrophobic chain area or can only stay in the interface of lipid bilayer. Here, x-ray diffraction is applied to answer the question with great accuracy. The thickness of a phospholipid bilayer can be calculated by measuring its phosphate-to phosphate distance (PtP) across the bilayer. When peptides are added into the system, the membrane thickness will be changed and
the level of changes mainly depends on the binding site depth at the membrane. The binding sites can be distinguished between a low energy-binding state that causes thinning and a higher energy-binding state that has little thinning effect.

When few initial amphipathic molecules approach a lipid bilayer, the interface is expected to the low energy-binding site (figure 4.3 A). Especially for most antimicrobial peptides, they initially bind to the interface of lipid bilayer due to hydrophobic effect. The membrane surface area stretches due to peptides binding. For this bilayer system, it requires lots of energies to maintain the empty space below the binding site in the chain region. Thus the fatty acid chains of the lipids that are adjacent to the peptide will naturally fill the space due to their fluid nature (figure 4.3 B). As a result of conservation of lipid volume, the thickness of chain regions, $h$, decreases.

Figure 4.3  Membrane thinning due to peptide interface binding. (A) The membrane surface is stretched by a peptide binding to the interface of the bilayer. (B) Fluid hydrophobic chains fill in the space that stretched by the binding peptide and cause the membrane thinning.
Another binding site with higher energy is the interior of the hydrocarbon chain region. In this case, no extra empty space in the chain region. The membrane surface is stretched by the peptide but the membrane does not thin as much as it does in the case of interface binding.

Based on previous studies of membrane thickness changes in our laboratory, antimicrobial peptides, such as curcumin, alamethicin, and melittin, show two-phase binding corresponding to two energy states mentioned in the previous two paragraphs. The transition from a low energy-binding state to a high energy-binding aggregation state can be explained by the energy cost of membrane thinning [39]. Peptide binding to membranes is due to a negative binding energy to the bilayer interface. However the binding causes an expansion in the membrane area and thinning of the hydrocarbon region. This elastic energy of membrane thinning is proportional to the square of the peptide concentration. Thus the energy level of interfacial binding increases linearly with the peptide concentration. When the bound peptide concentration reaches a critical value, a non-linear transition to the high-energy aggregation state occurs, very much like a phase transition.
References


