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Investigating the influence of lipid ion pairing on daptomycin activity in model staphylococcal membranes

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Abstract

Infections caused by *Staphylococcus aureus* remain a major global health concern, particularly due to the increasing prevalence of multidrug-resistant strains that compromise the effectiveness of standard antibiotic therapies. Among last-resort antibiotics, daptomycin plays a crucial role in the treatment of severe Gram-positive infections. Its antibacterial activity depends on calcium-mediated binding to negatively charged lipids, primarily phosphatidylglycerol (PG). However, resistance to daptomycin has increasingly been associated with alterations in membrane lipid composition, especially the conversion of PG to lysyl-phosphatidylglycerol (LPG) mediated by the MprF protein, which dampens membrane charge and is thought to impair drug binding.

In this study, the influence of LPG on calcium-dependent daptomycin-membrane interactions was systematically investigated using liposomal model membranes with defined lipid compositions. To ensure reproducibility, chemically stable LPG analogues were used, allowing controlled variation of lysylated lipid content. Experiments were conducted across different calcium concentrations and under both neutral and mildly acidic pH conditions.

Daptomycin binding was assessed via fluorescence spectroscopy based on its intrinsic kynurenine (Kyn) residue, enabling sensitive detection of membrane association. Binding was strictly dependent on the presence of PG, confirming its essential role in mediating daptomycin-membrane interactions. Incorporation of lysylated lipids did not abolish binding but resulted in a pronounced shift of the binding equilibrium towards higher Ca^{2+} concentrations and reduced binding efficiency at low calcium levels. These findings are consistent with partial charge neutralization of PG through reversible interactions with lysylated lipids, which limit the availability of negatively charged binding sites.

In addition, zeta potential measurements revealed a general dampening of membrane surface charge upon addition of calcium ions across all lipid compositions, indicating a modulation of membrane electrostatics. Binding was further influenced by pH, with reduced interaction observed under mildly acidic conditions.

Overall, the results demonstrate that LPG modulates, rather than prevents, daptomycin-membrane interactions by altering membrane charge properties and shifting calcium requirements. These findings support a model in which LPG contributes to resistance in a quantitative manner and is unlikely to represent the sole determinant of daptomycin resistance in *Staphylococcus aureus*.

Zusammenfassung

Infektionen durch *Staphylococcus aureus* stellen weiterhin ein bedeutendes globales Gesundheitsproblem dar, insbesondere aufgrund der zunehmenden Verbreitung multiresistenter Stämme, die die Wirksamkeit standardmäßiger Antibiotikatherapien beeinträchtigen. Unter den Reserveantibiotika spielt Daptomycin eine zentrale Rolle bei der Behandlung schwerer Infektionen durch Gram-positive Bakterien. Die antibakterielle Aktivität beruht auf einer calciumvermittelten Bindung an negativ geladene Lipide, vor allem Phosphatidylglycerol (PG). Allerdings wird Daptomycin-Resistenz zunehmend mit Veränderung der Lipidzusammensetzung der Membran in Verbindung gebracht, insbesondere mit der durch das MprF-Protein vermittelten Umwandlung von PG zu Lysyl-phosphatidylglycerol (LPG), wodurch die Oberflächenladung reduziert und die Bindung des Antibiotikums beeinträchtigt wird.

In der vorliegenden Arbeit wurde der Einfluss von LPG auf die calciumabhängigen Wechselwirkungen zwischen Daptomycin und Membranen systematisch unter Verwendung liposomaler Modellsysteme mit definierter Lipidzusammensetzung untersucht. Zur Gewährleistung reproduzierbarer Bedingungen wurden chemisch stabile LPG-Analoga eingesetzt, die eine kontrollierte Variation des Anteils lysierter Lipide ermöglichten. Die Experimente wurden über verschiedene Calciumkonzentrationen hinweg sowie unter neutralen und leicht sauren pH-Bedingungen durchgeführt.

Die Bindung von Daptomycin wurde mittels Fluoreszenzspektroskopie unter Ausnutzung seines intrinsischen Kynurenin-(Kyn)-Restes untersucht, wodurch eine sensitive Detektion der Membranassoziation möglich war. Die Bindung war strikt von der Anwesenheit von PG abhängig, was dessen essenzielle Rolle bei der Vermittlung der Daptomycin-Membran-Interaktion bestätigt. Das Vorhandensein lysylierter Lipide führte nicht zum vollständigen Verlust der Bindung, bewirkte jedoch eine deutliche Verschiebung des Bindungsgleichgewichts hin zu höheren Ca^{2+}-Konzentrationen sowie eine verringerte Bindungseffizienz bei niedrigen Calciumkonzentrationen. Diese Beobachtungen sind konsistent mit einer teilweisen Ladungsneutralisierung von PG durch reversible Wechselwirkungen mit lysierten Lipiden, wodurch die Verfügbarkeit negativ geladener Bindungsstellen eingeschränkt wird.

Zusätzlich zeigten Zetapotentialmessungen eine generelle Abschwächung der Membranoberflächenladung nach Zugabe von Calciumionen über alle untersuchten Lipidzusammensetzungen hinweg, was auf eine Modulation der Membran-Elektrostatik

hinweist. Darüber hinaus wurde die Bindung durch den pH-Wert beeinflusst, wobei unter leicht sauren Bedingungen eine reduzierte Interaktion beobachtet wurde.

Insgesamt zeigen die Ergebnisse, dass LPG die Wechselwirkungen zwischen Daptomycin und Membranen nicht verhindert, sondern durch Veränderungen der Membranladung und der Calciumabhängigkeit moduliert. Die vorliegenden Daten unterstützen ein Modell, in dem LPG in quantitativer Weise zur Resistenz beiträgt, jedoch wahrscheinlich nicht als alleiniger bestimmender Faktor für die Daptomycin-Resistenz bei *Staphylococcus aureus* angesehen werden kann.

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Abbreviations

$CaCl_2$	calcium chloride
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol
LPG	lysyl-phosphatidylglycerol
CL	cardiolipin
DP3adLPG	1,2-O-dipalmitoyl-3-aza-dehydroxy lysylphosphatidylglycerol
PO3adLPG	1-palmitoyl-2-oleyl-3-aza-dehydroxylysyl-phosphatidylglycerol
S. aureus	Staphylococcus aureus
LUV	large unilamellar vesicles
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NaCl	sodium chloride
Kyn	kynurenine
Trp	tryptophan
MprF	Multiple Peptide Resistance Factor

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1 Introduction

Infections caused by *Staphylococcus aureus* represent a persistent global health concern, placing significant strain on healthcare resources due to their high prevalence and the potential for severe clinical outcomes. This challenge has been further intensified by the rising incidence of strains with reduced susceptibility to multiple antibiotics, which were considered standard therapy for those infections.[1], [2]

The increasing prevalence of resistant strains is a consequence of the remarkable evolutionary adaptability of bacteria in response to selective antibiotic pressure. Resistance can be intrinsic, based on inherent structural or functional traits, or acquired through chromosomal mutations and horizontal gene transfer. These processes enable bacteria to evade antimicrobial activity through mechanisms such as enzymatic drug inactivation, reduced drug accumulation, or modification of antibiotic targets.[3] In *Staphylococcus aureus*, the accumulation of such resistance determinants has significantly limited therapeutic options and contributed to its continued clinical relevance. The escalating challenge of antimicrobial resistance in *Staphylococcus aureus* emphasizes the urgent need for the development of novel last-resort antibiotics, among these, daptomycin represents a particularly important therapeutic option.[4], [5]

Although the exact mechanism of action of daptomycin has not been fully elucidated, its antibacterial activity is known to depend on calcium ions (Ca^{2+}), which induce conformational changes and promote oligomerization of the negatively charged lipopeptide. The resulting daptomycin-calcium complex exhibits a high affinity for the anionic phospholipid phosphatidylglycerol (PG), a major constituent of the cytoplasmic membrane of Gram-positive bacteria, such as *Staphylococcus aureus*.[6], [7]

Despite extensive research on daptomycin interactions with model bacterial membranes[8], [9], [10], important experimental parameters are often simplified or insufficiently addressed. In particular, the role of lysyl-phosphatidylglycerol (LPG), a key lipid involved in resistance mechanisms, is frequently studied using simplified membrane models without considering whether LPG remains stable under experimental conditions.[11]

Since daptomycin works by interacting with negatively charged phosphatidylglycerols in bacterial membranes, changes in membrane composition can significantly affect susceptibility. In particular, mutations in the *mprF* gene are often found in daptomycin-resistant strains. This gene codes for the membrane-associated lysyl-transferase MprF, which is involved in modifying the bacterial membrane charge through the conversion of PG

to LPG, which is thought to reduce daptomycin binding affinity and therefore contribute to resistance.[12]

However, despite the recognized importance of LPG, its precise role in modulating daptomycin-membrane interactions remains insufficiently characterized und controlled and stable experimental conditions. In particular, it is not fully understood how increasing levels of lysylation affect calcium-dependent binding behaviour, nor how environmental factors such as pH influence this interplay. This represents a key gap in current mechanistic models of daptomycin resistance.

The aim of this study was to examine how variations in membrane lipid composition, with particular emphasis on increasing LPG content, affect the binding of daptomycin to model membranes of *S. aureus*. Liposomes with varying phospholipid compositions, including different proportions of LPG, were prepared to mimic changes in membrane charge.

It was hypothesized that increasing LPG content would reduce daptomycin binding in a calcium-dependent manner by decreasing the availability of negatively charged phosphatidylglycerol, thereby shifting the binding equilibrium towards higher Ca^{2+} concentrations. Furthermore, it was expected that acidic pH conditions would further weaken membrane association due to reduced electrostatic interactions, thereby amplifying the effects of lipid composition.

One of the main challenges in these experiments was the limited chemical stability of native LPG.[13], [14] To address this issue and ensure reproducibility and comparability of the results, a chemically stable analogue of LPG was used as a substitute for the natural lipid, allowing reliable investigation of membrane interactions. Furthermore, measurements were performed under both neutral and mildly acidic pH conditions to better approximate the extracellular environment of *Staphylococcus aureus* [15], thereby mimicking the physiological proton gradient across the cytoplasmic membrane.[16]

Daptomycin binding was assessed using fluorescence measurements based on its intrinsic kynurenine residue, with fluorescence intensity used as a measure of membrane association.[12]

By systematically exploring how different membrane-mimicking liposome compositions, particularly variations in LPG content, and pH conditions influence daptomycin-membrane interactions, this work aims to provide valuable insights into the contribution of this lipid to daptomycin resistance development.

1.1 Daptomycin

Daptomycin, originally designated LY146032, is a calcium-dependent cyclic anionic lipodepsipeptide obtained by fermentation of *Streptomyces roseosporus*.[17], [18] Development of the drug began in the mid-1980s but was initially discontinued after Phase II clinical trials indicated the occurrence of potential drug-induced myopathic adverse events. In 1997, several years after its discontinuation, clinical development was resumed following licensing by another pharmaceutical company, with particular emphasis on optimizing the dosing regimen in order to reduce the risk of muscle toxicity.[19] Following successful clinical development, daptomycin was approved in 2003 by the U.S. Food and Drug Administration (FDA) and in 2006 by the European Medicines Agency (EMA) for intravenous administration in the treatment of complicated skin and soft tissue infections caused by Gram-positive bacteria.[18]

1.1.1 Characteristics of daptomycin

Lipopeptides, of which daptomycin is by far the most studied, are a structurally diverse class of compounds, consisting of a peptide core covalently linked to a lipid tail.[20] The structure of daptomycin is the secret to the distinctive mechanism of action.[21] Its peptide core consists of 13 amino acids, six of which are non-proteinogenic, including kynurenine (Kyn) which along with a single tryptophan (Trp), act as intrinsic fluorophores.[9] The C-terminal ten amino acids form a macrocyclic depsipeptide ring, while the N-terminal tripeptide carries a variable fatty acyl residue attached to Trp, influencing antibacterial activity and toxicity.[9] Daptomycin contains six ionizable residues, four acidic and two basic, resulting in a predominantly negatively-charged molecule at physiological pH.[9] The intrinsic fluorescence of Kyn and Trp has been widely used to study daptomycin´s interactions with lipid membranes, making these structural features particularly relevant for understanding its mechanism of action.[9], [20]

At physiological pH (~7.4), daptomycin carries an overall net charge of approximately -3 due to four deprotonated acidic residues, three aspartic acid residues and one 3-methylglutamic acid residue, and one basic ornithine residue. Calcium binding reduces the negative charge, masks the anionic nature, and confers an overall amphiphilic character, inducing structural changes that promote membrane interaction.[17], [22] The aliphatic amine of the ornithine side chain has a reported pKa of 10, indicating pH-dependent protonation and a

predominantly positive charge under physiological conditions. Therefore, the overall net charge of daptomycin may vary with environmental pH [23], potentially altering calcium-binding affinity or stoichiometry and thereby affecting membrane association and antibacterial efficacy.

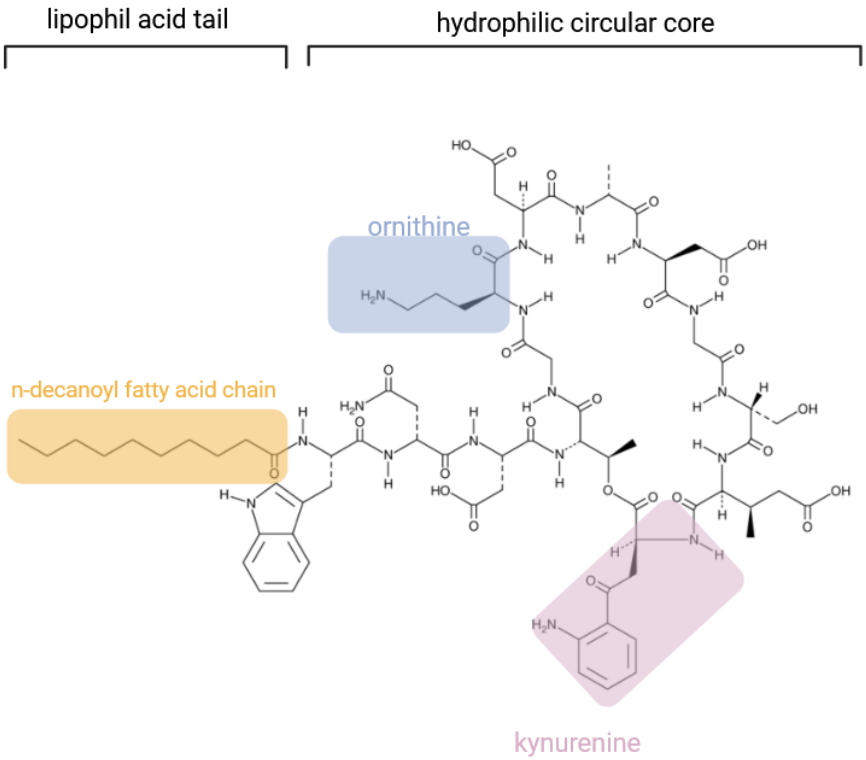

Figure 1 Daptomycin structure; created with Biorender.com

1.1.2 Mechanism of action

To date, studies on the mechanism of action of daptomycin have produced seemingly conflicting results. Early reports indicated that daptomycin inhibits peptidoglycan biosynthesis, accompanied by potassium leakage from *Staphylococcus aureus* cells; however, analysis of killing kinetics later suggested that membrane leakage is likely a consequence, rather than the primary cause of cell death.[17]

The antibacterial activity of daptomycin requires the presence of both calcium ions and the negatively charged membrane lipid phosphatidylglycerol (PG), which is abundant in the membranes of Gram-positive cells, thereby promoting its preferential activity against Gram-positive cells compared with mammalian cells.[21], [24] Daptomycin is inherently inactive against Gram-negative bacteria, as their outer membrane prevents the drug from penetrating.[21]

The mechanism of action cannot be attributed solely to a single membrane-associated process. Rather, daptomycin exhibits a dual and concentration-dependent mechanism of action. At low concentrations, particularly in actively dividing bacteria, daptomycin interferes with peptidoglycan biosynthesis.[12] In the presence of Ca^{2+}, the drug inserts into the cytoplasmic membrane and forms a tripartite complex with phosphatidylglycerol and the cell wall precursor lipid II.[6], [17] This interaction results in sequestration of lipid II, thereby preventing its incorporation into the growing peptidoglycan network and disrupting cell wall assembly.[12] In this context, lipid II is not merely a structural building block but a functional scaffold whose spatial organization is essential for coordinated cell wall synthesis and cell division.[17] In contrast, higher daptomycin concentrations are required to exert bactericidal activity against metabolically quiescent, stationary-phase cells, in which cell wall synthesis is reduced. Under these conditions, daptomycin primarily targets the bacterial membrane itself.[12] Oligomerization within the membrane leads to permeabilization, dissipation of ion gradients, and leakage of cytoplasmic contents.[12] Consequently, daptomycin combines lipid II-dependent inhibition of cell wall biosynthesis with a distinct membrane-disruptive mechanism. Although mechanistically independent, both activities strictly require calcium ions, which promote membrane association by mediating binding to PG, thereby stabilizing the daptomycin-lipid II complex.[12], [17]

Calcium triggers two key structural transitions in daptomycin. Initially, Ca^{2+} binds to the peptide in solution, inducing a more defined conformation and increasing its amphiphilic character, which promotes insertion into membrane bilayers.[25] In the presence of both Ca^{2+} and the negatively charged lipid PG, a second structural transition occurs, facilitating oligomerization and stable membrane association.[24] Therefore, the bactericidal activity of daptomycin depends on calcium-mediated membrane association and is strongly influenced by the phospholipid composition of the bacterial membrane, particularly the presence of phosphatidylglycerol.[25]

The PG-dependent mechanism of action of daptomycin involves a stereospecific interaction between the daptomycin-calcium complex and phosphatidylglycerol. This stereoselectivity highlights that not only the presence but also the structural organization of membrane lipids play a crucial role in mediating membrane binding and subsequent antibacterial activity.[26] These PG interactions, in combination with lipid II, facilitate the formation of the tripartite complex with lipid II under growth conditions, linking membrane binding to inhibition of cell wall synthesis.[10], [12]

1.2 Lipid composition of *Staphylococcus aureus* cell membranes

In the Gram-positive bacterium *Staphylococcus aureus*, the cytoplasmic membrane plays a central role in maintaining cellular integrity and regulating the exchange of molecules between the cell and its environment. Structurally, the cytoplasmic membrane consists of a phospholipid bilayer that provides a diffusion barrier, while transport across the membrane is primarily mediated by membrane proteins, which largely determine its selective permeability and influence the uptake of harmful substances, including certain antibiotics.[27] Although the membrane is surrounded by a thick peptidoglycan cell wall that helps maintain cell shape and resistance to osmotic pressure, the membrane itself is essential for controlling transport processes and cellular homeostasis. Consequently, the lipid composition of the membrane is a key determinant of its structural and functional properties.

Although the membrane is surrounded by a thick peptidoglycan cell wall that contributes to cell shape and resistance to osmotic pressure, it is essential for cellular homeostasis due to its role in protein-mediated transport. The lipid composition of the membrane influences its structural properties and modulates permeability, particularly for molecules that directly interact with the lipid bilayer.[28]

The lipid component of plasma membranes consists mainly of amphiphilic lipids, most commonly glycerophospholipids, which consist of two fatty acid chains, a glycerol backbone, a phosphate group, and a variable head group.[29] In *Staphylococcus aureus,* the main phospholipids – phosphatidylglycerol (PG), cardiolipin (CL), and lysyl-phosphatidylglycerol (LPG) – share the basic glycerophospholipid structure but differ in chain number and specific modifications.[30] PG carries a negative charge due to its phosphate group, while CL contains four fatty acid chains, contributing to the structural diversity of the membrane. LPG is formed from PG through a transesterification reaction that uses lysyl-tRNA as a cosubstrate. This reaction is catalysed by the membrane protein MprF, which not only adds the lysine to PG, neutralizing its negative charge, but also translocates LPG from the inner to the outer leaflet of the plasma membrane. By altering the surface charge, LPG influences interactions with antimicrobial peptides thus and plays a role in the bacterium´s antibiotic defence mechanisms.[9], [28]

The composition of phospholipids in the *Staphylococcus aureus* membrane is highly dynamic, allowing the bacterium to adapt to environmental stressors such as pH shifts, temperature changes, and exposure to antimicrobial agents, which in turn can enhance its pathogenic potential.[31], [32]

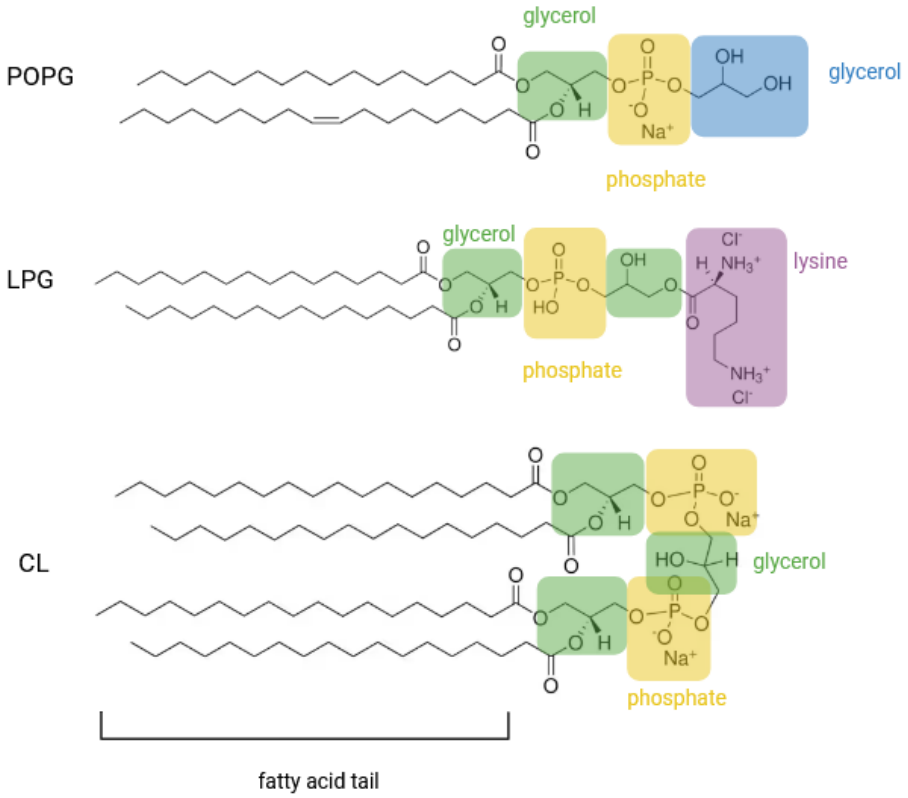

Figure 2 Structures of the common phospholipids in the S. aureus cell membrane: POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol), LPG (lysyl-phosphatidylglycerol) and CL (cardiolipin); created with Biorender.com

1.2.1 Daptomycin resistance in *Staphylococcus aureus*

Because of its distinctive membrane-associated mechanism of action, the development of resistance to daptomycin was initially considered unlikely.[33] However, in recent years, an increasing number of treatment failures have been reported due to high-level resistance to daptomycin among clinically relevant Gram-positive bacteria.[34] This rise in resistance represents a serious challenge, especially given the limited alternative treatment options available for multidrug-resistant infections.[35] In *Staphylococcus aureus*, daptomycin resistance has become an important problem that complicates the treatment of severe infections. [19], [20] Current evidence suggests that resistance arises mainly through bacterial adaptations of the cytoplasmic membrane, with mutations that alter phospholipid composition and membrane charge playing a key role in reducing daptomycin efficacy.[36], [37]

The central determinant of these adaptations is the *mprF* (multiple peptide resistance factor) gene, which encodes a bifunctional membrane protein with a C-terminal lysyl-phosphatidylglycerol (LPG) synthase domain and an N-terminal floppase domain that translocates LPG to the outer leaflet of the cytoplasmic membrane.[7], [12] Gain-of-function mutations in *mprF* increase LPG levels and reduce PG content, promoting the formation of PG/LPG ion pairs that neutralize the membrane and thereby decrease daptomycin binding and insertion, whereas deletion or loss-of-function mutations sensitize bacteria to the drug.[11], [38]

Other genes, such as *pgsA*, which encodes a phosphatidylglycerol synthase, and *cls2*, which encodes a cardiolipin synthase, can further influence membrane composition, decreasing PG level or converting PG into cardiolipin (CL).[35], [39] Although these genes have been implicated in altering membrane lipid composition, this work focuses primarily on the LPG-mediated adaptations as a central mechanism of daptomycin resistance.

Daptomycin exerts its bactericidal effect by binding to anionic phospholipids in a calcium-dependent manner, preferentially targeting regions of increased membrane fluidity.[8] The alterations associated with MprF, including the conversion of PG to LPG, reduce available binding sites and introduce electrostatic repulsion. In addition, through PG/LPG ion pairing, these changes can affect membrane organization and properties, thereby interfering with daptomycin oligomerization and insertion.[13] These combined changes in membrane properties provide a robust mechanism for resistance, which may explain why gain-of-function mutations in *mprF* are so frequently observed in clinical daptomycin-resistant *Staphylococcus aureus* isolates.

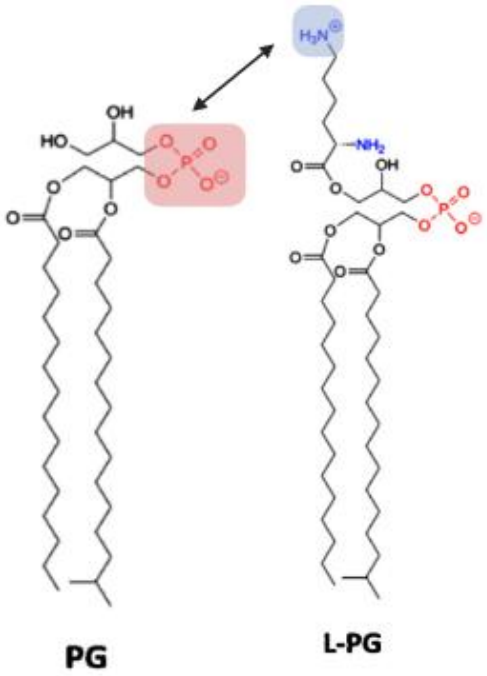

Figure 3 Structures of phosphatidylglycerol (PG) and lysyl-phosphatidylglycerol (LPG), highlighting ion pair formation via electrostatic interaction between the phosphate group of PG and the ε-amino group of the lysyl-residue; created with Biorender.com

1.3 Liposomes as model membranes

Liposomes are widely used as simplified model systems to study the physicochemical properties of the lipid components of biological membranes and their interaction with membrane-active compounds. They are spherical vesicles consisting of one or more phospholipid bilayers that enclose an aqueous core.[40], [41] Their formation is driven by the amphiphilic nature of phospholipids, which contain hydrophilic head groups and hydrophobic hydrocarbon chains. When dispersed in aqueous environments, these molecules spontaneously arrange into bilayer structures with hydrophobic chains facing inward and polar headgroups oriented towards the aqueous environment.[42]

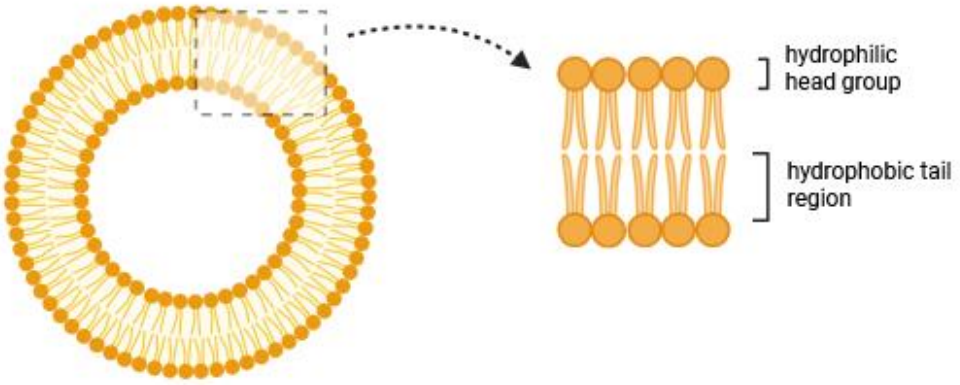

Figure 4 Schematic representation of a liposome and the corresponding phospholipid bilayer structure; created with Biorender.com

An important advantage of liposomes is that their membrane properties can be easily modified by altering the lipid composition. By adjusting their lipid composition, liposomes can replicate key features of bacterial membranes, offering a controlled and precisely defined system to investigate membrane properties.[43] Therefore, liposomes are an ideal model system to study how specific lipid alterations, such as changes in PG and LPG content, influence membrane behaviour and modulate daptomycin-membrane interactions.

1.4 Fluorescence properties of daptomycin

As described above, daptomycin is a cyclic lipopeptide antibiotic whose antimicrobial activity depends on its interaction with bacterial membranes. To investigate these interactions in vitro, fluorescence spectroscopy provides a highly sensitive tool, allowing the monitoring of peptide binding and insertion into liposomal model membranes. Daptomycin contains two aromatic residues, tryptophan (Trp-1) and kynurenine (Kyn-13), both intrinsically fluorescent.[9], [22] While the Trp residue exhibits a relatively low fluorescence yield, partly due to Förster-type energy transfer to the Kyn residue, Kyn-13 provides the dominant fluorescent signal, making it particularly suitable for fluorescence-based liposome binding assays.[44]

Fluorescence spectroscopy is a robust and sensitive method for studying peptide-membrane interactions.[45] Fluorophores absorb light at a specific excitation wavelength and, after a

short excited-state lifetime, emit energy at longer wavelengths. The fluorescence of aromatic residues is strongly influenced by the polarity of their surrounding environment.[22], [45] This environmental sensitivity enables the detection of membrane insertion events, as the chromophores move from a polar aqueous phase to the more hydrophobic lipid environment.

Upon insertion into phospholipid membranes, the environment of daptomycin´s lipophilic residues becomes less polar, which can lead to changes in fluorescence properties, including an increase in emission intensity and, in some cases, a blue shift of the emission maximum.[22], [35] In particular, the fluorescence of Kyn-13 is characterized by a substantial increase in emission intensity upon membrane interaction.[35], [44]

To quantitatively assess membrane binding, liposome-based fluorescence assays were performed in a 96-well plate format using liposomes of defined lipid compositions. At a fixed daptomycin concentration, Ca^{2+} was titrated to promote membrane association, and binding was monitored via the increase in Kyn-13 fluorescence intensity. Under these controlled conditions, the fluorescence signal can be correlated with the fraction of membrane-bound peptide.[12]

Calcium ions modulate these interactions. Weak or transient interactions may occur in the absence of calcium, whereas stable membrane binding and insertion strictly require Ca^{2+}. Subsequent calcium addition promotes membrane insertion, resulting in a pronounced increase in fluorescence intensity, reflecting stronger peptide-membrane association. [10], [44] By plotting normalized fluorescence intensity versus calcium concentration, a binding curve can be obtained, allowing estimation of the relative affinity of daptomycin for different lipid compositions.[10]

Overall, the intrinsic fluorescence of daptomycin, dominated by Kyn-13 and modulated by Trp-1, provides a reliable indicator for membrane insertion, calcium-dependent interactions, and lipid specificity. The spectral changes observed, including both intensity increases and blue shifts, reflect the transition of the aromatic residues from polar to hydrophobic environments, linking peptide structure to membrane binding behaviour.[22], [44]

2 Materials and methods

2.1 Materials

The lipids 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (16:0-18:1 PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (16:0-18:1 PG), and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (chloride salt) (18:1 LPG) were all supplied by Avanti Polar Lipids (Alabaster, AL, USA). The lysylphosphatidylglycerol analogues 1-palmitoyl-2-oleyl-3-aza-dehydroxylysyl-phosphatidylglycerol (PO3adLPG) and Dipalmitoyl-3-aza-dehydroxylysyl-phosphatidylglycerol (DP3adLPG) were custom synthesized as described by Rehal *et al*. 2019 [13].

The lipopeptide antibiotic Daptomycin was obtained from Combi-Blocks (San Diego, CA, USA). Sodium chloride (NaCl, purity ≥ 99%), methanol, and chloroform were sourced from Carl Roth (Karlsruhe, Germany). HEPES and calcium chloride were supplied by Sigma-Aldrich (Darmstadt, Germany).

For all experiments, the ultrapure milli-Q water with a specific resistivity above 18.2 MΩ was used. It was obtained from a Purelab® Flex 3 water deioniser from Elga LabWater (High Wycombe, UK).

2.2 Methods

2.2.1 Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared from different lipid mixtures composed of POPC, POPG, DPLPG, PO3adLPG and DP3adLPG. It should be noted that POPC was included solely as a non-interacting structural matrix to facilitate the incorporation of the remaining lipid components and to ensure stability.

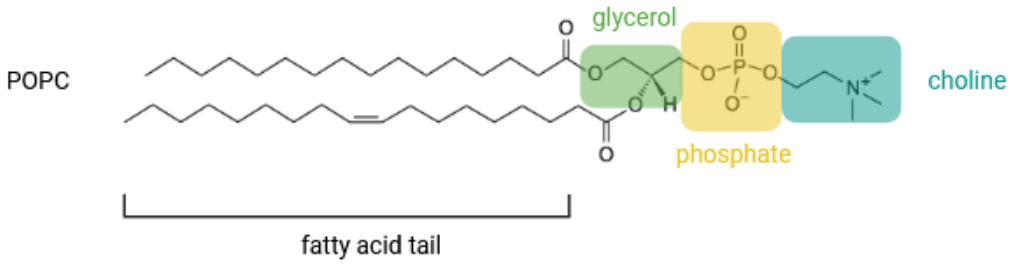

Figure 5 Structure of POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine), which was used as a non-interacting structural matrix for liposome preparation; created with Biorender.com

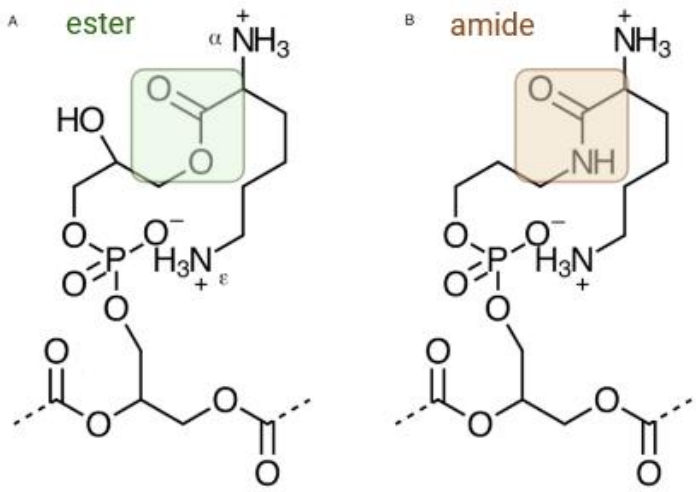

Figure 6 Headgroup of native LPG (A) and synthesised 3adLPG (B); created with Biorender.com

The required amounts of lipids to obtain a final lipid concentration of ~5 mM were weighed out and dissolved in a chloroform/methanol mixture (8:2). The lipid solutions were combined in defined ratios, as listed in Tables 1 and 2, using microsyringes (Hamilton, Switzerland) to generate the specific lipid composition needed for the daptomycin binding assays. The organic solvent was completely removed by rotary evaporation (Rotavapor® Hei-VAP Expert Control, Heidolph Instruments, Germany) at 40°C and under vacuum at 150 mbar at 300 rpm for 30 minutes, resulting in the formation of a thin, dry lipid film.

Table 1 The ratios of lipid mixtures used in daptomycin binding assays at pH 7.4.

POPC/POPG 4:1
POPC/POPG/DP3adLPG 4:1:0.8
POPC/POPG/PO3adLPG 4:1:0.8
POPC/POPG/LPG 4:1:0.8

Table 2 The ratios of lipid mixtures used in daptomycin binding assays at pH 5.3.

POPC/POPG 3:1	POPC/POPG/PO3adLPG 4:1:0.6
POPC/POPG 4:1	POPC/POPG/PO3adLPG 4:1:0.8
POPC/POPG/DP3adLPG 4:1:0.8	POPC/POPG/PO3adLPG 4:1:1.2
POPC/PO3adLPG 3:1	POPC/POPG/PO3adLPG 4:1:1.4
POPC/POPG/PO3adLPG 4:1:0.2	POPC/POPG/LPG 4:1:0.8
POPC/POPG/PO3adLPG 4:1:0.4	

The lipid films were rehydrated in 20 mM HEPES-buffered saline (150 mM NaCl). Unless stated otherwise, experiments were performed at pH 5.3; where indicated, the pH was adjusted to 7.4. Rehydration resulted in multilamellar lipid dispersions, which were subsequently processed to obtain large unilamellar vesicles.

The dispersions were extruded under compressed nitrogen pressure using LIPEX® Flow extruder (Evonik Inc., BC Canada) at 25°C. Extrusion was performed for three passes through polycarbonate membranes with a pore size of 100 nm (AMD Manufacturing Inc., ON Canada) to obtain LUVs with a defined size distribution.

2.2.2 Characterization of liposomes

The hydrodynamic diameter and zeta potential of the prepared liposomes were determined using a Zetasizer Pro (Malvern Panalytical Ltd, UK) at room temperature (25°C). For the zeta potential measurements, disposable folded capillary cells (DTS1070) were used, while size measurements were performed in disposable plastic cuvettes containing 250 µL of sample at a concentration of 1 mM. For the zeta potential measurements, a 20 mM HEPES buffer without sodium chloride was used to prepare a 5 mM liposome dispersion, since the presence of salt ions increases the ionic strength of the medium, which screens the electrical double layer around the liposomes and can lead to reduced or distorted zeta potential values.[46] Particle size was measured by Dynamic Light Scattering (DLS) in general-purpose mode with an equilibration time of 10 seconds. Both size and zeta potential were

measured in triplicate, and data analysis was performed using ZS Xplorer software (Malvern Panalytical Ltd, UK).

2.2.3 Daptomycin binding assays

The binding assays were performed according to the method previously described by Moreira and Taylor [8]. All solutions were prepared in 20 mM HEPES-buffered saline. Previously prepared liposomes (5 mM) were diluted to approximately 556 µM, and a daptomycin solution of 11 µM was prepared in the same buffer.

By mixing equal volumes of the liposome dispersion (~556 µM) with the daptomycin solution (11 µM), a 10:1 liposome/daptomycin molar ratio was achieved.

Each well of an Invitrogen™ 96-well black-walled, clear-bottom fluorescence microtitre plate was pre-loaded with 10 µL of $CaCl_2$ solution at concentrations tenfold higher than the desired final concentrations, covering a range from 0,01 to 100 mM. Subsequently, 90 µL of the liposome/daptomycin mixture was added to each well (in triplicate), resulting in a final volume of 100 µL per well containing approximately 250 µM total lipid, 5 µM daptomycin, and $CaCl_2$ concentrations ranging from 0,001 to 10 mM.

Fluorescence measurements were performed using an Infinite 200 Pro fluorimeter (Tecan group Ltd, Grödig, Austria) at 25°C, with an excitation wavelength of 360 nm (9 nm bandwidth). The emission wavelength was adjusted for each liposome mixture to match the fluorescence maximum, ranging from 434 to 452 nm (20 nm bandwidth), in order to optimize signal detection.

2.2.4 Data analysis

Following the fluorescence measurements, the recorded intensities were background-corrected and normalized. The normalized fluorescence intensity at 448 nm was plotted as a function of $CaCl_2$ concentration. Data analysis and curve fitting were performed using the non-linear regression tool in GraphPad Prism (version 10.4.0 for macOS, GraphPad Software, MA, USA).

The measured data was fitted using a four-parameter Hill equation to characterize the calcium-dependent binding of daptomycin:

$$I_{448} = I_{min} + \frac{x^a \cdot (I_{max} - I_{min})}{x^a + K_{0,5}^a}$$

I_{min} represents the baseline fluorescence intensity, while I_{max} denotes the maximum fluorescence intensity. The parameter a corresponds to the Hill coefficient and reflects the steepness of the curve. $K_{0,5}$ is the half-saturation constant, indicating the concentration of $CaCl_2$ at which sufficient daptomycin binding to PG occurs to produce half of the maximal fluorescence intensity.[12]

IC_{50} values and Hill coefficients were derived from averaged fluorescence data. All results are presented descriptively, and no statistical comparisons were performed.

3 Results

3.1 Characterization of LUVs

As the interaction of daptomycin with model membranes critically depends on membrane composition and surface properties, liposomes were characterized by measuring their size and zeta potential prior to binding experiments. These measurements were used to confirm the successful formation of liposomes and the reproducibility of the preparation method.

The dynamic light scattering (DLS) method was used to verify that the extrusion process produced vesicles with the expected mean diameter of ~100 nm and an appropriate polydispersity index (PDI) of 0.13 ± 0.07, to confirm the formation of monodisperse samples and enabling comparable results across liposomes of different compositions.[47]

In addition, the surface charge properties of different liposome compositions were analysed using a Zetasizer to assess how it was affected by the addition of calcium ions (figure 7), whose inclusion was necessary for the binding assay. Changes in zeta potential upon calcium incorporation were observed, indicating that calcium ions influence the electrostatic properties of the liposomes and may modulate their interaction with daptomycin.

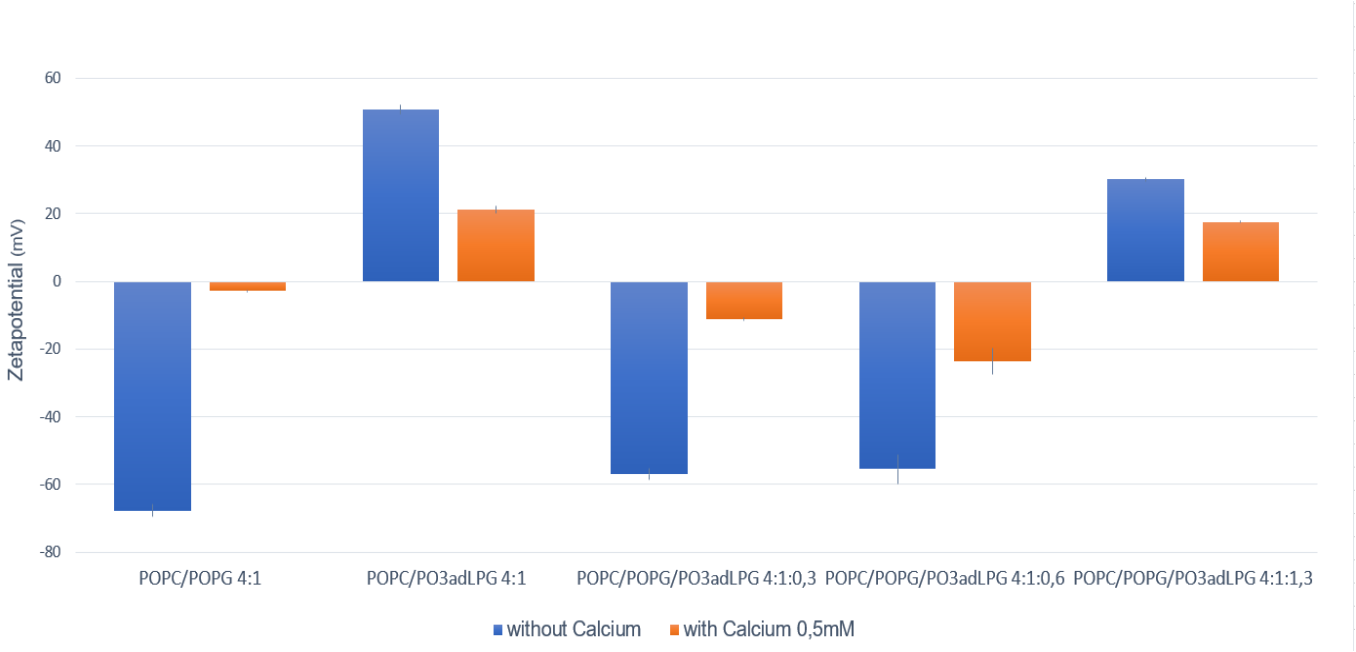

Figure 7 Comparison of zeta potential values of different liposome formulations measured at 22°C, with and without calcium ions (n=3), illustrating the influence of calcium on the surface charge of the liposomes.

In the absence of calcium ions, liposome zeta potentials appear to be wholly influenced by the presence and proportion of the opposingly charged lipids PG and 3adLPG. When PG is the only charged lipid present, or when it is in excess in liposomes containing both PG and 3adLPG, the zeta potential is negative (from -55 to -68 mV) and appears to be related to the amount of unpaired PG. The addition of calcium ions produces a clear dampening effect on the zeta potentials of all liposome compositions, shifting them to lower positive or negative values. This demonstrable influence of calcium ions on the overall surface charge may in turn modulate the binding of daptomycin to the liposomes.

3.2 The role of POPG for daptomycin interaction with model membranes

To determine the role of POPG as a key determinant for daptomycin binding to bacterial membranes, model membranes composed of POPC/POPG were compared with those lacking POPG, and those containing only POPC and PO3adLPG (figure 8).

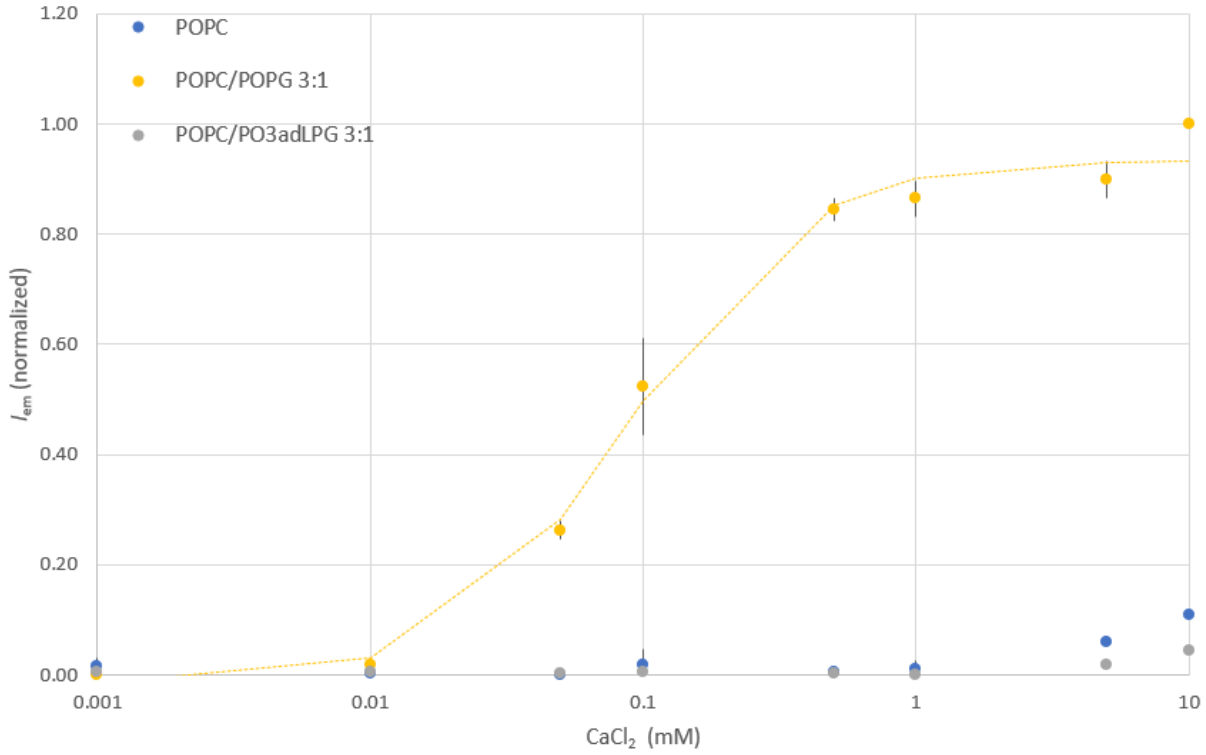

Figure 8 Normalized fluorescence-based binding of daptomycin to POPC, POPC/POPG (3:1), and POPC/PO3adLPG (3:1) membranes measured at 22°C (n=3). The dashed line represents the four-parameter Hill fit for POPC/POPG (3:1).

Table 3 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG (3:1) membranes, derived from four-parameter Hill fits.

POPC/POPG 3:1	
Hill slope	1.36
IC_{50}	0.089
R^2	0.993

In the binding assay, the POPC/POPG 3:1 liposomes showed a significantly larger increase in fluorescence intensity at the highest concentration of calcium, with a plateau indicating maximal daptomycin binding at ~0.5 mM $CaCl_2$. Since both of the other liposome types showed only modest increases in kynurenine fluorescence emission intensity, at high calcium concentrations, their results were normalized against that of the PG-containing liposomes.

The pronounced calcium concentration-dependent increase in kynurenine fluorescence intensity observed with the POPG-containing, is consistent with daptomycin binding to the model membranes in significant quantities. Fitting of the data using a four-parameter Hill equation revealed a sigmoidal dose-response relationship with an IC_{50} of 0.089 and a Hill slope of 1.36. The maximal fluorescence intensity (I_{max}) reached 0.94 a.u. while baseline

fluorescence remained close to zero. The R^2 value of 0.993 confirms that the model accurately captures the observed calcium concentration-dependent daptomycin/PG interaction.

In contrast to the POPC/POPG 3:1 liposomes, POPC and POPC/PO3adLPG 3:1 systems showed only small increases in fluorescence intensity upon addition of daptomycin, remaining almost at baseline levels across the tested calcium concentration range. This indicates the absence of measurable daptomycin binding under these conditions. Consequently, no significant calcium concentration-dependent response was observed, and the data could not be fitted using a four-parameter Hill equation.

Together, these findings establish POPG as a critical component for daptomycin binding and provide a robust baseline for further investigations into how additional lipids, such as LPG, may modulate this interaction.

3.3 Influence of pH on daptomycin-membrane interaction

The effect of pH on the calcium-dependent interaction of daptomycin with model membranes was investigated by comparing kynurenine fluorescence emission intensities at acidic (pH 5.3) and neutral (pH 7.4) conditions across different liposome compositions, including analysis of the corresponding IC_{50} values and Hill slopes, derived from fitting the data with the Hill function.

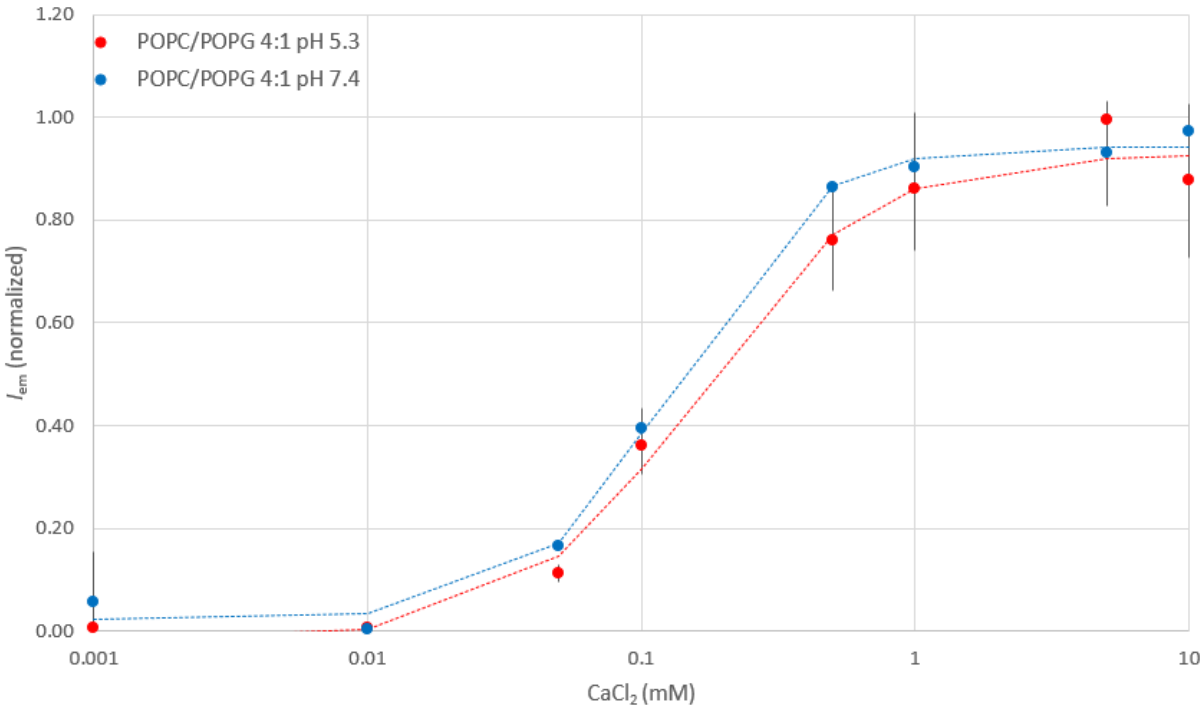

Figure 9 Normalized fluorescence-based analysis of daptomycin binding to POPC/POPG (4:1) membranes at pH 5.3 and pH 7.4 measured at 22°C (n=3). Dashed lines correspond to four-parameter Hill fits.

Table 4 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG (4:1) membranes at pH 5.3 and pH 7.4, derived from four-parameter Hill fits.

POPC/POPG 4:1		
	pH 5.3	**pH 7.4**
Hill slope	1.391	1.768
IC_{50}	0.1551	0.1272
R^2	0.989	0.9973

For POPC/POPG (4:1) liposomes (figure 9, table 3), the IC_{50} at pH 7.4 (0.1272) is lower than at pH 5.3 (0.1551), suggesting that lower calcium concentrations may be sufficient to reach half-maximal binding at neutral pH. In addition, the higher Hill slope at pH 7.4 indicates a potentially more cooperative binding process, reflected in a steeper transition from low to high fluorescence intensity.

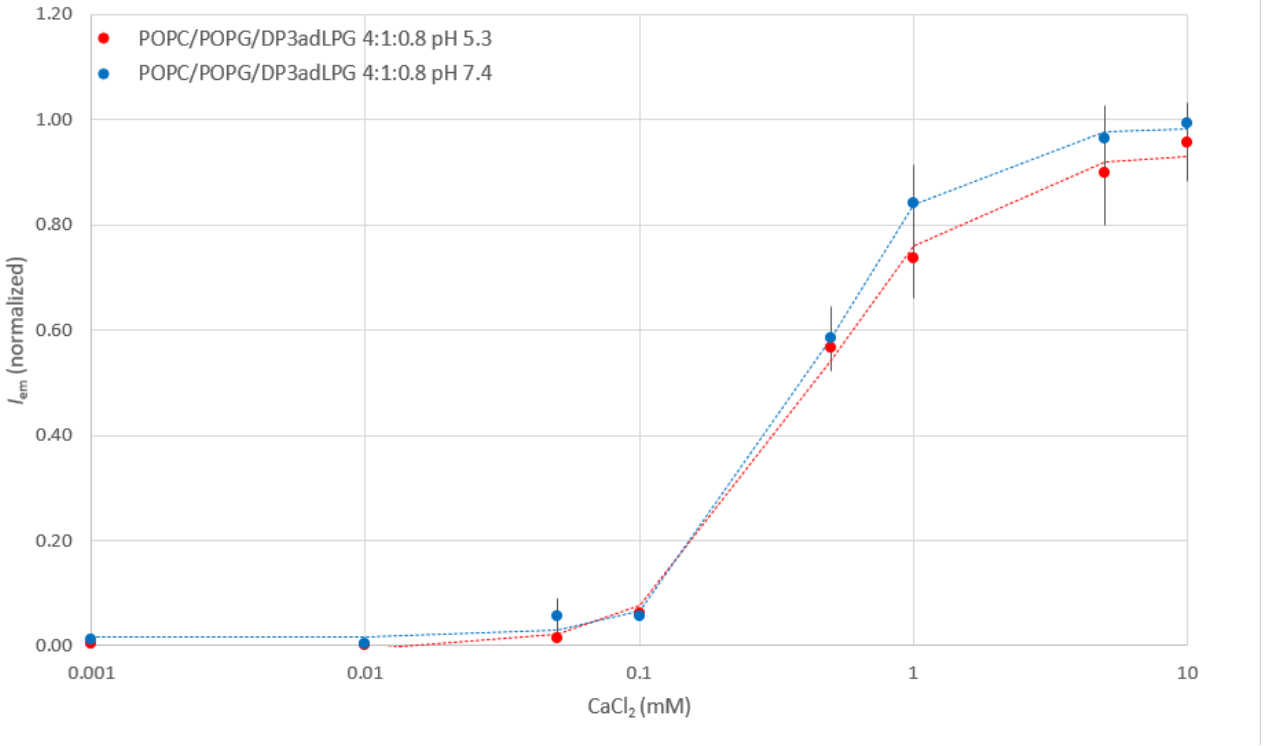

Figure 10 Normalized fluorescence-based analysis of daptomycin binding to POPC/POPG/DP3adLPG (4:1:0.8) membranes at pH 5.3 and pH 7.4 measured at 22°C (n=3). Dashed lines correspond to four-parameter Hill fits.

Table 5 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/DP3adLPG (4:1:0.8) membranes at pH 5.3 and pH 7.4, derived from four-parameter Hill fits.

POPC/POPG/DP3adLPG 4:1:0.8		
	pH 5.3	**pH 7.4**
Hill slope	1.645	2.016
IC_{50}	0.4053	0.4204
R^2	0.9978	0.9991

In contrast, POPC/POPG/DP3adLPG (4:1:0.8) liposomes showed similar IC_{50} values at both pH conditions, suggesting that the calcium concentration required for half-maximal binding is largely unaffected by pH in this system. However, the Hill slope increased from 1.645 at pH 5.3 to 2.016 at pH 7.4, indicating a steeper transition and thus potentially increased cooperativity of binding at neutral pH, while the overall calcium sensitivity remains

comparable between conditions. This change may reflect pH-dependent effects on membrane-daptomycin interactions.

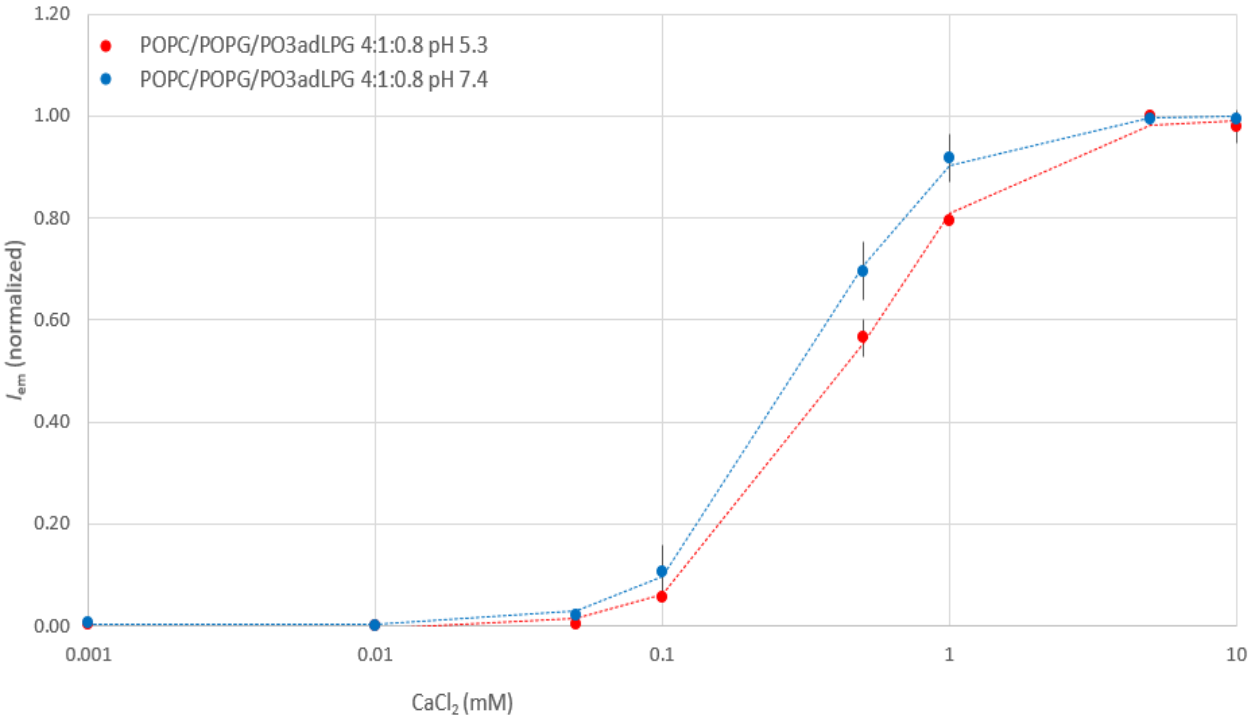

Figure 11 Normalized fluorescence-based analysis of daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.8) membranes at pH 5.3 and pH 7.4 measured at 22°C (n=3). Dashed lines correspond to four-parameter Hill fits.

Table 6 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.8) membranes at pH 5.3 and pH 7.4, derived from four-parameter Hill fits.

POPC/POPG/PO3adLPG 4:1:0.8		
	pH 5.3	**pH 7.4**
Hill slope	1.78	1.933
IC_{50}	0.435	0.3199
R^2	0,9993	0,9997

For POPC/POPG/PO3adLPG (4:1:0.8) liposomes, a pH-dependent trend was observed. The IC_{50} decreased from 0.4357 at pH 5.3 to 0.3199 at pH 7.4, suggesting that the calcium concentration required for half-maximal binding is lower at neutral pH. The Hill slope increased slightly from 1.78 at pH 5.3 to 1.933 at pH 7.4, indicating a modest increase in cooperative binding at the higher pH.

3.4 Comparison of native LPG and the stable analogue PO3adLPG on the binding of daptomycin

Daptomycin interactions with membranes containing either native DPLPG or the stable analogue PO3adLPG were compared under acidic (pH 5.3) and neutral (pH 7.4) conditions.

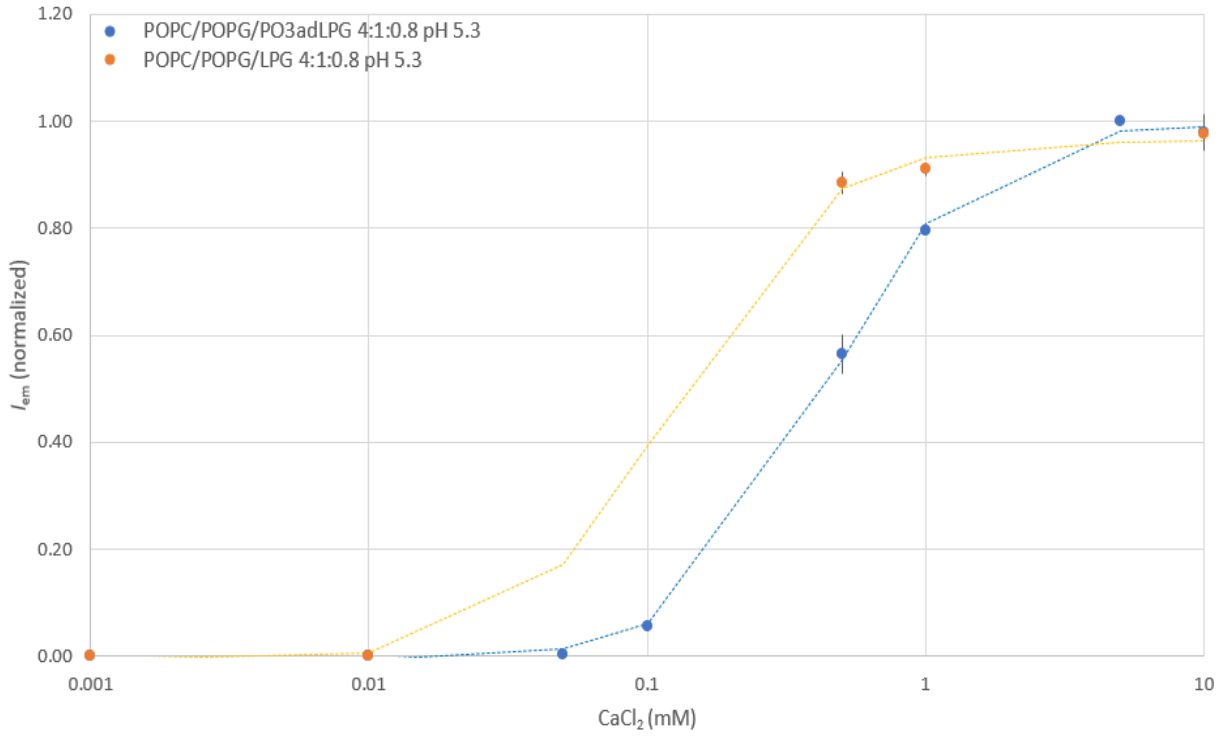

Figure 12 Normalized fluorescence-based analysis of daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.8) and POPC/POPG/LPG (4:1:0.8) membranes at pH 5.3 and pH 7.4 measured at 22°C (n=3). Dashed lines correspond to four-parameter Hill fits.

Table 7 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.8) and POPC/POPG/LPG (4:1:0.8) membranes at pH 5.3, derived from four-parameter Hill fits.

	POPC/POPG/PO3adLPG (4:1:0.8)	POPC/POPG/LPG (4:1:0.8)
Hill slope	1.78	1.646
IC_{50}	0.4357	0.1237
R^2	0.9993	0.9991

At pH 5.3 (figure 12), clear differences in the calcium-dependent binding behaviour of daptomycin were observed between membranes containing native LPG and those containing PO3adLPG.

For native DPLPG-containing membranes, the binding curve was characterized by a Hill slope of 1.646 and an IC_{50} of 0.1237. In contrast, PO3adLPG-containing membranes exhibited a slightly steeper Hill slope of 1.78, indicating a modest increase in binding affinity. However, the IC_{50} was increased to 0.4357, indicating reduced calcium binding affinity, as a higher calcium concentration was required to reach half-maximal fluorescence, which corresponds to an approximately 3.5-fold increase relative to DPLPG-containing membranes.

Thus, the substitution of LPG with PO3adLPG resulted in a clear right shift of the binding curve, reflected by the increased IC_{50} value, indicating a reduced affinity of daptomycin for PO3adLPG-containing membranes compared to native DPLPG-containing membranes.

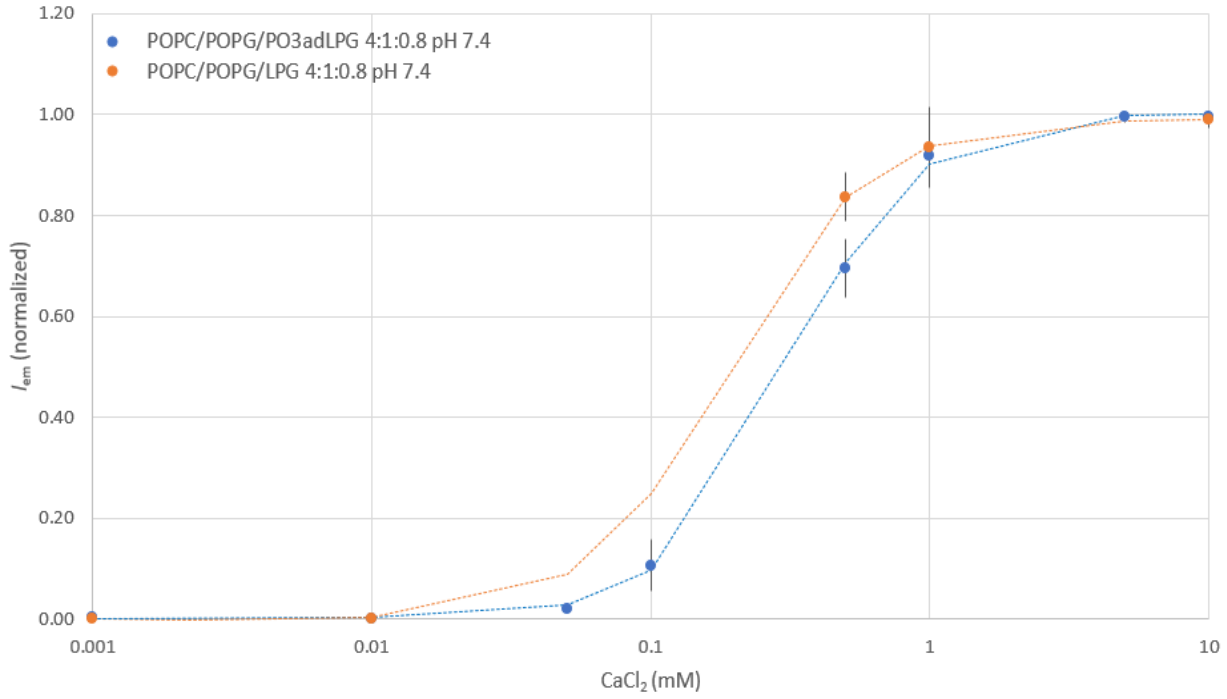

Figure 13 Normalized fluorescence-based analysis of daptomycin binding to POPC/POPG/LPG (4:1:0.8) and POPC/POPG/PO3adLPG (4:1:0.8) membranes at pH 7.4 measured at 22°C (n=3). Dashed lines correspond to four-parameter Hill fits.

Table 8 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.8) and POPC/POPG/LPG (4:1:0.8) membranes at pH 7.4, derived from four-parameter Hill fits.

	POPC/POPG/PO3adLPG (4:1:0.8)	POPC/POPG/LPG (4:1:0.8)
Hill slope	1.933	1.726
IC_{50}	0.3199	0.1871
R^2	0.9997	1

At pH 7.4 (figure 13), a similar trend was observed to that under mildly acidic conditions, although the differences between the lipid systems were less pronounced.

LPG-containing membranes showed a Hill slope of 1.726 and an IC_{50} of 0.1871. For PO3adLPG-containing membranes, the Hill slope increased to 1.933, again suggesting slightly enhanced cooperativity. The IC_{50} was higher (0.3199), corresponding to an approximately 1.7-fold difference compared to LPG-containing membranes.

As at pH 5.3, PO3adLPG led to a rightward shift of the binding curve, indicating reduced binding affinity. However, the magnitude of this shift was smaller under neutral conditions.

When comparing both pH conditions, the effect of lipid composition on daptomycin binding was clearly pH-dependent.

For LPG-containing membranes, the IC_{50} increased from 0.1237 at pH 5.3 to 0.1871 at pH 7.4, indicating reduced binding affinity at higher pH. In contrast, for PO3adLPG, the IC_{50} decreased from 0.4357 at pH 5.3 to 0.3199 at pH 7.4, suggesting that the difference between lipid systems is most pronounced under acidic conditions.

3.5 Effects of increasing amounts of PO3adLPG on daptomycin binding

Fluorescence emission was measured as a function of $CaCl_2$ concentration to quantify daptomycin binding to model membranes containing increasing proportions of the stable LPG analogue PO3adLPG at a pH of 5.3.

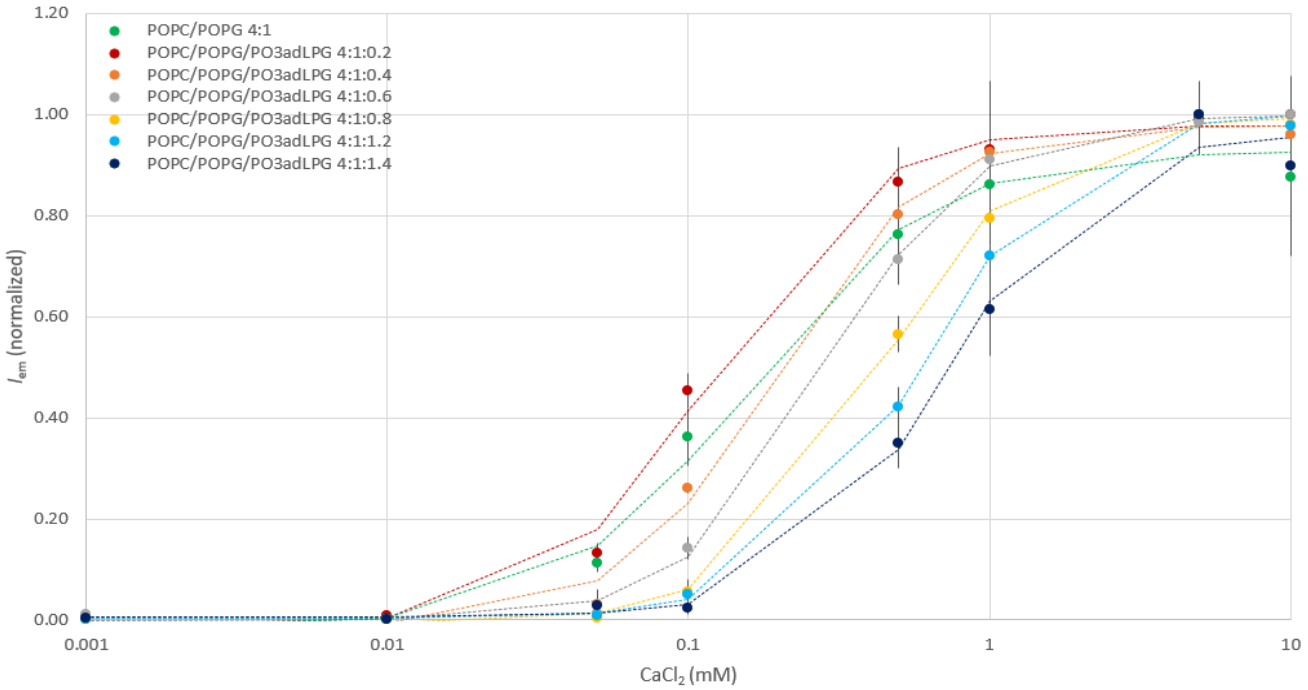

Figure 14 Normalized fluorescence-based analysis of daptomycin binding to membranes composed of POPC/POPG 4:1 and POPC/POPG/PO3adLPG in varying ratios at pH 5.3 measured at 22°C (n=3). Dashed lines correspond to four-parameter Hill fits.

Table 9 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG (4:1) membranes, derived from four-parameter Hill fits.

POPC/POPG 4:1	
Hill slope	1.315
IC_{50}	0.1798
R^2	0.9552

Table 10 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.2) and POPC/POPG/PO3adLPG (4:1:0.4) membranes, derived from four-parameter Hill fits.

	POPC/POPG/PO3adLPG 4:1:0.2	POPC/POPG/PO3adLPG 4:1:0.4
Hill slope	1.651	1.709
IC_{50}	0.118	0.193
R^2	0.9957	0.997

Table 11 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/PO3adLPG (4:1:0.6) and POPC/POPG/PO3adLPG (4:1:0.8) membranes, derived from four-parameter Hill fits.

	POPC/POPG/PO3adLPG 4:1:0.6	POPC/POPG/PO3adLPG 4:1:0.8
Hill slope	1.797	1.78
IC_{50}	0.2921	0.4357
R^2	0.9991	0.9993

Table 12 IC_{50} values and Hill slopes for daptomycin binding to POPC/POPG/PO3adLPG (4:1:1.2) and POPC/POPG/PO3adLPG (4:1:1.4) membranes, derived from four-parameter Hill fits.

	POPC/POPG/PO3adLPG 4:1:1.2	POPC/POPG/PO3adLPG 4:1:1.4
Hill slope	1.809	1.846
IC_{50}	0.5968	0.7062
R^2	0.9994	0.9936

For all membrane compositions, fluorescence intensity increased in a sigmoidal manner with rising $CaCl_2$ concentrations (figure 14), remaining close to baseline at ≤ 0.01 mM and reaching a plateau at higher concentrations.

An incremental rightward shift in the binding curves was observed with increasing PO3adLPG content, and consequently with an altered PG-to-PO3adLPG ratio within the membranes. Membranes containing lower proportions of PO3adLPG and correspondingly higher PG content reached higher fluorescence intensities at lower $CaCl_2$ concentrations, whereas membranes with higher PO3adLPG content required higher $CaCl_2$ concentration to achieve comparable kynurenine fluorescence emission intensity levels. This shift was evident across the entire calcium concentration range of the curves.

Quantitative analysis of the dose-response relationships revealed a progressive increase in IC_{50} values with increasing PO3adLPG content, corresponding to an associated decrease in the relative proportion of PG within the membrane. The lowest PO3adLPG fraction (0.2) exhibited an IC_{50} of 0.1188. Increasing the proportion to 0.4 and 0.6 resulted in IC_{50} values of 0.193 and 0.2921. Further increases in PO3adLPG content led to IC_{50} values of 0.4357 for 0.8, 0.5968 for 1.2 and 0.7062 for 1.4, demonstrating a continuous shift towards higher $CaCl_2$ concentrations needed to achieve half maximal daptomycin binding.

The steepness of the binding curves, as described by the Hill slope, showed only moderate variation across the different membrane compositions. Hill slopes ranged from 1.651 for membranes with 0.2 PO3adLPG to 1.846 for membranes with 1.4 PO3adLPG.

At higher $CaCl_2$ concentrations (≥ 1 mM), fluorescence intensities approached similar maximal levels across all membrane compositions, with only minor differences observed between conditions.

4 Discussion

A central finding of this study is that the incorporation of PO3adLPG into PG-containing liposomal model membranes significantly modulates the calcium-dependent binding behaviour of daptomycin. This effect can be explained by considering that ion pairs are able to form between negatively charged PG and the net positively charged lysylated headgroup of PO3adLPG.[12] These ion paired lipid complexes would theoretically reduce the pool of PG available for interaction with daptomycin. Since daptomycin requires negatively charged PG for membrane association [6], [24], the incremental reduction of freely accessible PG in liposomes with increasing proportions of 3adLPG, is what most likely leads to our observed decreased binding affinity. This dampening effect on daptomycin binding became increasingly pronounced with rising proportions of PO3adLPG, indicating that the extent of ion pair formation strongly depends on membrane composition.

The requirement of PG for daptomycin binding observed in this study is fully consistent with previous studies describing the PG-dependent mechanism of action of daptomycin.[6], [24] Membranes lacking PG did not show detectable binding, confirming that negatively charged phosphatidylglycerol is essential for the initial membrane interaction. This finding supports the current understanding that electrostatic attraction between the daptomycin-calcium complex and anionic phospholipids represents a necessary condition for membrane binding and antibacterial activity.[12] Importantly, our results further suggest that PG-PG complexes stabilized by calcium ions are not static structures, as these calcium-bridged complexes can be disrupted by daptomycin even at relatively low calcium concentrations through binding to individual PG molecules, thereby competing with and displacing PG-PG interactions. This provides an additional mechanistic layer for how membrane association can still occur in the presence of calcium-mediated lipid organization.

Our results further demonstrate that Ca^{2+} ions substantially influence the interaction behaviour within the lipid mixtures. In agreement with previously proposed models, calcium appears to interact directly with PG headgroups and promotes the formation of calcium-mediated PG-complexes.[6], [31] Ca^{2+} may thereby act as a bridging element between PG molecules, resulting in the formation of charge-neutralized domains. These interactions alter the electrostatic properties of the membrane and influence the equilibrium between free PG, PG-PO3adLPG ion pairs, and calcium-associated PG species. The data suggests that at increased calcium concentrations, Ca^{2+} appears to partially disrupt PG-PO3adLPG ion pairs by displacing PO3adLPG, thereby increasing the availability of free PG for daptomycin

binding. This interpretation is supported by the fact that membrane association is restored at higher Ca^{2+} concentrations despite lysylated lipid content.

An additional important aspect of this study is the investigation of daptomycin binding under mildly acidic conditions at pH 5.3. Compared to the commonly used physiological pH of 7.4, pH 5.3 more closely reflects the proton gradient across the cytoplasmic membrane of *Staphylococcus aureus*.[15], [16] The adoption of mildly acidic conditions therefore provides a more biologically relevant model system for investigating the interactions of drugs which target components of the Gram positive plasma membrane.

In PG-containing membranes without PO3adLPG, a slight difference in IC_{50} values was observed between pH 5.3 and pH 7.4. This effect is likely related to pH-dependent changes in the protonation state of the phosphate groups of PG, which may influence the net membrane surface charge.[9] At lower pH, partial protonation of the phosphate groups reduces the overall negative surface charge of the membrane, thereby weakening electrostatic interactions with the daptomycin-calcium complex and slightly decreasing binding affinity. Interestingly, this pH-dependent effect was largely absent in membranes containing PO3adLPG. One possible explanation is that, due to ion pair formation between PG and PO3adLPG, the amount of freely accessible PG is already reduced, regardless of pH. As a result, additional changes in PG protonation have only a minor influence on the overall membrane charge available for daptomycin interaction.

The comparison between native LPG and the chemically stabilized analogue PO3adLPG also provided important insights into the interpretation of previous experimental studies. Daptomycin showed a higher apparent affinity towards membranes containing native LPG than towards membranes containing PO3adLPG. A likely explanation is the chemical instability of native LPG.[11], [12], [14] Due to its labile nature, native LPG may undergo degradation during sample preparation, potentially resulting in the formation of PG or PG-containing degradation products. This would increase the proportion of free PG available for daptomycin binding and thereby artificially enhance membrane association.[12] In contrast, experiments performed with the chemically stable analogue PO3adLPG led to consistent results, supporting its suitability as a reliable model system for investigating the role of lysylated phospholipids in daptomycin resistance.[12]

Taken together, the findings of this study support a model in which ion pair formation between PG and PO3adLPG modulates daptomycin-membrane interactions by reducing the availability of negatively charged PG. This dampening effect becomes more pronounced at higher PO3adLPG concentrations but can partially be overcome at elevated Ca^{2+} concentrations due to calcium-mediated displacement within the membrane. Rather than

completely preventing daptomycin binding, lysylated lipids therefore shift the equilibrium of membrane association and alter the calcium dependence of the interaction. These results contribute to a more detailed mechanistic understanding of how changes in membrane lipid composition influence daptomycin-membrane interactions, which may be one of the several factors affecting daptomycin susceptibility in *Staphylococcus aureus*.

5 Conclusion

This study demonstrates that membrane lipid composition strongly influences the calcium-dependent interaction of daptomycin with model *Staphylococcus aureus* membranes. In particular, lysylated phospholipids were shown to modulate membrane association by reducing the availability of negatively charged phosphatidylglycerol required for daptomycin binding. The results further indicate that calcium ions affect these interactions and partially compensate for the inhibitory effects of membrane lysylation.

In addition, the findings highlight the importance of physiologically relevant experimental conditions. The observed pH-dependent differences in membrane association emphasize that environmental factors can substantially influence antibiotic-membrane interactions and should therefore be carefully considered in mechanistic studies.

The use of the chemically stable analogue PO3adLPG enabled reproducible investigation of lysylated lipid effect and suggests that the instability of native LPG may influence the interpretation of previous experimental data. Overall, this work supports the concept that daptomycin resistance arises in part from dynamic alterations in membrane physicochemical properties rather than from complete inhibition of membrane binding.

While the liposome-based model system used in this study allowed controlled investigation of specific membrane parameters, it represents only a simplified approximation of the complex bacterial cell envelope and does not fully capture the dynamic physiological environment of living cells. By providing a more detailed understanding of how lipid composition, calcium ions, and environmental conditions collectively influence daptomycin-membrane interactions, this study contributes to a more nuanced understanding of antibiotic resistance in *Staphylococcus aureus*.

Declaration of AI-assisted technologies:

Throughout the writing process of this work, the author used OpenAI ChatGPT and DeepL Translate in order to improve readability, grammar, punctuation, and spelling. After using these tools/services, the author reviewed and edited the content as needed and takes full responsibility for the content of this thesis.

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