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Investigating of the influence of Cardiolipin on the behavior and Daptomycin interaction of a model Staphylococcus aureus membrane

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Abstract

Resistance to daptomycin, an important antibiotic for the treatment of Gram-positive *Staphylococcus aureus* infections, is a growing problem in clinical practice. Although DAP is considered an antibiotic of last resort, resistance has nevertheless emerged. The lipid composition of the cell membrane in staphylococci, is thought to play a role in their ability to resist daptomycin. This project therefore investigated the role of the lipids posphatidylglycerol, lysyl-phosphatidylglycerol and in particular cardiolipin, which are important components of the *Staphylococcus aureus* membrane, in daptomycin resistance. By using biophysical methods such as Langmuir-trough monolayer compression, Isothermal Titration Calorimetry and Zeta potential analyses, the interactions and structural properties of different mixtures of these lipids were investigated. Interestingly, the results showed that cardiolipin exhibits an important influence on monolayer flexibility and structural complexity despite being present in low quantities in the membrane. Nevertheless, cardiolipin was found to have no significant effect on the binding affinity of daptomycin. Monolayer pressure-area isotherm analysis suggested that cardiolipin facilitates negative curvature but tolerates a planar monolayer interface in the presence of LPG. These results provide insights into the roles of specific lipids on the molecular mechanisms of daptomycin resistance and emphasize the complex role of lipids in antibiotic action.

Zusammenfassung

Die Resistenz gegen Daptomycin, ein wichtiges Antibiotikum für die Behandlung grampositiver *Staphylococcus aureus* Infektionen, ist ein wachsendes Problem in der klinischen Praxis. Obwohl DAP als Antibiotikum der letzten Wahl gilt, ist dennoch eine Resistenz entstanden. Es wird angenommen, dass die Lipidzusammensetzung der Zellmembran von Staphylokokken eine Rolle bei ihrer Fähigkeit spielt, gegen Daptomycin resistent zu sein. In diesem Projekt wurde daher die Rolle der Lipide Phosphatidylglycerol, Lysyl-Phosphatidylglycerol und insbesondere Cardiolipin, die wichtigen Bestandteile der Membran von *Staphylococcus aureus* sind, bei der Daptomycin-Resistenz untersucht. Mit Hilfe biophysikalischer Methoden wie Langmuir-Trog-Monolayer-Kompression, Isothermal Titration Calorimetry und Zeta-Potential-Analysen wurden die Wechselwirkungen und strukturellen Eigenschaften verschiedener Mischungen dieser Lipide untersucht. Interessanterweise zeigten die Ergebnisse, dass Cardiolipin einen wichtigen Einfluss auf die Flexibilität und strukturelle Komplexität der Monoschicht hat, obwohl es nur in geringen Mengen in der Membran vorhanden ist. Dennoch wurde festgestellt, dass Cardiolipin keinen signifikanten Einfluss auf die Bindungsaffinität von Daptomycin hat. Die Analyse der Druck-Flächen-Isotherme der Monoschicht deutet darauf hin, dass Cardiolipin eine negative Krümmung begünstigt, aber eine ebene Monoschicht-Grenzfläche in Gegenwart von LPG toleriert. Diese Ergebnisse geben Einblicke in die Rolle spezifischer Lipide bei den molekularen Mechanismen der Daptomycin-Resistenz und unterstreichen die komplexe Rolle der Lipide bei der Antibiotikawirkung.

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Abbreviation

DAP	Daptomycin
ITC	Isothermal Titration Calorimetry
mprF	multiple peptide resistance factor
POCL	1',3'-bis[1-palmitoyl-2-oleoyl-sn-glycero-3-phospho]-glycerol (sodium salt)
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1´-rac-glycerol) (sodium salt)
S. aureus	*Staphylococcus aureus*

1. Introduction

Daptomycin, a cyclic lipopeptide antibiotic effective against Gram-positive bacteria such as *Staphylococcus aureus* for treating complicated skin and skin structure infections, has been a lifesaving antibiotic for almost two decades. However, its precise molecular mechanism of action remains unclear [1]. Two primary mechanisms have been proposed for Daptomycin's bactericidal action: one involves the disruption of cell wall synthesis, and the other suggests that Daptomycin forms pores in the bacterial membrane, leading to cell death [2].
Building on a proposed mechanism, a more recent study by Grein et al. (2020) found that Daptomycin, in the presence of Ca^{2+} and the anionic phospholipid phosphatidylglycerol, specifically interacts with undecaprenyl-linked cell envelope precursors, forming a tripartite complex and primarily binding at the *Staphylococcus* septum, thereby interrupting cell wall synthesis [3]. This leads to the delocalization of peptidoglycan biosynthesis components and massive membrane rearrangements, explaining the specificity of Daptomycin for bacterial cells and supporting a concise model for its mechanism of action [3]. Despite these findings, the role of cell membrane components in influencing these mechanisms, particularly in the context of antibiotic resistance, remains an area of active research.

Although Daptomycin is a lifesaving antibiotic, resistance to it is not impossible. In *Staphylococcus aureus*, resistance mechanisms primarily involve changes in the membrane composition. One hypothesis suggests that the increased lysyl-phosphatidylglycerol (LPG) content in the membrane, due to the upregulation of the multiple peptide resistance factor gene (*mprF*), reduces the availability of phosphatidylglycerol (PG) for Daptomycin binding [4],. Another hypothesis posits that the cationic nature of LPG creates an electrostatic barrier, hindering Daptomycin attachment. However, recent studies indicate that electrostatic repulsion alone may not fully explain the resistance mechanism. Additionally, an increase in cardiolipin (CL) concentration in the membrane has been observed, which might further interfere with Daptomycin's ability to disrupt the membrane [5].
These findings highlight the complexity of resistance mechanisms in S. aureus and underscore the need for further research to fully understand the role of cell membrane components in influencing these mechanisms and to combat this issue.
In this work, the influence of phospholipids with a main focus on cardiolipin (CL) was investigated. The aims of this master thesis were firstly to investigate the effect of different model staphylococcal membrane lipid mixtures on the mixing properties of the lipids, both with and without Ca^{2+}, and secondly to evaluate the effect of model membrane components on Daptomycin interactions.
Cardiolipin, a phospholipid commonly found in bacterial membranes, has been implicated in the development of resistance to Daptomycin. One study showed that CL inhibits the pore-forming activity of Daptomycin in a liposome model [6].
Increased cardiolipin (CL) content in bacterial membranes can enhance resistance to Daptomycin by effectively suppressing its membrane translocation and pore formation. The proposed model describes pore formation in four steps: binding of Daptomycin to PG in the outer leaflet, formation of a tetramer, translocation to the inner leaflet, and formation of a functional pore [7]. These results suggest that increased CL content may inhibit pore formation in vivo, contributing to bacterial resistance [7].

To study the binding affinity and the influence of membrane lipids on Daptomycin, ITC should be used [8]. Previous studies have utilized ITC to explore Daptomycin-membrane interactions, revealing crucial insights into the mechanism of action. For example, the study by Kotsogianni et al. (2021) demonstrated the phospholipid specificity and calcium-dependent binding mechanism of daptomycin using ITC [9].

Their findings highlighted that Daptomycin has a clear preference for binding to target bacterial phospholipids, primarily phosphatidylglycerol (PG), and that calcium ions play a critical role in this binding process [9].

1.1 Characteristics of the Daptomycin activity

Daptomycin (Cubicin, Cubist Pharmaceuticals) *(Figure 1)* is a calcium-dependent cyclic lipopeptide antibiotic [10]. It was discovered in 1987 but was only approved for therapeutic use in 2003 by the Food and Drug Administration (FDA) due to serious side effects such as myopathy [10]. However, because of the drastic increase of antibiotic resistance, Daptomycin was finally approved 16 years after its discovery [11].

Daptomycin is used for the treatment of complicated skin and soft-tissue infections caused by Gram positive bacteria such as methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*, *Streptococcus* and *Enterococcus* species [12].

Daptomycin *(Figure 1)* was isolated from the Gram-positive soil organism *Streptomyces roseosporus* and is a depsipeptide with 13 amino acids included in a ten-membered cyclic structure [10], [12]. The cyclic lactone core contains kynurenine and 3-methylglutamic acid, which are crucial for the effectiveness of Daptomycin [12]. Another structure, which increases the activity of Daptomycin is the ester bond between kynurenine and threonine [13]. The amino acids tryptophan, asparagine and aspartic acid link the cyclic lactone ring to the decanoyl fatty acid side chain [12], [13].

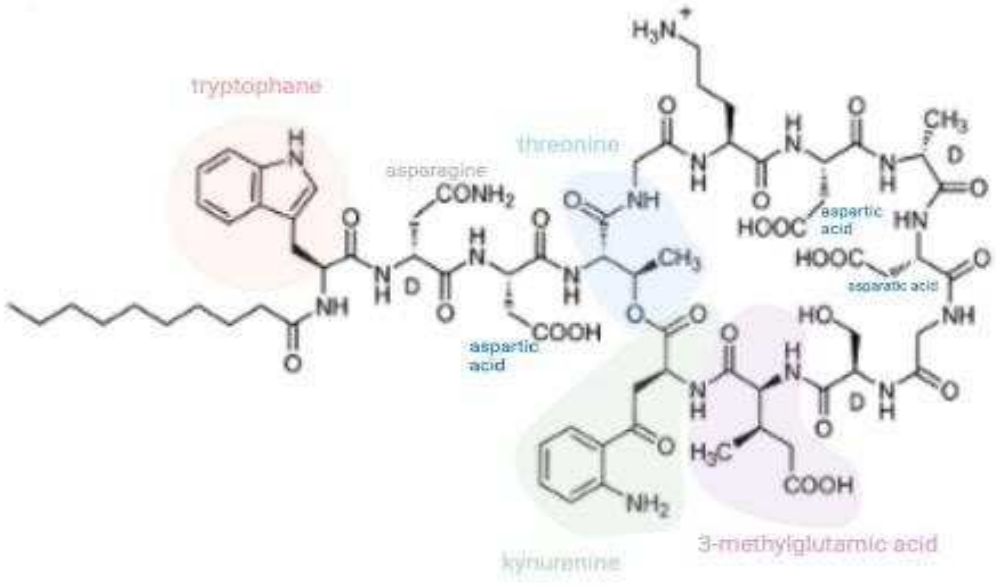


Figure 1 *chemical structure of Daptomycin adapted from [1]*

Aside from the structural properties, Daptomycin has a net charge of -3 at neutral pH, and its physiochemical properties are dependent on the presence of the doubly positively charged calcium ions [13].

1.1.1 Mechanism of action

Although the mechanism of action of Daptomycin is still unclear [6], there are several hypotheses. One of these hypotheses is from an older study by S. K. Straus and R. E. W. Hancock (2006) which claims that Daptomycin binds to the cytoplasmic membrane in a calcium-dependent manner, oligomerizes there, and causes potassium efflux, disrupting macromolecular synthesis and leading to cell death [14].
The study by Scott D. Taylor et al. suggests that two tetramers, one in the inner and one in the outer leaflet of the membrane, form oligomeric pores in the membrane, which also leads to membrane depolarization [7]. Another study by J. Pogliano (2012) explains that Daptomycin binds to PG-rich areas of the cell membrane, altering its properties and impairing crucial processes like cell wall production and cell division. [15].
A recent study found that Daptomycin targets fluid microdomains in the cell membrane and disrupts cell wall biosynthesis by binding to Lipid II, a bactoprenyl-bound cell wall precursor, in PG-rich membranes [3].This binding occurs only in the presence of calcium ions, which are essential for its antimicrobial activity [16]. As a result, there is a restructuring of the membrane and a redistribution of the biosynthetic enzymes crucial for cell wall production [3].

1.1.3 Daptomycin resistance

Although bacterial resistance to Daptomycin is rare, some clinical cases have been reported, with hypotheses suggesting that bacteria may alter the composition of their cytoplasmatic membrane to develop resistance [17].
A study comparing DAP-sensitive and DAP-resistant bacterial strains found that resistant *Staphylococcus aureus* strains have increased lysyl-phosphatidylglycerol (LPG) content in their cytoplasmic membranes due to the upregulation of the multiple peptide resistance factor (*mprF*), a gene encoding a membrane-integral lysyl-transferase [4]. The exact mechanism by which the increased lysyl-PG content facilitates resistance is still unclear, but two main hypotheses exist: Firstly, the overproduction of lysyl-PG could reduce the amount of PG available to bind Daptomycin [4], [18]. Secondly, the cationic nature of lysyl-PG could create an electrostatic barrier around the membrane, making it more difficult for Daptomycin to bind [18]. However, recent research by Khatib et al. (2016) suggests that electrostatic repulsion by lysyl-PG alone is not sufficient to fully explain the inhibitory effects [19].
Another resistance mechanism involves increased concentrations of cardiolipin (CL) in the cytoplasmic membranes of the bacteria. Here, too, there are two main theories: The first states that CL may confine Daptomycin to the outer membrane layer, which interferes with the formation of cation-selective pores [20]. The second, less explored hypothesis, proposes that the production of CL formed from two PG molecules reduces the amount of PG available for binding [7].

1.2 Major phospholipids of the *Staphylococcus aureus* membrane

The phospholipid composition of the plasma membrane is important for bacteria to cope with environmental hazards and plays a role in bacterial infections by acting as a target and barrier for antibiotics and host defense mechanisms [21]. However, changes in membrane phospholipid composition influence important cellular processes such as metabolism, stress response, antimicrobial resistance and virulence [21].

The three main phospholipids *(Figure 2)* in *Staphylococcus aureus* membranes are phosphatidylglycerol (PG), lysyl-phosphatidylglycerol (LPG) and cardiolipin (CL), with PG dominating at 40 to 70 % and LPG at 30 to 55 %. CL is only present in the membrane at 4 to a maximum of 9 % [22], [23].

LPG is a positively-charged *(Figure 2a)* lipid in which a lysyl group is transferred from a specific lysyl-tRNA to PG [24]. Mutations in the *mprF* gene alter the charge on *Staphylococcus aureu*s cell surfaces by affecting LPG synthesis, thereby increasing sensitivity to cationic antimicrobial peptides (AMP) [25].

In this study, instead of LPG with its highly labile ester group in the headgroup, which easily hydrolyzes at neutral pH, the more stable analog PO3adLPG, was used, containing 3-aza-dehydroxy group (3adLPG) in the headgroup [22].

Phosphatidylglycerol (PG) *(Figure 2b)* differs from lysyl-phosphatidylglycerol (LPG) in the absence of the positively-charged lysyl group and is therefore a negatively charged lipid *(Figure 2a)* [26].
PG plays a crucial role in the oligomerization of Daptomycin in the membrane, forming functional lesions essential for bacterial susceptibility and the antibiotics efficacy [27]. In the case of resistance, the increased production of LPG reduces the proportion of PG in the cytoplasmic membrane. The modification of PG with positively-charged LPG reduces the net negative charge of the membrane, which leads to a weakened binding affinity of cationic compounds such as Daptomycin and thus impairs the binding and efficacy of the antibiotic [26].

Cardiolipin (CL) *(Figure 2c)*, a unique dimeric, is a negatively-charged phospholipid and is activated by cardiolipin synthase, which catalyzes the condensation of two PG molecules [28]. Although cardiolipin has a single negative charge, both phosphate groups in the head group share the negative charge [29]. Cardiolipin, with its small head group and large fatty acid tails, has a conical shape, which induces a negative curvature in the membrane [30].
In addition to the protective effect of LPG against Daptomycin, a study has shown that incorporating 10 % CL into artificial PG membranes reduces the oligomerization of the antibiotic, inhibits the translocation of the oligomers across the membrane, and consequently inhibits the activity of Daptomycin [7].

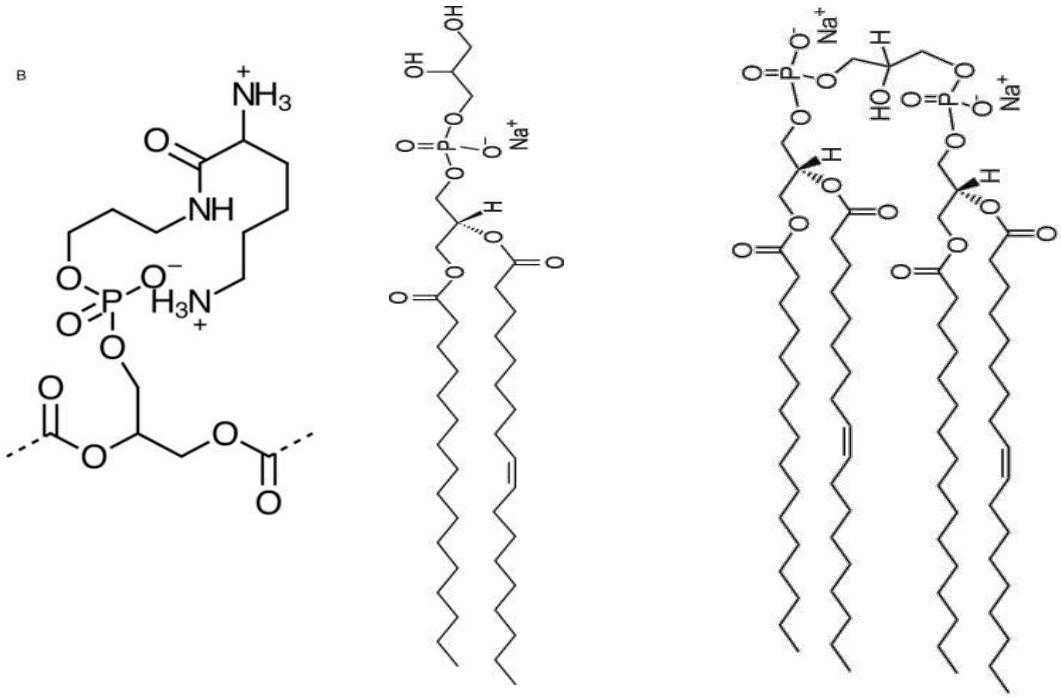


Figure 2 *a head group of PO3adLPG with the three ionizable units phosphate, epsilon amine and alpha amin; reproduced from [22] ,b chemical structure of POPG, reproduced from [56] and c chemical structure of POCL, reproduced from [57]*

All three staphylococcal phospholipids are not evenly distributed in the membrane, but asymmetrically between the inner and outer membrane sheets [26]. 70-80% of LPG is localized in the inner leaflet of the cell membrane [31]. However, a mutation in mprF was observed in Daptomycin-resistant *Staphylococcus aureus* strains, which increases the enzyme activity. Thus, more LPGs are synthesized and flipping causes the LPGs to accumulate in the outer leaflet of the membrane and thus impair the interaction of the Daptomycin [32]. PG and CL are mostly localized more than 75 % in the outer leaflet of the bacterial cell membrane [31].

1.3 Model membrane for studying lipid interactions

1.3.1 Lipid monolayer

Lipid monolayers are formed when phospholipids are introduced into an aqueous subphase, with the hydrophilic head groups oriented towards the aqueous side and the hydrophobic tails positioned at the boundary between water and air [33]. These monolayers provide a simplified platform for analyzing the asymmetric distribution of lipids, which is central to understanding the properties of bilayers in biological membranes. Surface pressure is an important parameter in the study of lipid monolayers as it influences the interactions and packing density of lipids at the water/air interface [34]. This asymmetry plays a decisive role in the function and stability of membranes, as it can influence various physical and chemical properties [33]. Although monolayers are practically only half a bilayer with a flat and non-curved structure, the knowledge gained from studying them, can be effectively applied to liposomal systems due to structural and thermodynamic similarities to the bilayer [34]. The investigation of pure and mixed lipid monolayers, as well as their internal interactions and interfacial interactions with drugs in the aqueous subphase is made possible by the use of a Langmuir trough [34].

1.3.1.1 Langmuir trough

The Langmuir trough *(Figure 3)* is a device which allows us to understand and characterize lipid/lipid and lipid/drug interactions at the air/liquid interface [35]. The trough is equipped with two movables barriers made from polytetrafluoroethylene (PTFE), which compress deposited lipid monolayers [35]. The dyne probe is attached to the microforce balance and measures the surface pressure throughout compression of the lipids, at a constant temperature, to produce a pressure/area isotherm [35].

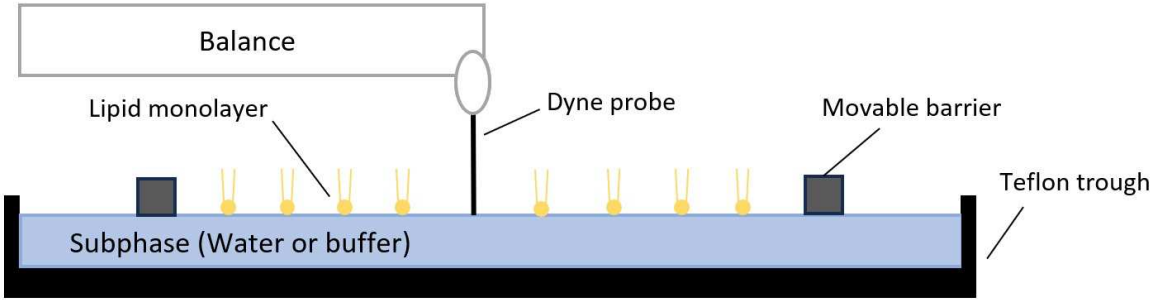


Figure 3 *An illustration of the Langmuir trough*

The trough is filled with an aqueous subphase, and a lipid solution is applied dropwise in an organic volatile solvent such as chloroform [35]. Upon solvent evaporation, the lipids form an insoluble monolayer at the air/liquid interface [35]. The barriers then compress the monolayer, reducing its surface area per molecule and altering the surface pressure [35]. When the concentration of the lipid solution and the number of molecules are known, surface pressure can be plotted against the average area per molecule, resulting in compressional isotherms [35].

Compression isotherms offer insight into the arrangement, dimensional properties, and phase transitions of molecules within the monolayer [35]. As the monolayers are compressed, the distance between molecules decreases, leading to different phases: gaseous phase, liquid expanded, liquid condensed and solid phase *(Figure 4)* [35]. In the gaseous phase, lipids exhibit the largest molecular area. With further compression, the liquid expanded phase forms, followed by the liquid condensed phase, where both phases coexist temporarily, visible as a plateau in the isotherm [36]. In the solid phase, molecules are tightly packed with parallel-aligned hydrophobic tails. Additional compression causes the monolayer to collapse, forming multilayers or micelles [36].

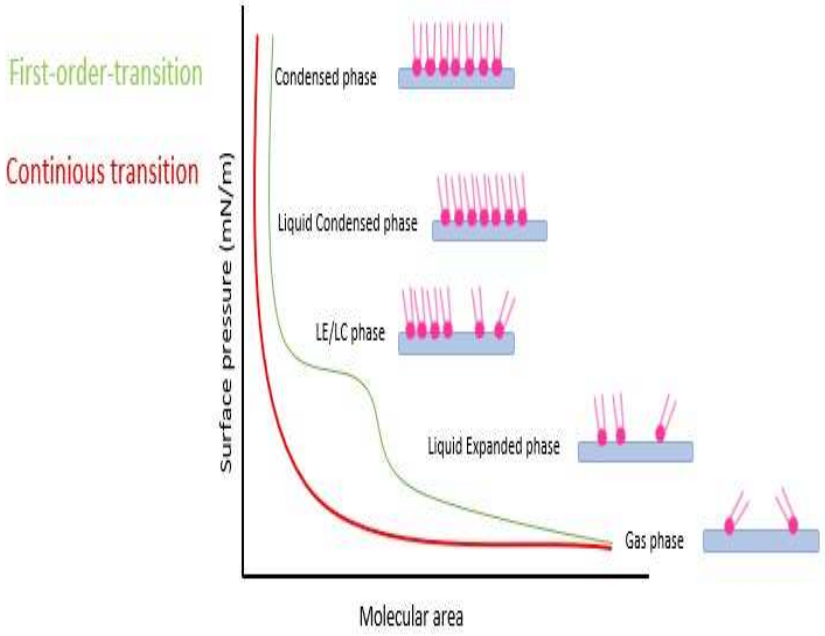


Figure 4 *Langmuir trough isotherms show the transition of each lipid mixtures*

The isotherms in the monolayer at the air/liquid interface can be either expanded or more condensed *(Figure 5)*. An expanded isotherm indicates repulsive forces caused by properties such as charges on the headgroups, the length and structure of the molecule, or steric hindrance to crystal packaging [37]. These forces prevent the formation of two-dimensional crystal structures [38]. In contrast, condensed isotherms indicate lower repulsive forces [37]. In condensed isotherms, the surface pressure remains minimal over a long period of time and then increases within a small molecular area, which is typically observed in continuous transitions [37].

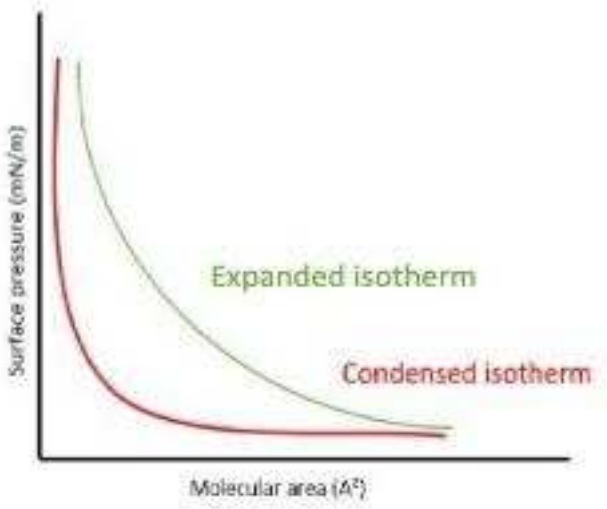


***Figure* 5** *An example of a condensed and an expanded isotherm*

Langmuir monolayers best mimic bilayer behavior at a surface pressure of 30-35 mN/m, aligning with the lateral pressure of lipid bilayers [38]. This pressure range is crucial for predicting the effects on the cell membranes and understanding the role of specific lipids [39].

1.3.1.2 Compressibility

Mathematical analyses provide quantitative description of the isotherms and help to better understand the behavior of the monolayer [40]. The surface compressional modulus with units mN/m is derived from the compression isotherms and is a valuable tool for characterizing monolayers [41]. The mathematical formula for compressibility modulus is [41]:

$$Cs^{-1} = -A\left(\frac{d\pi}{dA}\right)$$

Where A is the area per molecule at a certain pressure and $\left(\frac{d\pi}{dA}\right)$ is the increase in the surface pressure isotherm [39].

The value of the compressional modulus is indicative of the phase of the monolayer, which means that higher values indicate a denser monolayer [41]. For example, the gaseous phase is characterized by compressibility values of 0 to 12.5 mN/m, the liquid expanded phase (LE) by 12.5 to 50 mN/m, the coexisting LE/LC phase by 50 to 100 mN/m, the liquid condensed phase (LC) by 100 to 250 mN/m and the solid phase by values above 250 mN/m [40], [42].

1.3.1.3 Compressional Work

To further understand monolayer characteristics, compressional work is analysed as it calculates the energy required to compress lipid monolayers [43]. This analysis provides insights into the stability and interaction dynamics of lipid films by evaluating the area under the isotherm between specific surface pressures. Compressional work $W(kJ)$ is determined by the following formula [43]:

$$W(kJ) = \sum_{A(\pi_0)}^{A(\pi_x)} \pi \cdot \delta A$$

Where δA defines the change in molecular area and π is the surface pressure [43].

1.4 Liposoms

1.4.1 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) *(Figure 6)* is a powerful technique used to evaluate the thermodynamic interactions between molecules, providing insights into binding affinity, enthalpy, and entropy changes [9]. In our study, ITC is employed to investigate the interactions between Daptomycin and various lipid membranes, particularly focusing on the role of different lipid components such as POCL, POPG, and PO3adLPG. Ligands are injected drop by drop into the sample cell, ensuring that all solutions used have the same buffer and pH value [9]. The heat generated or absorbed during the interaction between Daptomycin, and the lipid components is measured, providing detailed information

on the binding affinity (Kd), enthalpy change (ΔH), and entropy change (ΔS) of the interaction *(Figure 8)* [9], [44].
In our research, we aim to build on these findings by examining how different lipid compositions, influence the binding of Daptomycin.

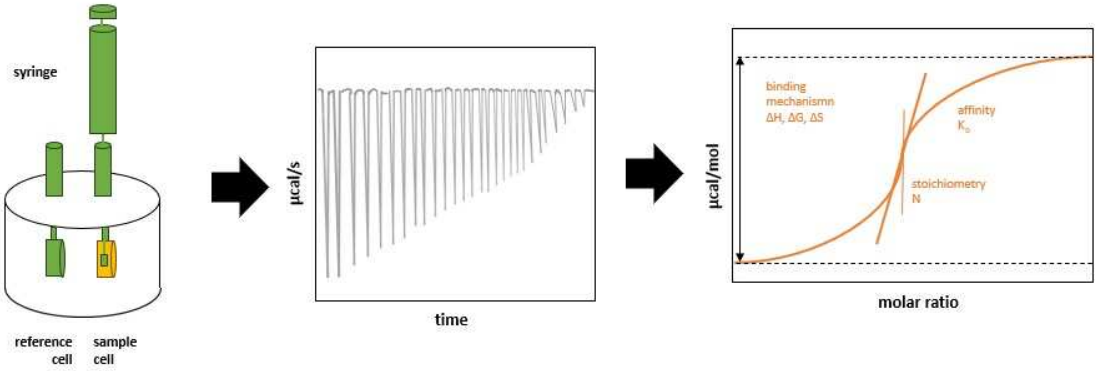


Figure 6 *ITC: Schematic representation and results of an Isothermal Titration Calorimetry (ITC) experiment. Left: The experimental setup with a syringe, reference cell, and sample cell. Middle: Heat signals (µcal/s) over time. Right: Binding curve plotting the enthalpy change (ΔH) against the molar ratio, showing the parameters of binding affinity (K_D), stoichiometry (N), and enthalpy (ΔH).*

1.4.2 Zeta sizer

A Zeta Sizer *(Figure 7)* is an analytical instrument used to determine the zeta potential and particle size in dispersions, which is crucial for the characterization of liposomes in biomedical research [45]. When particles are dispersed in bulk solutions, they move by Brownian motion, creating a layer of opposite charges around each particle called the Stern layer [46]. The zeta potential is the charge that arises on the slip plane of the Stern layer, the transition region to the aqueous environment and the diffuse charge layer of the particle [46], [47].

The zeta potential provides information about the electrical charge on the surface of particles and is an important indicator of the stability of colloidal dispersions [48]. The particles move through an electric field while light rays are incident on them, and they move in the direction of the electrode with the opposite charge to their net charge [45]. The acceleration is proportional to the force of the applied electric field on the charges around the particle, and this information is used to measure the zeta potential [47], [45]. A high zeta potential, above ±30 mV, indicates high stability due to strong electrostatic repulsive forces preventing particle aggregation [45].

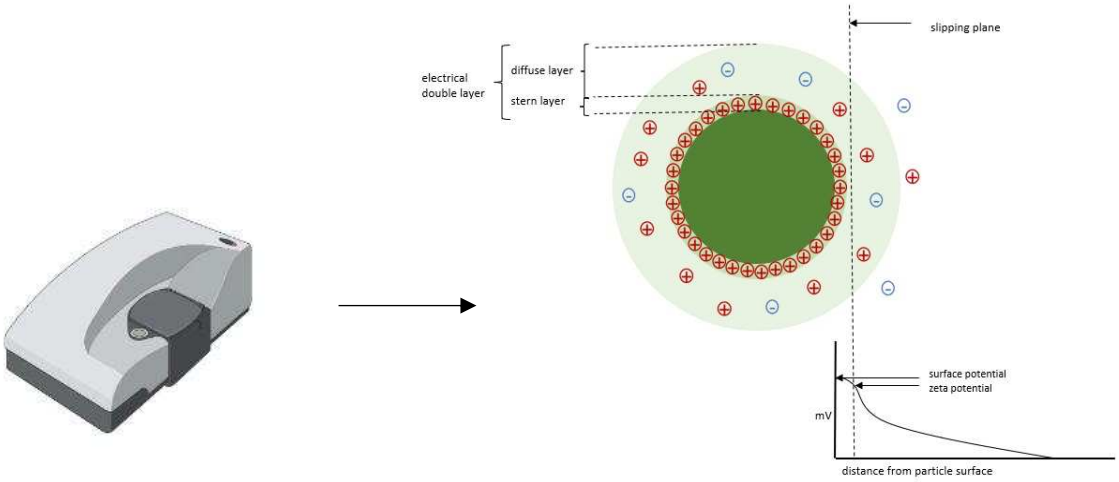


Figure 7 *An illustration of the analytic instrument of the Zeta sizer and a schematic illustration of the zeta potential. Created in BioRender.com.*

The measurement of liposome size provides information about the average diameter and size distribution of liposomes in a sample, which influences their biological distribution, drug release rate, and cell uptake [49]. Dynamic light scattering (DLS) is a commonly used method for determining particle size, measuring the frequency shift of a laser beam scattered by the moving particles in the dispersion [50]. DLS provides two main values: the Z-average, which is the intensity-weighted mean of the particle distribution, and the PDI (polydispersity index), which indicates the variance in size. In this work, only the obtained Z-average in nm is reported [49], [50].

These measurements are valuable for research on Daptomycin resistance and the influence of phospholipids on the antibiotic. The zeta potential helps to understand how changes in lipid composition affect the interaction and efficacy of Daptomycin.

2. Material and Methods

The lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1´-rac-glycerol) (sodium salt) (16:0-18:1 PG), and 1',3'-bis[1-palmitoyl-2-oleoyl-sn-glycero-3-phospho]-glycerol (sodium salt) (16:0-18:1 Cardiolipin) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The lysylphosphatidylglycerol analogue 1-palmitoyl-2-oleyl-3-*aza*-dehydroxylysyl-phosphatidylglycerol (PO3adLPG) was custom synthesized at King's College London, as described by Rehal *et al*. [22]. The lipopeptide antibiotic Daptomycin was purchased from Combi-Blocks (San Diego, CA, USA). Chloroform (analytical grade) and methanol Optigrade® were purchased from Promochem (Wesel, Germany). Sodium chloride was supplies by Carl Roth (Karlsruhe, Germany), calcium chloride by Sigma-Aldrich (Darmstadt, Germany) and HEPES solution by Merck (Darmstadt, Germany). For all experiments ultrapure water (MiliQ) with a specific resistivity of 18.2

2.1 Lipid mixtures for the Langmuir trough

Before deposition at the air/liquid interface, all lipid solutions were prepared in chloroform/methanol 9:1, at a concentration of 1 mM. Lipid mixtures were prepared according to Tables 1 and 2.

The various lipid mixtures were deposited as monolayers on subphases consisting of 20 mM Hepes with 150 mM NaCl at a pH of 7.4. Since daptomycin is a calcium-dependent antibiotic, the measurement of each lipid mixture was repeated with the same buffer but with adding 0.3 mM $CaCl_2$, in order to determine its effect on lipid/lipid interactions
The Dyne probe was calibrated using the surface tension of water at the operating temperature (22 ± 0.5°C).
After spreading the lipid mixtures *(Tables 1 and Table 2)* dropwise with a micro syringe (Hamilton, Switzerland) onto the subphase surface, the monolayer was allowed to stand for 20 minutes to ensure solvent evaporation before compression, with a barrier speed of 10 mm/minute.
All isotherms were measured at least three times to confirm their reproducibility.

Table 1 *The percentage ratios of binary lipid mixtures used in monolayer experiments.*

PO3adLPG/POPG 67:33	PO3adLPG/POCL 67:33	POCL/POPG 75:25
PO3adLPG/POPG 50:50	PO3adLPG/POCL 50:50	POCL/POPG 67:33
PO3adLPG/POPG 33:67	PO3adLPG/POCL 33:67	POCL/POPG 50:50
PO3adLPG/POPG 25:75	PO3adLPG/POCL 25:75	POCL/POPG 33:67
PO3adLPG/POPG 20:80	PO3adLPG/POCL. 20:80	POCL/POPG 25:75

Table 2 *The percentage ratios of the ternary lipid mixtures used in monolayer experiments*

POCL/PO3adLPG/POPG 6:47:47	POCL/PO3adLPG/POPG 16:42:42
POCL/PO3adLPG/POPG 6:63:31	POCL/PO3adLPG/POPG 16:56:28
POCL/PO3adLPG/POPG 6:31:62	POCL/PO3adLPG/POPG 16:28:56
POCL/PO3adLPG/POPG 10:60:30	POCL/PO3adLPG/POPG 20:40:40
POCL/PO3adLPG/POPG 10:60:30	POCL/PO3adLPG/POPG 20:30:50
POCL/PO3adLPG/POPG 10:30:60	POCL/PO3adLPG/POPG 20:50:30

2.3 Preparation of liposomes

For liposome preparation, the lipids were dissolved in chloroform/methanol 9:1 to create a 10 mM solution. The liposomes consisted of the lipids POPG, PO3adLPG and POCL, whereby the proportion of POCL varies. The ratios of PG to LPG are either 7:3 or vice versa in order to investigate the influence of cardiolipin. The total volume of each liposome preparation was 1 mL. Then each solvent was evaporated at 40°C, 250 rpm and 150 mbar using the Hei-Vap Rotavapor, (Heidolphs Instruments; Germany) for 50 minutes. As soon as a thin lipid film was formed, 100 mg of yttrium -stabilized zirconium oxide beads (0.1-0.2 mm) were added to the vial. Then the vial is filled with 50 µl of buffer (20 mM Hepes, 150 mM NaCl and 0.3 mM $CaCl_2$). The vial is placed in the Dual Centrifuge (ZentriMIX 380 R, Hettich GmbH, Germany) at 30°C, 2500 rpm for 20 minutes. To achieve an equilibration during this process a counter-balancing vial was added to the centrifuge. It is important that the Zentrimix reached 30°C before starting to centrifuge the vial. If this is not the case, the Dual Centrifuge can should be heated up to the desired temperature for 40 minutes. After 20 minutes the lipid films get destroyed by the beads and results in the formation of vesicular phospholipid gels to which 1000 µl of the same buffer was added. Then the vial was dual-centrifuged a second time at 30°C, 1500 rpm for 2 minutes. After this time the

beads sedimented on the bottom of the vial. Before storing the liposomes in the fridge, they were first allowed to equilibrate at room temperature.

2.4 ITC: Sample preparation and experimental set up

Isothermal titration calorimetry measurements were performed using a TA Instruments Nano ITC (Waters LLC, Germany). The POPG/PO3adLPG 7:3 (additionally prepared with the addition of 0.5 and 1 mol% POCL) liposomes used in the ITC samples, were centrifuged for 2 minutes using a benchtop centrifuge in order to separate the liposome dispersions from any residual zirconium beads. Prior to loading into the ITC, the liposomes, Milli-Q water for the reference cell and 50 µM Daptomycin-solution were all degassed under vacuum for 20 minutes, at 30°C and 450 mmHg. Both the liposomes and the daptomycin solution were prepared in 20 mM Hepes buffer, with 150 mM NaCl and 0.3 mM $CaCl_2$. Then ITC sample cell was loaded with 300 µl of a 0.05 mM Daptomycin solution and the burette syringe was loaded with 50 µl of a specific 10 mM liposome dispersion. For each titration run, 40 injections were carried out, with 0.52 µl liposomes being injected into the antibiotic every 300 seconds, with a stirring rate of 350 rpm. Each liposome type was titrated at least twice to assess reproducibility.

3. Results

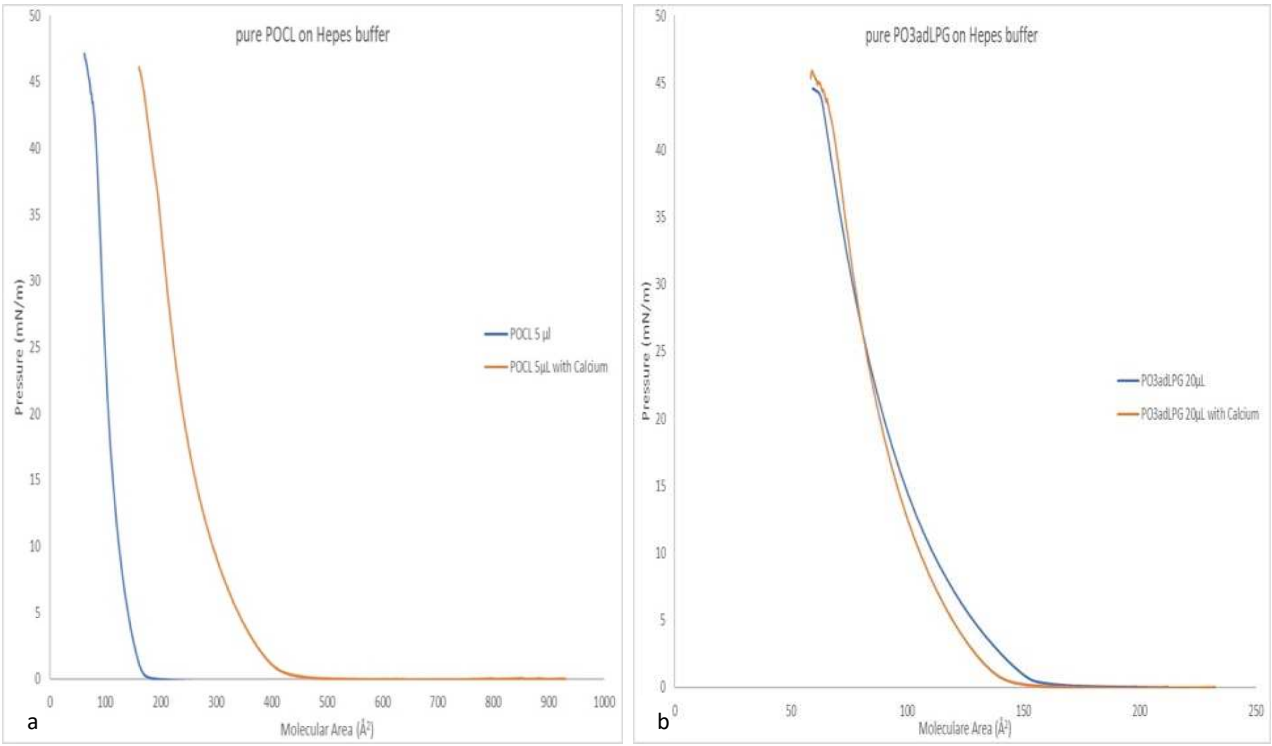
3.1 Langmuir trough

3.1.1 Compressibility of pure lipids

Starting with the pure lipid POCL, the isotherm initially shows a long gas phase range *(Figure 8 a)*. The lift-off area, i.e., the point at which the isotherm begins to rise from the x-axis, is approximately 180 Å². The surface pressure then increases and reaches a maximum of about 47 mN/m at a molecular area of about 100 Å². The difference between the lift-off area and the collapse point is therefore 80 Å². The POCL molecules are in the liquid-expanded (LE) phase, as the surface compressional modulus does not exceed 100 mN/m *(Figure 9 a)*. In the presence of Ca^{2+}, POCL shows similar behavior, but the molecular area is larger. The lift-off area here is around 420 Å², which is due to the interaction between the oppositely charged POCL molecules and calcium ions. The maximum pressure is reached at about 46 mN/m, but with a molecular area of about 200 Å². The difference between the lift-off area and the monolayer collapse area, is about 220 Å². The larger difference in molecular area when Ca^{2+} is added indicates that the ions influence the packing of the POCL molecules and promote a more expanded monolayer structure. In the presence of calcium ions, the POCL molecules remain in the liquid-expanded phase *(Figure 9 b).*

With PO3adLPG *(Figure 8 b)*, there is no difference between the isotherms with and without calcium ions, as repulsive forces are dominant in both cases. This is because PO3adLPG carries a net positive charge, so there is no electrostatic attraction between the lipid and the calcium ions. Both isotherms start with a short gas phase region, and the lift-off area is at 150 Å². The surface pressure reaches a maximum value of about 45 mN/m at a molecular area of 60 Å². Because of the large difference of 90 Å² between the lift-off area and the point of maximum pressure, the isotherms are more expanded, so a denser packing of the lipids in the monolayer is not possible. Like POCL, PO3adLPG is in the liquid-expanded (LE) phase, as the surface compressional modulus does not exceed 100 mN/m *(Figure 9 a-b).*

The differences in the isotherms of POPG *(Figure 8 c)* in the presence and absence of calcium ions are significant and provide important insights into the intermolecular interactions and the resulting structure of the lipid monolayer. Without Ca^{2+}, the lift-off area is around 200 Å². The repulsive forces between the like-charged head groups leads to a larger molecular area and a predominantly liquid-expanded phase up to the collapse pressure of about 47 mN/m at a molecular area of 90 Å². In the presence of calcium ions, the lift-off area is around 140 Å², and the maximum pressure of 46 mN/m is reached at a molecular area of 60 Å². The attractive electrostatic interactions between the head groups and Ca^{2+} ions enable a denser packing of the molecules, which is why the difference between the lift-off area and the point of maximum pressure of 80 Å² is smaller than in the isotherm without the calcium ions, which is 110 Å². Despite the possibility of cross-linking of the head groups by Ca^{2+}, the lipid layer does not form a condensed phase due to the steric hinderance of the oleoyl chains and remains flexible under compression.



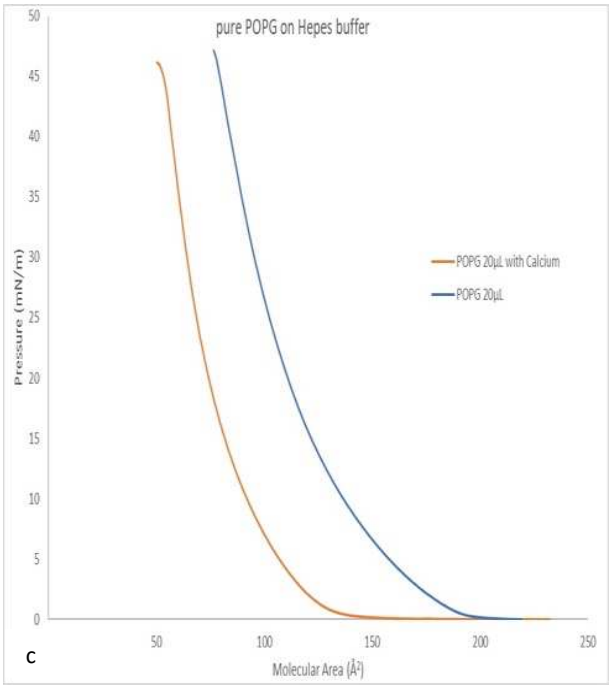
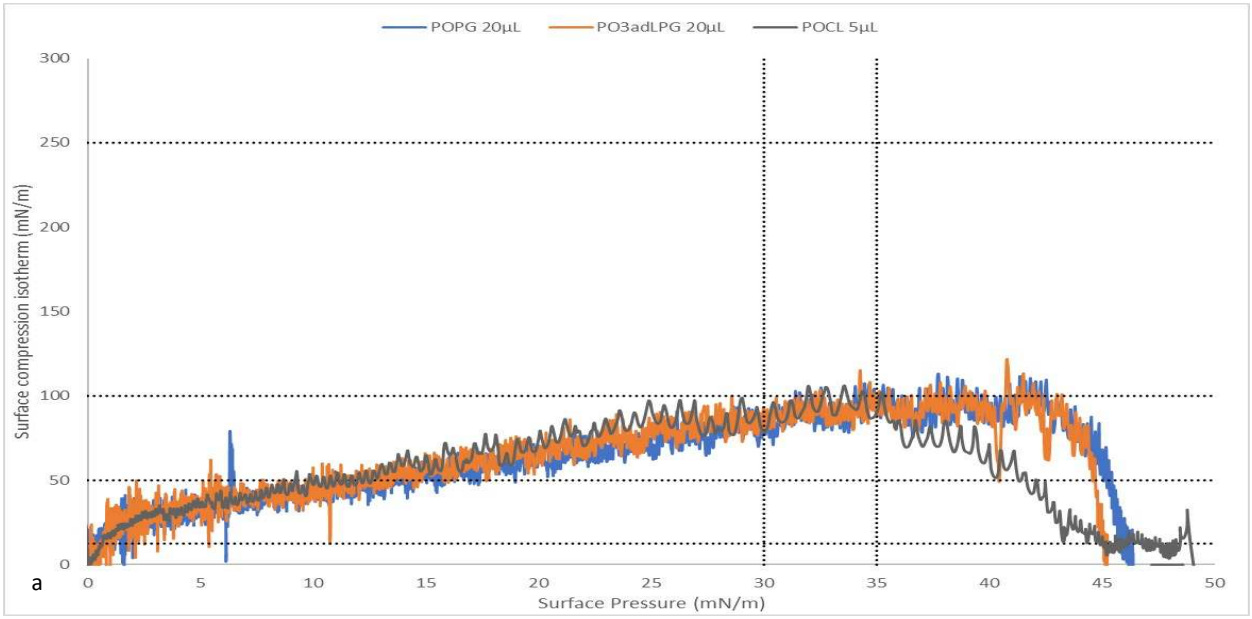


Figure 8 *Surface pressure/area isotherms of the pure lipids a PO3adLPG b POPG and c POCL with and without Ca^{2+} each*



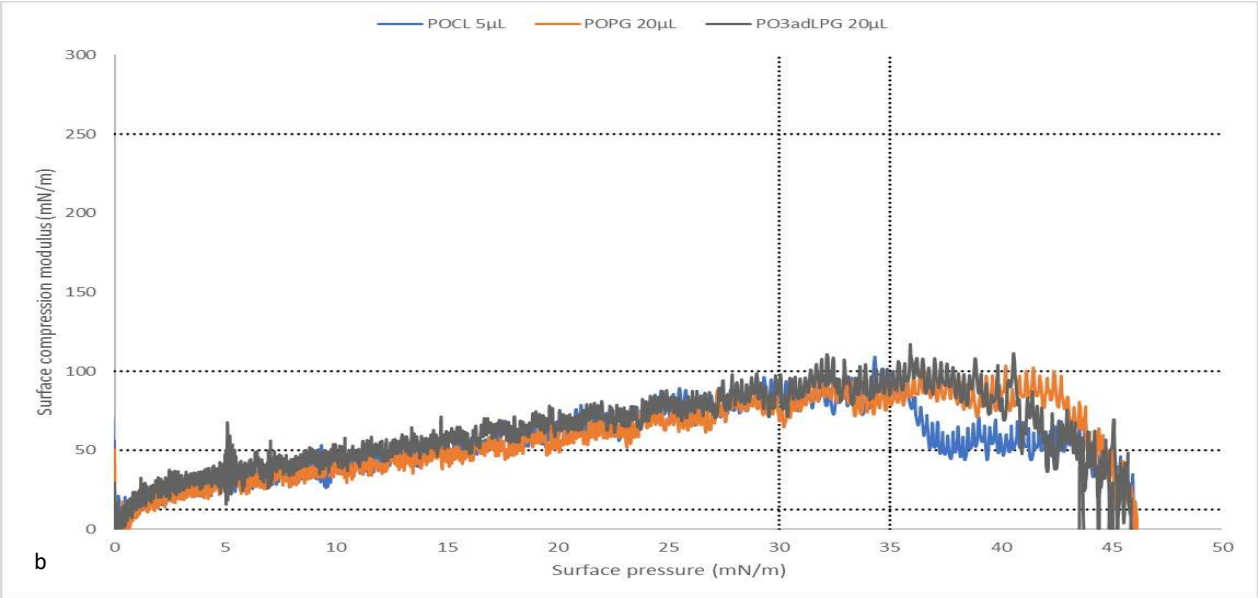


Figure 9 *Compressional modulus of the pure lipids of POPG, PO3adLPG and POCL on Hepes buffer a without Ca^{2+} and b without Ca^{2+} as well as the pure lipids compared*

3.1.2 Compressibility of binary lipid mixtures

The isotherms of the binary lipid mixtures of POPG and PO3adLPG without Ca^{2+} ions are less expanded than the pure lipids *(Figure 10)*. The lift-off area range is between 150 Å² and 170 Å² and the maximum pressure of approximately 40 mN/m is reached at an average molecular area of 100 Å². Due to the smaller difference between the lift-off range and the point of maximum surface pressure, the isotherms are less expanded, which means that they are less extended than the isotherms of pure lipids. In the presence of calcium ions, the isotherms are less expanded, which means that the molecular area is smaller than without calcium ions. In particular, the lipid mixtures POPG/PO3adLPG in the ratios 1:1 and 4:1 show the highest expansion, as the lift-off range is approximately 230 Å², while the collapse pressure of approximately 45 mN/m is reached at a molecular surface area of 130 Å². The lipid mixture PO3adLPG:POPG ratio 1:4 is less condensed as the lift off area is bigger at 220 Å² and the surface pressure is reached at 140 Å.
For the surface compressional modulus without Ca^{2+} *(Figure 11 a)*, all mixing ratios start out the same, so that they remain in the liquid expanded phase up to a surface pressure of 10 mN/m. From a range of 30-35 mN/m, differences in the mixing ratios can be recognized. The lowest value of the surface compressional modulus is seen with POPG/PO3adLPG 1:2, whereby all mixing ratios remain in the LE phase. With increasing POPG content, the surface compressional modulus slowly increases and reaches its highest value at POPG/PO3adLPG 4:1 and is correspondingly more stable. It is noticeable that the monolayer with a higher POPG content has a higher collapse pressure at approx. 43 mN/m.
It becomes interesting when Ca^{2+} is added *(Figure 11 b),* as POPG/PO3adLPG 4:1 has the lowest value of the surface compressional modulus. Here too, all mixing ratios remain in the LE phase at a surface pressure of 30-35 mN/m. The highest surface compressional modulus and thus the highest stability is achieved with POPG/PO3adLPG 1:2, which means that the lipids in the monolayer could be more densely packed. With Ca^{2+} ions present, all curves are slightly dispersed in the LE phase compared to without the divalent ions, but all lipid mixtures collapse at 40 mN/m.

The work of compression without Ca^{2+} *(Figure 12 a)* tends to decrease with increased POPG content. The addition of calcium ions *(Figure 12 b)* shows a stabilizing effect by reducing the change in the work of compression as a function of the molar proportion of POPG.

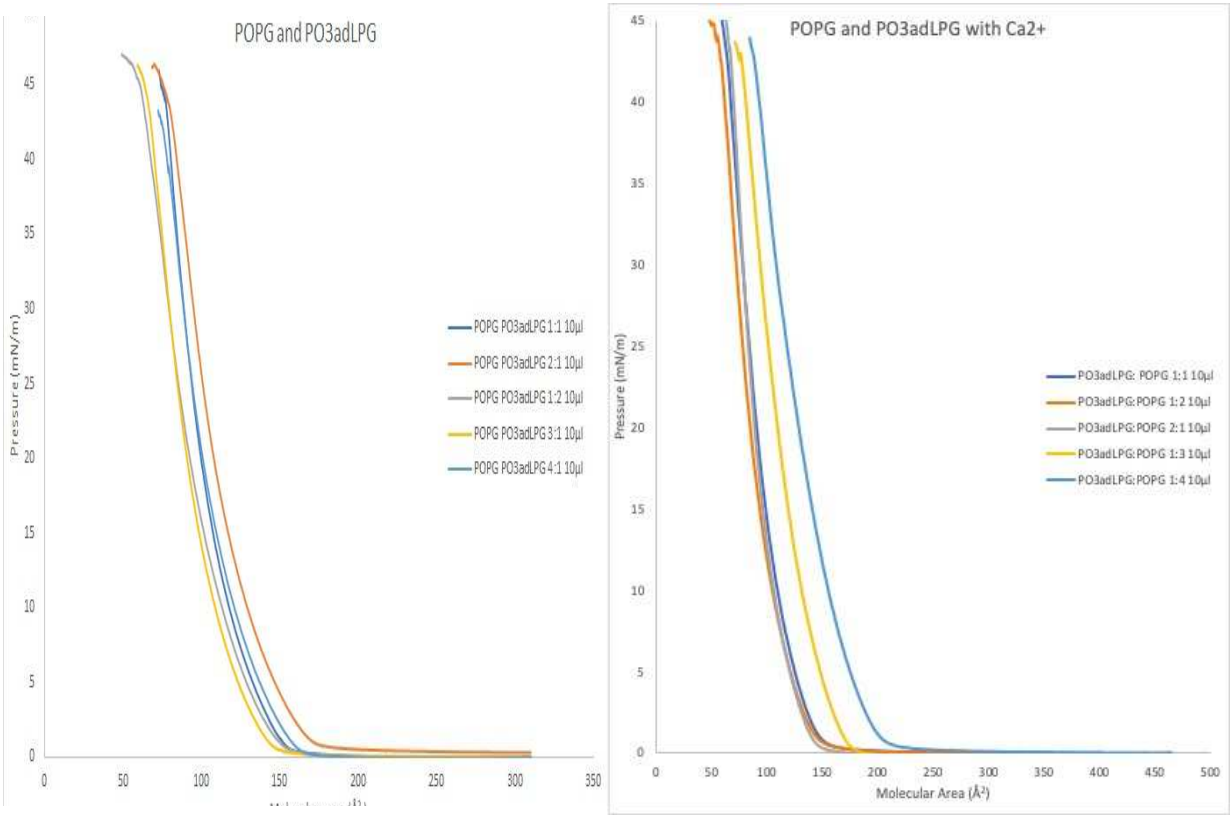
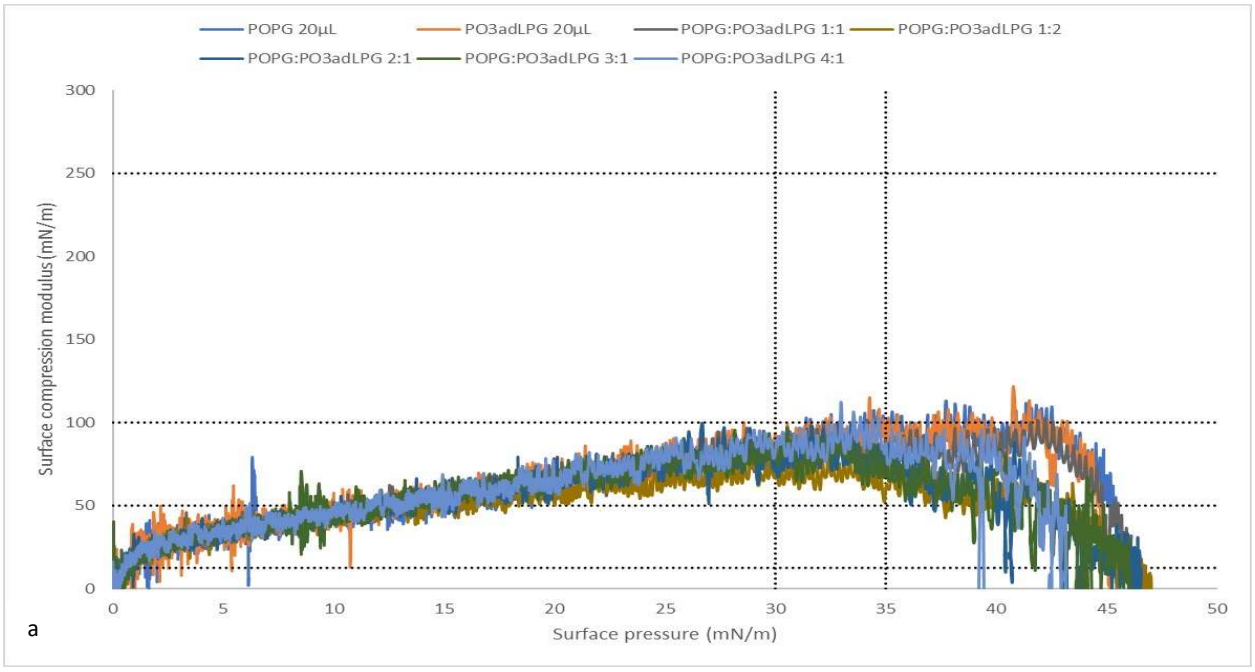


Figure 10 *Surface pressure/area isotherms of POPG and PO3adLPG on Hepes buffer with and without Ca^{2+}*



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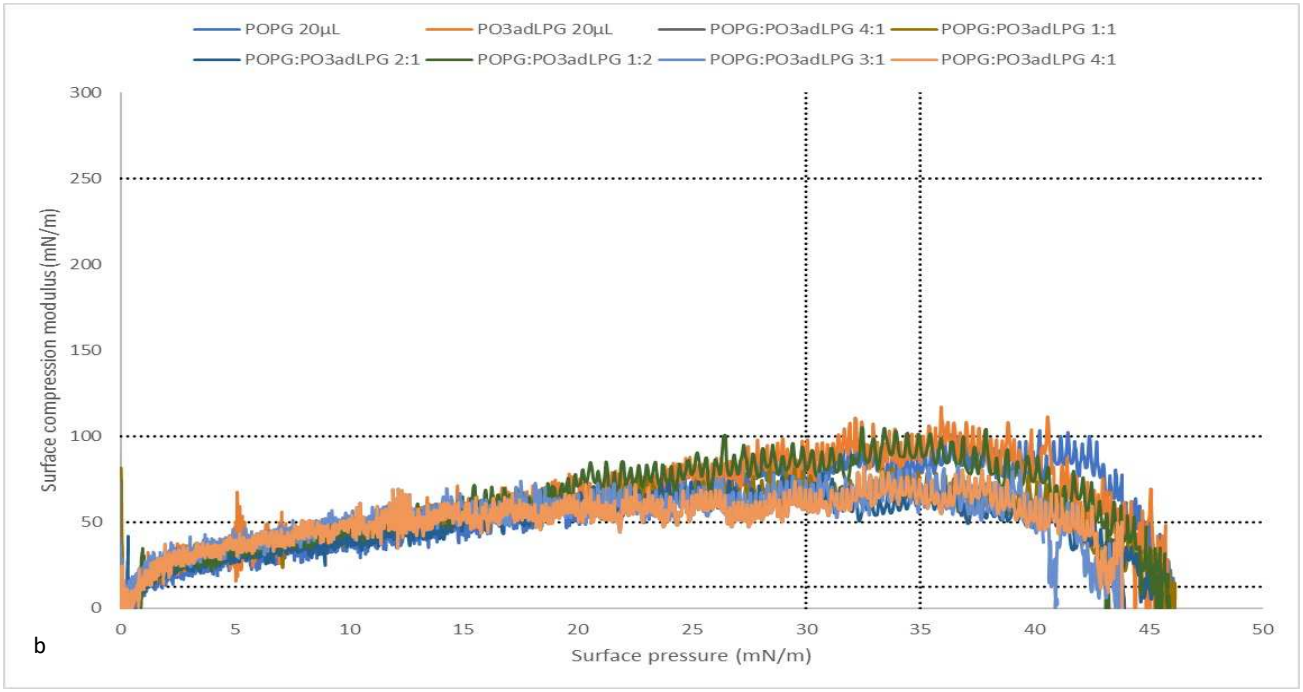


Figure 11 *Compressional modulus of POPG and PO3adLPG in different ratios on Hepes buffer a without Ca^{2+} and b without Ca^{2+} as well as the pure lipids compared*

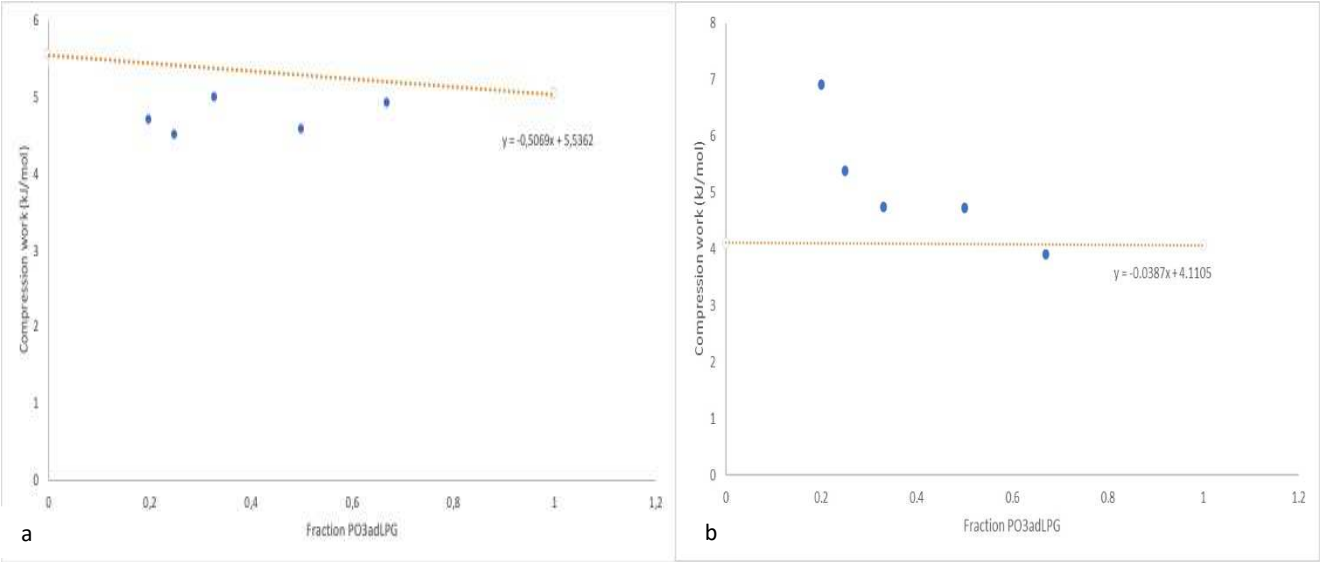


Figure 12 *Compressional work of PO3adLPG and POPG in different ratios on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

Compared to the pure POCL lipids, the mixtures of POCL and PO3adLPG have a smaller average molecular area of about 150 Å² *(Figure 13),* despite the large fatty acid chain area of POCL. It appears that PO3adLPG allows tighter packing of the lipids in the monolayer, resulting in a smaller average molecular area. All lipid mixtures show an average lift-off area of approximately 200 Å² and reach a maximum surface pressure of 45 mN/m with an average molecular area of 80 Å², with the PO3adLPG/POCL 1:4 lipid mixture being the only isotherm to reach a collapse pressure of 50 mN/m. In

the presence of calcium ions, the monolayers expand, with the expansion being more pronounced at higher PO3adLPG values. This indicates a greater effect of the Ca^{2+} ions in this mixture, possibly due to the higher POCL content. This increase in the surface area of the molecule being likely due to increased repulsive steric forces between the lipid chains.

All lipid mixtures remain at a surface compressional modulus of less than 100 mN/m both with and without Ca^{2+} *(Figure 14 a-b)*. Without Ca^{2+} all curves are similar and reach their maximum in the range of 30-35 mN/m. The lipid mixture PO3adLPG/POCL 1:3 shows the smaller maximum value and PO3adLPG/POCL 1:4 the larger one, after which the curves drop, which can be attributed to destabilization and finally to a breakdown of the structural order of the monolayer at 40 mN/m.

The presence of Ca^{2+} leads to higher and more widely distributed values of the compression modulus. The maxima shift to the range of 35-40 mN/m and the monolayers are more stable at higher surface pressures.

For the work of compression, it can be shown that Ca^{2+} has an amplifying effect on the work of compression and the relationship between the molar fraction of lipids and the required work is stronger *(Figure 15 a-b).*

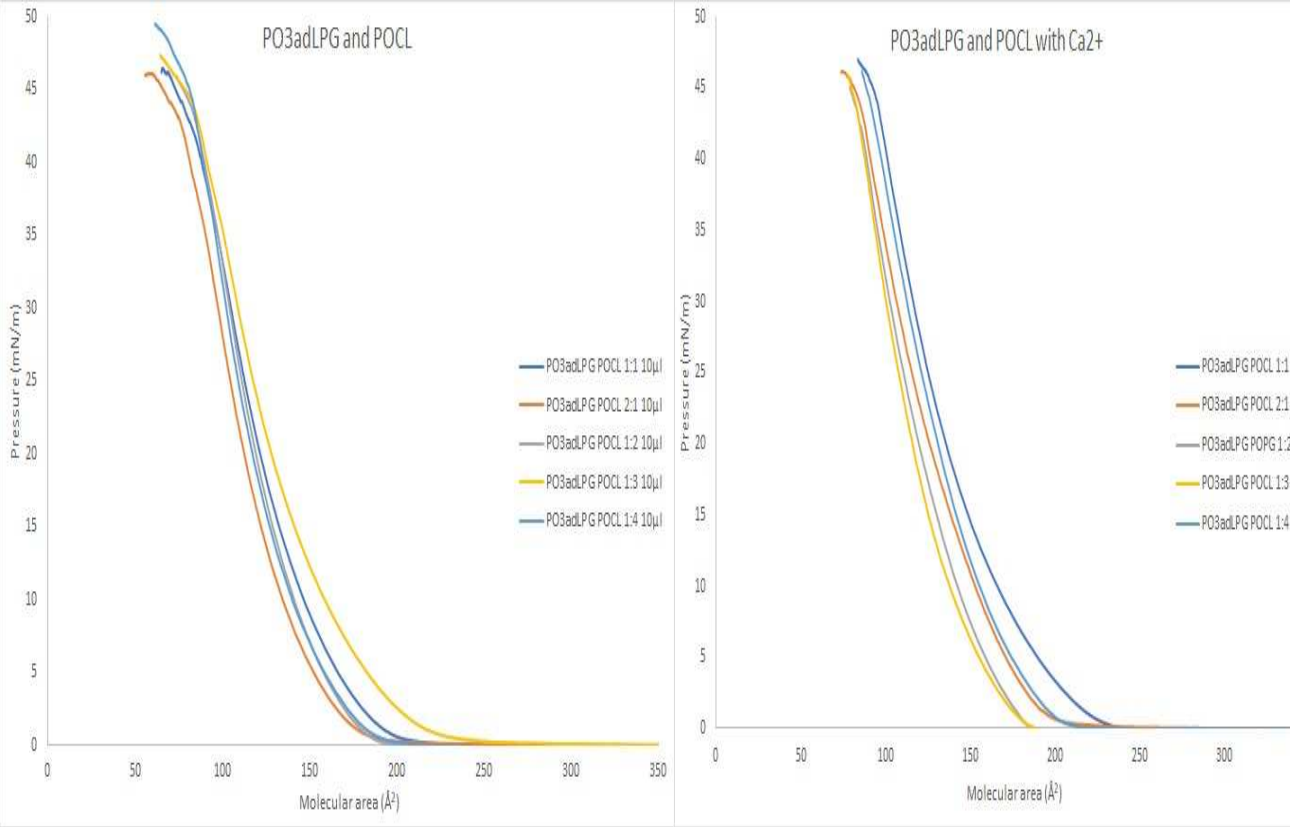
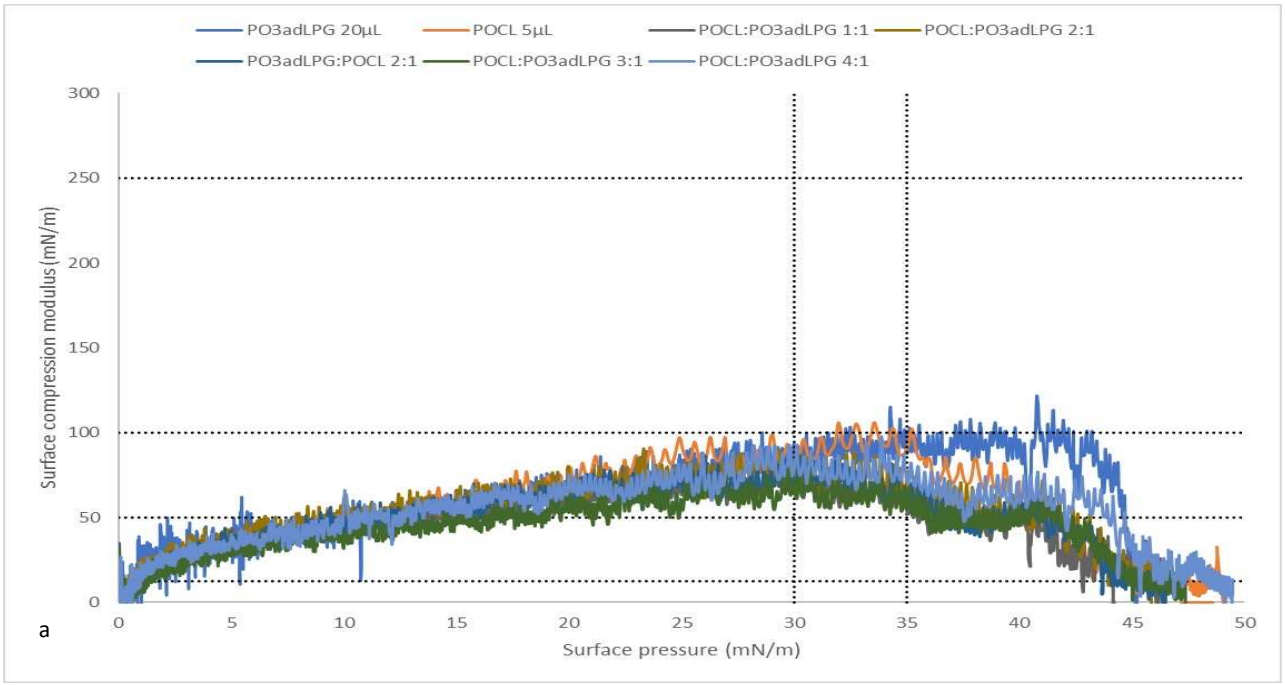


Figure 13 *Surface pressure/area isotherms of POCL and PO3adLPG on Hepes buffer with and without* Ca^{2+}



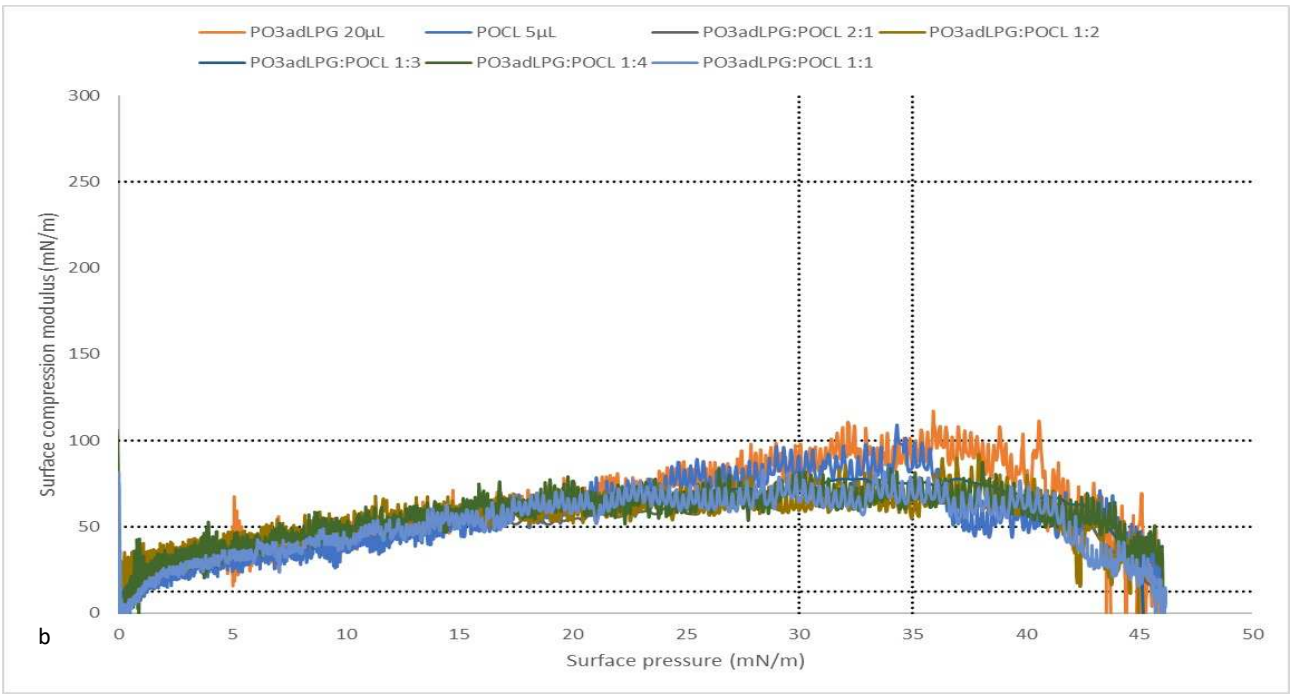


Figure 14 *Compressional modulus of POCL and PO3adLPG in different ratios on Hepes buffer a without Ca^{2+} and b with Ca^{2+} as well as the pure lipids compared*

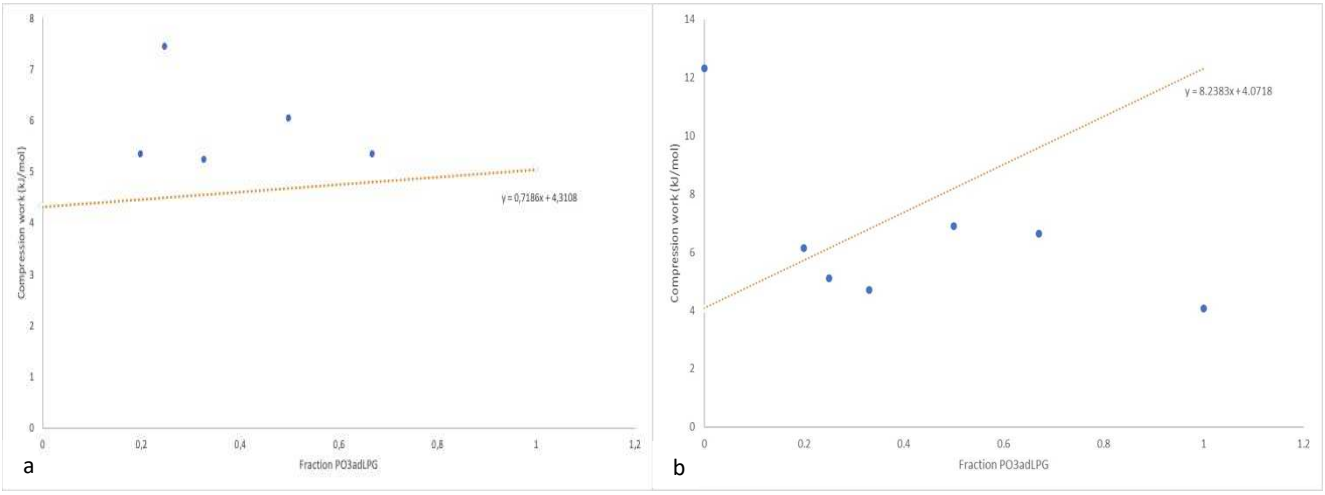


Figure 15 *Compressional work of PO3adLPG and POCL in different ratios on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

The isotherms of the different mixing ratios of POPG and POCL clearly show that the lipid mixtures have different compressional properties *(Figure 16)*. Although both lipids carry the same charges and therefore repel each other in the monolayer, the molecular areas in each lipid ratio are smaller than for pure cardiolipin. This makes sense as there is less cardiolipin, which increases the packing density. Without the addition of Ca^{2+}, the lipid mixture in the ratio POPG/POCL 1:2 shows the highest surface pressure before collapse. This indicates stronger intermolecular interactions and denser packing of the lipids in the monolayer. In contrast, the POPG/POCL 1:3 and 3:1 ratios show larger molecular areas and wider isotherms, which is probably due to stronger electrostatic repulsive forces and steric hindrances that prevent denser packing.

When Ca^{2+} is added, the lift-off areas of almost all lipid mixtures shift to larger molecular areas, which indicates a more expansive effect of the calcium ions on POCL. A notable exception is the POPG/POCL 3:1 ratio, where denser packing and a smaller lift-off area can be observed.

The compressional modulus shows that the lipid mixtures, POPG/POCL 1:3 and 3:1, are less stable than the other lipid mixtures, as they collapse at a surface pressure of less than 35 mN/m, whereas the other lipid mixtures only collapse at pressures over 40 mN/m *(Figure 17 a).*

On the other hand, the addition of Ca^{2+} promotes denser packing in the lipid mixtures POPG/POCL 1:3 and 3:1 and thus a higher stability *(Figure 17 b).* This time POPG/POCL 1:1 and 2:1 show more expanded isotherms and therefore indicate less stability. This analysis can also be observed in the surface compressional modulus. The curves of POPG/POCL 1:1 and 2:1 are much smoother than those of the other lipid mixtures, which are noisier. Although all lipid mixtures collapse at approximately the same pressure at over 40 mN/m, these two lipid mixtures have the lowest maximum values in the LE phase.

In the compressional work without Ca^{2+} *(Figure 18 a),* a steep increase in work with the POPG fraction can be observed due to the expanded nature of the monolayer. Without calcium ions, the interactions between the lipids are less stable, which leads to a higher variability and a stronger increase in the work of compression. In contrast, Ca^{2+} has a stabilizing effect on the lipid monolayer *(Figure 18 b),* which leads to a more moderate increase in the work of compression with the increasing POPG fraction.

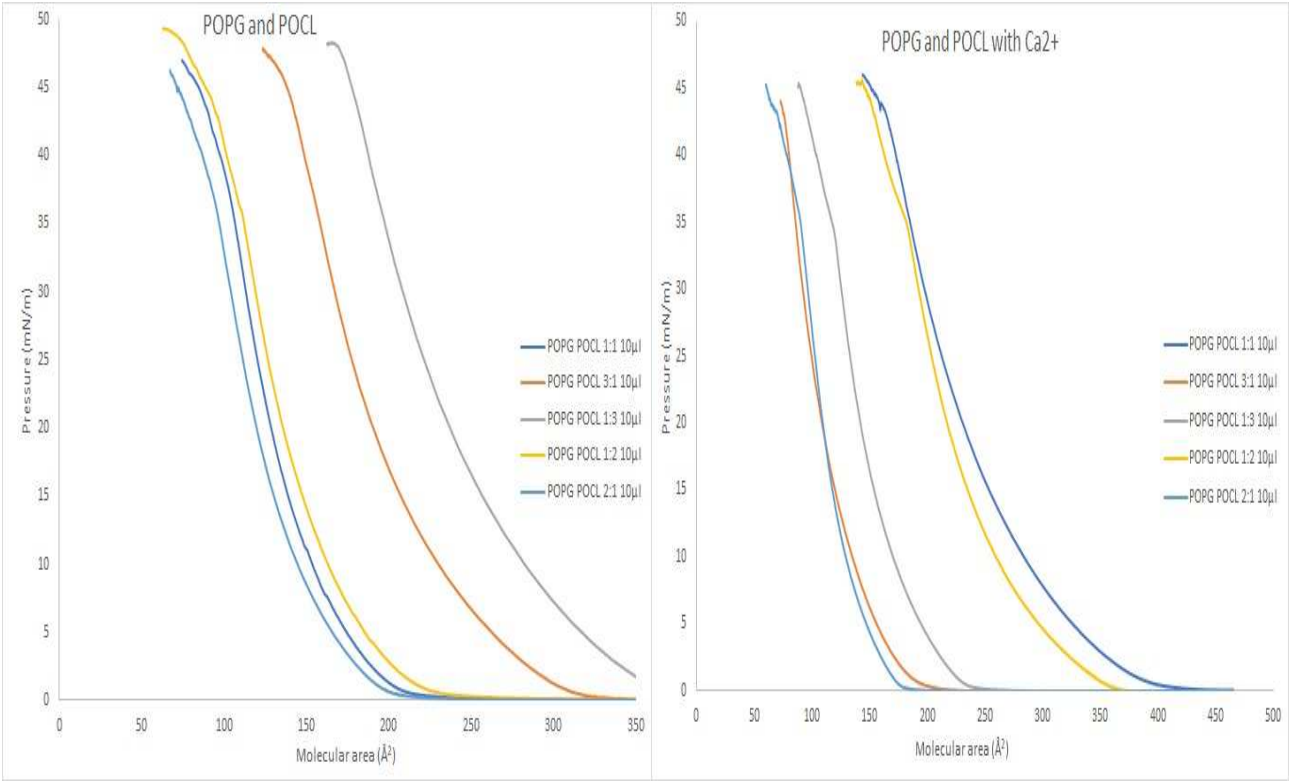
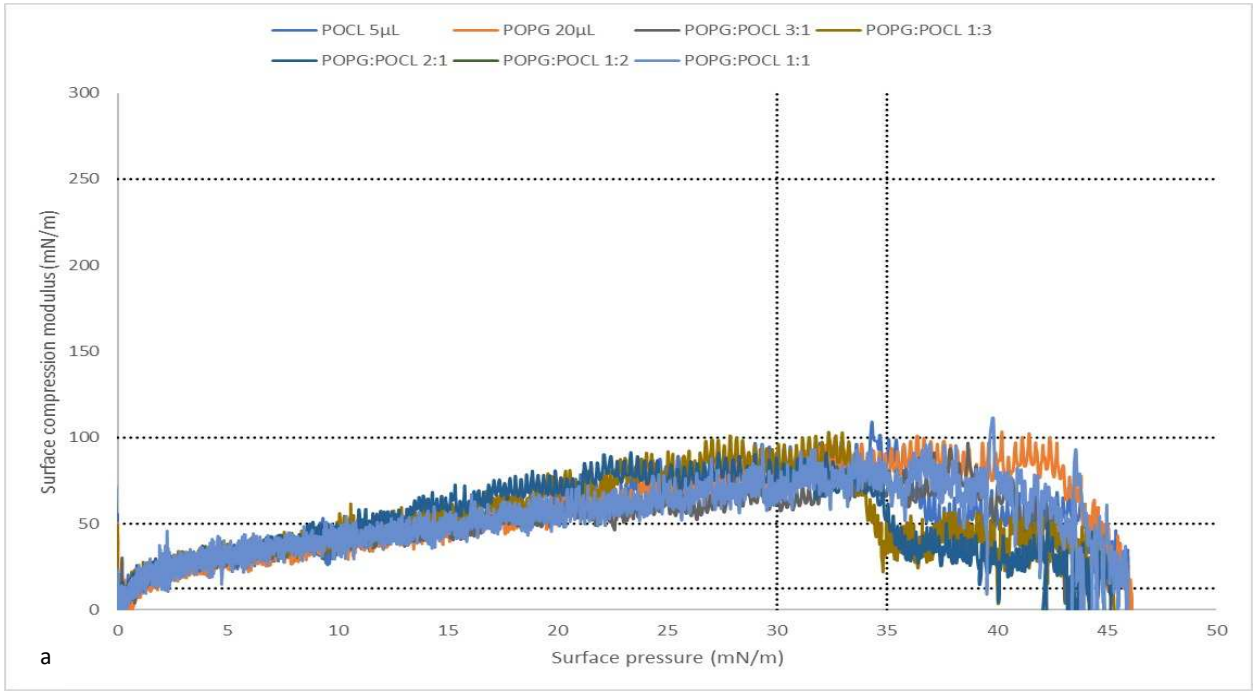


Figure 16 *Surface pressure/area isotherms of POPG and POCL on Hepes buffer with and without Ca^{2+}*



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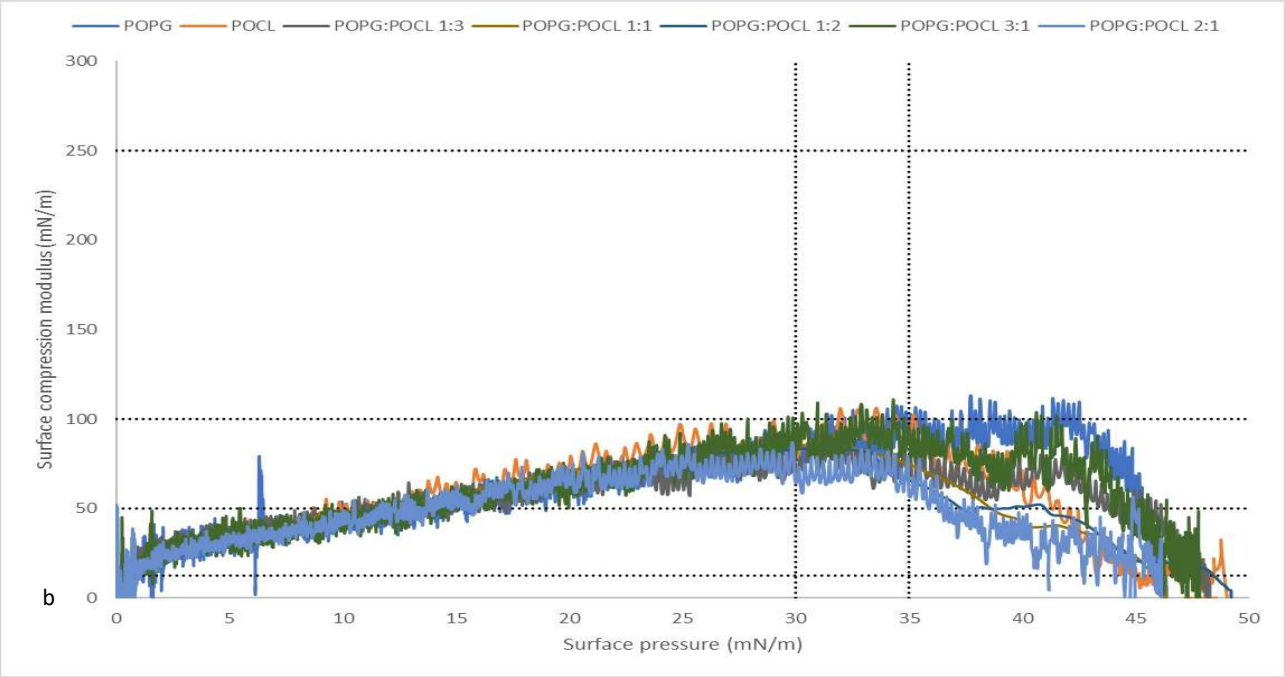


Figure 17 *Compressional modulus of POPG and POCL in different ratios on Hepes buffer a without Ca^{2+} and with Ca^{2+} as well as the pure lipids*

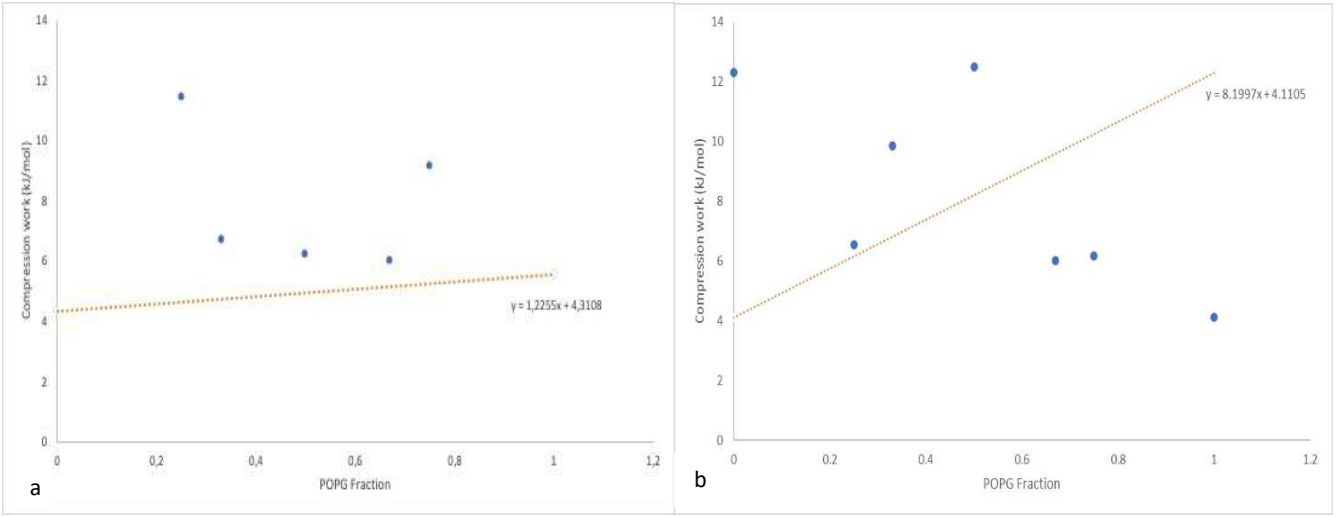


Figure 18 *Compressional work of POPG and POCL in different ratios on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

3.1.3 Compressibility of the ternary lipid mixtures

The curves of the ternary lipid mixtures with 6 % POCL without calcium ions are less expanded and all isotherms reach approximately the same maximum pressure of 45 mN/m at a molecular area of approximately 90 Å² *(Figure 19)*. The two lipid mixtures POCL/POPG/PO3adLPG in the ratios 6:47:47 and 6:31:63 both show a lift-off region at around 220 Å². This indicates that the lipid molecules in these mixtures begin increase in density at similar surface pressures. Both isotherms reach a maximum surface pressure of 45 mN/m at an average molecular area of about 100 Å², indicating that the lipid monolayers in these blends exhibit similar collapse behavior under the given conditions. In contrast, the lipid mixture POCL/POPG/PO3adLPG in the ratio 6:63:31 shows a smaller lift-off range at about 180 Å², which indicates a denser initial packing of the lipids. In this mixture, the maximum surface pressure of 45 mN/m is also reached at a molecular area of 100 Å², before the lipids collapse.
The addition of calcium ions results in significant shifts in the isotherms of all lipid mixtures. A notable effect is the enlargement of the lift-off areas with the lipid mixtures POCL/POPG/PO3adLPG in the ratio 6:63:31 and 6:31:63, which indicates that the lipid molecules are packed less densely by the presence of Ca^{2+} ions. The lipid mixture POCL/POPG/PO3adLPG in the ratios 6:47:47 shows a smaller lift-off area of 150 Å² under the influence of calcium ions. The lipid mixtures POCL/POPG/PO3adLPG in the ratios 6:47:47 and 6:63:31, the maximum surface pressure remains constant at about 45 mN/m, similar to the isotherms without calcium ions. The average molecular area at which this maximum pressure is reached is between 100 Å² and 140 Å². In contrast, the mixture ratio POCL/POPG/PO3adLPG 6:31:63 shows a slightly lower maximum pressure, which is achieved with a larger average molecular area of about 160 Å². This indicates that the lipid molecules in this mixture are more expanded than the other two isotherms.
The surface compressional modulus curves show a gradual increase in value at the beginning (0-10 mN/m), indicating the presence of a highly expanded phase in which the lipid molecules can still move relatively freely. With increasing pressure, especially in the range of 10-25 mN/m, the surface compressional modulus increases further, indicating that the lipids become more densely packed and form a liquid-expanded (LE) phase. The curves reach their maximum in the range of 30-35 mN/m, whereby the surface compressional modulus is particularly pronounced at higher PO3adLPG contents (up to 63 %), but without transition to a condensed phase. This is possibly due to increased ion-pairing in the monolayer *(Figure 20 a).* The presence of calcium ions leads to a significantly further increase in the surface compression modulus for the mixture with the highest PO3adLPG content (63%), which indicates an increased stabilization of the lipid monolayer. The effect of Ca^{2+} is particularly evident in the range of 30-35 mN/m, where the curve reaches its maximum *(Figure 20 b).* The other lipid mixtures show a slightly higher maximum, which indicates that the influence of Ca^{2+} is somewhat weaker in these mixtures.
The work of compression without calcium ions shows a moderate increase from 5.5 mJ/m² to 6.5 mJ/m² with increasing PO3adLPG content, which indicates higher stability and compressibility of the monolayer. With Ca^{2+}, the work of compression shows a minimal decrease from 2 mJ/m² to 1.8 mJ/m² with increasing POPG content, which indicates the stabilizing effect of the calcium ions on the monolayer *(Figure 21 a-b).*

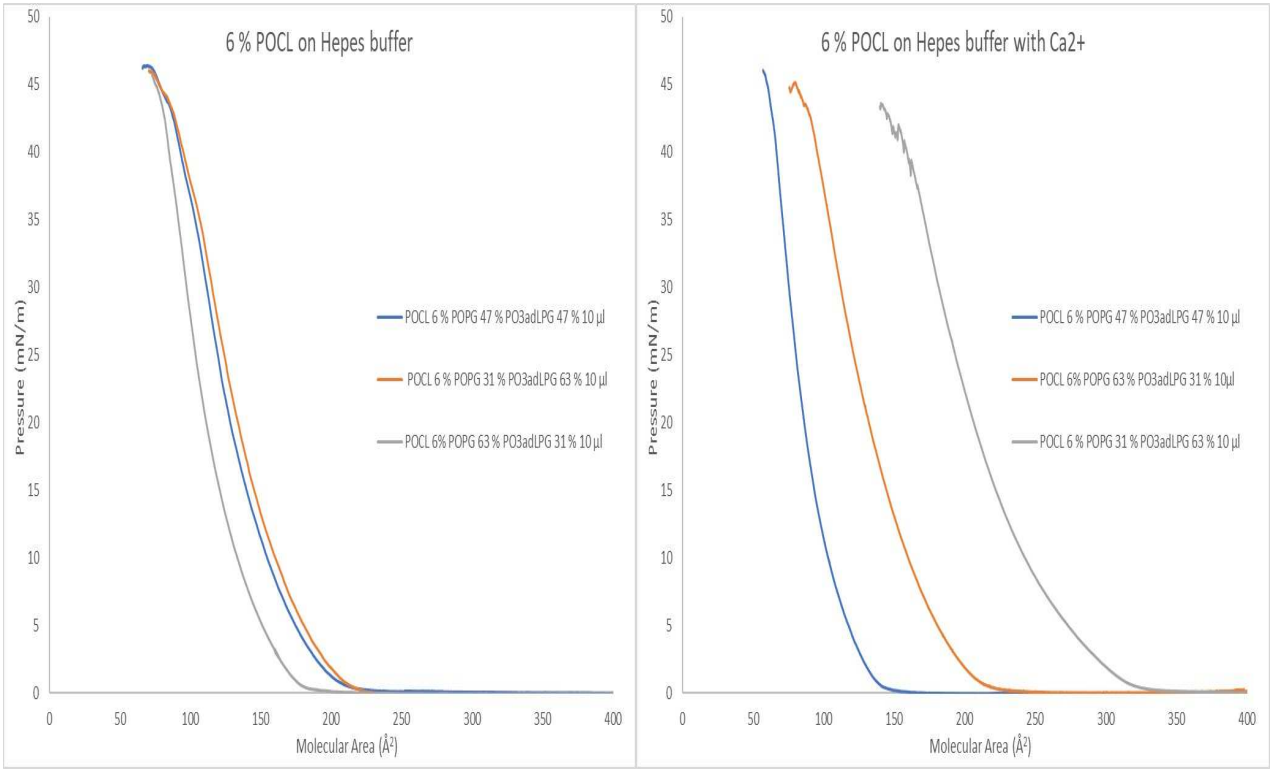
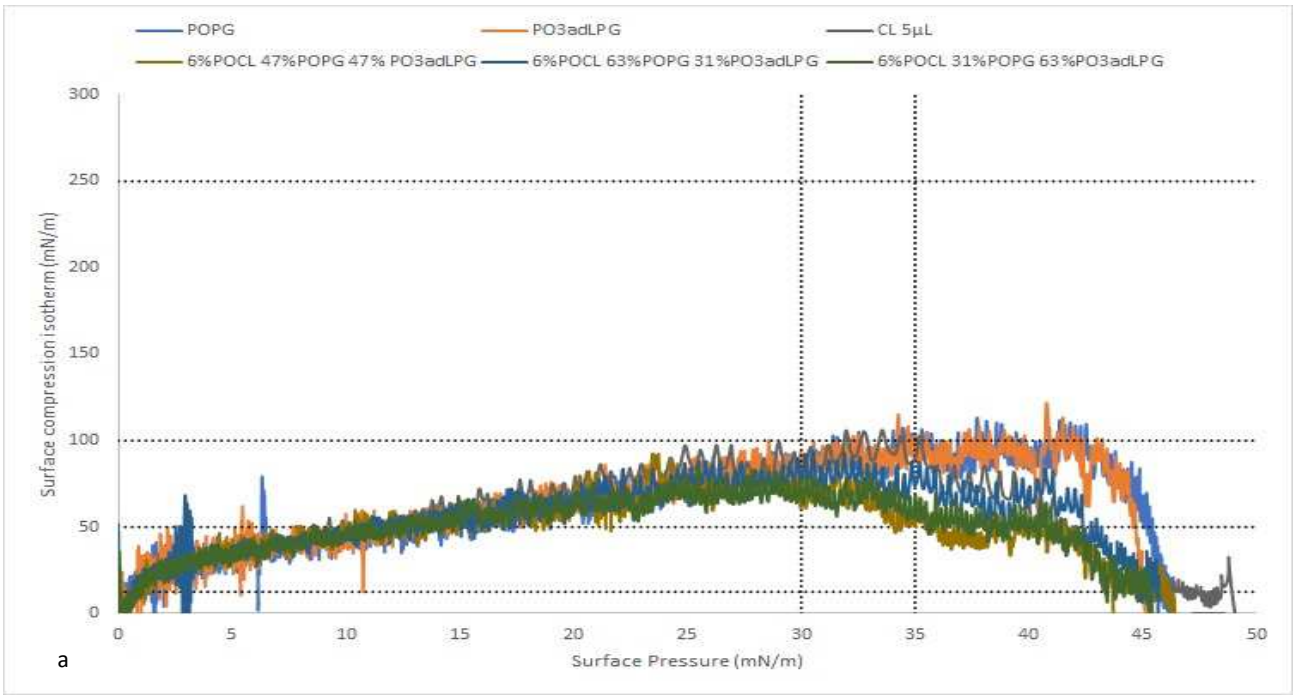


Figure 19 *Surface pressure/area isotherm with 6 % POCL and different ratios of POPG and PO3adLPG on Hepes buffer with and without* Ca^{2+}



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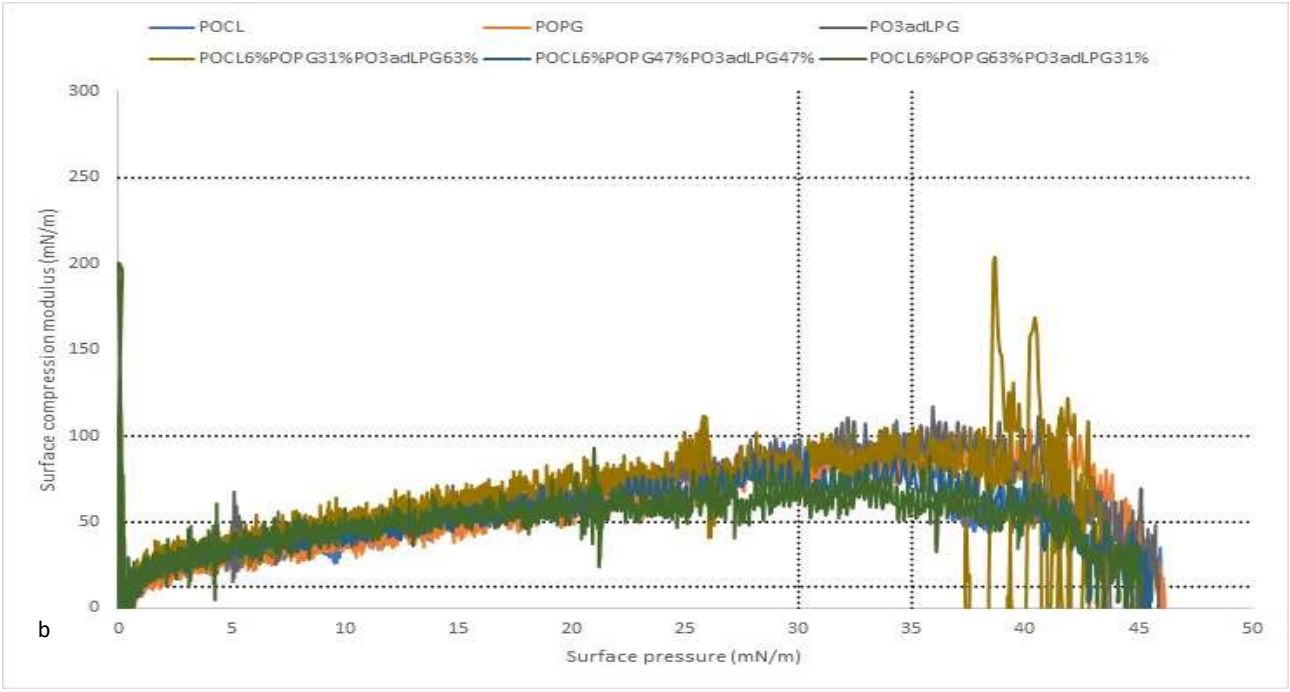


Figure 20 *Compressional modulus of 6 % POCL with different ratios of POPG and PO3adLPG on Hepes buffer a without Ca^{2+} and b with Ca^{2+} as well as the pure lipids compared*

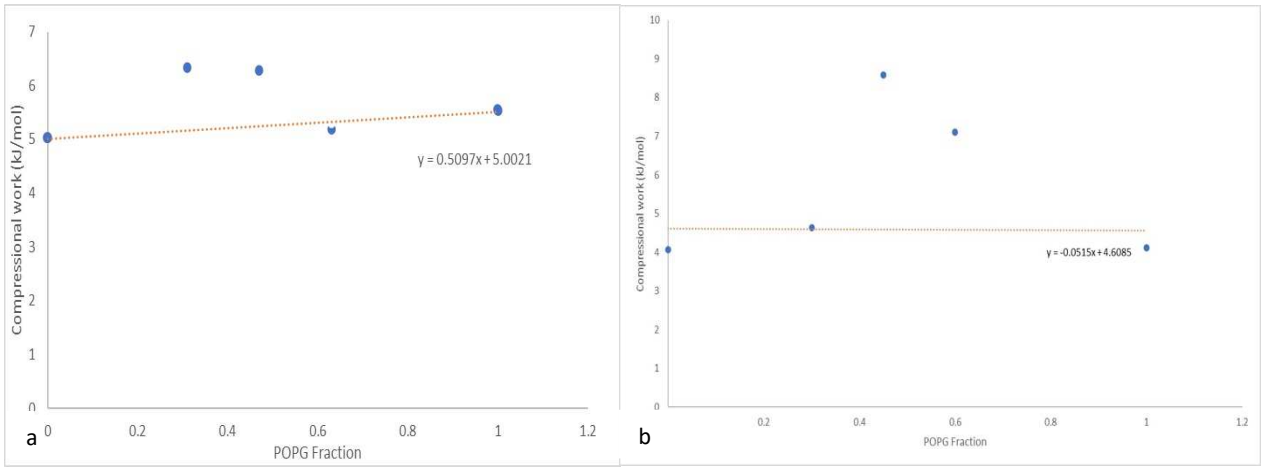


Figure 21 *Compressional work of POPG and PO3adLPG in different ratios with 6 % POCL on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

At a POCL of 10 % without calcium ions, the mixtures show different lift-off ranges between 160 Å² and 240 Å² *(Figure 22).* Particularly striking is the mixture POCL/POPG/PO3adLPG in the ratio 10:30:60, which achieves the highest maximum surface pressure of 47 mN/m with a smaller average molecular area of approximately 60 Å². This means that the lipids form a tightly packed structure on the monolayer and are therefore less expanded. The two other isotherms POCL/POPG/PO3adLPG in the ratios 10:60:30 and 10:45:45 show a similar profile, but with slightly lower maximum pressure values and somewhat larger molecular areas of about 100 Å². The lift-off range with a higher proportion of POPG (60%) nevertheless shows a higher expansion, as the lift-off range is 240 Å², whereas for POCL/POPG/PO3adLPG 10:45:45 is

180 Å². With the addition of calcium ions, the lift-off areas of the mixtures POCL/POPG/PO3adLPG 10:45:45 and 10:30:60 shift to larger molecular areas, which indicates that the lipid molecules are packed less densely due to the effect of the calcium ions on POCL and the isotherms become more expanded. In the lipid mixture POCL/POPG/PO3adLPG 10:60:30, the lift-off area is the only isotherm that shifts to a smaller lift-off area of 210 Å² to approximately 350 Å². The maximum surface pressure remains at about 45 mN/m in all cases, but with slightly larger molecular areas (between 120 and 140 Å²) than in the isotherms without calcium.

In the surface compressional modulus without calcium ions, all lipid mixtures show a similar curve *(Figure 23 a).* They reach their maximum values in the range of 30-35 mN/m, remaining in the liquid-expanded (LE) phase and showing no transitions to a liquid-condensed (LC) phase, as the surface compressional modulus stays below 100 mN/m. The lipid mixture POCL/POPG/PO3adLPG in the ratio 10:60:30 shows the lowest maximum value, and the surface compressional modulus begins to slowly decrease at a pressure of 35 mN/m before ultimately collapsing at 40 mN/m. The other lipid mixtures POCL/POPG/PO3adLPG in the ratios 10:30:60 and 10:45:45 show slightly higher maximum values and collapse at a pressure of 40 mN/m. In the presence of calcium ions, the overall behavior of the lipid mixtures is similar to that without calcium *(Figure 23 b).* All mixtures reach their maximum compressional work values at around 30 mN/m, with the surface compressional modulus remaining below 100 mN/m. As with the situation without calcium ions, the lipid mixture POCL/POPG/PO3adLPG in the ratio 10:60:30 shows the lowest maximum value, indicating lower stiffness and stability of the monolayer. In contrast to the situation without calcium, however, this mixture collapses at a surface pressure of over 40 mN/m, similar to the other lipid mixtures. This could indicate that calcium ions have a slight stabilizing effect that increases the collapse pressure.

Without calcium ions, the work of compression shows a positive trend with increasing POPG content, indicating that the monolayers are more expanded and therefore less stable, as more energy is required to compress them. In the presence of calcium ions, on the other hand, the work of compression remains constant, indicating a stabilizing effect of the calcium ions, which limits the expansion capacity of the monolayer and stabilizes its structure, regardless of the PO3adLPG content *(Figure 24 a-b).*

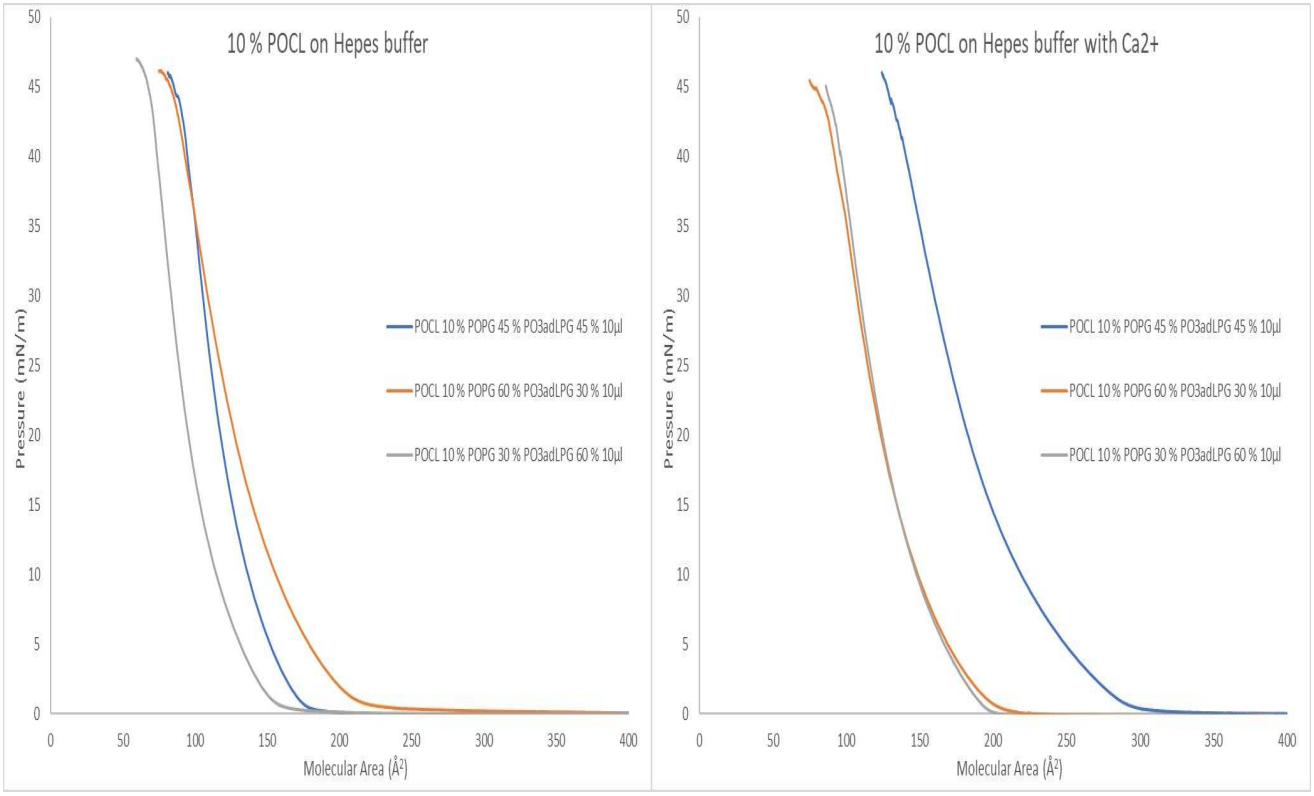
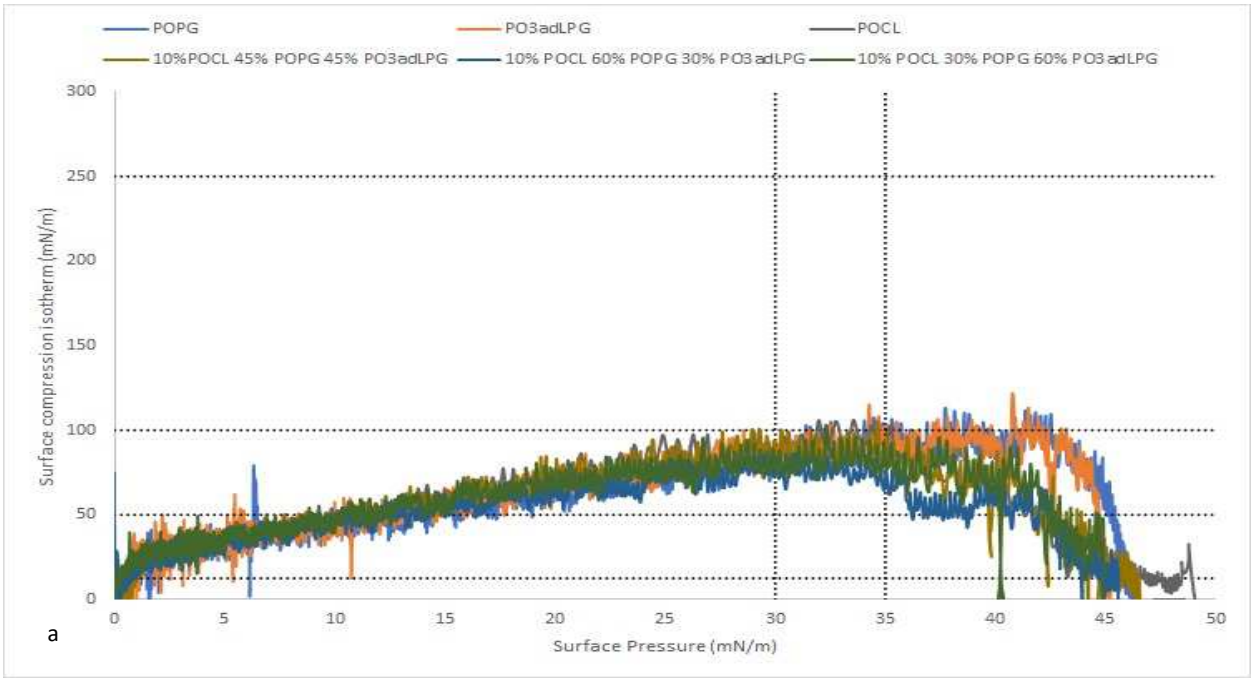


Figure 22 *Surface pressure/area isotherms of 10 % POCL with different ratios of POPG and PO3adLPG on Hepes buffer with and without Ca^{2+}*



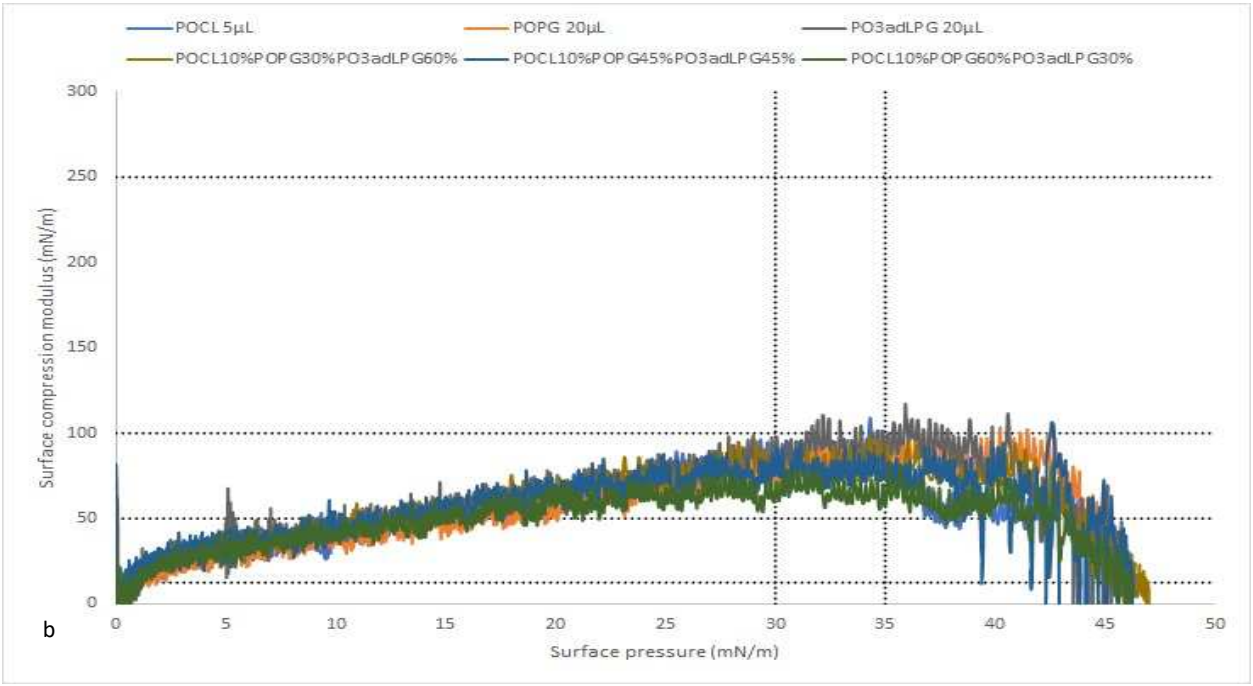


Figure 23 *Compressional modulus of 10 % POCL with different ratios of POPG and PO3adLPG on Hepes buffer a without Ca^{2+} and b with Ca^{2+}as well as the pure lipids*

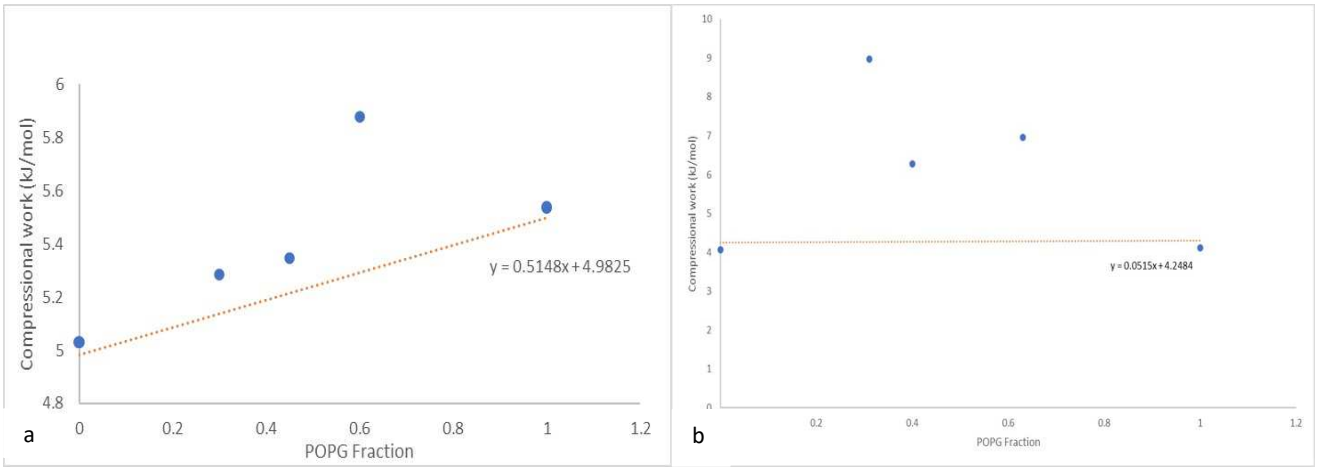


Figure 24 *Compressional work of POPG and PO3adLPG in different ratios with 10% POCL on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

At 16 % POCL without calcium ions, the isotherm with a higher PO3adLPG content of 56 % shows the highest collapse pressure at a molecular area of 90 Å² *(Figure 25)*. The lift-off area is around 170 Å², resulting in a difference of 80 Å², which indicates that this mixture is less expanded and more densely packed. In comparison, the other mixtures POCL/POPG/PO3adLPG in the ratios 16:56:28 and 16:42:42 show a similar maximum pressure at a larger molecular area, indicating looser packing and greater expansion, especially as the lift-off areas range between 250-290 Å². In the presence of calcium ions, the differences between the isotherms are less pronounced. All mixtures reach the same maximum surface pressure of about 45 mN/m at an average molecular area of 120 Å². The lift-off area is around 200 Å², resulting in a difference of 80 Å² between the lift-off area and the point at which the maximum surface pressure is reached. This means that the isotherms are less expanded in the presence of calcium ions, indicating that the lipid molecules are more densely packed by the calcium ions, and the monolayers become overall more stable and less flexible.

The compressional modulus without calcium ions shows different maximum values of the compressional modulus for the different lipid mixtures *(Figure 26 a)*. Mixtures with a higher PO3adLPG content (56 %) tend to achieve lower maximum values, which indicates a looser packing of the lipids and a lower stability of the monolayer. In contrast, the lipid mixtures with a higher POPG content (56 %) show higher maximum values, indicating a denser packing and a more stable monolayer. For all lipid mixtures, the curves begin to fall gradually from around 35 mN/m until they finally collapse at around 40 mN/m. This indicates that all mixtures have similar collapse stability, despite the differences in packing density. In the presence of calcium ions, it can be observed that the mixture with 56 % PO3adLPG becomes more stable after the addition of calcium, which is reflected in higher collapse pressure values *(Figure 26 b).* In contrast, the stability of the mixture with 56 % POPG appears to decrease in the presence of calcium ions, as lower collapse pressure values are achieved.

The work of compression without calcium ions shows an increase in the energy required with increasing POPG content, indicating greater expansion and higher compressibility of the monolayers, but not necessarily greater stability. With calcium ions, the work of compression remains almost constant, indicating that calcium ions stabilize the interactions within the monolayer and reduce flexibility, resulting in denser packing and more uniform compression *(Figure 27 a-b).*

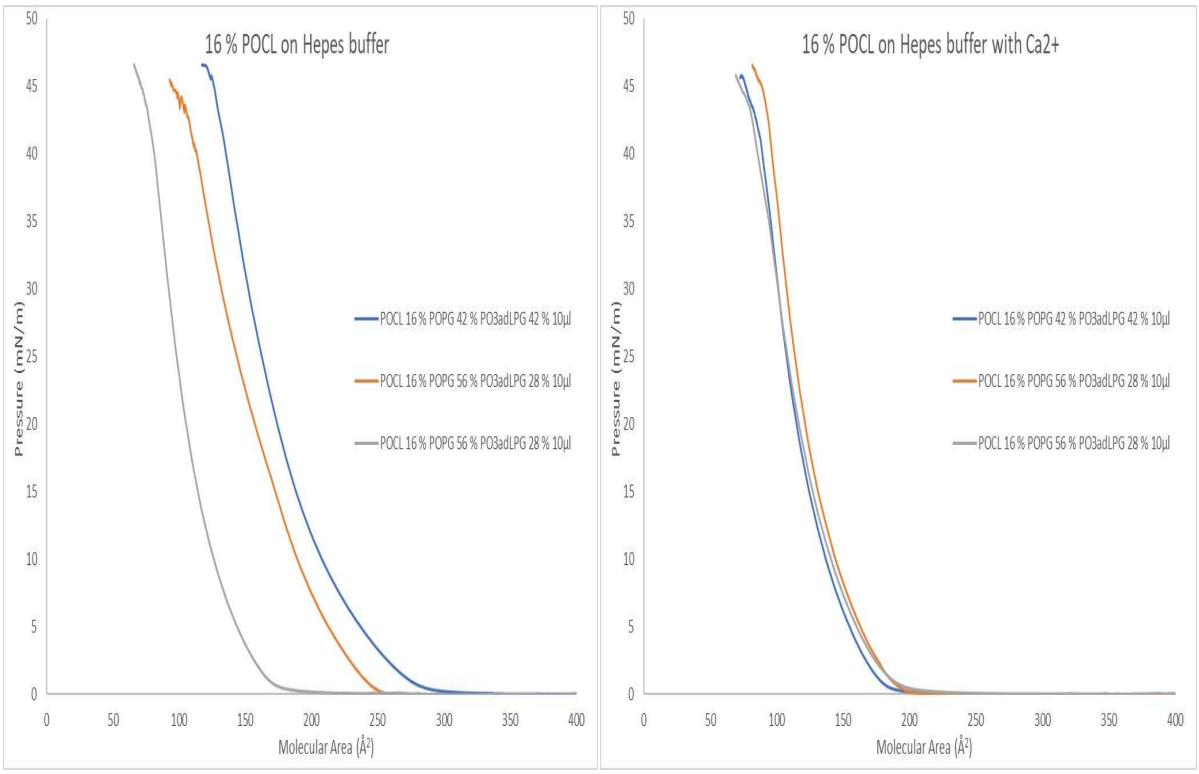
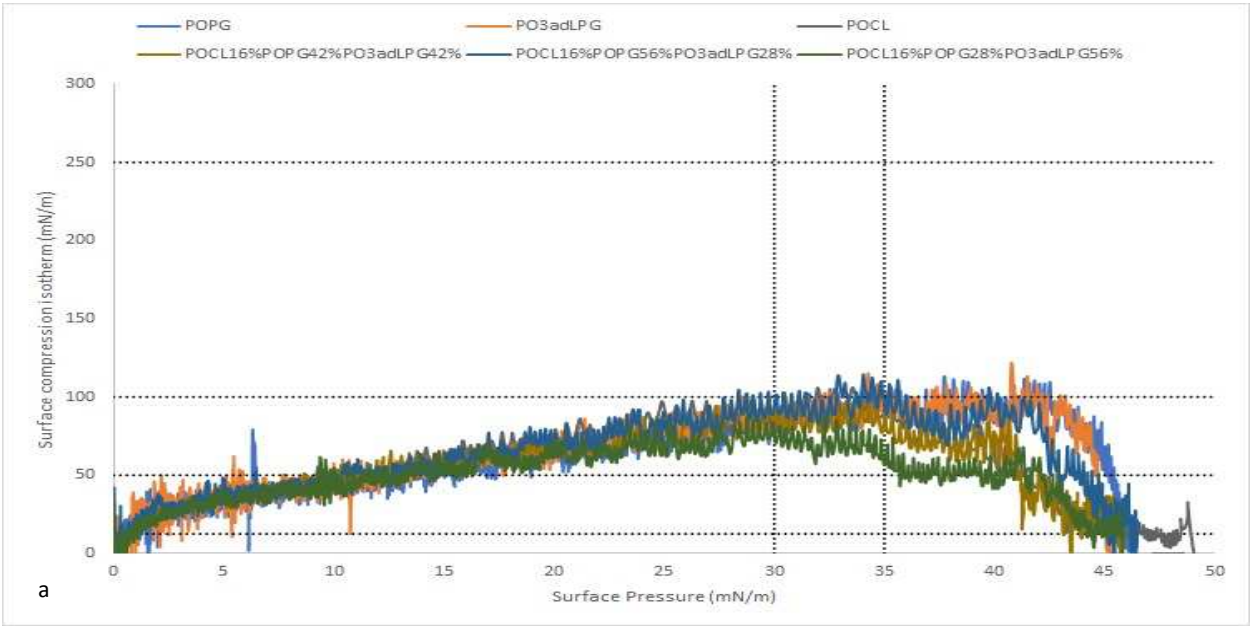


Figure 25 *Surface pressure/area isotherms of 16 % POCL with different ratios of POPG and PO3adLPG on Hepes buffer with and without* Ca^{2+}



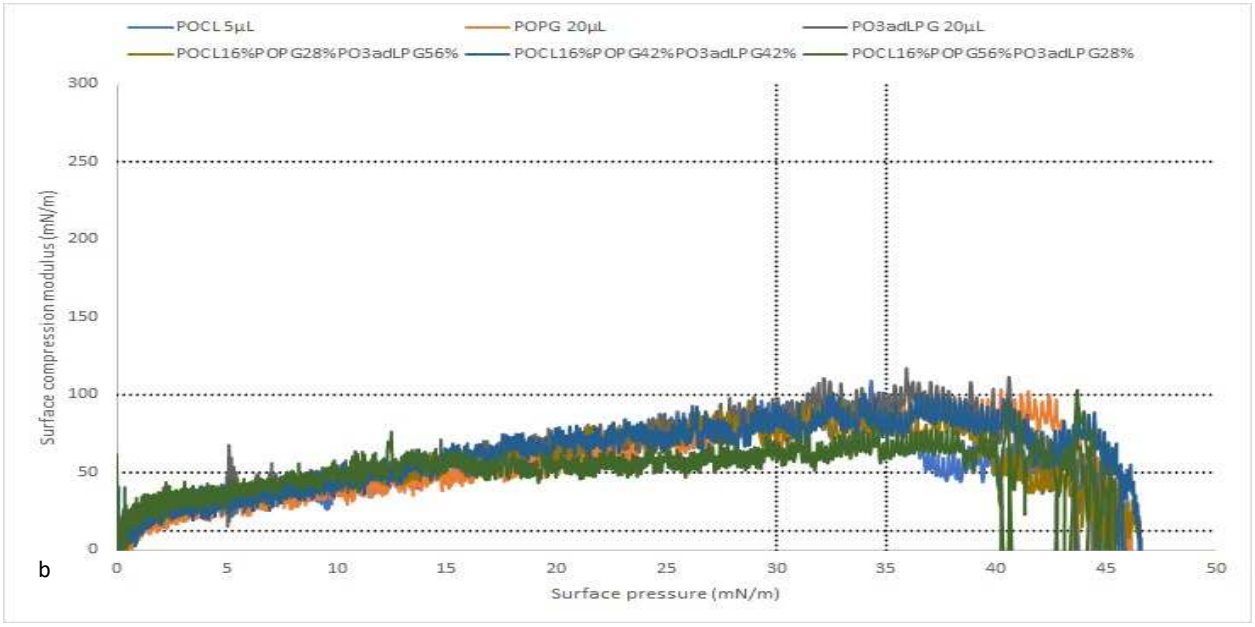


Figure 26 *Compressional modulus of 16 % POCL with different ratios of POPG and PO3adLPG on Hepes buffer a without Ca^{2+} and b with Ca^{2+} as well as the pure lipids compared*

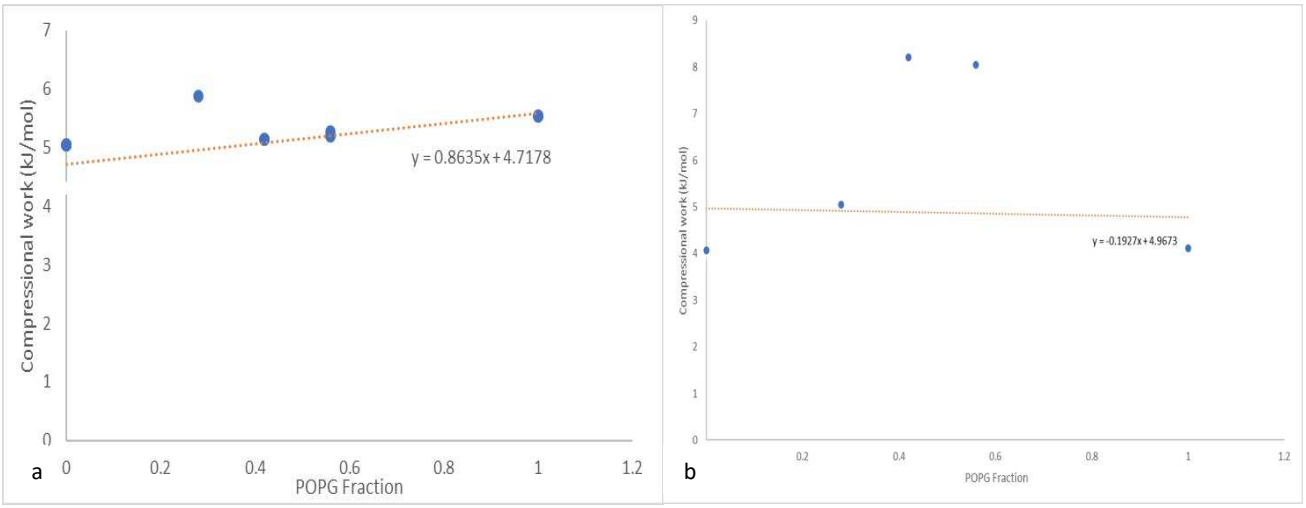


Figure 27 *Compressional work of POPG and PO3adLPG in different ratios with 16 % POCL on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

Compared to the lipid mixtures with 16 % POCL, the isotherms with 20 % POCL show greater expansion, particularly in the presence of calcium ions *(Figure 28).* Without calcium ions, the isotherm with a ratio of 40 % PO3adLPG and 40 % POPG shows the highest collapse pressure of 47 mN/m at a smaller molecular area of 80 Å² and a smaller lift-off area of 210 $Å^2$, indicating that this lipid mixture allows for the densest packing of the lipids. The lipid mixture POCL/POPG/PO3adLPG 20:30:50 shows a similar trend, but it reaches a slightly lower maximum surface pressure of 45 mN/m. The mixture POCL/POPG/PO3adLPG in the ratio 20:50:30 shows the greatest expansion of all the isotherms, with a lift-off area of approximately 270 Å² and a surface pressure of 45 mN/m at a molecular area of 140 Å². In the presence of calcium ions, the isotherms are overall more expanded, due to their higher POCL content. The lift-off areas generally shift to larger values; however, the areas where the maximum surface pressures are reached remain unchanged, similar to the isotherms without calcium ions. Thus, all the isotherms are more expanded, with the lipid mixture in the ratio POCL/POPG/PO3adLPG 20:50:30 showing the greatest expansion due to the larger lift-off area.

In the compressional modulus data without calcium ions, the lipid mixtures show different maximum values of the compressional modulus *(Figure 29 a).* Mixtures with a higher PO3adLPG content (50 %) tend to achieve higher maximum values, which indicates a denser packing and potentially higher stability of the monolayer. In contrast, the lipid mixtures with the ratios POCL/POPG/PO3adLPG 20:40:40 and 20:50:30 show lower maximum values, indicating looser packing and lower stability. However, all mixtures remain in the liquid-expanded (LE) phase at a surface pressure of 30-35 mN/m. The monolayers collapse at a surface pressure of over 40 mN/m, indicating relatively uniform collapse stability. In the presence of calcium ions, all lipid mixtures show significant changes in the compression modulus *(Figure 29 b).* This time, the lipid mixture with an increased POPG content (50 %) achieves the highest maximum value of the compression modulus, which indicates a denser packing and a more stable monolayer. In comparison, the other lipid mixtures show significantly lower maximum values. However, none of the compression modulus values exceed the value of 100 mN/m, which indicates that all mixtures remain in the liquid-expanded (LE) phase. Overall, the lipid mixtures collapse at a surface pressure of over 40 mN/m. It is noticeable that the lipid mixture POCL/POPG/PO3adLPG 20:30:50 already shows a gradual decrease in the compressional modulus from 30 mN/m, which indicates an increasing instability of the monolayer before it finally collapses at over 40 mN/m.

At 20 % POCL without calcium ions, the work of compression shows an increase with increasing POPG content, indicating that more energy is required for compression as the monolayers are more expanded. With calcium ions, on the other hand, there is an almost negligible negative correlation, which means that less energy is required for compression, indicating denser packing and less expanded monolayers *(Figure 30 a-b).*

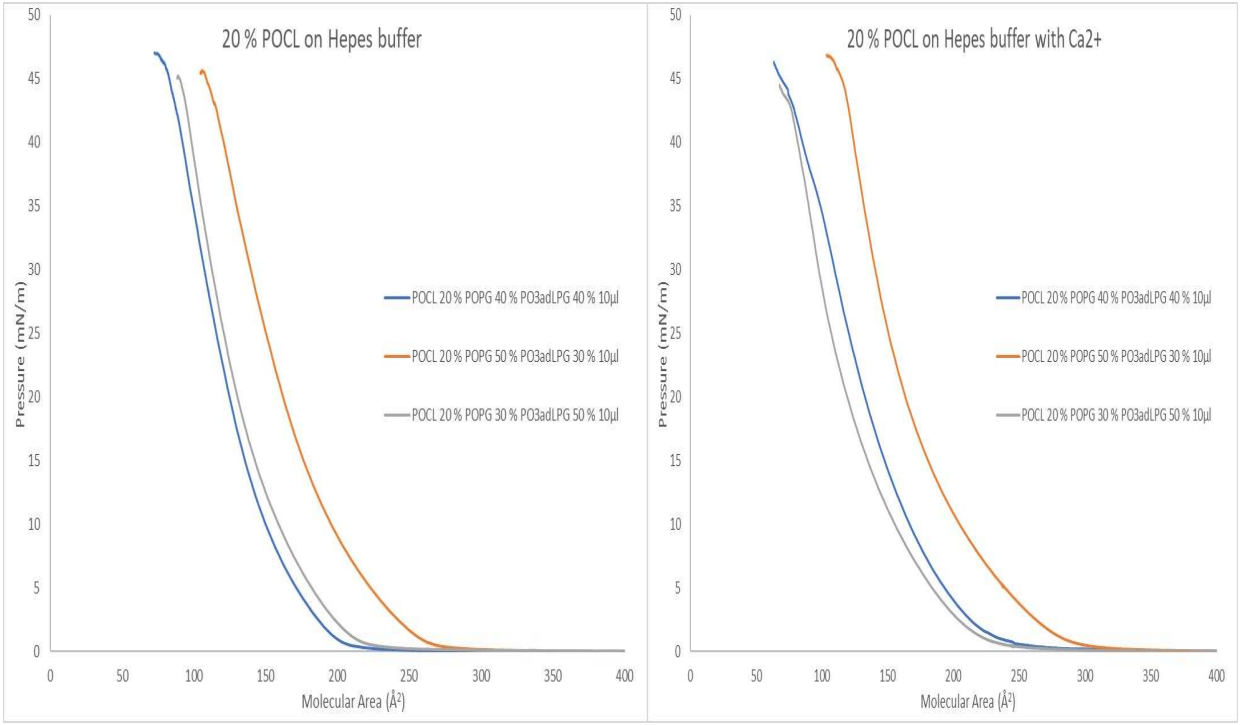
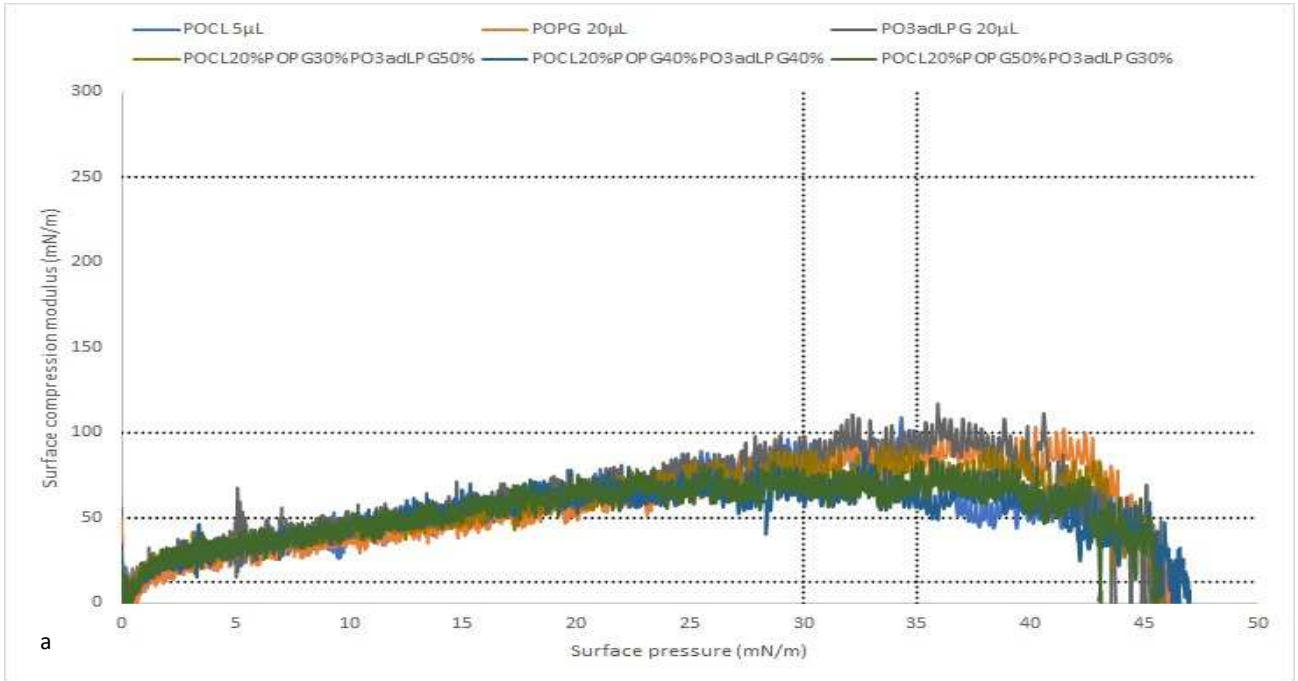


Figure 28 *Surface pressure/area isotherms of 20 % POCL and different ratios of POPG and PO3adLPG on Hepes buffer with and without* Ca^{2+}



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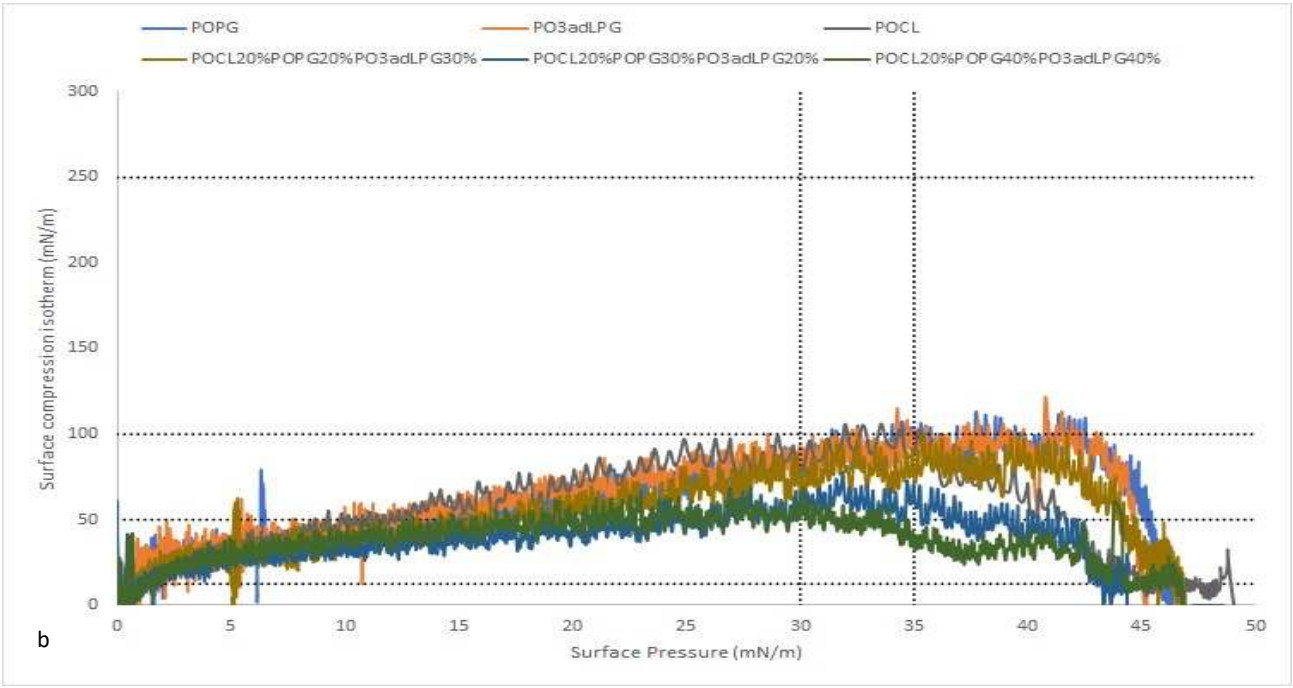


Figure 29 *Compressional modulus of 20 % POCL with different ratios of POPG and PO3adLPG on Hepes buffer a without Ca^{2+} and b with Ca^{2+} as well as the pure lipids compared*

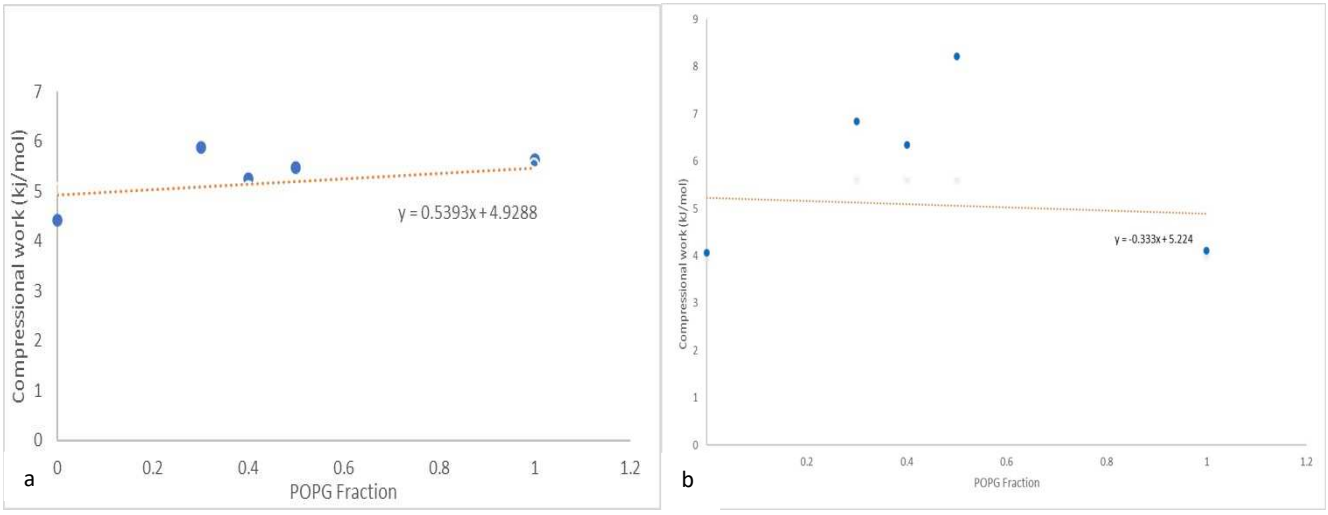


Figure 30 *Compressional work of POPG and PO3adLPG in different ratios with 20 % POCL on Hepes buffer a without Ca^{2+} and b with Ca^{2+}. The blue points represent the individual measured values, while the orange regression line shows the ideal values.*

3.2 Zetasizer data

The influence of Daptomycin and POCL on the size and surface characteristics of liposomes made from POPG/PO3adLPG mixtures with 7:3 and 3:7 molar ratios, was investigated in the Zeta Sizer *(Figure 31)*. POPG/PO3adLPG 7:3 shows a smaller particle size of 106 nm and a negative zeta potential of -44.6 mV, which is typical for vesicles with a high proportion of an anionic lipid like POPG. POPG/ PO3adLPG 3:7

has a larger particle size of 142 nm and a zeta potential of +48.5 mV, which is due to the larger proportion of cationic PO3adLPG.
The addition of DAP leads to a slight increase in particle size. For example, POPG/PO3adLPG 7:3 and 50 µl DAP shows a size of 112 nm and a less negative zeta potential (-32.3 mV), indicating that the interaction between DAP and the lipids has a charge neutralizing effect. With POPG/PO3adLPG 3:7 and 50 µl DAP, the zeta potential decreases to +33.9 mV, again indicating a slight neutralizing effect of the antibiotic.
The addition of POCL leads to varying particle sizes and zeta potentials. For example, POPG/PO3adLPG 7:3 with 0.5 % POCL shows a size of 122 nm and a zeta potential of -45 mV.
Higher concentrations of POCL (up to 5 %) in combination with DAP generally increase the particle size and the zeta potential with POPG/PO3adLPG 7:3 increases while decreases with POPG/PO3adLPG 3:7.

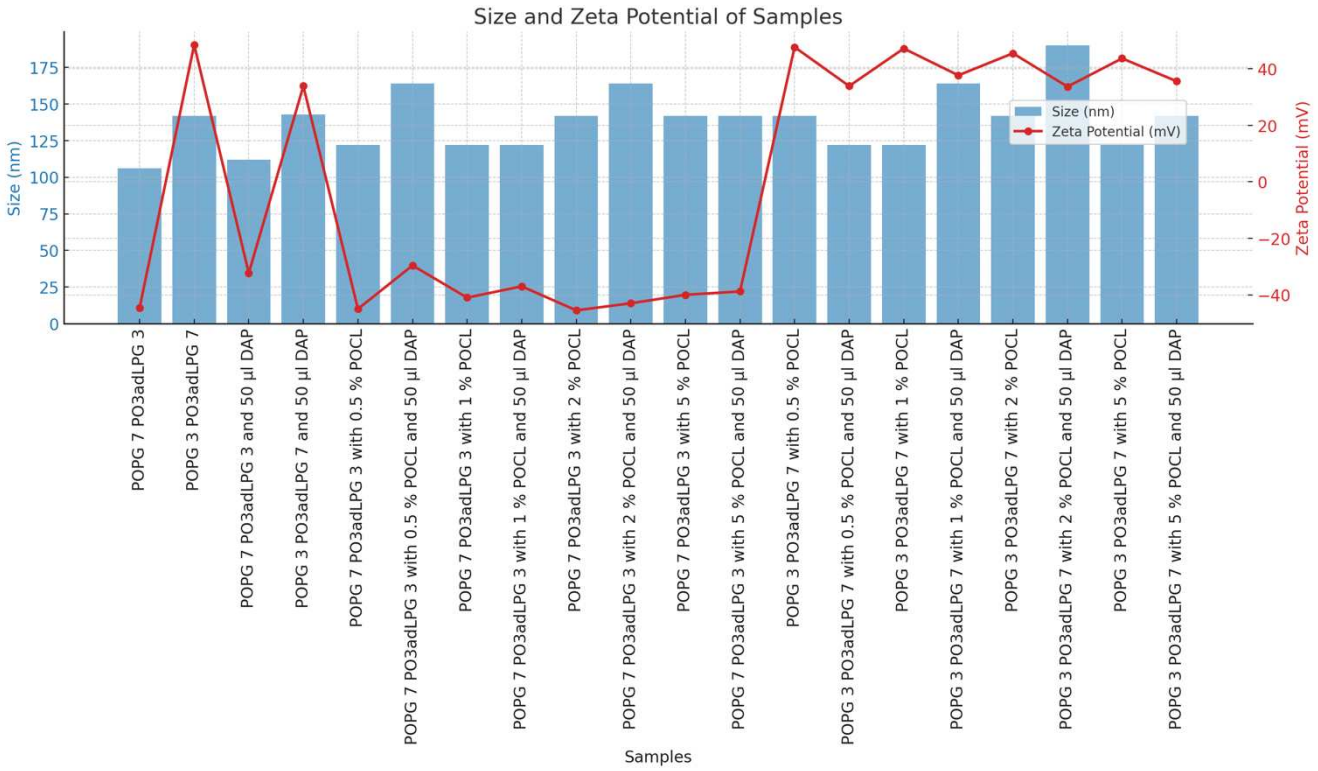


Figure 31 *Comparison of the influence of POCL and DAP of the Zeta potential (red) and the Particle size (blue) of the different lipid ratios*

3.3 Isothermal Titration Calorimetry

The present ITC data show the thermodynamic interactions between the liposome components POPG/PO3adLPG in a ratio of 7:3 and the liposomes after addition of 0.5 % and 1 % cardiolipin (POCL) during titration in daptomycin. All experiments indicate an exothermic reaction, which means that heat is released during the binding process between the liposomes and daptomycin. This release of heat corresponds to the negative change in enthalpy (ΔH) during the binding process *(Table 3).*
In the first experiment, where pure POPG/PO3adLPG (7:3) *(Figure 32)* was used, the ITC measurement shows a series of heat pulses at the beginning of the titration, the level of which decreases continuously

over time. This indicates that the free binding sites on the liposomes are gradually becoming saturated. After the 6th injection a plateau is reached, indicating that no more significant heat effects occur and the binding between the molecules is saturated.
The Kd value of 2.045E^{-5} M indicates a high binding affinity between daptomycin and the liposomes. The exothermic reaction is confirmed by the negative enthalpy change of -11.81 kJ/mol, which indicates that the binding is energetically favourable. The stoichiometry (n) is 1.35, indicating that each liposome molecule binds on average about 1.65 daptomycin molecules. This indicates several possible binding sites.
After the addition of 0.5 % POCL *(Figure 33)*, the binding behaviour changes slightly. The heat pulses at the beginning of the titration are similar to the previous experiment but reach a plateau at a higher molar ratio. The Kd value of 1.502E-5 M shows that the binding affinity has slightly decreased compared to the measurement without POCL, indicating that POCL slightly weakens the binding. This could be due to structural changes in the liposome membrane caused by the introduction of POCL.
The enthalpy change (ΔH) is -10.66 kJ/mol, which shows a slightly less exothermic response compared to the measurement without POCL. This indicates that the binding is less energy intensive. Interestingly, the value of stoichiometry (n) increases to 1.74, indicating that the addition of POCL makes more binding sites available on the liposomes. This could indicate a change in membrane structure caused by POCL, which makes more binding sites accessible for daptomycin, although these bonds are less efficient.
With the further increase of the POCL content to 1 % *(Figure 34)*, the ITC experiment again shows a decrease in binding affinity, as evidenced by the Kd value of 1.204E-5 M. This decrease in affinity indicates that the addition of POCL changes the structure of the liposomes in such a way that the interaction with daptomycin becomes even weaker. The enthalpy change of -12.31 kJ/mol indicates that this binding is still exothermic, but energetically somewhat more favorable than the reactions without POCL or with 0.5 % POCL. The stoichiometry value (n) increases further to 2.023, which shows that there are even more binding sites for daptomycin at higher POCL concentrations. This could indicate that POCL further destabilizes the liposome membrane due to its particular conical shape and creates more binding sites, although these bonds are less specific and less efficient.

Table 3 *Parameters of the ITC Experiments*

Samples	Kd (M)	ΔH (kJ/mol)	Stoichiometry (n)
POPG/PO3adLPG 7:3	2.045E^{-5}	-11.81	1.35
POPG/PO3adLPG 7:3 with 0.5 % POCL	1.502E^{-5}	-10.66	1.74
POPG/PO3adLPG 7:3 with 1 % POCL	1.204E^{-5}	-12.31	1.89

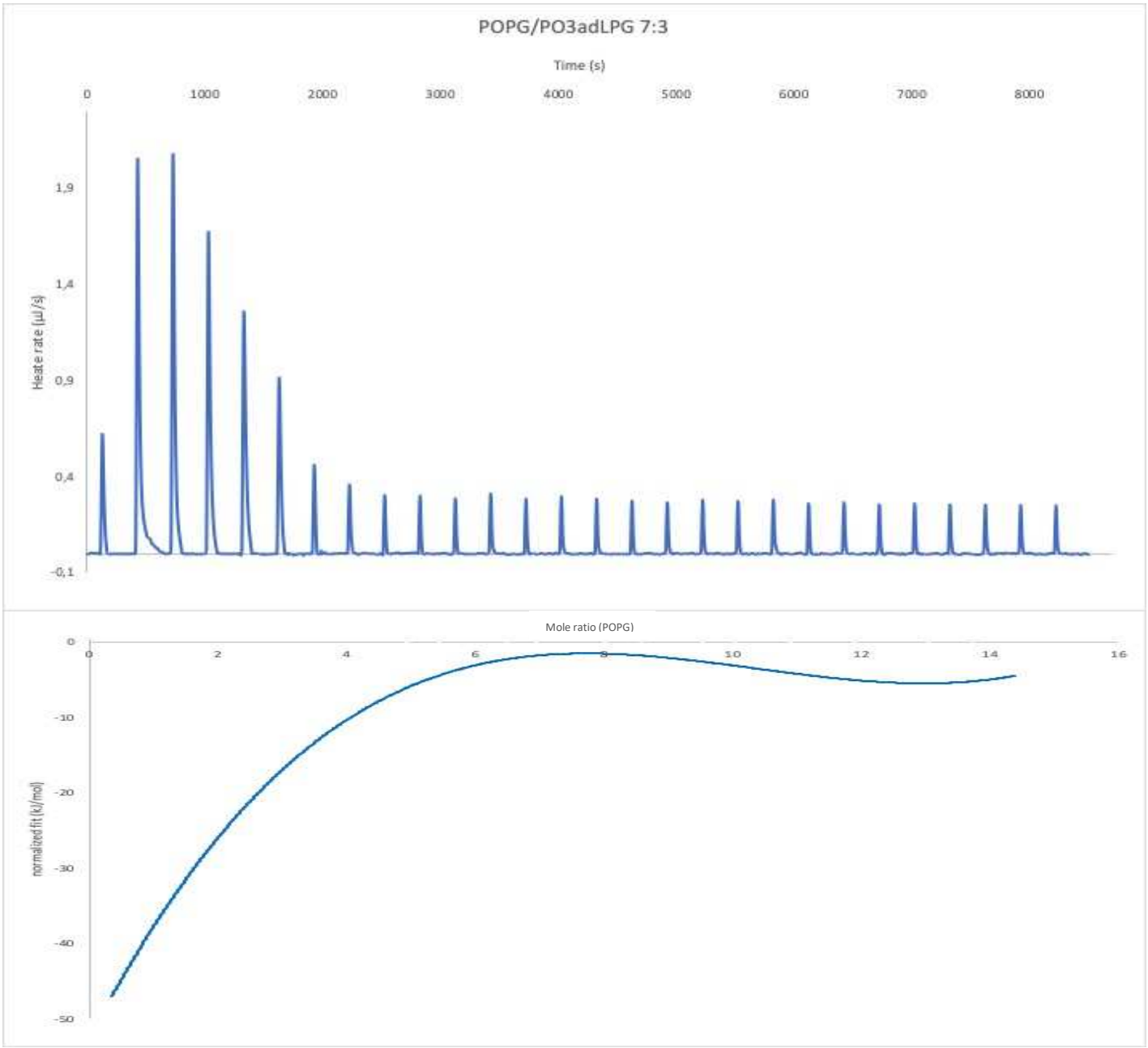


Figure 32 *ITC measurement of POPG/PO3adLPG 7:3*

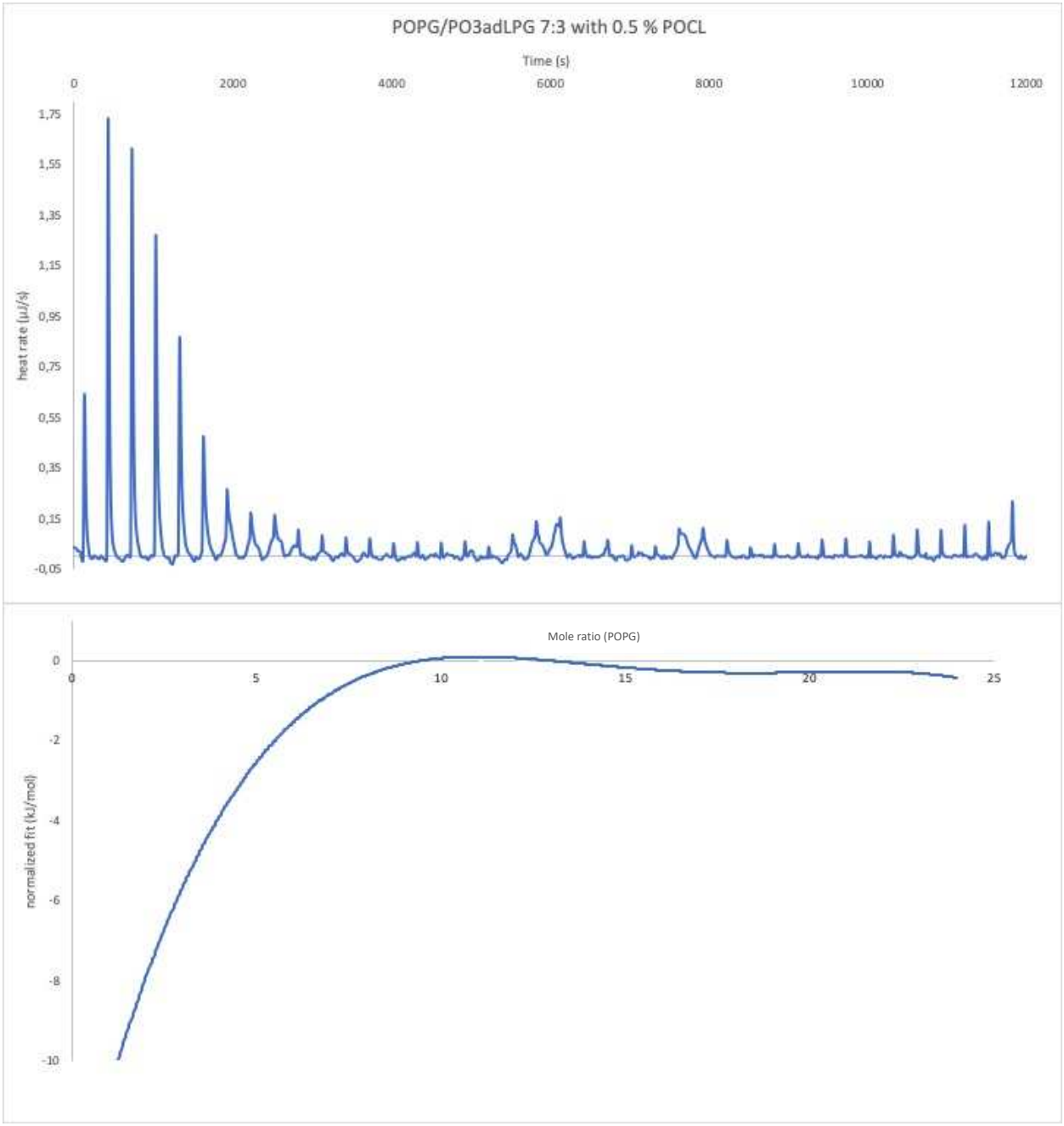


Figure 33 *ITC measurement of POPG/PO3adLPG 7:3 with 0.5 % POCL*

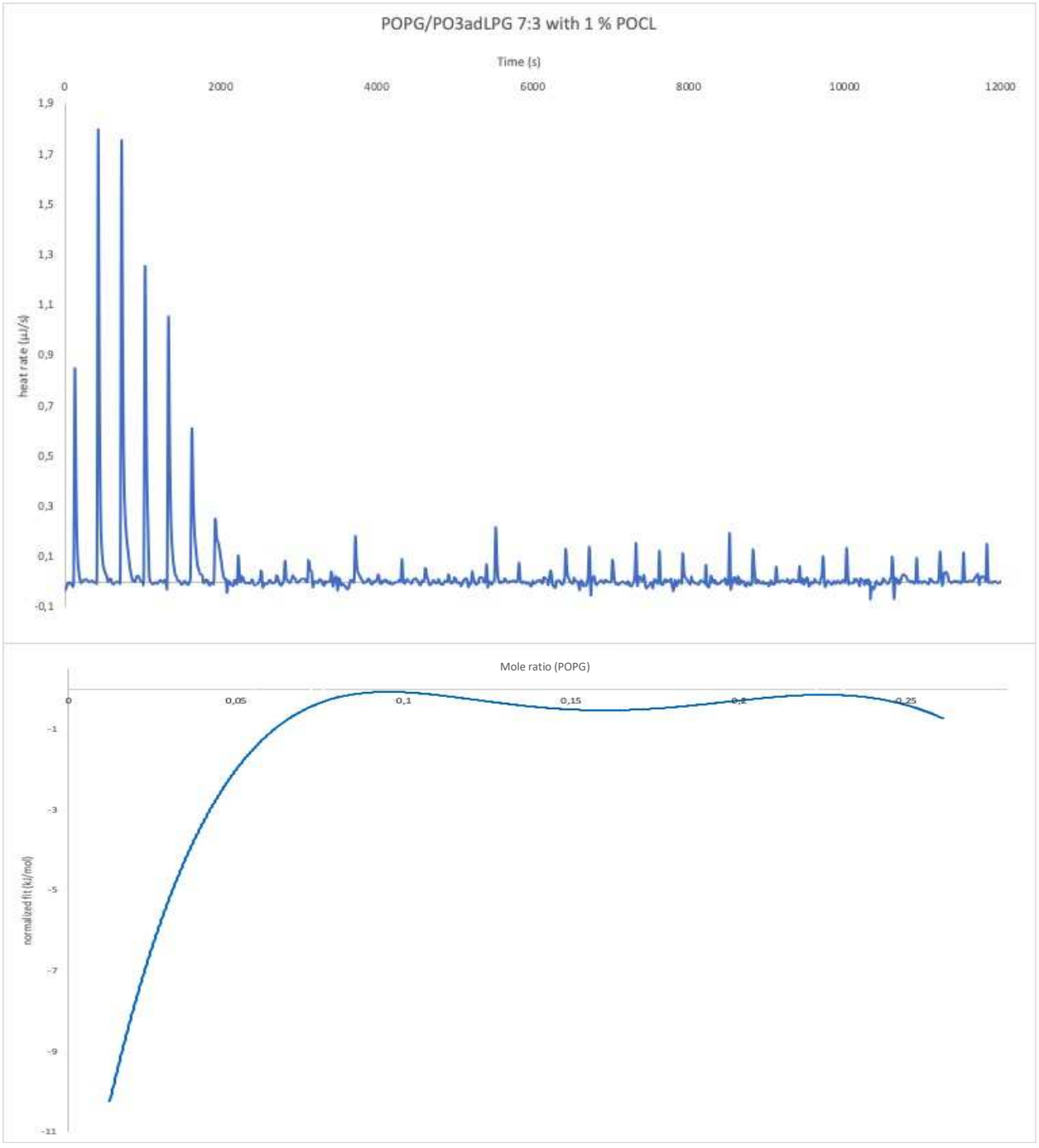
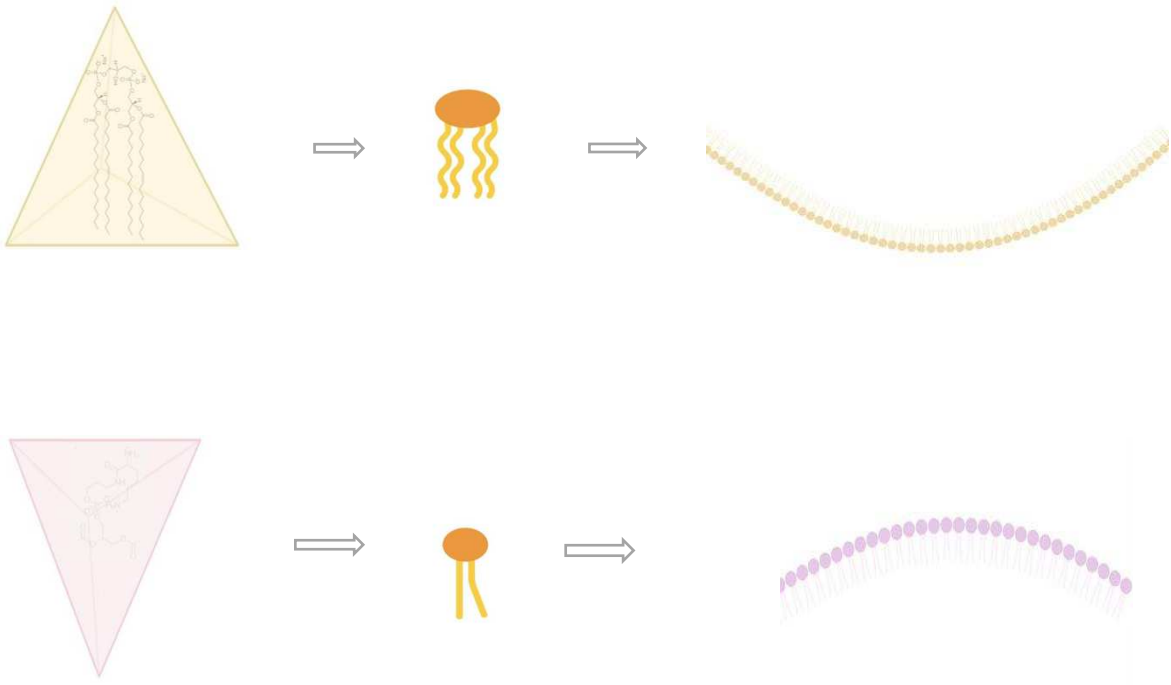


Figure 34 *ITC measurement of POPG/PO3adLPG 7:3 with 1 % POCL*

Discussion

The aims of this study were firstly to investigate the effect of different model staphylococcal membrane lipid mixtures on the mixing properties of the lipids, both with and without Ca^{2+}, and secondly to evaluate the effect of model membrane components on daptomycin interactions. The main focus is always on the lipid cardiolipin, to assess the effect of its presence on lipid packing and also on daptomycin binding.

To better understand the results of the experiments, it is helpful to imagine the lipids as small pieces of a puzzle. Not only do the electrostatic interactions and charges of the head groups determine the behavior of the different lipid mixtures, but the shapes of the individual lipids also play a crucial role. Although POCL constitutes only a small fraction of the membrane lipids in *Staphylococcus aureus*, it exerts a surprisingly significant influence on the model systems used in these experiments. This is due to its complex structure, which can subtly yet decisively affect the organization and stability of the lipid membrane. Lipid shapes determine how they arrange themselves relative to one another, how densely they can be packed, and what interactions they engage in—all factors that ultimately manifest in the experimental results.
Starting with CL, this lipid possess an overall inverted truncated cone because of its small head group relative to the four fatty chains [51], causing membranes with high CL content to adopt negative curvature [52] *(Figure 35).*
LPG *(Figure 35),* on the other hand, has exactly the opposite molecular shape compared to CL. With its larger head group compared to its smaller fatty chains, this lipid takes the shape of a truncated cone. Since POCL causes a negative curvature with its shape, it is expected that LPG facilitates positive curvature of the membrane [25], [52]. POPG, like PO3adLPG, has a cone-shaped structure, but its head group is somewhat smaller due to the absence of a lysyl group [22]. Since the POPG used in this thesis contains an unsaturated fatty acid chain, it tends to have a more cylindrical shape *(Figure 35).*



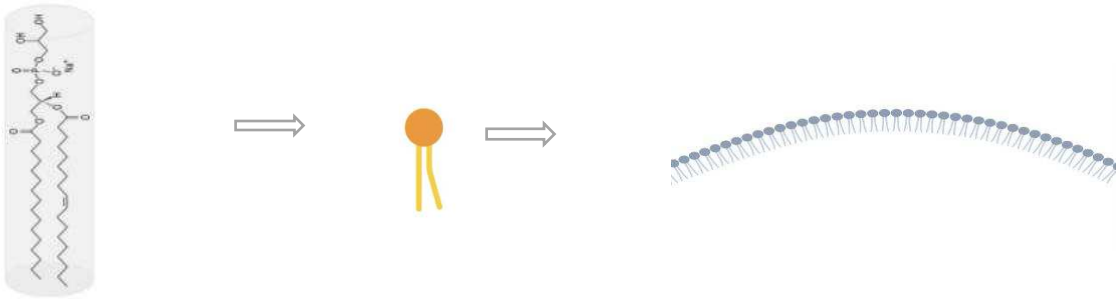


Figure 35 *A schematic illustration of POCL, PO3adLPG and POPG and their influence on the membrane curvature. Created in BioRender.com.*

In the pure POCL isotherm, it was observed that the molecular area is larger in the presence of Ca^{2+} than without. Normally, one would expect the molecular area to be smaller because CL and calcium ions have opposite charges, and the attractive forces should pack the lipids more tightly. Here, too, the structure of cardiolipin plays a significant role. This lipid is very flexible in its structure. CL has a negative charge shared by both phosphate groups in the head group, making them both negatively charged. In the presence of Ca^{2+}, this ion binds to the head group, and both phosphate groups surround Ca^{2+} due to the attractive forces, and as a result the chains splay outward, producing a larger molecular volume which in turn produces a larger area per molecule in the monolayer [29] *(Figure 36).*

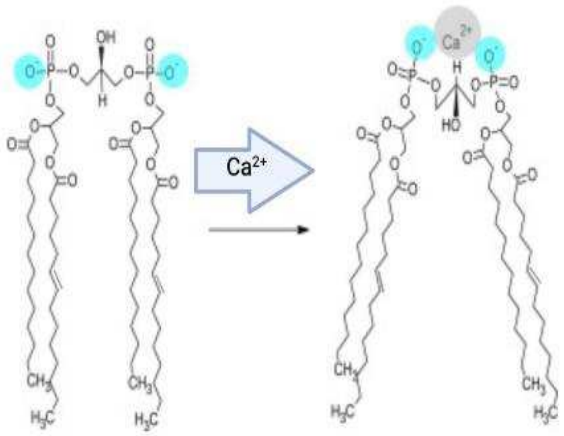


Figure 36 *The influence of calcium ion on the lipid chain splay of POCL; adapted from [29].*

In the lipid mixture of PO3adLPG and POCL, the average molecular area is smaller than that of pure cardiolipin (POCL), indicating that PO3adLPG restricts the freedom of movement of POCL molecules. This reduction in molecular area could indicate attractive interactions between the lipids, particularly because PO3adLPG fits as a 'puzzle piece' between the POCL molecules due to its complementary molecular shape. This leads to a denser packing of the lipids in the monolayer and an increased planarization of the membrane, even in the presence of calcium ions. These results are supported by the observation that the surface pressure is increased in the presence of Ca^{2+}, indicating a more stable structure at higher pressures *(Figure 37).*

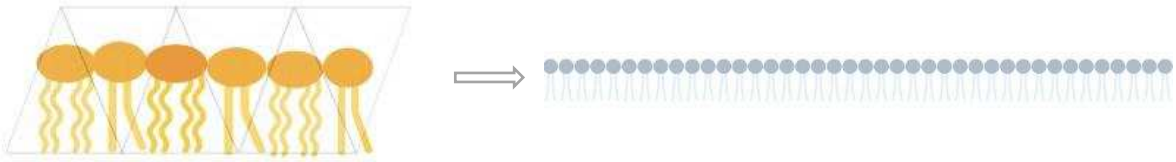


Figure 37 *An illustration of how the lipids POPG und PO3adLPG influence the membrane. Created in BioRender.com.*

The results show that in the binary lipid mixtures of POPG and PO3adLPG without Ca^{2+}, the isotherms lie in a narrower molecular area range compared to the pure lipids, which indicates a lower expansion of the lipids. In particular, the range between the lift-off pressure and the collapse pressure is narrower for the lipid mixtures than for the pure lipids, indicating a denser packing of the molecules. This effect could be due to the fact that PO3adLPG has a positively charged head group [24], whereas POPG has a negatively charged head group [26]. In the absence of calcium ions, these opposite charges lead to an electrostatic attraction between the molecules. When the head groups bind, the lipid tails splay outwards, resulting in a somewhat loose packing of the molecules. The addition of Ca^{2+} further reinforces a denser packing as the calcium ions act as bridges to POPG and partially neutralize the charge differences. This increases the packing density of the lipid molecules and increases the molecular surface area.

Another explanation is the difference in the size of the headgroups of PO3adLPG and POPG. The headgroup of PO3adLPG is larger than that of POPG due to the additional lysyl group [51]. This larger headgroup could cause the lipid molecules to be less tightly packed, which reduces the stability of the monolayer. Without Ca^{2+}, the surface compressional modulus shows that all mixing ratios remain in the liquid-expanded phase (LE phase) until a surface pressure of about 10 mN/m is reached. At a surface pressure of 30-35 mN/m, the POPG/PO3adLPG 1:2 ratio shows the lowest value of the surface compressional modulus, indicating that the larger headgroup of PO3adLPG leads to less stable lipid packing. As the proportion of POPG increases, whose smaller headgroup allows for tighter packing, the surface compressional modulus increases, indicating higher stability of the monolayer.

The influence of the lipid POCL in the ternary lipid mixtures, especially at higher concentrations of 16% and 20%, was recognizable. Increased POCL concentrations led to less expanded isotherms, indicating a denser lipid packing. This effect was especially pronounced in the absence of calcium ions, where the isotherms with 20% POCL exhibited higher collapse pressure stability with a small lift-off area, suggesting a dense and a stable lipid packing. As the POCL concentration increased without Ca^{2+}, it was observed that the compression work also increased, which indicates looser packaging.

One possible explanation for these results is that the overall molecular volume plays a significant role, especially with the presence of more 3adLPG. As the concentration of POCL increases, the increased molecular volume, rather than just hydrophobic interactions, likely leads to a denser packing of the lipid molecules. This denser packing would require more energy to compress the lipids, which could explain the increased compression work observed. Another hypothesis is that at a high POCL concentration, the ratio of cardiolipin to PO3adLPG becomes balanced. Since these two lipids are structurally complementary and possess opposite charges, this could result in denser lipid packing, aided by attractive electrostatic forces. This denser packing would also require higher compression work, as the fatty acid chains, due to the enhanced interactions, would attempt to extend outward. Evidence for this is seen in the higher surface compression modulus at 20 % POCL, indicating denser packing and greater monolayer stability, particularly in mixtures with high PO3adLPG content.
The influence of calcium ions was also noteworthy, as they led to larger lift-off areas, indicating less dense lipid packing. Calcium ions have a high affinity for the negatively charged head groups of lipids such as POCL and POPG. By binding to these head groups, calcium ions reduce the repulsion between the head groups, which might initially suggest closer lipid packing. However, the larger lift-off areas observed are likely due to increased tail disorder and potential phase separation within the lipid monolayer *(Figure 38)*, rather than reduced headgroup repulsion alone. This tail disorder and phase separation result in greater expansion and larger molecular areas in the lift-off regions.

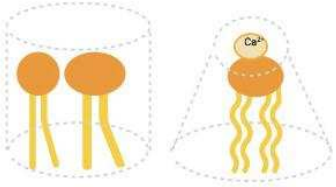


Figure 38 *Phase separation: spatial separation between POPG/PO3adLPG cluster and POCL/Ca^{2+} complex. Created in BioRender.com.*

A possible hypothesis is that calcium ions may facilitate crosslinking between the negatively charged POPG molecules. By forming bridges between two POPG molecules, calcium could reorganize the structure of the lipid monolayer, leading to a more flexible yet stable membrane. This crosslinking could stabilize the membrane without enforcing dense packing. Similar to the study by Wölk et al. (2020), which observed specific interactions between positively charged PO3adLPG, an analog of LPG, and negatively charged DPPG, calcium ions in my system could have similar stabilizing effects [23]. Interestingly, in the presence of calcium ions, the surface compression modulus values in lipid mixtures with high PO3adLPG content increase, despite both being positively charged. This could be explained by complex electrostatic interactions where calcium ions indirectly interact with PO3adLPG through negative groups of other lipids like POPG or POCL. These interactions could stabilize the membrane and increase the surface compression modulus values. Additionally, calcium near the PO3adLPG molecules might induce dipoles in the surrounding lipids, leading to attractive interactions that make the membrane denser and more stable. Finally, it is conceivable that calcium ions form specific bonds or complexes with certain groups within the PO3adLPG head groups, further stabilizing the membrane.

The zeta sizer measurements provided valuable insights into the behavior of the liposomes, particularly with regard to their size and surface charge. An important point is that the buffer used did not contain

$NaCl_2$, as the electrode would otherwise have been damaged during the measurement. However, the absence of $NaCl_2$ has a direct effect on the charge distribution of the liposomes. $NaCl_2$ would normally dissociate in the solution to form sodium ions (Na^+) and chloride ions (Cl-), which could shield the surface charges of the liposomes [53]. Without these ions, the charge on the liposome surface remains unshielded and has a stronger effect [53]. This means that the negative or positive charges of the liposomes are measured directly, resulting in stronger zeta potential values.
The results show that the liposomes with a higher proportion of PO3adLPG (7:3 ratio to POPG) were larger compared to the POPG-rich liposomes (7:3 ratio to PO3adLPG). This size difference can be explained by the larger head group of PO3adLPG, which contains an additional lysyl group, making the liposomes more voluminous. In addition, the data showed that liposomes with a higher proportion of PO3adLPG had a positive zeta potential, while those with a higher proportion of POPG had a negative zeta potential. This emphasizes that the charge of the liposomes strongly depends on the relative amount of positively and negatively charged lipids. Since the buffer does not contain $NaCl_2$, the charges on the liposomes remain completely unshielded [53], which makes the differences in zeta potential more visible.

The POPG-dominant liposomes showed that the zeta potential decreased under the influence of Daptomycin. Daptomycin binds together with calcium ions to the negative charges of POPG and neutralizes them through electrostatic interactions. This neutralization leads to a reduction in the negative surface charge, which makes the zeta potential less negative. At the same time, there was a slight increase in particle size, suggesting that Daptomycin may promote liposome aggregation. The absence of $NaCl_2$ could also play a role here, as there are no shielding ions to smooth out the new charge distributions on the liposomes [53]. This makes the change in zeta potential more pronounced. The addition of cardiolipin at concentrations up to 2 % had a minimal effect on the zeta potential. Since the liposomes already carry a strong negative charge due to the high proportion of POPG, POCL at these low levels does not add enough additional negative charge to significantly affect the zeta potential. Only at 5 % POCL was there a slight decrease in the zeta potential, which could indicate a more even distribution of cardiolipin in the membrane. However, the overall effect remains small, as the negative charge of POPG continues to dominate. Again, the absence of $NaCl_2$ enhances the effect of unshielded negative charges, which means that additional negative charges at low concentrations do not cause noticeable changes as the POPG membrane is already highly negatively charged. The combination of Daptomycin and cardiolipin led to an interesting behavior. Although POCL alone had little effect on the zeta potential, a noticeable effect was seen when added together with Daptomycin. In particular, at 0.5 % cardiolipin in combination with Daptomycin, the antibiotic interacted efficiently with the negative charges of POCL, which led to a strong decrease in the zeta potential. At higher cardiolipin concentrations of 1 %, 2 % and 5 %, the decrease in zeta potential was also present, but not as pronounced as with 0.5 % POCL. This could indicate a saturation effect, where Daptomycin is no longer able to completely neutralize all negative charges, especially at higher concentrations of cardiolipin. This saturation effect indicates that at lower concentrations of POCL, neutralization by Daptomycin is more effective as there are fewer negative charges to neutralize. However, at higher concentrations of cardiolipin, a greater amount of un-neutralized negative charges remain, which explains why the decrease in zeta potential is less pronounced at higher concentrations.

The addition of cardiolipin showed no significant effect on the zeta potential of the PO3adLPG-dominant liposomes. This could be due to the fact that the negatively charged cardiolipin is not sufficient at these concentrations to significantly influence the strong positive surface charge of the liposomes. PO3adLPG

remains the dominant lipid, and the positive charges predominate even with increasing cardiolipin concentrations. Since the buffer does not contain $NaCl_2$, which would normally equalize and screen the charges, the positive charges of PO3adLPG remain strongly pronounced and the addition of cardiolipin does not lead to a measurable change in the zeta potential.
Interestingly, the PO3adLPG-dominant liposomes showed a slight decrease in zeta potential with the addition of Daptomycin. Normally, Daptomycin binds preferentially to negatively charged lipids such as POPG, which are only present in small amounts in this liposome composition. A more likely explanation for the slight decrease in the zeta potential is that Daptomycin interacts directly with the negatively charged LPG at areas of high surface charge density. This interaction may involve the displacement of some calcium ions, as Daptomycin typically relies on calcium for binding to lipid membranes.

The study from Scott Taylor and Michael Palmer (2014) demonstrated in their study that as little as 10 % cardiolipin in liposome models is sufficient to inhibit the pore-forming activity of daptomycin, suggesting that an increased concentration of cardiolipin in bacterial cell membranes could potentially increase resistance to daptomycin [7]. However, in this study, only up to 1 % POCL was used, resulting in only minor effects on binding affinity. The ITC data, which show the interaction between the lipid components POPG/PO3adLPG 7:3 both without and with the addition of 0.5 % and 1 % POCL during the titration with daptomycin, indicate a slight influence of cardiolipin on binding behavior. As mentioned, POCL could cause a negative curvature of the membrane; however, its concentration relative to POPG and PO3adLPG is too low to bring about a significant change in membrane curvature. The reason why POCL, despite its complex structure, shows minimal changes could be that the electrostatic interaction and molecular geometry of the existing lipid components, POPG, already provide optimal binding sites for daptomycin, so the addition of POCL does not create additional binding sites and possibly POCL is not directly involved in resistance. On the other hand, POCL might have an indirect influence on binding affinity by altering the distribution of charges on the membrane surface, thereby changing the liposomes' binding ability for the antibiotic. Another hypothesis is that POCL might affect the packing density of the lipids in the membrane, even in small amounts. POCL has larger fatty acid chains, and its presence could disrupt the local organization of the lipids, leading to a slight change in the binding sites. A final hypothesis is that the concentrations of 0.5 % and 1 % POCL are too low to cause a noticeable change in the thermodynamic parameters.

Conclusion

The results of this study show the significant influence of the molecular shape and charge of individual lipids on the membrane structure and behaviour. While cardiolipin (POCL) is present in small amounts in the *Staphylococcus aureus* membrane, its complex structure plays a crucial role the lipid organization and stability [51]. The shape of an inverted truncated cone of POCL promotes negative membrane curvature, while other lipids like LPG and POPG contribute differently due to their different shapes and charge distributions [52]. One of the key observations was the impact of calcium ions on lipid packing. Calcium ions generally led to less dense lipid packing by neutralizing the negative charges on the lipid head groups, resulting in larger molecular areas. However, in ternary lipid mixtures, particularly at increased concentrations of POCL, a denser lipid packing was observed, suggesting that POCL can enhance hydrophobic interactions and possibly lead to stronger electrostatic attractions in the presence of complementary lipids like PO3adLPG. The study also provided insights into the interactions between daptomycin and the lipid mixtures. The results indicated that even a lower concentration of POCL could slightly influence daptomycin binding, although its effects were not as pronounced as previously thought. The ITC data suggested that the existing lipid components, POPG and PO3adLPG, already provide optimal binding sites for daptomycin, potentially diminishing the impact of POCL at the tested concentrations.
Overall, this work demonstrates the complex interplay between lipid composition, membrane structure, and antibiotic interaction. The findings contribute to a deeper understanding of how specific lipid components, especially cardiolipin, influence the membrane behavior and could inform future studies on bacterial resistance mechanisms and the development of more effective antimicrobial treatments. Further research with different concentrations and combinations of lipid components may provide additional insights into the role of cardiolipin in membrane dynamics and its interaction with antibiotics like Daptomycin.

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