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# Permeabilization of reconstituted Lipid Bilayers containing mycobacterial TDM through the AMP LL32: Insights into Membrane-Peptide Interactions

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**Abstract**

This study focuses on the permeabilization behavior of lipid bilayer membranes composed of natural and synthetically produced phosphatidylcholines, in the presence of mycobacterial glycolipid trehalose-6,6'-dimycolate (TDM). The experiments were conducted in the presence of the alpha-helical antimicrobial peptide (AMP) LL32, known for its ability to interact with and disrupt lipid bilayers. Reconstituted lipid bilayers were used to model the biological membrane and to investigate the influence of TDM on the interactions between LL32 and the lipid bilayer. By evaluating membrane integrity, the results provide insights into the effects of TDM on membrane stability and peptide-lipid interactions. This research helps to understand the modulation of membrane integrity by lipids and their role in peptide-mediated permeabilization, with implications for antimicrobial mechanisms and potential future therapeutic applications.

## I. Introduction

The human cathelicidin hCAP18, which originates from the intracellular granules of neutrophilic granulocytes, contains an antimicrobial peptide (AMP) called LL37 [1], [2]. The LL32 peptide fragment lacks an unstructured region of the full-length peptide, which may explain its enhanced antimicrobial activity. This cationic peptide can bind to and permeabilize membranes through electrostatic interactions [3],[4].

Phosphatidylcholines (PC) are a major component of eukaryotic membranes and are essential for maintaining their structure and function [5]. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) is a PC variant. PC and POPC differ in their composition and origin, which makes them valuable for studying membrane behavior. PC, which was derived from chicken egg yolk in this study, is a natural mixture of phosphatidylcholine molecules with varying fatty acid chains, closely resembling biological membrane diversity. In contrast, POPC is a synthetic, purified phospholipid with uniform fatty acid chains, providing a controlled and reproducible model system. Comparing these two lipids allows us to investigate the effects of molecular heterogeneity versus uniformity on membrane stability, permeability, and interactions with antimicrobial peptides like LL32.

Trehalose-6,6'-dimycolate (TDM), also known as *cord factor*, is a extraordinary large glycolipid found predominantly in the cell walls of mycobacteria, including *Mycobacterium tuberculosis*. TDM plays a critical role in the structural stability and functionality of mycobacterial membranes, contributing to their extraordinary resis-

tance and ability to endure harsh environmental conditions [6], [7]. This stability may explain why mycobacteria can persist in hostile environments and resist external stress factors.

Given its structural properties, TDM is hypothesized to be the key factor behind the high stability of mycobacterial membranes. To investigate this, TDM can be incorporated into model membranes composed of phosphatidylcholines (e.g. PC, POPC) that are serving as carrier lipids. By studying how the antimicrobial peptide LL32 interacts with and permeabilizes these membranes containing TDM, we aim to better understand the stabilizing role of TDM and its influence on AMP-membrane interactions.

## II. Methods and materials

The electrophysiological measurements were performed using the Orbit Mini device (Nanion Technologies GmbH, Munich, Germany), which uses MECA-4 chips (Ionera Technologies GmbH, Freiburg, Germany) to create planar lipid bilayers and record the current flow. These chips have a chamber in which 150 µL buffer can be added to. The chamber contains four channels, each with a diameter of 100 µm, allowing the simultaneous measurement of four experiments. The channels contain a integrated electrode and a silver/silver chloride reference electrode to establish a potential difference across the membrane for the measurements. With this method we investigated the permeabilization of lipid bilayers by adding the peptide LL32. Current flow through the membrane was measurable as a consequence of pore formation.

The buffer system was prepared using 100 mM NaCl and 10 mM HEPES at pH 7. All four lipid solutions (from now on called POPC, PC, POPC/TDM, PC/TDM) (POPC: Avanti 850457P, Alabama, USA; PC: Egg, Chicken, Avanti 840051P; TDM: Bioclot GmbH, Aidenbach, Germany) were prepared at a concentration of 1 mg/ml in octane. This was achieved by evaporating the original solvent (chloroform) under nitrogen and then adding octane as the new solvent. Octane was used as recommended by the manufacturer, as the experiments could only be conducted successfully with this solvent. It is important to note that the solvent will be incorporated into the membrane, thereby exerting an effect on the measurement.

For the peptide solution, the desired peptide was diluted in the aforementioned buffer solution to create a highly concentrated solution. Subsequently, the stock was diluted and utilised for the measurements to ensure the desired concentrations. Prior to the administration of the measurements, the solution was subjected to vigorous stirring in order to ensure complete dissolution and homogenous distribution of the components.

TDM was prepared in the same manner, and the appropriate volumes of the lipid solutions were combined to obtain solutions of POPC and PC, each containing 10 wt% TDM (POPC/TDM, PC/TDM). Lipids were introduced into the channels of the MECA-4 chip through pipetting, which resulted in membranes being formed in each channel with a diameter that matches that of the chip.

The quality of the membrane was tested by applying voltage pulses in ± 50 mV steps up to ± 150 mV. This was done to make sure that the membrane can withstand certain voltages without breaking and thus causing a current flow, so any changes in the current during the measurement can then be attributed to the membrane-peptide interaction. After this the membrane capacitance and resistance were measured. The membrane capacitance remained at 4000 ± 1000 pF across all measurements, while the current was below 1 pA. The LL32 peptide solution (Bartels, Borstel, Germany) was prepared in the before mentioned buffer and added to the *cis*-side of the chip. After this preparation, the measurements would begin.

Multiple measurements of the current flow through the membrane were performed at -60 mV for 20 minutes at a temperature of 37° C. The temperature was set with a temperature control unit. In the following, exemplary measurements of the conducted experiments are presented.

## III. Results and discussion

Figure 1 shows the current response (in nA) as a function of time (in seconds). (a) shows the current curve induced by pore formation in a POPC-membrane treated with 20 µM LL32 peptide at a constant voltage of -60 mV. (b) shows the current trace for a POPC/TDM-membrane. A comparison between the two membranes shows the significant reduction in pore formation when TDM is present in the membrane. While the membrane without TDM undergoes permeabilization with a current up to -195 nA, the current trace for the POPC/TDM-membrane shows a much lower peak with a maximum current not exceeding -40 nA.

Figure 2 shows the current response for a pure PC-membrane (a) and a PC/TDM-membrane (b) after the addition of 20 µM LL32 peptide at a constant voltage of -60 mV. In both cases, the current measured is greater than -200 nA, exceeding the measurement limit of the Orbit Mini device. However, there are two differences. For the pure PC-membrane, the current starts decreasing from -200 nA toward zero after 120 seconds, whereas for the PC/TDM membrane, this process takes over twice as long, beginning after approximately 350 seconds. The second difference is observed in the current curve during the decay phase, as shown in the smaller insert plots. For the pure PC-membrane (a), the current trace shows fluctuations, whereas for the PC/TDM-membrane (b),

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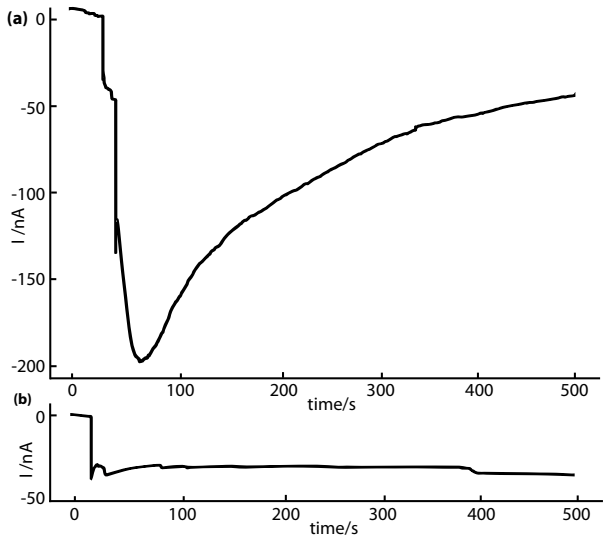


**Figure 1:** Current measurements were conducted at -60 mV for 20 minutes on a pure POPC membrane (a) and a POPC/TDM membrane (b) followed by the addition of LL32 in a 100 mM NaCl and 10 mM HEPES buffer system (pH 7). The peptide concentration was 20 µM. The peptide was added at t=0 s. The presented curves are representative of three measurements performed under identical experimental conditions. It is evident that the POPC/TDM membrane is more resistant to permeabilization compared to the pure POPC membrane, as indicated by the reduced current trace.

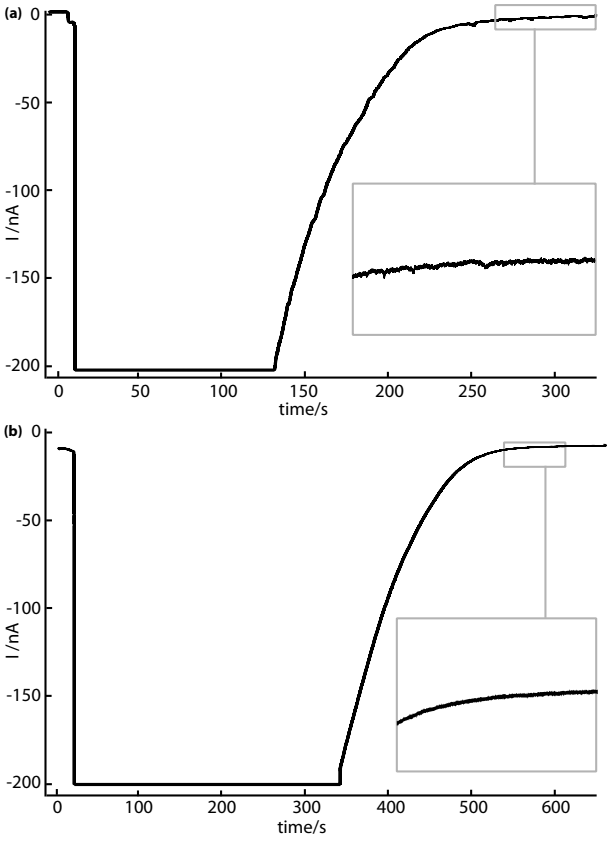


**Figure 2:** Current measurements were conducted at -60 mV for 20 minutes on a pure PC membrane (a) and a PC/TDM membrane (b) following the addition of LL32 in a 100 mM NaCl and 10 mM HEPES buffer system (pH 7). The peptide concentration was 20 µM. The peptide was added at t=0 s. The presented curves are representative of three measurements performed under identical experimental conditions. It is clear to see that the PC/TDM-membrane takes longer to recover after permeabilisation and to reassemble into an intact membrane. This recovery process also occurs with fewer fluctuations.

the current decreases more smoothly without major interuptions.

These observations suggest that a 10% TDM content in a PC-membrane is not sufficient to drastically reduce the current flow. However, TDM appears to prevent the healing of membrane defects, resulting in fewer disturbances during the process. This highlights the role of TDM in modulating membrane stability, although the reduction in current flow is not as pronounced as expected.

## III.I. Discussion

In this study, we investigated the effect of TDM on membrane permeabilization induced by the antimicrobial peptide LL32 in lipid bilayers composed of different phospholipids. The following is a summary and discussion of the data obtained.

### The Role of TDM in Membrane Permeabilization

The data obtained from the current measurements in the figures 1 and 2 show a significant reduction in membrane permeabilization when TDM is present inside the membrane. For example, in the case of the pure POPC-membrane (fig.1), the current increases rapidly indicating strong permeabilization by LL32. However, when TDM is present (10 wt%), the current only reaches a lower maximum. This large difference underlines the role of TDM in stabilizing the membrane and limiting the extent of permeabilization. The reduced current flow suggests that TDM interacts with the membrane in a way that inhibits the formation of pores induced by LL32. As Spargo et al. (1991) also hypothesise, the stabilisation of the membrane and the prevention of pore formation can be interpreted as the result of steric hindrance caused by the TDM [6]. Consequently, the peptide is unable to approach the membrane.

Furthermore, the observation that TDM does not completely prevent the permeabilization process, but rather moderates it, suggests that the effect of TDM on the membrane is more complex than simply blocking pore formation.

### The Role of TDM in Membrane Recovery and Stability

The results from the PC- and PC/TDM-membranes in figure 2 reveal additional insights into the stabilizing effects of TDM. After the addition of LL32, both membranes initially exhibit a sharp increase in current. The current of the pure PC-membrane decreases faster than in the case of the PC/TDM-membrane. Furthermore, the decline in current for the PC/TDM-membrane is smoother with fewer fluctuations. This suggests that TDM not only inhibits pore formation but also helps with the smoother reassembly of the membrane after permeabilization.

That the PC/TDM-membrane takes longer to recover from permeabilization may be because TDM is slowing the spontaneous aggregation of phospholipids into a fully intact bilayer. TDM could be preventing the rapid reorganization of the lipid molecules and thereby contributing to a more controlled repair of the membrane.

### Implications for Membrane Modulation

The results demonstrate that TDM acts as a stabilizer in lipid membranes. This could be if TDM forms specific interactions with the lipid bilayer, altering its properties in a way that reduces the size or frequency of pores formed by LL32 [6]. In addition, TDM may alter the phase behaviour of the membrane, preventing the rapid reorganization of lipids that often follows peptide-induced permeabilization. This could also explain the difference in observations between the POPC- and PC-membranes. It appears that LL32 induces a stronger permeabilization in the POPC-membrane, which could be attributed to its homogeneity. In contrast, the diverse composition of PC might hinder the function of TDM to some extent. This could suggest that TDM is more effectively incorporated into ordered membranes, like those formed by POPC, allowing it to exert its stabilizing effect more efficiently.

It should be mentioned that the measurement limit of the Orbit Mini and the octance as a solvent did on the one hand hide valuable data about the permeabilization and on the other hand change the properties and behaviour of both the lipids and the peptide. Therefore it is necessary to use alternative solvents and methods to support the acquired data.

## IV. Conclusion

In conclusion, our study highlights the complex interaction between antimicrobial peptides, phospholipids and glycolipids in modulating membrane integrity. The results indicate that TDM plays an important role in stabilizing membranes composed of different phospholipids, including POPC and PC, and reduces the extent of permeabilization induced by LL32. These findings provide valuable insights into the mechanisms of membrane permeabilization and stabilization, with potential applications in the design of therapeutic agents that require a fine-tuned control of membrane dynamics to reduce host-toxicity. Nonetheless further studies are required to deepen the understanding of the molecular interactions between TDM and lipid bilayers.

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## Author's statement

Authors state no conflict of interest. Informed consent has been obtained from all individuals included in this study. The research related to human use complies with all the relevant national regulations, institutional policies and was performed in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. DeepL was used for the linguistic fine-tuning of this article.

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