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# Daptomycin forms cation- and size-selective pores in model membranes

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ABSTRACT

Daptomycin is a lipopeptide antibiotic that is used clinically to treat severe infections caused by Gram-positive bacteria. Its bactericidal action involves the calcium-dependent binding to membranes containing phosphatidylglycerol, followed by the formation of membrane-associated oligomers. Bacterial cells exposed to daptomycin undergo membrane depolarization, suggesting the formation of channels or pores in the target membranes. We here used a liposome model to detect and characterize the permeability properties of the daptomycin pores. The pores are selective for cations, with permeabilities being highest for $Na^+$, $K^+$, and other alkali metal ions. The permeability is approximately twice lower for $Mg^{++}$, and lower again for the organic cations choline and hexamethonium. Anions are excluded, as is the zwitterion cysteine. These observations account for the observed depolarization of bacterial cells by daptomycin and suggest that under typical in vivo conditions depolarization is mainly due to sodium influx.



## 1. Introduction

Daptomycin is the first clinically approved lipopeptide antibiotic; it has been available for injection under the trade name Cubicin® since 2003 [1]. It is used to treat severe infections caused by Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis*, including strains that are resistant to β-lactam antibiotics and vancomycin [2,3]. Daptomycin consists of a cyclic peptide moiety with 10 amino acids, from which the N-terminal three amino acids protrude; the N-terminus carries a decanoyl fatty acyl side chain (Fig. 1). The antibiotic is extraribosomally synthesized by *Streptomyces roseosporus* [4,5] and contains several non-standard amino acids.

Daptomycin acts at the bacterial membrane. Various molecular targets and action modes have been proposed, including the inhibition of peptidoglycan [6] or lipoteichoic acid synthesis [7]. However, the only effect consistently reported in studies from different laboratories consists in the depolarization of the bacterial cell membrane [8–11]. Concomitantly with membrane depolarization, bacterial cells lose the ability to accumulate amino acid substrates, while leaving glucose uptake intact, indicating a selective nature of the membrane permeability defect [9]. No membrane discontinuities have been observed by electron microscopy [12], also supporting the notion that the functional membrane lesion is discrete and small.

Based on precedent from other membrane-damaging peptides and proteins, it was proposed early on that daptomycin acts through the formation of oligomeric transmembrane pores [10]; however, experimental evidence was only obtained more recently. Using various fluorescently labeled, functionally active daptomycin derivatives, oligomerization was observed both on model liposomes and on bacterial membranes [13–15], and it was subsequently shown that oligomerization is required for antibacterial action [16]. The model liposomes used in those studies contained only phosphatidylcholine (PC), which is largely inert to daptomycin [13], and phosphatidylglycerol (PG), which was required to induce binding at physiologically relevant calcium concentration, as well as to trigger oligomer formation.

It is noteworthy that the abundance of phosphatidylglycerol in bacterial cell membranes is also a major determinant in the susceptibility of bacteria to daptomycin [17–19]. It was therefore of interest to determine whether the PC/PG liposome model also suffices to support the formation of the functional daptomycin pore, and if so, to characterize the pore's permeability properties. This study reports the corresponding experiments. The results show that daptomycin forms discrete pores on liposome membranes that are selective for cations of limited size.

## 2. Materials and methods

### 2.1. Preparation of indicator-loaded large unilamellar vesicles

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1′-glycerol) (DMPG; both

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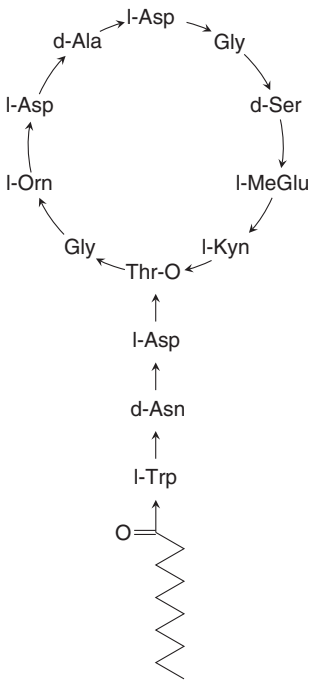


**Fig. 1.** Schematic of the structure of daptomycin. Arrows indicate amide bonds, except between kynurenine and threonine, where an ester bond between the carboxyl group of kynurenine and the side chain hydroxyl group of threonine closes the ring.

from Avanti Polar Lipids, Alabaster, AL, USA) were used to prepare the vesicles. Equimolar amounts of DMPC and DMPG were weighed into a round-bottom flask and dissolved in chloroform/methanol (3:1). The solvent mixture was then evaporated with nitrogen to produce a lipid film, which was further dried under vacuum for 3 h and then dispersed in buffer. The resulting lipid suspension was extruded through a 100 nm polycarbonate filter 15 times, using a nitrogen-pressurized extruder to produce indicator-loaded large unilamellar liposomes [20].

The buffers used for dispersing the lipid film contained either pyranine or dithio-bis-nitrobenzoic acid (DTNB) as an indicator for the permeabilization assays, and they varied in pH and salt composition as detailed in Table 1. Following polycarbonate membrane extrusion, the liposomes were subjected to gel filtration on a Bio-Rad P-6DG column (Bio-Rad, Richmond, CA, USA) in order to remove unentrapped indicator. The column buffers used in the gel filtration step matched those used for lipid film rehydration, except for the absence of pyranine or DTNB.

As indicated in Table 1, all loading buffers also contained 250 mM sucrose. This experimental detail was adopted from a previous study [21] and was found to improve the stability of the indicator-loaded liposomes.

### 2.2. Fluorescence measurements

With pyranine-loaded liposomes, time-based emission scans were acquired on a PTI QuantaMaster 4 system, the sample holder of which was thermostatted at 30 °C. Liposome suspensions were diluted to a final concentration of 250 μM of total lipids into reaction buffer with 5 mM $CaCl_2$. Daptomycin (Cubist), carbonyl cyanide m-chlorophenyl hydrazine (CCCP, Sigma), and valinomycin or gramicidin (Sigma) were added as indicated to final concentrations of 1 μM, 5 nM, 0.5 μM, and 10 nM, respectively, in order to initiate the reaction. The fluorescence emission at 510 nm was recorded for 5 min, with excitation at 460 nm. Triton X-100 was then added to a final concentration of 0.1%, followed by one more minute of recording; this was done in order to disrupt the liposomes and so to establish the fluorescence intensity equivalent to 100% test solute permeation.

With cationic test solutes, the reaction buffer was similar to the corresponding liposome hydration buffer (see Table 1), but the concentration of the test solute was increased to 100 mM, and the pH value from 6.00 to 8.00. With the anionic test solute ($Cl^-$), the reaction buffer was similar to its hydration buffer, but choline chloride was added to 100 mM, and the pH value was lowered from 8.00 to 6.00. Using the intrinsic fluorescence of the kynurenine residue, we confirmed that daptomycin binds quantitatively to the model membranes under these experimental conditions (see Fig. S1).

### 2.3. Spectrophotometric measurements

The permeation of thiols into the liposomes was measured through reduction of entrapped DTNB, which was measured by its absorption at 412 nm ($\varepsilon = 13{,}400\ M^{-1}\ cm^{-1}$). DTNB-loaded liposomes were diluted to a final concentration of 250 μM total lipids into the corresponding hydration buffer (Table 1) supplemented with 100 mM cysteine and 5 mM $CaCl_2$. 1 μM daptomycin was added to initiate the reaction.

### 2.4. Assessment of flow rate of different cations through daptomycin pores

To convert observed changes of pyranine fluorescence to permeation rates of cations, a calibration curve for pyranine fluorescence as a function of pH was recorded. 200 mL of buffer (5 mM MES, 5 mM Tricine, 5 mM NaCl, 5 mM KCl, 250 mM sucrose, pH 6.00; the same as used for the liposome interior when testing cation permeation) containing 2 μM pyranine was titrated with 1 M NaOH, while measuring both the pyranine fluorescence intensity at 510 nm and the pH value after each successive addition. The measured curve was fitted with the empirical polynomial function:

$$y = ax + bx^{1/2} + cx^{1/3} + dx^{1/4}.$$

The fitted function was then used as a calibration curve to convert the measured time-based fluorescence traces to changes in the intraliposomal proton concentration.

## 3. Results

### 3.1. Effect of daptomycin on cation permeability

The permeability of liposomes for cations was measured using a coupled fluorescence assay that is based on the pH-sensitive indicator pyranine, whose fluorescence increases with the pH as its phenolic OH group dissociates between pH 6 and 8.5 [21]. The experimental rationale, with potassium as an example, is illustrated in Fig. 2. Dilution of

**Table 1**
Buffers and indicators used to detect membrane permeabilization toward different solutes. The indicators were dissolved in the corresponding hydration buffer, and the solution was then used to rehydrate PC/PG lipid films. After extrusion of the lipid dispersion through polycarbonate membranes, unentrapped indicator was removed by gel filtration using the corresponding hydration buffer. In experiments with anionic or cationic test solute, pH gradients were established by diluting the liposome samples into reaction buffers with different pH levels. Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid) and DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) were obtained from Sigma.

| Test solutes | Hydration buffer | Entrapped indicator | pH value |
|---|---|---|---|
| Cations | 5 mM MES, 5 mM Tricine, 5 mM NaCl, 5 mM KCl, 250 mM sucrose | 5 mM pyranine | 6.00 |
| Anions | 5 mM MES, 5 mM Tricine, 5 mM NaCl, 5 mM KCl, 250 mM sucrose | 5 mM pyranine | 8.00 |
| Neutral solutes (thiols) | 20 mM HEPES, 150 mM NaCl, 250 mM sucrose | 5 mM DTNB | 7.00 |



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