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Oligomerization of daptomycin on membranes

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ABSTRACT

Daptomycin is a lipopeptide antibiotic that kills Gram-positive bacteria by membrane depolarization. While it has long been assumed that the mode of action of daptomycin involves the formation of membrane-associated oligomers, this has so far not been experimentally demonstrated. We here use FRET between native daptomycin and an NBD-labeled daptomycin derivative to show that such oligomerization indeed occurs. The oligomers are observed in the presence of calcium ions on membrane vesicles isolated from *Bacillus subtilis*, as well as on model membranes containing the negatively charged phospholipid phosphatidylglycerol. In contrast, oligomerization does not occur on membranes containing phosphatidylcholine only, nor in solution at micromolar daptomycin concentrations. The requirements for oligomerization of daptomycin resemble those previously reported for antibacterial activity, suggesting that oligomerization is necessary for the activity.

1. Introduction

Daptomycin is a lipopeptide antibiotic produced by *Streptomyces roseosporus*. It is a cyclic molecule with a decanoyl fatty acid side chain attached to the exocyclic, N-terminal single tryptophan residue [1]. It contains thirteen amino acid residues, some of which are non-standard ones, including a kynurenine and an ornithine (Fig. 1). Daptomycin possesses rapid bactericidal activity against a broad spectrum of Gram-positive pathogens [2,3]. It is active against difficult pathogens such as vancomycin-resistant staphylococci and enterococci [4]. Daptomycin is approved for clinical use in the treatment of *Staphylococcus aureus*, including MRSA, in bacteremia, right-sided endocarditis, and complicated skin and skin structure infections.

The bactericidal activity of daptomycin is mediated by membrane permeabilization and depolarization [5]. It is calcium-dependent and applies to both resting and proliferating cells [6]. Susceptibility of bacteria to daptomycin is correlated with the membrane content of phosphatidylglycerol; it is decreased by mutations that promote the conversion of phosphatidylglycerol to lysylphosphatidylglycerol [7]. On bacterial cell membranes, fluorescently labeled daptomycin localizes to phosphatidylglycerol-rich membrane areas [8].

Membrane-permeabilizing proteins and peptides commonly form oligomeric structures on the target membrane. Daptomycin oligomers have been detected in solution at relatively high (0.75 mM) concentrations, and the formation of oligomers prior to membrane binding has been interpreted as an integral step in daptomycin activity [9]. However, there is as yet no experimental evidence of daptomycin oligomers on lipid membranes.

In the present study, we have examined the oligomerization of daptomycin both in solution and on membranes. To this end, we used daptomycin derivatives fluorescently labeled with NBD (7-nitro-2,1,3-benzoxadiazol) and with Alexa Fluor 350. Oligomerization was detected through fluorescence resonance energy transfer (FRET) as well as through the self-quenching of NBD fluorescence. We detected oligomerization on liposome membranes containing phosphatidylglycerol, combined with either phosphatidylcholine (PC) or phosphatidylethanolamine (PE) plus cardiolipin (CL), as well as on membrane vesicles prepared from *Bacillus subtilis* (ATCC 6633) cells. In contrast, no oligomerization was observed with liposomes containing phosphatidylcholine alone, nor in solution at daptomycin concentrations similar to those required for antibacterial activity. The requirements that we find for oligomerization correlate with those for membrane permeabilization and bacterial susceptibility [1,7], suggesting that the membrane lesion is caused by the daptomycin oligomer.

2. Materials and methods

2.1. Preparation of NBD–daptomycin

Unlabeled daptomycin was supplied by Cubist Pharmaceuticals Inc. (Lexington, MA, USA). NBD-Cl (4-chloro,7-nitro-2,1,3-benzoxadiazol, or 7-chloro-4-nitrobenzofurazan) was obtained from Fluka (Fluka, St. Louis, MO, USA). All other chemicals were obtained from Bioshop (Burlington, ON) and were of analytical grade. Daptomycin (0.6 mM) was dissolved in 50 mM sodium borate buffer (pH 8)

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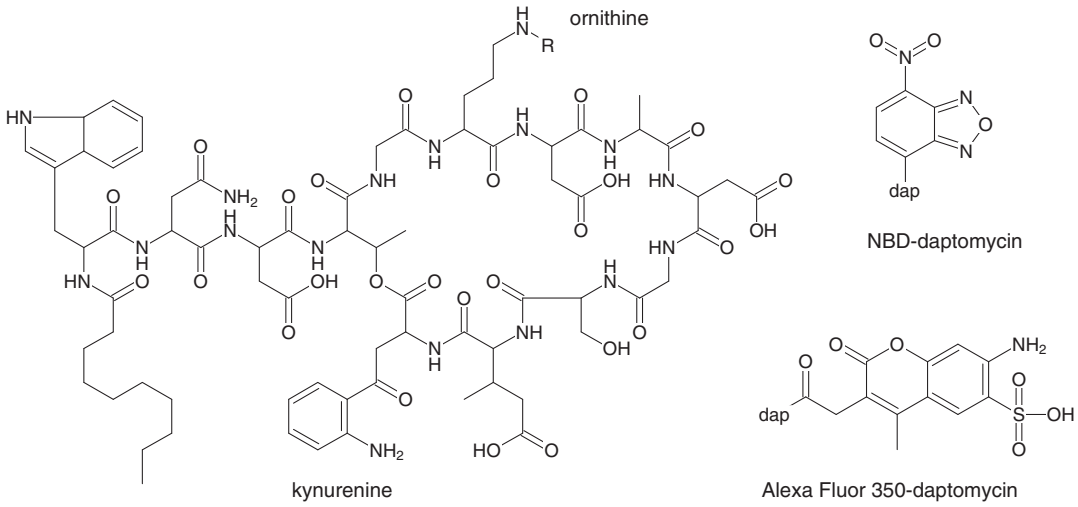

Fig. 1. Structure of daptomycin and of the labeled derivatives used in this study. The decanoyl residue is attached to the exocyclic N-terminal tryptophan residue. The ornithine residue carries the sole free amino group, which is free in daptomycin (R = H) but labeled with the fluorophores NBD or Alexa Fluor 350 in the two derivatives. The kynurenine residue possesses intrinsic fluorescence.

containing 20 mM EDTA. NBD-Cl (25 mM) was dissolved in acetonitrile. 300 µl of daptomycin solution and 100 µl of NDB-Cl solution were mixed in a reaction vial and incubated at 60 C for two hours. The mixture was then cooled in an ice water bath for 2 min, and finally acidified by adding 400 µl of acetic acid (50 mM) to stop the labeling reaction.

An HPLC system (Waters 625 LC) was used to purify the labeled daptomycin on a reversed-phase column (Agilent, Eclipse XDB-C8, 150 × 4.6 mm). The mobile phase was 20 mM ammonium acetate (pH 5.5) with an acetonitrile gradient from 30 to 40% and a flow rate of 1 ml/min [10,11]. Fractions were examined by spectrophotometry, spectrofluorometry (see below), and mass spectrometry using a Micromass Q-TOF Ultima GLOBAL mass spectrometer. The molecular weight of the purified NBD-labeled daptomycin determined by mass-spectrometry was 1783.7 Da. This corresponds to the expected molecular weight of 1784 Da for the stoichiometric adduct (Fig. 1). To determine the concentration, we assumed a molar extinction coefficient at 476 nm of 24,000 as previously determined for the NBD-adduct of alanine [12].

2.2. Preparation of Alexa Fluor 350–daptomycin

Daptomycin trifluoroacetate salt (20.34 mg, 0.013 mmol) was dissolved in DMF at 0 °C. Alexa Fluor-350 succinimide (Molecular Probes, 5 mg, 0.013 mmol) was added and stirred for 5 min. Hünig's base (*N,N*-diisopropyl-ethylamine) was added and the reaction mixture was stirred for 60 min. The reaction solution was poured into a flask containing cold diethylether (35 ml), which led to precipitation. The precipitate was collected by centrifugation (3500 rpm for 5 min), washed with diethyl ether and again recovered by centrifugation. The material was dried under high vacuum then purified via HPLC.

2.3. Preparation of large unilamellar vesicles (LUV) and of bacterial membrane vesicles

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DMPG), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanol (POPE), 1,2-dioleoyl-sn-glycerol-3-phospho-(1-rac-glycerol) (DOPG), and 1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (cardiolipin; TOCL) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The required amounts of DMPC and DMPG, or of POPE, POPG and cardiolipin, were weighted, dissolved in chloroform and transferred to a round-bottom flask. The solvent was evaporated with nitrogen and the resulting lipid films further dried under vacuum for three hours. The lipids were then suspended in HEPES buffer (20 mM, pH 7.4) containing 150 mM sodium chloride. Finally, the suspension was extruded 10–15 times through 100 nm polycarbonate filters, using a nitrogen-pressurized extrusion device [13]. The liposomes were employed in the fluorescence experiments at a final concentration of 250 µM of lipid. Bacterial membranes were prepared from *Bacillus subtilis* ATCC 6633, grown in LB broth, according to the procedure described in Ref. [14], except that the Potter homogenization was omitted, and the membranes were treated with a probe sonicator on ice for 2 min instead. Again as in [14], the membrane vesicles obtained were quantitated by assaying for membrane protein using the Lowry method.

2.4. Antibacterial activity test for daptomycin and NBD–daptomycin

NBD–daptomycin and unlabeled daptomycin were tested for antibacterial activity by broth dilution. LB broth was supplemented with calcium (5 mM) and daptomycin and NBD–daptomycin, respectively, at concentrations of 10, 5, 3, 2, 1.5, and 1 µg/ml. Growth controls without antibiotic were also included. Each tube was inoculated with *Bacillus subtilis* ATCC 6633 and incubated overnight at 37 °C. Growth inhibition was evaluated visually by turbidity.

2.5. Fluorescence studies

Emission spectra and time-based scans were acquired on a QuantaMaster 4 spectrofluorimeter (Photon Technology International, London, ON). Kynurenine fluorescence was excited at 365 nm and the emission recorded from 400 to 600 nm. NBD fluorescence was excited at 465 nm and the emission recorded from 490 to 600 nm. Alexa Fluor 350 was excited at 350 nm and emission recorded from 400 to 600 nm. Bandpasses for excitation and emission were between 2 and 5 nm. The steady state spectra shown were acquired at 37 °C; spectra acquired at room temperature were virtually indistinguishable.

The concentrations of calcium, where present, were 200 mM with PC liposomes and 5 mM in all other cases. For the calculation of the Förster radius [15] for kynurenine and NBD on PC/PG membranes, the quantum yield of kynurenine (0.039) was obtained by comparison to a quinine sulfate standard. The spectral overlap integral for donor emission and acceptor absorbance was determined from the absorption spectrum of NBD–daptomycin (see Fig. 2) and the emission

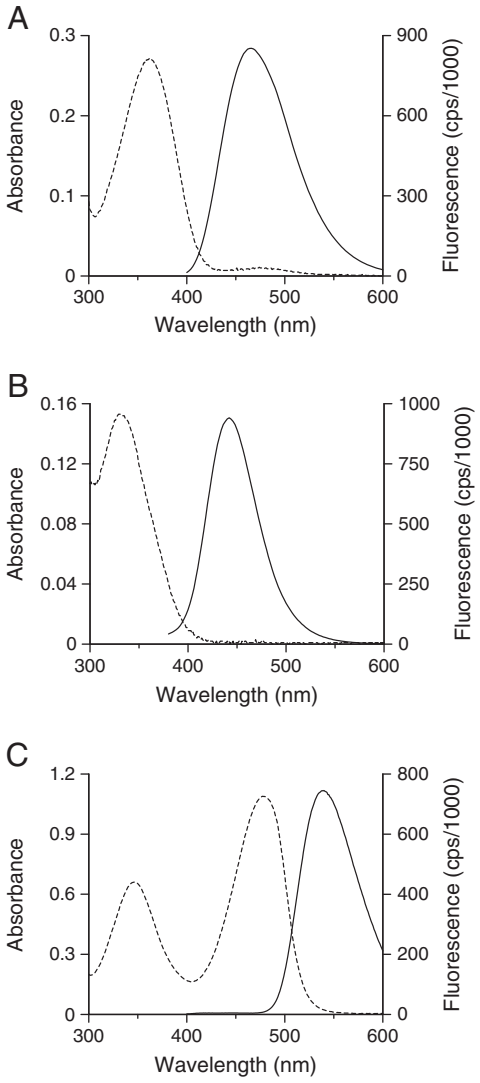

Fig. 2. Absorbance (dashed lines) and fluorescence emission (solid lines) spectra of (A) daptomycin, (B) Alexa Fluor 350–daptomycin and (C) NBD–daptomycin. The kynurenine residue in daptomycin causes the absorbance peak around 360 nm and the emission peak at 460 nm. The absorbance and emission peaks of Alexa Fluor 350–daptomycin occur in similar positions. NBD–daptomycin has an absorbance peak at 475 nm that overlaps the emission peaks of the other two; this overlap causes fluorescence resonance energy transfer (FRET) from daptomycin and Alexa Fluor 350–daptomycin to NBD–daptomycin. Direct excitation at 475 nm allows the observation of NBD fluorescence alone.

spectrum of daptomycin on DMPC/DMPG membranes in the presence of calcium, with correction for the wavelength-dependent sensitivity of the spectrofluorimeter. For the orientation factor κ^2, a value of 2/3 was used, which applies in the commonly assumed case of randomly distributed orientations of donor and acceptor [15].

Fluorescence lifetime measurements were performed on a FluoTime 100 Lifetime spectrometer using a P-C-370 diode laser light source (PicoQuant, Berlin, Germany). The lifetime instrument did not have a thermostatted cell holder, and therefore lifetime measurements were performed only at room temperature. Kynurenine emission was isolated using a 460 ± 10 nm band pass filter (Melles-Griot, Brossard, QC). Experimental decays were numerically fit with three exponentials, with resulting χ^2 values typically below 1.2. From these three components, average lifetimes were calculated according to the equation

$$\langle \tau \rangle = \frac{\sum \alpha_i \tau_i}{\sum \alpha_i}$$

were α_i represents the amplitude at time zero and τ_i the lifetime of the ith component.

3. Results

3.1. Characterization of NBD–daptomycin

The absorption and emission spectra of purified NBD–daptomycin are shown in Fig. 2C. The main absorbance peak of NBD–daptomycin occupies a very similar spectral position as the kynurenine fluorescence emission peak of unlabeled daptomycin (Fig. 2A). This spectral overlap indicates that fluorescence energy transfer (FRET) will occur from kynurenine to NBD. Indeed, when the kynurenine residue in NBD–daptomycin is excited (at 365 nm), the only fluorescence peak observed is that typical of NBD, indicating that all photons absorbed by kynurenine are transferred to NBD through FRET.

The antibacterial activity of NBD–daptomycin was identical to that of unmodified daptomycin (MIC = 3 μg/ml). Alexa Fluor 350–daptomycin was found to be 8- to 16-fold less active than unlabeled daptomycin.

3.2. Calcium-dependent membrane interaction of daptomycin and NBD–daptomycin

It has previously been reported that the kynurenine fluorescence of daptomycin increases in intensity and undergoes a blue shift upon interaction with calcium and negatively charged phospholipid membranes, composed of phosphatidylcholine (PC) and phosphatidylglycerol (PG). Such changes are observed with many fluorophores upon transition from a more polar environment to a less polar one, such as the hydrophobic interior of a lipid bilayer. A similar change in the fluorescence signal also occurs on neutral (PC) membranes. However, the gain in intensity is less, and the position of the fluorescence peak is different from that observed on PC/PG membranes, suggesting a different environment of the kynurenine residue and, hence, a different conformation of the daptomycin molecule [16,17]. Also note that with PC alone the fluorescence change occurs only at far higher concentrations of calcium.

NBD–daptomycin also gains in fluorescence intensity in the presence of membranes and calcium (Fig. 3). The fluorescence gain appears larger on PC membranes than on PC/PG membranes, although the opposite is actually true after NBD self-quenching on PC/PG membranes is accounted for (compare Fig. 5, below). The spectral difference indicates a different environment of the labeled residue (ornithine) on the two types of membranes.

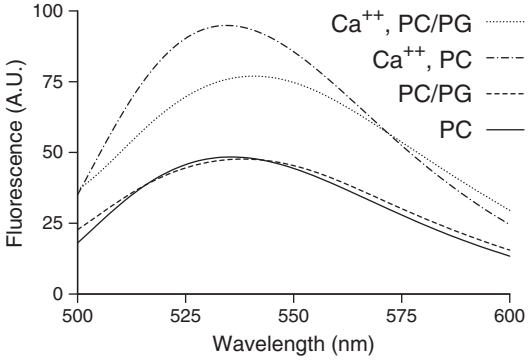

Fig. 3. Fluorescence of NBD–daptomycin (0.91 μM) incubated with liposomes, with or without calcium. The liposomes consisted of phosphatidylcholine and phosphatidylglycerol (PC/PG) or phosphatidylcholine only (PC). The excitation wavelength was 465 nm. The two samples without calcium are scaled to the same maximum intensity; the two samples containing calcium were scaled accordingly, so that the relative gain in intensity upon addition of calcium can be compared.

3.3. Detection of daptomycin oligomerization on membranes by FRET

The efficiency of FRET is distance-dependent, and it can approach 100% if the distance between the donor and an acceptor is well below the Foerster radius (R_0). For kynurenine in unlabeled daptomycin, bound to DMPC/DMPG membranes, as the donor and NBD–daptomycin as the acceptor, we estimated R_0 as 2.7 nm (see Materials and methods). Since the daptomycin molecule is smaller than that, formation of hybrid oligomers from daptomycin and NBD–daptomycin should cause FRET from the unlabeled to the labeled species. However, some degree of FRET should also occur without oligomerization, due to the proximity of some donors and acceptors when both are randomly distributed in the membrane plane [18].

To distinguish between these two effects, we measured FRET at different ratios of daptomycin to NBD–daptomycin, while keeping the absolute concentration of NBD–daptomycin constant. The rationale of this experiment is as follows: if donors (daptomycin) and acceptors (NBD–daptomycin) remain monomeric and separate, the efficiency of FRET, as measured by the reduction of donor emission, varies only with the acceptor concentration but not the donor concentration [18]; hence, the extent of FRET should not vary between the samples in our experiment. On the other hand, if donor and acceptor form hybrid oligomers, the probability for a donor to end up within close proximity to an acceptor molecule will increase with the relative amount of acceptors present. Therefore, at a fixed concentration of NBD–daptomycin, the efficiency of FRET should increase with decreasing concentration of unlabeled daptomycin.

FRET can be observed both with time-resolved (Fig. 4A) and with steady-state fluorescence measurements (Fig. 4B). In the time-resolved experiment, an increase in FRET corresponds to a decrease in the donor's excited state lifetime. Fig. 4A shows that, on DMPC membranes, the lifetime is almost the same at donor-acceptor ratios of 16:1 and 1:1, indicating no change in FRET and therefore no oligomerization. In contrast, on PC/PG membranes, the same change in the donor to acceptor ratio causes a fourfold reduction in the kynurenine lifetime, indicating that oligomerization occurs on these membranes.

Also shown for comparison are the lifetimes of unlabeled daptomycin alone. On both PC and PC/PG membranes, the kynurenine lifetime is significantly longer than with those samples that contain the equivalent amount of donor but with NBD–daptomycin present. This difference reflects FRET between molecules that are not part of the same oligomers, and thus is not an indication of oligomerization.

In steady-state fluorescence spectra (Fig. 4B), the kynurenine peak was much lower with mixtures of daptomycin and NBD–daptomycin than with daptomycin only, indicating extensive FRET. This FRET should occur in part within oligomers and in part between oligomers. When daptomycin and NBD–daptomycin were applied to PC/PG membranes sequentially, with time allowed for the oligomerization of NBD–daptomycin before addition of the daptomycin, the drop in donor intensity was reduced by half. The most likely interpretation is that in this sample the labeled and the unlabeled molecules had formed separate oligomers, thus removing the component of FRET that occurred within hybrid oligomers, and leaving only the component between separate oligomers. The spectra of such samples did not change substantially over two hours, suggesting that most oligomers remained intact over this length of time.

3.4. Detection of oligomerization by NBD self-quenching; oligomerization on bacterial membranes

Like many other fluorophores, NBD undergoes self-quenching at high concentration, which likely involves FRET from monomeric NBD molecules to non-fluorescent aggregated pairs [19]. In oligomers consisting of NBD–daptomycin only, the local concentration of NBD groups, and thus the likelihood of aggregation will be higher than in mixed oligomers with unlabeled daptomycin.

Therefore, the fluorescence signal of the same amount of NBD–daptomycin – after direct excitation at 465 nm, eliminating any FRET from kynurenine – should increase in the presence of unlabeled daptomycin. This is indeed observed with PG/PC membranes (Fig. 5A). On the other hand, without oligomerization, the extent of self-quenching should be unaffected by the presence of unlabeled daptomycin, and therefore the NBD-fluorescence should remain unchanged. This is indeed the case with PC alone (Fig. 5B), which confirms that oligomerization does not occur on these membranes.

The composition of *Bacillus cereus* and *Bacillus subtilis* cell membranes has been modeled with liposomes consisting of cardiolipin (TOCL, 17%), phosphatidylglycerol (DOPG, 40%) and phosphatidylethanolamine (POPE, 43%) [20,21]. On such liposome membranes, oligomerization is again observed (Fig. 5C). The same applies to membrane vesicles prepared from *Bacillus subtilis* bacterial cells (Fig. 5D).

3.5. Daptomycin does not form oligomers in solution at antimicrobially active concentrations

It has been previously reported that daptomycin forms oligomers in solution in the presence of calcium. This was observed by both NMR [9,16] and ultracentrifugation [22], and accordingly the concentration of daptomycin was in the millimolar range. Fluorescence allows the observation of oligomerization at much lower concentrations, similar

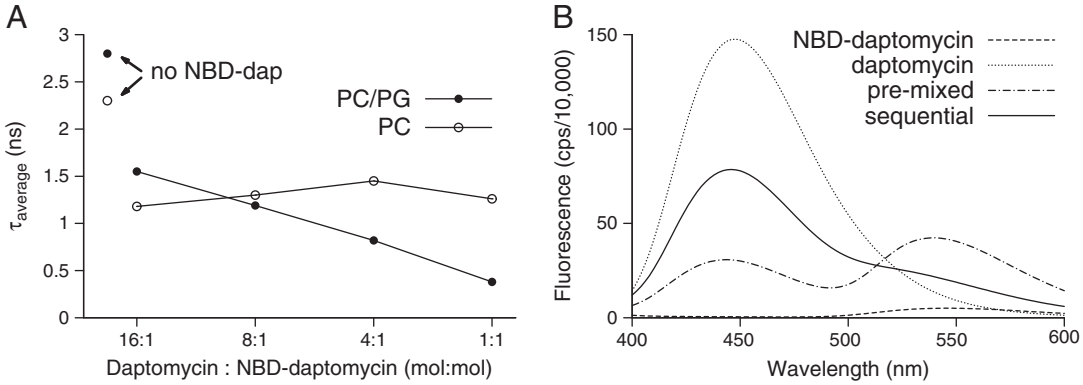

Fig. 4. FRET experiments on the oligomerization of daptomycin. A: Kynurenine fluorescence lifetimes in mixtures of daptomycin and NBD–daptomycin, on PC/PG and PC membranes with saturating concentrations of calcium. The concentration of NBD–daptomycin was 1.36 μM, where present; the concentration of unlabeled daptomycin is implied by the indicated molar ratios. The two unconnected data points were obtained with unlabeled daptomycin only. B: Fluorescence emission spectra of daptomycin (4.8 μM), and of NBD–daptomycin (0.91 μM), either alone or together, on DMPC/DMPG membranes. In the *premixed* sample, daptomycin and NBD–daptomycin were mixed before addition of membranes and calcium. In the *sequential* sample, daptomycin and calcium were added to the membranes and incubated for 30 min before application of NBD–daptomycin.

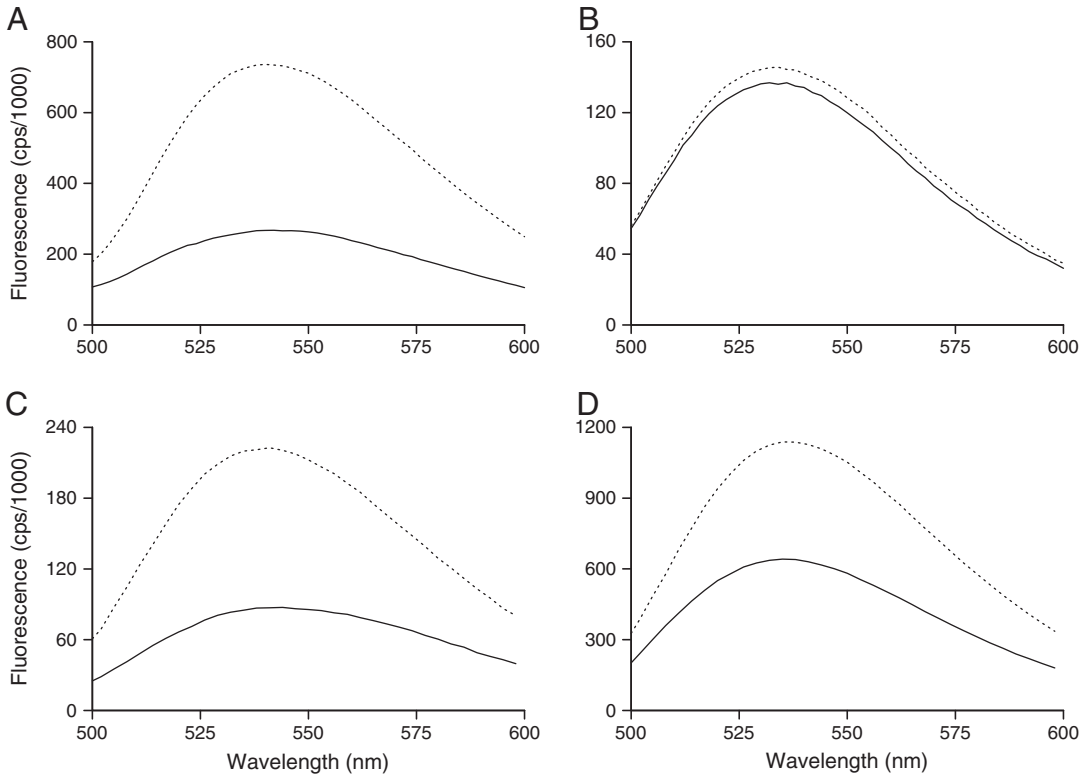

Fig. 5. Fluorescence of NBD–daptomycin (0.91 μM) on (A) DMPC/DMPG liposomes, (B) PC liposomes, (C) POPE/DOPG/TOCL liposomes and (D) *Bacillus subtilis* membrane vesicles, without (solid lines) or with (dashed lines) a fourfold excess with unlabeled daptomycin. Since the excitation wavelength (465 nm) was outside the absorbance spectrum of kynurenine, differences in intensity are not due to FRET from kynurenine but to NBD self-quenching within oligomers.

to those required for antimicrobial activity. Because kynurenine has a very low fluorescence intensity in aqueous solution, we here used daptomycin labeled with Alexa Fluor 350, which has a high intensity in both polar and apolar environments. The spectral positions of excitation and emission of Alexa Fluor 350 are such that this labeled derivative can again be combined with NBD–daptomycin in FRET experiments [23].

Fig. 6A shows the interaction of Alexa Fluor 350–daptomycin and NBD–daptomycin in solution and on PC/PG membranes, in the presence of calcium. On membranes, Alexa fluorescence is strongly reduced by FRET to NBD, which is consistent with oligomerization. In contrast, no FRET occurs when the membranes are missing. This indicates that no oligomerization is happening; calcium-mediated oligomerization in solution therefore is restricted to much higher concentrations and not germane to the antimicrobial effect of daptomycin (without NBD–daptomycin, the fluorescence of Alexa Fluor 350–daptomycin on membranes is still somewhat lower than without membranes, suggesting that Alexa Fluor 35o is subject to some degree of self-quenching upon oligomerization, too).

Fig. 6B shows the interaction of Alexa Fluor 350–daptomycin and NBD–daptomycin on PC membranes, with and without calcium. With calcium, there is FRET, albeit less than on PC/PG membranes. Since no oligomerization occurs on PC, this FRET is due to membrane binding of both Alexa Fluor 350–daptomycin and NBD–daptomycin; membrane

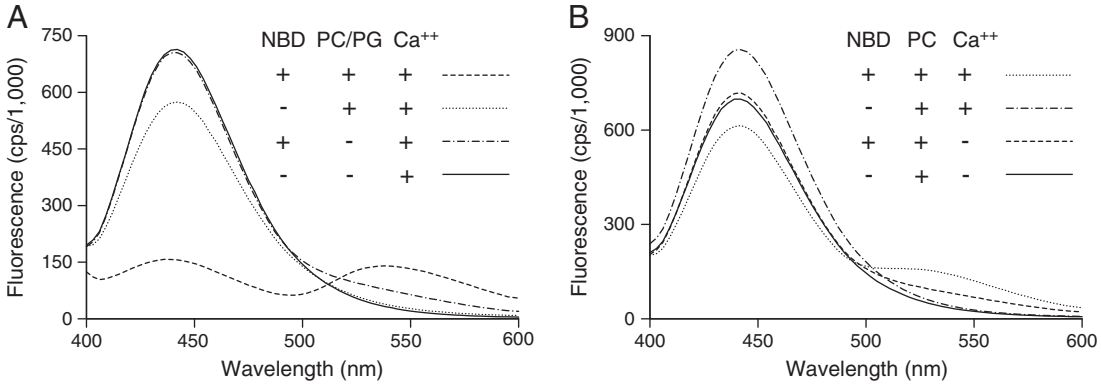

Fig. 6. Requirements for daptomycin membrane binding and oligomerization. A: Alexa Fluor 350–daptomycin (0.175 μM; A) was incubated with Ca^{++} (3 mM), with or without NBD–daptomycin (0.88 μM; N) and with or without PC/PG membranes (125 μM; M). The two labeled species readily form mixed oligomers on PC/PG membranes, as evident from the suppression of Alexa Fluor fluorescence through FRET. Without membranes, no FRET is observed, indicating absence of oligomerization. B: Alexa Fluor 350–daptomycin (A) was incubated with PC membranes (M), with or without NBD–daptomycin (N) and with or without Ca^{++} (200 mM; all other concentrations as in panel A). In the presence of both PC membranes and calcium, Alexa Fluor 350–daptomycin undergoes an increase in fluorescence intensity, which is likely due in part to kynurenine. In contrast, with the two labeled species, the emission at 450 nm is diminished through FRET. Since daptomycin does not oligomerize on PC membranes, this FRET is due to membrane binding alone. The absence of FRET in the sample with NBD–daptomycin but without calcium indicates absence of membrane binding.

binding brings them much closer to each other than they are in solution. No FRET occurs with PC but without calcium, indicating that calcium is required for membrane binding.

4. Discussion

While it has been proposed previously that daptomycin forms oligomers on membranes, this study provides the first such experimental evidence. Oligomerization on membranes correlates with the presence of phosphatidylglycerol, which coincides with the involvement of phosphatidylglycerol in bacterial susceptibility to daptomycin [7] and suggests that the daptomycin oligomer is the functional membrane lesion.

Our findings can be summarized in the model of daptomycin activity that is shown in Fig. 7. In this model, binding of calcium to daptomycin causes a conformational change that in turn triggers membrane binding, or, at sufficiently high concentration, oligomerization in solution [16]. Membrane-bound daptomycin then interacts with phosphatidylglycerol, resulting in a second conformational transition that leads to the formation of the oligomer. We propose that the oligomer forms the functional membrane lesion, possibly enclosing a central aperture as is the case with many pore-forming proteins and peptides, although differences in the rate of daptomycin-induced membrane depolarization [5] and the absence of non-specific membrane permeabilization [24] suggest that a classic pore may not be formed.

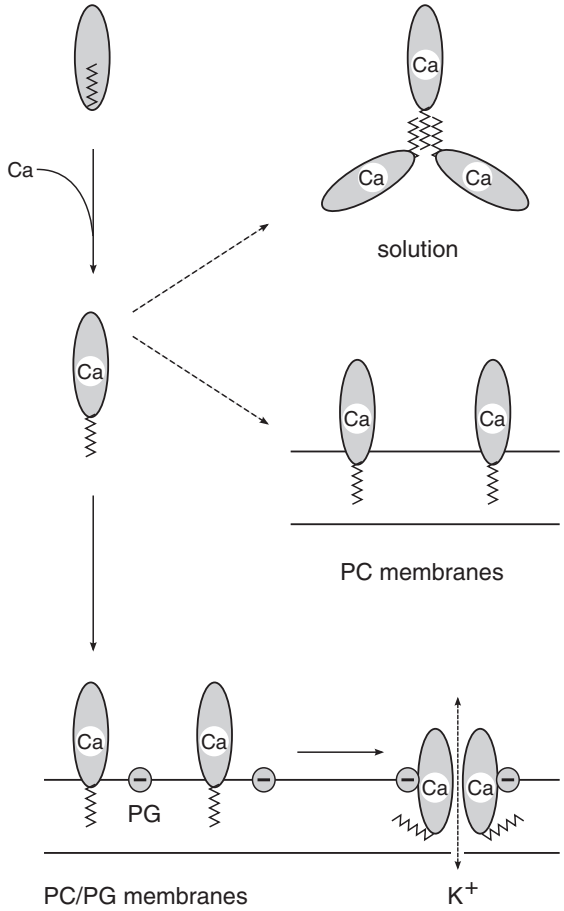

Fig. 7. Model of daptomycin oligomerization on membranes. Calcium binds to daptomycin in solution and causes a conformational change, which at high concentration facilitates oligomerization in solution. It also facilitates binding to PC membranes, on which however no oligomerization occurs. On PC/PG membranes, as well as on bacterial membranes, the interaction of daptomycin/calcium with the negatively charged headgroup of phosphatidylglycerol induces a second conformational change that induces oligomerization and deeper membrane insertion.

The key aspects of this model, in particular the roles of calcium in membrane binding and of phosphatidylglycerol in oligomerization, are supported by evidence; however, several details remain hypothetical. The postulated changes in conformation are inferred from those in function. The initial change triggered by calcium binding may lead to an increased exposure of hydrophobic moieties, possibly the N-terminal acyl chain; such a change would promote both oligomerization in solution and binding to membranes. The second change, triggered by phosphatidylglycerol, is depicted as causing not only oligomerization but also deeper membrane penetration. Both kynurenine [17] and NBD in NBD–daptomycin show a greater gain of fluorescence intensity on PC/PG membranes than on PC membranes, indicating a more hydrophobic environment on the former. However, on PC/PG membranes, daptomycin will not only interact with surrounding lipid molecules but also other daptomycin molecules within the oligomer that forms only on these membranes. It should also be noted that PC/PG membranes may be subject to lateral phase segregation, which is further promoted by calcium ions. If this occurs, membranes may become leaky, which may expose additional sites for hydrophobic interaction. For these reasons, the increase in environmental hydrophobicity cannot be unequivocally ascribed to deeper membrane penetration.

In Fig. 7, the interaction of daptomycin with phosphatidylglycerol is assumed to be mediated by calcium. Daptomycin has only one intrinsic positive charge, which is the amino group in the side chain of the ornithine residue. With NBD–daptomycin, as well as with previously characterized ornithine-modified derivatives [25,26], even this single charge is masked (see Fig. 1); nevertheless, these derivatives retain antimicrobial activity, which leaves calcium as the only available positive charge available to interact with the negative charge of phosphatidylglycerol. Not many more conclusions can be drawn as to the structure of the oligomer, other than that it is fairly stable on a time scale of minutes to hours, as indicated by the experiment shown in Fig. 4B. The structure of the membrane-associated oligomer thus remains to be determined.

Regarding the experimental strategies used in this study, it should be noted that FRET between daptomycin (or Alexa Fluor 350–daptomycin) and NBD–daptomycin can be quite extensive even in the absence of oligomerization, as is evident from Figs. 4A, B and 5C, D. The extent of FRET will also vary with the ratio of total daptomycin to membrane lipids, and it will also be affected by uneven distribution of membrane-bound daptomycin, as has been demonstrated by fluorescence with bacterial cells [8]. In contrast, the relief of self-quenching of NBD–daptomycin, caused by the addition of unlabeled daptomycin, is much less sensitive to such influences, as long as the amount of NBD–daptomycin remains constant. Without oligomerization, the addition of unlabeled daptomycin should cause no change in quenching, since the unlabeled daptomycin would simply not interfere with the NBD–daptomycin in any way. In contrast, with oligomerization, the addition of unlabeled daptomycin must reduce quenching, since within the hybrid oligomers the NBD–daptomycin molecules will be less likely to closely interact with one another. Therefore, the more robust quenching assay was preferred to detect oligomerization on bacterial membranes.

In summary, we have shown that daptomycin oligomerizes on liposomal and bacterial membranes. The requirements for oligomerization resemble those established for membrane permeabilization, suggesting that the oligomer is the functional membrane lesion. The detailed structure of the oligomer remains to be established.

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