Estimation of the subunit stoichiometry of the membrane-associated daptomycin oligomer by FRET

Jawad K. Muraih and Michael Palmer*

Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada

*Corresponding author. Email: mpalmer@uwaterloo.ca

Abstract

Daptomycin is a lipopeptide antibiotic that kills Gram-positive bacteria by depolarizing their cell membranes. This antibacterial action of daptomycin is correlated with the formation of membrane-associated oligomers. We here examine the number of subunits contained in one oligomer using fluorescence resonance energy transfer (FRET). The results suggest that the oligomer contains approximately 6 to 7 subunits, or possibly twice this number if it spans both membrane monolayers.

Introduction

The lipopeptide daptomycin is an important reserve antibiotic for the treatment of infections by otherwise resistant strains of Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* [1-3]. Its bactericidal effect involves membrane depolarization due to permeabilization for potassium ions [4].

Daptomycin has recently been shown to form oligomers on bacterial membranes and on liposome membranes containing phosphatidylglycerol [5, 6]. Oligomerization is dependent on calcium. The requirements of oligomerization for both calcium and phosphatidylglycerol mirror those of bactericidal action [7], which suggests that the oligomer forms the functional membrane lesion, as is the case with many other membrane-permeabilizing peptides and proteins.

At high concentrations and in the presence of calcium, daptomycin also undergoes oligomerization in solution. Such oligomers have been structurally characterized by NMR and by density gradient centrifugation, and they reportedly consist of 14-16 subunits [8]. On the other hand, the action of daptomycin has also been compared to that of cationic antimicrobial peptides, many of which do not form discrete oligomers but rather follow the carpet model of membrane destabilization [9]. In the current study, we therefore set out to determine whether the membrane-associated oligomer has a defined subunit stoichiometry.

Daptomycin contains several non-standard amino acid residues. One of these, kynurenine, exhibits an experimentally useful intrinsic fluorescence that responds with a significant gain in quantum yield to membrane interaction [10]. Another one, ornithine, carries the molecule's single free amino group. Derivatization of this ornithine residue can be used to prepare

fluorescent derivatives such as NBD-daptomycin, which retains antibacterial activity and forms hybrid oligomers with unlabeled daptomycin [5]. In NBD-daptomycin, kynurenine fluorescence is entirely suppressed due to fluorescence resonance energy transfer (FRET) from kynurenine to NBD. Moreover, in mixed oligomers, FRET also occurs between unlabeled daptomycin and NBD-daptomycin. The experimental approach used here to determine the subunit stoichiometry of the membrane-bound oligomer is based on the latter effect. The results suggest that the membrane-associated oligomer is smaller and structurally different from the oligomer observed in solution.

Materials and Methods

Materials. Large unilamellar liposomes (LUV) were prepared from an equimolar mixture of dimyristoyl-phosphatidylcholine (DMPC) and dimyristoyl-phosphatidylglycerol (DMPG) by extrusion through polycarbonate membranes as described [5]. NBD-daptomycin was obtained by reaction of daptomycin with nitrobenzoxadiazole-chloride (NBD-chloride, Fluka) and purification by HPLC also as described before [5]. Unlabeled daptomycin was supplied by Cubist Inc (Lexington, MA). Other reagents were of analytical grade and were obtained from Bioshop (Burlington, ON).

Fluorescence measurements. Samples contained sample buffer (HEPES 20 mM, 150mM NaCl, pH 7.4), LUV (250 μM total lipid), calcium chloride (6 mM), and either perylene-daptomycin [6] or daptomycin and NBD-daptomycin in various proportions and total concentrations. Steady state fluorescence emission spectra were obtained on a PTI QuantaMaster 4 instrument. For daptomycin and NBD-daptomycin, the excitation wavelength was 365 nm, and emission was recorded between 400 and 600 nm. For perylene-daptomycin, the excitation wavelength was 430

nm, and emission was recorded from 440 nm to 600 nm. Excitation and emission slit widths were adjusted to keep the maximal emission recorded below 10^6 counts/second; this latter value was determined to be within the linear range of the instrument's response.

Determination of the corrected relative kynurenine fluorescence of daptomycin in mixed oligomer samples. The purpose is to measure the kynurenine fluorescence of unlabeled daptomycin molecules within oligomers that have formed from mixtures of daptomycin with NBD-daptomycin, relative to the fluorescence of the same number of daptomycin molecules within oligomers formed without NBD-daptomycin, and corrected for FRET between pure daptomycin oligomers and NBD-daptomycin-containing oligomers.

Mixtures of daptomycin and NBD-daptomycin, in various proportions as indicated in the Results section, were prepared and added at a combined final concentration of 10 μM to liposomes and calcium and incubated for 180 seconds to induce oligomerization before measuring the kynurenine emission.

To correct for FRET between (as opposed to within) oligomers, 4 μM unlabeled daptomycin was then added to the same sample and allowed to oligomerize for 180 seconds, and the kynurenine fluorescence was read again. The rationale for this calibration procedure is explained in the Results section. The relative kynurenine fluorescence was calculated using the following equation:

$$F_r = \frac{d_2}{d_1}\left(\frac{I_1 - I_0}{I_2\frac{V_2}{V_1} - I_1}\right) \qquad (1)$$

In equation (1), F_r is the relative kynurenine fluorescence, and d_1 and d_2 are the molar amounts of

unlabeled daptomycin added in the first and second step, respectively. I_0, I_1 and I_2 are the emission intensities at 445 nm of a liposome blank, and of the oligomer sample after the first and second addition of daptomycin, respectively. V_1 and V_2 are the sample volumes before and after that addition, respectively; the term V_2/V_1 corrects for the dilution caused by the second addition of daptomycin, which amounted to 4% of the sample volume.

Numerical simulation of hetero-oligomer formation. The simulation shown in Figure 5 was implemented as a Python program. The code of the program, with explanations, is contained in the supplementary file to this manuscript.

Results

Rationale of the experimental approach to the determination of oligomer subunit stoichiometry. The experimental approach used in this study is based on fluorescence resonance energy transfer (FRET) between the kynurenine in unlabeled daptomycin and NBD-daptomycin in hybrid oligomers. FRET involves the kynurenine residue in unlabeled daptomycin as the donor, and the NBD moiety of NBD-daptomycin as the acceptor. We made the following assumptions:

1. All daptomycin oligomers have the same number of subunits, n.
2. Conversion of monomeric daptomycin to oligomers is quantitative on the time scale of the experiment.
3. When native daptomycin and NBD-daptomycin are mixed before application to membranes, they will form oligomers randomly, without any positive or negative mutual discrimination.

4. In hybrid oligomers, the kynurenine fluorescence of all unlabeled daptomycin molecules is completely quenched by FRET. Therefore, any remaining kynurenine fluorescence originates from oligomers that consist of unlabeled daptomycin only.

5. Oligomers are stable on the time scale of the experiment.

Let d be the fraction of native daptomycin in a mixture with NBD-daptomycin (whose fraction is 1-d). After this mixture has undergone oligomerization according to conditions 1 and 3, the fraction of oligomers that contain only native daptomycin subunits but no NBD-daptomycin will be d^n . Then, the fraction D of all native daptomycin molecules from the original mixture that were incorporated into these donor-only oligomers will be given by

$$D = \frac{d^n}{d} = d^{n-1} \tag{2}$$

According to assumption 4, only the oligomers represented by D will emit observable kynurenine fluorescence. Therefore, the corrected relative kynurenine fluorescence F_r of a given amount of daptomycin that was mixed with NBD-daptomycin before oligomerization, as defined in equation (1), equals D. Substituting F_r for D in equation (2) and solving or n yields

$$n = 1 + \log_d(F_r) = 1 + \frac{\ln(F_r)}{\ln(d)} \tag{3}$$

Therefore, we can obtain the subunit stoichiometry of the oligomer by comparing the kynurenine fluorescence of mixed oligomers to that of pure native daptomycin oligomers.

In the following, we will first present some experiments that test the assumptions underlying the experimental approach, followed by the experiments to measure the oligomer subunit

stoichiometry.

Kinetics and linear range of the fluorescence signal. Interaction of daptomycin with membranes causes a steep increase in the fluorescence emission of its single kynurenine residue [10]. Figure 1A shows the time course of kynurenine fluorescence after addition of daptomycin to PC/PG liposomes and calcium. The increase in the fluorescence signal is rapid and largely complete after 300 seconds. While the fluorescence increase suggests that the kynurenine residue has entered a more hydrophobic environment, such as the apolar interior of a lipid membrane, it may under certain conditions also be observed in the absence of oligomerization [5] and therefore does not prove that oligomerization has indeed occurred. Oligomerization itself can be monitored with perylene-daptomycin by way of concomitant formation of perylene excimers [6]. The fluorescence of perylene-daptomycin at 560 nm is predominantly due to excimers [6]. Figure 1B shows that, as with the kynurenine fluorescence, the perylene excimer fluorescence develops and stabilizes very rapidly after the addition of perylene-daptomycin to liposomes and calcium. Therefore, oligomerization proceeds rapidly and reaches completion on the time scale of the experiment.

Figure 1C shows that the kynurenine emission signal is linear across the range of concentrations and under the measurement conditions of the experiment. Thus, the kynurenine emission can be used to measure the amount of fluorescence-emitting oligomers.

Kynurenine fluorescence of daptomycin in the presence of NBD-daptomycin. When daptomycin and NBD-daptomycin are mixed and then added to membranes, the kynurenine fluorescence of the unlabeled species is strongly suppressed (Figure 2A). The strong reduction of the kynurenine emission in this sample suggests that most unlabeled daptomycin has indeed been incorporated

into hybrid oligomers (assumption 3), and that its kynurenine fluorescence is effectively quenched within these hybrids (assumption 4).

Figure 2A also shows the fluorescence of a sample that was prepared sequentially, such that unlabeled daptomycin was applied to the membranes first, and NBD-daptomycin was applied 3 minutes later. Under these conditions, daptomycin and NBD-daptomycin should form oligomers separately. Here, the kynurenine signal is higher. It remains virtually unchanged after 10 minutes, indicating that the two oligomer species have remained separate and have not rearranged into hybrid oligomers; thus, individual oligomers are stable on this time scale (assumption 5).

On the other hand, the kynurenine fluorescence of the sequentially prepared sample in Figure 2A is below that of a sample containing no NBD-daptomycin at all. This indicates that, in the sequentially prepared sample, the kynurenine fluorescence of the daptomycin-only oligomers is partially quenched by FRET to NBD-daptomycin oligomers in the vicinity. Raising the ratio of lipid to daptomycin reduces but does not eliminate FRET between oligomers (Figure 2B).

For our intended use of equation (3), we need to determine the FRET that occurs *within* hybrid oligomers, as opposed to *between* them. According to assumptions 2 and 5, we can correct for FRET between oligomers experimentally by adding a fixed quantity of unlabeled daptomycin to the same sample after the hybrid oligomers have been formed. The principle of this correction method is illustrated in Figure 3A; its details are given in the Methods section.

The experiments described so far support our assumptions 2 and 5. Assumptions 3 and 4 are supported to a degree: Hybrid oligomer formation and strong quenching by FRET are evident, but perfect randomness of oligomerization and quantitative FRET are hard to prove, as is the

uniformity of the oligomer subunit stoichiometry (assumption 1). For now, these latter assumptions may be taken as working hypotheses; we will revisit them in the Discussion.

Kynurenine fluorescence in hybrid oligomers formed on membranes. In the experiments shown in Figure 4, NBD-daptomycin was combined with unlabeled daptomycin at various molar ratios; the total of both species was kept constant at 10 μM. The mixtures were added to PC/PG membranes (250 μM total lipid) and calcium (6 mM). The kynurenine fluorescence of the ensuing oligomers was measured and corrected for FRET between oligomers as described above, and the oligomer subunit stoichiometry, *n*, was calculated using equation (3). The calculated individual values for *n* vary between 5.7 and 7.5.

In sum, the results shown in Figure 4 support the notion that daptomycin forms oligomers with approximately six to seven subunits on membranes. This interpretation must be qualified in several ways, as discussed below.

Discussion

This study aimed at determining the subunit stoichiometry of the membrane-associated daptomycin oligomer. The approach taken here is similar to that used previously for measuring the number of lipid II molecules that are incorporated into the transmembrane pores formed by the lantibiotic nisin [11]. In both cases, random mixing of a fluorescently labeled derivative with the native species was assumed, and the oligomer subunit stoichiometry was extracted from the probability of mutual interaction of two fluorophore molecules. Both approaches also assume the interaction between two fluorophore molecules to occur with equal and complete efficiency, regardless of their respective positions and distance within the oligomer. In [11], the interaction

in question was the formation of pyrene excimers, whereas FRET was employed in the current study. Heteromer analysis using FRET has also previously been used to characterize the dimeric nature of membrane-associated gramicidin [12].

Both excimer formation and FRET are sensitive to distance. In the case of FRET, the distance dependency is given by the Förster radius, R_0, which for unlabeled daptomycin and NBD-daptomycin was previously estimated to 2.7 nm [5]. The thickness of a lipid bilayer is on the order of 4 nm. While the fluorescence changes of kynurenine in native daptomycin [10] and of NBD in NBD-daptomycin [5] suggest that both fluorophores insert into the membrane, it is possible that they both remain close to the membrane surface. It is currently unknown whether a single daptomycin oligomer interacts with one or both membrane leaflets. If indeed it interacts with both, subnunits located in opposite leaflets may be beyond the reach of mutual FRET (see Figure 3B). The apparent oligomer stoichiometry obtained with the present method would then reflect the number of subunits in only one of the two monolayers, and the total number of subunits might in fact be twice as high. To resolve this question, it would be desirable to use probes that provide a greater effective R_0; however, several other fluorescent derivatives that we tested showed a significant reduction in their specific activity, which suggests that their oligomerization may be impaired, too, and therefore that they may not be able to randomly co-oligomerize with native daptomycin.

The extraction of the subunit stoichiometry from FRET within hybrid oligomers requires correction for FRET occurring between donor-only and acceptor-containing oligomers. In principle, it should be possible to separate oligomers from one another by dilution, that is, by making the ratio of daptomycin to lipid very small. However, this approach is not feasible in

practice, because the fluorescence intensity of kynurenine is low; its molar extinction coefficient is 4000, and its quantum yield is 0.04 [5]. Furthermore, at high lipid concentration and in the presence of calcium, the PG-containing liposomes become very turbid, which interferes with the accurate measurement of fluorescence intensities. It was therefore necessary to correct for, rather than physically eliminate, FRET between oligomers. Our correction procedure assumes that all daptomycin oligomers are distributed evenly in the membrane, or alternatively that the native daptomycin applied in the second step will be distributed in the membrane in the same manner as the mixture of the native and NBD-daptomycin that was applied in the first step. In this context, it is noteworthy that the extent of FRET between oligomers (cf. Figure 2B) is greater than predicted for randomly distributed donors and effectors by the pertinent formula given by Wolber and Hudson [13] with the parameters of our system (R_0=2.7 nm, surface area of 0.7 nm^2 per lipid molecule). It is known that acidic phospholipids within mixed membranes tend to cluster in the presence of calcium, and this tendency is further promoted by proteins and peptides that bind to them multivalently [14]. With bacterial cells, daptomycin clusters have indeed been observed on bacterial membranes (J. Pogliano, personal communication; manuscript in preparation). Importantly, however, in those experiments, two samples of daptomycin that carried two different labels and were added to the same cells at different times were found enriched and superimposed in the very same locations on the membrane. We assume that the same happens on our liposome membranes.

In the previously obtained NMR structure of daptomycin in water [15], the diameter of the peptide moiety, in a plane perpendicular to the extended fatty acyl chain, ranges from 0.8 to 1.2 nm. Therefore, even if we confine the consideration to subunits within the same monolayer, it is

conceivable that the cumulative diameter of the oligomer subunits may exceed the range within which FRET can be assumed to be quantitative, as was done in the present study. In this context, however, it is interesting to note that, upon prolonged exposure of daptomycin oligomers that were formed on membranes to 10 mM CHAPS, an apparent oligomer subunit stoichiometry of up to 15 was observed. This finding suggests that within the membrane – or, as discussed above, within a membrane monolayer – it is not the R_0 that limits the extent of FRET but rather the actual number of subunits present. (CHAPS was also found to induce rapid destabilization of oligomers, as evident from scrambling of donor and acceptor subunits, and therefore CHAPS-solubilized samples cannot be considered a valid model of the membrane-associated state of daptomycin.)

Another caveat concerning the method employed in this study concerns the relative rates of oligomer incorporation of the donor and the acceptor; these rates cannot currently be measured accurately. Figure 5 shows simulated data to illustrate what happens if the donor oligomerizes either more rapidly or more slowly than the acceptor. While native daptomycin and NBD-daptomycin have the same specific activity, it still seems possible that their rates of oligomer incorporation may be slightly different, which may then cause the estimated number of subunits to deviate from the true value.

Our analysis is also based on the assumption of uniform oligomer subunit stoichiometry. While our data do not provide direct evidence for or against this assumption, it seems to fit with the previously reported selective and specific nature of the permeability lesion caused by daptomycin [4, 16]. Less selective and possibly dose-dependent leakage of larger marker molecules might be expected in the case of inhomogeneous subunit stoichiometries of oligomers

and associated membrane defects.

In sum, the results of our study indicate that the membrane-associated daptomycin oligomer contains approximately six to seven subunits. Whether this number applies to the entire oligomer or rather to a part of it that is embedded in one of the two membrane leaflets remains to be determined. In either case, the findings support the notion that daptomycin acts through the formation of oligomers of discrete size, rather than through detergent-like or carpet-mode wholesale membrane disruption. This conclusion is in line with the previously observed absence of morphological disruption of bacterial cells exposed to daptomycin [17], as well as with the selective inhibition of membrane potential-dependent, but not of phosphotransfer-dependent membrane transport [18].

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Figure Legends

Figure 1: Time course and linear range of daptomycin fluorescence responses to interaction with liposomes. **A**: Time course of kynurenine fluorescence of 10 μM native daptomycin upon addition of PC/PG liposomes (250 μM total lipid) and calcium (6 mM) at time=0. The small initial lag is caused by the shutter of the instrument. **B**: Time course of perylene fluorescence (λex=430 nm, λem=560 nm) of 2.5 μM perylene-daptomycin to liposomes and calcium as before. The emission at 560 nM is mostly caused by excimers [6], indicating that oligomer formation is instantaneous. C: Linear range of kynurenine fluorescence upon addition of native daptomycin to liposomes and calcium as before and incubation for 300 seconds.

Figure 2: FRET within and between daptomycin oligomers. **A**: Fluorescence emission spectra of 2.4 μM daptomycin only, and of daptomycin (2.4 μM) plus NBD-daptomycin (0.9 μM), pre-mixed before addition to liposomes and calcium or added sequentially to the latter, with incubation for 3 minutes between the additions of the two species. Solid lines are spectra taken 5 minutes after sample preparation, and dashed lines are spectra acquired after an additional incubation for 10 minutes after the first measurement. The absence of an intensity decrease in the sequentially prepared sample indicates that the donor and acceptor oligomers remain distinct and stable on the time scale of the experiment. **B**: Kynurenine fluorescence intensity of samples containing native daptomycin and NBD-daptomycin at 2:1 molar ratio, and sequentially incubated with liposomes and calcium, at 250 μM total lipid and the indicated ratio of lipid to total daptomycin, relative to samples containing the same amount of native daptomycin but no NBD-daptomycin.

Figure 3: Illustration of the the experimental approach used to measure oligomer subunit stoichiometry (A), and of hypothetical oligomer structures (B). **A**: When a mixture of donor (D; native daptomycin) and acceptor molecules (A; NBD-daptomycin) is applied to membranes, they randomly combine into oligomers. The fraction D_1 of donor monomers that become incorporated into pure donor oligomers is related to the number of oligomer subunits according to equation (2). In order to determine D_1 from the donor fluorescence, the latter must be corrected for FRET between donor-only oligomers and acceptor-containing oligomers in the vicinity. To this end, a second sample containing native daptomycin only is added subsequently. These molecules will all be converted to donor-only oligomers; that is, D_2=1. At the same time, they will be quenched by FRET to acceptor-containing oligomers to the same extent as the oligomers represented by D_1. Their fluorescence intensity can therefore be used to calibrate the determination of D_1 from the donor fluorescence measured after the first step.

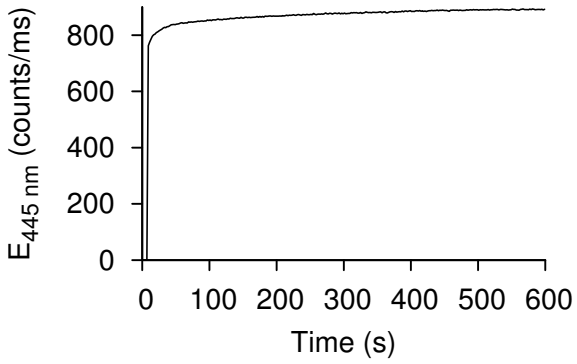
B: Hypothetical daptomycin oligomer structures. Is it presently unknown whether daptomycin becomes embedded only into the outer monolayer (top) or into both monolayers (bottom). In the latter case, it is conceivable that the distance between two subunits embedded in different monolayers exceeds the Förster radius for FRET between kynurenine and NBD (2.7 nm). In this case, the actual subunit stoichiometry may be up to twice the value obtained by FRET experiments.

Figure 4: Determination of oligomer subunit stoichiometry by FRET. **A**: Kynurenine fluorescence intensities of 4 individual experiments, each performed at 4 different donor/acceptor ratios. The fluorescence intensities were corrected according to equation (1). The curves represent theoretical values calculated according to equation (2). **B**: Subunit

stoichiometries calculated using equation (3) from the values shown in A (averages ± standard deviation).

Figure 5: Numerical simulation of oligomerization with varying bimolecular rate constants for the incorporation of donor and acceptor monomers into growing oligomers. The oligomer is assumed to contain 6 subunits. The bimolecular rate constant k_A, which controls the addition of an acceptor monomer to an incomplete oligomer, was held at 1 in each case, whereas the rate constant k_D, which controls the addition of a donor monomer to an incomplete oligomer, was varied between 0.1 and 10. The program used to perform the calculation is listed in the supplementary file. **A**: For each combination of rate constants, the fraction *D* of donors that are incorporated into donor-only oligomers is plotted as a function of the fraction of donor momoners *d* in the initial mixture (compare equation (2)). **B**: The apparent subunit stoichiometries that would be inferred from the data shown in A according to equations (2) and (3) are plotted as functions of *d*.

A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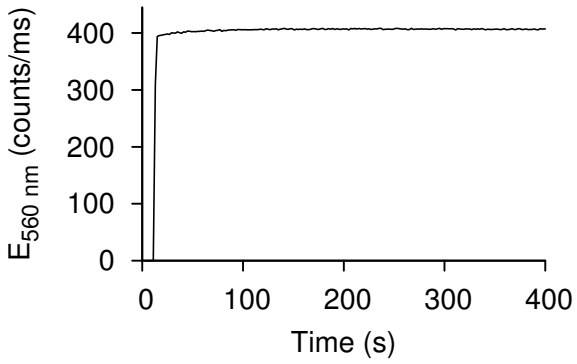
200
300
400
500
600
Time (s)

B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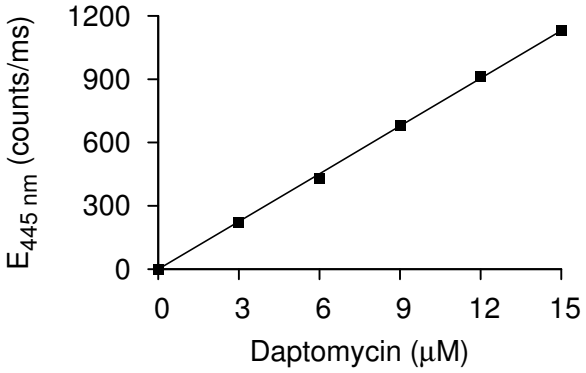
C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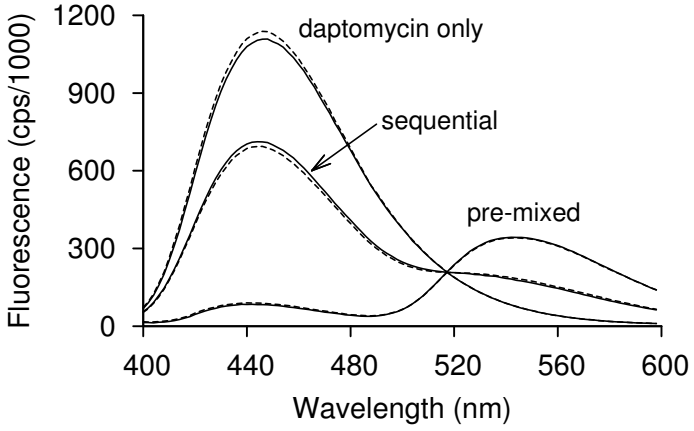
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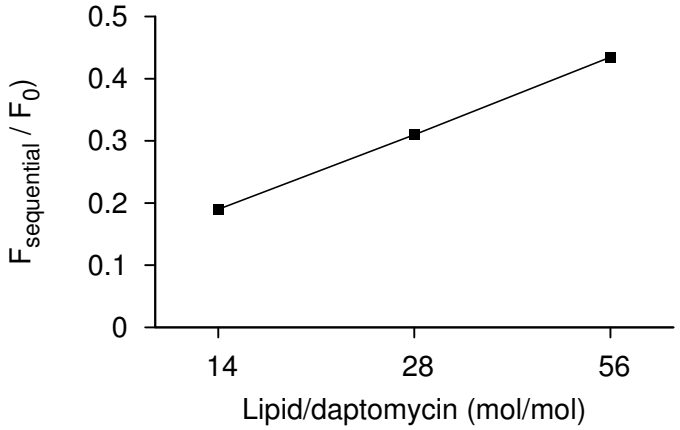
B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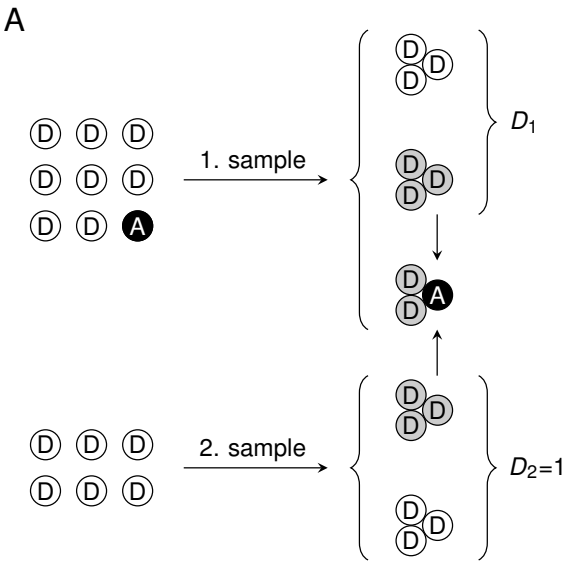
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D
A
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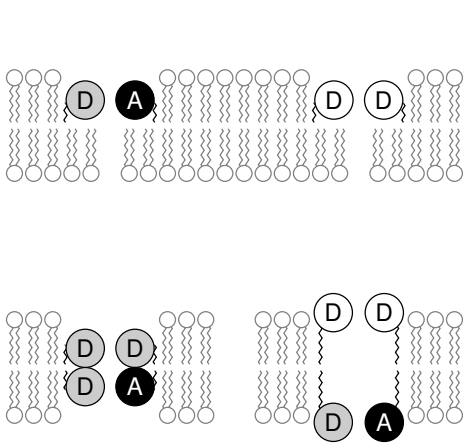
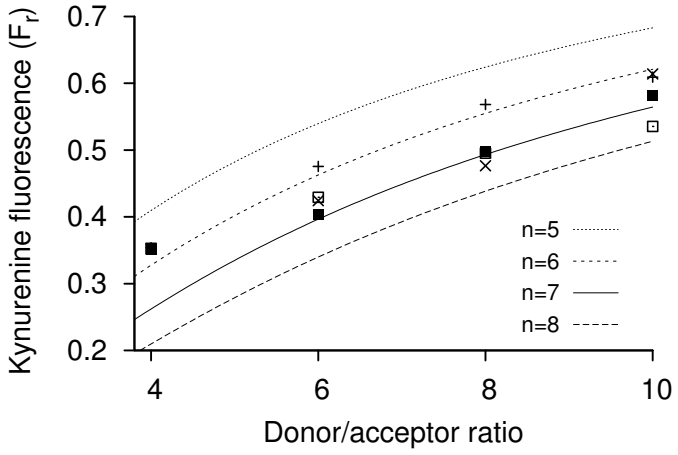
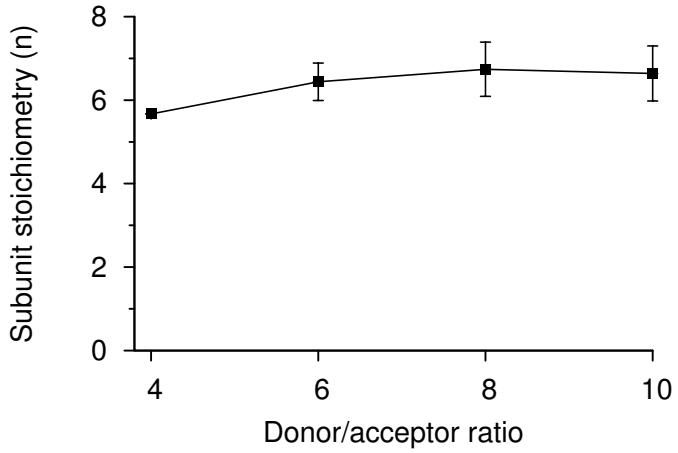
2. sample
D_1
D_2=1


B
D
A

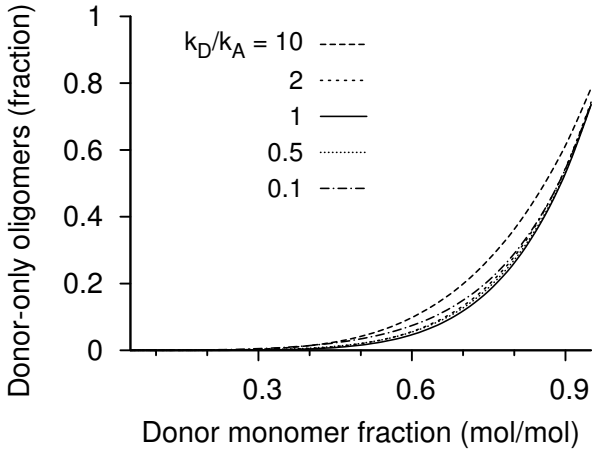
A


Kynurenine fluorescence (F_r)
0.7
0.6
0.5
0.4
0.3
0.2
4
6
8
10
Donor/acceptor ratio
n=5
n=6
n=7
n=8

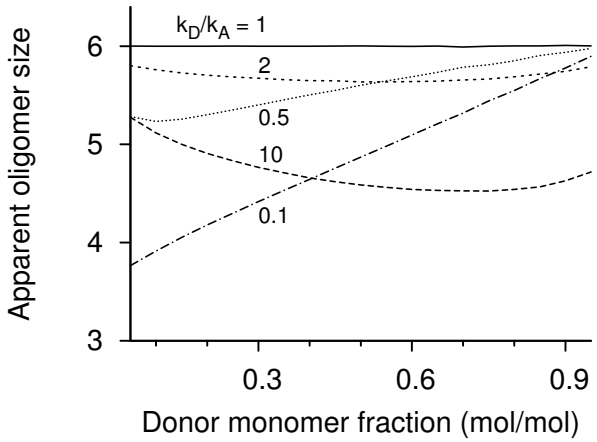
B


Subunit stoichiometry (n)
8
6
4
2
0
4
6
8
10
Donor/acceptor ratio

A


Donor-only oligomers (fraction)
1
0.8
0.6
0.4
0.2
0
k_D/k_A = 10
2
1
0.5
0.1
0.3
0.6
0.9
Donor monomer fraction (mol/mol)

B


Apparent oligomer size
6
5
4
3
k_D/k_A = 1
2
0.5
10
0.1
0.3
0.6
0.9
Donor monomer fraction (mol/mol)