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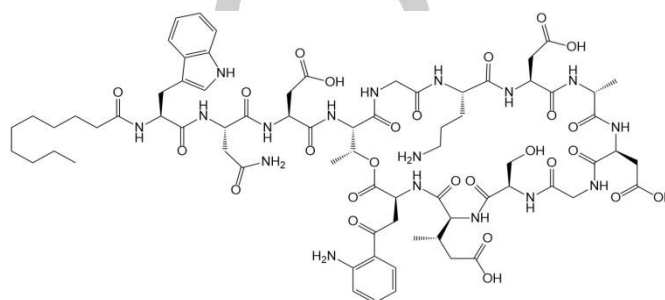
Self-Assembly of the Cyclic Lipopeptide Daptomycin: Spherical Micelle Formation does not Depend on the Presence of Calcium Chloride

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Abstract: The cyclic lipopeptide Daptomycin, used as a treatment for infections where antimicrobial resistance is observed, is shown to self-assemble into spherical micelles above a critical aggregation concentration. Micelles are observed either in the absence or presence of CaCl₂, in contrast to claims in the literature that CaCl₂ is required for micellization.

Daptomycin is a cyclic lipopeptide drug produced by the gram-positive bacterium *Streptomyces roseosporus*.^[1] It is also known under the tradename Cubicin and is used as an antibiotic to treat serious infections caused by Gram positive bacteria.^[2] It is effective against infections where antimicrobial resistance (AMR) is a problem, including methicillin-resistant *Staphylococcus aureus* (MRSA)^[1b] and vancomycin-resistant Enterococci.^[2-3] As shown in figure 1, Daptomycin is composed of 13 amino acids, 10 making up the cyclic structure and the other three forming a chain. The cyclic section of the molecule is linked through an ester bond to the tail through the terminal kynurenine residue, an unusual non-canonical amino acid product of tryptophan metabolism, and the threonine hydroxyl group.^[2] Although Daptomycin is negatively charged, it is able to interact with the negatively charged bacterial cell membrane, in the presence of cations. In particular it is indicated that its *in vitro* antibiotic activity requires the presence of calcium ions.^[4]

The consensus mechanism of action of Daptomycin involves disruption of the bacterial cell membrane by insertion of the lipophilic N-terminal decanoyl chain into the membrane.^[3, 5] This then allows the flow of potassium ions out of the cell, which causes depolarisation, and is proposed to be the cause of cell death.^[6] There is also the possibility of an additional factor



Scheme 1. Molecular structure of Daptomycin

contributing towards its activity that results from oligomerisation in the presence of calcium ions, causing disruption of cell membrane when the oligomer dissociates at the bacterial membrane.^[7] The antimicrobial activity is calcium-dependent, although the reasons are poorly understood. It has been reported that a 1:1 ratio of Ca²⁺ to Daptomycin leads to aggregation, although this does not lead to a change in Daptomycin conformation.^[7-8] In the absence of calcium ions, three different structures have been proposed (based on NMR experiments) for Daptomycin, none of which are highly amphiphilic.^[7] At one molar equivalent of added Ca²⁺ ions, it is proposed (based on ultracentrifugation and NMR experiments) that 14-16 daptomycin monomers oligomerise, likely into a micelle.^[7, 9] When the oligomer is in close proximity to the bacterial membrane, it dissociates and the insertion process described before occurs.

Despite this prior work, there is no direct prior evidence for the nature of the self-assembled structures of Daptomycin. In the present paper we use a powerful combination of *in situ* techniques to examine micellization of Daptomycin. Cryogenic-transmission electron microscopy (cryo-TEM) provides direct images of small spherical micelles, the structure of which is also elucidated using small-angle X-ray scattering (SAXS). The critical micelle concentration is determined using an auto-fluorescence assay. The kynurenine residue in Daptomycin, containing an aniline component, is responsible for a strong fluorescence emission peak at 460 nm.^[5a] Another emission peak at 355 nm is due to Trp-1.^[5a] These peaks enable fluorescence assays based on intrinsic fluorescence, without the use of probes sensitive to the local hydrophobic environment such as pyrene widely used in critical micelle concentration assays. We also investigate the secondary structure of Daptomycin using a combination of spectroscopic methods including circular dichroism (CD) and FTIR.

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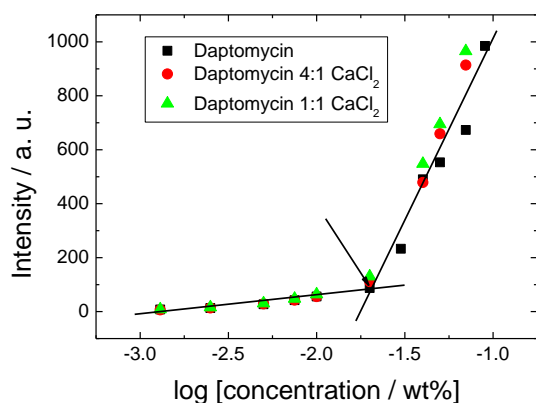


Figure 1. Critical aggregation concentration assay for Daptomycin in 100 mM KCl solution with no CaCl_2 or with Daptomycin: $\text{CaCl}_2 = 4 : 1$ or $1 : 1$.

Results from fluorescence assays based on the self-fluorescence peak of Daptomycin at 466 nm are shown in Fig.1 (the original spectra are shown in Fig. S1). The data shown in Fig.1a performed for Daptomycin in the presence of 100 mM KCl reveal a discontinuity at a concentration of $(0.020 \pm 0.003 \text{ wt}\%)$ ($0.02 \text{ wt}\% \equiv 0.12 \text{ mM}$), remarkably independent of CaCl_2 content. These molar ratios of added CaCl_2 were selected based on prior work^[3, 7, 9]. These values indicate that Daptomycin undergoes a CaCl_2 -independent critical aggregation concentration (cac). The value obtained from our measurements is the same as the $\text{cac} = 0.12 \text{ mM}$ obtained previously in pH 4 aqueous solution (without salts) on the basis of auto-fluorescence with similar values obtained from dynamic light scattering.^[5a] It has also been reported that Daptomycin aggregates above 1 mM in aqueous solution, although the (salt) conditions were not specified.^[4b]

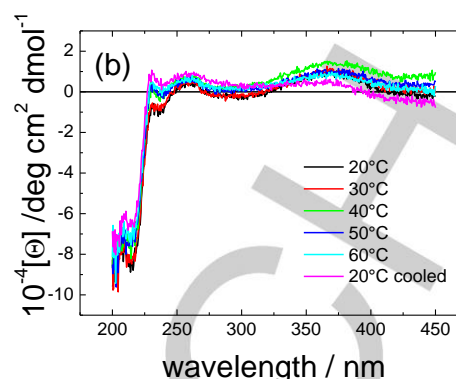
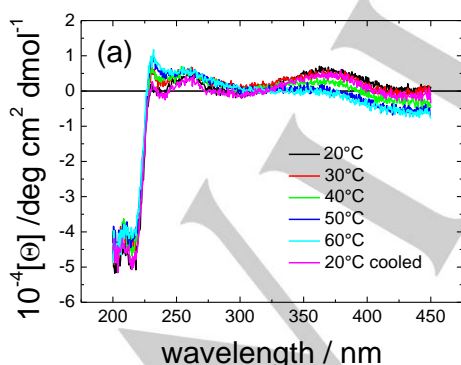


Figure 2. Circular dichroism spectra for 0.1 wt% Daptomycin solutions in (a) 100 mM KCl solution, (b) 100 mM KCl solution with Daptomycin $\text{CaCl}_2 = 4 : 1$.

The secondary structure of Daptomycin in aqueous (100 mM KCl) solution was investigated in the absence and presence of CaCl_2 using circular dichroism (CD) and FTIR spectroscopies. Fig.2 and Fig.S2 show CD spectra which are very similar in shape with or without CaCl_2 (at 4:1 molar ratio Daptomycin: CaCl_2 or at a 1:1 molar ratio). In addition, there is very little temperature dependence in the range examined, 20 – 60 °C. The spectra in the far UV region are consistent with a reported spectrum for $6 \mu\text{M}$ Daptomycin in 5 mM CaCl_2 .^[3] The main features are the maximum at 231 nm, consistent with previously reported spectra,^[3] and a peak at 258 nm, consistent with the measured absorption spectrum of Daptomycin and kynurenine.^[4b, 5a] In the near UV region (Fig.2b,c), each spectrum shows a minimum near 300 nm and a maximum at 360-370 nm. The latter feature is ascribed to absorbance from the kynurenine unit.^[4b, 5a] A key feature of the CD spectra in Fig.2 is the similarity in shape for solutions with or without CaCl_2 .

As shown in Fig.3, the FTIR spectra measured for Daptomycin solutions with or without CaCl_2 are very similar in the 1250 – 2000 cm^{-1} range. Strong peaks are observed at 1454 cm^{-1} and 1648 cm^{-1} with a weak additional peak at 1580 cm^{-1} along with a shoulder at 1720 cm^{-1} . The 1454 cm^{-1} peak is assigned to HDO stretching deformations, the formation of HDO resulting from H/D exchange between protons and D_2O ,^[10] although there is also a Trp side group deformation possible at this wavenumber.^[11] The peak in the amide I' band at 1648 cm^{-1} indicates an unordered peptide conformation.^[11b, 12] The 1720 cm^{-1} peak is assigned to carbonyl stretch.^[13]

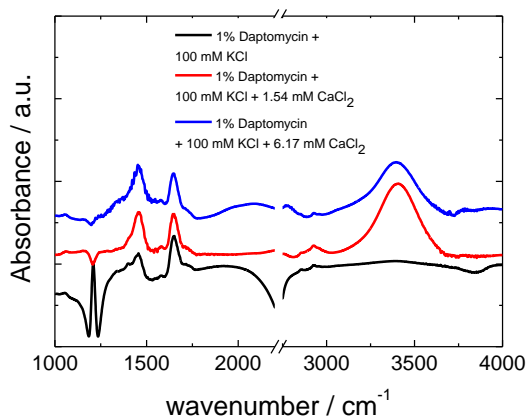


Figure 3. FTIR spectra from 1 wt% Daptomycin solutions in 100 mM KCl D₂O solutions with and without CaCl₂, as indicated.

There is a significant difference in the intensity of the peak at 3400 cm⁻¹, which is substantially enhanced in the CaCl₂ solutions. This peak is assigned to the –OH stretch vibration^[13] and its reduced intensity for the salt solution reflects the salt-induced disruption of hydrogen bonds involving –OH groups in the acidic residues within Daptomycin. The FTIR data supports the results from CD, showing an unordered peptide conformation which is not changed in the presence of CaCl₂.

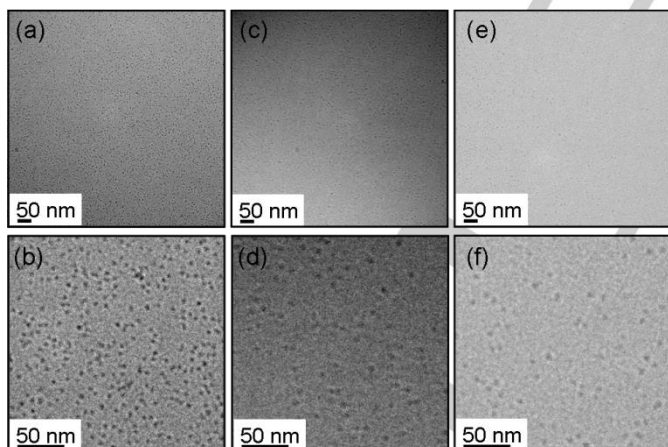


Figure 4. Cryo-TEM images from 0.5 wt% Daptomycin solutions with (a, b) 100 mM KCl (c, d) 100 mM KCl + 0.77 mM CaCl₂, (e, f) 100 mM KCl + 3.09 mM CaCl₂.

The self-assembly of Daptomycin in the presence and absence of CaCl₂ was examined using cryogenic-TEM. Representative images are shown in Fig.4. These clearly reveal the presence of spherical micellar structures in the presence and absence of

CaCl₂. The micelles have a diameter estimated to be approximately 5 nm.

The presence of micellar structures resulting from the self-assembly of Daptomycin in aqueous solution is consistent with the observed absence of ordered secondary structure. We have previously shown for Toll-like receptor agonist like lipopeptides,^[14] and other model lipopeptides,^[15] that micellar structures are correlated to unordered peptide conformation. In contrast, β -sheet conformation is observed for extended fibrillar or nanotape structures.^[14, 16]

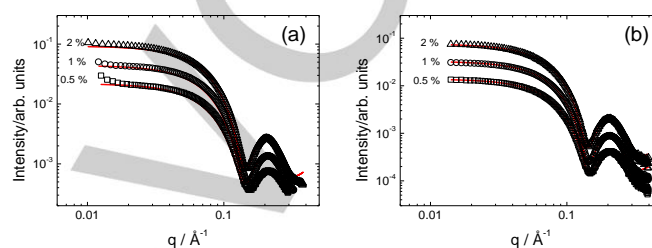


Figure 5. SAXS data at the concentrations of Daptomycin indicated (a) in CaCl₂ solutions with 100 mM KCl, molar ratio Daptomycin: CaCl₂ = 4 : 1). As a reference 1 wt% Daptomycin = 6.17 mM, (b) in 100 mM KCl with no added CaCl₂. For clarity of presentation, only every 5th measured data point is shown.

SAXS was used to obtain quantitative information on the internal structure of Daptomycin micelles. The data were modelled using a core-shell sphere model using the software SASfit.^[17] The data along with the model form factor fits are shown in Fig.5 and the fit parameters are listed in Table 1. The outer micelle radius was found to be $R = (27.2 \pm 0.1)$ Å independent of concentration in the range 0.5 – 2 wt% Daptomycin, and in the presence or absence of CaCl₂. In the form factor fits, the only concentration-dependent terms are those relating to the scattering contrast of the shell and core and the inner core radius. In particular, at 2 wt%, the inner core radius is larger and the relative scattering contrast of the inner core is more negative, indicating increased packing of hydrophobic units (which have a negative scattering density relative to the solvent) in the micelle core. Using the micelle radius $R = (27.2 \pm 0.1)$ Å obtained from fitting SAXS data (SI Table S1), along with a molecular volume 1435 Å³ (calculated at www.molinspiration.com), we estimate an association number of 59 ± 6 for Daptomycin (concentration range 0.5 – 2 wt%) in 100 mM KCl solution with or without 0.25 molar equivalents of CaCl₂. In a previous study, the aggregation number was estimated from static light scattering data and found to be (18 ± 2) .^[5a]

In summary, in contrast to previous literature^[7] we find clear evidence that Daptomycin can self-assemble into well-defined spherical micelles in the absence or presence of CaCl₂ (at least up to 1:1 molar ratio of added CaCl₂, i.e. covering the range previously studied). The structure of the micelles appears to be the same, with or without the added salt. It is remarkable that a

molecule such as Daptomycin with a large cyclic "headgroup" can self-assemble into spherical micelles with a relatively large association number. It seems likely that the conformation of the molecule (Scheme 1) will be such as to favour sequestration of the lipid chain and aromatic residues in the micelle core, with the polar residues forming the micelle corona. As indicated by the *cac* assay, micellization occurs at the same concentration with or without added CaCl_2 .

Experimental Section

Materials

Daptomycin was obtained from Merck Millipore (USA) with a molecular weight of $1620.7 \text{ g mol}^{-1}$ and a purity of 100% determined by HPLC. Potassium chloride was from Fisher Scientific (UK) and calcium chloride from Sigma-Aldrich (USA).

Following previous reports,^[3, 7, 9] Daptomycin was dissolved in 100 mM KCl solutions in the presence and absence of CaCl_2 , adding 0.25 molar or 1.00 equivalents of CaCl_2 . For reference, a sample containing 0.5 wt% Daptomycin with 100 mM KCl and 0.77 mM CaCl_2 had pH 4.1, while a sample diluted to 0.05 wt% had pH 4.68.

Cryo-TEM

Experiments were carried out using a field emission cryo-electron microscope (JEOL JEM-3200FSC) operating at 200 kV. Images were taken using bright-field mode and zero loss energy filtering (omega type) with a slit with 20 eV. Micrographs were recorded using a Gatan Ultrascan 4000 CCD camera. The specimen temperature was maintained at $-187 \text{ }^\circ\text{C}$ during the imaging. Vitrified specimens were prepared using an automated FEI Vitrobot device using Quantifoil 3.5/1 holey carbon copper grids with 3.5 μm hole sizes. Grids were cleaned using a Gatan Solarus 9500 plasma cleaner just prior to use and then transferred into an environmental chamber of FEI Vitrobot at room temperature and 100% humidity. Thereafter, 3 μl of sample solution at 0.5 wt% concentration was applied on the grid, blotted once for 1 second and then vitrified in a 1/1 mixture of liquid ethane and propane at $-180 \text{ }^\circ\text{C}$. Grids with vitrified sample solutions were maintained in a liquid nitrogen atmosphere and then cryo-transferred into the microscope.

Pyrene fluorescence

Fluorescence spectra were recorded with a Varian Cary Eclipse Fluorescence Spectrometer with samples in 4 mm inner Quartz cuvettes. The assays were performed using 1.3×10^{-3} – 0.13 wt.% Daptomycin, in 2.3×10^{-5} wt.% pyrene solution. The samples were excited at $\lambda_{\text{ex}} = 380 \text{ nm}$, and the fluorescence emission was measured for $\lambda = (400\text{--}550\text{nm})$.

Circular Dichroism Spectroscopy

CD spectra were recorded using a Chirascan spectropolarimeter (Applied Photophysics, UK). Each sample (0.1 wt% Daptomycin in 100 mM KCl and 0.154 mM or 0.617 mM CaCl_2) was placed in a cover slip cuvette (0.1 mm thick). Spectra are presented with absorbance $A < 2$ at any measured point with a 0.5 nm step, 1 nm bandwidth, and 1 second collection time per step at $20 \text{ }^\circ\text{C}$. The Daptomycin solution was acclimatised at each temperature point for 10 minutes before measurements were taken. The CD signal from the background was subtracted from the CD data of the daptomycin solution.

SAXS

Experiments were performed on beamline B21 at Diamond Light Source, Harwell, UK. Solutions of Daptomycin (0.5, 1 and 2 wt% with 100 mM KCl and with or without CaCl_2) were loaded into the 96 well plate of an EMBL BioSAXS robot. Aliquots of solutions (25 μL) were then injected via an automated sample exchanger at a slow and very reproducible flux into a quartz capillary (1.8 mm internal diameter) in the X-ray beam. The quartz capillary was enclosed in a vacuum chamber, in order to avoid parasitic scattering. After the sample was injected in the capillary and reached the X-ray beam, the flow was stopped during the SAXS data acquisition. SAXS frames were collected with duration 20 s or 100 s). B21 operated with a fixed camera length (4.01 m) and fixed energy (12.4 keV). The images were captured using a Pilatus 2M detector. Data processing (background subtraction, radial averaging) was performed using the dedicated beamline software Scatter.

SAXS data were modelled using the software SASfit^[17] with model "Spherical Shell i". A flat background was added in the case of the data from Diamond beamline B21, it was necessary to allow for a linear sloping background to fit the data from Maxlab beamline I911. The fit parameters are provided in Table S1.

FTIR Spectroscopy

Spectra were recorded using a Thermo Scientific Nicolet IS5 and a Nexus-FTIR spectrometer, both equipped with a DTGS detector. A 40 μl drop of the sample (6.17 mM Daptomycin in 100 mM KCl with or without 1.54 mM or 6.17 mM CaCl_2 in D_2O) was sandwiched between two CaF_2 plate windows, with a 0.006 mm thick Mylar spacer) in a Specac GS20500 sample cell holder. Cells were heated using a Specac 4000 series high stability heating controller, and a Specac electrical heating jacket. The sample was equilibrated at each temperature point for 10 minutes before measurements were taken. Spectra were scanned 128 times over the range of $900\text{--}4000 \text{ cm}^{-1}$.

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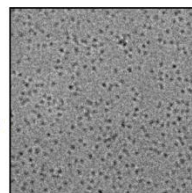
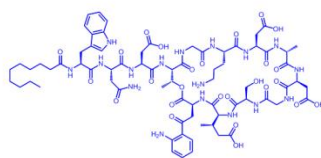
Keywords: Antimicrobial Peptide • Micelle • Lipopeptide • Self-Assembly • Secondary Structure

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COMMUNICATION

The antimicrobial lipopeptide Daptomycin is shown to self-assemble into spherical micelles in the presence or absence of CaCl_2 .



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