Evaluation of Lipopeptide (Daptomycin) Aggregation Using Fluorescence, Light Scattering, and Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: The aggregation behavior and critical aggregation concentration (CAC) values of daptomycin in aqueous solutions were evaluated under the external factors of pH, temperature, daptomycin concentration, and calcium ions concentration by using the complementary characterization techniques, fluorescence, dynamic and static light scattering, and nuclear magnetic resonance (NMR) spectroscopy. On the basis of the intrinsic fluorescence resonance energy transfer of daptomycin, the CAC values were identified by an upward inflection of the fluorescence emission from Kyn-13 at 460 nm. The pH-dependent CAC values were determined to be 0.14 mM at pH 3.0, 0.12 mM at pH 4.0, and 0.20 mM at pH 2.5 and 5.0. The CAC values obtained by fluorescence spectroscopy were confirmed by dynamic light scattering and NMR spectroscopy. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:853–861, 2014

Keywords: peptide aggregation; daptomycin; lipopeptide antibiotic; fluorescence spectroscopy; dynamic light scattering; static light scattering; spectroscopy; analytical chemistry; NMR spectroscopy; zwitterion

INTRODUCTION

Daptomycin, derived from *Streptomyces roseosporus* fermentation, is an anionic cyclic lipopeptide antibiotic for the treatment of complicated skin, soft tissue, and bloodstream infections with activity against drug-resistant Gram-positive pathogens,[1,2] including vancomycin-resistant *enterococci*, vancomycin-resistant *Staphylococcus aureus*, methicillin-resistant *S. aureus*, penicillin-resistant *Streptococci*, and coagulase-negative *Staphylococci*.[3,4]

Daptomycin is a cyclic lipopeptide composed of 13 amino-acid residues. The C-terminus is connected with the hydroxyl group of Thr-4 via an ester bond, forming a 10-membered ring. The N-terminus is acylated with a decanoyl aliphatic chain and situated between the lipid chain and Kyn-13 residue. Without terminal amine and carboxylic groups, daptomycin contains six side-chain ionizable groups: four carboxylic residues (three aspartic acids, Asp-3, Asp-7, and Asp-9, one methyl-glutamic acid, mGlu-12) and two amines (aliphatic amine in ornithine Orn-6 and aromatic amine in Kyn-13).

Most currently available antibiotics target enzymes responsible for formation and maintenance of cell wall structure or inhibition of protein synthesis, nucleic acid synthesis, or metabolic pathway.[5] Hypothetical mechanism of action of daptomycin involves direct binding to the Gram-positive cell membrane by its lipid tail, followed by calcium-dependent insertion and oligomerization. Daptomycin oligomers form ion channels, disrupting the functional integrity of the membrane, and trigger a release of intracellular ions. Calcium-induced daptomycin aggregation is essential for therapeutic activity of disrupting the bacterial membrane through the formation of transmembrane channels.[6–8] Details of daptomycin's mechanism of action have not been yet fully elucidated, that is, how the rapid bactericidal activity of daptomycin is related to its dynamic nature and its interaction with the cytoplasmic membrane and whether oligomerization in the membrane is crucial.[9–11]

Daptomycin's three-dimensional structure and its biologically active conformation have been investigated in a number of recent nuclear magnetic resonance (NMR) studies, but with different findings.[10–14]

The aqueous conformation of daptomycin has been reported by Rotondi and Gierasch[14] to be qualitatively similar to the Ca^{2+}-bound structure reported by Jung et al.[13] A preferred aqueous conformation for daptomycin proposed by Jung et al.[13] and Ball et al.[12] differs from that from Rotondi and Gierasch[14] primarily by the presence or absence of a cluster of the hydrophobic side chains of a decanoyl lipoidal tail, Trp-1, and Kyn-13. Jung et al.[13] showed that daptomycin undergoes significant calcium-dependent conformational changes upon association with model lipid membranes. In contrast, Ball et al.[12] demonstrated that the binding of calcium ions does not result in major conformational changes, but does induce aggregation. The subsequent studies results from Scott et al.[11] suggested that daptomycin undergoes only a minor conformational rearrangement upon binding with 1,2-dihexanoyl-sn-glycero-3-phosphocholine in the presence of calcium ions.

Various analytical methods have been used to investigate mechanism and stoichiometry of peptide/protein aggregation. Broadly, these analytical methods can be classified as size determination methods and molecular spectroscopic detection. Size determination methods include size-exclusion chromatography (SEC), static, and dynamic light scattering (DLS and SLS). Molecular spectroscopic detection includes circular dichroism, Fourier-transform infrared, fluorescence spectroscopy, and NMR.[15,16] Daptomycin "aggregation" is reversible

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"self-association". Thus, the analytical techniques involving dilution such as SEC and field flow fractionation may not be suitable for the determination of the critical aggregation concentration (CAC) of daptomycin.

According to the literature, daptomycin aggregation is induced by calcium ion, pH variation, or substrate concentration.[6,12,17,18] However, a systematic determination of CAC values of daptomycin has not been made. Lakey and Ptak[17] observed that the ratio of fluorescence yield Kyn-13/Trp-1 increased at daptomycin concentrations of 5 mM, possibly because of the aggregation effects increasing the fluorescence energy transfer from Trp-1 to Kyn-13; moreover, ^{1}H NMR results from aqueous solutions were reported to show significant line broadening consistent with aggregation although representative spectra were not published. Calcium-induced daptomycin aggregation was observed using NMR.[11–13] The pronounced broadening of resonances in NMR pH titration of daptomycin was observed as decreasing pH up to 2.[12] The CAC value of daptomycin in borate buffer solution (pH 10) was determined to be 2 mM by surface tension measurement.[18] An observed deviation in hydrolytic reaction order at higher substrate concentration in alkaline solutions was attributed to daptomycin aggregation.

Daptomycin has two aromatic fluorescence residues at the N-terminus and at the C-terminus. Because of the proximity of these two fluorophores connected by ester bond between Thr-4 with Kyn-13 residues and the overlap of the donor Trp-1 emission spectrum and acceptor Kyn-13 excitation spectrum, fluorescence resonance energy transfer (FRET) occurs between the two fluorophores, which has been observed and confirmed.[17] In this study, the CAC values of daptomycin were identified using fluorescence spectroscopy based on FRET, and further evaluated with complimentary analytical techniques, DLS, SLS, and NMR spectroscopy.

Investigations of daptomycin aggregation at different pH values in aqueous solution would be helpful for reconciling the different findings regarding daptomycin conformation and understanding the effects of conformation and aggregation on its pharmaceutical properties. The primary research objectives are to evaluate the conformational transition and aggregation behavior of daptomycin under the different external factors, and to elucidate the different findings of daptomycin conformational structures of NMR study.

EXPERIMENTAL MATERIALS AND METHODS

Materials

Daptomycin obtained from Eli Lilly Research Laboratories (Indianapolis, Indiana) was used as received. L-Kynurenine was purchased from MP Biomedicals (Santa Ana, California). Sodium chloride, calcium chloride, 0.01 or 0.1 M sodium hydroxide solutions, 0.1 or 1 M hydrochloric acid solutions, toluene (HPLC grade), and phosphate-buffered saline (PBS) were purchased from Fisher Scientific (Fair Lawn, New Jersey). Deuterium oxide (100% D_2O) was purchased from Cambridge Isotope Laboratories (Cambridge, Massachusetts). Standardized pH buffers of 2, 4, 7, and 10 were obtained from Fisher Scientific. All other chemicals used were reagent grade from Fisher Scientific (Pittsburgh, Pennsylvania).

Methods

Preparation of Daptomycin Solutions

Daptomycin aggregation behavior was studied in aqueous solutions in the pH range 2.5–7.4 and in the concentrations ranged from 0.06 to 21.21 mM. Daptomycin solutions were prepared by directly dissolving the proper amount of daptomycin into water, adjusting to the target pH values with diluted sodium hydroxide or hydrochloric acid solution, and diluting to the targeted concentrations with the same pH values of deionized water, except at pH 7.4 using PBS. The pH measurements were made at room temperature by using an Accumet Model 25 pH/Ion Meter and an Accumet 3 mm Ingold combination electrode with an AgCl reference (Fisher Scientific). The pH meter was calibrated with three certified standards pH 2, pH 4, and pH 7 (Fisher Scientific). Sample pH was measured before and after analytical measurements.

To compare the fluorescence properties of kynurenine and Kyn-13 in daptomycin, daptomycin and kynurenine aqueous solutions at pH 4.0 were separately prepared at 0.23 and 0.24 mM, respectively.

The temperature effect on daptomycin aggregation was performed in the concentration range from 0.06 to 3.11 mM in pH 4.0 aqueous solutions. The samples in fluorescence quartz cuvette were thermostated at 25°C and 40°C using circulating water with a Thermo Hakke C10-B3 Heating Circulator Bath (Thermo Hakke, Newington, New Hampshire).

The calcium ion effect on daptomycin aggregation was carried out in daptomycin concentration range from 0.0026 to 0.26 mM at pH 7.4 and 6.5 in the presence of calcium ion concentrations at 1.0 and 10 mM, respectively.

Fluorescence Spectroscopy

Fluorescence spectra were recorded using a LS55 luminescence spectrometer (PerkinElmer Instruments, Norwalk, Connecticut). Daptomycin solutions were excited at 285 nm, and the emission spectra were collected from 250 to 550 nm to observe the scattered intensity at excitation peak and the emission intensities from both Trp-1 at 355 nm and Kyn-13 at 460 nm. The scattered intensity at 285 nm decreased with increasing daptomycin concentration due to the dominant absorption from Trp-1 residue. The scan rate was set at 250 nm/min. The excitation and emission slit widths were set to 4 and 3 nm, respectively. A quartz cuvette with a 10, 3, or 1 mm path length (Starna Cells, Inc., Atascadero, California) was used depending on the concentration in solution to expand the measured concentration range in the presence of self-quenching.

Ultraviolet Spectroscopy

Ultraviolet (UV) absorbance spectra of 0.10 mM daptomycin and 0.12 mM kynurenine in pH 4.0 solutions were collected on a Hewlett-Packard 8453 diode-array UV–Visible spectrophotometer (Agilent, Palo Alto, California).

DLS and SLS

Dynamic light scattering experiments were conducted at 25°C with an ALV-NIBS High Performance Particle Sizer (Model No ALV-NIBS/HPPS, ALV-Laser Vertriebsgesellschaft m.b.H Langen, Germany). Scattered light intensity (He–Ne laser 632.8 nm) was detected at an angle of 173° with an ALV-5000/E

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