CHAPTER III

MATERIALS AND METHODS

Materials

1,4-diaminobutane core PAMAM generation 3 solution 20% (w/v), generation 5 solution 10% and generation 6 solution 10% in methanol were purchased from Aldrich (St. Louis, MO). Daptomycin (Lot# 212LK9) was obtained from Lilly Research Laboratories, Indianapolis, IN. Sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2PO_4), tris(hydroxymethyl)aminomethane (Tris buffer), standardized sodium hydroxide and hydrochloric acid solutions (0.1 N NaOH and 0.1 N HCl), and acetonitrile (HPLC grade) were obtained from Fisher Chemical, Co. (Fair Lawn, NJ). Standardized buffer solutions were from VWR® Scientific (West Chester, PA). All other chemicals were of analytical grade or better and double-distilled water was used throughout.

Methods

1. Formation of Daptomycin and PAMAM Dendrimer complexes

Daptomycin and dendrimer complexes were typically prepared as followed. An appropriate aliquot of PAMAM dendrimer in methanol was evaporated under nitrogen gas. The residues were reconstituted with media to make of stock PAMAM dendrimer solution. Daptomycin stock solution was separately prepared in the same solvent.

Daptomycin-dendrimer complexes were prepared by either mixing aliquots of both stock solutions to the desired concentrations or titration of PAMAM dendrimer to daptomycin solution or vice versa. The mixtures were stirred and allowed to reach equilibrium.

2. Selection of Characterization Techniques

2.1. Characterization of daptomycin and PAMAM dendrimer complex using Ultrafiltration technique

The use of ultrafiltration to determine binding was investigated using an Amicon® micropartition system (Amicon® MPS). The Amicon® MPS equipped with regenerated cellulose membrane molecular weight cut off 10 kiloDaltons (kDa) (Ultracel YM-10) was used to separate free daptomycin from dendrimer-daptomycin complexes.

Aliquots of daptomycin and dendrimer stock solutions in 1.0 mM phosphate buffer were mixed to obtain the mixtures of daptomycin and PAMAM dendrimer. The mixtures were shaken at least 3 minutes and then allowed to reach equilibrium for approximately 30 minutes. Typical ultrafiltration was performed by placing 1.0 mL of well-mixed sample mixtures in the upper reservoir of Amicon® MPS. The apparatus was then centrifuged using Fisher Scientific Marthon 22K centrifuge at 2000 rpm for 15 minutes. The ultrafiltrate was carefully removed from the lower reservoir. The assay of the free amount of daptomycin in ultrafiltrate was done by UV spectroscopy. Ultrafiltration of daptomycin solutions at the same concentrations were performed as control. The loss of daptomycin from control sample during ultrafiltration process was used as a correction factor for each point of data.

UV spectroscopy was employed to investigate the unbound concentration of daptomycin. The ultrafiltrate was directly placed in a 1 cm path length cuvette. Absorption spectrum of ultrafiltrate solution was obtained using a HP 8453A UV/ visible spectrophotometer at ambient temperature. The absorption maxima were selected in order to use for the further study.

2.1.1. Approaches for improving ultrafiltration technique

2.1.1.1. Control of a daptomycin aggregation

Daptomycin tends to form aggregates at high concentrations (Muangsiri, 2000). The aggregation is diminished in the presence of 10% acetonitrile. Therefore, 10% acetonitrile was added to the sample prior to ultrafiltration.

Various concentrations of daptomycin (0.02 to 0.7 mM) were combined with PAMAM generation 5 dendrimer (0.07 mM) to prepare mixtures of dendrimer-daptomycin complexes in 10% acetonitrile and 1.0 mM phosphate buffer. The well mixed solutions were ultrafiltrated using method described earlier. After ultrafiltration the ultrafiltrates was collected and then analyzed using UV spectroscopy.

2.1.1.2 Determination of effect of phosphate on free concentration of daptomycin

The mixtures of PAMAM generation 5 dendrimer (0.47 mM) and various concentrations of daptomycin (0.5 – 0.7 mM) were prepared in 10% acetonitrile at the absence of phosphate buffer at pH 7.0. The pH value of the system was kept constant by titration with 0.10 N sodium hydroxide and/or 0.10 N hydrochloric acid solutions. The pH value was monitored using a Metrohm Multi-Dosimat E415 equipped with an Ag/AgCl combination electrode. The mixtures were well mixed, and allow to equilibrate for 30 minutes. The solutions were ultrafiltrated using method described earlier and the ultrafiltrates was then analyzed for free daptomycin concentration using UV spectroscopy.

2.1.1.3 Effect of the daptomycin and PAMAM dendrimer concentration on binding

In order to avoid precipitation, the concentration of the mixtures of daptomycin and PAMAM generation 5 dendrimers in 10% acetonitrile were reduced to be in a range of 10 to 70 μM and 7.1 to 13.9 μM, respectively. The mixtures were then ultrafiltrated using the method described earlier. After ultrafiltration, the ultrafiltrate was collected for analysis of free daptomycin concentration using UV spectroscopy.

The concentration of unbound daptomycin was determined HPLC. The HPLC system consisted of a degaser (OGU-14A, Shimadzu), pump (FCV-10AL vp, Shimadzu), system controller (SCL-10A vp, Shimadzu), UV detector (SPD-10A vp, Shimadzu) and auto injector (SIL-10AD vp, Shimadzu). The HPLC method has been reported elsewhere (Kirsch et al., 1989). A 250 mm x 4.6 mm, 5 μm particle size, C-8 column (Zorbax 300SB-C8, Hewlett Packard) was employed at room temperature with a solvent flow rate of 1 ml/min. The mobile phase was acetronitrile : 0.05 M

phosphate buffer pH 5 (30:70). Sample injection volume was 10 µl. Analytical wavelength was 214 nm with a run time of 30 minutes. The column was equilibrated for at least 3 hours prior to use. Samples were analyzed in duplicate.

2.1.1.4 Determination of optimized ultrafiltration operating parameters

Daptomycin (50 µM) solution was prepared in 10% acetonitrile and 1.0 mM Tris buffer and the solutions were then ultrafiltered using various conditions described by the following full factorial design to determine the effect of samples volumes, centrifugal force, and centrifugal intervals on the recovery of daptomycin in the absence of dendrimer (Table 2). In this experiment, loss of daptomycin could be attributed to non-specific binding, retention of aggregated daptomycin (unlikely in the presence of acetonitrile) or failure to recovery all of the filterable solution.

Table 2 Full factorial design with the center point for determination of effects of ultrafiltration operating parameters

Run number	Centrifugal force (x g)	Time (min)	Volume (mL)
1	2000	25	0.8
2	2000	25	0.4
3	2000	15	0.8
4	2000	15	0.4
5	1500	25	0.8
6	1500	25	0.4
7	1500	15	0.8
8	1500	15	0.4
9	1750	20	0.6

The experiments were performed in duplicate. After ultrafiltration, the ultrafiltrates were collected and further analyzed using HPLC. The percent recovery was calculated according to equation 13.

$$\%\ \text{recovery} = \frac{[\text{Dap}]_{\text{After ultrafiltration}}}{[\text{Dap}]_{\text{Before ultrafiltration}}} \times 100 \qquad \text{equation 13}$$

The optimum parameters were determined based on the percent yield using the full factorial analysis by JMP software version 5.0.1 from SAS institute. The prediction profiles were plotted in order to determine the main and interaction effect on the percent recovery.

2.1.2. Determination of dendrimer-daptomycin association constant

The association constant (K) was evaluated using equation 5 as previously described (Conners, 2002: 243-245):

$$f_{11} = \frac{K \times [\text{Dap}]}{1 + K \times [\text{Dap}]} \qquad \text{equation 5}$$

where f_{11} is the fraction of substrate present in the complexed or bound form. [Dap] represents a free concentration of daptomycin obtained from HPLC analysis. Binding isotherm was constructed by plotting f_{11} against [Dap] in order to determine the binding parameter, the association constant (K). Nonlinear regression was used to obtain the best estimates for the association constant in equation 5 (WinNonlin version 5.0.1, Pharsight Corporation).

2.2. Characterization of daptomycin and PAMAM dendrimer complex using UV difference spectroscopy

A series of sample containing 10 μM daptomycin at various concentrations of PAMAM dendrimer generation 5 (0, 1, 10 and 50 μM) were prepared as described above. UV spectra of all mixtures at room temperature were obtained using a HP 8453A UV/ Visible spectrophotometer equipped with a 1 cm path length cuvette.

The association constants (K) were estimated from equation 12 as described earlier (Corners, 1987: 147-148):

$$\frac{\Delta A}{b} = \frac{S_T K \Delta \varepsilon_{11} [\text{Dap}]}{(1 + K[\text{Dap}])} \qquad \text{equation 12}$$

where ΔA are the different in absorbance values of daptomycin in the absence and presence of dendrimer, respectively. $\Delta\varepsilon_{11}$ are different molar absorptivity constant. S_T is total concentration of dendrimer. Binding isotherm was constructed by plotting ΔA against [Dap] in order to determine the binding parameter, the association constant (K). Curve fitting of equation 12 allowed one to determine the $\Delta\varepsilon_{11}$ and K value.

2.3. Characterization of daptomycin and PAMAM dendrimer complex using fluorescence spectroscopy

2.3.1. Determination of fluorescence response upon complex formation

The PAMAM generation 5 in methanol was pretreated by drying under nitrogen gas as previously described. 5 μM daptomycin solution and 68.5 μM PAMAM generation 5 solutions were separately prepared in aqueous solution. The pH value of the mixture was kept constant by titrating to pH 7.0 with standardized dilute sodium hydroxide or hydrochloric acid solutions. A Metrohm Multi-Dosimat E415 and an Ag/AgCl combination electrode were used to monitor pH. Daptomycin-PAMAM dendrimer complex was formed by titrating daptomycin with PAMAM. A 3 mL of 5.0 μM daptomycin was placed in a fluorescence quartz cell equipped with a magnetic stirrer. An aliquot of 68.5 μM PAMAM generation 5 solutions was added to the solution using a Hamilton syringe. The mixture was stirred for 3 minutes. During titration the temperature was controlled at 25°C using a circulating water bath. The complex formation was monitored using a Perkin Elmer LS-55 spectrofluorimeter. The excitation wavelength was set at maxima absorption wavelength (260 and 285 nm) and the emission spectra were scan from 320 to 540 nm in order to observe the emission response from both tryptophan and kynurenine. Excitation/emission slit width was varied on a range of 4/10 and 4/15. Fluorescence spectrum of 5.0 μM daptomycin solution was used as a control.

2.3.2 Determination of concentration range

The titrations of daptomycin (1.0 and 3.0 μM) in 1.0 mM phosphate buffer were titrated by adding aliquots of 82.2 μM PAMAM generation 5 solution. The fluorescence spectroscopy was performed as described earlier.

3. Investigation of pH Effect on the Binding

3.1. The effect of pH on daptomycin-dendrimer complex formation

3.1.1. Titration of PAMAM dendrimer to daptomycin solution at various pH values

3.0 μM daptomycin solutions were prepared at pH values of 3, 4, 5, 6, 6.5, 7 and 8. The pH values of all solutions were monitored using a Metrohm Multi-Dosimat E415, and an Ag/AgCl combination electrode. The aliquot was titrated to the predetermined pH value using 0.10 N hydrochloric acid or 0.10 N sodium hydroxide solutions. A 3 mL aliquot of daptomycin solution was placed in a fluorescence quartz cell equipped with a magnetic stirrer. Five microliter aliquots of 73.1 μM PAMAM dendrimer generation 5 solution were added to the daptomycin solution using a Hamilton syringe. The mixture was stirred for 3 minutes after each addition. The fluorescence responses were taken as previously mentioned. Titrations of double-distilled water to daptomycin solution at each pH were performed as control studies.

3.1.2 Titration of daptomycin to PAMAM dendrimer solution at various pH values

Titration order was also investigated by titration of PAMAM dendrimer solution with daptomycin solution. The PAMAM generation 5 solutions was pretreated as described earlier and reconstituted in double-distilled water at concentration of 0.05 μM for the solutions pH values of 3.5, 4 and 4.5, and at concentration of 0.27 μM for the solutions pH values of 3, 5, 6, 7, 8 and 9. A 3 mL aliquot of PAMAM generation 5 dendrimer was placed in a fluorescence quartz cell equipped with a magnetic stirrer. The aliquot was titrated to the predetermined pH value using 0.1N NaOH or 0.1N HCl. Five microliter aliquots of daptomycin solution (179.8 or 360 μM for dendrimeric solution at the concentration range of 0.05 and 0.27 μM, respectively) were added to the dendrimeric solution using a Hamilton syringe. The mixture was stirred for 3 minutes after each addition. The fluorescence responses were taken as mentioned above. Control studies were also done by titration of aliquots of daptomycin to blank solvent at the same pH range.

3.1.3 Binding isotherm construction

In order to construct the binding isotherm, the fluorescence intensity obtained from the titration mixtures was subtracted by that of daptomycin solution at the same concentration. The resultant fluorescence intensity differences (ΔF) were plotted against total titrant concentration.

3.2. The effect of pH on the binding parameters

3.2.1. The estimation of the binding parameters; molar signal coefficient (ΔE), dissociation constant (K_d) and capacity constant (n)

Various mathematical models were derived based on fluorescence intensity differences (ΔF), the nature of the binding reactions and the total concentration of titrant. The ability of each model to described the observed experimental changes in ΔF as a function of titrant concentration was used to select the correct binding model and to estimate the binding parameters, including molar signal coefficient (ΔE), dissociation constant (K_d) and capacity constant (n). The nonlinear regression analysis was performed using various model equations (WinNonlin software version 5.0.1, Pharsight Corporation). Descriptions of the mathematic models and their physical meaning are presented below.

3.2.2. Assessment of relationship between the ionic properties of daptomycin, PAMAM dendrimer and binding capaciy

The ionization profiles of both daptomycin and dendrimer were constructed based on the relationship between the fractions of each ionic species and pKa values obtained from literature. Comparison of the ionization profiles with the pH-dependence of the estimated binding parameters provided insight into the physical chemical nature of the intermolecular interactions between daptomycin and PAMAM dendrimers of various sizes (generations).

4. Investigation of the Effects of Dendrimer Size on daptomycin-dendrimer interactions

4.1. The effect of dendrimer generation size on complex formation

Additional two sizes of PAMAM polymers (generation 3 and 6) were employed in this study. Binding studies of PAMAM generation 6 and daptomycin were conducted in the pH range of 3.5 to 7, and the binding studies between PAMAM generation 3 and daptomycin were carried in pH range of 4 and 7. Titration procedure were performed as describe above.

4.2. The effect of generation size on the binding parameters

4.2.1. The estimation of molar signal coefficient (ΔE), dissociation Constant (K_d) and capacity constant (n)

Derivation of the mathematical model was performed as describes earlier based on the experimental conditions and the characteristics of the binding isotherm. The binding parameters, molar signal coefficient (ΔE), dissociation (K_d) and capacity (n) constants, were estimated using nonlinear regression according to the derived mathematical model. The nonlinear regression analysis were performed by WinNonlin software version 5.0.1 (Pharsight Corporation).

4.2.2. Evaluation of the relationship between ionic form of reactants and binding constants

The ionization profiles of daptomycin and dendrimer were constructed as previously described. The determinant species in the binding was determined from the comparison of ionization profile and the estimated binding parameters.

4.2.3. Evaluation of the effects of pH and dendrimer size using multivariate linear regression

A general linear model was used to quantitate the effects of pH and generation size on binding parameters. The values of the binding parameters for each

generation of dendrimer and at pH values of 4 and 7 were compared using a linear model with two-way interaction terms. Analysis was performed using statistical software (JMP version 5.0.1, SAS Institute).

4.3. Development of molecular model for the interaction between daptomycin and PAMAM dendrimer

The conformations of calcium free daptomycin (apodaptomycin) and calcium-conjugated daptomycin in aqueous solution have been predicted using information obtained by the Nuclear Overhauser Enhancement Spectroscopy (NOESY) experiments and have been provided in the RCSB protein data bank (Jung et al., 2004). These data were used to generate molecular models of aqueous daptomycin (calcium-free) in space filling or CPK representation Chem3D Ultra 10.0 included in ChemOffice Ultra 2006 package. This type of molecular model shows atoms as three-dimensional spheres whose radii are scaled to the atoms' van der Waals radii and are usually considered useful indicators of the three-dimensional size and shape of macromolecules. The different crossectional diameters of the daptomycin were estimated following the different views of chemical structure using measurement tool in Chem3D software and used to predict binding capacities based on spatial constrains of dendrimer surface area and daptomycin cross-sectional area in different orientations.

The daptomycin cross sectional area was estimated from the different dimensional diameters based on the estimation of free daptomycin in aqueous solution using Chem3D software. The equation of the surface area of a circle was used to calculate the cross sectional area of daptomycin.

$$\text{Daptomycin cross sectional area} = \pi r^2$$

The PAMAM diameter reported by Jackson et al. was used to calculate the surface area of PAMAM (Jackson et al., 1998). According to the assumption that PAMAM are almost in spherical shape, the equation describing the surface area of a sphere was used to calculate the surface area of PAMAM dendrimer.

$$\text{Surface area of PAMAM} = 4\pi r^2$$

where r represent a radius of PAMAM dendrimer molecule.

The highest theoretical binding capacity of daptomycin per each of dendrimer molecule was calculated using equation 14.

$$\text{Theoretical capacity} = \frac{\text{Surface area of PAMAM}}{\text{Daptomycin cross sectional area}} \quad \text{equation 14}$$

4.4. Relationships between binding models, parameter values and binding isotherm shapes

The simulation data was simulated in spreadsheets according to derived one and two type binding model. All parameters including molar signal coefficient (ΔE), dissociation constant (K_d) and the total number of independent binding sites (R) and those of each type were varied one parameter at times. The range of each parameters were varied from 100 to 300, -100 to 40, 0.001 to 2, 0.005 to 10, 1.5 to 5.5 and 0 to 4 for ΔE_1, ΔE_2, K_{d1}, K_{d2}, R_1 and R_2, respectively.

5. Prediction of Optimum Total Dendrimer Concentration

The data was analyzed in order to predict the optimum conditions for complex formation. Mathametical equation was derived to calculate fraction of bound daptomycin (fraction of daptomycin-dendrimer complex) based on the previously determined binding parameters.

Plots between fractions of bound daptomycin against total concentration of dendrimer were constructed to evaluate the optimum combination of daptomycin and dendrimer to provide the highest complexed fraction of drug.