CHAPTER V

CONCLUSIONS

Selection of Characterization Techniques

Daptomycin is a cyclic lipopeptide agent that highly active against a broad spectrum of vital gram-positive pathogens. However, initial clinical trials resulted in treatment failure due to the high degree of daptomycin protein binding, rapid renal clearance or inadequate distribution to the target sites. In order to improve therapeutic outcomes of daptomycin, a non-covalent carrier system was developed using PAMAM dendrimers. Considering the feasibility of using PAMAM dendrimers for efficient daptomycin carriers, it is beneficial to understand their binding properties. Several characterization techniques were utilized in order to probe the binding interaction between PAMAM dendrimer and daptomycin.

1. Characterization of daptomycin and PAMAM dendrimer complex using ultrafiltration technique

Low percent yield obtained from the ultrafiltration of daptomycin solution indicated that the characterization using ultrafiltration appeared to have unidentified interferences. The UV light scattering obtained from ultrafiltrates of daptomycin and dendrimer mixtures suggested the self aggregation of the daptomycin during ultrafiltration process. 10% acetonitrile was used to alleviate the daptomycin aggregation. Consequently, the working concentration range of daptomycin was extended to a higher concentration range. The depletion of bound concentration and the precipitation were observed with increase in total daptomycin concentration and this was suspected to be a consequence of either the displacement of phosphate ion or the precipitation of mixtures. Without using phosphate in the system, the depletion and the precipitation were disappeared. Phosphate was suggested to induce the PAMAM self aggregation resulting in the precipitation and depletion of bound concentration of daptomycin at high daptomycin concentration range. To avoid the precipitation, the working concentration ranges were reduced. However, decrease in daptomycin concentration brought to the sensitivity problem of daptomycin analysis using UV spectrometer. HPLC was used to

measure the free concentration of daptomycin. The optimized ultrafiltration operating parameters was done using factorial design. From the statistical analysis operating time was a main effect and was suggested to use at 25 minute. On the other hand, centrifugation force and initial volume did not affect the ultrafiltration technique.

Due to indistinct difficulties arising from either non-specific binding of daptomycin to ultrafiltration device or self-aggregation of daptomycin itself, the binding study using ultrafiltration technique did not show consistent and reproducible results. Therefore, ultrafiltration technique was not a proper method for characterization of dendrimer and daptomycin complexes.

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

Daptomycin showed UV characteristics over the range of 270 to 430 nm. The UV difference technique was use as a quanlitative tool to determine the binding interaction, but it did not a proper technique to quantitative determination of daptomycin and dendrimer complex. Since, the differences were too small and unable to use for the determination of binding parameters.

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

Daptomycin contains two fluorophores (tryptophan and kynurenine residues). Tryptophan residue transfers fluorescence energy to kynurenine residues due to the overlapping region of tryptophan emission and kynurenine absorption. Kynurenine emission data were sensitively related to binding interaction. Due to a high sensitivity and selectivity of fluorescence technique, the proper working concentration ranges and proper fluorescence parameters were determined. The results obtained from this technique suggested that this technique was suitable for characterization of the interaction between daptomycin and dendrimer. Moreover, probing interaction using fluorescence spectroscopy provided more information on the detail of how daptomycin interact to the dendrimer molecule.

In addition, the one site and two site binding model were successfully developed according to the fluorimetric titrations. The model described the relationship between the changes in fluorimetric signal and total concentration of the ligand.

Therefore, these binding models will be benefit to the other binding study which the binding system possesses the similar spectroscopic behavior.

Investigation of pH Effect on the Binding

Titration of daptomycin with dendrimer showed the biphasic binding isotherm which was suggest to be a result of the occurrence of the second type binding interaction. The binding parameters were unable to obtain from the titration of dendrimer to daptomycin due to unknown total binding site and the interference of second binding type.

The titrations were performed in reverse direction by addition of daptomycin to dendrimer. All isotherms from the binding of PAMAM dendrimer generation 5 were similar in shape excluding the curves from sample at pH 3.0 and pH 9.0. In addition, the increase in intensity was highest in a pH range of 4 to 4.5. Typical curve fitting using the modified one type binding equation well matched the experimental data in a hyperbolic behavior. The binding parameters were successfully determined following our modified equation. The molar signal coefficient and dissociation constant were not pH dependent, whereas the capacity constants were highest at pH range of 4 to 4.5. Comparison of the binding capacity with the ionization profiles of daptomycin and dendrimer revealed that zwitterionic form of daptomycin appeared to be a determinant of the binding interaction between daptomycin and PAMAM dendrimer generation 5.

Investigation of the Effect of Dendrimer Size on Dendrimer-Daptomycin interactions

The binding isotherm of interaction between PAMAM generation 6 and daptomycin was pH dependent. At low pH region (pH range of 4-5) the binding isotherms were in a hyperbolic behavior following the one type binding interaction. Whereas the binding curves between PAMMA generation 6 and daptomycin at the high pH values (6.0 and 7.0) showed biphasic behavior involving a second binding site. The binding constants were perfectly determined using our one type binding and two type binding mathematical model. Relationships between the estimated capacity constants and ionic states of daptomycin and dendrimer were complex. The capacity values for PAMAM dendrimer generation 6 related to both daptomycin and dendrimer ionization.

Additional studies were conducted to evaluate the cross interaction effect of pH and generation size by using PAMAM generation 3. The binding isotherms appeared to be in similar behavior as seen in the binding isotherm generated from binding interaction of daptomycin and PAMAM generation 6. The full factorial consisted of two factors; (i) PAMAM generation size with three levels including PAMAM generation 3, 5 and 6, and (ii) pH with two levels including pH 4 and pH 7. The leverage plot indicated both PAMAM generation size and pH were significant main effects. On contrary, there were no cross interaction effect between generation size and pH was significant main effects.

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

Sizes of daptomycin were estimated from its molecular model as latitudinal and longitudinal dimensions. Comparing the theoretical binding capacity with the estimated binding capacity obtained from previous study supported that daptomycin used its latitudinal dimension in the interaction with the dendrimer. Additional fluorescence information indicated the decanoyl chain of daptomycin insertion into dendrimer molecule. Mapping all information together, daptomycin in bound state was suggested to be in latitudinal dimensional residue with the lipid tail insertion. As mention earlier, several researchers attempt to propose daptomycin mode of action. The latest version proposed that daptomycin insert its tail into the bacterial membrane in a calcium dependent manner. However, there was no solid information that point out the tail insertion of daptomycin. The piece of information observed in this study may fulfill the suspicion in the nature of daptomycin interactions with charged biomolecules. In addition, this may help to understand more in the mechanism of daptomycin activity.

Prediction of Optimum Total Dendrimer Concentration

The simulated profiles were hyperbolic in shape. The plateau region indicated the highest complex yield or the optimum total dendrimer concentration. In order to gain highest complex yield, total concentration of dendrimer generation 6 must be over than 0.057 μM and the complexes must form at pH value of 4.

Future aspects

In order to apply the knowledge of daptomycin-dendrimer complex to be more practical, several aspects of daptomycin-dendrimer complex study are considered including dosage formulation and release study in physiological condition. For the formulation, powder dosage form appears to be a most appropriate formulation of daptomycin-dendrimer complex due to stability issue of daptomycin in aqueous environment. Lyophilization is suggested to be a proper manufacturing process among the other drying process, since it does not require high temperature in the procedure.

In order to evaluate the usefulness of the daptomycin-dendrimer complex, the release study of daptomycin from complex in physiological conditions is necessary. In physiological environment, the biological substances such as ions, biopolymers, and proteins, have been shown to alter the binding interaction. Especially Drug-protein interaction is the normal consideration when the drug is needed to enter the blood stream before reaching the target site. In case of daptomycin, there are several reports indicated that daptomycin has high albumin binding (approximately 92% reported by Dvorchik et al. and approximately 63% reported by Craig et al.) (Dvorchik et al., 2003; Craig, Kiem and Andes, 2004). Daptomycin in the complex form with dendrimer may alter the accessibility of daptomycin to albumin. Therefore, the investigation of the daptomycin-dendrimer complex system at the presence of albumin may be fascinated.

Taken together, the development of suitable dosage formulation and the daptomycin-dendrimer complex profile in physiological conditions may need to be further investigated in order to fulfill the usefulness of non-covalent daptomycin-dendrimer macromolecular system as a potential drug delivery system for daptomycin.