CHAPTER II

LITERATURE REVIEWS

Daptomycin and Pharmaceutical Issues

1. Bactericidal activity of daptomycin

Daptomycin possesses a potent bactericidal activity against broad spectrum of gram-positive bacteria including vancomycin-resistant *enterococci*, methicillin-resistant *staphylococci*, glycopeptide intermediately susceptible *staphylococci aureus*, coagulase-negative *staphylococci* and penicillin-resistant *streptococci* (Snydman et al., 2000; Rybak et al., 2000; Akins and Rybak, 2001). Mode of action of daptomycin is the subject of considerably debate. However, several researches agree that daptomycin antibacterial activity depends on the presence of calcium ion (Eliopolous et al., 1985; Snydman et al., 2000). Recently, a significant calcium-dependent conformational change of daptomycin was reported upon association with model lipid membranes (Jung et al., 2004). These statements suggested that the calcium alters daptomycin conformation into an active form. In addition, interactions of daptomycin with model membranes of dipalmitoleoylphosphatidylethanolamine (DiPoPE) show that daptomycin can induce curvature strain (Straus and Hancock, 2006). Jung and coworkers provide evidence that membrane depolarization occurs after cell death and is a consequence of the bactericidal activity of daptomycin (Jung et al., 2004). Taken together, the findings presented above support a model proposed by Straus and Hancock as illustrated in Figure 1. In the first step, calcium-dependent daptomycin binds and inserts its lipophilic tail into the cytoplasmic membrane. Then, daptomycin molecules form an oligomeric channel on the membrane. Consequently, leakage occurs leading to cell collapse and cell death.

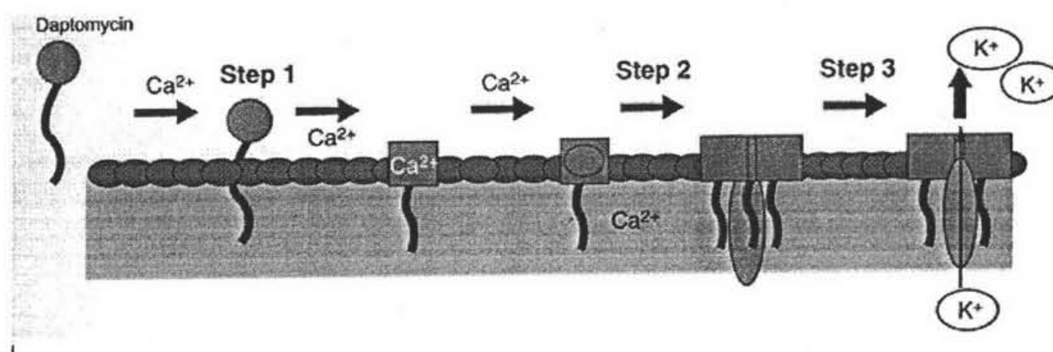

Figure 1 Mode of action of daptomycin

Daptomycin has demonstrated significant *in vitro* bactericidal activity with a post-antibiotic effect (PAE) (Hangerber et al., 1991; Bush et al., 1989). However, initial Phase II clinical trials were terminated (using a recommended regimen, 2 mg/kg body weight per day) because of significantly clinical failures. These failures were attritubed to high daptomycin plasma protein binding, rapid renal clearance, or inadequate distribution to the target sites (Garrison et al., 1990; Lee, Sachdeva and Chambers, 1991; Rybak et al., 1992; Bergeron, 1986).

2. United States Food and Drug Administration approval

In September, 2003 the United States Food and Drug Administration (USFDA) approved a 4 mg/kg IV once daily dose of daptomycin in a for the treatment of complicated skin and skin structure infections (cSSSI) caused by susceptible isolates of the following gram-positive microorganisms including *Staphylococcus aureus* (including methicillin-resistant isolates), *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae subsp. equisimilis*, and *Enterococcus faecalis* (vancomycin-susceptible isolates only). In 2006, the New Drug Approval (NDA) for daptomycin was extended to include treatment of bloodstream infections (bacteremia) and right-sided endocarditis caused by methicillin-susceptible and methicillin-resistant isolates at an approved dose of 6 mg/kg (Cubist Pharmaceuticals, 2006).

3. Pharmacokinetic parameters of daptomycin

In vitro and *in vivo* analysis revealed that daptomycin is effective in concentration-dependent manner. At the concentration of 4mg/kg, daptomycin processed several relatively good pharmacokinetic parameters including a long half-life of 8.1 hours,

a maximum plasma concentration (C_{max}) of 57.8 mg/L, a time to maximum plasma concentration (T_{max}) of 0.8 hours, an area under the concentration-time curve from 0 to 24 h (AUC_{0-24}) of 494 mg.h/L, an apparent volumne of distribution (V) of 0.096 L/kg, a systemic clearance (CL_T) of 8.3 mL/h/kg, a renal clearance (CL_R) of 4.8 mL/h/kg, and a percentage of dose recovered in urine over 24 hours as unchanged daptomycin following the first dose (Ae_{24}) of 53.0%. For the higher concentration of 6 mg/kg, pharmacokinetic parameters of daptomycin were as followed; a half-life of 8.9 hours, a C_{max} of 98.6 mg/L, a T_{max} of 0.5 hours, an AUC_{0-24} of 747 mg.h/L, a V of 0.104 L/kg, a CL_T of 8.1 mL/h/kg, a CL_R of 4.4 mL/h/kg, and an Ae_{24} of 47.7 % (Steenbergen et al., 2005; Brauers et al., 2006).

4. Daptomycin toxicity

Preclinical studies indicate that skeletal muscle is the primary target of dose-related toxicity for daptomycin. In Phase 1 studies, two of five subjects receiving daptomycin at 4 mg/kg twice daily for 7 – 12 days had acute creatinine phosphokinase (CPK) elevations (more than 10 times the upper unit of normal range), associated with muscle weakness and myalgia (Tally et al., 1999). Recently, Veligandla also reported that daptomycin at 6 mg/kg daily induced myalgia (Veligandla et al., 2004). These effects are rapidly and completely reversible after discontinuation of the drug. Subsequent studies in dogs indicated that the frequency and severity of muscle effects increased with divided dosing and decreased with once-daily dosing (Oleson et al., 2000).

5. Daptomycin structure and ionic properties

Daptomycin is a 13 amino acid cyclic peptide acylated to a dedecanoyl side chain (Figure 2, Debono et. al, 1988). The molecular weight of daptomycin is 1620 Da (Kirsch et. al, 1989).

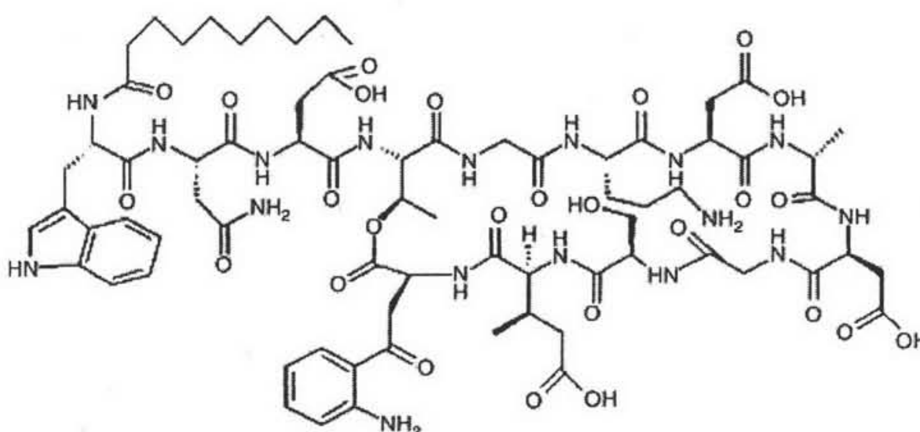

Figure 2 Chemical structure of daptomycin

The cyclic structure is due to the formation of an ester bond between the C-terminal amino acid residue of the peptide, kynurenine, and the hydroxyl side chain of the fourth amino acid, threonine. The N-terminal tryptophan is acylated to a decanoyl lipid chain. Daptomycin is amphiphilic and has a propensity to aggregate at a concentration above 5 mM in water at pH 6 (Lakey and Ptak, 1988). The presence of both the lipid side chain and the cyclic peptide is essential for antibiotic activity (Woodworth et. al, 1992). In pH range of 0 and 14, daptomycin contains six ionizable groups which are four carboxylic acid side chains (three aspartic acids and one methyl-glutamic acid) and two primary amines (kynurenine and ornithine). Aspartic acid residue 3 has pKa of 3.0 while other carboxylic side chains have overlapping pKa's of 5.3 (Kirsch et. al, 1989). An aromatic amine on kynurenine residue has pKa of 0.8 and an aliphatic amine on ornitine has pKa of 10.0 (Muangsiri, 2000).

6. Spectroscopic properties of daptomycin

Daptomycin has UV absorption over the range of 200 to 420. The adsorption maxima due to aromatic rings of tryptophan and kynurenine residues are 282 or 365 nm. An ionization-dependent change in adsorption maxima of daptomycin spectra was used to determine pKa value of kynurenine residue on the daptomycin by Muangsiri (Muangsiri, 2000). The protonated kynurenine had absorption maxima at 280 nm, while the unprotonated form had absorption maxima at 370 nm.

In addition, daptomycin contains two fluorophores at tryptophan residue situated between the decanoyl side chain and the cyclic peptide, and kynurenine residue

located in the cyclic peptide (Figure 2). When daptomycin is excited at 285 nm, the tryptophan residue emission is less than 4% of the expected emission of free L-tryptophan under the same conditions (Lakey and Ptak, 1988). Furthermore, a second emission at 465 nm is observed, and it was more intense when excited at 365 nm, indicating that kynurenine residue is the second emission source. The relative quantum yield of kynurenine residue is 7.6 times greater that of free L-kynurenine under the same conditions. The decrease in tryptophan and increase in kynurenine emissions is a consequence of Forster-type energy transfer resulting from the combination of the proximity of two fluorophores and the overlapping of the tryptophan emission spectrum and kynurenine adsorption spectrum (Weinryb and Steiner, 1971).

Polyamidoamine (PAMAM) Dendrimers in Drug Delivery

1. Dendrimer properties in host-guest aspect

The structural features of dendrimers offer a variety of defined sizes and shapes (i.e. cargo spaces) and permit extensive dendrimer surface modifications to effectively control drug delivery system.

A hydrophobic drug would be expected to associate with a dendrimer interior to achieve maximum contact between its hydrophobic components and comparable domains within the dendrimer. If the drug molecule is significantly large or incompatible with either the dimensions or hydrophilic/lipophilic characteristics of the dendrimer cavity, a complex might not form, or the guest might only be partially incarcerated within the dendrimer host. Analysis of a typical symmetrically branched dendritic lattice suggests that other subtle parameters that could control the interior space of a dendrimer and influence guest–host interactions (Naylor et al, 1989). These include crucial branch-cell components, such as: branching angles, rotation angles and repeat-unit segment length (Figure 3).

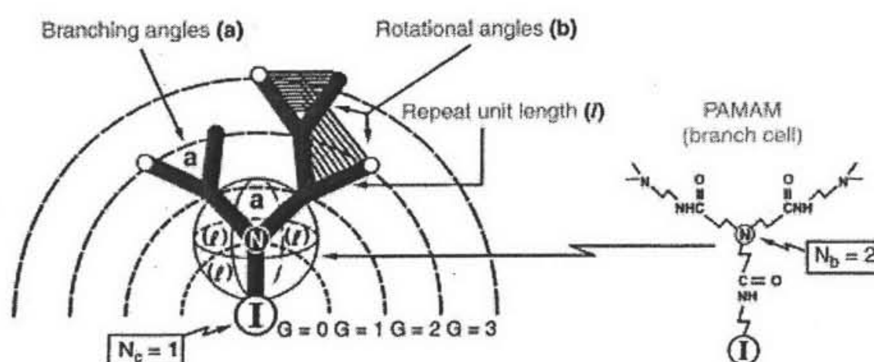

Figure 3 Branch cell parameters: branching angles (a), rotational angles (b), repeat unit length (l) and branch cell multiplicity (N_b) are the crucial parameters that determine cargo-space properties within the interior lattice of a dendrimer.

Dendrimer surface chemistry is another important aspect for drug delivery system. The presence of ionic end groups is responsible for their high solubility (Twyman et al., 1999). In addition, dendrimer can be synthesized with a variety functional end group for the specific purpose. For instance, the dendritic box was designed to control drug release by forming a stable shell on the surface of the dendrimer (Baker et al., 2001).

2. PAMAM characteristic

Synthetic polymers have many applications in biomedical area, because of their ease of manufacture, durability, and low immunogenicity. In last decade, dendrimer have become useful tools for drug delivery by molecular encapsulation and for gene therapy. Polyamidoamine (PAMAM) dendrimers is first reported by Tomalia et al in the early 1980s (Tomalia and Frechet, 2002). When PAMAM dendrimers molecules are synthesized in a stepwise manner, each new step doubles in molecular weight, the number of end groups, and the number of branch points. Dendritic structure possesses three distinguishing architectural components which are an interior core, interior layers often called "generations" which are composed of repeating units radially attached to the initiator core, and extending to the outermost surface (Figure 4). The well defined, highly branched, compartmentalized structures in nanometer size range exhibits unique properties such as high degree of molecular uniformity, narrow molecular weight

distribution, specific size and shape characteristics, and a highly functionalized terminal surface.

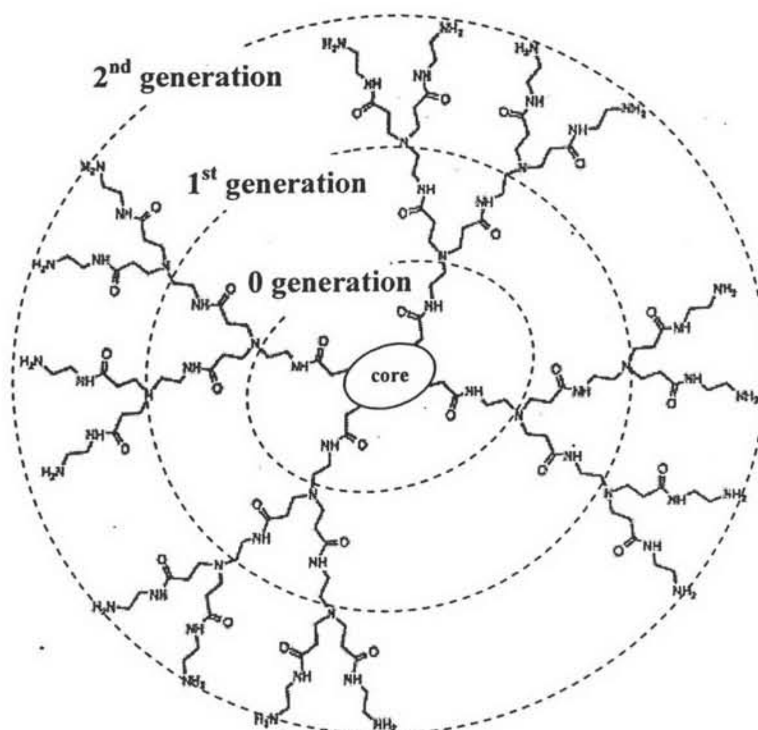

Figure 4 Chemical structure of PAMAM generation 2

3. PAMAM size and shape

The average radius of gyration (R_g) and the shape tensor of monomer provide a quantitative characterization of dendrimer size and shape. Table 1 shows a detailed comparison of the R_g of PAMAM dendrimers with various generation from 2 techniques, SANS and SAXS. As the number of PAMAM generation increased, its size increasing.

Table 1 Radius of gyration (R_g) as a function of generation for PAMAM in methanol using small angle x-ray scattering (SAXS) (Prosa et al., 1997) and small angle neutron scattering (SANS) ((a) Topp et al., 1999a; (b) Topp et al., 1999b)

Generation	Radius of gyration (Rg) (Å)	
	SAXS	SANS
3	15.8	
4	17.1	
5	24.1	24.3[a]
6	26.3	
7	31.3	34.4[b]
8	40.3	39.5[a]
9	49.2	
10	57.4	

Size of PAMAM generation 5 to 10 has been reported to be 4.3 to 14.7 nm, respectively, by using transmission electron microscopy (TEM) (Jackson et al., 1998). The mean diameters of the dendrimers measured by TEM on the stained specimens are consistent with those from SAXS measurements in methanol as shown in Figure 5.

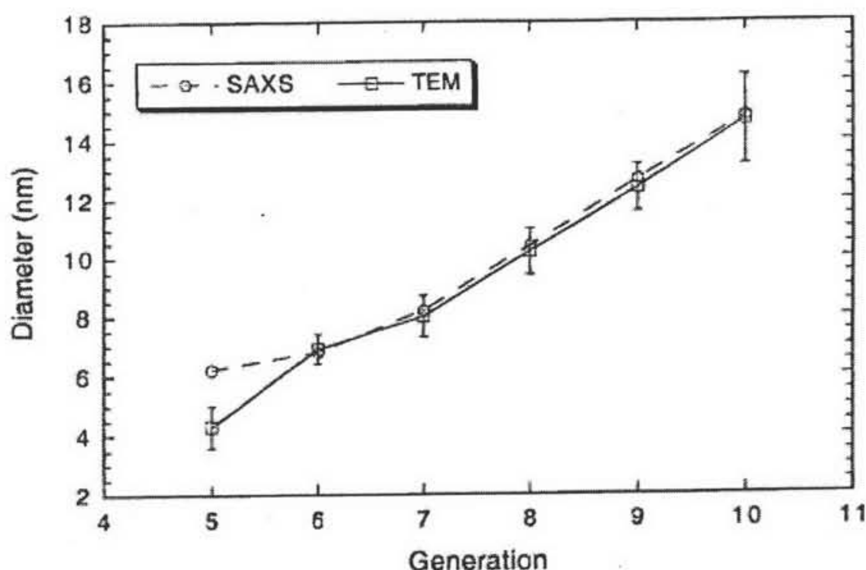

Figure 5 Comparison of mean dendrimer diameters measured by TEM (□) and small-angle X-ray scattering (O) techniques, as a function of generation number and molecular weight.

TEM indicates that a higher generation dendrimer (generation 7 to 10) are spherical in shape, with some molecules showing "edge" or slightly polyhedral shape. For the lower generation (generation 5 to 6) the shapes in TEM images become less distinct.

Partial deuterated PAMAM generation 7 were prepared to reveal end-group back-folding structures. The results show that the distribution of dendrimer terminal groups is not uniform throughout the interior of dendrimer, but rather the terminal groups are localized near the periphery of the dendrimer much as predicted by de Gennes et al (Topp et al., 1999a; deGennes and Hervet. 1983).

4. PAMAM ionic properties

Full generation PAMAM dendrimers consist of primary amine end groups on the surface and tertiary amine groups situated at the branching points in the core. Therefore, the ionization behavior of PAMAM involves two independent deprotonation steps at the primary amine and tertiary amine groups (Figure 6). The mechanism of dendrimer protonation with ionizable groups is under debate. However, the data from the acid-base titration suggest that (i) the tertiary and primary amines of ethylenediamine

(EDA) core PAMAM dendrimers independently protonate and (ii) dendrimer generation and terminal group chemistry do not have a significant impact on the extent of protonation of the tertiary amine groups of EDA core PAMAM dendrimers (Diallo et al., 2004). The measured pKa values of tertiary amine and primary amine in PAMAM generation 3, 4 and 5 are ranging from 6.70 – 6.30 and 9.00 – 9.23, respectively (Niu, Sun and Crooks, 2003; Cakara, Kleimann and Borkovec, 2003).

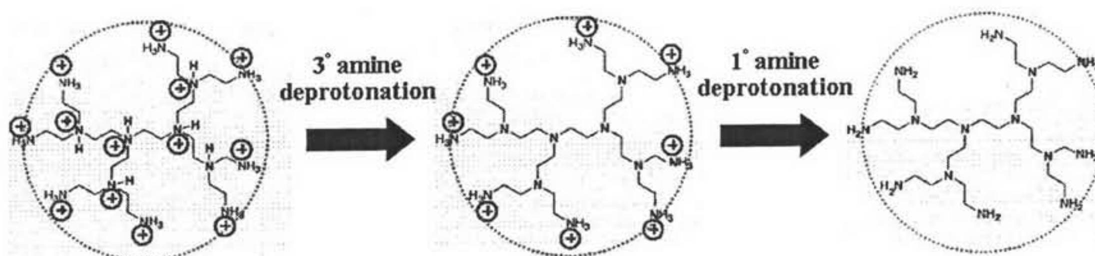

Figure 6 Deprotonation step of PAMAM dendrimer

5. Binding properties of PAMAM

Based on the PAMAM structure, it possesses a high amount of amine surface groups. The amino groups are excellent electronic complexing agents because of ionized state. In addition, the branch-cell components containing tertiary amine and amide linkage provide a proper internal cavity in the PAMAM dendrimer. Therefore, drug molecule can either be attached to the end groups of the PAMAM dendrimer or encapsulated in the macromolecule interior.

The internal cavities of PAMAM dendrimer can host small guest molecules. Fluorescence probes such as ANS and tryptophan are used to probe an ability of loading small molecule into dendrimer cavity (Klajnert and Bryszewaka, 2002). Not only charges of the molecule appear to play the great role on the interaction, but hydrophobic forces are also involved. The vicinity of the tryptophan indole ring is hydrophilic, and is found to locate on the surface of the dendrimers, whereas the ANS aromatic rings are placed more deeply in a more hydrophobic environment. In addition, the size and structure of PAMAM is also an important determinant in the host-guest interaction. Two types of dendrimer binding centers is characterized by different affinity towards ANS and the observed binding capacities and the location of ANS molecule are strongly influenced by the size of dendrimers (Klajnert et al., 2006).

The interaction of a large molecule with PAMAM has also been investigated. However, the interaction appears to be more complicate than the small guest system. The detailed analysis of the interaction between PAMAM G4 dendrimer and serum albumins was performed using circular dichroism, isothermal titration calorimetry, capillary electrophoresis, zeta-potential and fluorescence polarization and showed that the anionic regions associated with serum albumins can bind to positivelyicharged dendrimers (Scharbin at al., 2007).

Driving Force of Protein Adsorption at Solid Surface

Proteins are copolymers of amino acids with various amino acids of varying hydrophobicity. As a consequence, proteins possess regions of hydrophobic and hydrophilic characteristics, and therefore, are usually highly surface active. Moreover, ionizable amino acid side chains and terminal moieties make the proteins amphoteric polyelectrolytes.

Proteins in aqueous solution possess conformations in which the atomic packing density, expressed in volume fraction, reaches a value of 0.70-0.80. In such a conformations, the rotation freedom along the polypeptide chain may be severely restricted, implying low conformational entropy. The restricted structure is possible only if interactions within the protein molecule and interactions between the protein molecule and its environment are sufficiently favorable to compensate for the low conformational entropy. These interactions are due to various forces including coulombic, hydrophobic, hydrogen-bonding and Lifshitz-van der Waals interactions.

1. Coulomb or electrostatic interaction

Coulombic force or electrostatic forces is a force creating between two charged molecules. Opposite charges produce attractive force while similar charges produce a repulsive force. The coulomb interactions are largely dependent on the charged density on the molecule and the distance between two charged molecules.

Most of the standard amino acids found in proteins have uncharged side chains, although histidine, lysine and arginine each have a positive charge at neutral pH and both glutamic and aspartic acids normally carry a negative charge. The basic residues in a

protein can have electrostatic interactions with negatively charge surface, whereas, acidic residues in a protein cause electrostatic repulsion to negatively charge surface.

2. Hydrophobic interaction

Hydrophobic "bonding", first proposed by Kauzmann, is not actually "bond formation", but rather the tendency of hydrophobic molecule or hydrophobic part of molecules to avoid water because hydrophobic regions are not readily accommodated in the hydrogen-bonding structure of water (Kauzmann, 1959). Thus large molecules with extensive hydrophobic regions such as protein tend to avoid the water molecules in an aqueous solution insofar as possible by associating into micelle-like structures with the nonpolar portions in contact in the inner regions of the micelles and the polar ends facing the water molecules. This attraction of hydrophobic species, resulting from their avoidance of water, is known as hydrophobic bonding or hydrophobic interaction. The driving forces involve van der Waals forces, hydrogen bonding of water molecules in a three-dimensional structure, and other interactions. Hydrophobic interactions are favored thermodynamically because of an increased disorder or entropy of the water molecules that accompanies the association of the nonpolar molecules, which exclude water molecules.

3. Hydrogen bonds

The interaction between a molecule containing a hydrogen atom and a strongly electronegative atom such as fluorine, oxygen, or nitrogen is of particular importance. The small size of a hydrogen atom with its large electrostatic field can move closely to an electronegative atom and form an electrostatic type of union known as a hydrogen bond or hydrogen bridge. Such a bond, discovered by Latimer and Rodebush, exists in ice and in liquid water and accounts for many of the unusual properties of this ubiquitous solvent, including its high dielectric constant, abnormally low vapor pressure, and high boiling point (Latimer and Rodebush, 1920).

Bond energies serve as a measure of the strength of bonds. Hydrogen bonds are relatively weak, having bond energy of about 2 to 8 kcal/mole as compared with 50 to 100 kcal/mole for covalent bonds and >100 kcal/mole for ionic bonds. The total interaction energies between molecules are contributed by a combination of orientation,

induction, and dispersion effects. The nature of the molecules determines which of these factors is most influential in the attraction.

4. **Lifshitz-van der Waals interaction**

The existence of a general attractive interaction between a pair of neutral atoms is first postulated by van der Waals to explain the observed deviation of a real gas behavior from the ideal gas law. In spite of the qualitative character of the argument used by van der Waals in his derivation, the great success of his equation of state in explaining experimental data on the properties of gases spurs a though on the origin of the interatomic or intermolecular forces.

These non-covalent and non-electrostatic molecular forces are referred to as van der Waals force. Van der Waals force plays a central role in all phenomena involving intermolecular forces. It is not as strong as coulombic or hydrogen bonding interactions. The van der Waals forces originates from: (i) randomly orienting dipole-dipole (or orientation) interactions, described by Keesom, (ii) randomly orienting dipole-induced dipole (or induction) interactions, described by Debye and (iii) fluctuating dipole-induced dipole (or dispersion) interactions, described by London (Martin, 1993: 22-23).

Complex Characterization Techniques

The most common way to categorize the characterization methods of complexes formed by molecular interactions is direct or indirect techniques. Direct techniques are based on monitoring the change of a physicochemical property of the protein–probe system upon binding. Examples of direct technique include optical absorption spectroscopy, magnetic resonance spectroscopy, reaction kinetic, and solubility measurement. Indirect techniques require a separation of the bound and free probe molecular entities followed by their quantitation. Examples of these types of techniques include equilibrium and dynamic dialysis, ultrafiltration, and gel filtration. Among the direct techniques, spectroscopy is extensively used and considered to be superior to the indirect techniques because, to a first approximation, they do not disturb the binding equilibrium upon separation (Wang, Wei and Chuan, 2006).

1. Ultrafiltration

Ultrafiltration method is a simple and convenient indirect technique for a routine determination of drug-protein binding. The ultrafiltration method is similar to equilibrium dialysis in that a membrane is used to separate macromolecules from small molecules. However, hydraulic pressure or centrifugation is used in ultrafiltration to force the solvent and the small molecules, unbound drug or ligand, through the membrane while preventing the passage of the bound ligand complex (Oravcova, Bohs and Lindner, 1996). The concentration of unbound ligand in the ultrafiltrate is then analyzed by spectrophotometeric or chromatographic methods. Furthermore, the ultrafiltration technique receives short analysis time, and the ultrafiltration kits are commercially available. In addtion, further sample preparation such as dilution is unnecessary; therefore, the equilibrated system is not perturbed by dilution effect or volume shift.

An example of commercially available ultrafiltration kit is Amicon® micropartition system (Amicon® MPS). The Amicon® MPS is an plastic device, consisting of a narrow reservoir capped to prevent gas exchange with the atmosphere and mounted over a membrane support and receiving cup, which are all held together by means of two plastic clips (Figure 7). Except for the membrane, the entire device is reusable.

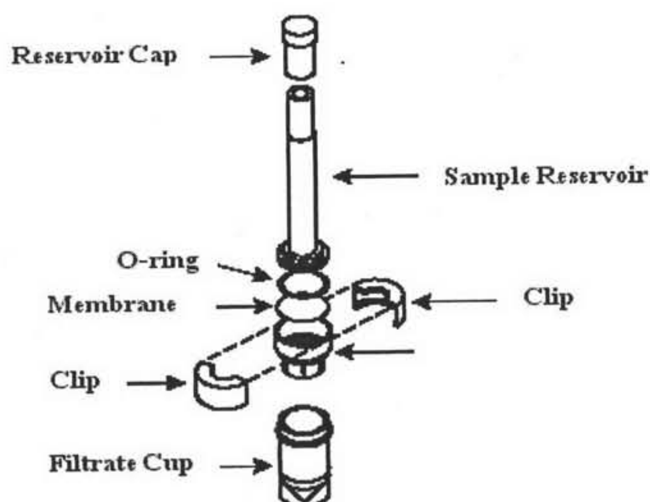

Figure 7 The Amicon® MPS

The ultrafiltration technique has been successfully used for a protein binding study of daptomycin due to the clinical failures of the first recommended regimen (2mg/kg). 95% recovery of drug was observed in the ultrafiltrate. On average, 90% of daptomycin was bound to serum protein and the average binding to serum in vivo (90%) was weaker than in vitro (94%) (Belle, Meena and Henry, 1991).

2. UV difference spectroscopy

A mixture of ligand and binding-macromolecule contains at least three molecular entities: unbound ligand and macromolecule and bound complex. Assuming that only macromolecule or ligand absorbs UV light, the spectra of the mixture is the consequence of the combined absorbance of both free and bound ligand at any specific wavelength. The difference between the spectra of complex mixture and that of free form is due to the concentration and absorptivity of the complex. The advantage of this technique is the simplicity of the principle and instrumentation. However, the major limitation is its selectivity in that an essential requirement is whether a significant UV molar absorptivity difference between free and bound ligand.

3. Fluorescence spectroscopy

Several types of fluorescence measurements can be used to measure binding. However, most applications use fluorescence intensity changes upon binding which manifest in either fluorescent enhancement or quenching.

3.1 Two component system; fluorescence enhancement

The fluorescence quantum yield of fluorescence of either substrate (S) or ligand (L) increases upon complex formation; therefore, the observed fluorescence intensity is increased. For instance, fluorescence probes, such as 2-toluidinylnaphthalene-6-sulfonate (TNS) or 8-anilino-1-napthalenesulfonate (ANS), have very low quantum yields in aqueous solution. On binding to proteins, their quantum yields increase markedly, indicating that the binding sites are relatively nonpolar (Edelman and McClure, 1968).

3.2 Two component system; fluorescence quenching

Complexation of substrate and ligand causes a decrease. This quenching phenomenon takes place when the emission band of fluorescent species overlaps the absorption band of quencher. The quenching phenomenon is more pronounced when

fluorescent species is in a closed-proximity to the quencher. At short intermolecular distances, a radiationless energy transfer from the excited state of fluorescence species to the ground state of quencher takes place; thus, the fluorescence intensity is reduced. For example, the fluorescence of serum albumin is quenched by thyroxine via this mechanism (Steiner, Roth and Robbins, 1966). In the case that quencher possesses fluorescence properties; the fluorescence intensity of the quencher can be enhanced.

3.3 Three system components; fluorescent substrate, nonfluorescent ligand ligand, and a quencher (Q)

In this scenario, both S and Q competitively bind to L, and at constant total substrate concentration the fluorescence intensity decreases as the concentration of quencher is increased. This technique has been used to study micellar binding (Encinas and Lissi, 1982).

Daptomycin contains two fluorophores, tryptophan and kynurenine, which interact to cause tryptophan emission quenching and kynurenine emission enhancement. It is not surprising that conditions that alter daptomycin conformations may also cause fluorescence changes. Lakey and coworkers show that the tryptophan emission of daptomycin is quenched by the absorption of kynurenine since tryptophan emission spectrum overlap with kynurenine absorption spectrum (Lakey and Ptak, 1988). The quenching effect is based on the proximity of two fluorophores. Moreover, they also observe that the kynurenine emission intensity is higher when kynurenine is a part of daptomycin than in the free amino acid form. The kynurenine fluorescence is a sensitive probe of the membrane interactions, and it is used in steady-state fluorescence measurements including fluorescence polarization anisotropy. Initial binding of daptomycin to 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and dipalmitoyl phosphatidylcholine phospholipid (DPPC) vesicles occurs in calcium-free solutions. When calcium is added, the resulting 10-fold fluorescent enhancement and 15-nm blue shift showed that it caused daptomycin to penetrate further into the lipid bilayer. Calcium ions have been shown to bind to daptomycin (at neutral pH) with an association constant (K) equal to 151 M^{-1} (Lakey and Ptak, 1988).

Binding Isotherms

In term of complex formation, the binding isotherm is a mathematical expression that relates the concentration of adsorbate (ligand) at the interface to its equilibrium concentration in the liquid phase. Typical desired information in complex formation are (i) the amount of ligand per unit mass or unit area of the substrate at a given temperature; (ii) the equilibrium concentration of ligand in the liquid phase required to produce a given surface concentration of ligand at a given temperature; (iii) the saturation concentration of ligand on the substrate at a given temperature; (iv) the orientation of the ligand and any other parameters that may shed the light on mechanism by which the complex is formed; and (v) the effect of complex formation on the other properties of the ligand or vice versa. Since most of the information can be obtained from adsorption isotherm, the isotherm is a usual method of describing complex formation.

The binding isotherm is derived on the basis of prior knowledge of the system which is tested against obtained experimental data. When information is not available to allow the construction of a reasonable model, first step may be to carry out exploratory studies to help define the problem. In the present context, a model is a statement of the possible complex affinity and capacity, perhaps with auxiliary statements that amplify the description.

1. 1:1 Binding isotherm described by indirect technique

The 1:1 stoichiometric model is the simplest of stoichiometric models. The reaction scheme of complex formation between substrate (S) and ligand (L) reaction is written by

$$S + L \overset{K}{\rightleftharpoons} SL \qquad \text{equation 1}$$

From equation 1, the equilibrium or association constant (K) is defined as

$$K = \frac{[SL]}{[S][L]} \qquad \text{equation 2}$$

where [S], [L] and [SL] are free substrate, free ligand and complex concentrations, respectively. Let; define f_{11} as the fraction of substrate present in the complex (bound) form, or

$$f_{11} = \frac{[SL]}{S_T} \qquad \text{equation 3}$$

where S_T state for total substrate concentration. From mass balance;

$$[S] = S_T - [SL] \qquad \text{equation 4}$$

Algebraic substitution of equation 3 to 4 gives a rectangular hyperbolic equation;

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

The f_{11} and [L], obtained from the experiment, is use to build up the binding isotherm in order to further evaluate association constant (K) by using linearized forms of equation 5 and linear regression analysis or by using nonlinear regression analysis.

2. 1:1 binding model described by direct technique

Spectroscopy is extensively used in binding study among the direct techniques. The following approach describes the use of data obtained from spectroscopic difference techniques. In this direct technique, the adsorption spectrum of free substrate is observed to be different from adsorption spectra of the complex. Beer's law is assumed to be applicable for all absorbing species. A wavelength is selected at which the molar absorptivities of substrate (ε_S) and complex (ε_{11}) are different. In the absence of ligand, the absorbance of substrate solution is

$$A_0 = \varepsilon_S b S_T \qquad \text{equation 6}$$

where S_T is total concentration of substrate and b is cuvette path length. In the presence of ligand, the total absorbance of a solution (A_L) is

$$A_L = \varepsilon_S b[S] + \varepsilon_L b[L] + \varepsilon_{11} b[SL] \qquad \text{equation 7}$$

which, combined with the mass balance, gives

$$A_L = \varepsilon_S b S_T + \varepsilon_L b L_T + \Delta\varepsilon_{11} b[SL] \qquad \text{equation 8}$$

where $\Delta\varepsilon_{11} = \varepsilon_{11} - \varepsilon_S - \varepsilon_L$. By subtracting the absorbance of ligand at the same total concentration (L_T), equation 8 becomes

$$A = \varepsilon_S b S_T + \Delta\varepsilon_{11} b[SL] \qquad \text{equation 9}$$

Combining equation 9 with the association constant definition (K; equation 3) gives

$$\Delta A = K \Delta\varepsilon_{11} b[S][L] \qquad \text{equation 10}$$

where $\Delta A = A - A_0$. From the mass balance expression (equation 4), association constant definition (equation 2) is rearranged so that

$$[S] = \frac{S_T}{(1 + K[L])} \qquad \text{equation 11}$$

Substitution of [S] in equation 10 with equation 11 gives the relationship between the changes in observed absorbance per path length in centimeter and free ligand concentration.

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

The binding isotherm is constructed by plotting the difference in spectroscopic signal (ΔA) against free ligand concentration [L]. Nonlinear regression analysis is used to fit the binding isotherm. Finally, association constant (K) and different molar absorptivity constant ($\Delta\varepsilon_{11}$) are estimated.

In the same manner as UV spectroscopy, fluorescence technique can be employed in order to directly quantitative binding interaction (Conners, 1987: 340). At low concentration, the fluorescence intensity is directly proportional to solute concentration as describe below

$$F = 2.3 I_0 \phi \varepsilon b c$$

where F is fluorescence intensity, I_0 is the intensity of the excitation source, ε is molar absorptivity at the excitation wavelength, b is path length, c is molar concentration, and ϕ is fluorescence quantum yield. This expressing is in a similar form as Beer's law (equation 6). Therefore, in the same fashion mathematical expression can be derived to explain binding isotherm between ΔF and free ligand concentration.

Possible Factors Affecting Binding Interaction

pH can cause significant changes in the interaction of ionic species. As the pH of aqueous phase is lower than pKa value, an ionic surface of a molecule will become more positive, and easily interact with anionic species. On the other hand, as pH value is higher than pKa, molecular surface becomes negative and electrostatically attractive to cationic species.

pH change also affects ligand molecules, especially those containing carboxylate groups or nonquaternary ammonium groups. For example, ligand containing carboxylic group looses its strong ionic interaction to positive substrate due to carboxylic protonation

at low pH value. Therefore, ligand becomes a neutral molecule and bind to positive charge substrate through weaker force of H-bonding or dispersion force (Rosen, 2004: 53).

Addition of neutral electrolyte, such as NaCl and KBr, causes a decrease in the interaction of ligand onto an oppositely charged substrate but induces binding of ligand onto a similarly charged substrate. High ionic strength decreases attraction between oppositely charged species and the decrease repulsion force between similarly charged species. Both the efficiency and effectiveness of the interaction of ionic surfactants onto similar charged substrates are increased by an increase in ionic strength of aqueous phase.

In general, as temperature increases, the efficiency and effectiveness of interaction of ionic species decreases. However, changes due to temperature effect are relatively small when compared to that caused by pH change.