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## Calorimetric Investigations of Non-Viral DNA Transfection Systems

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### 1. Introduction

Although the structural and energetic forces involved in DNA condensation have been studied for years, this area has experienced a resurgence of interest in recent years with respect to developing gene therapy protocols to combat a variety of human diseases. Despite an intense effort to study the mechanism(s) of DNA condensation using a variety of techniques such as microscopic, light scattering, fluorescence, and calorimetric techniques the precise details of the energetics of DNA nanoparticle formation and how these complexes assemble is not well understood at present. Isothermal titration calorimetry (ITC) has become an important technique for studying the binding energetics of biological processes, including protein-protein binding, protein-carbohydrate binding, protein-lipid binding, antigen-antibody binding, DNA-protein binding, and the DNA-lipid binding that is at the heart of non-viral transfection vectors. Given the large informational content of thermodynamic data (such as binding constants, stoichiometry, enthalpies of interaction, etc.) it is not surprising that ITC is being used for the elucidation of the binding mechanism of DNA with surfactants or lipids used in non-viral gene delivery vectors. This is described in more detail in this Chapter.

#### 1.1 Principle and experimental set up

One of the fundamental challenges in biophysical chemistry, as well as in all physicochemical events implying a solvent, is to attribute the contribution of different non-covalent interactions (electrostatic in the most general sense, solvation and hydrophobic interactions, hydrogen bonding, Van der Waals interactions) to the free energy change of a given molecule upon its interaction with a binding partner. Another challenge is to investigate how conformational changes in the three dimensional structure of a protein, DNA or RNA can modify the interaction between itself and its binding partner. Finally, the most important challenge is to relate structure (the high resolution X-ray or NMR structure of more and more proteins and nucleotides are available) to the “binding affinity” of these molecules with their binding partners. This means that the “binding affinities” have to be accurately measured for as many as possible systems to get a clear picture of structure-

activity relationships, which is particularly important for the design of new drugs or the design of gene vectors for gene therapy. Unlike any other method used to determine thermodynamic parameters associated with bimolecular interactions, calorimetry uses the direct measurement of the heat of interaction (or molar enthalpy  $\Delta H$ ) to probe the extent of binding between the molecules. When substances bind, heat is either generated or absorbed.

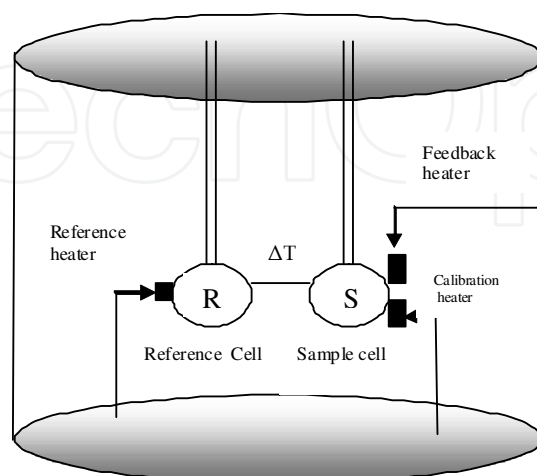


Fig. 1. Schematic diagram of an ITC instrument.

Isothermal microcalorimetry is a thermodynamic technique that directly measures the heat released or absorbed during a biomolecular binding event. Measurement of this heat allows accurate determination of binding constants, reaction stoichiometry ( $n$ ), enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ), thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment. The first modern isothermal microcalorimeter was designed by Calvet (Calvet and Prat, 1963). An ITC instrument consists of two identical cells composed of a highly efficient thermal conducting material (e.g. gold) surrounded by an adiabatic jacket (Figure 1). The jacket is usually cooled by a circulating water bath. Sensitive thermopile circuits detect temperature differences between cells and the jacket, while heaters located on both cells and the jacket are activated when necessary to maintain identical temperatures between all components. In a typical ITC experiment, the macromolecule is placed into the sample cell of the calorimeter and is titrated at constant temperature with the ligand in a syringe. The reference cell contains water or buffer. Prior to injection of the titrant, a constant power ( $< 1$  mW) is applied to the reference cell. This signal directs the feedback circuit to activate the heater located on the sample cell. This represents the baseline signal. During the injection of titrant into the sample cell, heat is taken up or evolved depending on whether macromolecular association reaction is endothermic or exothermic. For an exothermic reaction, the temperature in the sample cell will increase, and the feedback power will be deactivated to maintain equal temperature between the two cells. For endothermic reaction, the reverse will occur.

The heat released (in terms of molar enthalpy  $\Delta H$ ), upon interaction of the titrant is monitored over time. Each peak represents a heat change associated with the injection of a small volume of ligand solution into the ITC reaction cell. As successive amounts of the ligand are titrated into the ITC cell, the quantity of heat absorbed or released is in direct proportion to the amount of binding. When the system reaches saturation, the heat signal diminishes until only heats of dilution are observed. A binding curve is then obtained from

a plot of the heats from each injection against the ratio of ligand and binding partner in the cell. A molecular interaction between two ligands can be defined by the following equation which forms the basis for an ITC analysis:

$$\Delta G = -RT \ln K_A = \Delta H - T\Delta S \quad (1)$$

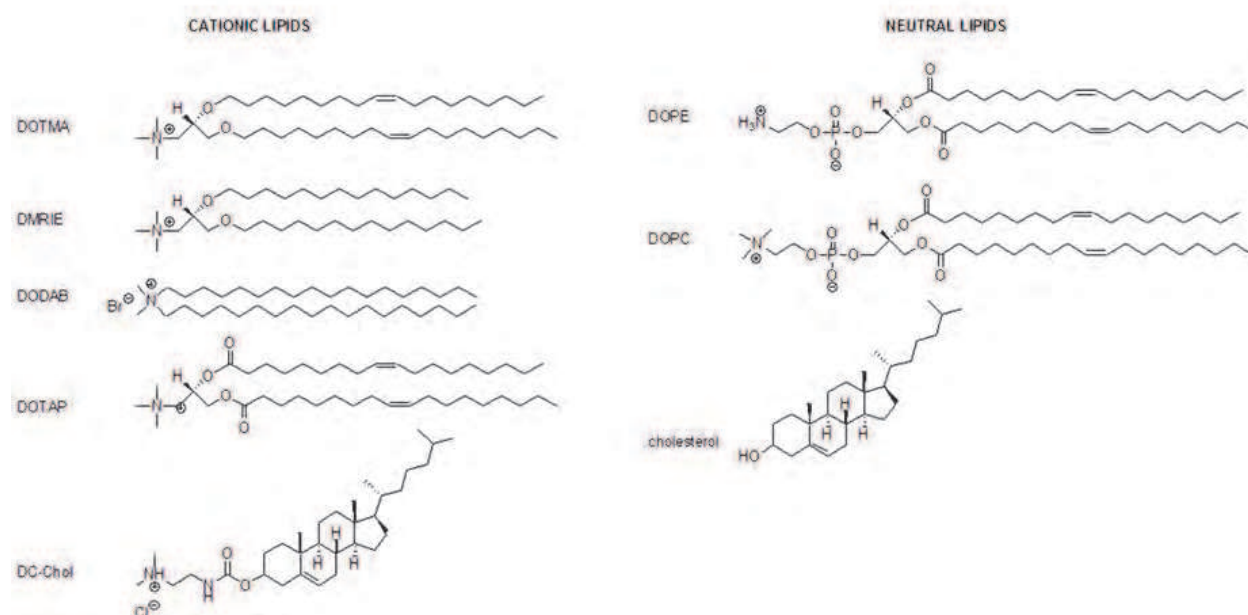
The first part of this equation implies that the change in the Gibbs free energy ( $\Delta G$ ) correlates with association constant  $K_A$  because  $R$ , the gas constant, and  $T$ , the absolute temperature, are constant. The dissociation constant  $K_D$ , which is commonly used to quantify the affinity between two ligands, is the inverse of  $K_A$ . The second part of this equation illustrates that the sum of enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ) changes define the free energy ( $\Delta G$ ) and consequently the affinity of an interaction. A major advantage of ITC experiments is the fact that  $K_A$  and  $\Delta H$  are measured in a single experiment. Having measured these two parameters the remaining variables  $\Delta G$  and  $\Delta S$  can be derived. In combination with structural information, the energetics of binding can provide a complete picture of the interaction and aid in identifying the most important regions of the binding interface and the energetic contribution (Pierce *et al.*, 1999).

## 2. Non-viral DNA transfection systems

An emerging trend in the treatment of disease is gene therapy, a broad term that includes any strategy to treat a disease by delivering exogenous gene, gene segments, or oligonucleotides into the cells of patients in order to restore normal functions to the cell. Such therapies are either DNA based (usually in the form of plasmids) or RNA based (usually in the form of small, interfering RNA or siRNA), both requiring some form of delivery system for the oligonucleotide(s) in question.

Two major classifications of delivery systems exist for DNA therapies, viral- and non-viral-based. Viral vectors typically demonstrate the highest gene transfer activity; however, they suffer from serious safety concerns particularly with respect to inflammatory and immune responses in patients. Non-viral vectors are generally comprised of a combination of cationic lipids, neutral lipids and/or cationic polymers, and have a much better safety profile compared to viral systems but suffer from low gene transfer activities. As such a great deal of effort is being expended towards the development of new non-viral vector systems with increased transfection capabilities.

Lipoplexes, complexes of liposomes with DNA, are the most investigated non-viral transfection vectors, and hold great potential application for gene delivery. They have been utilized in approximately 8% of the clinical trials for gene therapy mainly due to their advantages over viral vectors. They show low toxicity, high therapeutic gene capacity, cell-specific targeting capability, and straightforward production, modification, and functionalization procedures. A key problem in the advancement of lipoplex transfection vectors, yet to be efficiently overcome, is the above mentioned inadequacy of transfection efficiency of current cationic lipid systems, attributed to the incomplete understanding of interaction mechanisms involved. While this is driving research efforts toward the design and synthesis of novel amphiphilic cationic compounds, as well as approaches for formulation of liposomal based vectors (Giatrellis *et al.*, 2009), less effort is being directed towards better understanding the fundamental mechanism(s) by which liposome-mediated transfection occurs.



Scheme 1. Chemical structure of lipids commonly used in the formulation of DNA transfection complexes: DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide), DODAB (dioleoyldimethylammonium bromide), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane (chloride salt), DC-Chol (3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride, DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and cholesterol.

The first reported application of cationic liposomes (or vesicles) as DNA transfection vectors was in 1987, by Felgner *et al.* They synthesized a cationic lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), that forms small unilamellar cationic liposomes, closed bilayer membrane shells of lipid molecules, and that interact with DNA spontaneously via electrostatic attraction. These lipid-DNA complexes maintain an overall positive charge, facilitating uptake by cells again by electrostatic attraction, now between the cationic complexes and the predominantly negatively charged cellular membrane. Complexes are then internalized allowing the transfer of functional DNA into the cell followed by expression. The technique was simple, highly reproducible, and more efficient than some other, commonly used procedures such as use of polycations, calcium phosphate, microinjection, electroporation and protoplast fusion (Felgner *et al.*, 1987).

## 2.1 Mechanism of DNA – lipid Interactions

Due to electrostatic interactions between negatively charged phosphate groups along the DNA back bone and the cationic head group of the lipid, they compact the DNA plasmid into small particles. This compaction serves two purposes: 1) to more easily transport the DNA across negatively charged cellular membrane(s), both in terms of eliminating electrostatic repulsion between negatively charged DNA and the cell membrane (Barreleiro *et al.*, 2000, Safinya, 2001), and also by simply having a smaller size (relative to an uncomplexed DNA plasmid); and 2) to protect the DNA from degradation (primarily by DNAses) during the transfection process (Tranchant *et al.*, 2004; Elouahabi and Ruyschaert,



2005). The importance of the cationic lipid is however not restricted solely to the charge of its head group. (Cherezov *et al.*, 2002). Kreiss *et al.* believed that both the nature (or structure) of the cationic lipid as well as the DNA backbone exert a strong influence on lipid-DNA packing and complex morphology, with the formation of the lipoplexes resulting from competitive interactions between electrostatic forces of lipid-DNA and elasticity forces driven by the lipid hydrophobic moiety (Zuzzi *et al.*, 2007; Ma *et al.*, 2007; Kreiss *et al.*, 1999). It has been suggested that lipoplex formation is an endothermic process and the repulsion force between DNA molecules as well as the release of tightly bound counterions from DNA and the lipid mainly controls the formation of cationic lipid/DNA complex (Ma *et al.*, 2007). In the course of complex formation, DNA acts as a polyanion molecule interacting with the cationic headgroups at the surface of liposomes. The neutralization of charges seems to affect both the DNA and the liposome structures. First, DNA might adopt a more compact structure, which is less accessible to intercalating dyes or nucleases. Second, neutralization might induce lipid bilayer mixing and/or aggregation resulting in fusion of liposomes and/or multilamellar structure formation. The fact that different structures are observed in the same lipoplex preparation further complicates the interpretation (Zabner *et al.*, 1995). Apart from the electrostatic interactions between positively charged lipid head groups and the negatively charged DNA phosphate backbone, variations in lipid-lipid and DNA-DNA ionic repulsive forces, attractive lipid-lipid hydrophobic interactions, hydration forces and other structural properties of the liposome and plasmid DNA can result in a wide variety of macromolecular structures that can vary not only with concentration(s) but also as a function of time. Hence, upon mixing of cationic lipid and DNA, the supramolecular organization of the two components changes considerably. Despite these changes in organization, the molecular structures of both components are generally preserved, leading to a so-called multilamellar complex, in which DNA molecules are intercalated between intact lipid bilayers and form a tightly packed grid (Smisterova *et al.*, 2001; Pitard *et al.*, 1999; Cherezov *et al.*, 2002; Radler *et al.*, 1997).

## 2.2 DNA / lipid complex morphologies

The most commonly complex assemblies include DNA molecules sandwiched within liposomal bilayers, DNA electrostatically adsorbed onto the vesicles outer surface, DNA encapsulated in the aqueous phase of the liposomes, and DNA coated by a monolayer of cationic lipid envelop (Felgner and Ringold, 1989; Giatrellis *et al.*, 2009). Therefore, lipoplexes have a broad range of morphologies from a hypothesized “beads on a string” arrangement proposed initially by Felgner (Felgner and Ringold, 1989) in their seminal paper, to more complicated such as spaghetti and meatballs structure (Sternberg *et al.*, 1994), map-pin structures (characterized by spheroidal heads and tapering pins) (Sternberg *et al.*, 1998) (See Figure 2), multilamellar or inverted hexagonal phase structures confirmed by high-resolution synchrotron small-angle X-ray scattering (SAXS) experiments (Safinya, 2001) and sliding columnar phase (O'Hern, 1998).

These above mentioned morphologies; however, are less common than the lamellar, “sandwich” structure (Koltover *et al.*, 1998; Sternberg *et al.*, 1994). Under lipid excess conditions there is only macroscopic heterogeneity (i.e., lipoplexes may be of different sizes) whereas the microscopic structure of lamellar cationic lipid-DNA “sandwich” is uniform as demonstrated by diffraction techniques (Radtke *et al.*, 1997).

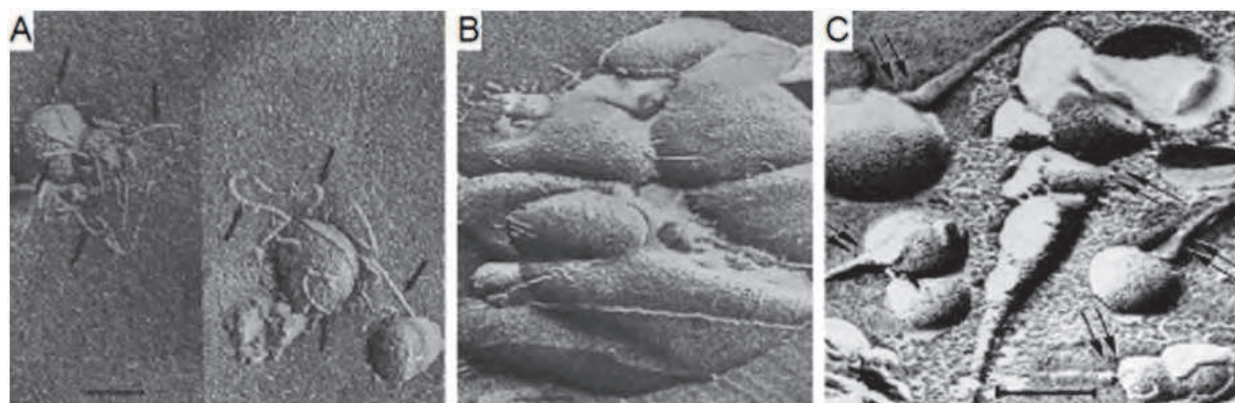


Fig. 2. A and B showing spaghetti-meatball assembly [adapted from (Sternberg *et al.*, 1994)] and C showing map-pin structures [adapted from (Sternberg *et al.*, 1998)].

### 2.3 Physiochemical properties, lipoplex structure, and their impact on transfection activity

Structure-activity studies suggested that biophysical properties, such as size, charge, and morphology of the resulting DNA complexes determine transfection efficiency within one class of vector. Several parameters effect transfection activity, such as structural variations of the cationic lipids (length and degree of unsaturation of the alkyl chains), the nature of the groups bound to the quaternary nitrogen, and the counterions. Variations in the lipid to DNA charge ratio and the presence or absence of helper lipids are other important formulation parameters of transfection efficiency (Lobo *et al.*, 2002;Malone *et al.*, 1989). In the majority of reported studies, lipid-DNA complexes function most effectively when a cationic lipid is mixed with a neutral or helper lipid such as DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) or DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) (Scheme 1). DOPE is by far the most commonly used helper lipid in non-viral DNA transfection applications (Ewert *et al.*, 2008;Hui *et al.*, 1996). The inclusion of neutral or zwitterionic lipids (co-lipids), such as cholesterol, DOPE and DOPC as well as PEG lipids, other cationic lipids, and non-lipid compounds like polymers or peptides in the cationic vesicle formulations has been shown to increase the transfection efficiency of the assemblies (at least in case of DOPE) by promoting membrane fusion in different cell lines (Koynova *et al.*, 2007).

#### 2.3.1 Size and zeta potential

In general, transfection efficiency (i) requires a positive or neutral zeta potential, (ii) is size dependent, e.g., it is higher for smaller size particles, and (iii) requires a vector that is stable in serum. The size of the DNA plays an important role in biodistribution, cellular internalization and intracellular trafficking (Pathak *et al.*, 2009). The correlation between lipoplex size and DNA transfection has been well studied; however, the most suitable size range (for transfection) still remains to be controversial. Early studies suggested the lipoplex sizes in the range of 400–1400 nm for more efficient transfection in cell culture than smaller (<400 nm) or larger (>1400 nm) aggregates (Kawaura *et al.*, 1998), but later results emphasize a smaller (<200 nm) lipoplexe size range (Zhang *et al.*, 2003). This controversy has been attributed to different size-dependent endocytic pathways involved in uptake of lipoplexes. As the gene transfection by the cationic liposomes was shown to be inhibited by wortmannin, an inhibitor of

endocytosis, it is suggested that the vesicles with moderate diameters are useful for gene transfection by endocytosis (Kawaura *et al.* 1998). Moreover, addition of non-lipid condensing agents such as polyethyleneimine, polylysine, protamine sulfate to the cationic lipid- DNA mixture have been reported to produce small homogeneous lipoplexes, in the range of 100 nm, more suitable to enter narrow capillaries than lipid-DNA alone. Further, the lipoplex particle size also plays a role in DNA release in which for lipoplexes with mean size of ~400–500 nm and lamellar distances of ~5–6 nm (and hence are composed of several tens of layers) extensive intermembrane interactions are required for their disassembly (Koynova, 2009). Zeta potential of lipoplexes is usually measured at different DNA/lipid ratios whereas pure cationic vesicles show a positive zeta potential of ~ 60 mV, and it decreases with addition of DNA and changes to negative at DNA/lipid charge ratio of more than 1 (Koynova *et al.*, 2007). Takeuchi *et al.* showed that transfection efficiency of plasmid DNA (pSV2CAT) into mammalian cultured cells was consistent with the values of zeta potential of cationic liposomes containing a tertiary amino head group (Takeuchi *et al.*, 1996). Therefore, lipoplexes with a small excess of positive zeta charges favours higher transfection efficiency (Elouahabi and Ruyschaert, 2005) and *vice versa*, for lipoplex formulations containing excess amount of DNA and negatively surface charge, the release of DNA is significantly hindered compared to lipoplexes with positive zeta potential (Koynova *et al.*, 2007).

### 2.3.2 Structure

The role of the cationic lipid molecular structure on transfection activity of resultant lipoplexes has recently been reviewed; with the conclusion that lipids having either saturated alkyl chains with a length of ~14 carbon atoms OR longer, monounsaturated chains having trans-isomerism are generally the most effective for transfection (Koynova and Tenchov, 2009; Koynova *et al.*, 2008). It is important to note that this is only a generalization, and factors such as the stoichiometry of the cationic lipid to DNA, the ionic strength of the formulation, the temperature of the formulation (both during and after formulation), and the incubation time can all have a significant effect on the resulting structure of the lipoplex (Giatrellis *et al.*, 2009).

Association of DNA with cationic lipids in a micellar or liposomal form leads to lamellar organization with DNA molecules sandwiched between lipid bilayers. Although the lamellar phase is the common described structure, as evidenced by small-angle X-ray scattering and electron microscopy, some cationic lipid combined with a hexagonal forming lipid could also result with DNA in an inverted hexagonal structure (Barteau *et al.*, 2008). Despite all the advances in biological and biophysical characterization of cationic lipid-DNA complexes, the relationship of their structural properties to their biological activity is still not well understood. Some earlier studies, especially those carried out on lipoplexes containing DOPE, have proposed the inverted hexagonal structure ( $H_{CII}$ ) is more efficient in transfection than the lamellar ( $L_{C\alpha}$ ) phase (See Figure 3) (Koltover *et al.*, 1998; Smisterova *et al.*, 2001). However, more recent studies have shown a general lack of correlation between lipoplex structures and transfection activity. Indeed, high lipofection not only depends on the structure and morphology of the vector assemblies, but also on other factors, (one important example of which is the type of cell being transfected; i.e. primary versus clonal, cancerous versus non-cancerous, etc.). The scope of accounting for all such factors makes it difficult for investigators to put together a comprehensive picture of effects such as variation of both cell types and cationic lipids within one study (Ma *et al.*, 2007).



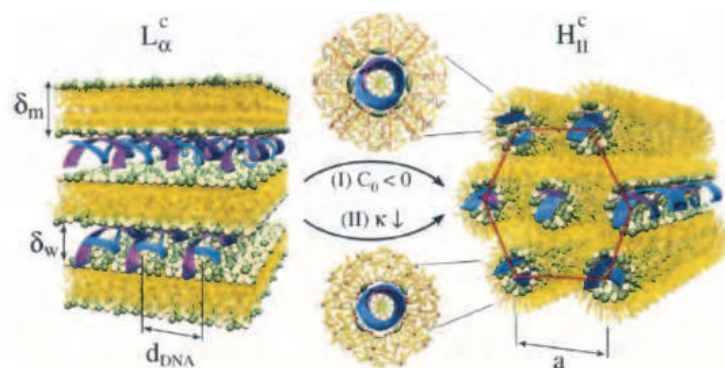


Fig. 3. Inverted hexagonal structure ( $H_{II}^c$ ) phase structure and multilamellar ( $L_{\alpha}^c$ ) phase structures [adapted from (Koltover *et al.*, 1998)].

## 2.4 Thermodynamics of lipoplex formation

Self-assembly of surfactants into aggregates, such as micelles, vesicles, etc., can be induced by increasing the surfactant concentration to above the critical micelle concentration (CMC) and/or by adjusting the temperature to exceed the critical micelle temperature (CMT). In a typical calorimetry experiment, heats of dilution are recorded. Initially, the dilution of the concentrated surfactant solution (usually well above the CMC) will result in the disassembly of micellar aggregates into monomers, and the observed heat of dilution will contain both the heat of solution of the monomer surfactant, and the heat associated with micelle dis-assembling, which is the negative of the heat of micelle formation. As the experiment proceeds, the surfactant concentration in the measurement cell increases to above the CMC, as described in section 1, integration of the measured heats for each injection provides an observed enthalpy of dilution, from which, for a typical surfactant, the enthalpy of micellization (or aggregation),  $\Delta H_{mic}$  can be obtained from the difference in the pre- and post-micellar enthalpies of dilution. The magnitude and sign (i.e., endo- or exothermic) of  $\Delta H_{mic}$  is governed by the types and magnitude of various contributions (hydrophobic and electrostatic) arising from surfactant-surfactant interactions.

The interaction of cationic aggregates (micelles and/or lipoplexes) with DNA, while being more complex than the aggregation of the surfactants or lipids alone, is governed again by a combination of hydrophobic and electrostatic contributions. The thermodynamics of cationic lipid – DNA lipoplex formation are found to be in most cases, endothermic (Pector *et al.*, 2000; Pozharski and MacDonald, 2002). Since, for any process to be thermodynamically favoured the free energy change must be negative, an endothermic lipoplex formation must be driven by a corresponding increase in entropy (recall equation 1). The interesting question then becomes “what exactly is the source of this entropy gain upon lipoplex formation”? It is widely accepted that the major contribution to this increase in entropy arises from counterion release. Bruinsma first pointed out that lipoplex formation is not simply driven by electrostatic attraction between oppositely charged DNA polymer and lipid membrane, but that in fact the situation is more complex given that both components exist in solution with oppositely charged counterions bound to them that are released upon complex formation (Bruinsma, 1998). Release of these counterions upon complex formation results in an entropy gain that is expected to be, according to Bruinsma,  $\sim 1 \text{ kT}$  per counterion. Both cationic lipid and DNA become partially dehydrated upon complex formation (Hirsch-Lerner and Barenholz, 1999) resulting in an additional entropy gain due to the additional degrees of freedom acquired by the released water. Hence, the entropy

based process is driven through the release of water molecules and of bound counter-ions via the ion-exchange process. It is important to note that the released counterions interact more strongly with water and restrict somewhat the mobility of the water molecules around them, an effect that must lead to some entropy loss. Additionally, the DNA and to a smaller extent the complexing lipids also experience some reduction in entropy because of loss of degrees of freedom due to increased rigidity of the complex relative to the separate components. Nevertheless, combined together the net result is entropically driven lipoplex formulation in general; however additional studies to further elucidate various contributions are still required.

As an example, Pozharski and MacDonald examined the strength of binding of DNA to the cationic lipid matrix upon lipoplex formation (Pozharski and MacDonald, 2003). In this study, the binding free energy was determined by monitoring lipoplex dissociation under conditions of increasing salt concentration. By using relatively short oligonucleotides, the investigators were able to determine the binding energy per nucleotide. A combination of calorimetry and fluorescence resonance energy transfer (FRET) was used in their work. For FRET studies a rhodamine labeled lipid was brought in proximity with carboxyfluorescein (CF) labeled DNA so that CF emission becomes quenched upon lipoplex formation. The decrease in the signal verified complex formation. A Microcal calorimeter was used to determine the cationic lipid-DNA binding enthalpy for the titration of 0.46 mM Dickerson dodecamer (5'-CGCGAATTCGCG) into 25  $\mu$ M EDOPC (O-ethyl dioleoylphosphocholine, cationic lipid) in HE-S buffer (of 20 mM HEPES, 0.1 mM EDTA, and 150 mM NaCl at pH 7.5). Figure 4 shows the degree of dissociation of complexes,  $\alpha$ , plotted against NaCl concentration, and the heat absorbed at various ratios of DNA/ Lipid (D/L).

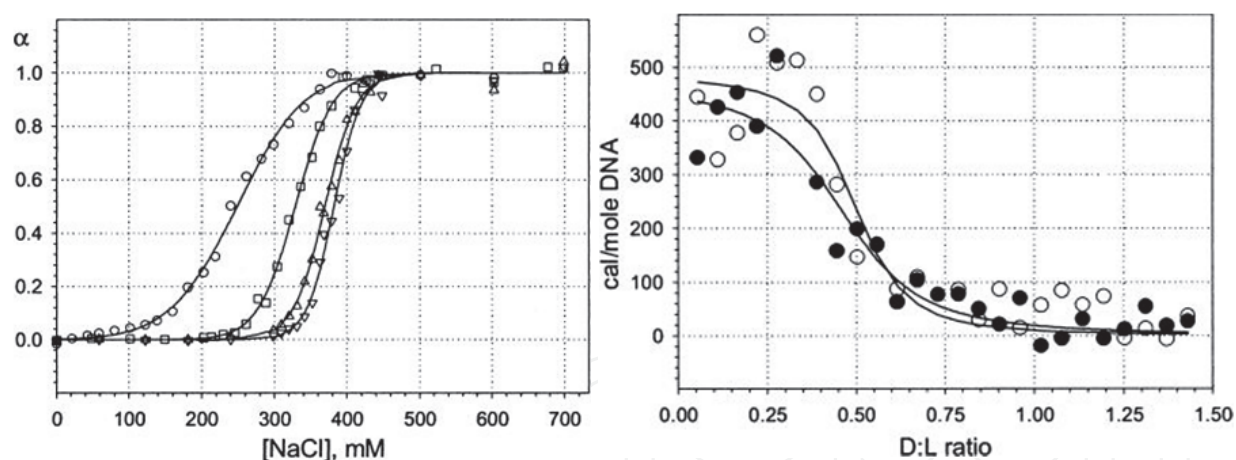


Fig. 4. DNA-EDOPC binding curves (LEFT) for lipid:DNA charge ratios of:  $\circ$  2:1 ;  $\square$  4:1 ;  $\Delta$  8:1 ; and  $\blacktriangledown$  16:1 . Calorimetric profile (RIGHT) of cationic lipid-DNA binding. 460  $\mu$ M of Dickerson dodecamer titrated into cationic lipid, EDOPC, suspension in HE-S buffer. Curves are from fitting to a one-site binding model; unfilled and solid circles correspond to two independent titrations. [Adapted from (Pozharski and MacDonald, 2003)].

Averaged over two runs, thermodynamic parameters of the binding were  $\Delta G = -445 \pm 20$  cal/mole, and  $\Delta H = 477 \pm 42$  cal/mole; it was observed that binding becomes more favorable with decreasing cationic lipid:DNA charge ratio. During lipoplex formation there is decrease of the apparent free energy with increasing length of the DNA fragment. It is suggested that there is strengthening of binding at higher lipid:DNA ratios, especially from

8:1 to 16:1, because of the continuing decrease of  $\Delta G$  up to a charge ratio of 16:1. Lipoplexes with excess lipid actually have stronger lipid-DNA interactions. For example (assuming that the free bilayer is actually part of the lipoplex particle and not as separate vesicles), there may be internal edge effects such that it is easier to accommodate the boundary of DNA-covered areas when there is more lipid.

## 2.5 Multipoint nature of cationic lipid-DNA interactions

A DNA molecule binds to the lipid membrane at many points and the relatively small free energies of the individual charges sum to a very large binding energy for the whole molecule. The binding of an individual DNA electrostatic charge to the lipid membrane is rather weak (Pozharski and MacDonald, 2003). Hence, the size of DNA does indeed affect binding energy of lipoplex formation. Single lipid molecules cannot dissociate from the complex since they are held in bilayers by strong hydrophobic interactions and thus form a continuous matrix to which DNA can bind. DNA bases are covalently linked to each other and the stiffness of the polymer is such that a molecule only about as short as a dodecamer binds as a single unit. As a result, much more free energy is released upon a binding event and this brings the apparent dissociation constant into the micromolar range at physiological ionic strength. Longer DNA molecules bind more tightly, but their energy of interaction is also limited by their flexibility. The entropy gain upon lipoplex formation is  $\sim 1$   $kT$  per released counterion, and in general, this positive  $\Delta S$  is the driving force of the lipoplex process, which is in good agreement with theoretical considerations. Moreover, very long DNA molecules will exhibit a lower tendency to remain attached throughout their length, but because the number of contact sites is high and because of their increased ability to bend, the extent of dissociation for long DNA molecules at moderate and low ionic strength is extremely small.

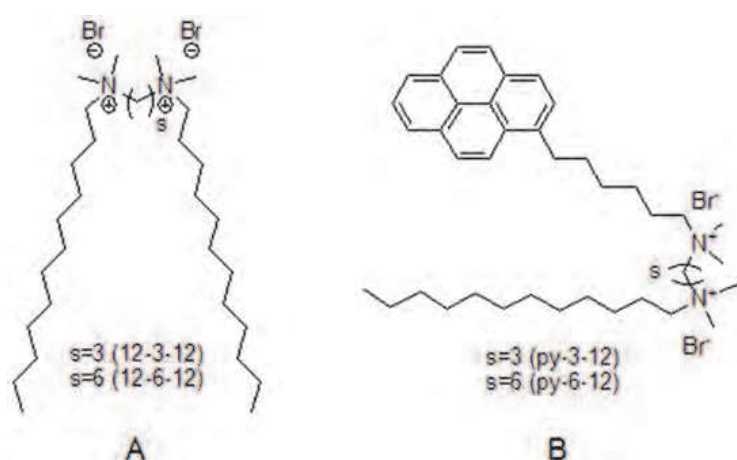
## 3. DNA – gemini surfactant systems

### 3.1 Unsubstituted gemini surfactants

We have recently reported the results of an isothermal titration calorimetric (ITC) study of the interaction of DNA with transfection systems based upon a family of dimeric surfactants, more commonly known as gemini surfactants (Wettig *et al.*, 2010; Yang *et al.*, 2010; Donkuru *et al.*, 2010). Gemini surfactants consist of two traditional surface active groups (i.e two hydrophobic chains and two polar headgroups) linked chemically by a spacer group. The most extensively synthesized and studied family of gemini surfactants are the N,N-bis- $\alpha,\omega$ -alkane-diammonium dibromides, having a general structure  $[C_mH_{2m+1}(CH_3)_2N^+(CH_2)_sN^+(CH_3)_2C_mH_{2m+1} \cdot 2Br^-]$ , hereafter referred as m-s-m, where m represents the carbon chain length of the alkyl tails and s the number of carbon atoms in the polymethylene spacer (See Scheme 2A). Compared to conventional surfactants having a single chain and a single head, gemini surfactants possess unique physiochemical properties, including lower critical micelle concentrations (CMCs) and better wetting properties. However, one of the most important advantages, in so far as gene therapy is concerned, is that the structure of gemini surfactants allows for expanded structural diversity through adjustment of the length of hydrophobic chains, the polarity of head groups, the structure of spacer and the counterions. The spacer length plays perhaps one of the most important roles in determining the properties of these surfactants, and therefore likely their interaction(s) with DNA. The effects of variations in the length of a polymethylene spacer group, as well

as the effects of incorporation of hydrophilic substituents have been extensively studied and are the focus of several reviews on gemini surfactants.

It is generally observed that the chemical arrangement of gemini surfactants provide a rather rich array of aggregate morphologies and solution properties that are dependent upon the nature and size of the linking group. Our previous studies indicated that the transfection efficiency and *in vivo* cutaneous absorption was dependent on the length of the spacer between the two positively charged head groups (Foldvari et al. 2006). Gemini surfactants having a trimethylene spacer demonstrated the highest activity, with the transfection efficiencies correlating to other physical properties (such as the head group area at the air/water interface, critical micelle concentration, etc.) that depend upon the size and/or nature of the spacer group. Moreover, variation in the length of the spacer group allows for a wider variation in the distance(s) between cationic head groups, which provides flexibility for designing surfactants with an optimal match with the anionic phosphate groups on the DNA backbone thereby increasing complexation efficiency.



Scheme 2. Structure of the A) 12-s-12 and B) py-3-12 (see discussion below) surfactants.

Microcalorimetry studies used alone and/or along with other techniques can be very helpful in understanding the DNA-gemini interactions in the systems described above. Lipoplexes of gemini compounds, where  $m = 12$ ,  $s = 3$ ,  $12$  and  $m = 18$  : 1 (Smisterova *et al.*, 2001),  $s = 2$ ,  $3$ ,  $6$ , with DNA were investigated using isothermal titration calorimetry, dynamic light scattering (DLS), zeta potential, atomic force microscopy (AFM) and circular dichroism (CD) techniques. ITC data showed that the interaction between DNA and gemini surfactants is endothermic and the observed enthalpy *vs.* charge ratio profile depends upon the titration sequence (Wang *et al.*, 2007a). Isoelectric points (IP) of lipoplex formation were estimated from the zeta potential measurements and show good agreement with the reaction endpoints (RP) obtained from ITC. DLS data indicated that DNA is condensed in the lipoplex whereas AFM images suggested that the lipoplex morphology changes from isolated globular-like aggregated particles to larger-size aggregates with great diversity in morphology. This work has recently been extended to the study of a non-symmetric gemini surfactant in which one of the alkyl tails is a dodecyl group (i.e.  $m = 12$ ) and the other has been replaced with a (pyren-6-yl)-hexyl group which has the same approximate length as a dodecyl group, but is significantly more hydrophobic in character (Wang *et al.*, 2007b). The interaction between these pyrene-based gemini surfactants (Scheme 2B) and DNA using ITC (Donkuru *et al.*, 2010), along with the 12-3-12 and 12-6-12 surfactants (for comparison), and



marked differences in the interactions with DNA were observed (Figure 5). The 2010 results for the interaction of the 12-3-12 and 12-6-12 with DNA showed a more complex interaction than that originally proposed in 2007, likely as a result of the difference in calorimeters used in the different studies. The major difference observed in the binding interaction(s) with DNA for the pyrenyl gemini surfactants lies in the ability of the pyrenyl group to intercalated between DNA base pairs; this intercalation was previously hypothesized based upon the results of fluorescence titration studies (Wang et al. 2007b).

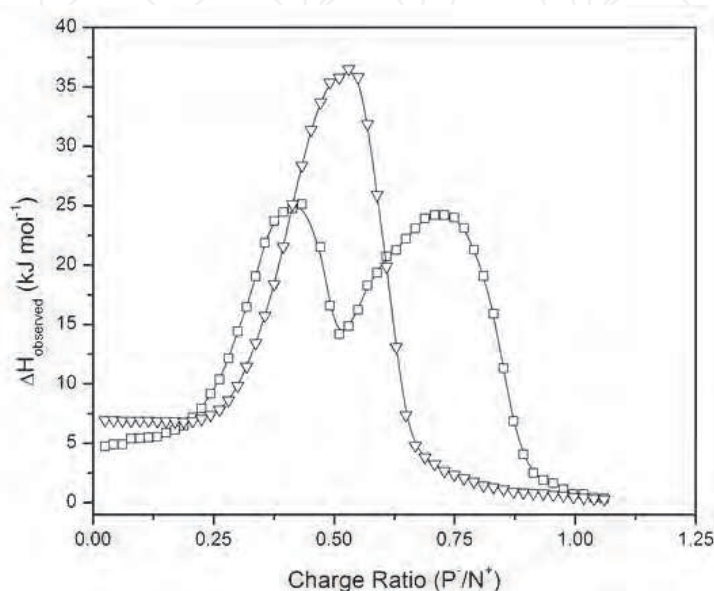


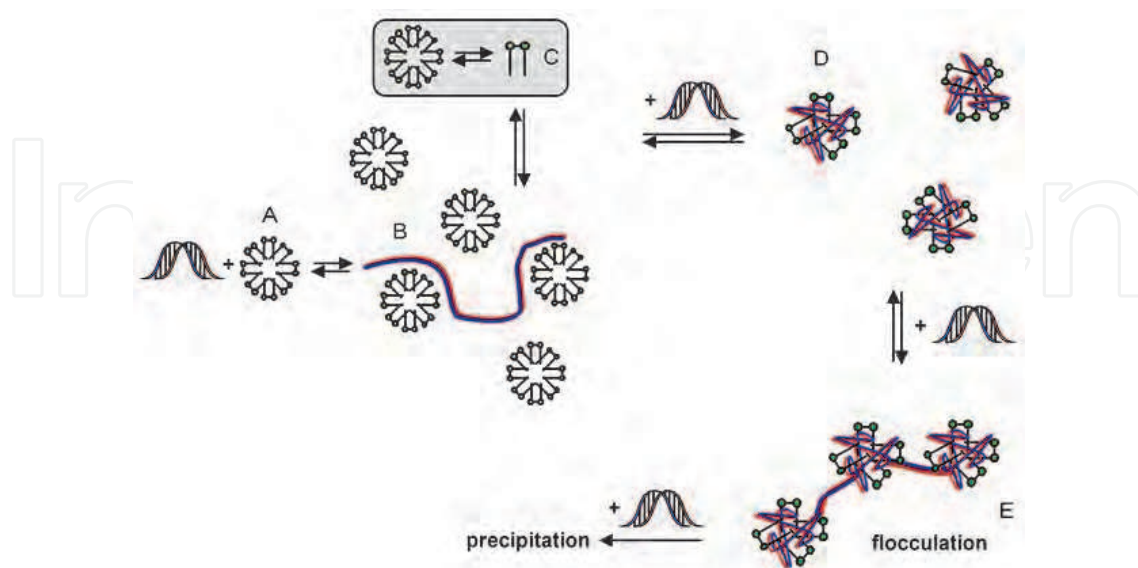
Fig. 5. Observed enthalpies for the titration of gemini surfactants with DNA: 12-3-12 at 25 °C (□) and py-3-12 at 25 °C (▽). Enthalpies are reported in kJ/mol of base pairs. [adapted from (Wettig et al., 2010)]

The double-peak feature observed in the enthalpogram for the titration of the 12-3-12 surfactant with DNA (Figure 5) is common to all of the symmetric gemini surfactants investigated (so far) by our group. The observed binding interactions have been rationalized in terms of the following steps (Wettig *et al.*, 2010):

1. An initial interaction between DNA and surfactant micelles; i.e., formation of the “beads on a string” complex – **endothermic**;
2. A significant **endothermic** contribution resulting from the disruption of the micelle-monomer equilibrium by continued addition of DNA;
3. A reorganization of the “beads on a string” complex into an approximately neutral complex which gives rise to an **exothermic** contribution from both the release of structured water and binding between DNA and surfactant monomer;
4. Flocculation of the now neutral surfactant – DNA complexes – **endothermic**;
5. Precipitation of the complexes (**exothermic**) followed by no net interaction.

This interaction mechanism is depicted in Scheme 3. The major difference between this mechanism and in the original work published (Scheme 2 in Wang et al., 2007) is that the micelle-monomer equilibrium can no longer be discounted; specifically, the continued addition of DNA to the system has a substantial effect on this equilibrium and results in a significant endothermic contribution to the observed enthalpy. Interestingly a simplification of the system is observed for the pyrenyl surfactants (Scheme 2B), such that a number of

intermediate equilibria described above do not seem to occur specifically as a result of the ability for the pyrenyl groups to intercalate between the DNA base pairs.



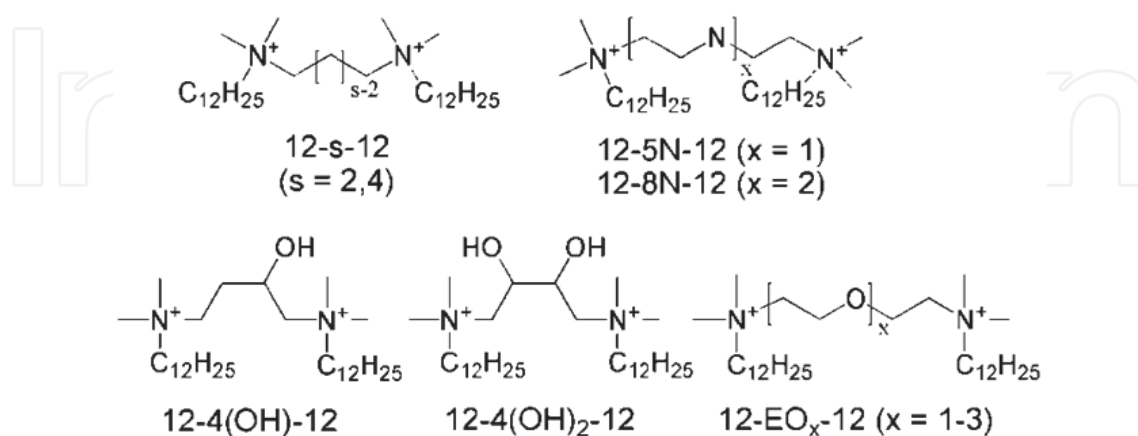
Scheme 3. Proposed interaction mechanism for m-s-m gemini surfactant - DNA systems. Initial binding of DNA to surfactant micelles (A) and formation of the beads-on-a-string complex (B). Continued addition of DNA results in a disruption of the monomer-micelle equilibrium (C) and the reorganization of the beads-on-a-string complexes into discrete DNA-surfactant aggregates (D) and eventual flocculation (E) and precipitation of these aggregates. [adapted from (Wettig *et al.*, 2010)]

Other studies have shown that the nature of the counterion significantly affects both micellization and aggregation critical for enhanced transfection. The micellization of six cationic gemini surfactants with various counterions,  $[C_{12}H_{25}(CH_3)_2N(CH_2)_6N(CH_3)_2C_{12}H_{25}]X_2$ , designated as  $C_{12}C_6C_{12}X_2$ , with  $X = F^-, Cl^-, Br^-, Ac^-, NO_3^-,$  and  $\frac{1}{2} SO_4^{2-}$  in aqueous solutions was investigated by microcalorimetry and conductivity measurements (Jiang *et al.*, 2004). The interaction of these surfactants with DNA in aqueous solutions was also investigated by microcalorimetry. The CMC, CAC (critical aggregation concentration) and the degree of micellar ionization, the saturation concentration of the aggregation, and the associated thermodynamic parameters were detected. Among the counterions examined,  $SO_4^{2-}$  was the most effective anion for decreasing the CMC (Koynova *et al.*, 2007). Both aggregation processes were mainly entropy-driven since the values of the entropy changes multiplied by temperature were much larger than the absolute values of the enthalpy changes. The binding of micelles to DNA was strongly dominated by the positive entropy gain on release of the small counterions from the micelles and from DNA. Further, the interaction of all of the surfactants with DNA was dependent on the DNA concentration and may be associated with each micelle interacting with more than one DNA molecule. In another microcalorimetric study, the interaction of a series of dissymmetric gemini surfactants, (designated as  $C_{(m)}C_{(6)}C_{(n)}Br_2$ , with constant  $m+n=24$ , and  $m=12, 14, 16,$  and  $18$ ) with DNA in 10 mM NaCl was studied and it was found that the dissymmetry degree has a marked effect on the interaction of the  $C_{(m)}C_{(6)}C_{(n)}Br_2$  surfactants with DNA since the CAC and saturation concentration tend to become smaller with increased  $m/n$  (Jiang *et al.*, 2005).

### 3.2 Substituted gemini surfactant systems

A more recent focus of research on gemini surfactants deals with the effect of substitution within the spacer group on the aggregation properties of the surfactant, and the rational design of surfactants with improved transfection activity (Kirby *et al.*, 2003; Wang *et al.*, 2004; Wettig *et al.*, 2002; Wettig *et al.*, 2003; Wettig *et al.*, 2007). Transfection activity is found to depend critically upon the structural elements present (Castro *et al.*, 2004). Recently, pseudoglyceryl gemini lipids bearing an oligooxyethylene  $(-\text{CH}_2-\text{CH}_2-\text{O})_m-\text{CH}_2-\text{CH}_2-$  spacer were found to be superior gene transfecting agents as compared to those bearing polymethylene  $(-\text{CH}_2)_m-$  spacers (Bajaj *et al.*, 2008). In this study, gemini lipids were found to be highly superior in gene transfer abilities as compared to their monomeric lipid and a related commercially available formulation (Bajaj *et al.*, 2008). The increased hydrophilicity of the ethoxylated spacer groups results in an increased water solubility of these compounds, and they preferentially locate at either air-water or micelle-water interface. In some cases, the efficiency of transfection has been correlated to a high extent of DNA condensation, which in turn depends heavily on the gemini spacer structure (Bombelli *et al.*, 2005). An important factor is the ability of the complexes to form polymorphic structures, which are not necessarily hexagonal. Absent from the above, is a thermodynamic characterization of the binding interaction(s) between DNA and gemini surfactants. While the determination of binding properties is complicated by the cooperative nature of the interaction between DNA and gemini surfactants, isothermal titration calorimetry has been used in a few reports.

In order to enhance gene transfection based upon structure-activity relationship, various gemini surfactants (Bombelli *et al.*, 2005) have been designed, synthesized and tested for gene delivery in our laboratory. Previously, we reported the results of a comprehensive study of the aggregation and thermodynamic properties of the 12-4-12 gemini surfactant with and without hydroxyl substitution as well as a series of ethoxylated gemini surfactants. Further, our group is using ITC to extend our work to better understand the thermodynamic properties of the complexation of DNA by the aza-, hydroxyl- and ethoxyl-gemini surfactants, specifically to examine how the binding interactions change as a result of substitution within the spacer group (Scheme 4).



Scheme 4. Structures of the unsubstituted and substituted gemini surfactants.

Enthalpograms for the titration of 2.5 mM bp DNA into 0.5 mM gemini surfactant solutions are shown in Figure 6. The interactions between unmodified gemini surfactants (12-3-12, 12-

12-12, 18:1-2-18:1, 18:1-3-18:1, 18:1-6-18:1) (Wang *et al.*, 2007a) and pyrenyl-modified gemini surfactants (Wettig *et al.*, 2010) with DNA have been studied previously in this manner, which provides important information on the nature of the interaction(s) between surfactant micelles and DNA for conditions of excess positive charge; i.e., conditions similar to those typically used to prepare complexes for transfection studies.

The same types of interactions observed between unsubstituted gemini surfactants and DNA (described above) will occur for the various substituted gemini surfactants studied in this investigation; however, since the various substitutions are made within the head group region of the surfactant, this should result in noticeable changes in the binding interaction(s) which is indeed the case as seen in Figure 6. The maximum in  $\Delta H_M^\circ$  for the 12-s-12 series has been attributed to energies associated with the configuration of the spacer group at the micelle-water interface. Enthalpies for the substituted gemini's in the presence of DNA were observed to decrease (becomes more exothermic), with increasing hydrophilicity of the spacer group in the order OH < EO < N (Table 1). The increased hydrophilicity of the ethoxylated spacer groups results in an increased water solubility of these compounds, and they preferentially locate at either air-water or micelle-water interface. Very recently, Anissa Bendjeriou-Sedjerar *et al.* (2009), from their surface tension measurements of gemini surfactants containing two quaternary ammonium groups bound by an ethylene oxide spacer chain, with  $x=1,3,7$  and 12, reported that the hydrophilic spacer with oxyethylene moieties was not fully extended at the air-water interface. With increasing the spacer group size  $x = 7-10$ , it became sufficiently flexible to adopt a particular conformation with a loop at the water side of the interface (Benjeriou-Sedjerari *et al.*, 2009). In addition, as observed for the 12-s-12 gemini surfactants, the micellization and binding process seems to be entropy driven for the 12-EOx-12 surfactants. In case of 12-8N-12 surfactant, the aza groups represent a bulkier, and perhaps more importantly, a more hydrophobic substituent as compared to oxygen for the case of the ethoxylated spacer groups. This has two direct effects on the ability of the spacer group to adopt conformations that would minimize the conformation energies: (i) steric hindrance between the aza methyl groups and the alkyl tails will not allow the spacer group to fold into the core of the micelle; and (ii) steric considerations also require that at least one of the aza methyl groups be oriented towards the aqueous phase, resulting in a net decrease in the energy associated with the transfer of the spacer from the aqueous to the micellar phase. The increase in CMC for the N-methyl substituted series compared to the epoxy series results from increased steric repulsion, particularly with respect to packing at the surface of the micelle, and the interference between the solvation shells around nitrogen and hydrocarbon regions within the spacer (Wettig *et al.*, 2007). A hydrophilic, flexible spacer prompts micelle formation, which leads to a smaller CMC, smaller  $\alpha$ , larger N, and more negative  $\Delta G_{mic}$  (Wang *et al.*, 2004). One would expect that the sources of contributions to the enthalpy of binding remain the same within the series. Since the length and composition of the alkyl tails is kept constant, the contribution to  $\Delta H_M^\circ$  arising from the transfer of the alkyl tails from the bulk to the micelle should also remain constant. Therefore, the observed differences between the gemini surfactants with and without EO/aza/OH groups in the spacer must be associated with differences in hydration of the spacers of these surfactant series, when they are monomers in the bulk phase or located in the micelle/water/solution interface and when they are interacting with DNA. The initial enthalpies ( $\Delta H_{init}$ ) and the enthalpies at the maximum of the main peaks observed in Figure 6 ( $\Delta H_1$  and  $\Delta H_2$ ), along with their positions (in terms of charge ratio (P-/N+)) are reported in Table 1. It is interesting to note that the



difference between the initial enthalpy (upon titration of the gemini surfactants with DNA) and the enthalpy at the first peak observed in Figure 6 ( $\Delta H_{init} - \Delta H_1$ ) is comparable to the enthalpies of micellization for the pure surfactant compounds (Figure 7, and also  $\Delta H_{mic}$  in Table 2) with a correlation of 0.72. As discussed above, one of the main differences in the mechanism proposed in our 2010 article (Wettig et al., 2010) and that originally proposed in our 2007 article (Wang et al, 2007) is the inclusion of the monomer-micelle equilibrium (Box C in Scheme 3). The monomer-micelle equilibrium is of course governed by the molecular structure of the surfactants, and therefore it is perhaps not surprising that we observe a correlation between the enthalpies of micellization and those observed upon interaction with DNA.

Surfactant	$\Delta H_{init}$ (kJ mol <sup>-1</sup> )	P <sup>-</sup> /N <sup>+</sup>	$\Delta H_1$ (kJ mol <sup>-1</sup> )	P <sup>-</sup> /N <sup>+</sup>	$\Delta H_2$ (kJ mol <sup>-1</sup> )
12-2-12	10.2	0.24	26.3	0.41	28.7
12-EO <sub>1</sub> -12	10.5	0.53	23.8	0.80	22.0
12-EO <sub>2</sub> -12	11.2	0.46	15.0	0.61	15.5
12-EO <sub>1</sub> -12	11.5	0.56	15.8	0.83	17.0
12-5N-12	13.8	0.38	21.5	0.69	20.1
12-8N-12	17.7	0.31	25.0	0.60	26.1
12-4-12	6.8	0.27	20.5	0.50	24.7
12-4(OH)-12	5.8	0.24	17.5	0.37	21.4
12-4(OH) <sub>2</sub> -12	4.0	0.30	18.7	0.48	19.0
12-3-12 <sup>a</sup>	4.7	0.41	25.1	0.73	24.2
12-EO <sub>3</sub> -12	12.1	0.42	18.9	0.68	17.6

Table 1. Thermodynamic properties for the titration of 0.5 mM gemini surfactant with DNA.  
<sup>a</sup>Data from Wettig et al., 2010.

The binding of cationic alkylammonium surfactants to DNA was studied by Matulis et al., who broke the interaction of the surfactants with DNA down into an electrostatic ( $\Delta_{elec}H$ ) and a hydrophobic contribution ( $\Delta_{h\phi}H$ ; Equation 2) (Matulis *et al.*, 2002).

$$\Delta H = \Delta_{h\phi}H + \Delta_{elec}H \tag{2}$$

The electrostatic component was estimated to be ~1.0 kJ/mol (obtained from the enthalpy of dissolution for ammonium phosphate which allowed for the determination of the hydrophobic contribution from experimental enthalpy measurements. Enthalpy titrations for the alkylammonium surfactant – DNA systems were carried out in the usual (forward) manner of adding concentrated surfactant solution to a solution of DNA, rather than the reverse manner of adding DNA to a concentrated surfactant solution used in our work above. This unfortunately precludes a more detailed analysis and comparison of our results for the substituted gemini surfactants to those for the alkylammonium surfactants (Matulis *et al.*, 2002) as well as the gemini surfactants (Bai *et al.*, 2001;Jiang *et al.*, 2005;Wang *et al.*, 2007; Pozharski and MacDonald, 2002). Forward titrations with the substituted compounds are currently underway to allow for such a comparison.

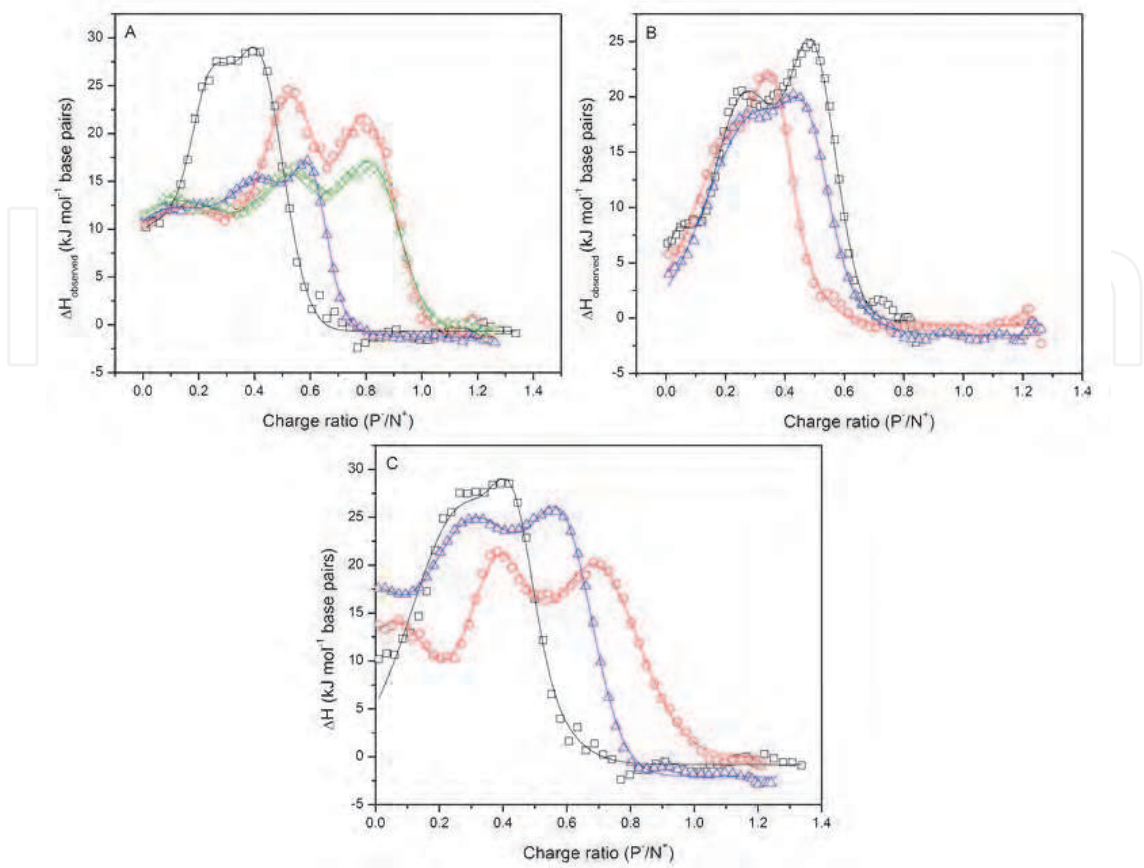


Fig. 6. Observed enthalpies for the titration of gemini surfactant solutions with DNA: A) 12-2-12 ( $\square$ , black), 12-EO<sub>1</sub>-12 ( $\circ$ , red), 12-EO<sub>2</sub>-12 ( $\triangle$ , blue) 12-EO<sub>3</sub>-12 ( $\diamond$ , green); B) 12-4-12 ( $\square$ , black), 12-4(OH)<sub>1</sub>-12 ( $\circ$ , red), 12-4(OH)<sub>2</sub>-12 ( $\triangle$ , blue); C) 12-2-12 ( $\square$ , black), 12-5N-12 ( $\circ$ , red), 12-8N-12 ( $\triangle$ , blue). Lines represent a fit of the experimental data to a multiple Gaussian peak model.

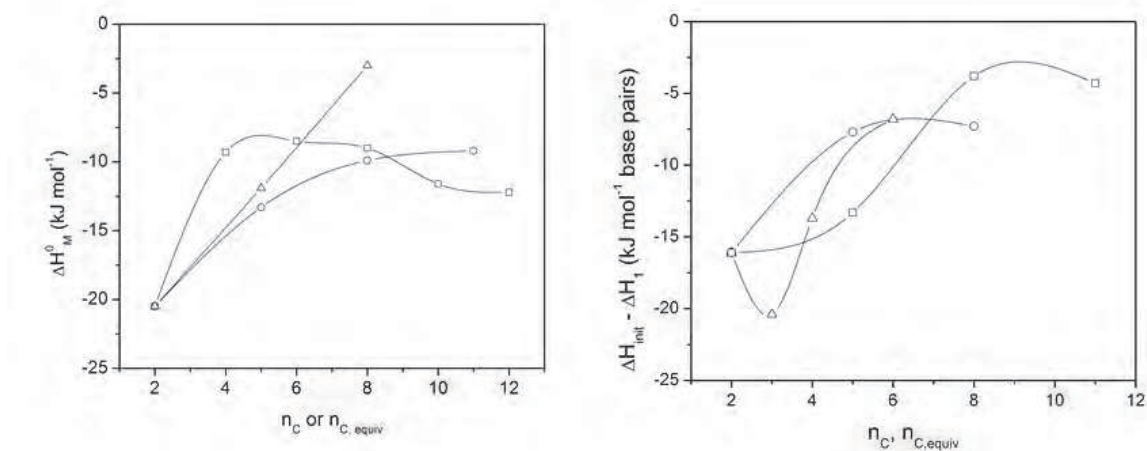


Fig. 7. Enthalpies of micellization ( $\Delta H_{mic}$ ) for the 12-s-12 the 12-EO<sub>x</sub>-12 and 12-Az<sub>x</sub>-12 gemini surfactants; and the difference in enthalpy at peak 1 and the initial enthalpy ( $\Delta H_{init} - \Delta H_1$ ) as a function of spacer length for the titration of DNA with the 12-s-12 , the 12-EO<sub>x</sub>-12 and 12-Az<sub>x</sub>-12 gemini surfactants.

Surfactant	$\Delta H_{mic}$ (kJ mol <sup>-1</sup> )	$\Delta H_{init} - \Delta H_1$ (kJ mol <sup>-1</sup> )
12-2-12	-20.5	-16.1
12-EO <sub>1</sub> -12	-13.3	-13.3
12-EO <sub>2</sub> -12	-9.9	-3.8
12-EO <sub>3</sub> -12	-9.2	-4.3
12-5N-12	-11.9	-7.7
12-8N-12	-3	-7.3
12-4-12	-9.2 <sup>a</sup>	-13.7
12-4(OH)-12	-12.1 <sup>a</sup>	-11.7
12-4(OH) <sub>2</sub> -12	-11.5 <sup>a</sup>	-14.3
12-3-12	-19.3 <sup>b</sup>	-16.1
12-6-12	-8.5 <sup>b</sup>	-6.8

Table 2. Enthalpies of micellization for aqueous gemini surfactants and the difference in the observed peak and initial enthalpies ( $\Delta H_{init} - \Delta H_1$ ) for the titration of gemini surfactants with DNA. <sup>a</sup> Data from (Wettig *et al.*, 2002); <sup>b</sup>Data from Wettig *et al.*, 2010.

The advantage of the reverse titration method is that it allows for an examination of complex formation under conditions of excess lipid (Kennedy *et al.*, 2000), potentially revealing differences in binding of DNA by lipid or surfactant aggregates (vesicles, micelles, etc.) rather than with monomer lipid or surfactant molecules that would be studied in the forward titration manner. Significant differences in how the resulting complexes assemble have been noted in the comparison of forward and reverse titration methods for the EDOPC – DNA systems introduced in section 2.3 (Kennedy *et al.*, 2000), indicating the potential to control the types, or more specifically structure, of lipid-DNA complexes simply by controlling the order in which the components are added to each other. Such an explanation likely explains the very common observation that transfection efficiencies are highly dependent upon the mixing order of the various components.

4. Conclusion

Isothermal microcalorimetry plays an important role in elucidating the binding mechanism of DNA with surfactants/ lipids used in non-viral gene therapy. Measurement of heats of interaction allows accurate determination of binding constants, reaction stoichiometry (n), enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ), thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment by employing an appropriate model. Structure-activity studies suggest that biophysical properties, such as size, charge, and morphology of the resulting DNA/lipid complexes determine transfection efficiency within one class of vector. Several parameters effect transfection activity, such as structural variations of the cationic lipids (length and degree of unsaturation of the alkyl chains), the polarity of head groups, the structure of spacer, and the counterions. It is generally observed that the chemical arrangement of gemini surfactants provide a rather rich array of aggregate morphologies and solution properties that are dependent upon the nature and size of the linking group. More recently, ITC has been used to study the interaction of DNA with transfection systems based upon a family of dimeric surfactants or gemini surfactants, which consist of two traditional surface active groups linked chemically by a spacer group. ITC data shows whether the

interaction between DNA and gemini surfactants is endothermic or exothermic and the observed enthalpy *vs.* charge ratio profile depends upon the titration sequence. The magnitude and sign (i.e., endo- or exo-thermic) of  $\Delta H_{\text{mic}}$  is governed by the types and magnitude of various contributions (hydrophobic and electrostatic) arising from surfactant-surfactant interactions. Since, for any process to be thermodynamically favoured the free energy change must be negative, an endothermic lipoplex formation must be driven by a corresponding increase in entropy. It is widely accepted that the major contribution to this increase in entropy arises from counterion release. The double-peak feature observed in the enthalpogram for the titration of the 12-3-12 surfactant with DNA is common to all of the symmetric gemini surfactants investigated (so far) by our group. Our group is using ITC to better understand the thermodynamic properties of the complexation of DNA by the aza-, hydroxyl- and ethoxyl-gemini substituted and unsubstituted surfactants, specifically to examine how the binding interactions change as a result of substitution within the spacer group. In addition, our group is interested in the study of a non-symmetric gemini surfactant in which one of the alkyl tails is a dodecyl group (i.e.  $m = 12$ ) and the other has been replaced with a (pyren-6-yl)-hexyl group which has the same approximate length as a dodecyl group, but is significantly more hydrophobic in character. The major difference observed in the binding interaction(s) with DNA for the pyrenyl gemini surfactants lies in the ability of the pyrenyl group to intercalated between DNA base pairs. Interestingly a simplification of the system is observed for the pyrenyl surfactants (py-3-12; py-6-12), such that a number of intermediate equilibria do not seem to occur. One of the main differences in the mechanism proposed in our 2010 article (Wettig et al., 2010) and that originally proposed in our 2007 article (Wang et al, 2007) is the inclusion of the monomer-micelle equilibrium which in turn is governed by the molecular structure of the surfactants. The significance of these results remains unclear; however, forward titrations of the substituted gemini surfactants with are underway. Ultimately these results will allow for a more complete picture of the importance of head group structure of the gemini surfactants for improved DNA transfection efficiency, and the rational design of improved non-viral transfection vectors.

## 5. References

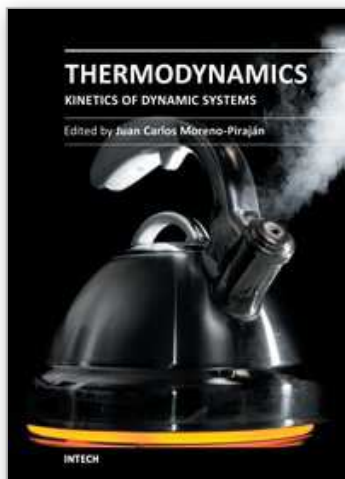
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