

Thermodynamic Studies of DNA-Cationic Components Interactions Using Titration Calorimetry

Shawn D Wettig^{1*} and Amany O Kamel^{1,2}

¹School of Pharmacy and Waterloo Institute of Nanotechnology, University of Waterloo University Ave. W., Waterloo, Canada

²Department of Pharmaceutics and Industrial Pharmacy, School of Pharmacy, Ain Shams University, Cairo, Egypt

Abstract

DNA delivery to cells has gained much attention as a powerful approach in gene therapy for treatment of inherited and acquired diseases. The transfection process depends on the nature of the binding interaction between DNA components of the non-viral delivery vector. The aim of this review is to explore the use of isothermal titration microcalorimetry (ITC) in the investigation of thermodynamic binding interactions between DNA and cationic components essential for DNA targeting to the cells. The review will focus on the interaction of DNA with block copolymers, surfactants and cationic lipids.

Keywords: Block copolymers; DNA; Isothermal titration microcalorimetry

Introduction

The study of the interaction of DNA with cationic lipids, polymers, and surfactants is of great importance for the development of DNA-based therapeutics for both acquired and genetic disease. Genetic therapies have the potential to revolutionize the treatment of disease; however, in order for this potential to be realized, efficient delivery of the DNA therapeutic into the target cell(s) of interest must be achieved. There are two widely accepted means of delivering DNA to cells within the body; 1) virus – based systems, and 2) systems based upon cationic lipids, polymers, and/or surfactants. The latter methods are grouped under the general heading of “non- viral” systems. A comprehensive review of these systems is beyond the scope of this article; there are numerous reviews [1-5] and texts [6-9] on this subject (in the case of non-viral systems since Felgner’s pioneering work in 1987 [10] that the reader is referred to).

In terms of delivery efficiencies, viral systems possess significant advantages by utilizing the infectious mechanism(s) inherent to viruses. That said, virus-based systems suffer from very real safety concerns, where their use can (and have) resulted in severe immune responses that can lead ultimately to patient death [11-13]. On the other hand, non-viral systems typically have low systemic toxicities, and low (or no) immunogenicity but suffer from much lower delivery efficiencies as compared to virus-based systems. Given the above considerations, a greater understanding of the mechanism(s) involved in non-viral delivery of DNA, the first step of which is the complexation of the DNA by the cationic component(s) of the delivery system, is needed. This review will explore the thermodynamics of the DNA-cationic component interactions, focusing specifically on the thermodynamics of cationic surfactant – DNA and cationic lipid – DNA systems.

Barriers to DNA Transfection

The delivery of DNA into the nucleus of a cell where it can be transcribed and expressed is a process known as transfection; however, for this to occur, the DNA therapeutic must overcome a large number of intra- and extra-cellular barriers specifically designed by nature to keep foreign genetic material out of the host body. While this review will focus specifically on the first of these barriers, it is important for the reader to understand the complexity of the entire process, and how optimizing a single step may (or may not) lead to improvements in the therapeutic system as a whole. Again, the following is not

meant to be comprehensive, for additional information the reader is referred to numerous reviews on these barriers [1,3-5,14].

Commonly recognized barriers to non-viral transfection include: cellular targeting/binding; internalization (cellular uptake); release of the complex into the cytoplasm; intracellular trafficking; and nuclear import leading to protein expression (for DNA based therapies as opposed to siRNA therapies) [3,4]. Unless specific targeting moieties have been incorporated into the non-viral vector, cellular targeting depends on non-specific binding to cellular membranes as a result of the overall net positive charge carried by the cationic transfection complexes. It should be noted that an additional barrier faced by non-viral transfection complexes is that of complexation by blood serum proteins, which also occurs as a result of their net positive charge. Incorporation of a layer of polyethylene glycol (PEG), usually through direct coupling to one of the lipid components of the transfection complex, has been successful in creating “stealth” liposomes [15,16] that do not interact with serum proteins.

Once localized at the membrane surface of the cell, the complex is taken up by the cell through what is generally recognized to be an endocytotic pathway, which may be: clathrin- mediated; caveolae dependent; macropinocytosis; or clathrin/caveolae independent endocytosis. Which specific mechanism(s) are involved is highly dependent upon the physical nature of the resulting transfection complexes, and in many cases has been observed to be highly dependent on the overall size of the complexes [4]. After uptake, the next barrier faced by the transfection vector is escape from the endosome which must occur before maturation of the endosomes into acidified lysosomes where the DNA will be degraded. Following endosomal escape the DNA cargo must: be released from the transfection vector; be imported into the cell nucleus and be transcribed into mRNA which

***Corresponding author:** Shawn D Wettig, School of Pharmacy and Waterloo Institute of Nanotechnology, University of Waterloo University Ave, Canada, E-mail: wettig@uwaterloo.ca

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can then be translated into the desired therapeutic protein (within the cytoplasm) (Figure 1) [3].

From the above description, there are two critical stages of the transfection process that depend upon the nature of the binding interaction(s) that occur between the DNA cargo, and the other components of the non-viral delivery vector; specifically complexation and release of the DNA by the vector particles. As will be described in the next section, isothermal titration calorimetry is perfectly suited to the study of such binding processes; albeit such study is complicated by the, generally, highly cooperative nature of such interactions.

Isothermal Titration Calorimetry (ITC)

A significant challenge in terms fundamental biophysics is being able to attribute the contribution of different non-covalent interactions (electrostatic, solvation and hydrophobic interactions, hydrogen bonding, Van der Waals interactions, etc.) to the free energy change of a given molecule upon its interaction with a binding partner [17]. In particular, the challenge is being able to “predict” how changes in molecular structure will ultimately impact the binding energetics between two binding partners. In highly cooperative processes, such as micelle formation and binding of cationic lipids or surfactants to DNA, such interpretations become even more difficult; however, such an understanding is crucial to the elucidation of the mechanism(s) involved for successful DNA transfection. Compared to other methods used to determine thermodynamic parameters associated with binding interactions, calorimetry uses direct measurement of the heat generated or absorbed during a binding event to then calculate molar enthalpies for the interaction that, for the titration method, can be examined as a function of concentration or stoichiometry. ITC measures the heat released or absorbed during a binding event as a function of ligand concentration. Measurement of this heat allows accurate determination of binding constants, reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS), thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment. A schematic of a basic ITC instrument is shown in Figure 2. In a typical ITC experiment, the substrate 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 heater of the reference cell. This in turn directs a feedback circuit

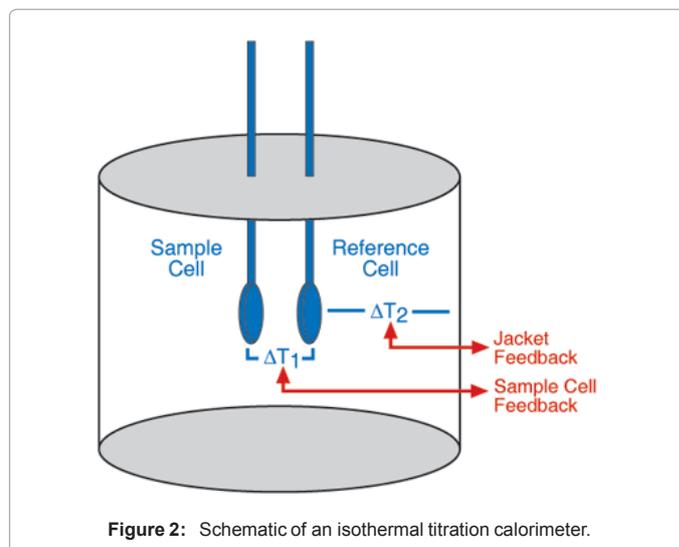


Figure 2: Schematic of an isothermal titration calorimeter.

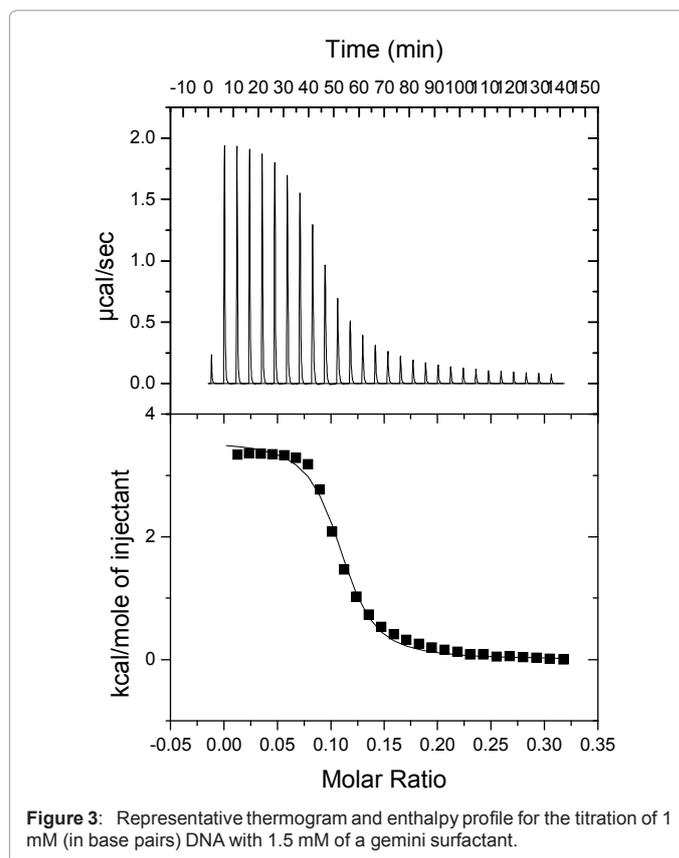


Figure 3: Representative thermogram and enthalpy profile for the titration of 1 mM (in base pairs) DNA with 1.5 mM of a gemini surfactant.

to activate the heater on the sample cell. During the injection of ligand into the sample cell, heat is taken up or evolved depending on whether the binding which occurs between substrate and ligand is endothermic or exothermic. This will result in an activation or deactivation of the feedback power (respectively) in order to maintain equal temperature between the two cells.

The heat absorbed or released (in terms of molar enthalpy ΔH), upon injection of the titrant is monitored over time (Figure 3). Each peak shown in Figure 3 represents a heat change associated with the injection of a small volume of ligand solution into the ITC reaction cell.

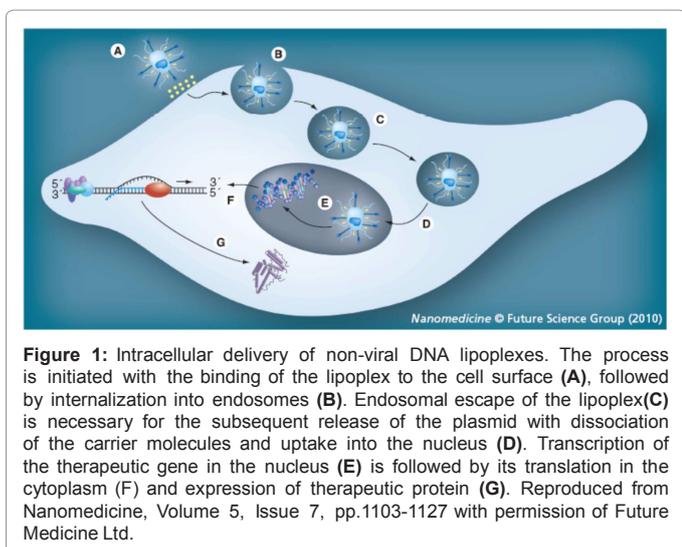


Figure 1: Intracellular delivery of non-viral DNA lipoplexes. The process is initiated with the binding of the lipoplex to the cell surface (A), followed by internalization into endosomes (B). Endosomal escape of the lipoplex (C) is necessary for the subsequent release of the plasmid with dissociation of the carrier molecules and uptake into the nucleus (D). Transcription of the therapeutic gene in the nucleus (E) is followed by its translation in the cytoplasm (F) and expression of therapeutic protein (G). Reproduced from Nanomedicine, Volume 5, Issue 7, pp.1103-1127 with permission of Future Medicine Ltd.

As successive amounts of ligand are titrated into the ITC cell, the heat absorbed or released is in direct proportion to the amount of binding. When the substrate is fully bound (i.e. saturated), the measured heat signal diminishes until only heats of dilution (of additional ligand) are observed [17]. The binding curve is then obtained from an integration of the heats (q) from each injection and the calculated enthalpies ($H=q/n$ where n is the number of mols) are plotted against the molar ratio of ligand and binding partner in the cell. Application of various binding models then allow for determination of the association constant for the ligand and substrate, K_A .

The molecular interaction between ligand and substrate 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 dissociation constant K_D , which is commonly used to quantify the affinity between two ligands, is the inverse of K_A , which is directly related to the Gibbs free energy. The Gibbs – Helmholtz equation states that the sum of enthalpy (ΔH) and entropy (ΔS) changes is equal to the free energy (ΔG) therefore also directly related to K_A . Therefore a major advantage of ITC experiments is the fact that K_A and ΔH are measured in a single experiment. Having measured these two parameters the remaining variables ΔG and ΔS 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 [18].

Calorimetric Investigations of Surfactant – DNA Binding

Non-viral vectors for DNA delivery contain cationic components (polymers, surfactants and/or lipids) that bind to DNA and form complexes that both condenses and protects the DNA during the transfection process. An excess of cationic component (relative to the DNA concentration in base pairs) is used to provide a net positive charge to the resulting particles, passively targeting cellular membranes via electrostatic attraction, and thus facilitating cellular uptake. The remainder of this article will examine various ITC studies of this binding, and the resulting implications with respect to the overall transfection mechanism(s).

Interactions between DNA and cationic surfactants

Cationic surfactants and lipids are efficient condensing agents for condensation of DNA. They initially bind to the DNA phosphates, and with increasing concentration can self-assemble into micellar aggregates bound to the DNA molecules. These aggregates will act as counter ions of very high valency that will interact strongly with DNA affecting its conformation. The interaction behavior of these cationic surfactants with DNA can be determined by ITC.

Zhu and Evans [19] studied the molecular mechanism and thermodynamics of the interactions between plasmid DNA and cationic surfactants benzyltrimethylammonium chloride, benzyltrimethyltetradecylammonium chloride, cetylpyridinium chloride, and cetyltrimethylammonium chloride. The thermal response signals showed that the reaction of DNA and surfactants is reversible, at high surfactant concentration a complex with DNA was rapidly and endothermically formed followed by exothermic dissociation after sufficient mixing. When the concentration of surfactants in the sample cell reached the CAC, the complex stopped dissociating. The ITC study

proved that the formation of the DNA surfactant complex was due to hydrophobic interaction of the surfactant molecules. Moreover, the DNA cationic surfactant interaction is an entropy-driven reaction shown in the positive value of ΔH , where the enthalpy reduction induced by charge-charge interaction of DNA and surfactant does not overcome the enthalpy increase caused by dissociation of counter ions and water molecules from DNA during the reaction. However, those dissociations largely increased the entropy of the system [19].

The hydrophobicity of the head group of cationic surfactants strongly affects their binding to DNA. Jadhav et al [20] studied the effect of the hydrophobicity of the surfactant head group on the interaction with DNA using different types of amino acid-based cationic surfactants (Alanine, Proline and Phenylalanine). By titrating DNA with the different surfactants, the enthalpy changes for the first injections were exothermic which then decreased steadily by further injection of the three surfactants. The enthalpy changes were saturated when the surfactant concentration in the cell reached 60, 80 and 100 μM for Phenylalanine, Proline and Alanine, respectively. This indicates that the surfactants with more hydrophobic head group, such as, phenylalanine interacted strongly with DNA (Phe > Pro > Ala) [20].

Gemini surfactants are surfactants having two head and two tail groups that are linked chemically. They have shown significant potential for use in non-viral transfection vectors for the delivery of genes into cells. Our group [21] studied the interaction of DNA with a series of N,N- bis(dimethylalkyl)- α,ω -alkanediammonium dibromide gemini compounds that simulates membrane components in terms of carbon chain length and chemical character, 18:1-s-18:1, where $s = 2, 3$, and 6 as well as 12-s-12, where $s = 3$ and 12. 18:1 signifies an 18-carbon chain with one double bond [22]. In aqueous solution, 18:1-2-18:1 and 18:1-3-18:1 gemini surfactants formed a vesicle structure while 18:1-6-18:1, as well as the compounds 12-3-12 and 12-12-12, formed micelles. The binding enthalpy for injection of DNA into 18:1-6-18:1 solution showed three distinct regions (Figure 4A). An initial endothermic region of nearly constant enthalpy (11–12 kJ mol due to interactions between DNA and 18:1-6-18:1 micelles and/or monomers. This was followed by a sharp rise in enthalpy to 32–33 kJ mol assigned to the formation of larger- sized aggregates derived from those formed in

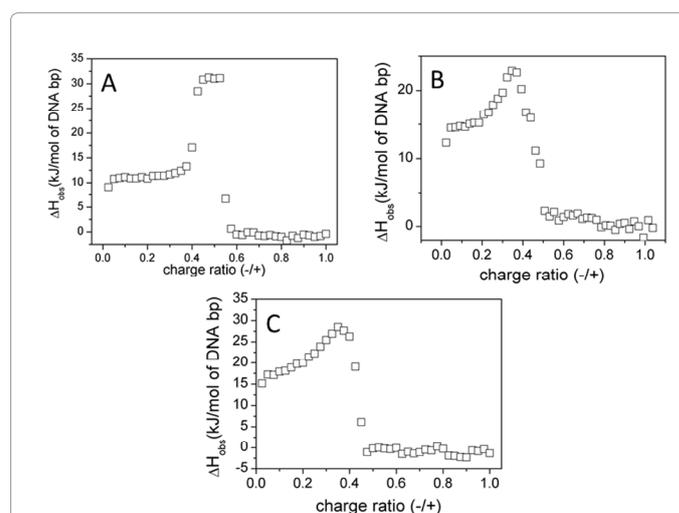


Figure 4: Profile of enthalpy vs. charge ratio, (-/+) upon titration of A) 18:1-6-18:1, B) 18:1-2-18:1, and C) 18:1-3-18:1 with DNA. Conditions: [DNA] = 3.0 mM, [18:1-6-18:1] = 0.5 mM; both in the presence of 10 mM NaBr. Adapted from Wang et al. [21].

the first region, further addition of negatively charged DNA induced aggregation of the isolated positively charged lipoplexes of the first region. The enthalpy of aggregation is almost three times as large as those of the first region and is due to the lower charge density and larger size of the lipoplexes. And finally a third region, where, after a sharp drop the measured enthalpy remains nearly constant at 0 kJ mol. This sharp drop in enthalpy at the transition is the complex reaction endpoint. Similar results were obtained upon addition of DNA to 12-3-12 and 12-12-12 at concentrations more their CMCs. Titrating DNA into 18:1-2-18:1 and 18:1-3-18:1 were different from those for 18:1-6-18:1 (Figure 4B and 4C, respectively). The initial enthalpy at low DNA concentration was 15kJ which increased monotonically to more than 20 kJ mol, then the enthalpy dropped rapidly to near zero and remains close to zero up to the highest DNA concentration studied [21].

Moreover, we used ITC measurements to study the effect of DOPE on the interaction between gemini surfactants and DNA by injecting DNA into reconstituted mixtures of 18:1-3-18:1/DOPE vesicles. The results showed that the presence of DOPE vesicles did not affect the enthalpy of complex formation between DNA and gemini surfactant [21].

We also studied the interaction of 12-s-12 Gemini surfactants with DNA after their modification with pyrene (py-s-12). This modified surfactant belongs to the dissymmetrically gemini surfactants. Initially, the interaction between DNA and py-3-12 micelles occurred in the same manner with a comparable endotherm as with 12-3-12 micelles because the head groups of the surfactants are the same, however, beyond this region significant differences have occurred. This might be due to the incorporation of a pyrenyl group into the gemini surfactant which led to changes in their binding interactions with DNA. The aggregation or flocculation observed with the 12-s-12 surfactants was eliminated due to strong intercalation of the pyrenyl group between DNA base pairs that might have forced the surfactant to adopt an orientation such that the dodecyl tails are oriented away from the complex, leading to a steric stabilization against Flocculation [22,23].

In another study Jiang et al. [24] used ITC to study the effect of dissymmetry degree of gemini surfactants on their interaction with DNA. They studied the interaction of a series of $C_mC_6C_nBr_2$ dissymmetric gemini surfactants having constant $m+n = 24$, and $m = 12, 14, 16$, and 18 with DNA. At the low surfactant concentration, addition of the gemini surfactants to the DNA solution showed a more positive observed enthalpy (H_{obs}) compared to those in the absence of DNA. This might be due the endothermic interaction between DNA and the monomers of the $C_mC_6C_nBr_2$ (Figure 5). Beyond the CAC a steep decrease in H_{obs} was observed followed by an exothermic hump, then H_{obs} increased upon further addition of surfactants after undergoing a minimum till the second critical H_{obs} is close to zero, indicating that only free micelles are diluted [24]. The CAC tended to become smaller with increased m/n . Moreover, the hydrophobic interaction between the hydrophobic chains of the surfactant molecules increased and the aggregation process was more spontaneous with increased m/n where, the enthalpy change (ΔH_{agg}) as well as the Gibbs free energy change (ΔG_{agg}) for aggregation became more negative down the series. The entropy changes of aggregation (ΔS_{agg}) were all positive indicating that the aggregation process is mainly entropy-driven [24]. The thermodynamic parameters ΔH_{agg} , ΔG_{agg} and ΔS_{agg} reflected the DNA surfactant aggregation process which is the contribution of the surfactant micellization as well as the binding of the micelle to the DNA [24].

In order to understand the interaction between the DNA and

surfactants, Jiang et al. calculated the thermodynamic parameters ΔH_{DS} , ΔG_{DS} and ΔS_{DS} reflecting the change of the aggregation behavior of the surfactants induced by the interaction of the surfactants (S) with DNA (D) [24]. Strong binding of the micelles to DNA has occurred as shown by all negative G_{DS} values (Figure 6). G_{DS} became less negative by increasing m/n indicating that the interaction tended to be weaker. The H_{DS} almost did not change with increasing m/n so the difference of G_{DS} is mainly due to the change of entropy S_{DS} with the change in m/n . In the absence of DNA, increasing m/n leads to a stronger hydrophobic interaction between the gemini surfactant molecules. In the presence of DNA, the electrostatic attraction between the head group of the gemini surfactant and DNA disrupt the hydrophobic interaction among the surfactant molecules. This disruption becomes more pronounced by increasing m/n . The ΔS tends to decrease by increasing m/n indicating the interaction tends to be less spontaneous [24]. Gemini surfactants can be used to obtain cationic liposomes which are very promising in non-viral gene delivery. Pullmannuva et al. [25] studied the interaction between cationic liposome obtained from the mixture of gemini surfactants, alkane- α,ω -diyl-bis(alkyldimethylammonium bromide) and helper lipid dioleoylphosphatidylcholine. Titration of DNA solution into the cationic liposome dispersion resulted in an endothermic process

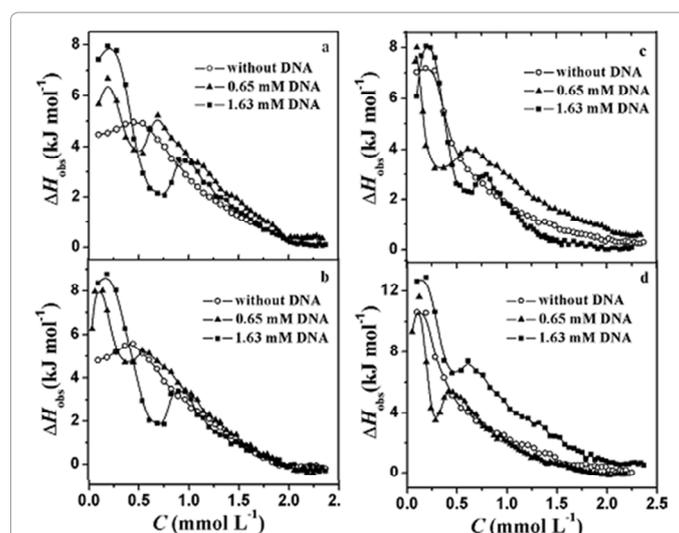


Figure 5: Curves of calorimetric titration of $C_mC_6C_nBr_2$ surfactants into 10 mMNaCl solution and DNA solutions at 298.15 K: (a) $C_{12}C_6C_{12}Br_2$; (b) $C_{14}C_6C_{10}Br_2$; (c) $C_mC_6C_nBr_2$; (d) $C_{16}C_6C_8Br_2$. The data points are the experimentally observed enthalpies per mol of surfactant. Adapted from Jiang et al. [24].

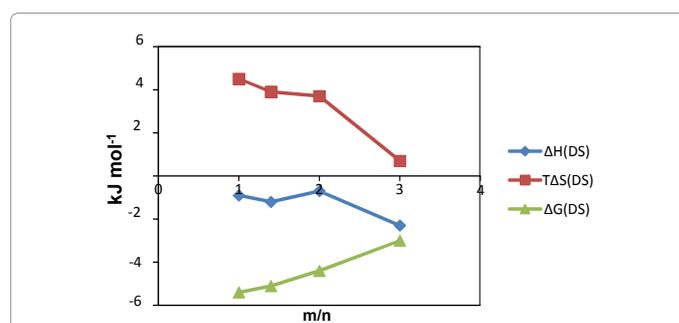


Figure 6: Relation between m/n and the thermodynamic parameters for the interaction between $C_mC_6C_nBr_2$ surfactants and DNA at 298.15 K. This plot is based on the data in Table 2 from Jiang et al. [24].

indicating that the interaction is driven by increase in entropy due to release of bound water and counter ion from surface of cationic liposomes and DNA. After a certain number of injections, addition of DNA showed up as small and constant exothermic changes, due to the dilution. At low ionic strength, several endothermic peaks were observed, which decreased fast to close to zero, and became negative in the last injections. At high ionic strength the number of endothermic peaks was very much reduced and their areas were smaller than those observed at low ionic strength due to the reduction of the amount of DNA binding to the cationic liposomes [25]. We highlighted the use of ITC in investigating the interactions of surfactants with DNA for its condensation and compaction. However, the decompaction of DNA condensate is as important as its compaction. The compaction of DNA into small particles is very important because it protects DNA from degradation by nucleases and aids in cellular uptake [26,27]. However, the decompaction can release the DNA inside the cell for transcription. Cao et al. [28] have used ITC measurements to study the decompaction of the DNA-gemini surfactant (hexyl- ω -bis(dodecyldimethylammonium bromide) condensate using β -cyclodextrin and the anionic surfactant sodium dodecyl sulfate. Their results showed that β -cyclodextrin and sodium dodecyl sulfate demonstrated different mechanisms in the decompaction of the condensate. The titration of sodium dodecyl sulfate into the DNA-gemini surfactant mixed solution is much more exothermic than that of β -cyclodextrin into the same mixture solution. This suggests that the decompaction of the condensate is due to the hydrophobic interaction involved in β -cyclodextrin and both electrostatic and hydrophobic interaction involved in sodium dodecyl sulfate [28].

Interaction between DNA and cationic polymers

ITC was used to study the condensation of plasmid DNA (pDNA)

by poly(ethylene glycol)-poly(L-lysine) block copolymer (PEG-PLL) [29]. The heat accompanied by interaction of pDNA with PEG-PLL was measured by changing the degree of lysine polymerization under different NaCl concentrations. The binding process was endothermic with a small increase in enthalpy, a large increase in entropy, and a large decrease in free energy. The ITC curves (Figure 7) show two distinct endothermic binding processes; the first being binding of PEG-PLL to pDNA, and the second due to the binding PEG-PLL to pDNA during a conformational transition. The binding constant K decreased by increasing NaCl concentration, moreover it became more dependant with the decrease in degree of polymerization of PLL (Figure 4) [29]. Tan et al. [30] studied the thermodynamics of binding between poly(ethylene oxide)-block-poly(2(diethylamino)ethyl methacrylate) block copolymers and plasmid DNA. They found that as the polymer solution was titrated into the DNA solution, a pronounced exothermic heat was observed showing that the interaction between the block copolymer and DNA was highly favorable enthalpically. This copolymer formed pH dependent micelles, at low pH, the amine groups of poly(2(diethylamino)ethyl methacrylate) are protonated, and the hydrophilic polymer did not aggregate in solution. At pH 7.4, these groups are partially deprotonated, so that the poly(2(diethylamino)ethyl methacrylate) chains are somewhat hydrophobic and drive the formation of micelles which are stabilized by the hydrophilic PEO segments. The remaining cationic poly(2(diethylamino)ethyl methacrylate) segments of the copolymer were able to bind with the negatively charged DNA. In another study, the interaction of calf thymus DNA with cationic polymers synthesized from methoxypoly(ethylene glycol) monomethacrylate and (3(methacryloylamino)propyl)-trimethylammonium chloride was studied using ITC. Low exothermic enthalpy changes have occurred due to the electrostatic interactions between the cationic

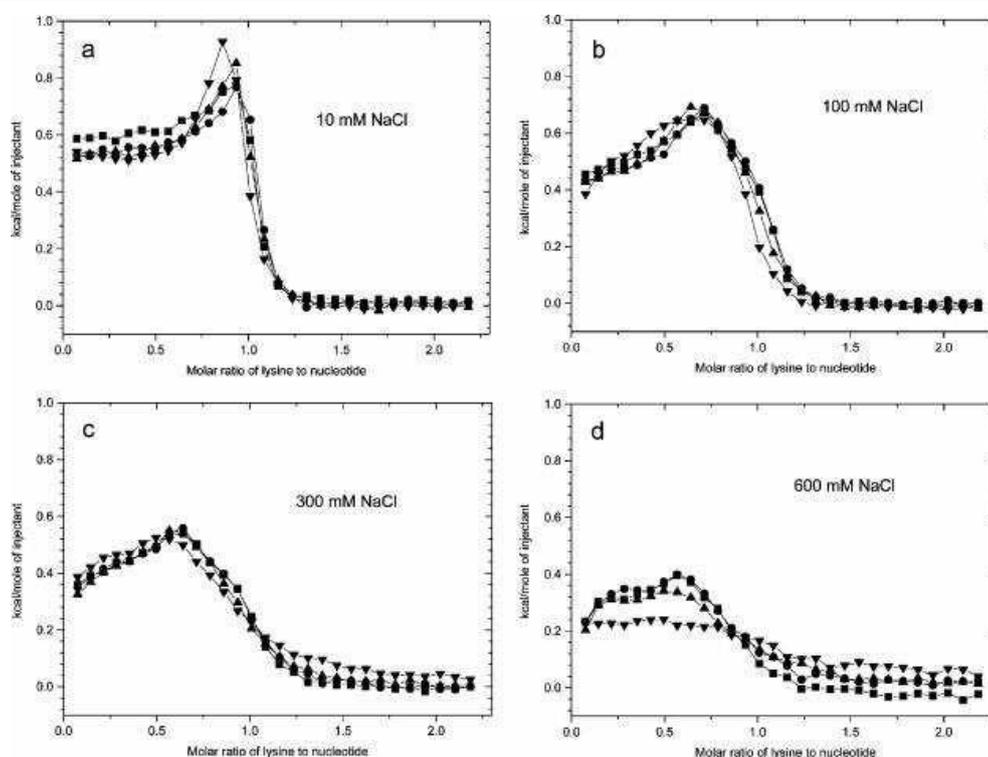


Figure 7: Dependence of integrated ITC curves of PEG-PLL binding to pDNA on degree of polymerization of PLL in various NaCl concentrations: (a) 10 mM, (b) 100 mM, (c) 300 mM, and (d) 600 mM; PEG-PLL (12-109; ■), PEG-PLL (12-73; ●), PEG-PLL (12-47; ▲), PEG-PLL (12-20; ▼). Adapted from Kim *et al.* [29].

units of the polymer and the negatively charged phosphate groups. The thermodynamic parameters showed that the binding process is entropy driven. The authors stated that the increase in the entropy of the system was due to the release of monovalent counter ions from the DNA phosphate groups [31]. ITC measurements were also used previously to investigate the interaction between amphiphilic copolymers and DNA. Roques et al. [32] investigated the interaction between pluronic L64 and tetronic 304 with DNA at 4, 20 and 37°C. The enthalpy of interaction between tetronic 304 and plasmid DNA was constant and endothermic (~ 0.2 kJ/mol) for all three temperature ranges. No interactions were observed between pluronic L64 and DNA at 4°C and 20°C; however it should be noted that no evidence of micelle formation was seen for L64 in the absence of DNA for these temperatures. When pluronic L64 self-assembled into micelles at 37°C, interactions with plasmid DNA were observed [32], with an enthalpy of interaction of ~ 2.1 kJ/mol. These results demonstrated that, for amphiphilic copolymer vectors, aggregation into micelles was crucial for successful transfection; likely due to weaker interactions between the neutral polymer monomers and DNA as compared to the stronger electrostatic interactions that occur for cationic polymers or lipids and DNA.

Summary

ITC measurement is a useful tool in investigating the binding interaction between DNA and other components in the non-viral DNA delivery system. This review showed that ITC experiments used direct measurement of heat absorbed or evolved during binding of DNA to cationic components like surfactants, polymers and lipids as a function of concentration. Measurements of this heat allowed accurate determination of binding constants, enthalpy and entropy. In most of the cases, the binding was due to electrostatic and/or hydrophobic interactions. Moreover, most of the investigated interactions were entropy driven.

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