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Thermodynamic analysis of binding and protonation in DOTAP/DOPE (1:1): DNA complexes using isothermal titration calorimetry

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Abstract

A better understanding of the nature of the interaction between various cationic lipids used for gene delivery and DNA would lend insight into their structural and physical properties that may modulate their efficacy. We therefore separated the protonation and binding events which occur upon complexation of 1:1 DOTAP (1,2-dioleoyl-3-trimethylammonium propane):DOPE (1,2-dioleoylphosphatidylethanolamine) liposomes to DNA using proton linkage theory and isothermal titration calorimetry (ITC). The enthalpy of DOPE protonation was estimated as -45.0 ± 0.7 kJ/mol and the intrinsic binding enthalpy of lipid to DNA as $+2.8 \pm 0.3$ kJ/mol. The pK_a of DOPE was calculated to shift from 7.7 ± 0.1 in the free state to 8.8 ± 0.1 in the complex. At physiological ionic strength, proton linkage was not observed upon complex formation and the buffer-independent binding enthalpy was $+1.0 \pm 0.4$ kJ/mol. These studies indicate that the intrinsic interaction between 1:1 DOTAP/DOPE and DNA is an entropy-driven process and that the affinities of cationic lipids that are formulated with and without DOPE for DNA are controlled by the positive entropic changes that occur upon complex formation.

Keywords: Isothermal titration calorimetry; Proton linkage; Cationic lipid; Plasmid DNA; Protonation; DOPE

1. Introduction

During the past decade, isothermal titration calorimetry (ITC) has been established as a direct method with which to characterize the energetics of biological associations including protein–protein, protein–DNA and protein–lipid interactions [1–3]. Additionally, ITC can be used to examine the interaction between DNA and various cationic lipids that are currently used to facilitate gene delivery [4–8]. This interaction is thought to be primarily electrostatic in origin and driven by an increase in entropy as a consequence of the release of bound counter-ions from the surface of both components upon complexation [7,8]. Although cationic lipids are promising agents for therapeutic gene delivery, their efficiency has yet to be correlated with structural or physical measurements of the DNA complexes [9,10]. ITC, however, has the

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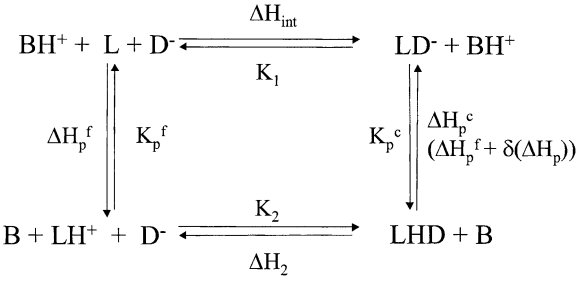
potential to determine if there might exist a thermodynamic basis for the relative effectiveness of gene delivery that is observed among various cationic lipids and their enhanced efficacy when mixed with neutral helper lipids such as DOPE [11]. Previous ITC studies have detected slight differences in the enthalpy of binding between several cationic lipid mixtures and DNA which may reflect the phase state of the lipid and the method of complex formation (lipid added to DNA vs. DNA added to lipid) [6–8]. These enthalpies of binding of quaternary ammonium lipids to DNA were all endothermic, implying an entropy-driven interaction. These findings, in combination with an observed decrease in the amount of lipid bound to DNA with increasing ionic strength [7] are the primary evidence for an electrostatic mechanism of binding between cationic liposomes and DNA.

Our initial calorimetric investigation of cationic lipid/DNA interactions (the results of which have been reproduced by others [6]) detected the protonation of DOPE upon binding of cationic lipid/DOPE liposomes to DNA and an exothermic enthalpy of binding at 25.0 °C, ionic strength (I) = 0.01 and pH 7.40 [7]. This proton linkage was detected calorimetrically by performing titrations of lipid mixtures into plasmid DNA in the presence of buffers of varying ionization enthalpy (ΔH_b^{ioniz}). These studies revealed that a systematic variation of binding enthalpy with buffer ionization enthalpy was observed only upon titration of DOTAP/DOPE 1:1 or DDAB/DOPE 1:1 liposomes into DNA and not upon binding of either cationic lipid alone to DNA. This finding supported the existence of proton linkage with DOPE-containing lipid mixtures and a net uptake of protons into the complex upon binding. Furthermore, this proton-linked binding event was abolished when DOPE was replaced by DOPC, in which the primary amine group of DOPE is replaced with a quaternary ammonium group. This suggests that proton linkage does, in fact, result from the protonation of the amine group of PE. This protonation event was qualitatively explained to be a result of an increase in the pK_a of the amine moiety of DOPE due to changes in surface charge and/or hydration at the lipid interface during binding to DNA [12]. This finding is provocative in that it suggests that the thermodynamics of complexation are considerably different between lipid mixtures that contain DOPE and those that do not. A further thermodynamic analysis of the interaction of DOPE-containing liposomes with DNA is presented in this report using proton linkage theory to separate the thermodynamics of protonation and binding.



Scheme 1. Thermodynamic square of binding and protonation of DOTAP/DOPE (1:1)(w/w) liposomes to plasmid DNA. The deprotonated and protonated forms of lipid are indicated as L and LH^+, respectively, DNA as D^- and the buffer species as B and BH^+. Also indicated are the protonation constants of the lipid in the free (K_p^f) and complexed (K_p^c) states and the binding constants of lipid to DNA in the deprotonated (K_1) and protonated (K_2) states. The enthalpy of binding of deprotonated lipid to DNA is defined as ΔH_{int} and of protonated lipid to DNA as ΔH_2. ΔH_p^f is the enthalpy of protonation of DOPE in the free state and ΔH_p^c the enthalpy of protonation of DOPE in the complex. $\delta(\Delta H_p)$ is the difference between these two protonation enthalpies.

Binding linkage theory attempts to define mathematically the effect of one ligand on the binding of another to the same receptor molecule [13,14]. Proton linkage theory addresses the specific case where the hydronium ion acts as a ligand and can protonate either the receptor or other ligand molecules [15–18]. The relative amounts of protonated and deprotonated species are dependent upon pH and each of these species has a different binding affinity for the remaining ligand/receptor molecules. As a result, the individual binding and protonation events are linked through their dependence on pH.

A suitable method to describe the pH dependence of these processes is to construct a thermodynamic square, as shown in Scheme 1. In this schematic, the binding processes are shown as the horizontal sides of the square and the protonation

events as the vertical sides. The protonated and deprotonated states of the ligand (lipid) are defined as LH^+ and L, respectively, the receptor (DNA) by D^- and the buffer species as B and BH^+. The principle of a thermodynamic square implies that a process defined by any two adjacent sides of the square is equivalent to a process defined by the remaining two sides. Therefore, each binding equilibria is coupled to an adjacent protonation event, such that the product of K_1 and K_p^c is equivalent to the product of K_2 and K_p^f. As a result, the ratio of binding affinities (K_2/K_1) is equivalent to the ratio of protonation constants (K_p^c/K_p^f). At high pH values the dominant equilibria is the upper horizontal binding process (ΔH_{int}) and at very low pH the lower binding equilibria will be dominant. Therefore, the apparent affinity constant will shift from the intrinsic affinity constant K_1 at high pH to K_2 at low pH values. It is also apparent from the schematic that proton linkage will only be significant at pH values near the free and complexed pK_a's (10^{-Kpf} and 10^{-Kpc}, respectively) of the ionizing group. The protonation change will be minimal at either extreme of pH, with a maximum value at a pH value midway between the free and bound p*K*a's of the ionizing group.

Proton linkage theory permits the binding and protonation events to be separated by fitting the pH dependence of the protonation change or the observed binding affinity to appropriate models [15–18]. The expressions for the pH-dependence of the protonation change (n) and the observed affinity constant (K_{obs}) of a single binding-linked protonation event are presented below [15,18]:

$$n = \left(\frac{10^{(pK_a^c)-pH}}{1+10^{(pK_a^c)-pH}} - \frac{10^{(pK_a^f)-pH}}{1+10^{(pK_a^f)-pH}} \right) \quad (1)$$

$$K_{obs} = K_1 \left(\frac{1+10^{(pK_a^c)-pH}}{1+10^{(pK_a^f)-pH}} \right) \quad (2)$$

In these equations, pK_a^f and pK_a^c are the pK_a's of the ionizing group in the free and complexed states and K_1 is the intrinsic affinity constant for the interaction. The previously described variations in the apparent affinity constant and protonation change with pH are also apparent from these equations. Returning to the thermodynamic square in Scheme 1, the enthalpies of interaction (ΔH_{int} and ΔH_2) can be directly obtained from an ITC experiment. These enthalpy values can then be used with the estimated affinity constants (K_1 and K_2) and the expression $\Delta G = -RT \ln K = \Delta H - T\Delta S$ to obtain all of the relevant thermodynamic parameters (ΔG, ΔH, ΔS) that characterize the individual binding and protonation events.

Although most proton linkage studies have investigated interactions between proteins and ligands that bind to specific sites on proteins [15,17–19], the non-specific electrostatic interaction between cationic lipids and DNA is also suitable for analysis by proton linkage theory. This theoretical treatment of the binding enthalpy effectively uncouples the processes of binding and protonation and allows a more mechanistic comparison between transfection lipids in their interaction with plasmid DNA.

2. Methods and materials

2.1. Materials

Purified supercoiled plasmid DNA pMB290 (5.1 kbp) was provided by Valentis, Inc. (Burlingame, CA) containing less than 5% of nicked and open-circular forms. DOTAP/DOPE 1:1 w/w (Transfection Reagent I) solution in chloroform was obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. All buffer components (acetate, piperazine, MES, cacodylate, BES, EDA, PIPES, EDA, ACES, imidazole, HEPES, TEA and TRIS) and NaCl were obtained from Sigma-Aldrich (St. Louis, MO). Milli-Q purified water was used in all solutions.

2.2. Methods

2.2.1. DNA preparation

Purified DNA solutions (approx. 8 ml of 200 mg/ml solutions) were dialyzed against 4.0 liters of buffer at 5.0 °C for 12 hours using a Pierce 10 K molecular weight cut-off dialysis cassette. DNA solutions post-dialysis were diluted to 100 mg/ml in dialysate buffer. DNA concentration was determined using UV-visible spectrophotometry with a Hewlett–Packard spectrophotometer employing an

extinction coefficient of 0.02 AU ml cm^{-1} mg^{-1}. An average molecular weight of 324.5 g/mol PO_4 was used for DNA concentration calculations. DNA solutions were stored at 5 °C and used on the day of preparation.

2.2.2. Liposome preparation

Stock solutions of lipids in chloroform were added to tared 5 ml vials, dried to a film using N_2 gas and desiccated overnight under vacuum. The amount of lipid present was determined gravimetrically and an appropriate volume of buffer was added to achieve a concentration of 3.72 mM cationic lipid. The resulting preparation was vortexed for 1 min and allowed to hydrate at room temperature for 30 min. Liposomes were extruded 11 times using an Avanti Mini-extruder (Avanti Polar Lipids, Alabaster, AL) and a 100 nm polycarbonate membrane at room temperature to produce large unilamellar vesicles (SUV) of approximately 100 ± 5 nm in diameter as determined by dynamic light scattering [7]. Liposome suspensions were used on the day of preparation.

2.2.3. pH adjustment of solutions

Liposome, buffer and DNA solutions used for ITC were adjusted to the desired pH (± 0.02 pH unit) at the experimental temperature of 25.0 ± 0.1 °C. pH measurements were made using a Mettler Toledo MP220 pH meter (0.01 pH unit sensitivity) and an accuTpH electrode (model #13-620-180). A 2 point electrode calibration was used (pH 4, 7 or 10 standards). Temperature control was obtained using a 20 ml Karl Fischer titration cell (Brinkmann Instruments, Westbury, NY) connected to a recirculating water bath. Water bath temperature was adjusted to provide a sample temperature of 25.0 ± 0.1 °C. Temperature measurements were made using a digital thermometer (Fisher Scientific).

2.2.4. ITC

All calorimetric titrations were performed with a CSC Model 4200 isothermal titration calorimeter (Calorimetry Sciences Corporation, American Fork, UT) at 25.0 °C. Calibration of the instrument and data analysis were previously described [7]. Titrations consisted of 10 μl injections of 3.72 mM cationic lipid into 1.41 ml (full cell) of 0.31 mM DNA until complex aggregation occurred and a loss of binding heat was observed, with 5 min between injections [7]. Previous ITC experiments performed at higher ionic strength have shown a reduction in the amount of lipid per injection that binds to DNA at higher ionic strength [7]. Therefore, to obtain the enthalpy of all injected lipid binding to DNA at 0.150 ionic strength, lipid and DNA concentrations were increased to 7.44 mM and 1.23 mM, respectively. Heats obtained from blank titrations of lipid into buffer were subtracted from each binding titration to obtain net binding heats which were normalized to the molar amount of cationic lipid added per injection, resulting in a net enthalpy per injection. The average enthalpy per injection up to a 0.8 charge ratio (cationic lipid to DNA phosphate) was used to calculate the observed enthalpy of binding of lipid to DNA (ΔH_{obs}).

2.2.5. pH titrations

HCl titrations of lipid preparations were performed using the same pH instrumentation and temperature control apparatus used for lipid and DNA solution pH adjustment for ITC (see above) at a temperature of 25.0 ± 0.1 °C. Titrations consisted of 30 μl additions of 0.010 M HCl in 0.010 M NaCl (spaced 15 min apart) into 4 ml of DOTAP/DOPE 1:1 w/w liposomes (0.4 mg/ml total lipid) prepared in 10 mM NaCl. The pH of liposome solutions was adjusted to ~8.9 with concentrated NaOH until stable at this pH for 30 min before the start of the titration. Titration curves represent the mean ± standard error of the mean (S.E.M.) of three acid titrations. The inverse second derivative of the titration curve was used to determine the buffer capacity of the system [20]. The buffer capacity data from three measurements were combined and the maximum buffer capacity (pK_a) was estimated from the center of an asymmetrical double sigmoidal function, a model that provided the best fit to the data.

3. Results

To further investigate the energetics of binding and protonation and the magnitude of the pK_a shift

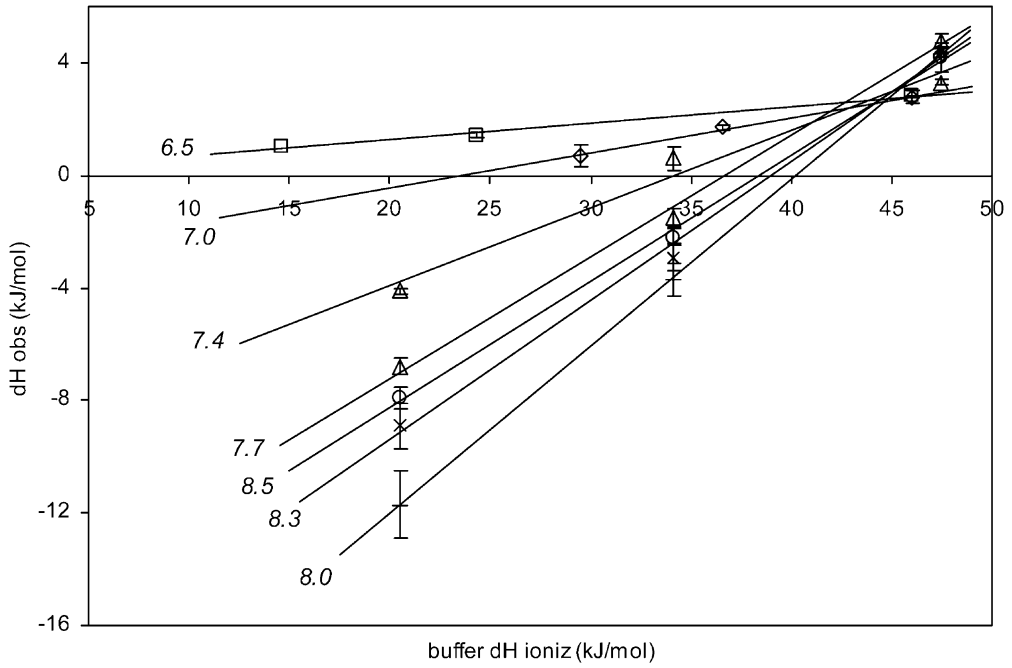


Fig. 1. Observed enthalpy of binding (ΔH_{obs}) determined by ITC vs. buffer ionization enthalpy (ΔH_b^{ioniz}) at various pH values of DOTAP/DOPE (1:1 w/w) liposomes (3.7 mM cationic lipid) titrated into plasmid DNA (0.31 mM) at 25.0 °C and $I = 0.010$. The pH of each data set is indicated in the figure.

of DOPE upon complex formation in DOTAP/DOPE 1:1/DNA complexes, ITC titrations of DOTAP/DOPE 1:1 (w/w) liposomes into plasmid DNA were performed at constant ionic strength (0.010) and temperature (25.0 °C) within the pH range of 6.5–8.5 and in the presence of buffers of varying ionization enthalpy (ΔH_b^{ioniz}). The upper range of pH investigation was limited to 8.5 due to the onset of colloidal instability of liposome preparations above this pH. The deprotonation of the amine group of DOPE at higher pH values reduces the cationic surface charge of the liposome mixture and the electrostatic repulsion between liposomes leading to extensive aggregation. The upper limit of liposome stability was determined using dynamic light scattering and zeta potential measurements of DOTAP/DOPE 1:1 liposomes as a function of pH. These measurements revealed a gradual reduction in liposome zeta potential with increasing pH, but only a slight increase in liposome hydrodynamic size and scattering intensity up to pH 8.5. Gross aggregation was observed at higher pH values (not illustrated).

According to proton linkage theory [16], binding titrations should display a linear relationship between ΔH_{obs} and ΔH_b^{ioniz}. The slope of this line reflects the degree of protonation of the ligand (n) while the y-intercept represents the buffer-corrected enthalpy of binding (ΔH_o), as shown in Eq. (3) below:

$$\Delta H_{obs} = \Delta H_o + n\,\Delta H_b^{ioniz} \quad (3)$$

As displayed in Fig. 1, ITC titrations of DOTAP/DOPE 1:1 liposomes into plasmid DNA within the pH range of 6.5–8.5 produced linear relationships between ΔH_{obs} and ΔH_b^{ioniz} at each pH. The slope of each best-fit line to the data (n) was greater with increasing pH up to pH 8.0 and then decreased above this pH. The variation of n with pH theoretically permits the determination of the pK_a's of DOPE in the free and bound states,

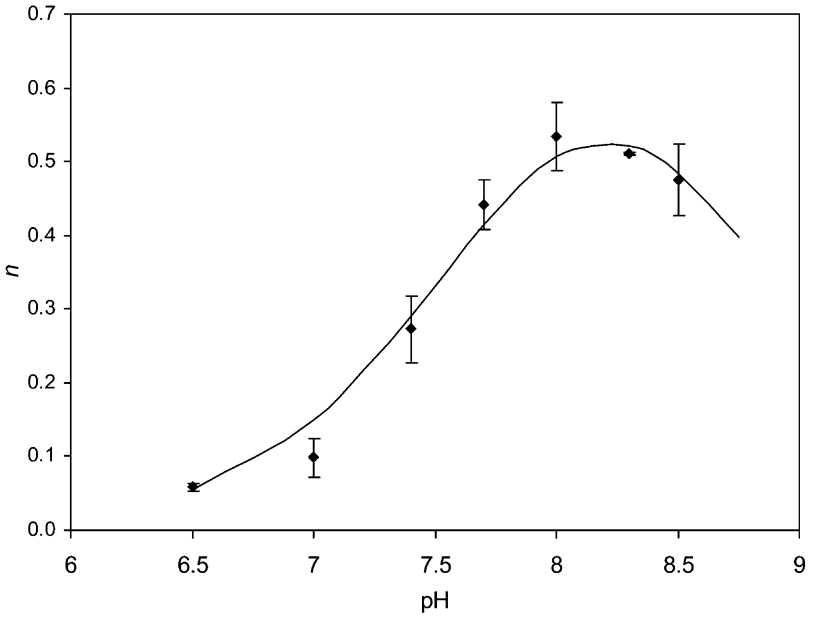


Fig. 2. The pH dependency of the degree of DOPE protonation (n) obtained from ITC titrations of DOTAP/DOPE (1:1 w/w) liposomes into DNA. The line represents the best fit of the data to a single ionizing group protonation model (Eq. (5) in the text) using non-linear least squares fitting.

employing the following equation for a single ionizing species:

$$n = H^{c} - H^{f} = \frac{K_{p}^{c} a_{H^+}}{1 + K_{p}^{c} a_{H^+}} - \frac{K_{p}^{f} a_{H^+}}{1 + K_{p}^{f} a_{H^+}} \quad (4)$$

where H^{c} is the protonated fraction of DOPE in the complex, H^{f} is the protonated fraction of DOPE in the free liposome, K_{p}^{c} is the proton binding constant for DOPE in the complex ($10^{pK_{ac}}$), K_{p}^{f} is the proton binding constant for DOPE in the free liposome ($10^{pK_{af}}$) and a_{H^+} is the activity of the hydronium ions, approximated here as the hydronium ion concentration. The pH-dependence of n for a single ionizing species should ideally be described by a bell-shaped curve. This results from the fact that the maximum degree of protonation occurs midway between the two pK_a's [16].

Fig. 2 presents the pH dependence of n for titrations of DOTAP/DOPE 1:1 liposomes into DNA and a best fit of Eq. (4) to the data by non-linear least squares analysis. The model fit to the data is better presented when the data is plotted with the x-axis in pH units (a linear scale) instead of the hydronium ion concentration, which would require a logarithmic scale. The calculated pK_a's of DOPE in the free and bound states using this model are approximately 7.7 ± 0.1 and 8.8 ± 0.1, respectively. The shift in pK_a of DOPE upon binding to DNA is therefore only 1 pH unit.

In order to determine the pK_a's of DOPE in the free and complexed states, independent of ITC, pH titrations were performed of DOTAP/DOPE 1:1 liposomes and of liposome/DNA complexes in unbuffered solution at 25.0 °C and $I = 0.010$. The resulting acid titration curve of liposomes alone is displayed as the mean $\pm$ S.E.M. of three individual measurements in Fig. 3a. The pK_a of DOPE in free liposomes can be estimated from the maximum effective buffering capacity of the system which is obtained from the reciprocal of the second derivative of the titration curve vs. pH [20], as shown in Fig. 3b. From this analysis, a pK_a of 7.55 ± 0.01 was obtained for DOPE in DOTAP/DOPE 1:1 liposomes, which is in reasonable agreement with the pK_{a}^{f} value obtained with ITC

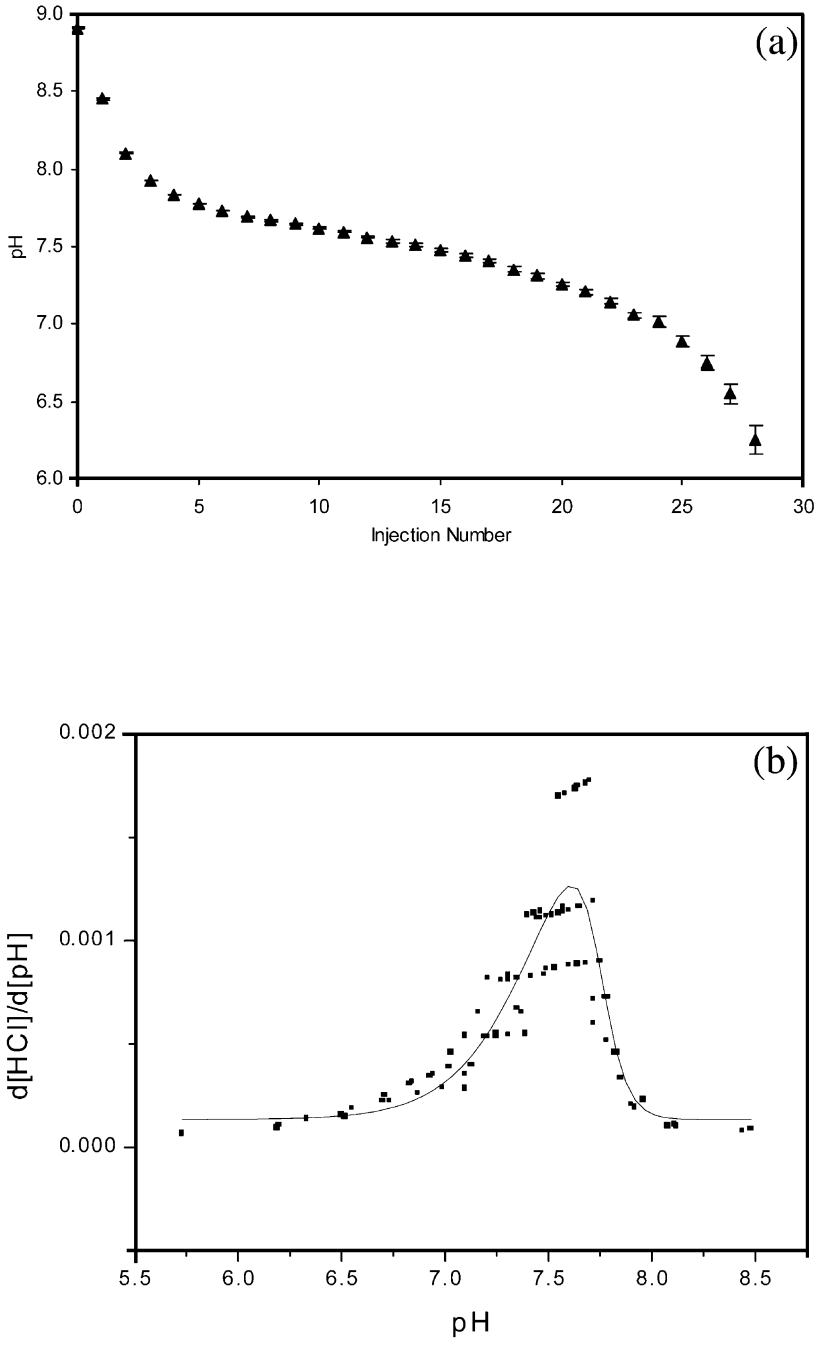


Fig. 3. (a) Acid titrations using HCl of 1:1 DOTAP/DOPE w/w liposomes at 25.0 °C and $I=0.010$. Data points and error bars represent the mean pH and standard error of the mean from three replicates. (b) Buffer capacity (d[HCl]/dpH) vs. pH from acid titrations of 1:1 DOTAP/DOPE liposomes at 25.0 °C and $I=0.010$. The line represents the best fit of an asymmetric double sigmoidal peak function through the combined data points of three titrations of liposomes with HCl.

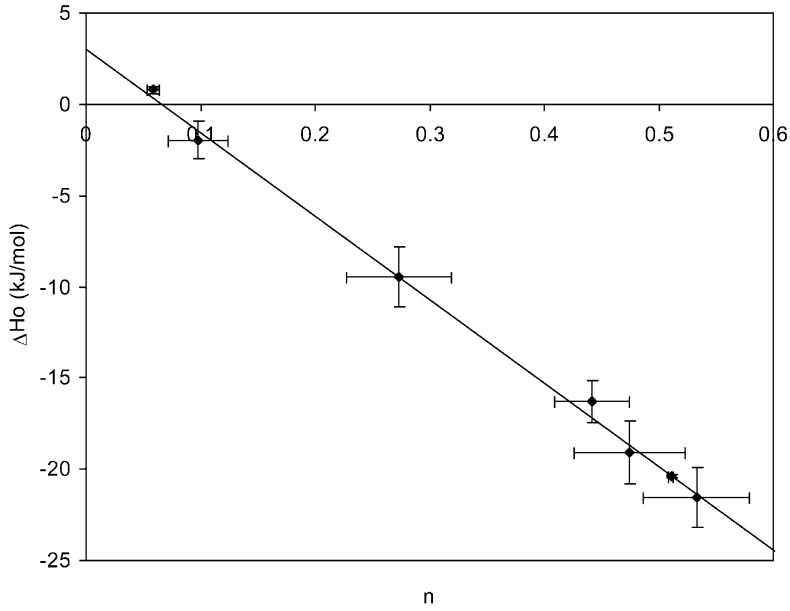


Fig. 4. Dependency of the buffer-corrected enthalpy of binding (ΔH_o) on the degree of DOPE protonation (n) derived from ITC titrations of DOTAP/DOPE (1:1 w/w) liposomes into DNA within the pH range of 6.50–8.50. The line through the data points represents a linear best fit to the data.

(7.7 ± 0.1). The pK_a of DOPE in the complex could not be obtained by pH titration due to colloidal instability of the complexes in unbuffered solutions, even at low cationic lipid-to-DNA charge ratios.

To resolve the separate enthalpic contributions from binding and protonation upon interaction of DOTAP/DOPE 1:1 liposomes with DNA, the variation of the buffer-corrected binding enthalpy (ΔH_o) with pH was analyzed. ΔH_o is actually a composite function of three enthalpic parameters representing the binding and protonation processes: ΔH_{int}—the intrinsic enthalpy of binding (the enthalpy of binding of the deprotonated liposome to DNA in the absence of proton linkage); ΔH_p^f—the protonation enthalpy of the free lipid and $\delta\Delta H_p$—the change in protonation enthalpy between the free and bound states of the lipid, as defined in Eq. (5) below [16]:

$$\Delta H_o = \Delta H_{int} + n(\Delta H_p^f) + H^c(\delta\Delta H_p) \quad (5)$$

The existence of a common intersection point of all the best fit lines in Fig. 1 (with the exception of pH 7.70) strongly suggests that the value of $\delta\Delta H_p$ is close to zero [16]. According to Eq. (5), if $\delta\Delta H_p$ is negligible, a linear relationship would exist between ΔH_o and n, with a slope of ΔH_p^f and a y-intercept of ΔH_{int}. This relationship implies that any change in n with pH produces a proportional change in ΔH_o, with the proportionality constant being the slope or protonation enthalpy of DOPE (ΔH_p^f). As shown in Fig. 4, an excellent linear relationship between ΔH_o and n was obtained ($R = -0.9994$), with a slope of -45.0 ± 0.7 kJ/mol (ΔH_p^f) and a y-intercept of $+2.8 \pm 0.3$ kJ/mol (ΔH_{int}). These values indicate that the intrinsic binding of DOTAP/DOPE 1:1 liposomes to DNA (in the deprotonated state and in the absence of proton linkage) is endothermic while the protonation of DOPE in the free and bound states is exothermic. The protonation enthalpy of DOPE obtained from the proton linkage model (-45.0 ± 0.7 kJ/mol) is in excellent agreement with reported values of the protonation enthalpy of primary amines: -46 ± 8 kJ/mol (-11 ± 2 kcal/mol) [21–23].

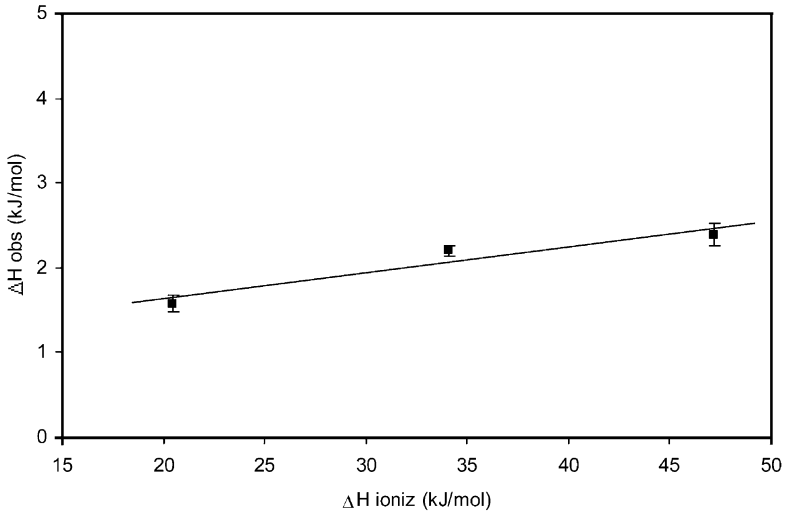


Fig. 5. Observed enthalpy of binding (ΔH_{obs}) determined with ITC vs. buffer ionization enthalpy (ΔH_b^{ioniz}) of DOTAP/DOPE (1:1) liposomes (7.44 mM cationic lipid) into plasmid DNA (1.23 mM) at pH = 7.40, 25.0 °C and $I = 0.150$. The line through the data represents the best fit from a linear least squares analysis.

To determine the binding enthalpy of DOTAP/DOPE 1:1 liposomes to DNA in the fully protonated state, an ITC titration of DOTAP:DOPE 1:1 liposomes into DNA was performed at pH 5.50 in the presence of acetate buffer ($\Delta H_b^{ioniz} \sim 0$) [24], a condition under which DOPE is fully protonated and the contribution of buffer ionization is minimal. An apparent endothermic enthalpy of $+1.8 \pm 0.2$ kJ/mol was obtained.

In the studies described above, the protonation of DOPE in DOTAP/DOPE 1:1 liposomes upon interaction with DNA were conducted under conditions of low ionic strength ($I = 0.010$). In order to determine if proton-linked binding exists at physiological ionic strength, titrations of DOTAP/DOPE 1:1 liposomes into DNA were repeated at pH 7.40 at an ionic strength of 0.150 in the presence of three buffers of different ionization enthalpy. A plot of ΔH_{obs} vs. ΔH_b^{ioniz} from these titrations is shown in Fig. 5. All observed enthalpies of binding were endothermic and resulted in a slope of 0.03 ± 0.01, suggesting the absence of proton linkage under physiological ionic strength. Furthermore, the buffer corrected enthalpy of binding under these conditions (1.0 ± 0.4 kJ/mol) was close to the value obtained at pH 5.50 in acetate buffer at low ionic strength (1.8 ± 0.2 kJ/mol), where the lipid was also in the fully protonated state and the extent of proton linkage was minimal.

4. Discussion

The goal of the current investigation is to determine if lipids that are prepared in the presence and absence of DOPE differ in their energetics and mechanism of binding to DNA. To accomplish this, the individual processes of binding and protonation that occur upon binding of DOTAP/DOPE to DNA must be separated.

A suitable procedure to separate the two processes of protonation and binding is to construct a thermodynamic square, as shown in Scheme 1. In this diagram, the binding equilibria of lipid in either the deprotonated or protonated states to DNA are represented in the horizontal planes and with association constants K_1 and K_2, respectively. The protonation of DOPE in the free and bound states are represented by the vertical sides of the square and are defined with protonation constants K_p^f and K_p^c, respectively. Employing the terminology adopted by Baker [16], the binding of lipid in the deprotonated form to DNA is regarded as the

intrinsic binding process, and the heat of this interaction is defined as the intrinsic binding enthalpy (ΔH_{int}). Furthermore, the enthalpy of protonation of DOPE in the free liposome is represented by the quantity ΔH_p^f, and the term $\delta\Delta H_p$ is a factor to account for any difference in protonation enthalpy of DOPE in the complex relative to the free state.

As described in our previous report [7], the occurrence of aggregation near charge neutrality during an ITC titration limits the binding process and renders the association constant and corresponding changes in binding free energy and entropy unavailable. Thus, with respect to the thermodynamic square, the quantities K_1 and K_2 remain unknown. Because these affinity constants are unavailable, a complete thermodynamic analysis of the binding equilibria of DOTAP/DOPE 1:1 to DNA cannot be achieved and the comparison of the energetics of binding must be limited to changes in enthalpy.

A complete thermodynamic description of the protonation events, however, can be obtained using the proton linkage model of Baker et al. [16]. From the definition of free energy, $\Delta G_p^c = -RT \ln K_p^c$ and $\Delta G_p^f = -RT \ln K_p^f$, the free energies of protonation in the complex and the free liposome are calculated as $\Delta G_p^c = -49.5 \pm 0.5$ kJ/mol and $\Delta G_p^f = -43.7 \pm 0.3$ kJ/mol. Since $\delta(\Delta H_p) \sim 0$, ΔH_p^c and ΔH_p^f are both -45.0 ± 0.7 kJ/mol. Therefore, the more favorable free energy of protonation in the complex relative to the free liposome results entirely from differences in the entropy of protonation between these two states. Entropy-driven ionization processes are common and may reflect differences in the entropy of ion solvation due to the electrorestriction of water [25]. The entropy changes for each protonation process can be calculated explicitly using the relationships $T\Delta S_p^f = \Delta H_p^f - \Delta G_p^f$ and $T\Delta S_p^c = \Delta H_p^c - \Delta G_p^c$, from which one obtains $T\Delta S_p^f = -1.3 \pm 1.1$ kJ/mol and $T\Delta S_p^c = +4.4 \pm 1.2$ kJ/mol. The protonation of DOPE in the free liposome is therefore entropically unfavorable ($T\Delta S_p^f < 0$) because protonation increases the cationic charge density of the liposome, which would require the accumulation of bound water and/or counterions at the lipid–water interface to hydrate or neutralize the cationic charge. In contrast, protonation of DOPE in the complex is entropically favorable ($T\Delta S_p^c > 0$) because protonation drives the complex towards charge neutrality, which would release bound water and/or counterions from the lipid–DNA interface.

The binding enthalpies of the deprotonated and protonated forms of lipid to DNA (ΔH_{int} and ΔH_2, respectively) are similar but should be identical [16]. There may be several reasons for this discrepancy. The headgroup conformation (especially the orientation of the P–N dipole of DOPE) and hydration state of the lipid bilayer is known to be sensitive to pH. These differences in lipid conformation and/or hydration may result in slightly different intrinsic enthalpies of binding at either extreme of pH. Alternatively, the use of ITC data that does not extend to high enough pH values may have resulted in some uncertainty in the estimation of ΔH_{int}.

The endothermic binding enthalpies of DOTAP/DOPE 1:1 to DNA observed when protonation is absent are consistent with an entropy-driven interaction and also support an electrostatic mechanism of binding. The binding enthalpies of DOTAP/DOPE 1:1 to DNA obtained at low pH in acetate buffer and at $I = 0.15$ at pH 7.40 (shown in Table 1) are approximately equivalent and reflect the binding enthalpy of the liposome in the protonated state to DNA. The similarity between these two measurements obtained at different ionic strengths also confirms that an electrostatic interaction exists between DOTAP/DOPE 1:1 liposomes and DNA. A minimal effect of ionic strength on the enthalpy of binding is consistent with electrostatic interactions because salt-dependent changes in the free energy of binding are manifested as alterations in the entropy of binding [26]. These two measurements of the binding enthalpy of DOTAP/DOPE 1:1 in the protonated state to DNA are also similar to the buffer-corrected binding enthalpy of 1:1 DOTAP/DOPC to DNA at low ionic strength (Table 1). This suggests that any difference in lipid surface curvature and/or hydration that exists between DOTAP/DOPE 1:1 and DOTAP/DOPC 1:1 liposomes and in their complexes with DNA, is not reflected in the enthalpy of complex formation. The existence of ion-dipole, van der Waals and hydrogen-bonding interactions may also be

Table 1
Binding enthalpies of cationic lipid mixtures to plasmid DNA at 25C using ITC

Lipid	ΔH (kJ/mol) (S.E.M)	Condition
DOTAP (ΔH_o)	7.0 (1.7)	$I=0.01$, pH = 7.40[a]
DDAB (ΔH_o)	11.6 (1.7)	$I=0.01$, pH = 7.40[a]
DOTAP/DOPE 1:1 (ΔH_{int})	2.8 (0.3)	$I=0.01$
DOTAP/DOPE 1:1 (ΔH_2)	1.8 (0.2)	$I=0.01$, pH = 5.50, $\Delta H_b^{ioniz}=0$
DOTAP/DOPE 1:1 (ΔH_o)	1.0 (0.4)	$I=0.150$, pH = 7.40
DOTAP/DOPC 1:1 (ΔH_o)	1.4 (0.2)	$I=0.01$, pH = 7.40[a]

[a] from ref. [7].

possible contributors to these interactions. This thermodynamic analysis, however, suggests that such possibilities are less likely. Hydrogen-bonding interactions tend to be strongly exothermic [27] while ion-dipole interactions typically possess an endothermic enthalpy much larger than that detected here ($\sim$16 kJ/mol) [28]. Van der Waals interactions are most frequently characterized by slightly exothermic enthalpies as well [27]. Thus, the slightly endothermic intrinsic binding enthalpy of DOTAP/DOPE to DNA detected here is most consistent with a primarily electrostatic contribution. Nevertheless, we cannot rigorously exclude minor contributions from these other types of interaction.

Overall, these measurements of the intrinsic binding enthalpy of equimolar DOTAP/DOPE to DNA are consistent with previous measurements which did not account for the protonation of DOPE [4], possibly because the deprotonation of TRIS buffer (+47.4 kJ/mol) [24] would offset the heat of DOPE protonation. A comparison of the buffer-corrected binding enthalpies of DOTAP, DDAB, DOTAP/DOPC 1:1 and the intrinsic binding enthalpy of DOTAP/DOPE 1:1 to DNA (Table 1) reveals that the largest endothermic binding enthalpies are observed with cationic lipids alone. This may reflect a slightly greater enthalpic penalty required to dehydrate a pure cationic lipid interface compared to a DOTAP/DOPE 1:1 or DOTAP/DOPC 1:1 surface, because the pure cationic lipid possesses twice the charge density of the lipid mixtures containing DOPE or DOPC.

These calorimetric findings are quite complementary to previous spectroscopic measurements that were made of the interfacial electrostatic properties of DOTAP/DOPE liposomes [29–31]. These studies monitored the pH-dependent dissociation of the fluorophore 4-heptadecyl-7-hydroxycoumarin (HC) in the lipid bilayer. The dissociation of DOPE was detected at bulk pH values above 7.9, which is consistent with the pK_a^f value of DOPE of 7.7 obtained in this present report.

In conclusion, this study has revealed an intrinsic endothermic binding process when DOTAP/DOPE 1:1 liposomes complex with DNA. This suggests that binding occurs by an electrostatic mechanism similar to that observed with pure cationic lipids. It is unknown, however, whether the enthalpically favorable protonation equilibria of DOPE produces an increase in affinity relative to that of DOTAP alone. The affinity constant of these lipids for DNA may prove to be the most important physical parameter for a mechanistic comparison of these lipid mixtures. Unfortunately, it has so far proved to be the most difficult parameter to obtain unambiguously. At physiological ionic strength, complex formation between 1:1 DOTAP:DOPE and DNA proceeds in the absence of proton linkage with an endothermic binding enthalpy. The favorable enthalpy of protonation which is observed at low ionic strength would therefore not exist under biological transfection conditions. This suggests that the affinity of these lipids for DNA at moderate ionic strength will be determined by the relative magnitudes of the entropy change upon complex formation.

References

[1] M. Pierce, C. Raman, B. Nall, Isothermal titration calorimetry of protein–protein interactions, Meth. Enzymol. 19 (1999) 213–221.

[2] J. Seelig, Titration calorimetry of lipid–peptide interactions, Biochim. Biophys. Acta. 1331 (1997) 103–116.
[3] K. Breslauer, E. Freire, M. Straume, Calorimetry: a tool for DNA and ligand-DNA studies, Meth. Enzymol. 211 (1992) 533–567.
[4] P. Baerreleiro, G. Olofsson, P. Alexandridis, Interaction of DNA with cationic vesicles: a calorimetric study, J. Phys. Chem. B 104 (2000) 7795–7802.
[5] V. Pector, J. Backmann, D. Maes, M. Vandenbranden, J.-M. Ruysschaert, Biophysical and Structural Properties of DNA-di C14-amidine Complexes, J. Biol. Chem. 275 (2000) 29533–29538.
[6] E. Pozharski, R. MacDonald, Thermodynamics of cationic lipid-DNA complex formation as studied by isothermal titration calorimetry, Biophys J 83, (2002).
[7] B. Lobo, A. Davis, G. Koe, J. Smith, C. Middaugh, Isothermal titration calorimetric analysis of the interaction between cationic lipids and plasmid DNA, Arch. Biochem. Biophys. 386 (2001) 95–105.
[8] M. Kennedy, E. Pozharski, V. Rakhmanova, R. MacDonald, Factors governing the assembly of cationic phospholipid–DNA complexes, Biophys. J. 78 (2000) 1620–1633.
[9] T. Stegmann, J. Legendre, Gene transfer mediated by cationic lipids: lack of a correlation between lipid mixing and transfection, Biochim. Biophys. Acta. 1325 (1997) 71–79.
[10] M. Ferrari, D. Rusalov, J. Enas, C. Wheeler, Trends in lipoplex physical properties dependent on cationic lipid structure, vehicle and complexation procedure do not correlate with biological activity, Nucl. Acids Res. 29 (2001) 1539–1548.
[11] J. Felgner, R. Kukmar, C. Sridhar, C. Wheeler, Y. Tsai, R. Border, et al., Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.
[12] S. Tatulian, in: G. Cevc (Ed.), Phospholipid Handbook, Ionization and ion binding, Marcell Dekker, New York, 1993, pp. 511–553.
[13] J. Wyman, Binding linkage and reciprocal effects in hemoglobin: a second look, Adv. Protein Chem. 19 (1964) 223–286.
[14] J. Wyman, S.J. Gill, Binding and linkage: functional chemistry of biological macromolecules, University Science Books, Mill Valley, CA, 1990.
[15] D. Xie, S. Gulnik, L. Collins, E. Gustchina, L. Suvorov, J. Erickson, Dissection of the pH dependence of inhibitor binding energetics for an aspartic protease:direct measurement of the protonation states of the catalytic aspartic acid residues, Biochemistry 36 (1997) 16166–16172.
[16] B. Baker, K. Murphy, Evaluation of linked protonation effects in protein binding reactions using isothermal titration calorimetry, Biophys. J. 71 (1996) 2049–2055.
[17] M. Crnogorac, G. Ullmann, N. Kostic, Effects of pH on protien association: modification of the proton-linkage model and experimental verification of the modified model in the case of cytochrome *c* and plastocyanin, J. Am. Chem. Soc. 123 (2001) 10789–10798.
[18] J. Bradshaw, G. Waksman, Calorimetric investigation of proton linkage by monitoring both the enthalpy and association constant of binding: application to the interaction of the Src SH2 domain with a high-affinity tyrosyl phosphopeptide, Biochemistry 37 (1998) 15400–15407.
[19] D.W. Bolen, J.L. Slightom, Calorimetric determination of linkage effects involving an acyl-enzyme intermediate, Biophys. Chem. 37 (1990) 303–312.
[20] A. von Harpe, H. Petersen, Y. Li, T. Kissel, Characterization of commercially available and synthesized polyethylenimines for gene delivery, J. Control. Release 69 (2000) 309–322.
[21] G. Beschiaschvili, J. Seelig, Peptide binding to lipid bilayers. Non-classical hydrophobic effect and membrane-induced p*K* Shifts, Biochemistry 31 (1992) 10044–10053.
[22] B. Martin, Introduction to Biophysical Chemistry, McGraw-Hill, New York, 1964.
[23] J. Christensen, L. Hansen, R. Izatt, Handbook of proton ionization heats and related thermodynamic quantities, John Wiley and Sons, New York, 1976.
[24] A. Cooper, C. Johnson, in: B. Mulloy, C. Jones, A.H. Thomas (Eds.), Microscopy, Optical Spectroscopy, and Macroscopic Techniques, Introduction to Microcalorimetry and Biomolecular Energetics, Humana Press Inc, Totowa, NJ, 1994.
[25] C. Mortimer, Reaction Heats and Bond Strengths, Pergamon Press, Reading, MA, 1962.
[26] V. Bloomfield, D. Crothers, I. Tinoco, Nucleic Acids: Structures, Properties and Functions, University Science Books, Sausalito, CA, 2000.
[27] P.D. Ross, S. Subramanian, Thermodynamics of protein association reactions: forces contributing to stability, Biochemistry 20 (1981) 3096–3102.
[28] S.E. McGraw, S. Lindenbaum, The use of microcalorimetry to measure thermodynamic parameters of the binding of ligands to insulin, Pharm. Res. 7 (1990) 606–611.
[29] N.J. Zuidam, Y. Barenholz, Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin, Biochim. Biophys. Acta 1329 (1997) 211–222.
[30] N.J. Zuidam, Y. Barenholz, Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery, Biochim. Biophys. Acta 1368 (1998) 115–128.
[31] N.J. Zuidam, Y. Barenholz, Characterization of DNA-lipid complexes commonly used for gene delivery, Int. J. Pharm. 183 (1999) 43–46.