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Thermodynamics of Lipoplex Formation: Relationship between the Lipid Alkyl Tail Length and Thermodynamic Functions

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The formation of a cationic-lipid/DNA complex (lipoplex), where the lipids that have a quaternary amine headgroup and alkyl tails of various lengths are newly synthesized, was investigated by use of isothermal titration calorimetry (ITC) and small-angle X-ray scattering (SAXS). SAXS showed that all of the samples formed a lamellar structure with DNA sandwiched between the lipid bilayers. For the hexyl tail, ITC showed an exothermic response, while for the nonyl and dodecyl tails, it was endothermic. We propose a lipoplex formation model to explain both SAXS and ITC results.

Cationic-lipid/DNA polyion complexes are called lipoplexes and applied to gene delivery.[1] Lipoplex formation is governed by combination and balance of multiple interactions, and their quantitative understanding is essentially important to develop better DNA carriers.[2] Safinya et al. were the first to carry out small-angle X-ray scattering (SAXS) from lipoplexes and to report that the transfection efficiency is strongly related to their structures.[3] According to them, lipoplexes take three predominant phases: (a) the multilamellar phase where DNA are intercalated between lipid bilayers (L_α^C), (b) the inverted hexagonal phase with DNA encapsulated within monolayers tubes and arranged on a two-dimensional hexagonal lattice (H_{II}^C), and (c) the cationic lipids form rod-like micelles arranged on a hexagonal lattice with DNA inserted within the interstices with honeycomb symmetry (H_I^C). They showed that H_{II}^C exhibits the highest transfection among others and its superior efficiency has been attributed to the unstable nature of the encapsulated DNA and bound lipids.

Independently from their studies, our group reported that aromatic amine and amidine derivatives can be used as a transfection reagent with a better efficiency than commercial products.[4–7] We reported that there could be another hexagonal phase where the DNA/cationic ion pairs are included inside of the cylinder and the cylinder surface is covered with the rest of the unbound lipids (we denoted the hexagonally packed bilayers DNA-inclusion cylinder: H_b^C).[4] Our data showed that the aromatic amine and amidine derivatives did not take H_{II}^C at any composition and H_I^C gave better transfection efficiency than H_b^C.[3] MacDonald et al.[8–10] came to a different conclusion after study of their own lipids. Although different systems can take a different structure after mixing with DNA, it seems that the general principle has not been established to relate transfection and lipoplex structures. One of the reasons complicating this issue is most transfection lipids are made by adding neutral lipids such as DOPE (dioleoylphosphatidylethanolamine) to cationic lipids and thus segregation between these two lipids may take place after binding to DNA. For example, in the H_b^C cylinder, the outer surface of the cylinder of H_b^C might be covered by the neutral lipids, while the cationic ones bind to DNA. In this work, as a model system, we focus on one component cationic system.

According to previous studies, lipoplex formation is endothermic and thus the reaction is entropy-driven. Bloomfield et al.,[11] who studied alkylammonium derivatives with isothermal titration calorimetry (ITC), proposed that the lipid tails lay down parallel to DNA and the dehydration of DNA and the hydrophobic interactions between the tail-coated DNAs are reasons for the endothermic reaction. According to them the lipoplex formation occurs at any ionic strength. Zhu and Evans also observed a large endothermic heat upon the lipoplex formation but they come to a different conclusion.[12] They showed that, when the solution ionic strength (I) is smaller than the critical ionic strength (I_C), defined by the ionic strength provided CAC = CMC; the micelles can bind through ion-pair formation, where CAC and CMC are the critical micellar concentration to form micelles and lipoplexes, respectively. On the other hand, there is no binding occurring at $I > I_C$. The above-mentioned thermodynamic and structural studies do not always provide coherent molecular pictures in the lipoplex formation. Therefore, in this paper, we study our aromatic cationic lipids by combining ITC and SAXS to present consistent features for both measurements. We synthesized a series of aromatic cationic lipids bearing quaternary amine as a headgroup and alkyl chains of various lengths as a hydrophobic tail (Figures 1A and S1[16]). We denote the lipids bearing hexyl, nonyl, and dodecyl tail as QA6, QA9, and QA12.

Figure 1B plots the ζ-potential and the hydrodynamic radius (R_h) against N/P for QA12/DNA complex. Here, N/P is

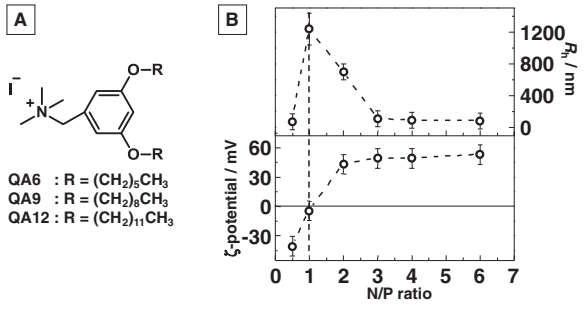

Figure 1. The chemical structure of the lipid used in this work (A) and N/P ratio dependence of the ζ-potential and the hydrodynamic radius determined with dynamic light scattering at [NaCl] = 50 mM.

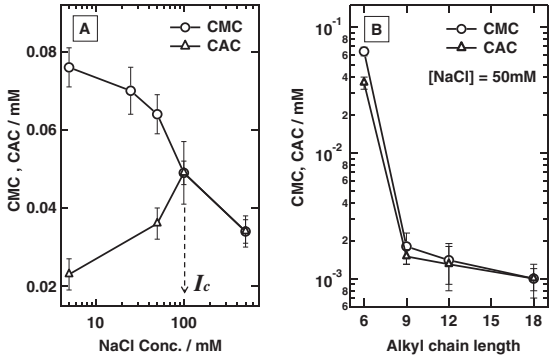

Figure 2. The salt concentration dependence of CMC and CAC for QA6 (A) and the alkyl tail length dependence of CMC and CAC.

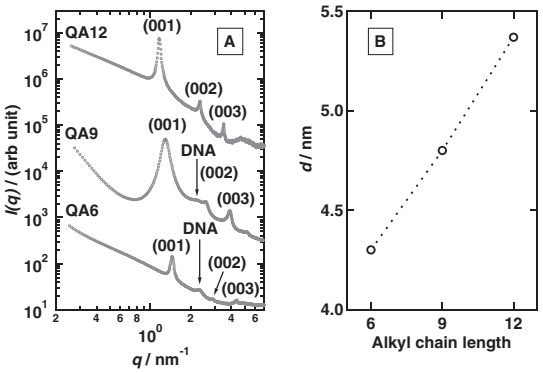

Figure 3. Comparison of SAXS profiles after being complexed with DNA, where the lamellar and inter-DNA diffraction are observed (A) and alkyl chain length dependence of d.

the molar ratio of the nitrogen in QA to the phosphate of DNA and represents the cation to anion ratio. At N/P = 1.0, potential became almost zero (i.e., isoelectric point) and R_h reached the maximum, which are commonly observed for other lipoplex formations. Similar results were obtained for the other QAs. Figure 2A plots CMC and CAC against I. In the range of $I < 100$ mM, CMC decreased with increase of I, while CAC increased with increase of I. These dependencies can be rationalized in that CMC is determined by the electrostatic repulsion between the heads and thus the shielding due to salts is favorable for the micellar formation, and that CAC is determined by how easily the ion-pair can occur and thus the salts act as a competitor for lipoplex formation. Above 100 mM, CAC merged with CMC. We confirmed with SAXS that the lipids bind to DNA even at $I > I_C$ (Figure S2).[16] This result did not agree with the conclusion of Zhu et al.[12] Figure 2B shows the tail length dependence of CMC and CAC. As reported by others, with increasing the tail length of n, both CMC and CAC decreased. For $n > 9$, all values were less than 2×10^{-3} mM and they were slightly decreased with increase of n, while $n = 6$, both were about 5–7 $\times 10^{-2}$ mM, about thirty times larger than others, indicating that the hydrophobic interaction of QA6 is much lower than the others.

The SAXS pattern of the lipoplexes at N/P = 1.0 and $I = 50$ mM is shown in Figure 3A, showing that the lipoplexes take lamellar structures and this feature is persistent for other salt concentrations (Figure S3).[16] There was another broad diffraction peak observed between the first and the second lamellae peaks for QA6 and QA9. This peak can be ascribed to diffraction of the sandwiched DNAs and thus it is evidence for $L_\alpha{}^C$ type lamella.[3] Figure 3B shows n dependence of the lamellar distance (d), showing that d increased linearly. The increments from $n = 6$ to 9 and from 9 to 12 were about 0.5 nm, which is almost equal to twice the fully stretched length of propane (i.e., increment of the carbon chain length). This fact confirms that all QA molecules form their lipoplex in the same manner; the only difference is the tail length. When we compare d with the sum of two QA molecular length and DNA diameter (Table 1), these two values are comparable, which is also consistent with the $L_\alpha{}^C$ model. Therefore, we can conclude that all QA takes $L_\alpha{}^C$ after mixing with DNA and there is no lying down of the alkyl chain occurring.

Table 1. Comparison of d and the molecular sizes

Alkyl chain length	d/nm	QA/nm	2 × QA + DNA (≈2 nm) /nm
6	4.3	1.2	4.4
9	4.8	1.5	5.0
12	5.37	1.7	5.4

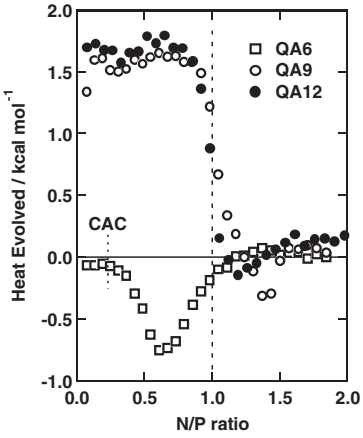

Figure 4. Comparison of ITC thermograms at 25 °C in [NaCl] = 50 mM.

Figure 4 summarizes ITC results when the QA solution (above CMC for QA9, QA12 and below CMC for QA6) was titrated with DNA solution, where the heat evolved is plotted against N/P. QA6 showed a bell-shaped exothermic profile, while others showed endothermic response similar to the previous studies. For QA6, the exothermic heat appeared when $C >$ CAC, where C is the lipid concentration. Below CAC, there was no interaction between QA6 and DNA, confirming the previous results.[12] By the composition where added DNA reached N/P = 1.0, the lipoplex formation was completed, indicating strong affinity to product formation.

The thermodynamics of lipoplex formation has been intensively studied by several groups,[11,13,14] which reveals that the thermodynamics reflects multiple processes, including ion-pair replacement, removal of DNA water, structural changes of

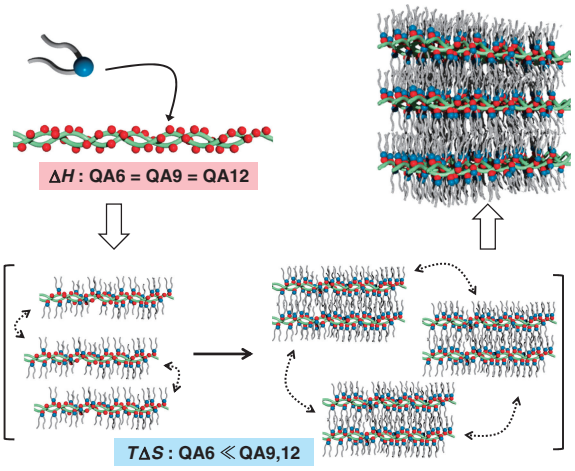

Figure 5. QA/DNA lipoplex formation model. At first, the negative charge of DNA binds to the positive QA heads (solid arrowed line). The primitive DNA/QA complexes are so unstable due to their hydrophobic tails facing water that they immediately aggregate and finally form the sandwiched lamellar structure (dotted arrowed line).

DNA as well as lipid micelles, and inclusion of DNA into the micelles, some of them occurring cooperatively. It is shown that its thermodynamics mainly involves (1) electrostatic interactions between lipid cation and DNA anion (ΔH), (2) hydrophobic interactions of alkyl tails ($T\Delta S$), and (3) dehydration of the DNA-bound waters (ΔH and $T\Delta S$). The third factor would be unchanged if the alkyl-chain lay-down does not happen and the head is the same. So we focused on the first and second factors. When the first factor is larger than second one ($\Delta H > T\Delta S$), the reaction is enthalpy-driven and we have an exothermic response. On the contrary, when the second factor is larger than first one ($T\Delta S > \Delta H$), the reaction is entropy-driven and we have an endothermic response. It is reasonable to assume that ΔH is almost constant if the headgroup consists of the same chemical structure, while ΔS increases with increase of the alkyl chain length.[11] Therefore, for the QA system, the difference between endo- and exothermal responses can be only due to the difference in ΔS. i.e., for QA6 $\Delta H > T\Delta S$ while for QA9 and QA12, $T\Delta S > \Delta H$. The large CMC for QA6 is also consistent with this argument, showing that its ΔS in the micellar formation is smaller than the others, because of its shorter tails.

All of the above discussion based on SAXS as well as this fact indicate that the hydrophobic interactions of the alkyl tails are the major factor to determine the lipoplex formation. If several lipid micelles attach on DNA surface and then the structures are changed so as to increase local concentration of the micelles, not all of the alkyl tails may be exposed to water to decrease ΔS. In fact, the morphological transition from spherical micelles to cylinders or lamellae does not cause large endothermic heat flows.[15] Therefore, we propose the following model for the lipoplex formation, as presented in Figure 5. Once DNA and QA micelles meet, the strong ionic interaction causes some QA molecules to pull from the micelle and to bind them to DNA. After the initial binding, the alkyl chains are facing toward water. This is a very unfavorable situation and thus, to cover the alkyl tails, more QA molecules cover DNA, and these primitive DNA/QA pairs stack with each other to form the sandwiched lamellar structure.

To sum up the present work, SAXS showed that all QA/DNA lipoplexes formed a L_{α}^{C} type structure which consists of the multilamellar phase where DNA is intercalated between lipid bilayers. On the other hand, ITC showed an exothermic response for $n = 6$, while it showed endothermic ones for $n = 9$ and 12. The lipoplex formation is governed by the hydrophobic interaction between the tail chains and we propose a model depicted in Figure 5.

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References and Notes

1 *Nonviral Vectors for Gene Therapy*, ed. by L. Huang, M.-C. Hung, E. Wagner, Academic Press, San Diego, **1999**.
2 P. C. Bell, M. Bergsma, I. P. Dolbnya, W. Bras, M. C. A. Stuart, A. E. Rowan, M. C. Feiters, J. B. F. N. Engberts, *J. Am. Chem. Soc.* **2003**, *125*, 1551.
3 J. O. Rädler, I. Koltover, T. Salditt, C. R. Safinya, *Science* **1997**, *275*, 810.
4 M. Sakuragi, S. Kusuki, E. Hamada, H. Masunaga, H. Ogawa, I. Akiba, K. Sakurai, *J. Phys.: Conf. Ser.* **2009**, *184*, 012008.
5 K. Koiwai, K. Tokuhisa, R. Karinaga, Y. Kudo, S. Kusuki, Y. Takeda, K. Sakurai, *Bioconjugate Chem.* **2005**, *16*, 1349.
6 T. Nishimura, H. Masunaga, H. Ogawa, I. Akiba, K. Sakurai, *Chem. Lett.* **2010**, *39*, 686.
7 T. Nishimura, T. Cho, A. M. Kelley, M. E. Powell, J. S. Fossey, S. D. Bull, T. D. James, H. Masunaga, I. Akiba, K. Sakurai, *Bull. Chem. Soc. Jpn.* **2010**, *83*, 1010.
8 R. Koynova, R. C. MacDonald, *Biochim. Biophys. Acta, Biomembr.* **2007**, *1768*, 2373.
9 R. Koynova, Y. S. Tarahovsky, L. Wang, R. C. MacDonald, *Biochim. Biophys. Acta, Biomembr.* **2007**, *1768*, 375.
10 R. Koynova, B. Tenchov, L. Wang, R. C. MacDonald, *Mol. Pharmaceutics* **2009**, *6*, 951.
11 D. Matulis, I. Rouzina, V. A. Bloomfield, *J. Am. Chem. Soc.* **2002**, *124*, 7331.
12 D.-M. Zhu, R. K. Evans, *Langmuir* **2006**, *22*, 3735.
13 M. M. Patel, T. J. Anchordoquy, *Biophys. J.* **2005**, *88*, 2089.
14 C. H. Spink, J. B. Chaires, *J. Am. Chem. Soc.* **1997**, *119*, 10920.
15 J. Zhang, H. Fan, D. A. Levorse, L. S. Crocker, *Langmuir* **2011**, *27*, 9473.
16 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

