Supramolecular Structures of Benzyl Amine Derivate/DNA Complexes Explored with Synchrotron Small Angle X-ray Scattering at SPring-8

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Abstract. Relationship between the transfection efficiency and the supramolecular structure of the lipoplexes consisting of DNA, a benzyl amine derivative (BA), and two neutral co-lipids (DOPE and DLPC) were examined. At a composition of BA:DOPE:DLPC = 1:2:1, where the highest efficiency was attained, SAXS showed that the micelles before adding DNA formed a hexagonally-packed cylinder and addition of DNA improved the packing order. This phenomenon may be interpreted by intercalation of DNA between the preformed micellar cylinders. At a composition of BA: DLPC=1:1, where the lowest efficiency was observed, the micelle adopted a spherical form and addition of DNA induce a structural transition from the sphere to a hexagonally packed cylinder, and then a lamellar form. Fitting with a multilayer model suggested that DNA was included into the hydrophobic layer of the complex. Comparison of these two compositions suggests that difference in the DNA location in the lipoplex is related to the transfection efficiency.

1. Introduction

The supermolecular complexes made from DNA and synthetic cationic lipids, called “lipoplex”, a coined term from the combination of “liposome” and “complex”, attracted a large amount of scientific and technological interest ever since the pioneering work done by Felgner et al [1], that cationic lipids can be used to transfer DNA into mammalian cells. One of the major reasons to invoke such attention is that they can replace viral vectors and thus possibly resolve safety issues inherent for them. Viral vectors are prepared by genetically modifying a highly infectious natural virus and the safety issues have been concerned in terms of immunity as well as possibility of genome-integration. Although suppliers do not disclose the chemical structure and ingredients, several types of cationic lipids are widely used as a transfection regent for in vitro studies in these days. However, they have still difficulties in extending in vivo use owing to low transfection and unknown mechanism of their expression.

Safinya et al [2]. are the first to investigate relation between the supramolecular structures of lipoplexes and their transfection efficiency with synchrotron small angle X-ray scattering (SAXS) and to show the efficiency is strongly related to lipoplex’s structures. According to them, there are two predominant phases, (a): multilamellar phase where DNA monolayers are intercalated between lipid bilayers (L_α^C) and (b): the inverted hexagonal phase with DNA encapsulated within cationic lipid monolayers tubes and arranged on a two-dimensional hexagonal lattice (H_{II}^C). It is believed that the later structure is more suitable for the transfection, because of the fact that inverted hexagonal structures are often observed in the region where biological membranes fuse [3,4].

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Independently from these previous studies, our group reported that aromatic amine and amidine compounds can be used as a transfection regent with a better efficiency and less toxic than commercial products [5]. Figure 1 presents the gene transfection efficiency for our benzyl amine (BA) system as a function of the composition, where the efficiency was evaluated with an established method called “luciferase assay” [6] which spectroscopically measures the amount of the light-producing protein expressed by foreign plasmid DNA (pDNA). The efficiency strongly depends on the composition: the highest one is achieved at BA: DOPE: DLPC = 1: 2: 1 (point B) and lowest ones are near the A point (BA: DOPE: DLPC = 1: 0: 1), where DOPE and DLPC are L-a-phosphatidyl ethanolamine dioleoyl and 1,2-dilauroyl-sn-glycero-3-phosphocholine, respectively. The coexisting DOPE and DLPC are considered to make BA to be compatible with water and to be less toxic for cells. However, there has been no information how these two colipids affect the micellar structure. In this paper we compares the supramolecular structures between A and B with SAXS.

![Figure 1](image.png)

**Figure 1.** Composition dependence of the luciferase activity for the BA/DOPE/DLPC system. N/P ratios were fixed at 3.3 for all samples.

2. Experiments and Fitting Equations

**Material:** DOPE and DLPC were purchased from Wako chemical and Cosmopolitan biology, respectively, and BA was synthesized with a reported method[5]. BA, DOPE and DLPC were dissolves in chloroform at a certain composition and freeze-dried in vacuum. The micelle solutions were prepared by adding a 50 mM NaCl solution to the freeze-dried lipid mixture and subsequently dispersed by ultra-sonication. Plasmid DNA coding pGL3 (bp = 5256) was amplified with E. coli competent cells and purified by using a QIAGEN HiSpeed Plasmid Maxi kit. The micellar solutions and pGL3 were mixed and stirred with a vortex for 40 s, about 20 minutes before the measurements. The micelle/DNA composition was expressed with N/P that is defined by the molar ratio of the nitrogen of BA to the phosphate of DNA.

**SAXS Set-Up:** SAXS measurements were carried out at 40B2 SPing-8 with a 1.5 m camera using a Rigaku imaging plate (30×30 cm, 3000×3000 pixels) as a detector. The wavelength of the beam was 1.0 or 0.72 Å, the exposure time was 5 min, and the relative X-ray intensity and the sample transmittance were determined with two ion-chambers located in front of and behind the sample. A bespoke chamber for SAXS measurements was constructed that enables a sample to be introduced in vacuum and scattering measured from it, as well as a vacuum-proof cell with a 0.10 mm quartz glass plate [7]. Combining these two instruments, we improved the S/N ratio by almost ten times in the entire q range and eliminated parasitic scattering, compared with conventional set-up, where q is the...
3. Results

3-1: Structural Transition at Point A

Figure 2a presents N/P dependence of SAXS at the composition A (the lowest transfection efficiency). The SAXS profile before adding DNA can be characterized as a multilayered spherical micelle and thus the data was fit with the corresponding model (a = 0 and k = 2 in Eq 1). Here, we adopted three fitting parameters: r1, r2, and \( \rho_c \) (the radii of the core and shell, and the electron density of the shell, respectively) under the condition that the core and solvent electron densities were fixed at \( \rho_c = 210 \text{ e/nm}^3 \) and \( \rho_s = 334 \text{ e/nm}^3 \), respectively. These densities were calculated from Eq 2 and can be treated as a constant with respect of other three parameters [10]. The obtained parameters are summarized in Table 1 and the best fit curve is shown as a solid line in Figure 2a, indicating that the model can well reproduce the experiment qualitatively. Based on these parameters, Figure 3a depicts the inner struc...

![Figure 2](image)

Figure 2. (a) N/P dependence of SAXS profile at the composition A (BA:DLPC=1:1). The micelle concentrations were 3.2 mM at N/P=3.3 - 26.4, 2.56 mM at N/P=2.64, and 1.07 mM at N/P=1.1. (b) N/P dependence of g (□) and 1-\( \chi \) (○).

The magnitude of the scattering vector. One of the advantages of this vacuum set-up is to enable to measure SAXS from the solutions with the same dilute conditions as those for bio assays.

Fitting Models and Electron Density Calculation:

The form factor for multilayer spherical (\( P_S \)) or cylindrical micelles (\( P_C \)) is given by Eq 1 [8].

\[
P_S(q) \propto \sum_i \frac{V_i(\rho_i - \rho_{sol}) F_i(qr_i)}{qr_i^2}
\]

Here, \( F_i \) is the first order Bessel function for cylinders or the first order spherical Bessel function for spheres, respectively. \( \rho_i, V_i, \) and \( r_i \) are the electron density of the \( i \)-th layer, the volume of the \( i \)-th layer, and the radius of the \( i \)-th layer, respectively. In Eq 1, \( \rho_{k+1} \) is the electron density of the solvent. When applied to the data, we assumed the Gaussian distribution for the radius. As the structural factor for the hexagonal packing [\( S_h(q) \)], we used the theoretical formula obtained by Hashimoto et al [9] that contains the paracrystalline distortion factor \( g \). The electron density of the solvent, core, and shell were calculated from the following equation;

\[
\rho = \sum_i \rho_{w_i} w_i
\]

Here, \( d \) is the density of the mixed solvent, \( \rho_{w_i} \), where \( w_i \) is the electron density per weight and the weight fraction for the \( i \) component, respectively.

The chemical structure of the lipids. The electron density of the shell (\( \rho_2 \)) is larger than the values estimated from the chemical structure, suggesting that the salt molecules are condensed on the head-groups.
When DNA was added to the micelle, a diffraction peak appeared at $q = 1.1 \text{ nm}^{-1}$ at $N/P = 26.4$, and, in the range of $N/P>4.4$, further addition caused to appear and increase other peaks at $q = 1.09, 1.92, 2.18$, and $2.89 \text{ nm}^{-1}$, as well as decrease of the convex curve which represents micellar scattering. These peak-positions satisfied the relation expected for hexagonally packed cylinders and the $N/P$ dependence indicates that all of the spherical micelles disappeared and only cylinders existed at $N/P = 4.4$. This fact implies that the conversion from the DNA-un-bound spheres to the DNA-bound cylinders was seemed to complete at $N/P = 4.4$. If this hexagonally packed cylinder takes $H^{C}_{II}$ (see the introduction), most of the lipids have to be bound to DNA unless the complex size is comparable with that of the cylinders [6]. We measured dynamic light scattering from the complex solution and analyzed the data with cumulant method [11]. The data showed that the size of the complex (c.a., 117 nm in diameter) was much larger than the cylinders, it can be concluded that there are almost 8 lipid molecules per one DNA phosphate anion from the relation of $BA : DOPE : DLPC = 1 : 0 : 1$ and $N/P = 4.4$. This lipid/DNA molar ratio indicates that $H^{C}_{II}$ requires one-to-one interaction between the phosphate and the cationic head. We fit the profile at $N/P = 4.4$ with $P_{c}(q) S_{h}(q)$, where $P_{c}(q)$ is the cylinder’s form factor and $S_{h}(q)$ is the hexagonal structural factor, assuming only hexagonally packed cylinders existing. The diameter of the cylinder is larger than that of the micelle and the cylinder contains a central core with the almost same electron density as that of the shell. This result suggests that once DNA formed an ion-pair with BA, the DNA must be included inside of the cylinder and the cylinder surface is covered with the rest of the free lipids, as illustrated in Figure 3b. This model can rationalize the lipid/DNA molar ratio more reasonably than $H^{C}_{II}$ model. Since the solutions at $26.4<N/P<4.4$ may be a mixture of the multilayered sphere given

![Figure 3](image-url)

**Figure 3.** (a) The core-shell sphere model used to fit the micellar data and its electron density profile, (b) The three layered cylinder model used to fit the profiles at $N/P = 4.4$ in Figure 2a, and (c) The core-shell cylinder model used to fit all the profiles in Figure 4. We used different values for the solvent density: 334 e/nm$^3$ for the micelle and 370 e/nm$^3$ for the lipoplex.

| Table 1. Structural Parameters for A or B |
|----------------------------------------|
| structure | $r_1$ [nm] | $r_2$ [nm] | $r_3$ [nm] | $\rho_1$ [e/nm$^3$] | $\rho_2$ [e/nm$^3$] | $\rho_3$ [e/nm$^3$] |
| A-micelles | sphere | 1.4 | 2.4 | - | 206 | 355 | - |
| A-lipoplexes | cylinder | 0.8 | 2.2 | 3.2 | 360 | 206 | 356 |
| B-micelles | cylinder | 2.2 | 1.6 | - | 220 | 470.5 | - |
| B-lipoplexes | cylinder | 2.3 | 1.6 | - | 220 | 470.5 | - |
by Figure 3a and the hexagonally packed cylinder given by Figure 3b, the scattering profiles was fit by assuming the following relation:

\[ I(q) = x_1 P_S(q) + x_2 P_C(q) S_h(q) \]  

(3)

Here, \( x_1 \) and \( x_2 \) are the relative composition ratios of the spherical and cylindrical micelles, respectively. The solid lines on the data points are resultant best fit curves, showing the Eq 3 well reproduces the experiments. Figure 2b plots \( X \) and \( g \) (the paracrystalline distortion factor) against N/P, showing that the structural order was increased upon addition of DNA. Here, \( X \) is the cylinder composition determined by \( 1-x_1/N_{micelle} \). \( x_1 \) is the relative composition of the spherical micelles before addition of DNA. The profiles at \( 3.3 > N/P \) in Figure 2a showed that further addition of DNA to the cylinders induced transition to a lamella structure. This transition was completed around \( N/P = 1.0 \) and further addition of DNA did not induce any changes. \( N/P = 1.0 \) means that one-to-one interaction is attained and thus all of the BA molecules are bound to DNA. Since DNA is included in the hydrophobic domain, DLPC molecules are seemed to face outside of the lamella as depicted in Figure 5.

3-2: Structural Transition at Point B

Figure 4a presents N/P dependence of SAXS at the composition B (the highest transfection efficiency). Before adding DNA, the profile had the peaks that can be characterized to a hexagonally packed cylinder with a convexed base line. This convex curve may be ascribed to an isolated scattering object (or a form factor). With adding DNA, the first diffraction peak becomes sharper and more intense and other higher-order peaks become obvious, while the convex curve becomes less obvious. These features can be interpreted by that the addition of DNA did not induce structural transitions such as the composition A, but to enhance the hexagonal ordering as well as reducing the amount of the isolated scattering object. The data was fit by assuming that the solution consists of both hexagonally-packed and isolated cylinders with the same structural parameters:

\[ I(q) = y P_C(q) + (1-y) P_C(q) S_h(q) \]  

(4)

The solid lines in the figure 4a represent the best fit and the parameters are summarized in Table 1. Figure 4b plots obtained \( g \) and \( 1-y \) values against N/P. As presented in Figure 4a, all of the profiles could be fit with the same cylinder with the inner structure depicted in Figure 5b, confirming that the addition of DNA did not induce structural transition but enhanced the packing of the cylinders.

![Diagram](image-url)
4. Concluding Remarks

Figure 5 depicted two models to explain N/P dependence of SAXS for the A and B points. At the point A, SAXS showed that drastic changes took place upon addition of DNA, from a spherical micelle to a hexagonally packed cylinder, then to lamella. During this process, DNA is considered to be incorporated into the hydrophobic domain. At the point B, the preceding cylinders are gathered by DNA and they are intercalated with each other, without undergoing major structural changes; presumably anionic DNA might neutralize and thus joint the cationic cylinders. With adding DNA, the inter-cylinder distance decreased from 7.16 nm to 6.85 nm, confirming the DNA’s role of an adhesion. Comparison of these two compositions suggests that difference in the DNA location in the complex is related to the transfection efficiency.

Figure 5. Schematic drawing of the morphological changes upon mixing (a) A-micelle and pDNA and (b) B-micelle and pDNA.

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References

[1] Felgner P L and Rhodes G 1991 Nature 349 351
[2] Radler J O, Koltouver I, Salditt T and Safinya C R 1997 Science 275 810
[3] Lin A J, Slack N L, Ahmad A, Koltover I, George C X, Samuel C E and Safinya C R 2000 Journal of Drug Targeting 8 13
[4] Huang L, Hung M C and Wagner E 1999 Nonviral Vectors for Gene Therapy (Academic Press London UK) p 91
[5] Sakurai K, Kusuki S, Hamada E and Takeda Y, JPA 2008-14384.
[6] Ewert K K, Samuel C E and Safinya C R 2008 DNA Interactions with Polymers and Surfactants
[7] Sakurai K, Synchrotron Small-Angle X-ray Scattering from Ultra-Dilute Solutions of pDNA/Cationic Lipid Complexes, X-ray and Neutron Techniques for Nano-Structural Research, SPring-8, Hyogo, Japan, 2008.
[8] Guinier A and Fournet G 1955 Small-Angle Scattering of X-rays (Inc.: New York)
[9] Hashimoto T, Kawamura T, Harada M and Tanaka H 1994 Macromolecules 27 3063
[10] Kawaguchi T, Hamanaka T, Kito Y and Machida H 1991 J. Phys. Chem. 95 (9) 3837
[11] Berne B J and Pecora R 2000 Dynamic Light Scattering with Applications to Chemistry, Biology, and Physics (Inc.:Dover Publications)