In Situ Fluorescence Microscope Measurements of the Phase Transfer Dynamics of Single DNA Molecular Ions with Dimethyldioctadecylammonium Chloride into Isooctane–1-Octanol Mixture

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The solvent extraction of single giant DNA molecular ions by \( \text{N}_2\text{N}_2\)-dimethyl-\( \text{N}_2\text{N}_2\)-dioctadecylammonium chloride was investigated under a fluorescence microscope with high resolution and their phase transfers from the water phase to the isooctane–1-octanol mixture (oil) phase were observed successfully. DNAs in the water phase were in the random-coil state, and those extracted in the oil phase were in the imperfect globule state, whereas those existing at the interface were almost in the globule state. From these conformation changes, the phase transfer mechanism of DNA at the interface was proposed.

1. Introduction

Deoxyribonucleic acid (DNA) is one of the essential biological macromolecules and it controls the lives of all creatures. DNAs have a large number of phosphate groups and thus they are polyanions in the neutral pH region. Natural double-stranded DNAs in aqueous solutions are in the random-coil state, being like long and thin threads, due to the intramolecular electrostatic repulsion. When hydrophobic polymers, cationic surfactants, or multivalent cations are added in the aqueous solutions, DNAs show a conformational change to the globule state, being like condensed threads [1-5], due to unfavorable solvation or reduced intramolecular repulsion. Like this, the conformation of single DNAs reflects their states in solutions and they can be observed only by optical microscope with high resolution [1-5]. From this restriction, almost all of the observations of the conformation of single DNAs have been carried out in one-phase systems, especially in aqueous solutions.

The analysis of the human genome has already finished, and recently genetic medicines and criminal investigations using human genes are increasing. Also, genetic engineering using other animals or creatures is often carried out. In these techniques, the sequence of various DNAs are analyzed and DNAs of various lengths are separated. The separation of shorter DNAs (less than about 10,000 base pairs (bp)) is commonly carried out by gel electrophoresis using a homogeneous electric field. However, longer DNAs (more than about 10,000 bp) are hard to separate by gel electrophoresis because such longer DNAs can barely migrate in a crowded gel network and thus only a slight difference in the mobility is generated. One of the effective separation methods for longer DNAs is pulse-field electrophoresis, but it takes a long time and its operation is complicated [6]. Therefore, other simple and easy separation methods for longer DNAs are required.
Solvent extraction is one of the powerful separation methods for hydrophobic compounds. As for the extraction of hydrophilic compounds, they are reacted with hydrophobic compounds or are surrounded by amphiphilic compounds, such as surfactants. Aqueous biological macromolecules, such as proteins or DNAs, are hydrophilic, and they cannot be converted to hydrophobic compounds easily. However, proteins can be extracted with amphiphilic compounds forming water-in-oil (W/O) microemulsion (reverse micelle) [7], such as Aerosol OT (sodium bis(2-ethylhexyl) sulfosuccinate). In this extraction system, proteins are considered to be dissolved in the W/O microemulsions in the oil phase. Goto et al. reported that DNAs were extracted into isooctane by \( N,N,N \)-dimethyl-\( N,N \)-dioctadecylammonium chloride (dC\(_{18}\)RCl) in the presence of 1-octanol, which was added as a co-surfactant [8]. Hydrated polyanionic DNA is surrounded by cationic dC\(_{18}\)R\(^+\) effectively, and the outside of DNA becomes hydrophobic with two long octadecyl-chains of dC\(_{18}\)R\(^+\), which stabilizes the DNAs in the oil phase. dC\(_{18}\)RCl is also known to form W/O microemulsion with a co-surfactant [9].

The oil/water interface is one of the keys to the extraction processes of proteins and DNAs because they are expected to be surrounded by the surfactants there. However, there have been no reports about the dynamics of their phase transfers through oil/water interfaces in solvent extraction systems, to the best of our knowledge.

We already examined the diffusion dynamics of single double-stranded DNA molecular ions near the toluene/water interface, and they were not adsorbed to the interface, as expected [10,11]. However, the addition of \( N,N,N \)-trioctylamine (tC\(_8\)N) into the toluene phase resulted in the adsorption of stretched DNA molecular ions to the interface [11]. tC\(_8\)N is protonated at the oil/water interface at pH = 8 [11], and the electrostatic attractive interaction between anionic DNAs and cationic protonated tC\(_8\)N (tC\(_8\)NH\(^+\)) causes the adsorption. However, tC\(_8\)NH\(^+\) cannot extract DNA.

The purpose of the present study is to investigate the phase transfer dynamics of single giant double-stranded DNA molecular ions from the water phase to the oil phase (isooctane–1-octanol mixture) in the extraction system with the cationic surfactant, dC\(_{18}\)RCl. We have also investigated the single double-stranded DNA conformations at the oil/water interface and in the oil and water phases with a fluorescence microscope with high resolution.

2. Experimental

2.1 Reagents and extraction system

T4GT7DNA (165,600 bp) and \( \lambda \) DNA (48,600 bp) were purchased from Nippon Gene and Toyobo, respectively. They were individually dissolved at a concentration of \( 4.0 \times 10^{-13} \) mol L\(^{-1} \) (1 L = 1 dm\(^3\) ) in an aqueous solution containing Tris (tris(hydroxymethyl)aminomethane) and EDTA (ethylenediaminetetraacetic acid) at 0.01 and 0.001 mol L\(^{-1} \), respectively. The pH was adjusted to 8.0 by the addition of dilute HCl. To visualize individual DNAs, a fluorescent dye, YO-PRO-1 (Molecular Probe), was added at \( 3.0 \times 10^{-7} \) mol L\(^{-1} \). This dye binds to DNA selectively, and the bound YO-PRO-1 shows stronger fluorescence (509 nm [12]). To reduce the photobleach of YO-PRO-1, 2-mercaptoethanol was added by 2% (by volume) to the DNA aqueous solution. An isooctane–1-octanol mixture (95:5 by volume) containing dC\(_{18}\)RCl at a concentration of 0.01 mol L\(^{-1} \) as an extractant was used as the oil phase. These experimental conditions corresponded to those that gave the best result in Goto’s study [8].
2.2 Microscope measurements

To observe the conformations of individual DNAs in solutions and their phase transfers through the interface, an inverted fluorescence microscope (IX51, Olympus) with a water-immersion microscope objective lens (UPlanApo60×W, numerical aperture (NA) 1.2; Olympus), an EMCCD camera (iXon, Andor) possessing ultrahigh sensitivity, a Xe excitation lamp (150 W), and a fluorescence mirror unit (U-MWIB2, Olympus) were mainly used. The objective lens shows high resolution in solutions due to its high NA value. The mirror unit consisted of an excitation filter (470 – 490 nm), a dichroic mirror (505 nm), and an emission filter (≥ 510 nm); the wavelength conditions suit the fluorescence of YO-PRO-1 bound to DNA.

The pre-equilibrium for the distribution of chemicals between the oil and water phases is important for the stable microscope observation of the oil/water interface. To do the pre-equilibrium, the oil and water phase solutions without DNAs and YO-PRO-1 were shaken by the common procedures. This, however, led to muddy phases with many bubbles and the phase separation was hard to attain by centrifugation. Instead of shaking, the oil and water phase solutions were mildly stirred with a magnetic stirrer for 24 h so as not to disturb the oil/water interface. After that, the water phase was filtrated with a membrane filter (pore size 0.2 µm, Minisart RC4, Sartorius) and mixed with DNA and YO-PRO-1 solutions. The resultant solution was used as the water phase for DNA extraction. The oil phase was also taken and filtrated to be used as the oil phase. Small dust disturbing the microscope observation can be removed by this filtration procedure effectively.

The two-phase extraction system was prepared in a homemade cylindrical two-phase microcell [13,14], as shown in Figure 1a. A glass tube (inner diameter 3 mm, height 15 mm) was adhered to a coverslip and it was placed on the microscope stage. The water phase solution (90 µL) was added into it. A glass capillary (inner diameter 1 mm, length 30 mm) was adhered to a metal bar, which was set to a manipulator (M-152, Narishige) placed on the microscope stage. The oil phase solution (5 µL) was put in the capillary by using the capillary action and its top-end was filled with Vaseline to prevent the vaporization of the organic solvents. Then, the capillary was inserted into the aqueous solution quietly and the formed interface was observed from the bottom. The position of the interface can be moved by the manipulator freely.

Since the oil/water interface is transparent and it has no scratches, one cannot focus on the interface. We reported a system that can detect the position (height) of oil/water interfaces precisely [9], and this system was also employed in the present study. Briefly, one end of an optical fiber was placed at a position conjugated to the image sensor of the CCD camera. White light was introduced from the other end of the fiber, and light emitted from the one end was reflected with a partially-

Figure 1. (a) Illustration of the used cylindrical two-phase microcell. (b) A clear image of the fiber end was observed when the interface was in focus. The image was created by the light reflected on the flat oil/water interface. Another microscope objective (10×) was used.
reflection mirror in the microscope and the light irradiated the interface. When a flat interface is in focus, a clear image of the fiber end is created by the reflected light, as shown in Figure 1b. If the interface is out of focus, only a blurred image of it is created. By changing the focus position, the conformations of individual DNAs near the interface were observed.

All measurements were carried out in a temperature-controlled room at 22–25°C.

3. Results and Discussion

3.1 Conformations of individual DNAs

The conformation change of DNAs from the random-coil state to the globule state is considered to be controlled by two factors mainly, that is, 1) a reduction of the intramolecular electrostatic repulsion and 2) an unfavorable interaction with surrounding molecules, which causes a decrease in the surface area of DNA [1-5].

T4GT7DNA and λDNA were not trapped at the oil/water interface and they passed through the interface from the water phase into the oil phase. Both DNAs showed translational and rotational diffusions in the oil and water phases, and at the oil/water interface.

Figures 2a–c show some examples of microscope fluorescence images of individual T4GT7DNAs at various positions. T4GT7DNAs have almost no convection in the oil and water phases, but slow convection occurred at the oil/water interface.

Figure 2a shows that T4GT7DNAs in the water phase are in the natural random-coil state. Their shapes were changed with time by the diffusions. dC18RCl is one of the highly hydrophobic organic salts, and thus almost all dC18RCl are distributed in the oil phase. This leads to the existence of a quite small amount of dC18R⁺ in the water phase. Therefore, there are no factors to induce the conformation change to the globule state in the water phase.

Smaller and brighter DNAs were observed at the oil/water interface, as shown in Figure 2b. Their shapes did not change by the diffusions and the convection, indicating that these DNAs were in the globule state. This conformation at the interface will be discussed in the following section 3.4.

Figure 2c shows the microscope fluorescence images of extracted DNAs in the oil phase. Compared with Figure 2a, the background is relatively high and the DNA images are not clear. The shape of the extracted
DNAs changed with time by the diffusions. If the DNAs are in the globule state, their shape is not changed by the diffusions. Figure 2c shows that the extracted DNAs are not in the globule state. The size of the extracted DNAs is smaller than that of the random-coiled DNAs in the water phase, and we call this state as imperfect globule state. The neutralization of the extracted DNAs by dC18R+ leads to an elimination of the intramolecular repulsion. The surface of the extracted DNAs becomes hydrophobic by surrounding dC18R+, and thus the interaction with oil phase solvent molecules is favorable. As a result, there is only one factor to induce the transition to the globule state, resulting in that the extracted DNAs are in the imperfect globule state.

In the λDNA case, there is almost no convection in the oil and water phases, but fast convection occurred at the oil/water interface.

Figures 3a and 3b show some examples of microscope fluorescence images of individual λDNAs. Random-coiled λDNAs smaller than T4GT7DNAs were observed in the water phase, as shown in Figure 3a, due to the shorter λDNA. In the oil phase, only darker images with a high background were obtained, as shown in Figures 3b. The DNA conformations cannot be recognized in Figure 3b, but smaller DNAs than the random-coiled DNAs (Figure 3a) were observed. The conformations of λDNAs at the oil/water interface cannot be recognized due to the fast convection, but they were not in the random-coil state (shown below).

In both (T4GT7DNA and λDNA) cases, the convection occurred only at the oil/water interface in spite of doing the pre-equilibrium. This would be a Marangoni convection; it could be caused by inhomogeneous interfacial concentrations of substances, such as dC18R+ and/or 1-octanol, which were consumed by the DNA extraction.

Figures 2 and 3 suggest that T4GT7DNA and λDNA show similar conformation changes during the phase transfer, that is, the random-coil state in the water phase, the globule state at the oil/water interface, and the imperfect globule state in the oil phase.

3.2 Diffusion rates of dC18RCl

To neutralize the negative charges of DNA at the oil/water interface, an equivalent amount of dC18RCl should be diffused toward the DNA in the oil phase and should be associated with DNA at the oil/water interface. The diffusion of dC18RCl would occur isotropically and thus we considered the diffusion of dC18RCl existing within a hemisphere as shown in Figure 4. The required amount of dC18RCl was calculated with the bp number of DNA. With the dC18RCl concentration (0.01 mol L⁻¹), the required volume was
obtained to be $5.5 \times 10^{-2}$ and $1.6 \times 10^{-2}$ $\mu m^3$ for T4GT7DNA and $\lambda$DNA, respectively. From these values, the radius of the hemisphere ($r_D$) was calculated to be 0.30 and 0.20 $\mu m$ for T4GT7DNA and $\lambda$DNA, respectively, by using the formula of hemisphere volume, $(2/3)\pi r_D^3$.

To obtain the diffusion time ($t_D$), the diffusion coefficient ($D$) of dC18RCl in the isooctane–1-octanol mixture is needed but its $D$ has not been reported. The following Einstein–Stokes equation is well used to estimate the $D$ value of a spherical solute in a solvent:

$$D = \frac{kT}{6\pi\eta r}$$

where $k$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the solvent, and $r$ is the radius of solute. If the shape of dC18RCl is approximated to be a sphere, its $r$ value can be calculated with its molar volume ($V_m$). However, $V_m$ of dC18RCl has also not been reported, and thus $V_m$ was estimated by using the additivity of molar volume [15]. $V_m$ values of some alkylammonium chlorides and alkylammonium bromides in benzene were reported, and it was clarified that the contribution of Cl$^-$ and Br$^-$ to $V_m$ was almost the same [14]. It was also obtained that the contribution of one CH$_2$ group of alkylammonium bromide was $17.3 \pm 0.10$ cm$^3$ mol$^{-1}$. $V_m$ values of tetrabutylammonium bromide ((CH$_3$)$_4$(CH$_2$)$_{12}N^+Br^-$) in benzene, CCl$_4$, and methanol were determined to be 290.0, 285.2, and 285.9 cm$^3$ mol$^{-1}$, respectively [17]. These values indicate that the solvents barely affect the $V_m$ of alkylammonium bromide. From these data, the $V_m$ value of dC18RCl ((CH$_3$)$_4$(CH$_2$)$_{12}N^+Cl^-$) was estimated to be 671 cm$^3$ mol$^{-1}$. By using this value, $r$ of dC18RCl was obtained to be 0.64 nm.

$\eta$ of the isooctane–1-octanol mixture was not reported, but $\eta$ values of octane–1-octanol mixtures exist [18]. $\eta$ values of octane and an octane–1-octanol mixture (94.7 : 5.3 by volume) are 0.518 and 0.554 mPa s, respectively. The addition of 1-octanol results in only a 7% increase in $\eta$, and thus $\eta$ of isooctane (0.473 mPa s at 25 $^\circ$C) was used for this estimation. As the result, $D$ of dC18RCl was estimated to be $7.2 \times 10^{-10}$ m$^2$ s$^{-1}$ with about a 10% error. The relation between a diffusion distance ($x$) and $t_D$ can be expressed as:

$$x^2 = 2Dt_D$$

By substituting $r_D$ for $x$, $t_D$ values were calculated to be 0.062 and 0.027 ms for T4GT7DNA and $\lambda$DNA, respectively, which are much shorter than the video interval (30 ms) of Figure 2.

dC18R$^+$ can be adsorbed at the oil/water interface, but these calculations suggest that the association of dC18R$^+$ with DNAs at the oil/water interface can be attained within one frame.  

### 3.3 Passing of DNA through the oil/water interface

The depth of focus ($d_s$) of a microscope system is expressed as:

$$d_s = \frac{n\lambda}{2(NA)}$$

where $n$ is the refractive index of medium and $\lambda$ is the wavelength of light. The $d_s$ value of the present microscope system was calculated to be 0.26 $\mu m$, respectively, with $n$ of isooctane (1.389 at 25 $^\circ$C [19]) and the maximum fluorescence wavelength of YO-PRO-1 bound to DNA (509 nm [12]).

If T4GT7DNA and $\lambda$DNA are fully stretched, their lengths are 57 and 17 $\mu m$, respectively, and their radius is 1 nm [10]. Their volumes were calculated to be $1.8 \times 10^{-4}$ and $5.3 \times 10^{-5}$ $\mu m^3$, respectively. When
the DNAs entered a spherical globule state, their radii \( r \) were 35 and 23 nm, respectively. \( D \) values of T4GT7DNA and \( \lambda \)DNA in the oil phase were calculated to be \( 1.3 \times 10^{-11} \) and \( 2.0 \times 10^{-11} \) m\(^2\) s\(^{-1}\), respectively, with eq. (1). By substituting \( d_z \) for \( x \), \( t_D \) values were calculated to be 2.5 and 1.7 ms for T4GT7DNA and \( \lambda \)DNA, respectively, which are much shorter than the video interval (30 ms) of Figure 2. These calculations mean that if the DNAs pass through the oil/water interface only by the translational diffusions, DNAs would exist at the interface within one frame.

Figure 5 shows the passing behaviors of individual \( \lambda \)DNAs at the oil/water interface. The focal plane was kept at the oil/water interface, and therefore DNAs existing at the interface are in focus and DNAs existing in the water or oil phases are out of focus. As mentioned above, we can see fast convection from the right-hand side to the left-hand side (about 80 \( \mu \)m s\(^{-1}\)) and each DNA image is stretched in the lateral direction by the effect of afterimage.

Under the present extraction conditions, the percent extraction of DNAs was reported to be 100% [8].

Figure 5. Continuous microscope fluorescence images of \( \lambda \)DNAs passing through the oil/water interface. The time interval is 30 ms.
This means that DNAs arriving at the oil/water interface from the water phase are extracted into the oil phase at about 100% possibility. Several λDNAs were observed at the oil/water interface in Figure 5. Three λDNAs, \(1, 2,\) and \(3\), were selected. As for \(1\), blurred images (out of focus) in the water phase were observed in Figure 5a–d, and then clear, small, and bright images (in focus) at the oil/water interface were obtained in Figure 5e–m. Finally, a blurred image (out of focus) in the oil phase was obtained in Figure 5n. In the \(2\) and \(3\) cases, similar behaviors were observed. A blurred image of \(2\) in Figure 5a, clear images of \(2\) in Figure 5b–k, and blurred images of \(2\) in Figure 5l–n were obtained. Blurred images of \(3\) in Figure 5a–d, clear images of \(3\) in Figure 5e–j, and blurred images of \(3\) in Figure 5k–n were obtained. This consideration indicated that \(1, 2,\) and \(3\) stayed at the oil/water interface for 0.27, 0.27, and 0.18 s (9, 9, and 6 frames), respectively.

Similar images were obtained for T4GT7DNAs near the oil/water interface. They stayed at the interface for a longer time than λDNAs, but long-time irradiation with the excitation light resulted in a photobleach of YO-PRO-1 [11]. Therefore, we cannot recognize the transfer of T4GT7DNAs into the oil phase from the microscope images at the interface.

The staying times of the DNAs at the oil/water interface are longer than the diffusion times of dC18RCl in the 3.2 section and those of the DNAs in this section, meaning that the other processes occurred at the interface.

### 3.4 Conformation changes of DNA at the oil/water interface

In our previous studies of DNAs at the pure toluene and water system, DNAs were not adsorbed at the toluene/water interface, and DNAs existing close to the toluene/water interfaces were in the random-coil state [10,11]. They showed a fast intramolecular motion and a fast rotational diffusion.

We speculated the following extraction mechanism of DNAs by dC18RCl. dC18R+ exists both in the oil phase and at the oil/water interface. When a DNA arrives from the water phase to the interface by the translational diffusion, a gradual association of dC18R+ and a gradual neutralization proceed at the interface, as shown in Figure 6a. This association is one of the ion-associations and hydration water molecules usually remain. In other words, there are water molecules between the DNA and dC18R+. On the other hand, DNAs existing at the interface show intramolecular and rotational motions. Therefore, after the association of dC18R+ (Figure 6a), this part will face the water side by the motions, leading to an unfavorable interaction with water molecules on this side, as shown in Figure 6b. As the result, the intramolecular repulsion of the

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![Figure 6](image-url)  
Figure 6. Illustration of the association of dC18R+ with DNA, rotation of DNA, and shrinking of DNA at the oil/water interface, and the extraction of DNA. DNA and dC18R+ would possess hydration water molecules around them. The broken line means hydrated DNA.
DNA decreases and the unfavorable solvation on the water side increases. These two factors would induce a shrinking of the DNA surface area (Figure 6b). Further association of dC18R⁺ causes a decrease in the intramolecular repulsion of the DNA and an increase in the unfavorable solvation (Figure 6c). Finally, the full association of dC18R⁺ is attained at the interface, resulting in the transition to the globule state and the DNA is extracted into the oil phase (Figure 6d). Such consideration about dynamic behaviors of DNA at the oil/water interface allows us to explain the conformation change of the DNAs at the interface. During the staying time of the DNAs shown in the 3.3 section, the shrinking and conformation change of DNAs would occur at the oil/water interface.

As mentioned above, N,N,N-trioctylammonium ions (tC₈RH⁺) cannot extract DNA [11]. At the interface, tC₈RH⁺’s bind only to the upper part (the oil side) of DNA [11]. Thus, favorable interactions with surrounding molecules are attained on both sides, and the electrostatic repulsion remains on the water side. As the result, the random-coiled and stretched DNAs were observed at the interface [11]. In this tC₈RH⁺ system, DNAs were also considered to show intramolecular and rotational motions, but tC₈RH⁺ may be released from DNA when this DNA part faced the water side.

The full surrounding of DNA with alkylammonium ions at the oil/water interface is one of the key processes to the extraction of DNA into the oil phase, and this is determined by several factors, for example, hydrophobicity of alkylammonium ions, the interaction of alkylammonium ions with DNA, the effect of co-surfactant.

4. Conclusion

The methodology for the observation of the phase transfer dynamics and conformational changes of single DNAs was successfully established in the present study for the first time. From the obtained conformations of individual DNAs, the reaction dynamics of DNAs with dC18R⁺ at the oil/water interface was proposed.

The phase transfers of T4GT7DNA and λDNA were considered to occur by a similar mechanism, and the difference in the phase transfer dynamics between them was not found out. The established methodology will enable us to find out a new extraction system that makes a difference in their phase transfer dynamics and attains their separation in the future.

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