PEG−PLL Block Copolymers Induce Reversible Large Discrete Coil−Globule Transition in a Single DNA Molecule through Cooperative Complex Formation

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Introduction

Polyion complexes (PIC) between DNA and cationic polymers (polyplexes) have been considered as a powerful tool to introduce foreign genes into living cells.[1−4] A series of PICs are ideal materials suitable not only for studies of transcriptional regulations of gene expressions but also for human gene therapy. The fundamental property required for polyplex-type gene vectors is a regulated compartmentalization of the complexed DNA: increased stability of DNA through complexation under physiological conditions, to achieve practically tolerable nuclease resistance, whereas smooth release of therapeutic DNA inside the target cells with proper timing and location to ensure a high transfection efficiency. Furthermore, the in vivo application of PICs is limited by their low solubility around the charge equivalent point. Recently, a major breakthrough has been made for improving the solubility of PIC under the charge neutralization condition by utilizing cationic block copolymers having hydrophilic segments, e.g., poly(ethylene glycol) (PEG).[5] PEG−PLL block copolymers composed of PEG and poly(L-lysine) (PLL) form water-soluble self-assembled structures (PIC micelles) with an oligonucleotide having a diameter of several tens of nanometers under charge neutralization conditions.[6] Most recently, the biological significance of PIC micelles of cationic block copolymers with an oligonucleotide or plasmid DNA was reported by several research groups.[7−12]

From another viewpoint, PICs of DNA with cationic polymers are often referred to as a model system of DNA condensation which is defined as the conformational change of DNA from an elongated coiled into a compact globular state. DNA condensation is considered to be a key process as the first step of gene therapeutics because DNA condensates have compact structures suitable for delivery and internalization into cells.[13,14] Although different kinds of chemicals have so far been investigated to clarify the physicochemical characteristics of DNA condensation, almost all PICs are not applicable for therapeutic purposes due to their low water solubility, cytotoxicity, etc. Here, PEG−PLL block copolymers that induce water-soluble DNA condensates can be received as a novel type of DNA condensing reagent that is applicable for actual clinical use as biocompatible polyplex vectors.[15]

Though both PEG and PLL are known as DNA condensing agents, their condensation mechanisms are quite different. While PLL binds to DNA through an electrostatic interaction, PEG acts through a crowding effect.[16] Thus, block copolymers of PEG−PLL might possess unique characters unexpected from individual homopolymers. The cooperative binding such as that observed during DNA condensation with cationic detergents[17] is also expected in our system. In this study, both the complexation and dissociation of PEG−PLL/DNA were investigated with emphases placed on the elucidation of the fundamental physicochemical properties required for polyplex vectors. The T4 phage DNA was used here as a model DNA to directly observe under fluorescence microscopy the conformational transition of a single DNA molecule in the presence of PEG−PLL. The long-term stability of PEG−PLL/DNA in an aqueous solution was confirmed from the comparison with PLL/DNA. Finally, DNA release was also demonstrated by the addition of the polyanion, dextran sulfate, into the PEG−PLL/DNA solution.

Experimental Section

Materials. The bacteriophage T4 dc DNA (166 kbp, contour length 57 μm) was purchased from Nippon Gene (Toyama, Japan) and used without further purification. The syntheses of the PEG−PLL block copolymer and PLL homopolymer were previously described.[5,18] Compositions and notations of PEG−PLL are listed in Table 1. The degree of polymerization of the PLL homopolymer was 22.5. A fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI),[19] and an antioxidant reagent, 2-mercaptoethanol (2-ME), were obtained from Wako Pure Chemicals (Osaka, Japan) and used as received for DNA observation under a fluorescence microscope. Dextran sulfate (M_w: ca. 500 kDa) for the polyanion exchange reaction was purchased from Wako Pure Chemicals.

Sample Preparations. DNA complexes with either the PEG−PLL or PLL were prepared by direct mixing of the polymer and fluorescence labeled DNA solutions in HEPES buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4). The mixing ratio r was defined as the charge ratio of the lysine residue to phosphate. Fluorescence microscopic observations were carried out at room temperature after a 24 h incubation. 2-ME was added to the complex solution just before the observation. Final concentrations of each component were as follows: 0.1 μg/mL T4 DNA, 0.6 μM DAPI, and 4% (v/v) 2-ME. It has been confirmed that the number of DAPI molecules per base pair is ca. 0.05 under this condition.[19] A DNA release induced by the polyanion exchange reaction was demonstrated only for PIC micelles of PEG-PLL(12-42)/DNA. Ten microliters of dextran sulfate was added to 100 μL of the PEG−PLL/DNA solutions. After a 2 h incubation, samples were tested under a fluorescence microscope.

Fluorescence Microscopy. For microscopic examination, each sample solution was placed between two microscope coverslips and sealed with nail enamel to prevent both convection and evaporation. The prepared specimen was illuminated by a band-passed mercury lamp at ca. 365 nm, and the fluorescence longer than 420 nm was monitored under

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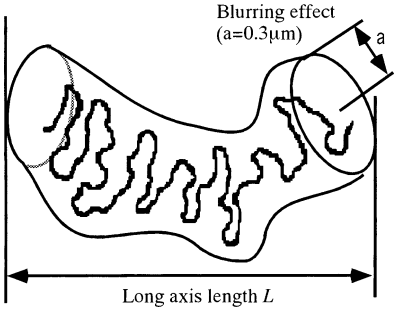

Figure 1. Schematic representation of the fluorescence image of the elongated coiled DNA. The long axis length L is defined as the longest distance of the image. As a microscopic view field of 43 × 43 μm^2 was transferred into an image file of 480 × 480 $pixel^2$, the raw data of L were evaluated by a spatial resolution of 90 nm and are given in the text without correction.

Table 1. Properties of the PEG−PLL Block Copolymers

PEG−PLL code name	M_w of PEG	av no. of lysine residues	mass per charge[a]	width of coexistence region
12-7	12 000	7	1590	$0.2 \le r \le 0.7$
12-19	12 000	19	802	$0.2 \le r \le 0.6$
12-28	12 000	28	574	$0.2 \le r \le 0.5$
12-42	12 000	42	485	$0.2 \le r \le 0.4$

[a] Determined by colloidal titration of lysine residues.

an inverted fluorescence microscope, Axiovert 135TV (Carl Zeiss, Germany), equipped with a ×100 oil-immersed objective and SIT tube (Hamamatsu, Japan). Fluorescence images of DNA complexes were recorded on videotape and analyzed using the image processor ARGUS-20 (Hamamatsu). As depicted in Figure 1, the fluorescence image of DNA appears to be a thick string due to both the diffraction limit of the optical microscope and the conformation of DNA.[20] To characterize the conformation of DNA, the apparent length of the long axis L that is defined as the longest distance in the outline of the DNA image was evaluated using the image processor.[20] In the absence of cationic polymers, an elongated coiled T4 DNA presented an intramolecular fluctuation and its long axis length L ranged around 2−5 μm. On the contrary, L for the compact globular DNA was observed to be smaller than 1 μm, including the blurring effect.[20] The actual size of globular DNA is much smaller than the apparent size. As expected from the Stokes−Einstein relationship, a marked Brownian motion was observed in the globular DNA. From the significant characteristics of DNA conformations, one can clearly distinguish the compact globule from the elongated coiled DNA. Therefore, values of L for coil and globule were independently evaluated. From the simple estimation with the DNA concentration, the number of DNA chains that should be observed on the monitor (43 × 43 μm^2) is approximately five.

Results and Discussion

Prior to examining the PEG−PLL/DNA system, the complexation of DNA with the PLL homopolymer was assessed using fluorescence microscopy. Figure 2a shows histograms of L at various mixing ratios r for randomly chosen 50 DNA molecules, and averages of L for both coiled and globular states are shown in Figure 2b. In the region $0.4 \le r < 0.7$, all of the DNA complexes take a globular state. A typical feature of the DNA conformational transition is the coexistence of the elongated coiled and compact globular states ($r = 0.2$ and 0.3). An intermediate state between the coil and globule which exhibits an intrachain phase segregated structure[21] was not detected. In the range of the higher mixing ratio, $r \ge 0.7$, the complexes disappeared from the medium due to the precipitation through an aggregation during the 24 h incubation at room temperature.

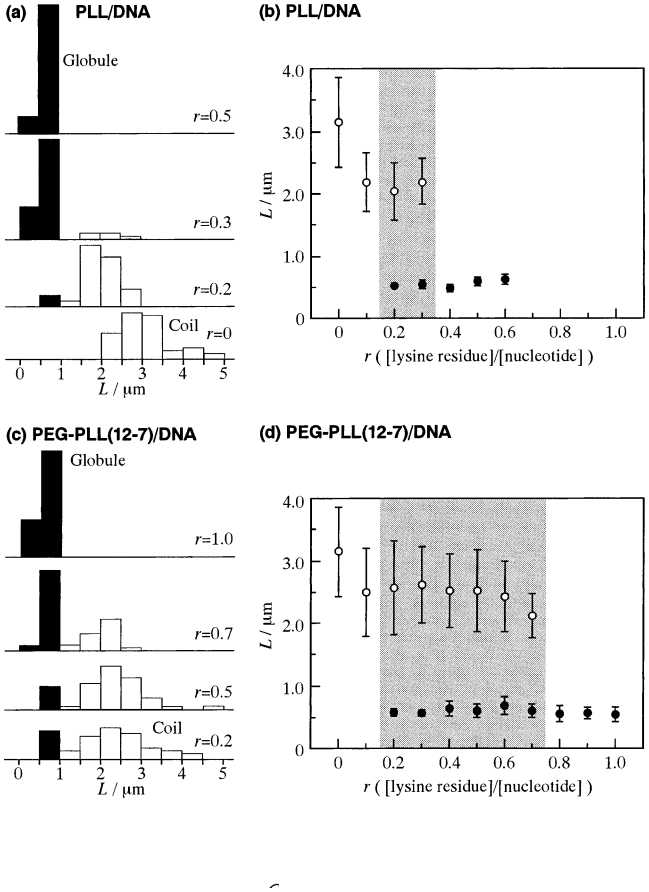

Figure 2. DNA conformational change in the complexation with either the PLL homopolymer or PEG−PLL(12-7) block copolymer. (a) and (c) are histograms of L at various mixing ratios r. Distributions for coil and globule are indicated by white and black pillars, respectively. (b) and (d) are phase diagrams for PLL/DNA and PEG−PLL/DNA. Average L values for the coil and globule are plotted by open and close circles, respectively. Both histograms and phase diagrams are independently constructed from the raw data of L.

Changing profiles of DNA size by the complexation with PEG−PLL were measured, and phase diagrams were compared with that of the PLL. Typical transition features are exemplified by the phase diagram for PEG−PLL(12-7)/DNA (Figure 2d). In this case, all the DNA chains underwent a compaction at $r \ge 0.8$, while no aggregation was observed. The number of globules observed inside the view field matched the predicted one, indicating that almost all the globular DNAs retained the so-called primary condensate including single DNA chain inside. The wide coexistence region ($0.2 < r < 0.7$) was confirmed during the formation of PIC micelle of PEG−PLL(12-7)/DNA.

Comparing the DNA conformational change induced by PLL and by PEG−PLL, a significant difference in the complex stability was recognized. The long-term stability of PEG−PLL/DNA is obviously superior to that of PLL/DNA. Although large aggregates are formed from the primary condensates of PLL/DNA under ordinary experimental conditions,[22] the hydrophilic PEG segments of PEG−PLL/DNA prevent the complex from aggregation in aqueous medium. The complex observed in the latter system retained its compact globular structure without further aggregation even after a 24 h incubation.

Despite the difference in complex stability, there exist several common features in the transition manner: (1)

DNA chains went into the compact globular state even where the mixing ratio was $r < 1$; (2) the discreteness between the coiled and globular states was confirmed in the transition. PLL, which is well-known as a DNA condensing regent, effectively induces the compaction of DNA chains even where a stoichiometric condition is not achieved. On the basis of the DNA condensation induced by spermidine (3+) or spermine (4+), the concentration of these reagents required to induce the transition is usually several tens higher than that of DNA phosphates.[23] Although the increase in the number of cationic residues enhances the electrostatic binding between DNA and cationic reagents, not only the electrostatic interaction but also the entropic contribution of the small released anions that were originally bound to the polycations play an important role in the complexation. The vicinity of DNA is thought to be in the same situation as predicted by the Oosawa−Manning theory, the so-called counterion condensation theory.[24,25] Thus, the complexation of DNA with polycations is also stabilized in terms of entropy.

As for the mixing ratio, the results obtained here coincide with those observed in the systems including relatively high salt concentrations by other researchers.[26,27] According to the two-variable counterion condensation theory that is applicable to actual experimental systems, e.g., a physiological condition, a small fraction of sodium ions bound to DNA also contributes to charge neutralization even where the degree of charge neutralization reached up to maximum.[28] Such a contribution of sodium ions becomes particularly important in the Ψ-DNA condensation (polymer and salt-induced DNA condensation). During Ψ-DNA condensation, PEG works as a condensing agent only when a certain amount of sodium ion coexists.[16,29] In our system, a similar effect is also produced by the fixation of PEG−PLL.

As Lai and Zanten pointed out that fluorescence microscopy is one of the most powerful tools to provide real-time monitoring of the complexation,[22] this technique actually has already clarified the structural bimodality during DNA condensation induced by most kinds of DNA condensing agents.[20,30,31] It is generally accepted that the bimodality in the DNA conformation results from the cooperative binding of the condensing agents onto DNA helices.[32] From the comparison between the PLL and PEG−PLL(12-7), it is suggested that the introduction of the PEG segment to PLL plays an important role in enhancing the cooperativity. This means that the dependency of the PLL length of the block copolymers on the width of the coexistence region provides information about the degree of cooperativity in the binding manner of PEG−PLL (Figures 2d and 3). Actually, as shown in Figure 3, the width of the coexistence region in PEG−PLL/DNA became narrower with the increase in the PLL fraction in the block copolymers. Thus, the ratio of the PLL segment length to that of the PEG controls the cooperativity of the PEG−PLL binding to DNA. In fact, the width of coexistence region in PEG−PLL(12-42)/DNA became comparable to that in PLL/DNA. Additionally, the linearity between the population of compact globular DNA and PEG-PLL concentration was not satisfied in the transition region (Figure 2c). The population of globular DNA suddenly increased for the mixing ratio $r = 0.7$. In this regime, PEG−PLL tends to preferentially bind to the globular DNA rather than to the coiled chain.

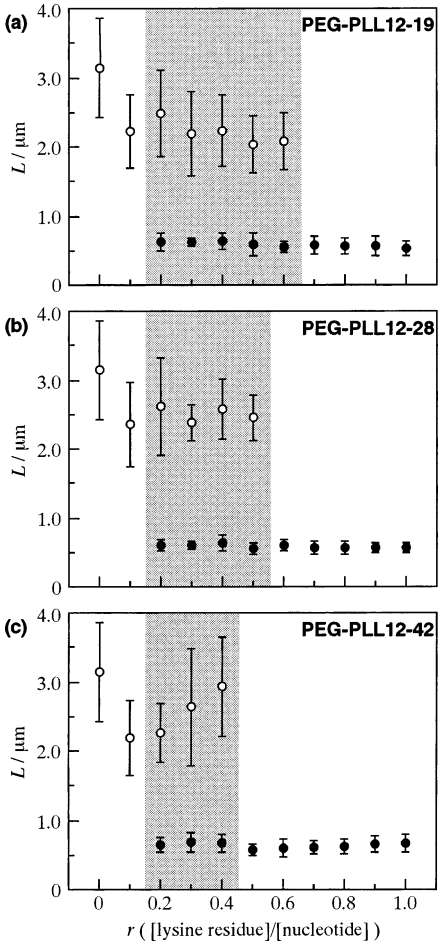

Figure 3. DNA conformational change in the complexation with the PEG−PLL block copolymers.

Such a strong cooperativity was also observed during the DNA condensation induced by detergents, e.g., cetyltrimethylammonium bromide (CTAB), in which the hydrophobic interactions of alkyl chains is essential for the cooperativity.[20] In our system, however, the complexation might be used to maintain the following balances: an entropic tradeoff for the DNA structure, a relationship between the charge neutralization and crowding effect produced by PEG, contributions to charge neutralization from sodium ions and PLL segments, and a compartmentalization between the hydrophobic PLL/DNA part and hydrophilic surrounding PEG part. It is still controversial as to understanding how these complicated interactions contribute to the formation of PIC micelles between DNA and PEG−PLL.

DNA release induced by the polyanion exchange reaction was also examined by fluorescence microscopy. Note that, after the internalization into the cell of the polyplex vectors, DNA release from the complexes should be necessary for gene expressions. Despite the presence of various kinds of polyanions, dextran sulfate (Dex-sul) was used as a model compound, expecting the following polyanion exchange reaction:

$$\text{PEG−PLL/DNA} + \text{Dex-sul} \rightarrow \text{PEG−PLL/Dex-sul} + \text{DNA}$$

As demonstrated in Figure 4, the compact globular DNA disappeared in the sample solutions where the mixing

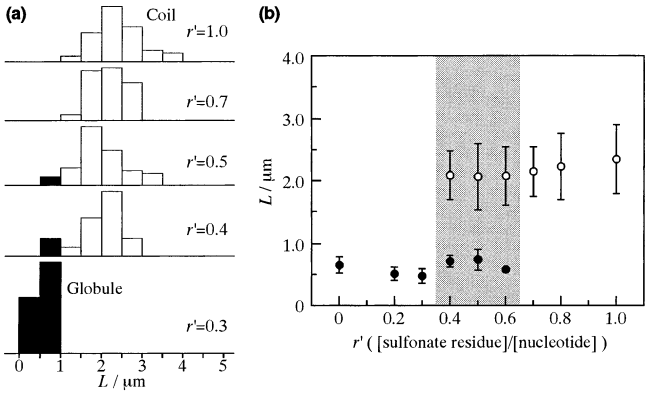

Figure 4. DNA release induced by polyanion exchange reaction with dextran sulfate. The changing profile of L was plotted vs the mixing ratio of the dextran sulfate r'.

ratio of dextran sulfate, $r' \geq 0.7$, indicating that the exchange reaction smoothly progresses under physiological conditions. Both translational and intrachain Brownian motions such as those observed in bare DNA were also observed in the released DNA chains. The reversibility observed in this study is considered to be a kind of switching responsiveness of PIC micelles to the environments that is favorable for gene vectors.

The bimodal profile observed in the DNA release reaction indicates the cooperative nature of this reaction. The absence of large aggregates allows the unequivocal observation of the reversible transition during the formation of PIC micelles. Note that this propensity of PEG−PLL/DNA micelle is a crucial advantage essential to understanding the molecular mechanism of the DNA condensation because a generation of secondary aggregates from primary condensates, which is unavoidable for the conventional condensing reagents, interferes with the observation of the condensation/decondensation processes. As Bloomfield pointed out, the size distribution of condensed particles may be determined kinetically rather than thermodynamically,[33] but it is still hard to understand the molecular mechanism for the primary condensate apart from the aggregation in the usual cases. Therefore, the term "condensation" is reserved for situations in which the aggregate is of finite size and ordered morphology.[29] A novel type of condensing reagents PEG−PLL block copolymers allows us to better understand the monomolecular DNA condensation process as a fundamental success in order to explore the intracellular gene expression mechanism of polyplex vector systems.

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