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# Effect of Molecular Weights of Polyethyleneimine on the Polyplex Formation with Calf Thymus DNA

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**Abstract:** In this study we investigated the spectral properties, including electric absorption, circular and linear dichroism (CD and LD), and fluorescence emission, of DNA in a DNA-branched polyethyleneimine (BPEI) polyplex at various polymer molecular weights ($M_w$) and BPEI-amine-to-DNA-phosphate ratios (N/P ratios). All BPEIs exhibited a common N/P dependence in their absorption and CD spectra. At N/P ratios < 1.0, we observed some hyperchromism in the absorption spectrum, red-shifts in CD bands, and decreases in LD intensity and fluorescence intensity of intercalated ethidium. At intermediate N/P ratios, complete collapse of all spectra occurred. As the N/P ratio increased further, the polyplex dissolved in water. From its characteristic CD spectrum obtained under these conditions, we conclude that the DNA exists in a B-like form. The fluorescence and LD intensities never recovered– even at high N/P ratios– which indicates that the dissolved polyplex possesses positive charges and the DNA in the polyplex is condensed despite its B-form CD spectrum. The N/P range in which the absorption and CD signals collapsed was wider when the BPEIs $M_w$ decreased. In the case where the BPEIs $M_w$ was 0.8 k, recovery of the absorption and CD spectral properties at a high N/P ratio was never achieved, which suggests that the molecular weight of the polymer plays an important role in its dissolution at a high N/P ratio.

*Keywords*: polarized light spectroscopy, polyplex, polyethyleneimine, gene carrier, DNA.

## Introduction

Importance of self-assembly between nucleic acids and bio-degradable polymers has become increasingly more recognized due to its potential application in biological transport and gene therapy.[1-4] Branched poly(ethylenimine) (BPEI, Figure 1), consisting of 25, 50 and 25% of primary, secondary and tertiary amines, respectively, has been known to condense DNA and to be an efficient gene carrier with the highest cationic charge density potential.[5-10] In the DNA-BPEI polyplex, the positively charged amine groups of BPEI interact with the negatively charged phosphate groups of DNA through electrostatic interaction. Furthermore, amines of BPEI exhibit a pH buffering effect in endosomal disruption after endocytosis. According to the proton sponge hypothesis, in order to explain the transfection efficiency of BPEI[11] it must be based on both the amine-phosphate interaction and the buffering effect of amine. The fact that lysosomes would swell and burst due to the osmolarity changes up on fusion with BPEI-containing endosomes, may be a reason for the high transfection efficiency of BPEI.[12,13] The transfection efficiency as well as the cytotoxicity of BPEI strongly depend on the molecular weights of the polymer. It is generally believed that BPEI with a molecular weight higher than 25 K displays a high transfection efficiency and cytotoxicity, while BPEI with a molecular weight less than 1.8 K shows almost no transfection but is less toxic.[14-18]

In this study, we investigated the spectral properties of DNA in the presence of BPEI with various molecular weights and at various N/P ratios. The spectroscopic

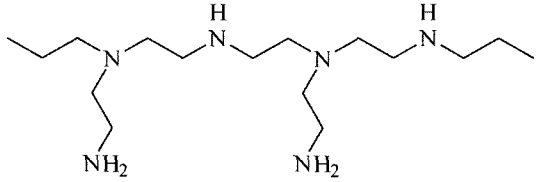


**Figure 1.** The chemical structure of BPEI.

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method utilized in this work includes electric absorption, CD, LD and fluorescence emission. All these conventional spectroscopic methods are easy to access and may provide different information about the conformation of DNA in the polyplex and for the polyplex itself.

## Materials and Methods

**Materials.** *Calf thymus* DNA ($M_w$ = 8.6 MDa, 13 kbp; referred to as DNA) was purchased from Sigma (St. Louis, MO) and dissolved in 5 mM cacodylate buffer solution at pH 7.0 containing 100 mM NaCl and 1 mM EDTA by exhaustive stirring, followed by several rounds of dialysis with 5 mM cacodylate buffer pH 7.0 at 4 °C. This buffer was used throughout this experiment. The concentration of DNA was determined using the extinction coefficient of 6700 $cm^{-1}M^{-1}$ at 258 nm. BPEI ($M_w$ = 0.8 K, 2 K, 25 K, 750 K) were purchased from Aldrich (Milwaukee, WI), BPEI ($M_w$ = 1.2 K, 70 K) from Polysciences (Warrington, PA) and BPEI ($M_w$ = 10 K) from Wako Pure Chemical Co. (Japan). Other chemicals were purchased from Sigma. Polymers and chemicals were used without further purification.

The concentrations of the BPEIs were determined as the molar concentration of nitrogen atoms per liter considering ($CH_2CH_2NH$) as a monomeric unit by potentio-metric titration with 0.1 M HCl, using a 702SM Titrino potentiometer (Metrhom, Swiss). Hence, the mixing ratio, N/P, in this study is defined by the ratio of nitrogen atoms of the PEI to DNA phosphate group or nucleobases. The polyplexes were prepared by mixing the corresponding concentrations of DNA and BPEI solutions and then left for two weeks at 4 °C because the formation of the polyplexes were very slow.

**Methods.** A JascoV-550 spectrophotometer was used to record absorption spectra and a Jasco FP-777 fluorometer for the fluorescence measurement. Changes in the fluorescence emission spectrum of the ethidium bromide (referred to as EB)-DNA complex were recorded under various polycation concentrations. The excitation wavelength was 535 nm. The silt widths for both excitation and emission were 5 nm.

The CD spectrum of DNA, is induced from the chiral arrangement of the electric transition moments of achiral DNA bases. The shape of CD spectrum represent the DNA conformation. For instance, B-form duplex is characterized by a moderate positive peak around 280 nm and a negative peak around 250 nm, while an A-form duplex shows an intense positive peak around 270 nm, a small negative peak around 235 nm, and large negative peak around 210 nm. LD is defined as the differential absorption of the light polarized parallel and perpendicular to some laboratory reference axis. In the case of flow LD, as in this study, the parallel direction is the flow direction.[19-21] The measured LD spectrum is then divided by the isotropic absorption spectrum to give the reduced LD spectrum ($LD^r$), which is related to the ability of the sample to orient within the flow and the angle of the electric transition moment relative to the flow direction. In the case of DNA, all the in-plane $\pi^* \leftarrow \pi$ transitions of the DNA base are expected to be perpendicular, resulting in a wavelength independent $LD^r$ in the DNA absorption region. CD and LD were recorded on a Jasco J715 spectropolarimeter. For LD measurements, a Wada type Couette cell was used to orient the sample.[19,20]

## Results

**Absorption and CD Spectra.** Absorption spectra of DNA in the presence of various concentrations of BPEI ($M_w$ 25 K) are depicted in Figure 2 as an example. Absorbance at 260 nm increases at a low N/P range ( N/P < 0.8). The overall appearance of the absorption spectrum is similar to that of DNA in the absence of BPEI, suggesting that the conformation of DNA in the presence of BPEI does not alter to a large extent. However, a tail above 300 nm is apparent in the absorption spectrum, suggesting some parts of DNA start to aggregate in the low N/P ratio range. The shape of the absorption spectrum at a high N/P ratio is similar to that in the low N/P ratio. At the intermediate N/P ratio (0.8 < N/P < 2.0), absorption spectra of DNA collapse: its absorbance at 260 nm collapsed and the long tail above 300 nm was apparent, which never reached to zero absorbance. This pattern of absorption change with respect to the BPEI concentration (and therefore, N/P ratio) is common for BPEI with $M_w$ 10 K or above (Figure 3). The N/P ratio range, in which DNA absorption collapses, is between 0.8 and 2.0. This N/P range exhibits the tendency to narrow as the $M_w$ of BPEI increases. The absorption minimum, at which the complete collapse of the absorption spectrum occurred, was at N/P = 1.0 for

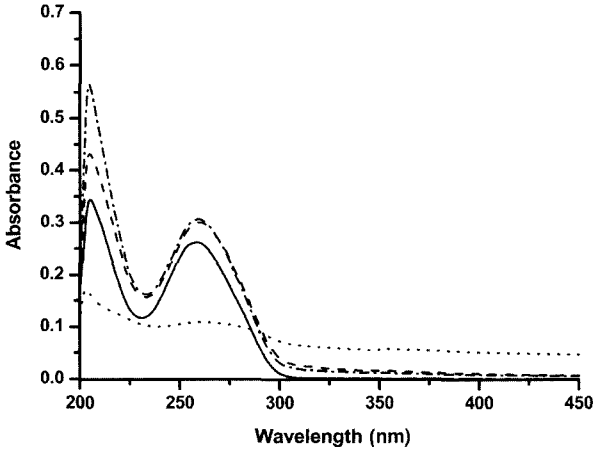


**Figure 2.** Changes in the absorption spectrum of DNA (40 μM) at the various N/P ratios. BPEI $M_w$ = 25 K. Solid curve: N/P = 0 (BPEI is absent); dashed curve: N/P = 0.8; dotted curve: N/P = 1.0, the absorption spectrum at N/P = 4.0 (dashed dotted curve) is identical to that recorded for N/P = 0.8.

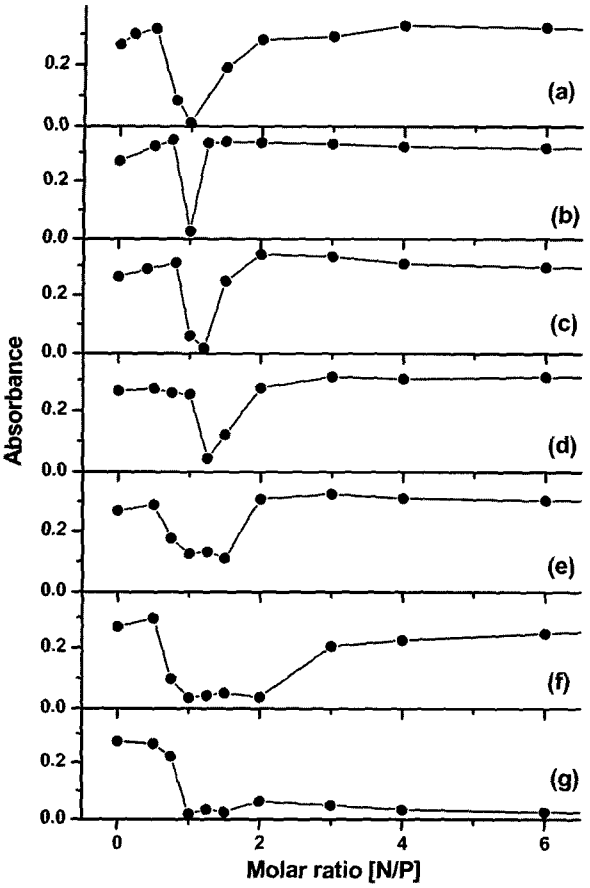


**Figure 3.** Changes in absorbance of DNA at 260 nm with respect to the N/P ratio. From the top, the $M_w$ of BPEI decreases as (a) 750 K, (b) 70 K, (c) 25 K, (d) 10 K, (e) 2 K, (f) 1.2 K and (g) 0.8 K. [DNA] = 40 $\mu$M.

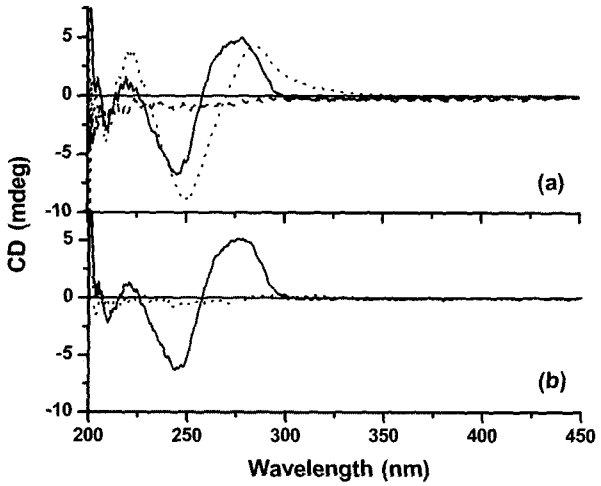


**Figure 4.** Representative CD spectra of the DNA-BPEI polyplexes at different BPEI $M_w$. Panel (a): BPEI $M_w$ = 1.2 K, and (b): $M_w$ = 0.8 K. BPEI with higher $M_w$ exhibited the same CD spectrum as in panel (a). Solid curve: N/P = 0.0 (in the absence of BPEI), dashed curve: N/P = 1.0, and dotted curve: N/P = 6.0. In panel (b), CD spectrum at N/P = 1.0 is identical with that at N/P = 6.0.

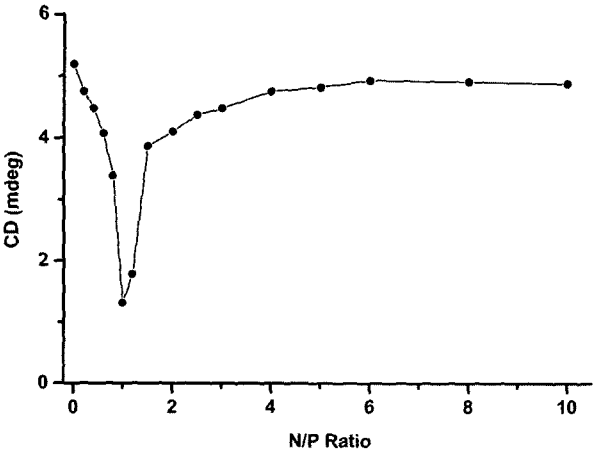


**Figure 5.** Changes in the intensity of CD spectrum of DNA at 275 nm with respect to the N/P ratio with an increasing N/P ratio. [DNA] = 40 $\mu$M, BPEI $M_w$ = 25 K. BPEI with its $M_w$ above 10 K exhibited a similar tendency while that with a $M_w$ of 0.8 K never recovered at a high N/P ratio (see text).

BPEI ($M_w$ > 10 K). In the case of BPEI, with its $M_w$ 2 K and 1.2 K, the range of the collapse is significantly wider compared to that of BPEI with high $M_w$. In the case of the BPEI with $M_w$ 1.2 K, the recovering N/P ratio reached 3.0 (Figure 3(f)). The BPEI with $M_w$ 0.8 K is an exception of the general pattern. As it is shown in Figure 3(g), the absorption spectrum at a high N/P ratio never recovered.

CD spectrum of the DNA-BEPI polyplex within the DNA absorption region in the presence and absence of polymers with two typical $M_w$ ($M_w$ 1.2 K in panel (a) and $M_w$ 0.8 K in panel (b)) at various N/P ratios are depicted in Figure 4. The change in CD spectrum of the DNA-BEPI polyplex above $M_w$ 1.2 K was similar to that of $M_w$ 1.2 K. The change in CD intensity at 275 nm of the polyplex ($M_w$ 25 K) with respect to the N/P ratios is also shown in Figure 5, as an example. At an N/P ratio below 1.0, a significant decrease in intensity of the CD band of DNA was apparent although the overall shape remains the same. As the N/P ratio increased, the CD signal started to collapse and at the N/P ratio of 1.0, complete collapse of the CD signal was observed. As the N/P ratio further increased (above N/P ratio of 2.0), recovering of the CD spectrum was apparent. All these changes in CD spectrum correspond to the change in the absorption spectrum.

It should be noted that the CD spectrum that reappeared at the high N/P ratios differs very little from the CD spectrum recorded at the low N/P ratio: both the negative and positive maximum red-shifted by ca. 6 nm and ca. 10 nm, respectively, and the magnitude of the negative band increased significantly. This suggests that the conformation of DNA at the high N/P ratio is essentially the same as that at the low N/P

ratio: they seem to remain in the B form helix. Although, the BPEI with a $M_w$ of 0.8 K was an exception, recovering of the CD spectrum at the high N/P ratio was never observed. The N/P range in which the CD spectrum starts to collapse and recover exhibits the tendency of being as wide as the $M_w$ of BPEI decrease, as it was observed from the absorption spectrum. It is also note worthy that the complete collapse occurred at N/P ratio of 1.0 disregarding the $M_w$ of the BPEI.

**Fluorescence Spectrum of Ethidium Bromide in the Complex.** The binding of BPEI to DNA was investigated by fluorescence titration of the ethidium-DNA complex (the ethidium/DNA base ratio was 0.025, corresponding to one ethidium per forty bases or twenty base pairs) with various $M_w$ of BPEI solution. Changes in the fluorescence spectrum of the ethidium-DNA complex (excitation at 535 nm and emission at 592 nm) in the presence of various concentrations of BPEI ($M_w$ 25 K) are depicted in Figure 6(a) as an example. Changes in fluorescence of the ethidium-DNA complex with BEPI at various $M_w$ were similar to that shown in this figure. As the N/P ratio increased, the fluorescence gradually decreased. Decrease in fluorescence intensity started at an N/P ratio of ca 0.8 and a complete loss of fluorescence were observed at the N/P ratio of 1.0. At a high N/P ratio, a strong scattering in the short wavelength region was observed, suggesting that undissolved large particles exist in the solution. Above the 1.0 N/P ratio, fluorescence intensity was never recovered, which is in contrast with the absorption and CD spectra

**Linear Dichroism.** As it was mentioned in the experimental section, the magnitude and the shape of LD and $LD^r$ depend on the ability of the sample to orient within the flow, and the angle of the electric transition moment relative to the flow direction. Representative LD and $LD^r$ spectra in the presence of BPEI ($M_w$ 25 K) at the N/P ratio of 0.0, 0.5 and 1.0 are depicted in Figures 7(a). Changes in LD magnitude with respect to the N/P ratio is depicted in Figure 7(c) for BEPI $M_w$ of 25 K, as an example. BEPI with other $M_w$ exhibited the same tendency. Upon increasing polymer concentration, the magnitude of the LD in the DNA absorption region gradually or almost proportionally decreased below the N/P ratio of 1.0. At the intermediate N/P ratio range, $LD^r$ in the DNA absorption region remains wavelength independent (Figure 7(c)), indicating that the part of the DNA where BPEI did not bind maintained normal conformation, i.e., the base (pairs) of DNA is almost perpendicular to the DNA helix. A decrease in $LD^r$ magnitude in this region may be understood as the part that formed the polyplex does not contribute to the $LD^r$. At the N/P ratio above 1.0, the LD signal completely collapsed. The zero LD and $LD^r$ signal can be observed for either the isotropic chromophore or all the electric transitions of the DNA base coincident with the magic angle, which is not conceivable in the present case. Therefore, the collapse of the LD signal observed at the N/P ratios above 1.0 conceivably can be attributed to the loss of

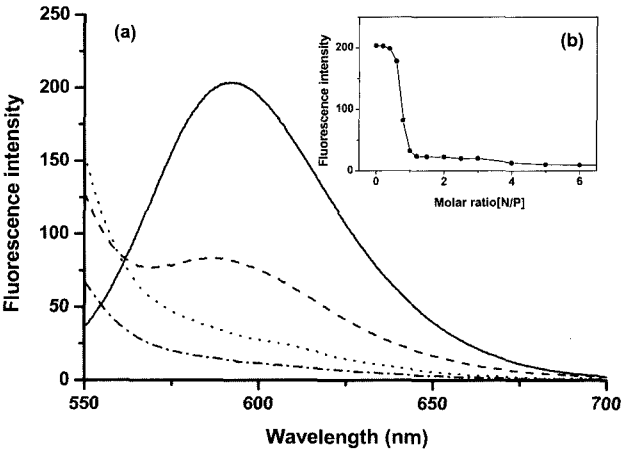


**Figure 6.** Fluorescence spectrum of DNA bound ethidium in the absence and presence of various amounts of BPEI (25 K). [DNA] = 40 $\mu$M, [ethidium] = 1 $\mu$M. Sample was excited at 535 nm. Solid curve: in the absence of BPEI, dashed curve: N/P = 0.8, dotted curve: N/P = 1.0, and dash-dotted curve: N/P = 4.0. Insertion: changes in the fluorescence intensity at 592 nm with respect to the various N/P ratios. The dependence of fluorescence intensity on the N/P ratio was identical for all BPEIs.

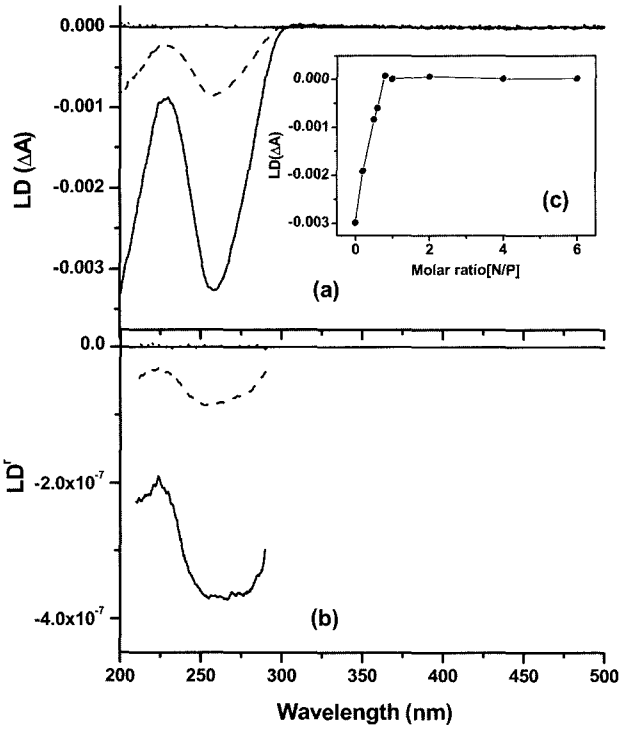


**Figure 7.** LD (a) and $LD^r$ (b) spectrums of DNA in the presence of BPEI $M_w$ 25 K. Solid curve: in the absence of BPEI; dashed curve: N/P = 0.5; dotted curve: complete collapse, exhibiting 0 LD and $LD^r$. Change in LD intensity at 260 nm with respect to the N/P ratio is inserted. All BPEIs with other $M_w$ displayed identical results.

the ability of the DNA to orient. The loss of orientability of the DNA suggests that DNA is no longer linear.

## Discussion

**Conformation of Polyplex at Low N/P Ratios ( < 1.0).** Spectral properties of the DNA-BPEI polyplex at N/P ratios lower than 1.0 can be summarized as hyperchromism in the absorption spectrum and the decrease in intensity of the positive CD band at 275 nm that was accompanied by a red-shift. Both in absorption and CD spectrum, a tail above 300 nm with increasing N/P ratios were pronounced. These observations indicate that DNA forms a polyplex with BPEI and at a low N/P ratio, the DNA-BPEI polyplex and DNA may co-exist. In other words, some part of the DNA forms the polyplex with BPEI while the rest, which is not associated with the polymer, remains in the B form. The part that forms the polyplex seems to cause a significant scattering of incident light, resulting in a tail in absorption and CD spectra above 300 nm. Considering that BPEI do not possess any aromatic moiety, which is required for the intercalation of any drug to DNA, the driving force to form a polyplex may be an electrostatic interaction in nature between the phosphate groups of DNA and the amine groups of the polymer. This partial aggregation results in the extrusion of intercalated ethidium as it is evident from the decrease in fluorescence intensity and the collapse in the LD spectrum. Among the two factors that determine the LD intensity, namely, the orientation factor and the optical factor, the latter remains the same at various N/P ratios: the only chromo-phores in the system are the bases of DNA that are not changed. Therefore, a decrease in LD spectrum reflects the decrease in the ability of the orientation of DNA as a result of forming a random aggregation, due to the loss of repulsive interaction between negatively charged phosphate groups of DNA. This type of aggregation/collapse in DNA naturally results in an extrusion of the intercalated ethidium. It should be noted that the $M_w$ of the BPEI does not affect the formation of the aggregation at low N/P ratios.

**Conformation of Polyplex at High N/P ratios.** At high N/P ratios, the absorption and CD spectra of the DNA-BPEI polyplex recover, indicating that the polyplex becomes soluble in water. As judged by its absorption and CD spectrum, DNA appears similar to the B form. Theories have been developed for precipitation and dissolution of the DNA-polymer polyplex.[22-24] Due to the association of multivalent cationic polyamines with DNA, DNA is considered to be in a nonpolar or less polar condition, which results in a phase separation from the polar aqueous solution. The fluidity of the ordered phase suggests that the probable binding of polyamine (spermine to be more specific) would be along the strands. We believe that the dissolution of the DNA-BPEI polyplex occurs by the positive charges of the BPEI, which are associated at the surface of the condensed DNA-BPEI complex through the hydrophobic interaction between the $-CH_2-$ groups of the BPEI. Since the surface of the dissolved complex is positively charged, it cannot provide the binding sites for ethidium. The LD signal, in contrast with the absorption and CD, did not recover. From this observation, it is suggested that DNA may not be capable of free movement in the dissolved complex at a high N/P ratio, i.e., collapsed DNA in the condensed complex remains in the condensed form. Considering that DNA CD is similar to B form, the water molecules in the polyplex may be abundant for DNA to exhibit a B form.

**Complete Aggregation of DNA at Intermediate N/P Ratios.** All spectra, including absorption, CD and LD collapse at the intermediate range of N/P ratios. These results indicate that all available DNA participate in the polyplex. The N/P range in which the spectral signals collapse depends on the molecular weight of BPEI. In the presence of BPEI with $M_w$ higher than 10 K, the N/P region of the collapse is narrow. Furthermore, a maximum collapsing ratio can be easily identified as N/P = 1.0. When the $M_w$ of BPEI is lowered, the N/P range, which collapsing occurs becomes wider: $1.0 < N/P < 3.0$ for BPEI $M_w$ 2 K and $1.0 < N/P < 4.0$ for 1.2 K. In the case of the BPEI with $M_w$ of 0.8 K, the dissolving of the polyplex never occurs. If the dissolution of the DNA-BPEI polyplex occurs by the surface positive charges of the BPEI, the difference in the aggregation range may be understood by the molecular weight or size of the BPEI itself. In other words, a smaller BPEI might be less efficient to form a polyplex whose surface is positively charged at a high N/P ratio.

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