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Polyplex Formation of Calf Thymus DNA with Branched and Linear Polyethyleneimine

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Abstract: We have investigated the spectral properties of DNA, including its electric absorption, circular and linear dichroism (CD and LD), and fluorescence emission, in the DNA-linear polyethyleneimine (LPEI) and DNA-branched polyethyleneimine (BPEI) complexes at various polymer concentrations. The spectral properties of both complexes are similar. We observed a relatively moderate change in the absorption and CD spectra at low amine/DNA phosphate (N/P) ratios (< 0.5), followed by a drastic collapse within the N/P range from 0.8 and 1.0. The absorption and CD spectra recovered as the N/P ratio increased to ca. 1.2. In contrast, the LD and emission of ethidium intercalated between the DNA bases decreased almost linearly at N/P ratios between 0.0 and 1.0. These spectra never recovered at higher N/P ratios. We believe that the moderate changes in the spectrum at low N/P ratios occurred because of electrostatic interactions between DNA and BPEI, while the collapsed spectra at N/P ratios between 0.5 and 1.5 occurred because of condensation/aggregation of the DNA. Considering the structure of the polymers, we suggest that the secondary amino group of LPEI and all three amino groups of BPEI are equally involved in DNA condensation.

Keywords: DNA, spectroscopy, polyplex, polyethyleneimine, gene carrier.

Introduction

The importance of self-assembly between nucleic acids and biodegradable polymers has become increasingly more recognized due to its potential application in biological transport and gene therapy.[1-4] Various cationic or neutral polymers including synthetic peptides, dendritic polyamidoamines and polyethyleneimine (PEI) as oligonucleotide carriers have been shown to increase the diffusion efficiency of poly- or oligonucleotides through cell membranes, an essential step for gene therapy.[1] PEI and modified PEI have been one of the widely studied families of DNA delivery vehicles.[2] It is believed that the polycations including PEI form a complex with DNA through, in general, the electrostatic interactions between the positively charged nitrogen of the polymer and the negatively charged DNA phosphate. The PEI-DNA complex has a tendency to form an aggregation of nano-size particles at intermediate polycation/DNA ratios.[5-7] The size of the particle correlates with its transfection efficiency[7-12]: small particles usually show lower transfection efficiency than larger ones.

Characterization of the physicochemical properties of both linear (LPEI) and branched PEI (BPEI) as DNA carriers and their derivatives[13,14] (Figure 1) and the PEIs (and their derivatives)-DNA complexes[15-20] has recently started using various methods including fluorescence techniques, and CD and electronic absorption spectroscopy.[5,6,13-20] Ottaviani and his co-workers described the superamolecular structures of the complex formed between the *calf thymus* DNA and starburst polyamidoamine dendrimers.[18] The structures depend on the "*r* ratio", which is defined as the

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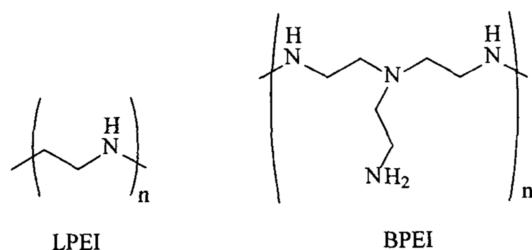

Figure 1. Structure of BPEI and LPEI.

ratio of the number of *surface* amine groups to DNA base pairs, and the size of the dendrimers, suggesting that the electrostatic interaction between DNA and amine groups at the surface of the dendrimers in the complex formation is important. Similarly, we compared the optical spectroscopic properties of the DNA-BPEI and DNA-LPEI complex, including circular and linear dichroism spectroscopy (CD and LD). BEPI and LPEI were chosen because LPEI has only secondary amines and BPEI possess all three kinds of amines thereby enabling us to compare the roles of the various amines. In order to avoid the possible unexpected effect from the polymer's size in the complex formation, polymers that have similar molecular weight were used.

Experimental

Materials. DNA was purchased from Sigma Chem. Co. (Product no. D1501, St. Louis, MO, USA) and then dissolved in 5 mM cacodylate buffer at pH 7.0 containing 100 mM NaCl and 1 mM EDTA by exhaustive stirring, and followed by several rounds of dialysis with 5 mM cacodylate buffer pH 7.0 at 4 °C. This buffer was used throughout this experiment. Other chemicals were purchased from Sigma Chem. Co. and used without further purification. The concentration of DNA was determined using an extinction coefficient of 6700 $cm^{-1}M^{-1}$ at 258 nm. LPEI was purchased from Polysciences (Warringtion, PA, USA) and BPEI from Aldrich Chem. Co. (Milwaukee, WI, USA) and used without further purification.

The concentrations of the PEIs were determined as the molar concentration of nitrogen atoms per liter considering -(CH_2CH_2NH)- as a monomeric unit by potentiometric 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 the phosphate group of DNA. The dissociation constants (the pK_a values) of the PEIs were determined also by potentiometric titration under N_2 environment using CO_2-free water. The BPEI-HCl and LPEI-HCl concentration for this titration was 5 mM. A 0.01 M KOH standard solution was used. From the potentiometric titration, the pK_a values were determined as 7.24 and 6.98 for BPEI and LPEI near pH 7.0, respectively, using the Henderson-Hasselbalch equation.[21] Therefore, for both PEIs, about a half of the amine group is protonated.

The polyplexes were prepared by mixing the concentrations of DNA and PEI solutions and left for at least two weeks at 4 °C, since the formation of the polyplexes were very slow as evident by slow change in spectral properties: no further change occurred after two weeks. The average molecular weight of both the BPEI and LPEI were 2,500 g/mole.

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

The CD spectrum of DNA, which is induced from the chiral arrangement of the electric transition moments of achiral DNA bases, was recorded on a Jasco J715 spectropolarimeter (Tokyo, Japan). 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, the parallel direction is the flow direction.[22-24] 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. LD was recorded on a Jasco J715 equipped with a Couette cell to orient the sample, and an Oxley prism was used to convert the circularly polarized light to linearly polarized light.[22,23]

Results and Discussion

Absorption and CD Spectrum. Absorption spectra of DNA in the presence and absence of various concentrations of BPEI and LPEI are depicted in Figures 2(a) and (b), respectively. Changes in absorbance at 260 nm with respect to the polymer concentration is also depicted (Figure 2(c)). As shown in Figures 2(a) and (b), some hypochromism was observed for both the BPEI- and LPEI-DNA complex under the N/P ratio of 0.8. An absorption tail above 300 nm, which is especially pronounced for BPEI, suggests that some of the the incident light was scattered by the aggregation of DNA. Between the N/P ratio of 0.8 and 1.2, the absorption signal collapsed. An unstructured weak absorption feature (for the BPEI-DNA complex) or a complete loss of absorbance (for the LPEI-DNA complex) was apparent in the absorption band at an N/P ratio of 1.0, indicating the complete aggregation of DNA. Surprisingly, the absorption signal of DNA reappears as the N/P ratio further increases (N/P

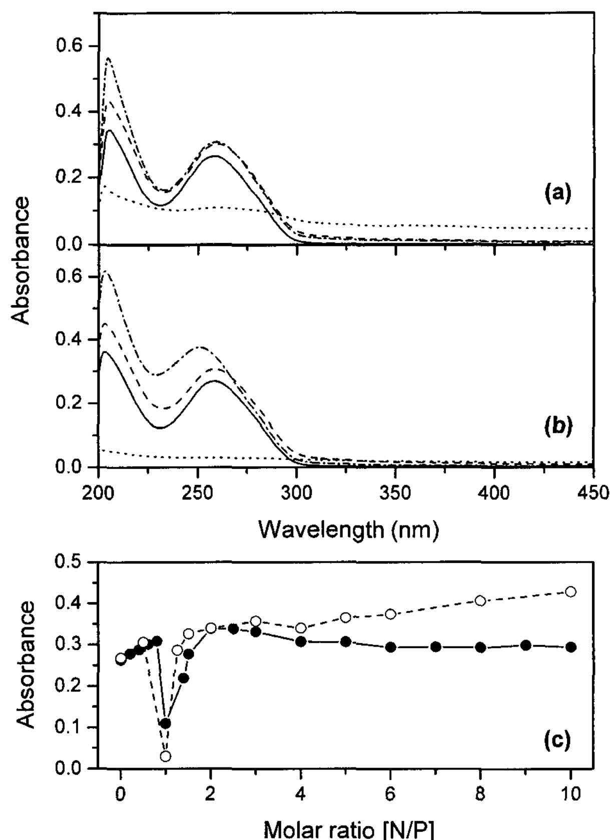

Figure 2. Selective absorption spectra of the DNA-BPEI (panel a) and DNA-LPEI (panel b) complex at various N/P ratios: DNA-BPEI N/P = 0.0 (solid), 0.4 (dashed), 1.0 (dotted) and 4.0 (dash and dotted): DNA-LPEI N/P = 0.0 (solid), 0.5 (dashed), 1.0 (dotted), 4.0 (dash and dotted). [DNA] = 40 μM. Panel (c): absorbance at 260 nm of the DNA-polymer mixtures in the presence of BPEI (closed circles) and LPEI (opened circles) with respect to the N/P ratios.

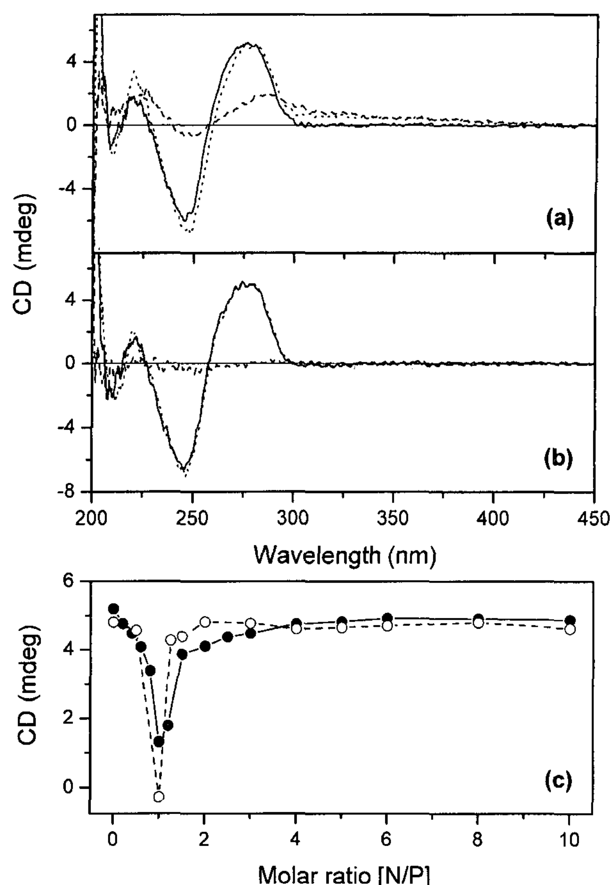

Figure 3. Selective CD spectra of the DNA-BPEI (panel a) and DNA-LPEI (panel b) complex at various N/P ratios: DNA-BPEI N/P = 0.0 (solid), 1.0 (dashed), and 4.0 (dotted): DNA-LPEI N/P = 0.0 (solid), 1.0 (dashed), and 4.0 (dotted). [DNA] = 60 μM. Panel (c): CD intensity at 276 nm of the DNA-polymer mixtures in the presence of BPEI (closed circles) and DNA-LPEI (opened circles) complex with respect to the N/P ratios.

> 1.2). At a high N/P ratio, some blue shift and hyperchromism in the DNA absorption band was observed for the LPEI-DNA complex (Figure 2(b)), whereas the BPEI-DNA complex exhibited no shift but some hyperchromism (Figure 2(a)), suggesting that the conformation of DNA at a high N/P ratio is somewhat different.

Changes in the CD spectrum within the DNA absorption region in the presence and absence of polymers at various N/P ratios are depicted in Figure 3. A significant change in the CD spectrum was observed when both the BPEI and LPEI concentration increased similarly in absorption spectrum. At an N/P ratio below 1.0, a significant decrease in intensity of the CD band of DNA was apparent. The CD signal collapsed at the N/P ratio of 1.0 and it recovered as the N/P ratio increased further. Changes in the CD spectrum are summarized in Figure 3(c): the CD intensity of DNA at 276 nm (at which the largest change occurs) is plotted as a function of the polymer concentrations. Similarly with the absorption spectrum (Figure 2(c)), the disappearance of the CD spectrum at the N/P ratio of 1.0 and then reappearance at a higher N/P is evident. At the N/P ratio of 1.0, both absorbance and CD spectrum of the LPEI-DNA complex was almost completely diminished. In contrast, the BPEI-DNA complex produces a featureless absorption even though the absorbance was very low with a non-zero CD spectrum having a tail above 300 nm, even at its minimum.

Fluorescence Spectrum of Ethidium Bromide in the Complex. Ethidium bromide has been used as a fluorescent probe to investigate the interaction between DNA and various dendrimers.[16] In the DNA-dendrimer complex case, there is one binding site for ethidium and the interaction between DNA and dendrimer was sufficiently strong that they cannot be displaced by ethidium.[16] Changes in the fluorescence intensity of the ethidium-DNA complex (excitation at 535 nm and emission at 592 nm) in the presence of various concen-

trations of LPEI and BPEI are depicted in Figure 4(a). Here, the ethidium/DNA base ratio was 0.025, corresponding to one ethidium per forty bases or twenty base pairs. A pronounced decrease occurred in the fluorescence intensity below an N/P of 1.0 for both BPEI and LPEI. Below the N/P ratio of 1.0, the shape of the emission spectrum of the ethidium-BPEI-DNA mixture was identical with that of the ethidium-DNA complex (data not shown). Above the N/P ratio 1.0, the emission spectrum of the mixture seemed to be similar to that of DNA free ethidium, although a strong scattering of light was detected, indicating that the polyplex formation resulted in the extrusion of ethidium. When the same experiment was performed at an N/P ratio of 0.25, which corresponds to one ethidium per two base pairs, at which condition ethidium saturates all available intercalation sites, a decrease in the fluorescence intensity started at very small N/P ratios of BPEI (Figure 4(b)). A similar result was obtained for LPEI (data not shown). Once again this observation indicates the extrusion of ethidium upon BPEI binding. At a high N/P ratio, fluorescence intensity was never recovered, which is in contrast with absorption and CD spectra.

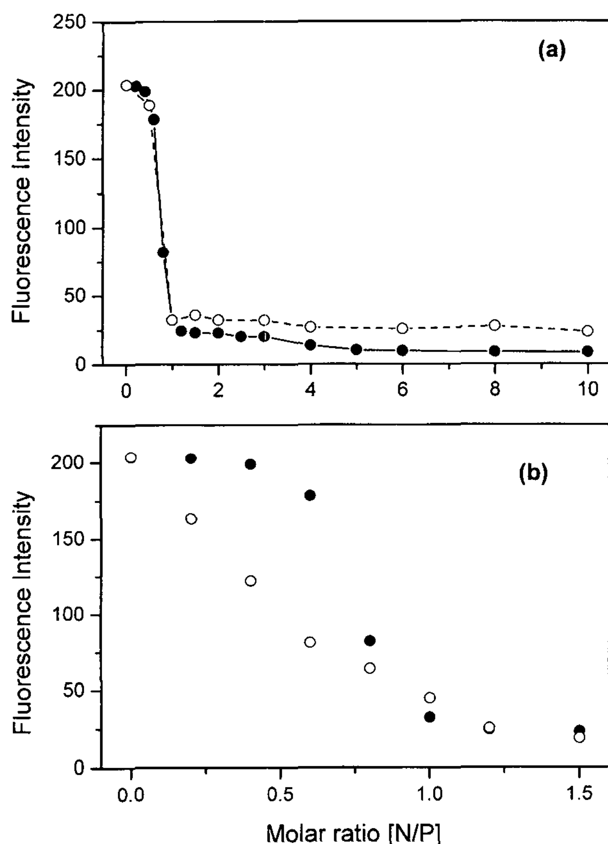

Figure 4. Panel (a): Changes in fluorescence intensity of the ethidium-DNA-polymer mixtures in the presence of various concentrations of BPEI (closed circles) and LPEI (opened circles). Excitation at 535 nm and emission at 600 nm. Slit widths for both excitation and emission were 5 nm. [DNA] = 40 μM, [ethidium bromide] = 2.0 μM. Panel (b): Changes in fluorescence intensity of the ethidium-DNA-BEPI complex in the presence of various BPEI concentrations for the ethidium/DNA base ratios of 0.025 (closed circles) and 0.25 (opened circles). [DNA] = 40 μM. This change is essentially the same as that of the DNA-LPEI complex.

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 that the angle of the electric transition moment relative to the flow direction. Representative LD and LD^r spectra in the presence of LPEI at the N/P ratio of 0.0, 0.5, 1.0 and 6.0 are depicted in Figures 5(a) and (b). Upon increasing polymer concentration, the magnitude of LD in the DNA absorption region gradually or almost proportionally decreased (Figure 3(c)) below the N/P ratio of 1.0. Above the N/P ratio of 1.0, the LD signal completely collapsed. The zero LD and LD^r signal is observed for either the isotropic chromophore or all the electric transition of the DNA base coincident with the magic angle, which is not conceivable in the present case. For an almost perpendicular orientation of nucleo-bases

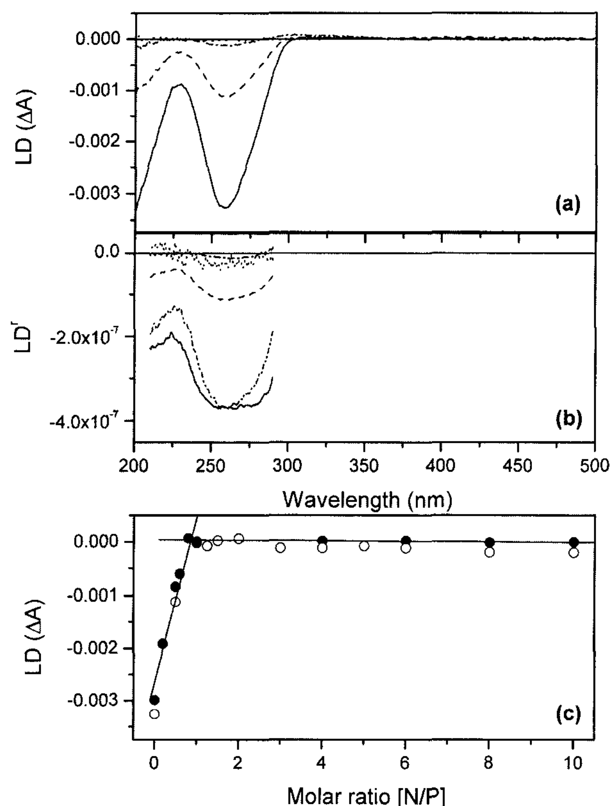

Figure 5. LD (panel a) and LD^r(panel b) spectrum of the DNA-LPEI complex at the representative N/P ratio: N/P = 0 (solid), 0.5 (dashed), 1.0 (dotted) and 6.0 (dash and dotted). [DNA] = 60 μM. Panel (c): LD intensity at 260 nm of the DNA-BPEI (closed circles) and DNA-LPEI (opened circles) complex with respect to the N/P ratios.

(and their $\pi^* \leftarrow \pi$ in-plane transitions) relative to the DNA helix axis results in a wavelength independent (constant) LD^r value between the wavelength region of ca. 250 and 280 nm (Figure 5(b)). The LD^r of both polyplexes at a N/P ratio around 0.5 are strongly wavelength dependent (Figure 5(b)), indicating the nucleo-bases are tilted. As previously observed for the fluorescence emission, LD did not recover at an extremely high N/P ratio, which is in contrast with absorption and CD spectra.

Condensation of DNA at an N/P Ratio below 1.0. The presence of either BPEI or LPEI results in drastic changes in the spectroscopic properties and consequently the DNA conformation. At an N/P ratio lower than 1.0, corresponding to one positively charged amine per two DNA phosphate, hyperchromism in the absorption spectrum, and a decrease and red shift in the positive CD band were apparent. We believe that these changes in the CD and absorption spectrum are related to the electrostatic interaction between the phosphate group of DNA and the protonated amine group of polymer. An increase in absorbance and a decrease in CD intensity are usually the result of a decrease in the π-π stacking interaction. Therefore, the association of DNA and polymers is likely related to destacking of DNA bases (pairs). However, a solid understanding for this conformational change requires more investigation. A tail above 300 nm in the absorption and CD spectrum, that is particularly apparent for the BPEI-DNA complex, suggests that some degree of aggregation may occur upon BPEI binding to the DNA. Both of the BPEI- and LPEI-DNA polyplexes are not capable of ethidium intercalation. Retained fluorescence intensity for the ethidium-DNA complex at low mixing ratio ($r = 0.025$) in the N/P ratio of below 0.5 may be understood by moving the ethidium to the next available intercalation site of DNA which is free from the polyplex. When ethidium initially occupies all intercalation sites ($r = 0.25$), the extrusion of ethidium appears even at a very low N/P ratio. This result is in contrast to the observation of the DNA-dendrimer complex, to which ethidium can bind with a considerably high equilibrium constant.[16] Extrusion of ethidium from the DNA-PEI complex may be understood by the loss of the negative charge of the phosphate group of DNA, also by the high concentration of NaCl. Neutralization of the phosphate group is also suggested by decrease in LD magnitude. Both the DNA-PEI complex do not provide usual intercalation site for ethidium conceivably due to a reduction of the negative charges of the phosphate.

Resolubilization of the DNA-PEI Complex at a N/P Ratio above 1.0. Although all of the spectral signals collapse at N/P ratios of 1.0 indicating the condensation/aggregation of the DNA-PEI complex, the absorption and CD signal recovered at N/P ratio greater than 1.0. The recovered absorption and CD spectrum indicates resolubilization of DNA. Theories have been developed for precipitation and resolubilization of DNA.[25-27] 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 resolubilization of the DNA-PEI complex occurs by the positive charges of the PEI, which are associated at the surface of the condensed DNA-BPEI complex through the hydrophobic interaction between the -CH_2- groups of the PEI. Since the surface of the resolubilized complex is positively charged, it cannot provide the binding sites for ethidium, which is also positively charged. 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 resolubilized complex, i.e., collapsed DNA in the condensed complex remained in the collapsed form.

In comparison of LPEI and BPEI, both PEIs can induce a condensation/aggregation of DNA at the N/P ratio of 1.0. The ratio of the protonated amine from potentiometric titration is estimated to be similar. We did not find a large difference in the protonation states between primary, secondary and tertiary amine. Therefore, it is logical to assume that all amines equally contribute in DNA condensation. Although the condensation of DNA on the dendrimer via electrostatic interactions between surface amines of dendrimer and phosphate groups of DNA have been reported,[16] the results observed in this work cannot be explained if it is only the primary amine that contributes to the electrostatic interaction with phosphate. Therefore, it is suggested that all amines, i.e., primary, secondary and tertiary amines contribute to the neutralization of DNA, resulting in DNA condensation/aggregation.

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