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Recognition of DNA Topology in Reactions between Plasmid DNA and Cationic Copolymers

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Abstract: This study for the first time demonstrates phenomenon of recognition of DNA tertiary structure by the synthetic polycationic molecules. Effects of DNA topology were evaluated using supercoiled and linearized forms of plasmid DNA (*sc*DNA and *l*DNA). Recognition is achieved by using relatively simple chemical structures interacting with the DNA. Two polycations modified with water-soluble poly(ethylene glycol) (PEG) chains, PEG−*block*−poly(*N*-methyl-4-vinylpyridinium sulfate) (PEG−*b*−PVP) and PEG−*graft*−polyethyleneimine (PEG−*g*−PEI) were used. When added to the mixtures of *l*DNA and *sc*DNA, PEO−*b*−PVP selectively bound to *sc*DNA, while *l*DNA remained free. In contrast, PEO−*g*−PEI interacted with both forms of the DNA present in the mixture. Distinct behavior of two copolymers was attributed to the differences in their structure, particularly, charge density of the polycation blocks. Relatively small variation in the polycation ionization state can result in drastic changes in its behavior upon interaction with DNA. Particularly, the change of pH from 7.0 to 5.0, increasing the charge density of PEI block in PEO−*g*−PEI, was also accompanied by the appearance of recognition phenomena. These findings uncover possibilities for the control of the processes of DNA incorporation in the complexes with cationic species by altering the DNA topology, which may have practical significance in using these complexes for gene delivery.

Non-viral gene delivery systems based on DNA complexes with polycations have recently attracted significant attention.[1] These complexes form spontaneously as a result of cooperative electrostatic interactions between phosphate groups of the DNA and oppositely charged groups of the polycation. Characterization of the reactions of polyion coupling between DNA and polycation and interactions of formed polycation/DNA complexes with charged species present in the biological milieu is essential for the use of these systems in gene delivery. In particular, reactions of polyion interchange may account for the release of the DNA in an active form inside cells.[2] Generally, these reactions involve transfer of the polyion chain, incorporated in the polymer complex, to another polyion present in the solution.[3] These processes are thermodynamically and kinetically controlled by the structure of the reacting species and environmental characteristics, including pH, ionic strength, and temperature.[3−5] While the reactions involving interchange of

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synthetic polyions have been studied intensively,[3−7] the processes involving transfer of polycations between different polynucleotide molecules have not been studied. This work examines polyion coupling and interchange reactions in DNA and polycation mixtures. To increase stability of DNA/polycation complexes in aqueous dispersions, polycations were modified with water-soluble poly(ethylene glycol) (PEG) chains. Using both linearized and supercoiled forms of plasmid DNA, this study for the first time demonstrates that the direction of interchange reactions depends on the tertiary structure of the nucleic acids.

Experimental Section

PEG−*g*−PEI (PEI = branched polyethyleneimine) (M_w = 16 600 g/mol; PEG/PEI ratio 1.7) was synthesized by conjugation of PEG, $M_n \approx 8000$, with randomly branched PEI, $M_w \approx 2000$.[8] PEG−*b*−poly(4-vinylpyridine) was synthesized by a sequential anionic polymerization process using modified method.[9] This sample was quaternized with dimethylsulfate to obtain PEG−*b*−PVP (PVP = poly(*N*-methyl-4-vinylpyridinium sulfate)) as described previously.[10] The block lengths in this copolymer were 200 for PEG and 55 for PVP as determined by gel permeation chromatography. The degree of alkylation of the PVP block was 96%. Plasmids, pCMV-luc (7.2 kb), and pCR3.0. (6.5 kb) were linearized with *Hind*III, and linearized DNA (*l*DNA) was purified from the reaction mixture using a Qiagen QIAquick gel extraction kit according to supplier's protocol. $[Poly(dA\text{-}dT)]_2$ was purchased from Pharmacia-LKB. Salmon testes DNA (*st*DNA) was purchased from Sigma. The quantity and quality of the DNA were assessed by spectrophotometric analysis at 260 and 280 nm. Free DNA and cationic copolymer/DNA complexes were analyzed by electrophoresis in an 0.8% agarose gel with 40 mM Tris-acetate buffer, pH 7.4, containing 1 mM EDTA, at 60 V for 90 min. Complexes were loaded in the gel in the same solutions as they were prepared (pH 5.0 or 7.0). The amount of DNA used was 0.5 μg per well. DNA was visualized by UV illumination following staining of the gels with ethidium bromide (EB) (0.5 μg/mL) for 30−60 min at room temperature. EB fluorescence was recorded using a Shimadzu P5000 spectrofluorimeter at excitation and emission wavelengths of 520 and 582 nm, respectively. DNA melting curves were obtained using a Varian Cary 3000 spectrophotometer at 260 nm.

Results

Characterization of Binding of Cationic Copolymers with DNA by EB Displacement Assay. Intercalation of EB into the DNA is accompanied with an increase in the fluorescence of this probe.[11] As is seen in Figure 1, addition of cationic copolymers to DNA−EB complex results in quenching of the fluorescence due to the displacement of EB by the copolymer. The degree of quenching depends on the ratio of the amounts of the nitrogen-containing groups of cationic copolymers to the phosphate groups of DNA (N/P ratio). All copolymers eventually quenched most of the EB fluorescence (80−95%). However, there were differences in the N/P ratios at which these degrees of quenching were observed with various copolymers. For example, in the case of PEO−*b*−PVP, in which practically all nitrogen-containing groups are charged, maximal quenching was observed at N/P ratio close to 1. In contrast, PEG−*g*−PEI quenched fluorescence to the same extent only at N/P ratios of ~1.6 at pH 5.0 and 2.2 at pH.7.0. This difference appears to correlate with the variation in the charge density in the PEI chain, since only ~65 and 45% of total amino groups of this copolymer are charged at pH 5.0 and 7.0, respectively.[8] Therefore, to account for the variation in the content of charged groups in cationic copolymers, the compositions of the complexes are expressed in terms of the charge ratio, *Z*, that is, the ratio of the ionized groups of polycation to phosphate groups of DNA. In terms of the charge ratio, the maximal quenching of fluorescence with all polycations was observed at equivalency of the polycation and DNA charges ($Z = 1$).

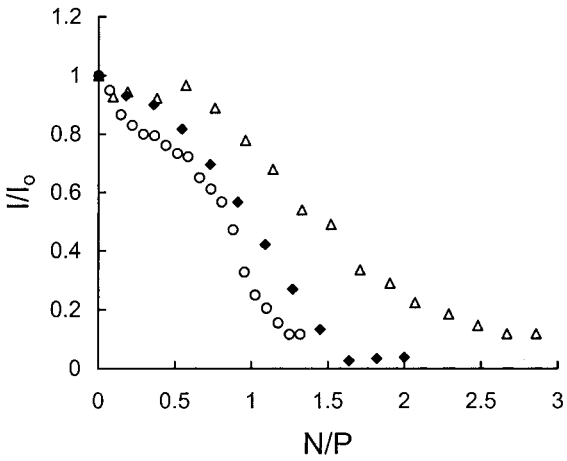

Figure 1. Binding of cationic copolymers with plasmid DNA. Supercoiled form of pCMV-luc (5 μg/mL) was mixed with EB (2 EB molecules per 5 bp) and then titrated with (○) PEG−*b*−PVP, (△,◆) PEG−*g*−PEI. All titration experiments were conducted at pH 7.0 (10 mM sodium phosphate buffer) except for (◆) PEG−*g*−PEI, which was conducted at pH 5.0 (10mM sodium acetate buffer). Dilution of samples was less than 2%. Sample fluorescence was determined after subtracting the baseline fluorescence of EB in the absence of the DNA. Data are presented as the ratio of fluorescence of the samples with and without copolymer (I/I_o).

Characterization of Complex Formation by Gel Electrophoresis. Complexes between DNA and polycations were prepared by simple mixing of the solutions of both components at various charge ratios. Upon mixing, these systems remained transparent, and no precipitation was observed over the entire range of charge ratios examined. Formation of complexes of cationic copolymers and plasmid DNA (supercoiled DNA (*sc*DNA)) was analyzed by gel electrophoresis. Figure 2a presents the gel electrophoresis pattern observed with PEG−*g*−PEI and *sc*DNA mixtures at pH 7.0. Migration of the *sc*DNA in the gel was retarded as the amount of the copolymers was increased. This demonstrated that the copolymer was binding to the DNA, neutralizing its charge. At stoichiometric compositions ($Z = 1$) complete neutralization of *sc*DNA by PEG−*g*−PEI was observed. Similarly, complete incorporation of *sc*DNA into the complex was observed at $Z = 1$ for PEG−*b*−PVP (data not shown in the figure). However, at lower charge ratios, the mechanism of binding of this copolymer to *sc*DNA was totally different from that of PEG−*g*−PEI. As is shown in Figure 2b, upon addition of PEG−*b*−PVP to the *sc*DNA at $Z = 0.3$, a portion of the *sc*DNA incorporated in the complex, while another portion of the *sc*DNA remained free (compare lines 1 and 2). In other words, uneven distribution of the copolymer chains among the *sc*DNA chains was observed ("disproportionation"). Lane 3 of the same figure presents for comparison the mixture of PEG−*g*−PEI and *sc*DNA at $Z = 0.3$ at pH 7.0. In this case all *sc*DNA observed in the gel was incorporated in

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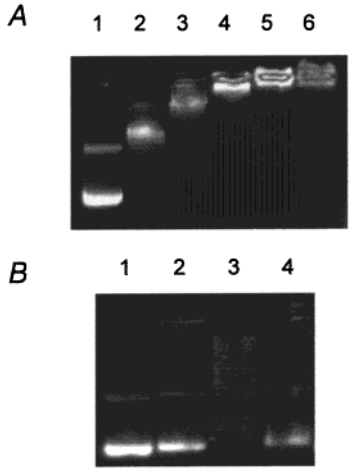

Figure 2. Characterization of formation of the complexes by gel electrophoresis. (A) Agarose gel electrophoresis of free *sc*DNA (lane 1) and its complexes with PEG−*g*−PEI at different charge ratios: 0.2 (lane 2), 0.4 (lane 3), 0.6 (lane 4), and 0.8 (lane 5), and 1.0 (lane 6). (B) Agarose gel electrophoresis of free *sc*DNA (lane 1) and its complexes with PEG−*b*−PEVP (lane 2); PEG−*g*−PEI prepared at pH 7.0 (lane 3) and pH 5.0 (lane 4). Charge ratio was 0.3 for all complexes.

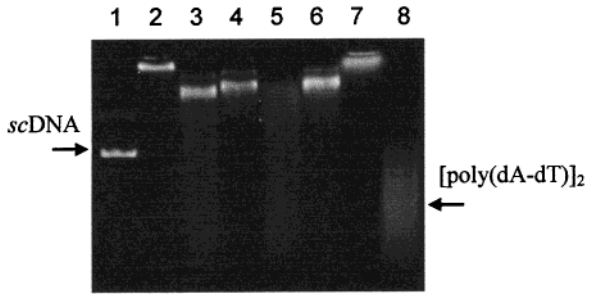

Figure 3. Study of polyion interchange reactions using agarose gel electrophoresis. Solutions of DNA and cationic copolymers (10 mM sodium phosphate buffer, pH 7.0) were mixed at charge ratios as indicated below and incubated 1 h prior to further experiment. To study interchange reactions, these complexes were then mixed with corresponding DNA samples and incubated for one additional hour. Gel presents free *sc*DNA (lane 1) and free $[poly(dA\text{-}dT)]_2$ (lane 8) as well as PEG−*g*−PEI/*sc*DNA complexes formed at charge ratio 1 (lane 2) and 0.5 (lane 4), PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complexes formed at charge ratio 0.5 (lane 5) and 1 (lane 7), mixture of PEG−*g*−PEI/*sc*DNA complex presented in lane 2 with 1 equiv of $[poly(dA\text{-}dT)]_2$ (lane 3), and mixture of PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$, complex presented in lane 7 with 1 equiv of *sc*DNA (lane 6).

the complex. Interestingly enough, disproportionation was observed with this copolymer when the complexes were prepared at pH 5.0 (lane 4). The behavior of *l*DNA upon interaction with the studied cationic copolymers was similar to that shown in Figure 2b for the *sc*DNA sample. Furthermore, similar results were obtained using another plasmid (pCR3.0.).

Study of Polyion Interchange Reactions by Gel Electrophoresis. To examine polyion interchange reactions, a synthetic linear DNA polymer, $[poly(dA\text{-}dT)]_2$ was added to the complex of PEG−*g*−PEI and plasmid DNA prepared at $Z = 1$. Concentration of $[poly(dA\text{-}dT)]_2$ added (base pairs) was equal to that of plasmid DNA. As is seen in Figure 3, this resulted in the shift of the position of the band of PEG−*g*−PEI/plasmid complex (compare lanes 2 and 3). The mobility of the resulting complex was the same as that of the PEG−*g*−PEI/plasmid complex at $Z = 0.5$ (lane 4). This suggests that polyion exchange reaction proceeded in the system resulting in the transfer of the copolymer chains from plasmid DNA to the added synthetic

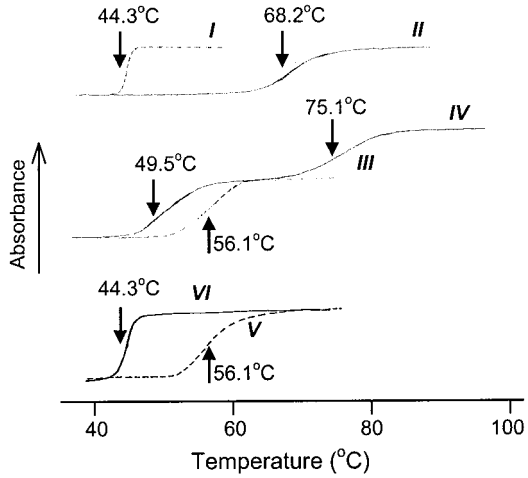

Figure 4. Study of polyion interchange reactions using UV-melting curves. The melting curves were recorded for (*I*) free $[poly(dA\text{-}dT)]_2$, (*II*) *st*DNA, (*III* and *V*) PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex at charge ratio 1, and mixtures of PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex (charge ratio 1) with (*IV*) 1 equiv of *st*DNA, or (*VI*) 1 equiv of heparin.

DNA chains. The equilibrium of this reaction did not depend on the sequence of mixing of the reacting species. Indeed, PEG−*g*−PEI/plasmid complex was formed following addition of one base pair equivalent of plasmid DNA to PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex (lanes 6 and 7).

Study of Polyion Interchange Reactions by DNA Melting. Binding of polycations to DNA increases the temperature of melting of DNA double strands.[7,12] Therefore, the melting curves can be used to study the reactions of polyion coupling and interchange involving DNA. Figure 4 presents the melting curves of several DNA samples and their complexes. Curves I and II correspond to melting of free $[poly(dA\text{-}dT)]_2$ and *st*DNA, respectively. Curve III corresponds to the melting of PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex at $Z = 1$. It is characterized by a helix-coil transition at $T_m = 56.1$ °C, which is almost 12 °C higher than the melting of the free $[poly(dA\text{-}dT)]_2$, ($T_m = 44.3$ °C). Curve IV corresponds to a mixture of *st*DNA and PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex prepared at one base pair equivalent of *st*DNA per base pair of $[poly(dA\text{-}dT)]_2$. In this case, two phase transitions are observed at $T_m = 49.5$ °C and $T_m = 75.1$ °C, which correspond to melting of the double helixes incorporated in PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex at $Z = 0.5$ and PEG−*g*−PEI/*st*DNA complex at $Z = 0.5$, respectively. This suggests that the exchange of the PEG−*g*−PEI chains occurred following addition of *st*DNA to stoichiometric PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$, resulting in even distribution of copolymer between the DNA molecules. The polyion interchange reactions can also proceed irreversibly in one direction. For example, following addition of one charge equivalent of heparin to the stoichiometic PEG−*g*−PEI/$[poly(dA\text{-}dT)]_2$ complex ($T_m = 56.1$ °C, curve V) the DNA is completely substituted by heparin resulting in the release of the free $[poly(dA\text{-}dT)]_2$ ($T_m = 44.3$ °C, curve VI).

Effect of DNA Topology on the Direction Of Polyion Interchange Reaction. The effects of the DNA topology on the direction of the polyion interchange reactions were studied using linearized and supercoiled forms of the plasmid DNA (pCMV-luc). Polyion exchange reactions were observed when *l*DNA was added to PEG−*g*−PEI/*sc*DNA complex or vice versa *sc*DNA was added to the PEG−*g*−PEI/*l*DNA at pH 7.0 (data

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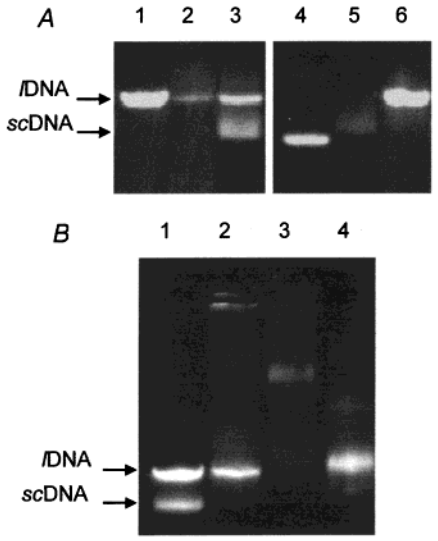

Figure 5. (A) Effect of DNA topology on the direction of polyion interchange reactions. Complexes of corresponding topological forms of pCMV-luc and PEG−*b*−PVP (10 mM sodium phosphate buffer, pH 7.0) were prepared at charge ratio 0.4, incubated at 20 °C for 1 h, then mixed with 1 equiv of the free DNA and incubated for 1 h prior to electrophoresis experiment. Gels present free *l*DNA (lane 1), PEG−*b*−PVP/*l*DNA (lane 2), mixture of PEG−*b*−PVP/*l*DNA with 1 equiv of *sc*DNA (lane 3), *sc*DNA (lane 4), PEG−*b*−PVP/*sc*DNA (lane 5) and mixture of PEG−*b*−PVP/*sc*DNA with 1 equiv of *l*DNA (lane 6). (B) Recognition of DNA topology by cationic copolymers. Equimolar mixture of *sc*DNA and *l*DNA (lane 1) was reacted with PEG−*b*−PVP (lane 2), PEG−*g*−PEI (lanes 3, lane 4), at charge ratio 0.3. Complexes were prepared at pH 7.0 (10 mM sodium phosphate buffer) except for PEG−*b*−PEI (lane 4) prepared at pH 5.0 (10 mM sodium acetate buffer).

not presented). In this system the direction of the reaction did not depend on the order of mixing of DNA samples, and PEG−*g*−PEI chains were evenly distributed between DNA chains, similar to the case of the linear synthetic and plasmid DNA presented above. Totally different behavior was observed in the case of PEG−*b*−PVP/DNA complexes (Figure 5). Upon addition of *sc*DNA to the PEG−*b*−PVP/*l*DNA complex the release of the *l*DNA was observed, suggesting that *sc*DNA displaced *l*DNA in the complex (compare lanes 1, 2, and 3). In contrast, no release of the free *sc*DNA was observed when *l*DNA was added to PEG−*b*−PVP/*sc*DNA complex (compare lanes 4, 5, and 6). This suggested that the polyion exchange reaction involving *sc*DNA and *l*DNA complexes was shifted completely toward formation of the PEG−*b*−PVP/*sc*DNA complex: {PEG−*b*−PVP/*l*DNA} + *sc*DNA → {PEG−*b*−PVP/*sc*DNA} + *l*DNA.

Furthermore, selective binding of PEG−*b*−PVP with *sc*DNA was observed when the copolymer was added to the equimolar mixture of linearized and *sc*DNA. As is shown in Figure 5b, upon addition of PEG−*b*−PVP to this mixture, the copolymer first binds with the supercoiled plasmid while significant amount of *l*DNA still remains free (compare lanes 1 and 2). In contrast, PEG−*g*−PEI at pH 7.0 binds to both linearized and *sc*DNA, resulting in disappearance of the free DNA bands (lane 3). It is noteworthy that the same copolymer, PEG−*g*−PEI, at pH 5.0 (lane 4) has elevated binding with *sc*DNA compared to binding with the *l*DNA. Similar effects of recognition of DNA topology by various polycations were observed using supercoiled and linearized forms of another plasmid (pCR3.0.).

Discussion

This work for the first time demonstrates the transfer of polycation chains between polynucleotide molecules of different types in aqueous media. The use of cationic copolymers containing polycation and PEG blocks facilitates this study by increasing the solubility of the interacting species. Indeed, coupling of DNA with these cationic copolymers results in formation of stable aqueous dispersions, even under the conditions of complete neutralization of the charges of the reacting polyions.[13,14] In such systems, the neutralized DNA and polycation chains form a hydrophobic core of the micelle-like species, while PEG chains form a hydrophilic corona. On the basis of the results of this work it is noteworthy that the reactions of the polyion exchange proceed with the participation of these complexes. This means that PEG chains do not prevent the interaction and the transfer of the reacting polyions.

This work uncovers two major phenomena, which can be illustrated using PEG−*g*−PEI and PEG−*b*−PVP as examples. First, there is a marked difference in the behavior of these cationic copolymers in the polyion coupling reactions with the DNA at pH 7.0. Namely, PEG−*b*−PVP and DNA complexes disproportionate, while PEG−*g*−PEI evenly distributes among DNA chains upon formation of the complexes. Second, those copolymers also reveal distinct behavior in the polyion interchange reactions involving DNA with different tertiary structures. The data show that the direction of the reactions is independent of the DNA structure as long as the topology of the participating DNA chains is not changed. However, PEG−*b*−PVP recognizes the tertiary structure of the DNA and selectively binds with the supercoiled plasmid when both supercoiled and linearized plasmids are present. In contrast, PEG−*g*−PEI does not exhibit such noticeable selectivity and is practically equally distributed between two forms of the DNA present in the mixture at pH 7.0. Both the disproportionation and recognition phenomena are observed when there is an excess of DNA in terms of charge ratio.

Distinct behavior of the copolymers is likely to be due to the differences in their molecular structure. Indeed, while both copolymers have practically the same number of the repeating units in the polycation blocks (46 and 55 units PEI and PVP respectively), PEI is branched and has a relatively hydrophilic backbone, while PVP is linear and have a hydrophobic backbone. Furthermore, the charge density in the case of PEI depends on the pH of the media, while PVP, as a strong polyelectrolyte, is completely charged at any pH. Comparing the behavior of PEG−*g*−PEI at different pH values reinforces the effect of the charge density. Indeed, the change of pH from 7.0 to 5.0, increasing the charge density of PEI due to additional ionization of secondary amino groups of the polycation,[8] is also accompanied by the appearance of both disproportionation and recognition phenomena.

We believe that the recognition phenomenon is dependent on electrostatic complementarity of the interacting chains. Release of the low molecular mass counterions condensed with the reacting polyions provides a favorable entropy contribution which is a driving force for the formation of the polyelectrolyte complexes.[15] The dependence of the direction and equilibrium of the polyion coupling and interchange reactions on the charge density of the reacting species at a constant charge ratio may be due to three major reasons. These reasons include (1) the effect of charge density of cationic copolymer on the release of

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counterions condensed with this copolymer, (2) the effect of charge density of DNA on the release of counterions condensed with DNA, and (3) the effect of charge density of cationic copolymer on the release of counterions condensed with DNA.

When DNA is present in excess, all counterions condensed with the polycation chain are released in the media independently of the charge density of this chain, assuming that each ionized group of the cationic copolymer participates in the formation of salt bonds with DNA. However, since the amount of the condensed counterions increases when the charge density of the polyion elevates, polycations with higher charge density can release more counterions upon binding with DNA (i.e., form more stable complexes) than polycations with lower charge density at the same number of salt bonds formed. On the other hand, it is known than supercoiled *sc*DNA has higher charge density than *l*DNA.[15] Consequently, more counterions are condensed with *sc*DNA and can be released upon binding with the polycations. This should result in a more favorable interaction of polycations with *sc*DNA than with *l*DNA, that is, topology recognition. Finally, the release of the counterions condensed with DNA can depend on the charge density of the copolymer. Indeed, it is possible that displacement of counterions condensed with DNA from the site of binding of a cationic copolymer with low charge density is incomplete. In other words, the system of salt bonds formed between DNA and polycation block have "mismatches" containing unpaired phosphates with some remaining condensed counterions. Conversely, polycation with high charge density, at the same number of salt bonds formed, should displace a greater portion of DNA counterions due to lower number of mismatches. As a result, the recognition phenomenon should be more pronounced in the case of polycations with high charge density, since a greater portion of the counterions condensed with the DNA are released in this case. In contrast, in the case of the polycations with low charge density (i.e., PEG−*g*−PEI at pH 7.0) the portion of the counterions released from the site of polycation binding with the DNA might be too low to provide for substantial distinction between the two forms of plasmid.

Hydrophobic interactions constitute another major contribution to the cooperative binding of DNA and cationic copolymers. Disproportionation has been previously observed during the reactions of polyvinylpyridinium homopolymers with double stranded DNA.[16,17] In those cases, neutralization of the charges of the phosphate groups of the DNA and pyridinium groups of the polycation resulted in the formation of hydrophobic sites, followed by precipitation of the stoichiometric complexes. In the case of PEG−*b*−PVP, precipitation is prevented due to the effects of the PEG chains. However, several PVP chains bound with the DNA can possibly interact with each other, forming hydrophobic clusters, which can explain the disproportionation behavior observed with this copolymer. Disproportionation in PEG−*g*−PEI and DNA mixtures is dependent on the pH of the media, which appears to be due to the change in the degree of protonation of PEI. At pH 7.0, the number of salt bonds formed at the site of attachment of PEI chain to DNA is lower than that at pH 5.0. As a result, these sites should be more hydrated and less hydrophobic, which probably explains the absence of disproportionation with this copolymer at pH 7.0. The tendency for disproportionation during binding of the cationic copolymer with the DNA should also contribute to recognition of the DNA tertiary structure by this copolymer. In this case, the preferential binding of one copolymer chain with *sc*DNA should increase the probability of binding of the next copolymer chain with the same DNA molecule and thus could amplify the recognition effect.

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Conclusions

The phenomenon of recognition of DNA tertiary structure by the synthetic polycation is unprecedented for polyelectrolyte complexes. It is remarkable that the recognition is achieved by using relatively simple chemical structures interacting with the DNA. Furthermore, a relatively small variation in the polycation ionization state can result in drastic changes in recognition and disproportionation behavior. The topological complementarity of the reacting DNA species appears to have a major influence on the direction of the process. While separated from each other, both *l*DNA and *sc*DNA can form complexes with the polycation; in contrast, in mixtures of the two DNA forms, selection of the more topologically acceptable (thermodynamically favorable) partner takes place. As a result, the cationic copolymer binds with the *sc*DNA while its linearized form remains free. This finding uncovers possibilities for the control of the processes of release of DNA incorporated in the complexes with cationic species by altering the DNA topology. Therefore, it is of potential theoretical and practical significance.

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