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Controlled DNA interpolyelectrolyte complex formation or dissociation via stimuli-responsive poly(vinylamine-*co*-*N*-vinylisobutylamide)

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ABSTRACT: Poly(vinylamine-*co*-*N*-vinylisobutylamide) or poly(VAm-*co*-NVIBA) was evaluated for its ability to stabilize double-stranded DNA (dsDNA) with the controlled formation or dissociation of polyion complexes. The poly(VAm-*co*-NVIBA) copolymer consists of the cationic poly(vinylamine) (VAm) that electrostatically binds to the anionic DNA and the thermally responsive poly(*N*-isobutylamide) (NVIBA) that helps limit the strength of the electrostatic interaction and prevents the alteration of the DNA helical structure. Agarose gel electrophoresis showed the successful complexation between dsDNA and poly(VAm-*co*-NVIBA). Moreover, DNA was released from the complex at 65 °C, but not at 25 °C. Thus, the NVIBA component in the copolymer played an important role in controlling the process of complex formation or dissociation according to the pH and temperature. The results showed that the molecular design of polycations with a thermoresponsive part is a potential strategy to allow the controllable formation and dissociation of the copolymer/dsDNA complex while avoiding changes to the DNA helical structure. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43852.

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INTRODUCTION

Deoxyribonucleic acid (DNA) is a double strand of biopolymer that has a sugar phosphate backbone connecting with nucleotides and twisted in the form of a double helix.[1] DNA is important for many applications, from forensic science[2] to medicine. DNA can be obtained in the laboratory from various methods, such as phenol-chloroform extraction[2] and salt extraction.[3] However, these methods might change the DNA conformation. Also, DNA is heat labile. Therefore, it is essential to propose an alternative method for DNA extraction by using a polyelectrolyte complex between polycations and DNA that does not change the DNA conformation and thermally stabilizes the DNA.

Research indicates that polycationic polymers can be used as a condensing agent for DNA because of their ability to bind the negatively charged DNA strand by electrostatic interaction.[4] Formation of a polyelectrolyte complex between polycationic polymers and DNA has been shown to increase the stability of DNA. Thus, DNA will be protected while handling before a forensic investigation or evaluation of genetic information by copying the DNA using polymerase chain reaction (PCR).[5] Moreover, the formation of the polyelectrolyte complex is variable and depends on various parameters, such as pH, temperature, chemical structure, and charge strength.[6]

Several researchers have paid attention to the complex formation of double-stranded DNA (dsDNA) or ribonucleic acid (RNA) with polycations, such as polyethyleneimine[7–9] and poly(L-lysine).[10–12] The reversible transition of DNA might be attainable by increasing the solubility of the complex and reducing the conformational changes of DNA. For instance, Murayama *et al.* regulated the interactions of polycations with DNA by conjugation of the polycations to the electrostatically neutral dextran. Modification of polycations by conjugation with hydrophilic chains was shown to improve the solubility of the dsDNA/polycation complex, leading to prevention of the phase separation of the complex from an aqueous medium. The modification of polycations with

dextran effectively stabilized the DNA duplex[13,14] and triplex[15–18] and suppressed conformational changes in the DNA. Stimuli-responsive polymers[19,20] are also candidates to develop other approaches to form a complex with DNA. For example, Maeda *et al.*[21,22] regulated an antisense DNA carrier by using the thermoresponsive poly(*N*-isopropylacrylamide) [poly(NIPAAm)]. Antisense single-stranded DNA (ssDNA) was bound to poly(NIPAAm) through the methacryloyloxysuccinamide precursor. The thermoresponsive nature of poly(NIPAAm) allows it to reversibly change its structure from a random coil to a globular structure above the lower critical solution temperature (LCST) and vice versa below it. This property can protect the specific binding messenger RNA (mRNA) from degradation.

In this research, we focused on the proper adjustment of the electrostatic interactions of the polycation and DNA and the prevention of formation of a compacted globular DNA complex with an approach to protect DNA at ambient temperature. Poly(vinylamine-*co*-*N*-vinylisobutylamide) or poly(VAm-*co*-NVIBA) copolymer is a copolymer of the polycationic vinylamine and the neutral hydrophilic *N*-vinylisobutylamide.[23,24] By controlling the ratio between these parts, we expect to be able to manipulate the poly(VAm-*co*-NVIBA) copolymer properties so as to have a relatively gentle interaction with DNA. Although it possesses thermosensitivity, it is noteworthy that poly(VAm-*co*-NVIBA) provides a constant solubility at body or ambient temperature because of the LCST variability from 39 °C to over 70 °C. Therefore, it should improve both the solubility of the DNA complex and reduce the conformational change of DNA at ambient temperature. Furthermore, this variable thermoresponsive property can be applied for collecting and thermally stabilizing DNA samples, which is necessary to further evaluate any *in vitro* genetic information. We aim to prove the concept of using a thermoresponsive polymer to protect DNA from denaturing by forming the complex, with the benefit of the controlled release of DNA under simple stimuli conditions. This concept might be applied for DNA extraction.

The work presented here is the first step in evaluating this copolymer and reports on the interactions that determine how the poly(VAm-*co*-NVIBA) copolymer is controlled to form or dissociate the DNA complex. Herein, we report on systematic studies on the poly(VAm-*co*-NVIBA)/DNA complex formation by varying (1) the copolymer/DNA molar ratio and (2) temperature.

EXPERIMENTAL

Materials

Salmon spermary deoxyribonucleic acid sodium salt with a molecular weight in the range of 2×10^7 and 3×10^7 or in the range of 30 and 45 kbp was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and λ DNA Hind III restriction enzyme fragments, ethidium bromide, bromophenol blue, xylene cyanol, and tris(hydroxymethyl)aminomethane (Tris) were supplied by Sigma Chemical Co., Steinheim, Germany. Boric acid and ethylenediaminetetraacetic acid (EDTA) were obtained from Nacali Tesque, Inc., Kyoto Japan. Agarose was a product of Takara, Kyoto, Japan.

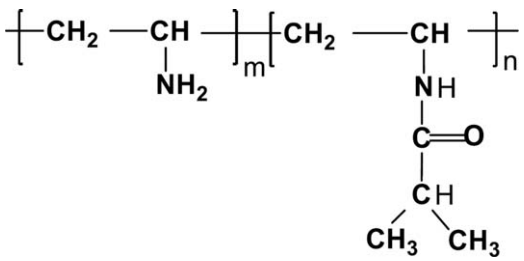

Figure 1. Molecular structure of poly(vinylamine-*co*-vinylisobutylamide) or poly(VAm-*co*-NVIBA).

Preparation of DNA Solution

Because of the limitations of the characterization method, salmon sperm DNA was fragmented in a TE buffer (10 m*M* Tris and 1 m*M* EDTA, pH 7.5) using a Tomy Seiko sonicator (Model UR-200P, Tokyo, Japan) to lower the molecular weight into the range where it could be evaluated. However, the whole DNA information still existed in the form of a fragmented DNA mixture. The relative molecular weight of the sonicated salmon sperm dsDNA was estimated by coresolution against a λ DNA Hind III restriction enzyme fragment by electrophoresis through a 1.0% agarose gel with visualization by ultraviolet (UV) transillumination following ethidium bromide (2 μg/mL) staining. The relative molecular weight of the sonicated DNA was found to be in the range of 100–3000 bps, while the DNA concentration, as determined by measuring the UV absorbance at 260 nm ($\varepsilon_{max} = 6600\ M^{-1}\ cm^{-1}$), was set to 50 μg/mL, corresponding to approximately 135 μ*M* in base pair equivalents. The denaturation or melting temperature (T_m) of the sonicated dsDNA was found to be at around 57 °C. Therefore, poly(VAm-*co*-NVIBA) with a VAm/NVIBA molar ratio that gave a LCST value higher but close to the T_m of DNA was selected so as to optimize the probability of thermally stabilizing the double-stranded DNA.

Synthesis of Poly(VAm-*co*-NVIBA)

Poly(VAm-*co*-NVIBA) was prepared by radical polymerization between poly(*N*-vinylformamide) [poly(NVF)] and poly(NVIBA) in methanol using 2,2′-azobisisobutyronitrile (AIBN) as an initiator at 60 °C for 24 h following the acidic hydrolysis of NVF to VAm. The chemical structure of poly(VAm-*co*-NVIBA) is shown in Figure 1. The molecular weight, polydispersity index (PDI), and LCST of the copolymers are summarized in Table I. The LCST was the minimum temperature that changed the solubility of the polymer solution (e.g., aqueous solution) from soluble to insoluble, that is, the molecular change from random coil (soluble) to compacted globule structure (insoluble). The LCST was defined as 50% transmittance (or turbidity) of the polymer aqueous solution during the heating process. The LCST of poly(VAm-*co*-NVIBA) was measured via a UV–Vis spectrophotometer (JASCO V-550, Tokyo, Japan) at 500 nm using a polymer concentration of 0.2 % w/v. The poly(VAm-*co*-NVIBA) did not show an LCST below pK_a (pK_a = 10), although the copolymer clearly exhibited an LCST and became turbid in pH 12 (above pK_a).[24] Poly(VAm-*co*-NVIBA) [M_n of 310,000, PDI (M_w/M_n) of 2.9] containing 76% NVIBA and 24% VAm content exhibiting an LCST of 60 °C at pH 12.0 was used in this experiment. The proton nuclear magnetic resonance (^{1}H-NMR) of poly(VAm-*co*-NVIBA) was

Table I. Poly(VAm-*co*-NVIBA) Copolymers and Their LCST

	In feed (mol %)				Before acid hydrolysis[a]		After acid hydrolysis[b]		
Run	NVF	NVIBA	$M_n \times 10^{-4}$	PDI	In copolymer (mol %) NVF content	LCST	In copolymer (mol %) VAm content[c]	LCST pH < pK_a[d]	LCST pH > pK_a[d] (pH 12)
0	0	100	2.6	2.4	—	39	—	—	—
1	10	90	2.8	3.4	8	45	8	—	48
2	20	80	3.4	2.8	18	52	18	—	56
3	25	75	3.1	2.9	24	55	24	—	60
4	30	70	3.3	2.7	26	59	26	—	66
5	40	60	3.1	2.5	39	70	39	—	70

[a] Ref. 23.
[b] Ref. 24.
[c] NVF units of copolymers were completely hydrolyzed by 2 *N* HCl.
[d] pK_a of VAm = 10.

characterized by a JEOL GSX-400, Massachusetts, USA (400 MHz). The chemical shifts (ppm) were side-chain methyl protons (1.0–1.2), main-chain methylene protons (1.5–2.0), side-chain methyne protons (2.4–2.6), and main-chain methyne protons (3.2–4.1).

Poly(VAm-*co*-NVIBA)/DNA Complexation and Controlled Dissociation

Poly(VAm-*co*-NVIBA)/DNA complexes were prepared at room temperature with a range of poly(VAm-*co*-NVIBA)/DNA molar ratios (r) between 0 and 10. To achieve this, poly(VAm-*co*-NVIBA), dissolved in the TE buffer (pH 7.5), was added dropwise into the DNA solution, mixed by vortexing, and incubated at room temperature (25 ± 2 °C) for 5 h to allow complete complex formation. To examine the controlled dissociation of DNA from poly(VAm-*co*-NVIBA)/DNA complexes, the pH was adjusted with 0.01 *M* NaOH and 0.01 *M* HCl.

Thermal Denaturation

The UV absorbance spectra and thermal denaturation experiments were performed on a JASCO V-550 model spectrophotometer in a quartz cell with a 1.0 cm path length equipped with a JASCO ETC-505T (Tokyo, Japan) temperature controller. Melting temperatures (T_m) corresponding to 50% transition of the complexes were measured by gradually heating the samples at a rate of 0.5 °C min^{-1} while monitoring the absorbance changes at 260 nm. The investigated interval of temperatures ranged from 25 to 80 °C.

CD Spectroscopy

Circular dichroism (CD) spectra were measured with a JASCO J-725 model CD spectropolarimeter. The CD apparatus consisted of a JASCO PS-450J spectropolarimeter power supply and a 450 W Xe lamp. CD measures the difference in absorption for left and right circularly polarized light by an asymmetric molecule ($\Delta\varepsilon$).

Band Retardation

Poly(VAm-*co*-NVIBA)/DNA complexes were prepared by mixing sonicated DNA of concentration 135 μ*M* in base pairs at various concentration ratios of poly(VAm-*co*-NVIBA). The complex formation was achieved by band retardation through gel electrophoresis. Gel electrophoresis was performed using Mupid mini gel electrophoresis (Seraing, Belgium) at a constant voltage (100 V) on a 1.0% agarose gel with 0.5 *M* TBE buffer (0.5 *M* Tris, 0.5 *M* boric acid, 0.5 *M* EDTA). Then the gel was stained with the intercalating dye of ethidium bromide to visualize the DNA under UV light. Photographs were taken with a Polaroid ACMEL CRT camera M-085D, Oregon, USA with Fuji FP-3000B film, Tokyo, Japan.

RESULTS AND DISCUSSION

Poly(VAm-*co*-NVIBA)/DNA Complexation

Thermal Denaturation. An insight into the effects of DNA stability to thermal denaturation when produced by complexation with the poly(VAm-*co*-NVIBA) copolymer was evaluated by observing the melting behavior of different molar ratio mixtures of this poly(VAm-*co*-NVIBA) copolymer with DNA. Melting curves were obtained by monitoring the absorbance change at 260 nm under a temperature range from 25 °C to 80 °C.

Figure 2 shows the melting behavior of sonicated dsDNA when complexed with poly(VAm-*co*-NVIBA)/DNA complexes with various molar ratios (r) of poly(VAm-*co*-NVIBA)/DNA in the range 0.1–10. The salmon sperm dsDNA was sonicated before use in order to fractionate the dsDNA into smaller molecules. This resulted in a molecular weight range of 100–3000 bps, as estimated by gel electrophoresis. The dsDNA shows a single phase melting step with a rather sharp transition at the melting temperature (T_m) of 57 °C [Figure 2(a)] that is due to the dsDNA transition to single-stranded DNA. Meanwhile, the poly(VAm-*co*-NVIBA) [Figure 1(e)] does not show any transition at 260 nm. The poly(VAm-*co*-NVIBA)/DNA complex derived from $r = 0.1$ [Figure 2(b)] shows a melting profile that is very similar to that of the dsDNA, while that obtained from $r = 1$ [Figure 2(c)] shows a biphasic melting transition. Here, the first transition corresponds to the noncomplexed dsDNA denaturation to ssDNA. The second higher T_m transition represents the complexation between poly(VAm-*co*-NVIBA) and dsDNA causing the thermally stabilized dsDNA to denature to

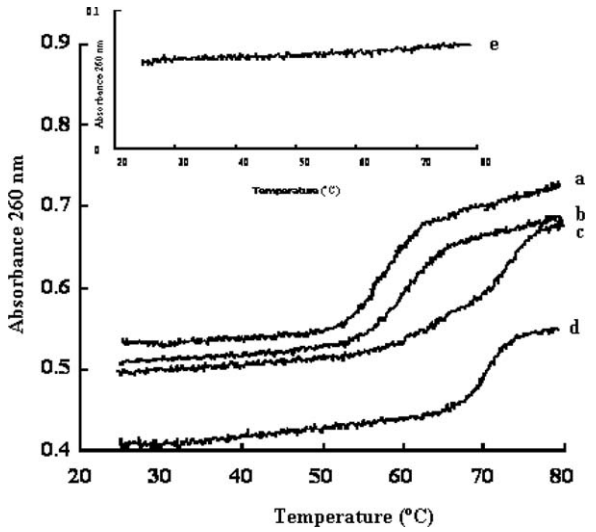

Figure 2. Ultraviolet melting curves of (a) salmon sperm DNA and the poly(VAm-*co*-NVIBA)/DNA complexes with molar ratios of (b) 0.1, (c) 1, and (d) 10, plus the (e) copolymer in TE buffer of pH 7.5.

ssDNA at this higher temperature. The complex formed at an even higher molar ratio of polymer, that is $r = 10$ [Figure 2(d)], showed only a single sharp T transition. This was at a higher temperature than that seen with dsDNA, since all the dsDNA is assumed to have formed a complex with poly(VAm-*co*-NVIBA) and was thermally stabilized, only denaturing to ssDNA at a higher temperature. The mechanism of such increased dsDNA thermal stability is unclear, but it is likely that poly(VAm-*co*-NVIBA) entangles along the dsDNA by ionic interactions between the ammonium ions of poly(-VAm-*co*-NVIBA) (pH 7.5) and the phosphate ions of DNA to form the complex and so, with the increased electrostatic and van der Waals forces, increased the required free energy to melt the complex.

Table II summarizes the T_m corresponding to a 50% transition of the sonicated dsDNA and poly(VAm-*co*-NVIBA)/DNA complexes. The melting temperature (T_m) of DNA, defined as the temperature at the transition midpoint where half of the base pairs are melted, was measured by gradually heating the samples at an increasing rate of 0.5 °C min^{-1} while monitoring the UV absorbance changes at 260 nm (Figure 2).

The UV absorption was found to increase as the dsDNA separates into the two single-stranded DNAs (ssDNA). The dsDNA was observed to have a T_m of 57 °C due to the denaturation of the DNA double helix (Table II, entry 1). The poly(VAm-*co*-NVIBA)/DNA complexes show a higher melting temperature (range 60–73 °C, Table II, entries 2–4) than the dsDNA, which indicates that poly(VAm-*co*-NVIBA) forms a complex with the DNA and protects the dsDNA from denaturing until at these higher temperatures. The poly(VAm-*co*-NVIBA)/DNA complex formed at $r = 0.1$ showed a melting profile that was similar to that of the dsDNA (Figure 2) and exhibits a T_m (Table II, entry 2) close to that of the dsDNA. This implied that most of the dsDNA was free and not complexed. In contrast, a biphasic T_m was observed at a poly(VAm-*co*-NVIBA)/DNA molar ratio of $r = 1$ (Table II, entry 3), where the first T_m corresponded to the free dsDNA denaturation to ssDNA, while the second higher T_m represents the complexation between poly(VAm-*co*-NVIBA) and DNA. This had thermally stabilized the dsDNA from denaturation until at this higher temperature. However, the complex formed at a molar ratio of $r = 10$ only revealed a single T_m (Table II, entry 4), and this was at a higher temperature than that for the dsDNA, suggesting that poly(VAm-*co*-NVIBA) has completely complexed and thermally stabilized all the dsDNA. At this pH (7.5), the poly(VAm-*co*-NVIBA) probably entangles along the dsDNA by ionic interactions between the cationic ammonium ions of poly(VAm-*co*-NVIBA) and the anionic phosphate ions of DNA to form the complex.

CD Spectroscopy.. The DNA conformation was also investigated with a circular dichroism spectropolarimeter. Figure 3 shows the CD spectra of the sonicated dsDNA [Figure 3(a)], the poly(VAm-*co*-NVIBA)/DNA complexes [Figure 3(b–d)], and the poly(VAm-*co*-NVIBA) copolymer alone [Figure 3(e)]. Sonicated dsDNA exhibited the typical B-form duplex with a peak of positive magnitude at approximately 273 nm, whereas the copolymer exhibited a plateau CD spectrum. The poly(VAm-*co*-NVIBA)/DNA polyion complexes also exhibited exactly the same CD pattern as that for the dsDNA, with a peak of positive magnitude at 273 nm, indicating that poly(VAm-*co*-NVIBA) and DNA could form polyion complexes without changing the helical structure of the dsDNA.

The present work shows a rare example for the case that even when a large molar ratio of copolymer is added to a DNA solution, it does not affect the DNA double-helical structure. This may be because the NVIBA part of the copolymer interferes with the close-contact electrostatic interactions between the positively charged VAm part of the copolymer and the negatively charged phosphate groups of the DNA.

Band Retardation. The notion of complex formation between the poly(VAm-*co*-NVIBA) copolymer and dsDNA was supported by the observed band retardation through gel electrophoresis on a 1.0% (w/v) agarose gel in 0.5 *M* TBE buffer (0.5 *M* Tris, 0.5 *M* boric acid, 0.5 *M* EDTA). The reason for this is that for an equal charge density, the smaller DNA molecules have a higher ability to move through the sieve like agarose under an electric field to the anode than the larger molecules. Of course, any reduction in the net negative charge density,

Table II. T_m of Sonicated dsDNA and of Poly(VAm-*co*-NVIBA)/DNA Complexes

Entry	Samples	r = [copolymer]/[DNA]	T_m (°C)
1	Sonicated DNA[a]	0	57
2	Poly(VAm-co-NVIBA)[b]/DNA complex	0.1	60
3		1	63, 73
4		10	70

[a] Molecular weight 100–3000 bps.
[b] Molecular weight 290,000.
[c] Obtained from the melting curves of each complex.

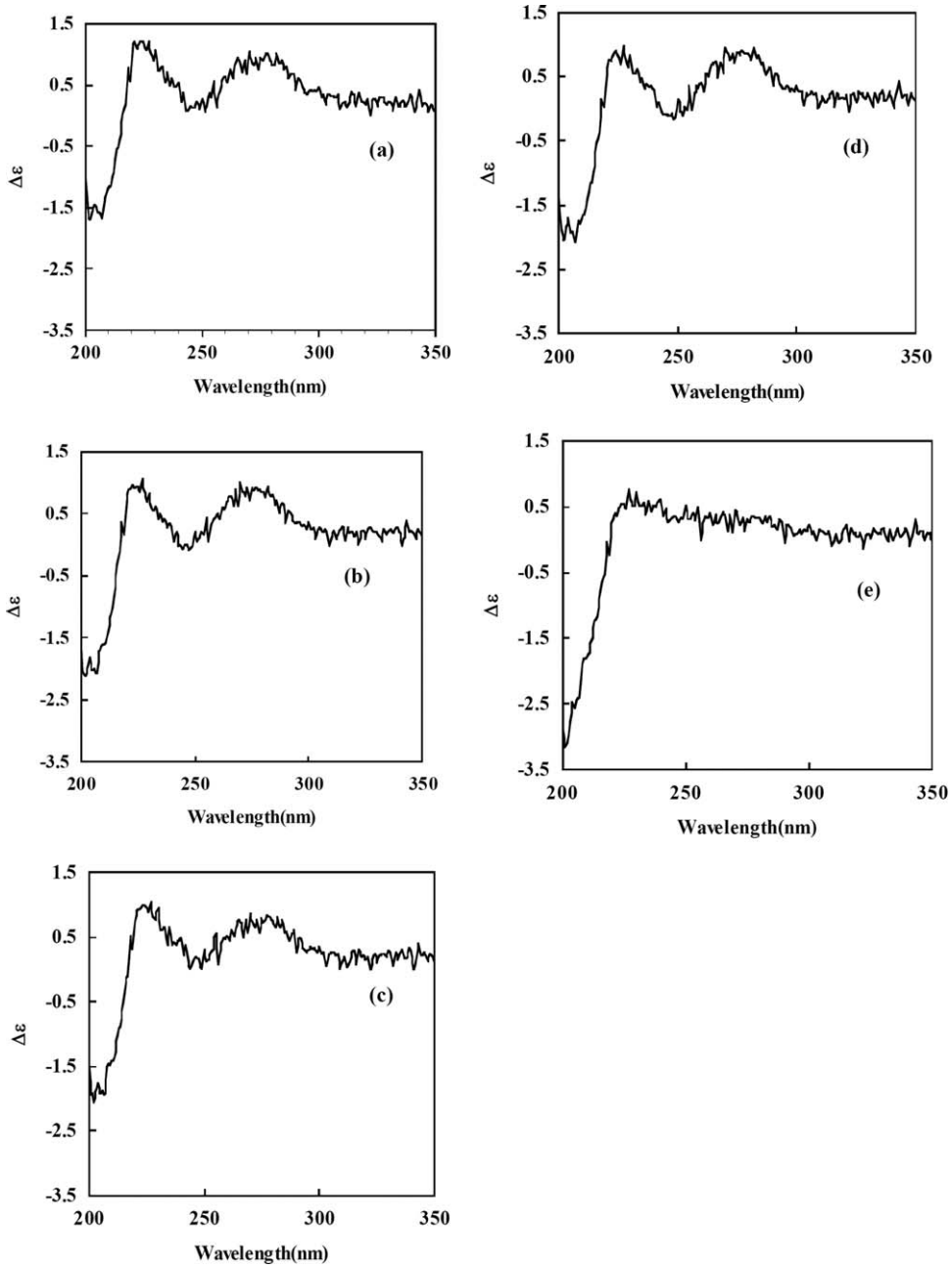

Figure 3. CD spectra of salmon sperm DNA (a) and poly(VAm-*co*-NVIBA)/DNA complexes with molar ratios of (b) 0.1, (c) 1, and (d) 10, plus the (e) copolymer in TE buffer of pH 7.5.

such as binding of the polycationic poly(VAm-*co*-NVIBA) copolymer, will also retard the relative migration rate through the gel. Therefore, gel electrophoresis was used to relate the relative migration rate (DNA band retardation) as a readout of DNA complexation to the poly(VAm-*co*-NVIBA) copolymer.

Figure 4 shows a typical agarose gel eletrophoresis result for the poly(VAm-*co*-NVIBA)/DNA complexes formed at room temperature. After electrophoresis, the gel was stained with ethidium bromide (2 μg/mL in TBE), and the DNA was visualized under UV transillumination. Relative to the λ DNA Hind III restriction enzyme fragments (lane 1), used as a discrete, known-size marker, the low molecular weight (fragmented by sonication) of salmon dsDNA shows high mobility and exhibits a continuous band in the range of 100–3000 bps (lane 2). The DNA mobility was then retarded as the molar ratio ($r = 0.1$–10) of poly(VAm-*co*-NVIBA)/DNA was increased from almost no discernable change at $r = 0.1$ (lane 3) to a loss of small fragments and an increased level of a large-mass DNA complex that cannot penetrate the gel at $r = 1$ (lane 4) and finally to only a large DNA complex that cannot penetrate the gel at $r = 10$ (lane 5). This retardation of the DNA, including the inability to penetrate the gel, is principally due to the increase in molecular weight caused by the complexation with the copolymer, but also as well by a decrease in the net anionic charge density of the complex brought about by the electrostatic

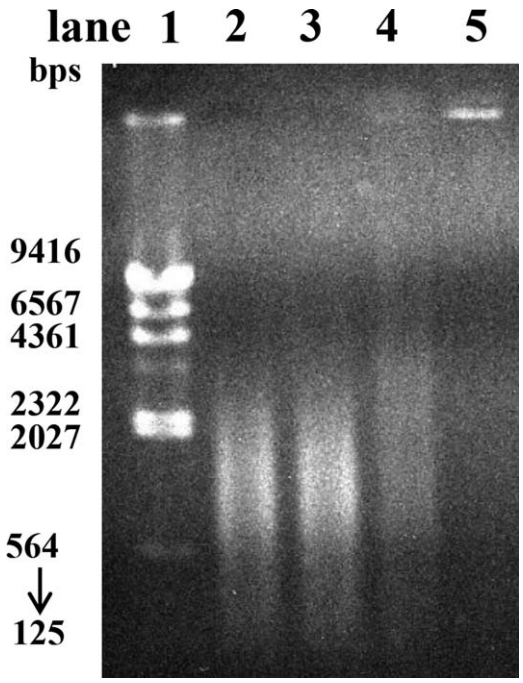

Figure 4. Electrophoresis of poly(VAm-*co*-NVIBA)/DNA complexes through a 1% (w/v) agarose gel. DNA was mixed with an increasing amount of poly(VAm-*co*-NVIBA). Lane 1: λ DNA Hind III restriction enzyme fragments; lane 2: sonicated DNA; lanes 3–5: poly(VAm-*co*-NVIBA)/dsDNA complex formed at molar ratios of $r = 0.1$, 1, and 10 for lanes 3, 4, and 5, respectively.

neutralization of the negatively charged phosphate groups of the DNA with the positively charged amino groups of the copolymer. Since DNA band retardation is the method used to visualize DNA by staining DNA with ethidium bromide, the excess amount of poly(VAm-*co*-NVIBA) that did not form a complex would not be disturbed in the visualization of the DNA band.

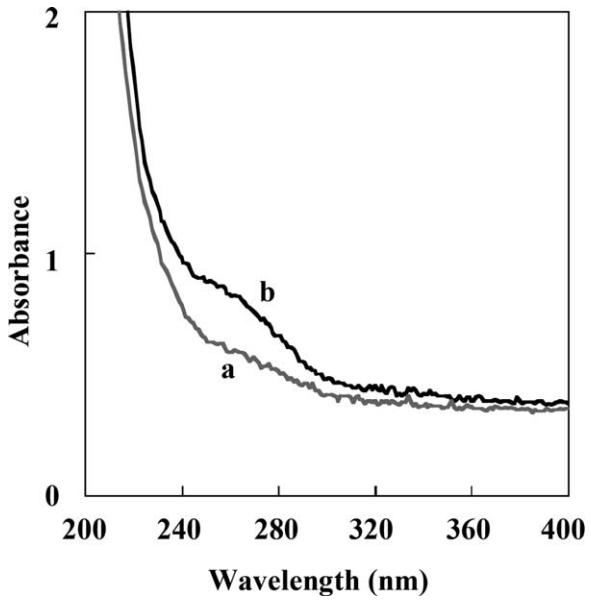

Figure 5. UV spectra of poly(VAm-*co*-NVIBA)/DNA complexes ($r = 10$) in an environmental pH 12 at (a) 25 °C and (b) 65 °C.

Controlled Dissociation of DNA from a Poly(VAm-*co*-NVIBA)/DNA Complex

It was of interest that the complexation between poly(VAm-*co*-NVIBA) was potentially thermally controllable due to the structural changes of the copolymer moiety from its stimuli-responsive properties, that is, the temperature-dependent conformational changes between a random coil and a globular structure and its pH-dependent changes in positive charge density that will affect its ability to interact with DNA. Indeed, the ability to bind DNA should be dramatically reduced as the pH value approaches and then exceeds that of its pK_a. At a pH > pK_a, the poly(VAm-*co*-NVIBA) copolymer showed an LCST of around 60 °C and changes its structure from a random coil to a globule state. Figure 5 shows the UV spectra of the poly(VAm-*co*-NVIBA)/DNA complex formed from a molar ratio (r) of 10, at a pH of 12 and at either 25 °C or 65 °C. No UV adsorption of the DNA was observed at 25 °C, implying the stability of the complex at this temperature. On the other hand, the absorbance of the DNA increased at 65 °C, which was due to the changes in copolymer structure from a random coil to a globule state, causing the release of the dsDNA.

Thus, the complex formation and dissociation is a controllable process. Therefore, poly(VAm-*co*-NVIBA) is capable of stabilizing the double-helix DNA without seemingly interfering with the nucleotide recognition and assembly. Interestingly, the complex formation or dissociation temperature and pH can possibly be adjusted by varying the VAm and NVIBA contents. Therefore, the molecular design of polycations with a thermoresponsive part is a potentially successful approach for the design of novel DNA protection and extraction methods.

CONCLUSIONS

In this study, the thermal and pH stimuli-responsive poly(VAm-*co*-NVIBA) copolymer consisting of defined molar ratios of VAm and NVIBA was used to evaluate complex formation or dissociation between a poly(VAm-*co*-NVIBA) copolymer and sonicated salmon sperm dsDNA. The polyion complexes, prepared with various poly(VAm-*co*-NVIBA)/DNA molar ratios (r) in a TE buffer of pH 7.5 at room temperature, showed complete complex formation at $r = 10$. Poly(VAm-*co*-NVIBA) formed a soluble complex with DNA without interfering with the DNA double-helix structure. This complex was very stable at ambient conditions, while the process was controllable, with complex dissociation controlled by the pH and temperature.

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