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Functional Monomers and Polymers CLXXI.† Synthesis and Interaction Studies on Water Soluble Nucleic Acid Analogs: Polyethyleneimine Derivatives Containing Cytosine and Hypoxanthine

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ABSTRACT: Nucleic acid analogs were prepared by grafting cytosine and hypoxanthine derivatives containing hydroxyl groups onto linear polyethyleneimine. The polymers contained about 90 unit mol% of the nucleic acid bases, and were easily soluble in water at neutral pH value. They were found to form polymer complexes with polynucleotides (PolyI and PolyC) by specific base-base interactions in aqueous solution.

KEY WORDS Nucleic Acid Analogs / Water Soluble Polymer / Cytosine / Hypoxanthine / Polyethyleneimine / Homoserine / PolyI / PolyC / Polymer Complex / Specific Interaction /

Synthetic polynucleotides are known to have biological activity. A synthetic double stranded polynucleotide complex of polyinosinic acid (PolyI) and polycytidylic acid (PolyC) (PolyI–PolyC Complex) is effective as an interferon inducer,[1-5] while it has a high level of toxicity.[6] On the other hand, a number of synthetic polymers containing nucleic acid bases have been prepared and their properties studied. These synthetic nucleic acid analogs are very stable and have been found to have biological activity.[7-17] Therefore a polymer complex of polynucleotides with the synthetic nucleic acid analogs may be stable and be expected to have enhanced bioactivity.[18]

The synthetic nucleic acid analogs, however, were hardly soluble in water at neutral pH values, because most of them consist of a hydrophobic polymer backbone and pendant nucleic acid bases. It is, therefore, very important to prepare water soluble synthetic nucleic acid analogs in order to apply the analogs to biological fields. The present article deals with the preparation of water soluble polyethyleneimine derivatives containing cytosine and hypoxanthine, interaction between these polymers and their interactions with PolyI or PolyC in aqueous solution. The bioactivity of polymer complex of the nucleic acid analogs and with polynucleotides will be shown elsewhere.[19]

EXPERIMENTAL

Materials

Ethyl 3-(Cytosyl-1-yl)propionate (**1C**)

To a suspension of N^4-acetyl cytosine[20] (27 g; 180 mmol) in ethanol (800 ml) with catalytic amount of sodium, ethyl acrylate (28 ml; 260 mmol) was added dropwise with stirring. The reaction was carried out at 90°C for 5 h to

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afford a clear solution. After the reaction, the solvent was removed under reduced pressure. The residue was recrystallized from ethanol to give ethyl 3-(cytosyl-1-yl)propionate (**1C**) in 94% yield (35 g); mp 199—201°C. IR (KBr, cm^{-1}): 1730 (ester), 1650, and 1610 (cytosine). ^1H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 1.10 (t, 3H), 2.50 (t, 2H), 3.90 (m, 4H), 5.55 (d, 1H), and 7.45 (d, 1H).

Anal. Calcd for $C_9H_{13}N_3O_3$: C, 51.17%; H, 6.20%; N, 22.73%. Found: C, 51.06%; H, 6.26%; N, 22.77%.

3-(Cytosyl-1-yl)propionic Acid (**2C**; *1-β-Carboxyethyl cytosine*)

The ester **1C** (1.7 g; 6.7 mmol) was hydrolyzed by 1 *N* NaOH aqueous solution at 60°C for 2 h. After the reaction, pH of the solution was adjusted to 5.0 by 1 *N* HCl to give a white precipitate. The precipitate was collected and recrystallized from water to give 3-(cytosyl-1-yl)propionic acid (**2C**) in 80% yield (960 mg); mp 264—266°C (dec). IR (KBr, cm^{-1}): 1710 (C=O), and 1650 (cytosine). ^1H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 2.60 (t, 2H), 3.85 (t, 2H), 5.65 (d, 1H), and 7.55 (d, 1H).

Anal. Calcd for $C_7H_9N_3O_3$: C, 45.90%; H, 4.95%; N, 22.94%. Found: C, 46.09%; H, 5.04%; N, 22.30%.

(±)-α-*N*-[*3-(Cytosyl-1-yl)propionyl*]*amino-γ-butyrolactone* (**4C**; Cyt-Hse-L)

To a solution of the 1-β-carboxyethyl cytosine (1.3 g; 7.0 mmol) in *N*,*N*-dimethylformamide (40 ml), pentachlorophenyl trichloroacetate (4.5 g; 11.0 mmol) and catalytic amount of triethylamine were added. The mixture was stirred at 80°C overnight. The precipitate was filtered and washed with diethylether to give 2.7 g of pentachlorophenyl 3-(cytosyl-1-yl) propionate (**3C**) in 90% yield. To a solution of (±)-α-amino-γ-butyrolactone hydrobromide (0.91 g; 5.0 mmol) in *N*,*N*-dimethyl formamide (20 ml), pentachlorophenyl 3-(cytosyl-1-yl)propionate (**4C**) (2.2 g; 5.0 mmol), triethylamine (0.7 ml; 5.0 mmol), and imidazole (340 mg; 5.0 mmol) were added and the reaction was carried out for one day at 50°C. After the reaction, the solvent was evaporated under reduced pressure. Acetone (150 ml) was added to the oily residue and stirred for 4 h to give a light brown precipitate. The precipitate was washed with water (50 ml) and dried under reduced pressure. The yield was 1.2 g (88%); mp 236—238°C. IR (KBr, cm^{-1}): 1770 (lactone), 1650, 1530 (amide), 1670, and 1620 (cytosine). Thin layer chromatography (TLC): *Rf* 0.44 (benzene–ethanol = 3 : 1). ^1H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 2.50 (t, 2H), 3.90 (t, 2H), 4.40 (m, 4H), 4.70 (m, 1H), 5.60 (d, 1H), 7.55 (d, 1H), and 8.45 (d, 1H).

Anal. Calcd for $C_{11}H_{14}N_4O_4$: C, 49.63%; H, 5.30%; N, 21.04%. Found: C, 48.93%; H, 5.40%; N, 21.31%.

(±)-α-*N*-[*3-(Hypoxanthyl-9-yl)propionyl*]-*amino-γ-butyrolactone* (*4H; Hyp-Hse-L*)

To a solution of (±)-α-amino-γ-butyrolactone hydrobromide (910 mg; 5.0 mmol) in *N*,*N*-dimethylformamide (20 ml), pentachlorophenyl 3-(hypoxanthyl-9-yl)propionate[21] (2.3 g; 5.0 mmol), triethylamine (0.7 ml; 5.0 mmol) and imidazole (340 mg; 5.0 mmol) were added. The mixture was stirred at 40°C for 20 h to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed with acetone and then with water. Recrystallization from ethanol–benzene gave crystals in 79% yield (1.1 g); mp 218—221°C. IR (KBr, cm^{-1}): 1770 (lactone), 1650, 1530 (amide), and 1680 (hypoxanthine). TLC: *Rf* 0.53 (benzene–ethanol = 3 : 1). ^1H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 2.60 (t, 2H), 4.20 (t, 2H), 4.50 (m, 4H), 4.70 (s, 1H), 7.80 (s, 1H), 8.00 (s, 1H), 8.50 (d, 1H), and 12.35 (s, 1H).

Anal. Calcd for $C_{17}H_{13}N_5O_3$: C, 38.83%; H, 4.07%; N, 22.63%. Found: C, 38.65%; H, 3.98%; N, 22.65%.

Polyethyleneimine (PEI)

Poly(2-ethyl-2-oxazoline) (PEOX presented by Dow Chemical Japan Co., M.W. = 50000, 40 g) was hydrolyzed in 6 *N* hydrochloric acid (200 ml) at 90°C for 48 h. After the reaction, the white precipitate was filtered and washed with 200 ml of ethanol to give polyethyleneimine hydrochloride. Then the polymer was dissolved in water (200 ml), and neutralized by NaOH (25 g; 0.36 mmol) at 90°C for 24 h. The solution was cooled in ice bath to give the polymer as a precipitate. The polymer was filtered and washed with 3 l of water and dried. The hydrolysis was found to be completed from NMR and IR spectra.[22]

Poly-N-[{2-[3-(cytosyl-1-yl)propionyl]amino-4-hydroxy}butanoyl]ethyleneimine (**5C**; *PEI-Hse-Cyt*)

To a suspension of Cyt-Hse-L (**4C**) (390 mg; 1.5 mmol) in water (5 ml), 1 *N* NaOH aqueous solution (1.5 ml; 1.5 mmol) was added. The mixture was stirred at 40°C for 1 h to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The resulting residue was washed with acetone (20 ml), then with diethyl ether (30 ml) to give a sodium salt of cytosine derivative (Cyt-Hse-Na) in 98% yield (430 mg, powder). TLC: *Rf* 0.00 (benzene–ethanol = 3 : 1), (ethanol), (*n*-butanol–acetic acid–water = 4 : 5 : 3).

To a suspension of Cyt-Hse-Na (430 mg; 1.5 mmol) in 10 ml of dry *N,N*-dimethylformamide, pentachlorophenyl trichloroacetate (910 mg; 2.2 mmol) and a catalytic amount of triethylamine were added. The mixture was stirred at 0°C for 2 h and then at 50°C until evolution of gas ceased to afford a light brown clear solution. After the reaction, the solvent was evaporated under reduced pressure. The residue was washed with diethyl ether (30 ml) to give a powder of pentachlorophenyl ester of cytosine derivative (Cyt-Hse-PCP) (yield 92%; 720 mg). TLC: *Rf* 0.82 (benzene–ethanol = 3 : 1).

To a solution of polyethyleneimine (70 mg; 1.5 mmol) in *N,N*-dimethylformamide (10 ml), Cyt-Hse-PCP (720 mg; 1.5 mmol) and imidazole (90 mg; 1.5 mmol) were added and stirred at 60°C for 48 h. After the reaction, the solution was concentrated under reduced pressure and poured into excess acetone to precipitate the polymer. The obtained polymer was purified by reprecipitation from *N,N*-dimethylformamide into excess acetone. The polymer was dissolved again in a small amount of water and freeze-dried to give a light brown powder. (360 mg, 78%); mp 210—215°C. IR (KBr, cm^{-1}): 1650 and 1550 (amide). TLC: *Rf* 0.0 (benzene–ethanol = 3 : 1), (ethanol). ^1H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 2.50 (t, 2H), 3.70 (m, 8H), 4.30 (m, 3H), 5.65 (d, 1H), 7.50 (d, 1H), and 8.60 (s, 1H).

Poly-N-[{2-[3-(hypoxanthyl-9-yl)propionyl]-amino-4-hydroxy}butanoyl]ethyleneimine (**5H**; *PEI-Hse-Hyp*)

This polymer was prepared from **4H** (1.0 g; 3.6 mmol) and polyethyleneimine (160 mg; 3.6 mmol) according to a similar procedure described for PEI-Hse-Cyt (**5C**) (yield 68%; 820 mg; mp 166—172°C). IR (KBr, cm^{-1}): 1650 and 1550 (amide). ^1H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 2.60 (t, 2H), 3.70 (m, 8H), 4.30 (m, 3H), 7.80 (s, 1H), 8.00 (s, 1H), and 8.60 (s, 1H).

Hydrolysis of the Polymer

The polyethyleneimine derivatives were hydrolyzed in 6 *N* hydrochloric acid at 80°C for 48 h, into polyethyleneimine hydrochloride and the carboxyethyl derivatives of nucleic acid bases. Quantitative calculation was made using the corresponding carboxyethyl derivatives as standard samples.

The nucleic acid base content in the poly-

Table I. Contents of PEI-Hse-Cyt and PEI-Hse-Hyp

	PEI-Hse-Cyt	PEI-Hse-Hyp
Contents (%)	86	92

mers is tabulated in Table I.

Interactions between the Polymers

Interactions of the polymers were determined from hypochromicity values in UV spectra as reported previously.[23] The UV spectra were measured with a JASCO UV-660 spectrometer equipped with a temperature controller at 20°C. PolyI (sodium salt) ($S^0_{20,w}$: 6—12), PolyC (sodium salt) ($S^0_{20,w}$: 6—12) were obtained from Yamasa Shoyu Co., Ltd. PEI-Hse-Cyt (**5C**), PEI-Hse-Hyp (**5H**) and polynucleotides were dissolved in Kolthoff buffer (pH 7.0) (1/10 M KH_2PO_4–1/20 M $Na_2B_4O_7 \cdot 10H_2O$). These solutions stocked for 2 days at 20 C, were mixed to give a polymer mixture of 10^{-4} M total concentration of nucleic acid base units in aqueous solution.

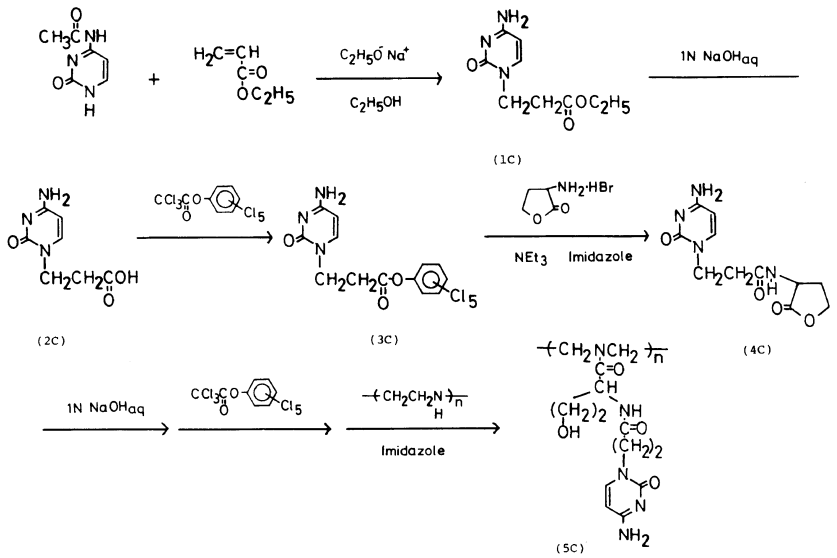
RESULTS AND DISCUSSION

Various kinds of synthetic nucleic acid analogs have been prepared. Most of these analogs, however, are not soluble in water, while polynucleotides are freely soluble in water. Therefore, interaction studies on nucleic acid analogs are limited in organic solvents or in mixture of water and organic solvents. A water soluble nucleic acid analog makes it possible to study interactions of a polymer with polynucleotides in an aqueous solution and biological activity of the analogs.

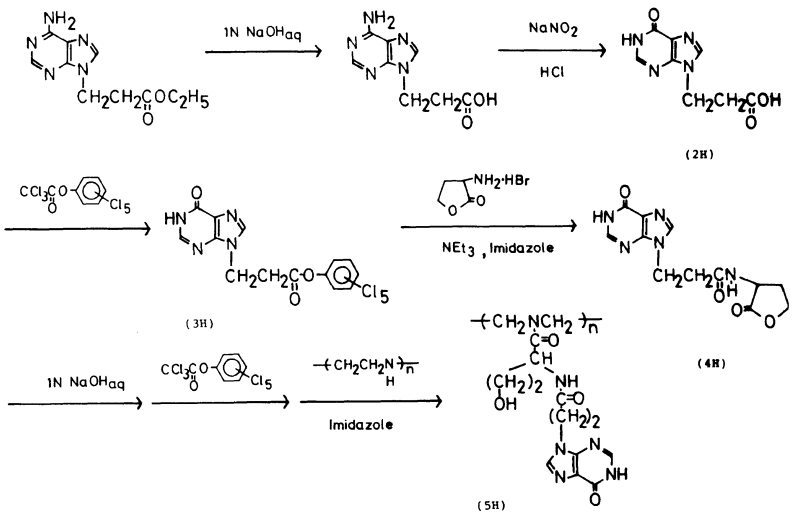
Overberger and Inaki prepared polyethyleneimine derivatives containing nucleic acid bases and amino acids.[24] One of these derivatives was a polyethyleneimine having a nucleic acid base and serine soluble in water. In the present study, water soluble polyethyleneimine derivatives containing nucleic acid bases were prepared. The derivatives contained homoserine units as a spacer between the polyethyleneimine and the nucleic acid base (Scheme 1 and Scheme 2).

Homoserine Derivatives of Nucleic Acid Bases

Starting with cytosine or adenine, the carboxyethyl derivatives of the nucleic acid bases were prepared by Michael type addition reaction of ethyl acrylate followed by hydrolysis. In the case of cytosine, the 4-amino group was protected by the acetyl group because of low solubility in ethanol, although the acetyl group was removed during the reaction. In the case of hypoxanthine, the addition occurred at the



Scheme 1.



Scheme 2.

7-position of the base. Therefore, the carboethoxyethyl derivative of hypoxanthine was prepared by the deamination reaction of carboxyethyl derivatives of adenine.[21]

The carboxyethyl derivatives of cytosine or hypoxanthine were reacted with (±)-α-amino-γ-butyrolactone hydrobromide to give γ-butyrolactone derivatives of cytosine or hypoxanthine. For the reactions, pentachlorophenyl ester derivatives were used with imidazole as a catalyst.[25] It was not necessary to protect the amino group of cytosine base.

Grafting onto Polyethyleneimine

The grafting of nucleic acid base derivatives having hydroxyl group onto polyethyleneimine backbone was carried out by the activated ester method. Since the reactivity of the γ-lactone is low, direct reactions of the lactone derivative with polyethyleneimine hardly occurred. Therefore, the lactone derivatives were hydrolyzed to 3-hydroxybutylic acid derivatives, followed by condensation with polyethyleneimine using the activated ester method. The grafting reaction was carried out in *N*,*N*-dimethylformamide, where a small amount of 4-pyrodino pyridine was an effective catalyst.

The nucleic acid base content of the polymer was determined by UV spectroscopy of the hydrolyzed sample. Quantitative calculations were made using the corresponding carboxyethyl derivatives as standard samples, and are tabulated in Table I.

Interactions of the Polymers

Natural and synthetic polynucleotides are known to form polymer complexes by specific base–base interactions with nucleic acid bases. Synthetic nucleic acid analogs such as polyethyleneimine and polylysine derivatives containing nucleic acid bases were also found to form polymer complexes with polynucleotides by specific base–base interactions.[7,8,26–28] Most of these nucleic acid analogs are slightly soluble in water. Therefore, these studies were carried out in organic solvents or water–organic mixed solvents, such as dimethyl sulfoxide, ethylene glycol, and water–propylene glycol.

The water soluble polyethyleneimine derivatives of cytosine or hypoxanthine make it possible to study interactions with polynucleotides in neutral aqueous solution. Formation of the polymer complex was observed for the PEI-Hse-Cyt–PEI-Hse-Hyp sys-

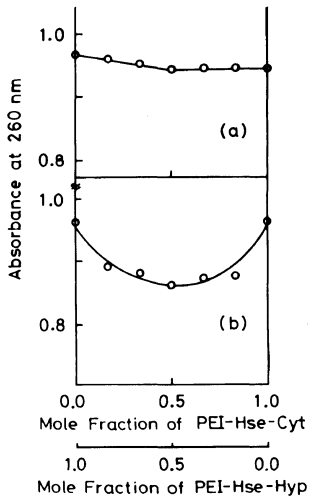


Figure 1. Continuous variation curve of PEI-Hse-Hyp and PEI-Hse-Cyt. Absorbance at 260 nm after (a) 3 h and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20°C. [PEI-Hse-Hyp] = 9.5×10^{-5} mol l^{-1}, and [PEI-Hse-Cyt] = 9.6×10^{-5} mol l^{-1}.

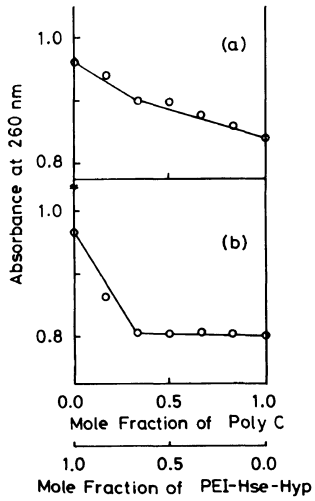


Figure 2. Continuous variation curve of PEI-Hse-Hyp and PolyC. Absorbance at 260 nm after (a) 3 h and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20°C. [PEI-Hse-Hyp] = 9.5×10^{-5} mol l^{-1}, and [PolyC] = 1.2×10^{-4} mol l^{-1}.

tem, and for polyethyleneimine derivatives–polynucleotides (PolyI, PolyC) systems.

Interactions of PEI-Hse-Cyt and PEI-Hse-Hyp

Figure 1 shows the mixing curve between PEI-Hse-Cyt (**5C**) and PEI-Hse-Hyp (**5H**) in the Kolthoff buffer solution (pH 7.0). The figure shows the highest hypochromicity value at a base unit ratio of about 1 : 1 (hypoxanthine–cytosine), suggesting the formation of a stable 1 : 1 polymer complex due to complementary nucleic acid base interactions. The hypochromicity value (21%) was higher than that of the PEI-Ade–PEI-Thy system in the literature.[26] As shown in Figure 1, time dependence of the hypochromicity value was observed. Therefore the conformational change of synthetic polymers may be important for formation of a stable polymer complex.[21,29]

Interactions of PEI-Hse-Hyp with PolyC

Interactions between PEI-Hse-Hyp (**5H**) and PolyC which contains the complementary nucleic acid base can be clearly observed in aqueous solution at pH 7.0, as shown in Figure 2. The overall stoichiometry of the complex based on the nucleic acid base units was approximately 2 : 1 (hypoxanthine–cytosine) under the conditions used. The maximum hypochromicity value (26%) was smaller than that of the PolyI–PolyC (33%) system but higher than the values of other synthetic polymer analog–polynucleotides systems.[7,26]

Formation of the PolyI–PolyC polymer complex was also studied under the same conditions used in this study (Kolthoff buffer solution). The stoichiometry of the complex based on the nucleic acid base units was 2 : 1 (hypoxanthine–cytosine) as in Figure 3. The maximum hypochromicity value of this system was 33%. The stoichiometry of PolyI–PolyC complex is reported to be 1 : 1 in Tris–HCl buffer solution.[30] It is known that the concentration of salt affects the stoichiometry of the PolyI–PolyC complex.[30-31] Therefore, the salt concentration may affect the stoichiometry observed for the PEI-Hse-Hyp–PolyC system in our case.

Another factor controlling the stoichiometry of the complex may be the structure

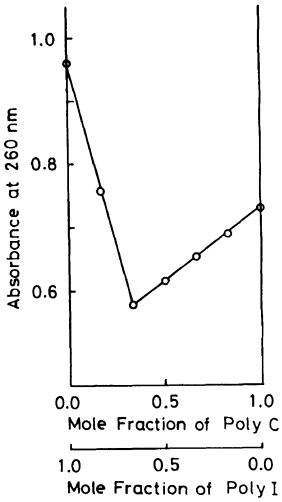


Figure 3. Continuous variation curve of PolyI and PolyC. Absorbance at 260 nm in 0.05 M Kolthoff buffer solution (pH 7.0) at 20°C. [PolyC] = 1.2×10^{-4} mol l^{-1}, and [PolyC] = 1.1×10^{-4} mol l^{-1}

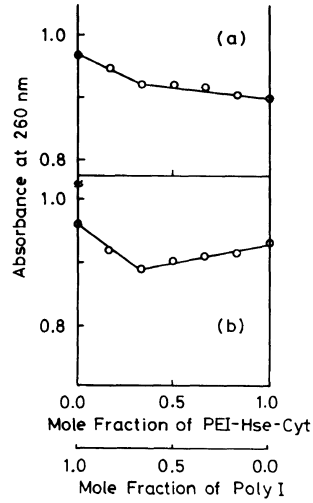


Figure 4. Continuous variation curve of PEI-Hse-Cyt and PolyI. Absorbance at 260 nm after (a) 3 h and (b) 3 days in 0.05 M Kolthoff buffer solution (pH 7.0) at 20°C. [PolyI] = 1.2×10^{-4} mol l^{-1}, and [PEI-Hse-Cyt] = 9.6×10^{-5} mol l^{-1}.

of the polymers. The base–base distance in PEI-Hse-Hyp is different from that in PolyC. This fact also affects the abnormal stoichiometry of the PEI-Hse-Hyp–PolyC complex.

Self association of the nucleic acid bases in the polymer is also an important factor for the formation of the polymer complex. The rate of complex formation for polyethyleneimine derivatives was slow compared with that of polynucleotide systems. The absorbance of a mixed solution of the polymers decreased slowly and became constant after 3 days. This was caused by self association of the nucleic acid bases in the polyethyleneimine derivatives, which dissociated slowly to form the intermolecular polymer complex.

Interactions of PEI-Hse-Cyt and PolyI

Figure 4 shows the mixing curve between PEI-Hse-Cyt (**5C**) and PolyI after 3 h and 3 days at pH 7.0 in the Kolthoff buffer aqueous solution. The stoichiometry of the complex was 2 : 1 (hypoxanthine–cytosine) and the hypochromicity value was 7%. The stoichiometry of the PEI-Hse-Cyt–PolyI complex was similar to that of PEI-Hse-Hyp–PolyC system.

The hypochromicity value was small compared to that of the PEI-Hse-Hyp–PolyC system. Polynucleotides containing purine base form very stable structures by self association in aqueous solution, while the structures of polynucleotides containing pyrimidine bases are not stable in aqueous solution. PolyI forms stable structures by self association in aqueous solution. Therefore, the intermolecular interactions of PolyI with PEI-Hse-Cyt become small because of enhanced intramolecular interactions of hypoxanthine bases in PolyI.[32]

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