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Synthesis and Application of Polyelectrolyte Graft Copolymers as DNA Carrier

Tokyo Institute of Technology

1999

Shoichiro Asayama

CONTENTS

ABBREVIATIONS

DDS, drug delivery systems; PLL, poly(L-lysine); PDEAEMA, poly[2-(diethylamino)ethyl methacrylate]; DMF, *N*,*N*-dimethylformamide; NCA, N^{α}-carboxyanhydride; CBZ, carbobenzoxy group; V-65, 2,2'-azobis(2,4-dimethylvaleronitrile); GPC, gel permeation chromatography; CD, circular dichroism; P_n, number-average degree of polymerization; PVIm, poly(1-vinylimidazole); PLL·Lac, "lactosylated" poly(L-lysine); HA, hyaluronic acid; SEC, liver sinusoidal endothelial cell; RI, refractive index; LS, light scattering; M_n, number-average molecular weight; M_w, weight-average molecular weight; ASGP-R, asialoglycoprotein receptor; PVLA, poly[*N*-(*p*-vinylbenzyl)-4-*O*-β-D-galactopyranosyl-D-gluconamide] or poly(vinylbenzyl-β-D-lactonamide); PAAm, polyacrylamide; THF, tetrahydrofuran; OPD, *o*-phenylenediamine dihydrochloride; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; VBA, *p*-vinylbenzylamine; TFA, trifluoroacetic acid; TEA, triethylamine; TNBS, trinitrobenzenesulfonic acid; ELISA, enzyme-linked immunosorbent assay; PLLS, succinylated poly(L-lysine); A_{274}, absorbance at 274 nm.

Chapter 1

General Introduction

Bioconjugate chemistry is a descriptive term for the joining of two different molecular functions by chemical or biological means (*1*). This includes, among other topics, the following:

Conjugation of...

antibodies (and their fragments)

nucleic acids and their analogs

liposomal components

other biologically active molecules (receptor–binding proteins, polysaccharides, hormones, peptides, ...)

with each other or with any molecular groups that add useful properties...

drugs, radionuclides, toxins, fluorophores, photoprobes, inhibitors, enzymes, haptens, ligands, etc.

In 1975, Ringsdorf (*2*) presented one famous design for bioconjugates (polymer–drug conjugates). As shown in Figure 1.1, his polymer carrier system is composed of five components; polymer backbone, transport system, solubilizer, drug, and the spacer which is the component for drug binding and release. The polymer backbone is made either of biodegradable or nonbiodegradable polymers. This selection between the two kinds of polymers is dependent on the desired characters for the drug in its actual use. The transport system includes two types of devices; a homing device and a nonspecific resorption enhancer. Homing devices utilize the specific interaction with the living system, and are exemplified by antibodies and hormones. Nonspecific resorption enhancers contribute to the efficient delivery of the drug by the interactions, based on their chemical and physical properties, with the living system. The solubilizer is classified into the following two types. Lipid-soluble types enhance the adsorption of the drug into the lipid bilayer of cell membranes. Water-

soluble types maintain the water solubility of this drug carrying system.

This solubilizing component is very important to actual applications as follows: The low water solubility of the polymer–drug conjugates often causes the problems that the precipitation of the bioconjugates is hazardous for the stability and the safety of the injection into the bloodstream. Hydrophobic solubilizers (oil-soluble) are considered to contribute to the increase of pharmacological activities by enhancing the permeation of the bioconjugates through cell membranes since the pharmacological activity may be obtained by the multiplication of the delivery efficiency to the target site and the uptake efficiency by the target cells (*3*).

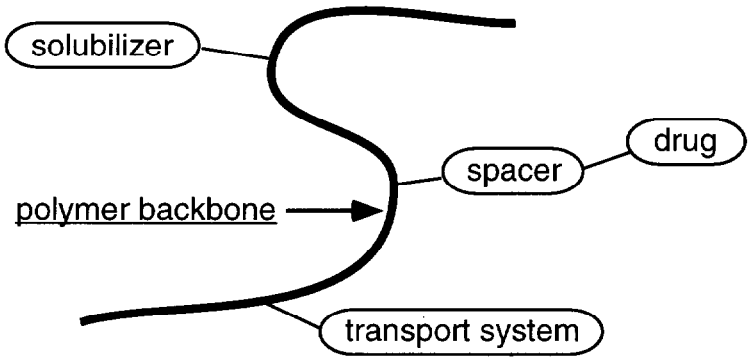

Figure 1.1. Ringsdorf's model for polymer-drug conjugates.

At the almost same time that Ringsdorf proposed the above model, in 1974, De Duve *et al.* presented the concept of novel bioconjugates, "lysosomotropic agents", in their review article (*4*). The term "lysosomotropic" designates all substance which is selectively taken up into lysosomes. By using the term of the lysosomotropic agents, they proposed the drug delivery into cells. The drug delivery proposed by them utilizes biological characters, transport, and the functions of the lysosomes. As shown in Figure 1.2, the lysosomotropic agent enters the lysosomes through three routs. Endocytosis or piggyback endocytosis is the main route for macromolecular bioconjugates. The term of the piggyback endocytosis is

used for the uptake of the substances that enter the lysosomes as part of an endocytizable complex. Therefore, the piggyback endocytosis is the most important route for the polymer–drug conjugates introduced into the lysosomes. In the lysosomes, the lysosomotropic agents are exposed to an acid milieu and many digestive enzymes. Then, the lysosomotropic agents express their pharmacological activities intralysosomally or extralysosomally. The most important significance of this concept for drug delivery systems lies in the alteration of the route for the drug entry into cells. The drugs which enter the cells by the only permeation through cell membranes can enter the cells by the piggyback endocytosis as drug–carrier conjugates. The other merit is that the character of the carrier controls the selectivity and the quantity of the drug dosing to the cells.

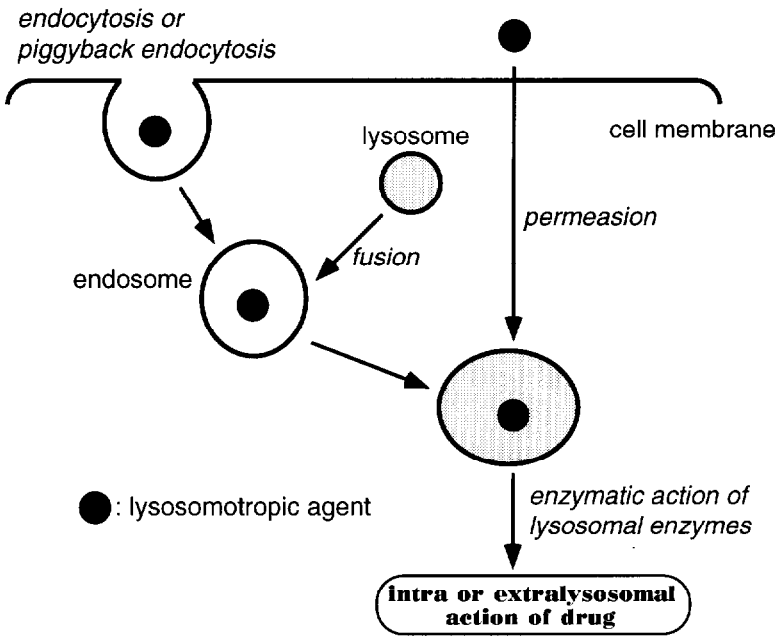

Figure 1.2. Concept of lysosomotropic agents (De Duve *et al.*).

Trouet and De Duve *et al.* reported DNA–daunomycin (anticancer drug) complexes as the first example of the lysosomotropic agents in 1972 (*5*). The daunomycin was physically bound to calf thymus DNA by the ionic interaction between the amino group of the

daunomycin and the phosphate group of DNA. The DNA is expected to be degraded by DNAase in lysosomes. Therefore, free daunomycin is recovered in the lysosome, expressing cytotoxic activity. In case of using DNA as drug, however, the degradation of DNA in the lysosomes is undesirable.

In the 1980's, many techniques have been developed for the DNA introduction into cells (*6*); for example, (i) the precipitation with calcium phosphate (*7*, *8*), DEAE-dextran (*9*), or polybrene (*10*, *11*), (ii) the electroporation (*12*, *13*) or microinjection (*14*, *15*) for the direct injection of DNA, and (iii) the DNA incorporation in reconstructed virus coats (*16–18*). Furthermore, liposomes have been used in transfection protocols (*19–21*). In the protocols, DNA molecules are either entrapped in the internal aqueous space of the liposomes or bound on their surface. Promising results have been obtained with pH-sensitive liposomes. The pH-sensitive liposomes are capable of fusing with the membranes of late endosomes and releasing DNA into the cytoplasm, which prevents DNA from accumulating and digesting enzymatically in lysosomes (*20*, *22*).

However, each of the above listed methods has various disadvantages and limitations. Although the techniques based on the precipitation with calcium phosphate or polycation are probably widespread in laboratory practice (*23*), they are characterized as follows: (i) Relatively low efficacy of transfection. (ii) Ineffectiveness for the introduction of RNA molecules into cells. (iii) Impossibility for the transfection *in vivo*. The techniques using retrovirus or adenovirus vectors (*17*, *18*) permit us to overcome some of these limitations. However, their application for gene therapy causes serious concern about the possible recombination with endogenous viruses, oncogenic effects, and immunologic reactions (*24*). To date, although the lipofection protocol seems to be most promising (*25*), there are complaints about its cytotoxicity (*26*) and its lack of homing devices. Therefore, despite a variety of methods existing already, the search of new possibilities for the transformation of

animal, plant, and procaryotic cells still continues.

In the 1990's (from the late 1980's), a new approach has been developed in order to enhance the delivery of genetic material into cells. This method uses the soluble polyelectrolyte complexes of DNA with linear polycations (*27–31*). These complexes assemble spontaneously as a result of mixing the components which form interchain electrostatic bonds by a cooperative system. Altering the composition of the complex and the chemical structure of the polyelectrolytes can vary the physicochemical properties of polyelectrolyte complexes (i.e., their solubility, dimensions, surface charges, etc.). The incorporation in the polyelectrolyte complexes results in the significant changes of DNA properties, leading to DNA compaction. DNA is tightly packed in the polyelectrolyte complex, so that the DNA chain is protected from the contact with the external medium. The resulting protection stabilizes DNA against the digestion by nuclease (*32*). By conjugating polycation molecules with homing devices, it is possible to transport polyelectrolyte complexes into cells via receptor-mediated endocytosis (*27*, *33–37*). Furthermore, the *in vivo* delivery of DNA complexes into target cells is expected (*38–40*).

Polyelectrolyte complexes are the self-assembling objects that are thermodynamically stable under a certain set of conditions, i.e., pH, ionic strength, temperature, medium composition, etc. This facilitates the preparation, storage, and application of polyelectrolyte complexes, as compared with, for example, the liposomes which are usually unstable and can not be stored for a long time (*29*). As further characteristic, the polyelectrolyte complexes belong to a class of self-assembling supramolecular system, e.g. block copolymer micelles by Kataoka *et al.* (*41*).

As well as delivering DNA effectively to the targeting cell (site-specific delivery), the release of DNA into the cytoplasm (cf. Figure 1.2) is important. In 1990, Okano *et al.* presented the system called "intelligent drug delivery systems (intelligent DDS)", which is

distinguished from the conventional DDS, as shown in Figure 1.3 (*42*, *43*). To realize the novel DDS, it is important to construct the system where the drug device itself senses environmental stimuli and responds as the drug release controlled appropriately. Namely, the intelligent DDS have been studied for the efficient delivery of drugs to the targeting site (site-specific control) and the drug release occurring only when the drug is required (temporal control). In the temporally-controlled drug delivery, there are chemically-responsive systems (glucose-responsive insulin delivery systems) (*44–47*) and physically[pH (*48–50*), electro (*51–53*), or thermo (*54–57*)]-responsive systems. The temporal control (self-regulating drug delivery) utilizes stimuli-responsive polymers widely.

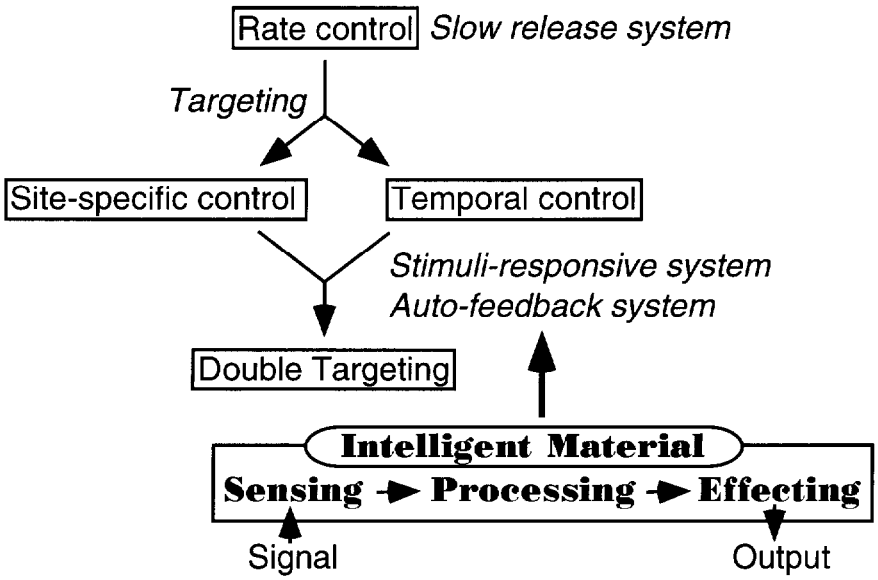

Figure 1.3. Strategy of novel drug delivery systems (Okano *et al.*).

This thesis is composed of six chapters, in which the synthesis and application of polyelectrolyte graft copolymers as bioconjugate are described in detail (Figure 1.4). These bioconjugates are mainly designed as DNA carrier; namely, the molecular design of the DNA carriers in this thesis is based on the graft copolymer such as micro-domain structured

polymer (*58*).

This chapter and Chapter 6 are general introduction and concluding remarks, respectively.

In Chapters 2 and 3, I have introduced stimuli-responsive polymers into the field of DNA delivery. Chapter 2 describes the novel concept of the DNA polyelectrolyte complex with pH-sensitivity. Chapter 3 describes the strategy of the escape of DNA polyelectrolyte complexes from endosome.

In Chapters 4 and 5, I have focused on site-specific control. Chapter 4 describes the DNA polyelectrolyte complex with solubilizer and homing devices utilizing the specific interaction with liver sinusoidal endothelial cells. Chapter 5 describes the novel concept of the affinity control of multivalent ligands for receptors by means of the prepolymer with a "specific" resorption enhancer.

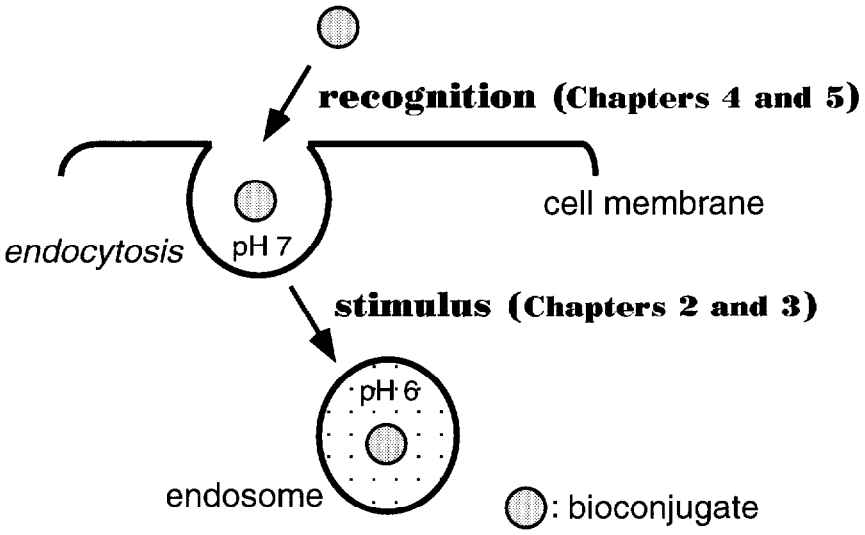

Figure 1.4. Brief standpoint of this thesis.

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Chapter 2

Design of Comb-Type Polyamine Copolymers for a Novel pH-Sensitive DNA Carrier

2.1 ABSTRACT

The comb-type polycation consisting of a poly[2-(diethylamino)ethyl methacrylate] (PDEAEMA) backbone and poly(L-lysine) (PLL) side chains has been prepared as a novel pH-sensitive DNA carrier. The comb-type copolymer PDEAEMA-*graft*-PLL was prepared by using the macromonomer method, in which a poly(N^{ε}-carbobenzoxy-L-lysine) macromonomer was radically copolymerized with DEAEMA. The comb-type copolymer exhibited a two-step proton dissociation and a dual ionic character owing to the two cationic segments in the copolymer, as determined by acid–base titration. In addition, the comb-type copolymer caused no significant turbidity even at pH 10, whereas PDEAEMA homopolymer suddenly precipitated out of the aqueous medium above pH 7.5 owing to the deprotonation of amino groups. Furthermore, a ^{1}H NMR study proved that protonated PLL segments solubilized the comb-type copolymer with a hydrophobic PDEAEMA core at higher pH. Finally, the pH-dependent behavior of the DNA complex with the comb-type copolymer was evaluated. The discontinuous turbidity change of the DNA/PDEAEMA-*graft*-PLL mixture at pH 7.5 suggested that the solubility of the complex varied in response to pH. By circular dichroism measurement, I also found that the comb-type copolymer was capable of varying DNA compaction pH-dependently. In conclusion, I have demonstrated that the comb-type copolymer is capable of sensing a pH signal and outputting the nonlinear change of the physicochemical properties of DNA polyelectrolyte complexes.

2.2 INTRODUCTION

Recently, the drug delivery systems using polymeric drug carriers have been studied to effect the efficient delivery of drugs to target sites (*1*,*2*). Among the drug delivery systems, the delivery of genes to target cells as a novel class of clinical application has become one of the topics. For the effective gene delivery by nonviral carriers, the special mechanisms for carrying genes to specific tissues or cells are required. Receptor-mediated gene transfer takes advantage of the ability of receptors on the surface of cells to bind and internalize a ligand (*3–8*). The components used in the receptor-mediated gene delivery systems include DNAs, receptor-targeting ligands, and linking polycations such as poly(L-lysine) (PLL). The first report of successful DNA transfer and gene expression via receptor-mediated endocytosis was made by Wu and Wu (*4*), who developed the methods used for the introduction of genes into the liver via the asialoglycoprotein receptor. Many ligands and their models were proposed and investigated as targeting moieties. The polycations conjugated with several ligands, such as transferrin (*5*), insulin (*6*), and antibody (*7*) molecules, have been studied for the efficient internalization of DNA–ligand complexes.

The major shortcomings of these delivery systems are, however, transient and low levels of transfection activity. These shortcomings are attributed to the barriers existing during the delivery to the final destination, i.e., cytoplasmic space or the nucleus of the target cells. One of the major barriers is the reticuloendotherial system that entraps and scavenges foreign materials in the blood stream. Because most of DNA complexes are the size of a few hundred nanometers, they are liable to be entrapped by the reticuloendotherial system before their arrival at the target tissues or cells (*9, 10*). Another major barrier of nonviral delivery is the fact that most endocytozed complexes remain entrapped in vesicles and are subsequently degraded by the lysosomal pathway (*11*).

On the other hand, many viruses have evolved various molecular mechanisms for gaining the efficient entry into cells and escaping lysosomal degradation. For example, some virus particles exhibit the fusogenic activity with endosomal membranes to translocate the viral DNA to

cytoplasmic space (*12*). The mechanics of the direct injection of DNA through the cellular plasma membrane were well documented for the infection of T4 bacteriophages (*13*). The fact that these viruses display such molecular mechanisms only after the arrival at the target cells is unique. The viruses, therefore, switch on these mechanisms by sensing environmental factors such as pH and/or specific molecules at the target sites.

Such "intelligence" of viral infection pathways has led us to create the nonviral carrier with intelligent functions. The materials internalized via receptor-mediated endocytosis are delivered into acidic endosomal vesicles so that they are subjected to the significant pH change (*14*). The polymer carriers which are capable of varying their functions in response to pH, therefore, enable us to construct intelligent DNA delivery systems.

In this chapter, I have prepared a novel comb-type polycation exhibiting a dual ionic property. The comb-type copolymer consists of stronger basic chains of PLL-*grafts* and a weaker basic backbone of poly[2-(diethylamino)ethyl methacrylate] (PDEAEMA). The PDEAEMA undergoes the drastic change in protonation degree and solution properties near neutral pH (*15*, *16*). Such a change in the PDEAEMA segment would give pH sensitivity to the DNA complex. It becomes possible to vary complex properties, such as the state of electrical charges and hydrophilic/hydrophobic balance, which are the factors determining the interaction with cellular or endosomal membranes. The higher-order structure of DNA in the complex, which is an influential factor in the transgene expression (*17*), can be also modified pH-dependently. Furthermore, the pH-sensitive DNA–polycation complexes may allow me to control the release of transgene enhancers, such as fusogenic substances and enzyme inhibitors, in a pH-sensitive manner.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Materials

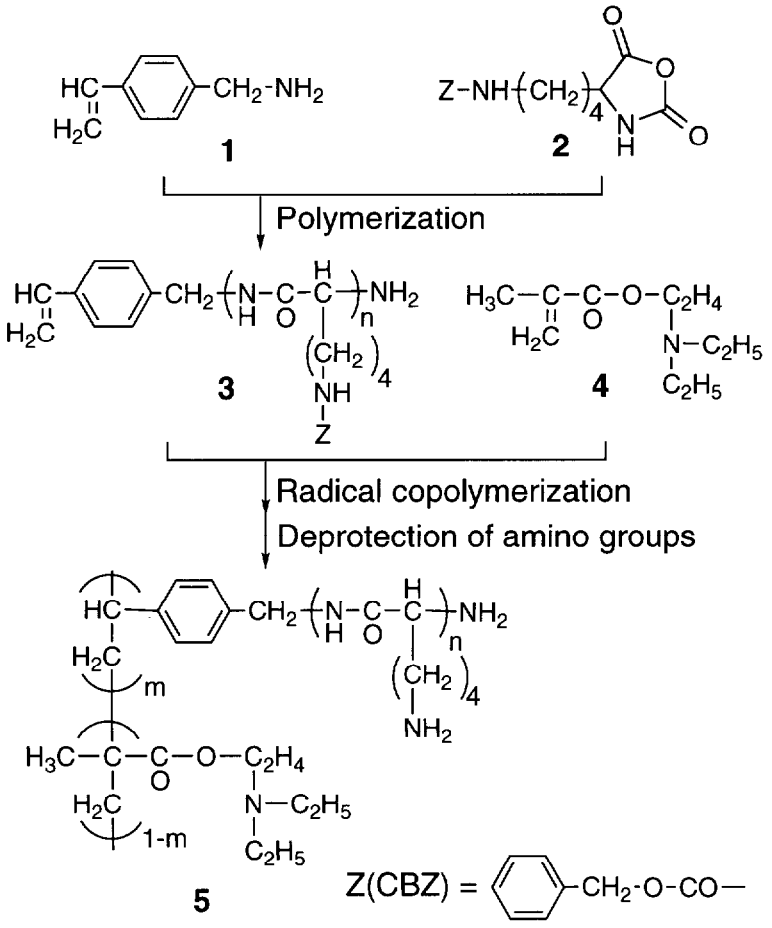
2-(Diethylamino)ethyl methacrylate (DEAEMA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and was distilled under reduced pressure after removing methacrylic acid with a 0.1 M NaOH aqueous solution. Dehydrated *N*,*N*-dimethylformamide (DMF) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), and the calf thymus DNA was from Sigma Chemical Co. (St. Louis, MO). All other chemicals of a special grade were used without further purification.

2.3.2 Synthesis of PDEAEMA-*graft*-PLL Comb-Type Copolymers

The synthetic route of the comb-type copolymers is shown in Scheme 2.1. The N^{α}-carboxyanhydride (NCA) of N^{ε}-carbobenzoxy(CBZ)-L-lysine (**2**) (*18*) was dissolved in dehydrated DMF. *p*-Aminomethylstyrene (**1**) (*19*) was dissolved in dehydrated DMF and added to the solution of **2**. The polymerization reaction was performed with 10 g of CBZ-L-lysine NCA (**2**) in 100 mL of DMF at a monomer (**2**)/initiator (**1**) molar ratio of 20 or 40 (for 72 h at 25 ℃). Then, the reaction mixture was poured into a 10-fold volume of diethyl ether. Precipitate was collected by filtration and washed with diethyl ether, followed by drying *in vacuo*.

The resulting macromonomer (**3**) and DEAEMA (**4**) were dissolved in 1 mL of DMF at the total monomer concentration of 300 mg/mL. The radical copolymerization reaction was carried out for 7 h at 47 ℃ in a sealed glass ampule using 20 mM of 2,2'-azobis(2,4-dimethylvaleronitrile) (V-65) as an initiator. After the reaction, the content was poured into a large excess of water, and precipitate was dried *in vacuo*. The crude polymers were dissolved in trifluoroacetic acid, followed by the addition of thioanisole to deprotect CBZ-amino groups. The final concentrations of thioanisole and the polymer were 1.6 and 0.32 M (based on the CBZ groups), respectively. The deprotection reaction was carried out for 3.5 h at 25 ℃. Then,

Scheme 2.1. Synthesis of PDEAEMA-*graft*-PLL Comb-Type Copolymers



the reaction mixture was poured into a large excess of diethyl ether. Precipitate was redissolved in trifluoroacetic acid and poured again into ether. The precipitate was dried *in vacuo* and dissolved in water, followed by dialysis against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10^4) to remove unpolymerized macromonomers. After dialysis, the resulting copolymer was obtained by freeze-drying.

2.3.3 Gel Permeation Chromatography (GPC)

GPC was carried out using a JASCO 880-PU pumping system (Tokyo, Japan) at a flow rate of 1.0 mL/min at 30 ℃ with Ultrahydrogel 500 and Ultrahydrogel 250 columns (Japan Waters Ltd., Tokyo, Japan). The aqueous solution containing 0.5 M CH_3COOH and 0.3 M Na_2SO_4 was used as a mobile phase. Three hundred microliters of 1 mg/mL samples was injected into the columns. The detection of the polymers was performed by a refractive index detector (830-RI, JASCO) and a multiangle light scattering detector (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA).

2.3.4 ^{1}H NMR Spectroscopy

Each polymer (3–5 mg) was dissolved in 700 μL of D_2O (99.95 at. % D; Merck, Darmstadt, Germany), and the pH of the solution was varied by adding a trace amount of a 1 M HCl or 1 M NaOH solution. The pH value was checked with a TOA HM-20E pH meter (Tokyo, Japan) before and after ^{1}H NMR analysis. ^{1}H NMR spectra (400 MHz) were obtained by a Varian Unity 400plus spectrometer (Palo Alto, CA), at a probe temperature of 298 K. The chemical shifts are expressed as parts per million using internal water molecules (δ = 4.7 ppm in D_2O) as a reference.

2.3.5 Acid–Base Titration and Turbidity Measurement of PDEAEMA-*graft*-PLL Solutions

To 1.5 mL of an aqueous solution of the polymer (3.8 mg/mL) was added a 1 M HCl

solution (20–30 μL) was added, and the acidic polymer solution (pH 2) was titrated with a 1 M NaOH solution. The titration was carried out by the stepwise addition of 1 M NaOH and stopped at pH 12. The reverse titration was then performed as above, except that 1 M HCl was used. The turbidity of the solution during the titration was measured by monitoring the absorbance at 500 nm with a Beckman DU-640 spectrophotometer (Fullerton, CA).

2.3.6 Circular Dichroism Spectropolarimetry and Turbidity Measurement of the Mixture of DNA and PDEAEMA-*graft*-PLL

The stock solutions of both DNA (1 mg/mL) and PDEAEMA-*graft*-PLL (4 mg/mL) in 0.15 M NaCl were mixed together at a final ratio of 1/10 or 10/1 for the lysine unit (PLL-*graft*) to the nucleotide unit (DNA). The final concentration of DNA was adjusted to 0.1 mg/mL (3×10^{-4} M based on the nucleotide units). The pH of the mixture was varied from 7 to 8.5 by adding either a 0.1 or 1 M solution of HCl or NaOH. The circular dichroism (CD) and the turbidity of the mixture were measured at each pH point. The CD was measured with a JASCO J-600 spectropolarimeter in the quartz cell with an optical path length of 1 cm at room temperature. The values of CD in the figure are expressed as molecular ellipticity, calculated for one nucleotide residue in the polynucleotide. The turbidity was measured by monitoring the absorbance at 500 nm. The pH value was also checked after the CD and turbidity measurement.

2.4 RESULTS AND DISCUSSION

2.4.1 Synthesis of PDEAEMA-*graft*-PLL Comb-Type Copolymers

The polymerizations of CBZ-L-lysine NCA (**2**) had progressed homogeneously in the solvent of dehydrated DMF. The vinyl groups were preserved in the resulting polymer (**3**), which was confirmed by ^{1}H NMR spectroscopy in $(CD_3)_2SO$ (DMSO-d_6): δ 5.2 (doublet), 5.8 (doublet), and 6.7 (quartet) ppm. The number-average degree of polymerization (P_n) of the macromonomer (**3**) determined by GPC was almost equal to that determined by ^{1}H NMR (see footnotes in Table 2.1) and was proportional to the feed ratio of monomer (**2**) to initiator (**1**). These results confirmed that the resulting macromonomers possessed one polymerizable vinyl end per molecule.

The radical copolymerizations of **3** with DEAEMA (**4**) had progressed homogeneously in DMF. Figure 2.1 shows the representative GPC profile of the resulting copolymer. The GPC profile indicated that unreacted macromonomers were removed by the purification process, including dialysis. The number-average molecular weight of the each copolymer determined by GPC was about 4.0×10^4. The ^{1}H NMR spectra (Figure 2.2) of the copolymer showed the characteristic signals of both PLL-*graft* and PDEAEMA backbone: δ 1.3 (methyl protons of the diethylamino group of PDEAEMA), 1.4–1.8 (β-, γ-, δ-methylene protons of PLL), 3.0 (ε-methylene protons of PLL), 3.3 (methylene protons of the diethylamino group of PDEAEMA), 3.5 (2-methylene protons of PDEAEMA), and 4.3 (α-methine protons of PLL) ppm. No residual CBZ groups were detected in the final polymer samples, indicating the successful deprotection of amino groups. From the signal ratio of ε-methylene protons (3.0 ppm) of the PLL-*grafts* and methylene protons (3.3 ppm) of the PDEAEMA backbone, the content (mole percent) of **3** in the copolymer was determined. As shown in Table 2.1, the content of **3** in the copolymer was higher than that in feed, which suggested the preferential incorporation of the macronomomers (**3**) in the copolymers under the experimental conditions. Thus, it is possible

Table 2.1. Characteristics of Prepared PDEAEMA-*graft*-PLL Comb-Type Copolymers[a]

sample	mol % of **3**[b]		yield (%)	[lysine] / [DEAEMA] in copolymer
	infeed	in copolymer		
1	1.9	2.5	25	0.46
2	3.7	4.3	24	0.80
3	7.1	8.8	6	1.7
4	1.2	1.5	11	0.44

[a] Initiator, 20 mM V-65; reaction temperature, 47 °C; reaction time, 7 h; solvent, DMF.
[b] For samples 1–3, P_n (determined by GPC) = 20 and P_n (determined by ^{1}H NMR) = 18; for sample 4, P_n (GPC) = 30 and P_n (^{1}H NMR) = 28.

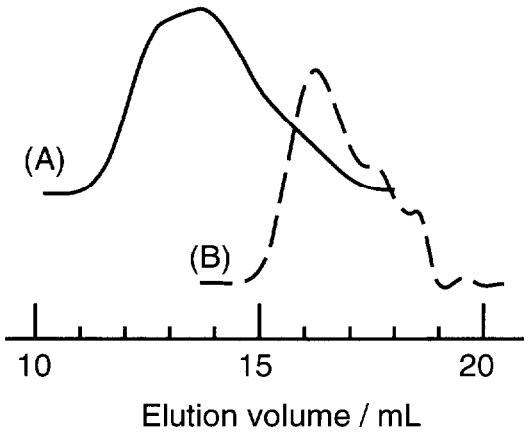


Figure 2.1. Gel permeation chromatograms of (A) the PDEAEMA-*graft*-PLL comb-type copolymer (sample 1) and (B) the deprotected CBZ-L-lysine macromonomer: column, Ultrahydrogel 500 + 250; eluent, 0.5 M CH_3COOH + 0.3 M Na_2SO_4; flow rate, 1.0 mL/min; temperature, 30 °C; detection, refractive index.

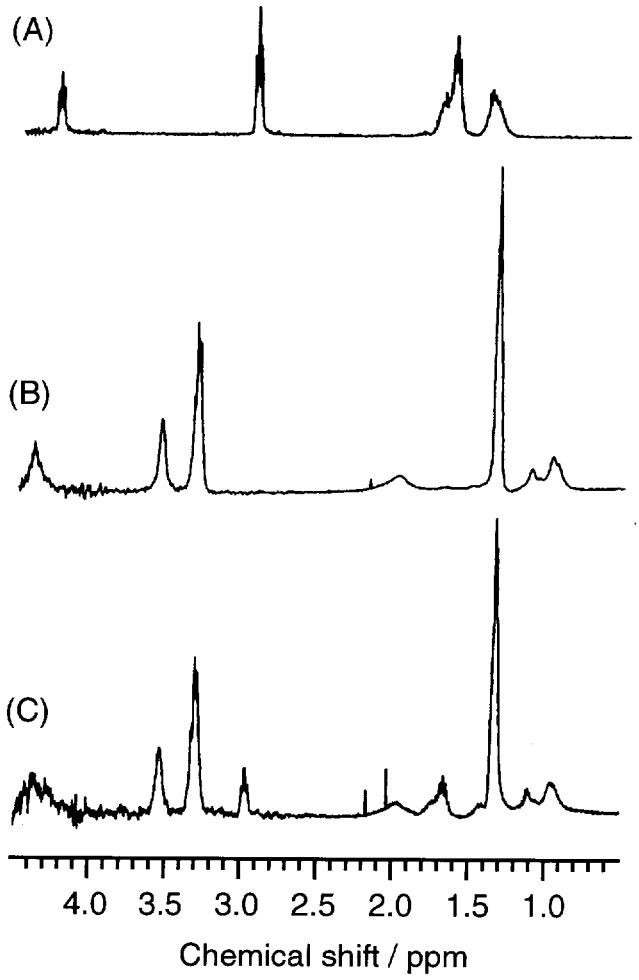


Figure 2.2. ^{1}H NMR spectra of (A) PLL, (B) PDEAEMA, and (C) PDEAEMA-*graft*-PLL in D_2O (pH 2).

to obtain the comb-type copolymers with well-controlled graft chains by using the macromonomer method.

2.4.2 pH-Dependent Behavior of PDEAEMA-*graft*-PLL Comb-Type Copolymers in Water

Figure 2.3 shows the acid–base titration curves of PDEAEMA-*graft*-PLL. The comb-type copolymer exhibited two-step proton dissociation. First and second proton dissociation was attributed to that of PDEAEMA (pH 7.5) and PLL (pH 10) segment, respectively. The dissociation profile varied according to the composition of the copolymer. Thus, the comb-type copolymer exhibited a dual ionic character owing to the two kinds of cationic segments, i.e., PDEAEMA and PLL, in the copolymer.

It should be noted that the solution behavior of the comb-type copolymer, as shown in Figure 2.4, was totally different from that of the PDEAEMA homopolymer. The PDEAEMA homopolymer exhibited precipitation above pH 7.5 owing to the deprotonation of the amino groups of the PDEAEMA. The precipitation of PDEAEMA was also seen in the polymer mixture of PDEAEMA and PLL. On the other hand, no significant turbidity was observed for the comb-type copolymer. The solution behavior of the comb-type copolymer was further evaluated by ^{1}H NMR measurement. As shown in Figure 2.5, the ^{1}H NMR signals of the copolymer varied drastically with the change of pH. The deprotonation of diethylamino groups was observed by the upfield shifts of the ^{1}H NMR signals with increasing pH. It is worth noting that the signals of the PDEAEMA segment disappeared at pH 8.4 where the amino groups of the segment were completely deprotonated. The peak area of the signals of the PDEAEMA segment decreased when more than 60% of the amino groups of the segment were deprotonated (Figure 2.6), whereas no significant change was observed for the PLL segment. As the PDEAEMA homopolymer became insoluble in higher pH, the PDEAEMA segment in the comb-type copolymer probably separated out of water and formed the microprecipitate (microparticle) that caused proton signal shielding (*20, 21*). The hydrophilic segments of the protonated PLL in the comb-type copolymer would stabilize the microprecipitate of the PDEAEMA segment and protect it from the bulk precipitation, leading to the transparent

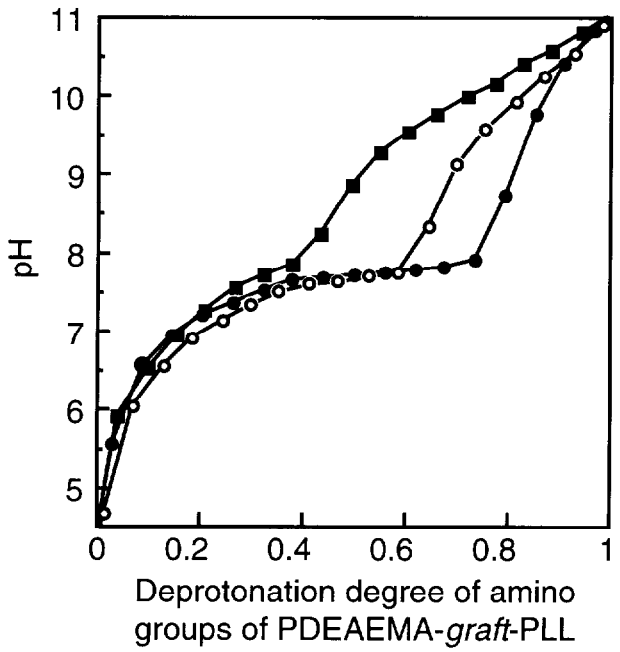


Figure 2.3. Acid–base titration curves of PDEAEMA-*graft*-PLL comb-type copolymers: (●) sample 1, (○) sample 2, and (■) sample 3. Acidic polymer solutions were titrated with the stepwise addition of 1 M NaOH. The horizontal axis is normalized as the deprotonation degree based on the total amino groups of the PDEAEMA-*graft*-PLL comb-type copolymer.

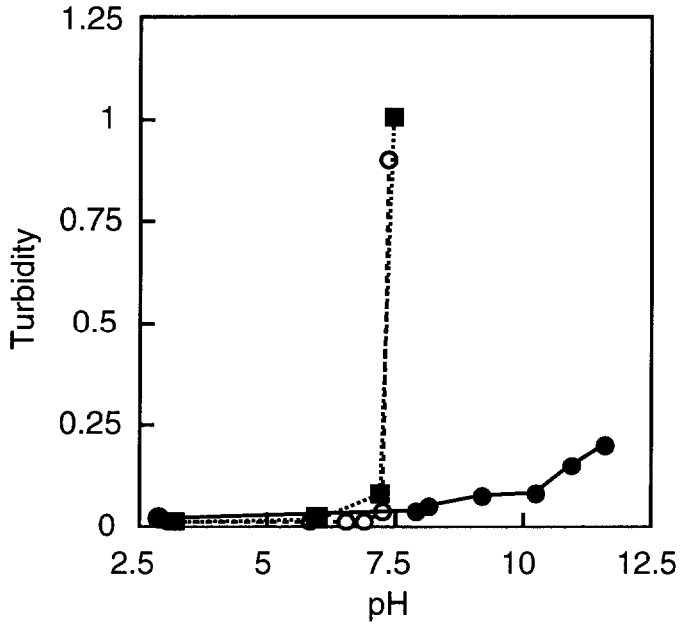


Figure 2.4. Effect of pH on the solubility of polymers in water: (●) PDEAEMA-*graft*-PLL comb-type copolymer (sample 1, 3.8 mg/mL), (■) PDEAEMA homopolymer (2.7 mg/mL), and (○) homopolymer mixture having the same composition (2.7 mg/mL PDEAEMA and 1.3 mg/mL PLL) as that of the copolymer. The turbidity was measured by monitoring the absorbance at 500 nm of the polymer aqueous solution during the acid–base titration.

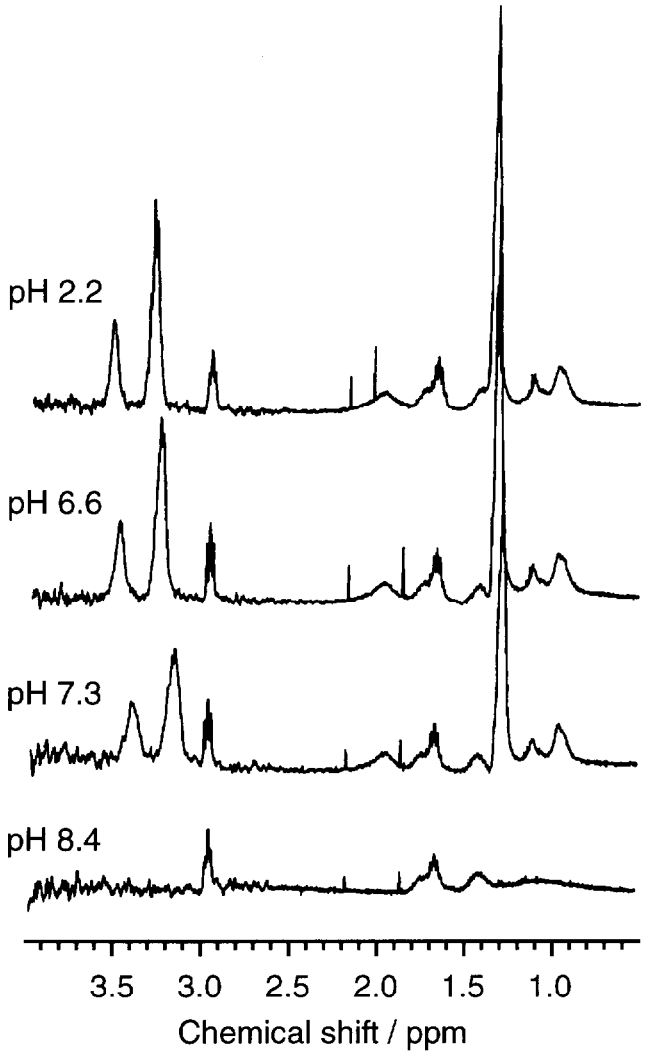


Figure 2.5. Effect of pH on the ^1H NMR spectra of the PDEAEMA-*graft*-PLL comb-type copolymer in D_2O. The pH was varied by adding a trace amount of 1 M HCl or 1 M NaOH.

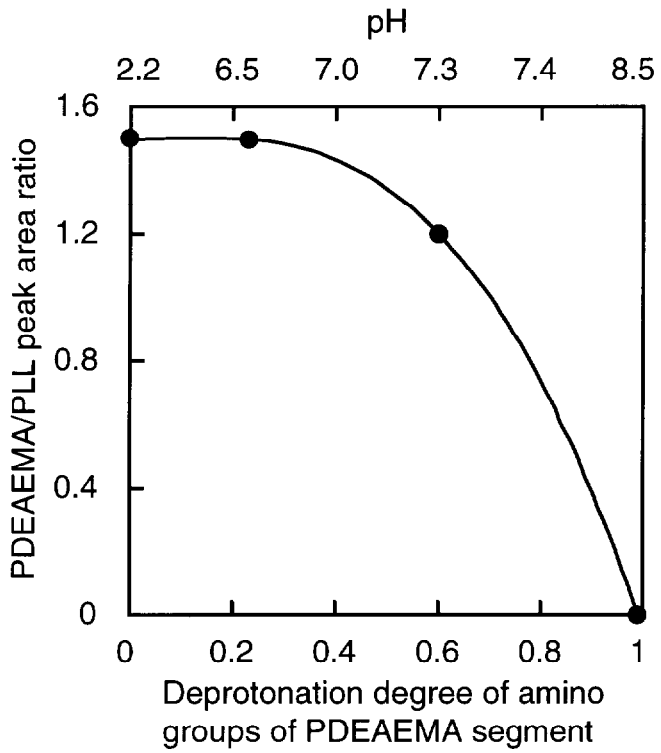


Figure 2.6. Plot of the ^{1}H NMR peak area ratio of the PDEAEMA signal at 2.0 ppm to the PLL signal at 3.0 ppm against the deprotonation degree of the PDEAEMA segment. The pH values are also indicated on the top horizontal axis.

solution even in higher pH. In this sense, the comb-type copolymer was capable of varying the hydrophilic–hydrophobic balance and higher-order structure, in addition to the total positive charges, in response to the small change of pH of the solution. The pH sensitivity of the comb-type copolymer observed with the turbidity and ^{1}H NMR measurement was shown to be reversible.

2.4.3 Solubility Change of the Polyelectrolyte Complex between DNA and the PDEAEMA-*graft*-PLL Comb-Type Copolymer in Response to pH

I examined how the pH sensitivity of the PDEAEMA-*graft*-PLL copolymers influenced the properties of their complex with DNA. Figure 2.7 shows the pH-dependent turbidity change of the DNA solution mixed with an excess amount of the PDEAEMA-*graft*-PLL. Neither the DNA solution nor the PDEAEMA-*graft*-PLL solution exhibited any turbidity. However, the DNA/PDEAEMA-*graft*-PLL mixture exhibited significant turbidity above pH 7.5, and the turbidity decreased discontinuously at pH 7.5. The pH-dependent change of turbidity was almost reversible. Since the PDEAEMA homopolymer became soluble below pH 7.5, the solubility change of the DNA/PDEAEMA-*graft*-PLL complex in response to pH was probably related to the protonation of the PDEAEMA segment in the copolymer. Maruyama *et al.* have already reported that the soluble complexes between DNAs and polycations are available by conjugating water soluble polymer chains such as dextran to polycations (*22*). In this case, the excess segments of the protonated PDEAEMA should contribute to increasing the solubility of the complex at lower pH.

2.4.4 pH-Dependent Conformational Change of DNA in the Presence of the PDEAEMA-*graft*-PLL Comb-Type Copolymer

To examine further the pH-dependent behavior of the DNA complex with the comb-type copolymer, I studied the structural change of DNA in the presence of the copolymer. Figure 2.8 shows the CD spectra of DNA in the presence of the comb-type copolymer under the DNA

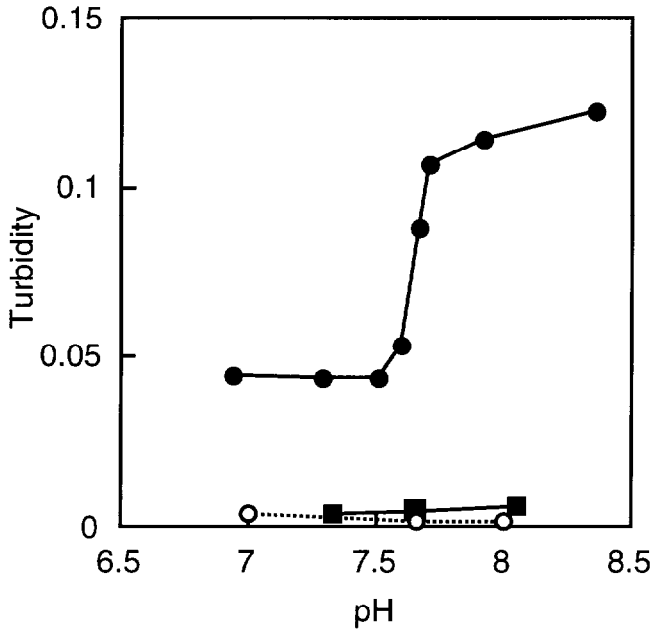


Figure 2.7. Effect of pH on the solubility of the mixture of DNA and the PDEAEMA-*graft*-PLL comb-type copolymer: (●) mixture of DNA (0.1 mg/mL) and PDEAEMA-*graft*-PLL (sample 1, 1.6 mg/mL) ([amino group]$_{PLL\text{-}graft}$/[phosphate group]$_{DNA}$ = 10), (○) DNA alone (0.1 mg/mL), and (■) PDEAEMA-*graft*-PLL alone (1.6 mg/mL) in 0.15 M NaCl. The pH was varied by adding a 1 M solution of HCl or NaOH. The turbidity was measured by monitoring the absorbance of the mixture at 500 nm.

excess condition. I found that the CD spectra of DNA in the presence of the comb-type copolymer varied between pH 7 and pH 9, whereas those of DNA alone did not vary. The CD spectrum of DNA exhibited the positive peak at around 275 nm and the negative peak at around 246 nm. The ratio of $-[\theta]_{275}/[\theta]_{246}$ is plotted against pH values in Figure 2.9A to estimate DNA distortion (*23, 24*). The structure of DNA in the mixture was distorted owing to the complex formation, and the distortion of DNA was enhanced at lower pH. Furthermore, the CD spectrum at lower pH exhibited non-zero values above 300 nm, as shown in Figure 2.9B. Such "tail" anomalies in the region of the spectrum above 300 nm (*25*), where the absorption of the uncondensed DNA goes to zero, are an indication of the formation of large chiral aggregates (*26, 27*). Taking these results into account, the comb-type copolymer was capable of varying DNA distortion and condensation at a particular pH. The CD spectra varied around pH 8.0, which was a little higher than the proton dissociation pH of the PDEAEMA segment (around pH 7.5). This is reasonable because the dissociation pH of DNA and the PDEAEMA segment should be higher than the proton dissociation pH of PDEAEMA (*28*). I also confirmed that the pH-dependent conformational change of DNA was reversible.

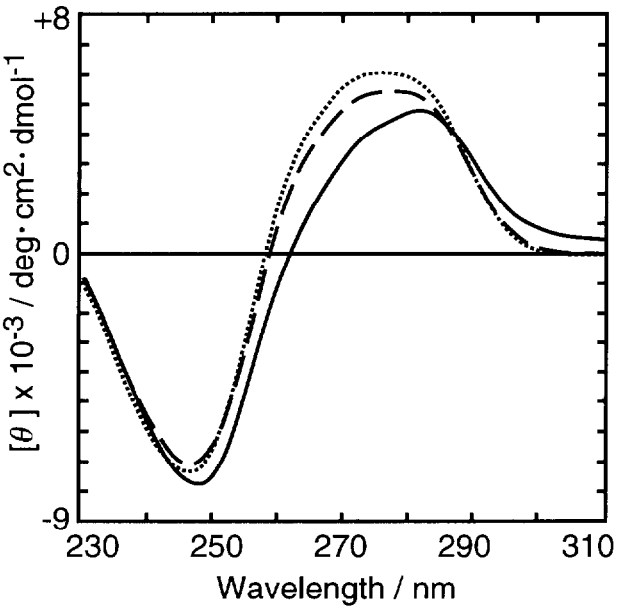


Figure 2.8. Effect of pH on the CD spectra of DNA in the presence of the PDEAEMA-*graft*-PLL comb-type copolymer: pH 7.7 (solid line), pH 8.2 (dashed line), and DNA alone (dotted line; the spectrum at pH 7.7 and 8.2 overlapped). DNA (0.1 mg/mL) and PDEAEMA-*graft*-PLL (sample 1, 17 μg/mL) were mixed in 0.15 M NaCl ([amino group]$_{PLL\text{-}graft}$/[phosphate group]$_{DNA}$ = 0.1). The pH was varied by adding a 0.1 M solution of HCl or NaOH. The molecular ellipticities based on nucleotide units (3×10^{-4} M) are represented.

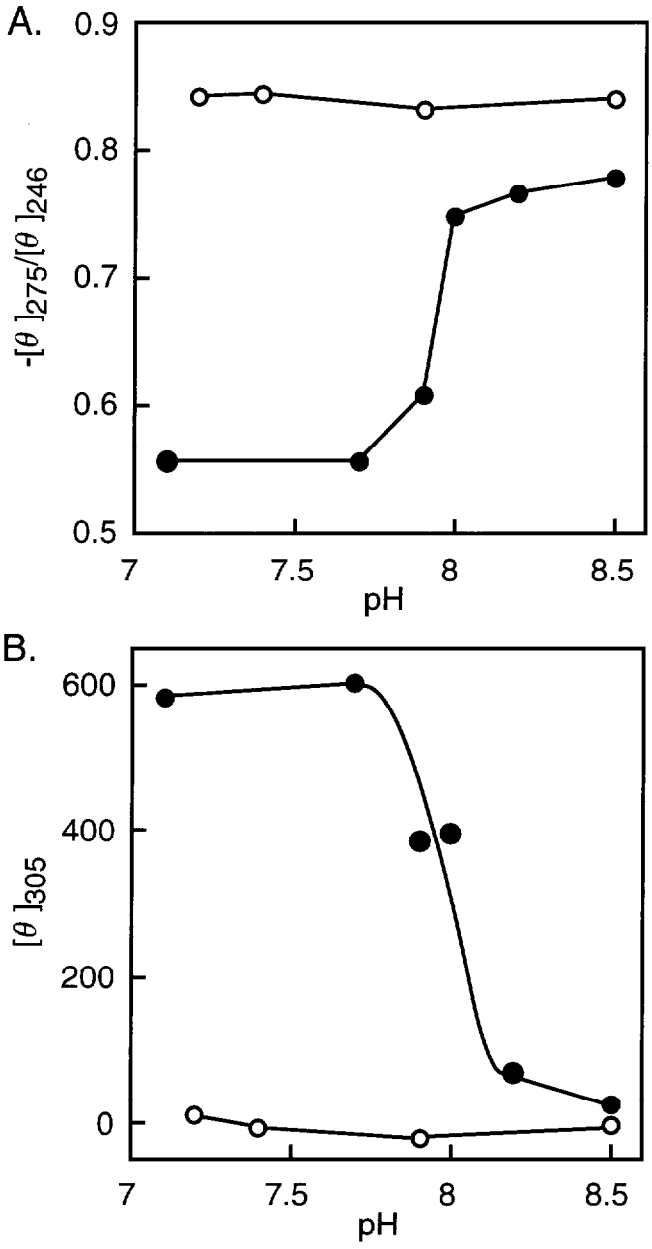


Figure 2.9. Plot of (A) $-[\theta]_{275}/[\theta]_{246}$ and (B) $[\theta]_{305}$ in the CD spectra against pH: (●) DNA/PDEAEMA-*graft*-PLL mixture and (○) DNA alone.

2.5 CONCLUSION

I have prepared the novel pH-sensitive comb-type copolymer PDEAEMA-*graft*-PLL and evaluated the solution properties of the comb-type copolymer. The comb-type copolymer exhibited unique solution properties in response to pH. The properties were totally different from those of each homopolymer and their mixture. By using the comb-type copolymer as a pH-sensitive polycation, the assembling structure of DNA complexes and the conformation of DNA were drastically varied at a particular pH. The properties of DNA complexes, such as the assembling structure and the compaction of DNA, have been described as factors which influence transfection activity (*29, 30*); therefore, the comb-type copolymer can be a unique tool for gaining insight into the structure–function relationship in the transfection activity. From a practical point of view, the transition pH value which triggered the response of the DNA complex was a little higher than that desired for sensing acidic endosomal vesicles. It is, however, possible to lower the transition pH value by introducing hydrophobic groups to the PDEAEMA segment (*31*) or by replacing PDEAEMA with other pH-sensitive polymers. In this chapter, I have demonstrated that the comb-type copolymer is capable of sensing a pH signal and outputting the nonlinear change of the physicochemical properties of DNA polyelectrolyte complexes. The achievement of the pH-induced nonlinear output can be the first step in mimicking the intelligent virus infection pathway with artificial polymer carriers.

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Chapter 3

Design of Bi-Phasic Polyelectrolytes Consisting of a Poly(1-vinylimidazole) Backbone and "Lactosylated" Poly(L-lysine) Graft Chains for the DNA Carrier with the pH Sensitivity at Endosomal pH

3.1 ABSTRACT

The bi-phasic polycation consisting of a poly(1-vinylimidazole) (PVIm) backbone and "lactosylated" poly(L-lysine) (PLL) side chains has been prepared as the DNA carrier with the pH sensitivity at endosomal pH. First, the comb-type copolymer PVIm-*graft*-PLL was prepared by using the macromonomer method, in which a poly(N^{ε}-carbobenzoxy-L-lysine) macromonomer was radically copolymerized with VIm. Then, ε-amino groups of PLL-*grafts* and the reducing end of lactose were covalently coupled by reductive amination. The resulting lactosylated PVIm-*graft*-PLL comb-type copolymer (PVIm-*graft*-PLL·Lac) exhibited a dual ionic character owing to the two cationic segments in the copolymer, as determined by acid–base titration. In agarose gel retardation assays, the DNA complex with the stronger basic segment PLL·Lac-*grafts* was solubilized by the weaker basic segment PVIm backbone which was water-soluble and inert to the interaction with DNA at physiological pH. Turbidity measurement showed that the DNA/PVIm-*graft*-PLL·Lac complex exhibited turbidity not at physiological pH (= 7.4) but at endosomal pH (= 5–6), owing to the basicity (protonation–deprotonation) of the imidazole groups. In addition, PLL·Lac-*graft* and not PLL-*graft* formed the DNA complex which exhibited the pH-dependent change of the solubility. It is worth noting that the solubility of the DNA/polycation complex in water decreased in spite of the increase of the net positive charges of DNA/polycation mixture. From a practical point of view, the DNA/PVIm-*graft*-PLL·Lac complex showed higher hemolytic activity at pH 6.0 than at pH 7.5 and significant transfection activity. In conclusion, I have demonstrated that the PVIm-*graft*-PLL·Lac comb-type copolymer is capable of sensing endosomal pH and outputting the unique change of both physicochemical and biological properties of DNA polyelectrolyte complexes.

3.2 INTRODUCTION

The concept of "lysosomotropic agents" was presented by De Duve *et al.* in 1974 (*1*). The term "lysosomotropic" designates all substances taken up selectively into lysosomes. In the lysosomes, the lysosomotropic agents are exposed to an acid milieu and many digestive enzymes. Trouet and De Duve *et al.* reported DNA–daunomycin (anticancer drug) complexes as the first example of the lysosomotropic agents (*2*). The drug was physically bound to calf thymus DNA by the ionic interaction between the amino group of the drug and the phosphate group of DNA. The DNA complexes are taken up into cells via endocytosis. Subsequently, DNA is expected to be degraded in lysosomes by DNAase, so that the drug becomes free in the lysosome. The free drug expresses cytotoxic activity. In case of using DNA as drug, however, the degradation of DNA in the lysosomes is undesirable.

Recently, many techniques have been developed for the DNA introduction into cells. Among the techniques, I focus on a system using the polyelectrolyte complexes between DNA and polycation (*3–7*) because the system has the following advantages. DNA is tightly packed in the polyelectrolyte complex, so that the DNA chain is protected from the contact with the external medium. The resulting protection stabilizes DNA against the digestion by DNAase (*8*). By conjugating polycation molecules with receptor-targeting ligands, it is possible to transport the polyelectrolyte complexes into cells via receptor-mediated endocytosis (*3*, *9–13*). The *in vivo* delivery of the DNA complexes into target cells is, moreover, expected (*14–16*). However, these DNA polyelectrolyte complexes tend to precipitate (*17–20*). Furthermore, most endocytozed complexes remain entrapped in endosomes and are subsequently delivered into lysosomes (*21*). Since the DNA–polycation complexes internalized via the receptor-mediated endocytosis are delivered into acidic

endosomes, they are subjected to the significant pH change from pH 7 to pH 5 (*22*). The DNA polyelectrolyte complexes which vary their properties in response to endosomal pH is expected to manage to get out of the endosomes before the arrival at lysosomes.

I have already presented the concept that the pH-dependent nonlinear change of the physicochemical properties of DNA polyelectrolyte complexes in Chapter 2 (*23*). In this chapter, I designed the DNA polyelectrolyte complex having the pH sensitivity at endosomal pH. The net charge of the DNA–polycation complex is even; nevertheless the complex keeps solubility at physiological pH. At endosomal pH, the solubility of the DNA–polycation complex in water decreased in spite of the increase of the net positive charges of DNA–polycation mixture. The polycation is the bi-phasic pH-sensitive polyelectrolyte consisting of a poly(1-vinylimidazole) (PVIm) (*24*) backbone and "lactosylated" poly(L-lysine) (PLL·Lac) side chains (PVIm-*graft*-PLL·Lac).

3.3 EXPERIMENTAL PROCEDURES

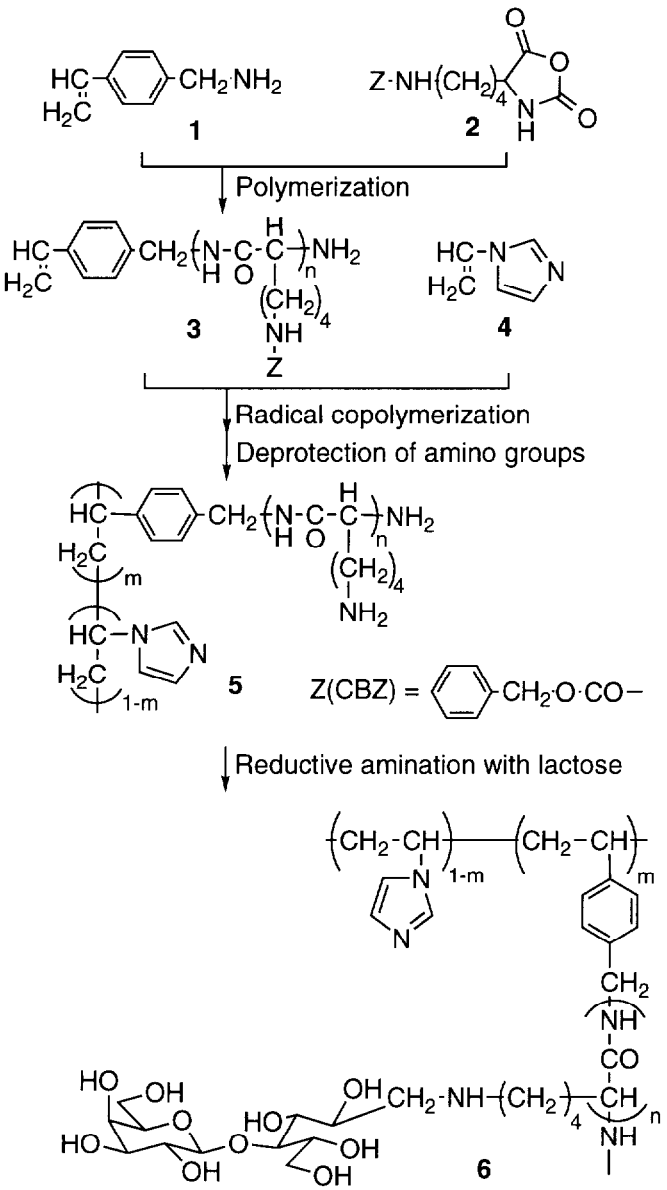
3.3.1 Materials

1-vinylimidazole (VIm) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was distilled under reduced pressure. 2,2'-azobis(2,4-dimethylvaleronitrile) (V-65) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and was crystallized from ethanol. Salmon sperm DNA (approximately 300 base pairs) was kindly provided by Nissan Chemical Industries, Ltd. (Osaka, Japan). All other chemicals of a special grade were used without further purification.

3.3.2 Synthesis of PVIm-*graft*-PLL·Lac Comb-Type Copolymers

The synthetic route of the comb-type copolymers is illustrated in Scheme 3.1. Poly[N^{ε}-carbobenzoxy (CBZ)-L-lysine] macromonomers (**3**) were prepared as described in Chapter 2 (*23*). The resulting macromonomer (**3**) (100 mg) and VIm (**4**) (1.2 mL) were dissolved in 1.9 mL of DMF. The radical copolymerization reaction was carried out for 7 h at 47 °C in a sealed glass ampule using 40 mM V-65 as an initiator. After the reaction, the content was poured into a large excess of diethyl ether, and precipitate was dried *in vacuo*. The crude polymers were dissolved in trifluoroacetic acid, followed by the addition of thioanisole to deprotect CBZ-amino groups. The final concentrations of thianisole and the polymer were 2 M and 83 mM (based on the CBZ groups), respectively. The deprotection reaction was carried out for 4 h at 27 °C. Then, the reaction mixture was poured into a large excess of diethyl ether. Precipitate was dried *in vacuo* and dissolved in water, followed by dialysis against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10^4) to remove unpolymerized macromonomers. After dialysis, the resulting copolymer (**5**)

Scheme 3.1. Synthesis of PVIm-*graft*-PLL·Lac Comb-Type Copolymers



was obtained by freeze-drying.

The resulting copolymer (**5**) (150 mg) and lactose (280 mg) were preincubated at 37 °C for 3 days in 7.5 mL of sodium borate buffer (0.1 M, pH 8.5). Then, a reducing agent, $NaBH_3CN$, was added to the mixture of **5** and lactose in 10 mL of the sodium borate buffer at a final concentration of 0.3 M. The reaction mixture was incubated at 37 °C for 3 days. The degree of amino group modification was examined by the primary amino group determination with fluorescamine (*25*). The reaction mixture was dialyzed against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10^3), followed by freeze-drying.

3.3.3 Gel Permeation Chromatography (GPC)

GPC was carried out using a JASCO 880-PU pumping system (Tokyo, Japan) at a flow rate of 0.8 mL/min at 25 °C with a Ultrahydrogel 1000 column (Japan Waters Ltd., Tokyo, Japan). The aqueous solution containing 0.5 M CH_3COOH and 0.2 M NaCl was used as a mobile phase. Three hundred microliters of 1 mg/mL samples was injected into the column. The detection of the polymers was performed by a refractive index detector (830-RI, JASCO) and a multiangle light scattering detector (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA).

3.3.4 ^{1}H NMR Spectroscopy

Each copolymer was dissolved in 700 μL of D_2O (99.95 at. % D; Merck, Darmstadt, Germany). ^{1}H NMR spectra (400 MHz) were obtained by a Varian Unity 400plus spectrometer (Palo Alto, CA), at a probe temperature of 298 K. The chemical shifts are expressed as parts per million using internal HDO molecules (δ = 4.7 ppm in D_2O) as a reference.

3.3.5 Acid–Base Titration

To 1.5 mL of aqueous solution of each polymer (3–4 mg/mL) was added a 1 M HCl solution (20–70 μL), and the acidic polymer solution (pH 2) was titrated with a 1 M NaOH solution. The titration was carried out by the stepwise addition of 1 M NaOH and stopped at pH 12. The pH value was checked with a Beckman Φ 210 pH meter (Fullerton, CA).

3.3.6 Agarose Gel Retardation Assay

Salmon sperm DNA was dissolved in 0.15 M NaCl at 1.9 mg/mL. The various concentrations (0.1–10 mg/mL) of polymer stock solutions were also prepared in 0.15 M NaCl. The DNA solution was diluted to about 50 μg/mL with 50 mM sodium phosphate buffer (pH 7.5 or pH 6.0). To the DNA solution was added a stock solution of the polymer (3–50 μL) at various polymer/DNA ratios. The final concentration of DNA was adjusted to 50 μg/mL. After 2 h of incubation at room temperature, 10 μL of the samples (corresponding to 0.5 μg of DNA) was electrophoresed through a 1% agarose gel containing 0.5 μg/mL ethidium bromide to visualize DNA using 50 mM sodium phosphate buffer (pH 7.5 or pH 6.0).

3.3.7 Turbidity Measurement of the Mixture of DNA and PVIm-*graft*-PLL·Lac

The stock solutions of both DNA (1.9 mg/mL) and PVIm-*graft*-PLL·Lac (or PVIm-*graft*-PLL) (10 mg/mL) in 0.15 M NaCl were mixed together at a final ratio of 2/1 for the lysine unit (graft chain) to the nucleotide unit (DNA). The final concentration of DNA was adjusted to 50 μg/mL. The pH of the mixture was varied from 7.5 to 4.5 by adding 0.1 M HCl. The turbidity of the mixture at each pH point was measured by monitoring the

absorbance at 500 nm with a Beckman DU-640 spectrophotometer.

3.3.8 Measurement of Hemolytic Activity

The stock solution (1.9 mg/mL) of DNA was diluted to about 50 μg/mL with 10 mM sodium phosphate buffer (pH 7.5 or pH 6.0) containing 130 mM NaCl. To the DNA solution was added the stock solution (10 mg/mL) of the PVIm-*graft*-PLL·Lac at a final ratio of 2/1 for the lysine unit (PLL·Lac-*graft*) to the nucleotide unit (DNA). The final concentration of DNA was adjusted to 50 μg/mL. Then, 150 μL of the DNA/PVIm-*graft*-PLL·Lac mixture was incubated with 20 μL of 50% sheep preserved blood (Cosmo Bio Co., Ltd., Tokyo Japan) for 13 h at 37 °C. After centrifugation, released hemoglobin was determined at 577 nm. One hundred percent hemolysis was achieved with hypotonic and surfactant treatment.

3.3.9 Transfection Protocol

In a typical 96-well plate experiment, 5000 cells/well HepG2 (human hepatoma) cells were transfected in 100 μL of medium containing 10% serum by the addition of 20 μL of phosphate-buffered saline (PBS) containing 2 μg of plasmid DNA encoded for the firefly luciferase reporter gene complexed with 2.4 μg of PVIm-*graft*-PLL·Lac. The above transfection was carried out for 4 h incubation and was repeated thrice. The DNA/PVIm-*graft*-PLL·Lac complexes were incubated for 14 h at room temperature, followed by the addition to cells. After the total 12 h of incubation, the medium was removed and replaced by fresh medium containing 10% serum. The cells were cultured for an additional 23 h and were assayed for luciferase expression.

3.4 RESULTS AND DISCUSSION

3.4.1 Synthesis and Characterization of Lactosylated PVIm-*graft*-PLL (PVIm-*graft*-PLL·Lac) Comb-Type Copolymers

The synthetic route of the PVIm-*graft*-PLL·Lac comb-type copolymer is shown in Scheme 3.1. I reported that the copolymerization of poly(CBZ-L-lysine) macromonomers (**3**) with comonomers results in the comb-type copolymer with a defined length and a defined density of PLL-*grafts* in Chapter 2 (and Chapter 5) (*23*, *26*). In this chapter, I chose VIm (**4**) as the comonomer. Figure 3.1 shows the GPC profile of the resulting copolymer PVIm-*graft*-PLL (**5**). The GPC profile indicated that unreacted macromonomers were removed by the purification process, including dialysis. The number-average molecular weight of the copolymer determined by GPC was about 4×10^4. The ^{1}H NMR spectra of the copolymer showed the characteristic signals of both PLL-*graft* and PVIm backbone: δ 1.4–1.8 (β-, γ-, and δ-methylene protons of PLL), 1.8–2.2 (methylene protons of PVIm), 3.0 (ε-methylene protons of PLL), 4.3 (α-methine protons of PLL), and 6.4–7.2 (imidazole protons of PVIm) ppm. No residual CBZ groups were detected in the final polymer samples, indicating the successful deprotection of amino groups. Furthermore, ^{1}H NMR signals revealed that the molar ratio of the imidazole groups to the primary amino groups was twelve to one. Then, the reductive amination between the ε-amino groups of PLL-*grafts* and the reducing end of lactose was carried out (*27*, *28*). The primary amino group determination with fluorescamine (*25*) confirmed that about 90% of the amino groups of the graft chains reacted with lactose. Figure 3.2 shows the acid–base titration curve of the resulting PVIm-*graft*-PLL·Lac (**6**). The comb-type copolymer (**6**) exhibited a dual ionic character owing to the two kinds of cationic segments, i.e., PVIm and PLL·Lac, in the copolymer. The titration curve

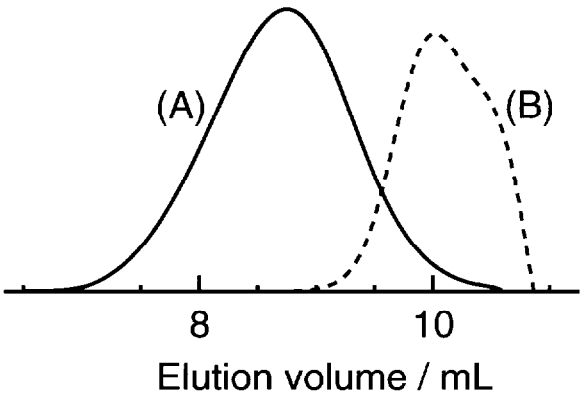


Figure 3.1. Gel permeation chromatograms of (A) the PDEAEMA-*graft*-PLL comb-type copolymer and (B) the deprotected CBZ-L-lysine macromonomer: column, Ultrahydrogel 1000; eluent, 0.5 M CH_3COOH + 0.2 M NaCl; flow rate, 0.8 mL/min; temperature, 25 °C; and detection, refractive index.

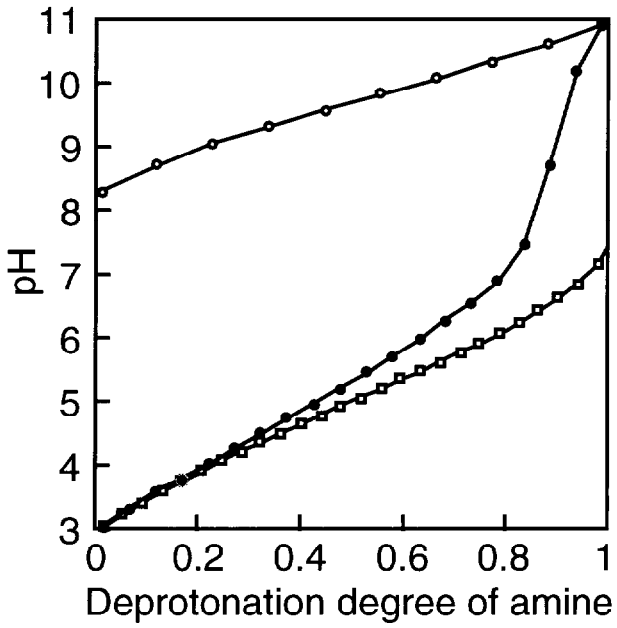


Figure 3.2. Acid-base titration curves of polymers: (●) PVIm-*graft*-PLL·Lac comb-type copolymer, (□) PVIm homopolymer, and (○) PLL·Lac homopolymer. Acidic polymer solutions were titrated with the stepwise addition of 1 M NaOH. The horizontal axis is normalized as the total basic groups of each polymer.

of **6** was consistent with the composition of the copolymer determined by ^{1}H NMR.

3.4.2 pH-Dependent Formation of the Polyelectrolyte Complex between DNA and the PVIm-*graft*-PLL·Lac Comb-Type Copolymer

I examined how the pH sensitivity of the PVIm-*graft*-PLL·Lac comb-type copolymer influenced the properties of their complex with DNA. First of all the pH-dependent formation of the DNA complex with PVIm homopolymer was assessed by agarose gel electrophoresis at neutral pH and acidic pH (Figure 3.3A). Each lane contains 7.5 μg of DNA, and lanes 2–5 (and lanes 2'–5') contain increasing amounts of PVIm resulting in unit ratios ([imidazole group]$_{PVIm}$/[phosphate group]$_{DNA}$) of 1, 6, 12, and 24, respectively. At pH 7.5, the DNA mixed with an excess amount of the PVIm (lanes 3–5) migrated into the gel, which suggested that DNA and PVIm did not form complexes owing to the complete deprotonation of PVIm. At pH 6.0, on the other hand, the DNA mixed with PVIm at a 1/1 unit ratio (lane 2') migrated partially into the gel. This is consistent the protonation degree of PVIm (about 25% of the imidazole groups were protonated at pH 6.0, as shown in Figure 3.2). When complexes were formed with an excess of positive charge (lanes 3'–5'), no band was visible. It is therefore suggested that the DNA did not enter the gel owing to the precipitation. No visible band was also observed in case of PLL·Lac and PLL homopolymer. PLL·Lac, as well as PLL (*29*), was completely protonated below pH 8.0 (Figure 3.2), so that the unit ratio ([amino group]$_{PLL\cdot Lac\ or\ PLL}$/[phosphate group]$_{DNA}$) was equal to the charge ratio (+/-) at pH 7.5. As shown in Figure 3.3B, the DNA complex with PLL·Lac or PLL presumably precipitated above a 1/1 (+/-) charge ratio (lanes 3, 4, 6, and 7) at pH 7.5. The same migration profile was observed at pH 6.0 (results not shown).

After confirming the pH-dependent properties of DNA/homopolymer complexes, I used

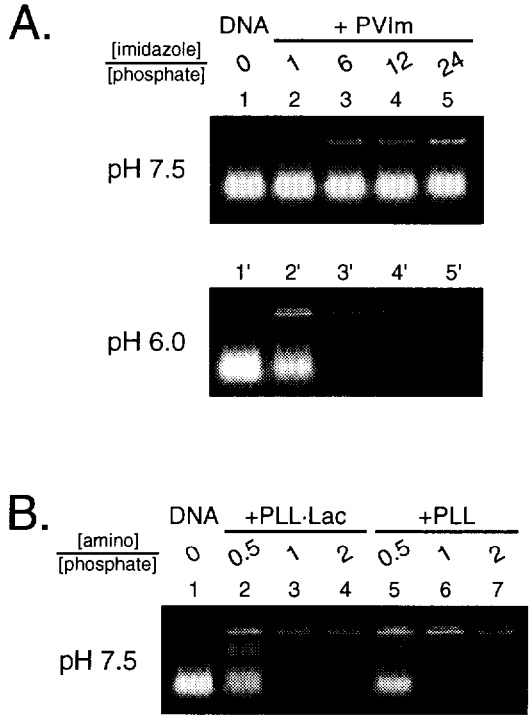


Figure 3.3. Analysis of the pH-dependent formation of the complex between DNA and homopolymers by agarose gel electrophoresis. (A) Interaction of PVIm with DNA at pH 7.5 (lanes 1–5) or pH 6.0 (lanes 1'–5'): Lanes 1 and 1', DNA alone; lanes 2–5 and 2'–5', DNA/PVIm mixtures at different unit ratios relative to imidazole groups of PVIm per phosphate group of DNA ([imidazole]/[phosphate]). Lanes 2 and 2', 1×; lanes 3 and 3', 6×; lanes 4 and 4', 12×; lanes 5 and 5', 24×. (B) Interaction of PLL·Lac (lanes 2–4) or PLL (lanes 5–7) with DNA at pH 7.5: Lane 1, DNA alone; lanes 2–7, DNA/polycation mixtures at different charge ratios relative to positive charges of polycations per negative charge of DNA (+/-). Lanes 2 and 5, 0.5×; lanes 3 and 6, 1×; lanes 4 and 7, 2×.

the comb-type copolymers of PVIm-*graft*-PLL·Lac and PVIm-*graft*-PLL for the gel retardation assay that was carried out under the same conditions as Figure 3.3. The result is shown in Figure 3.4. At pH 7.5, the bands resulting from DNA/copolymer complexes were visible and retained at the origin above a 1/1 (+/-) charge ratio (lanes 3, 4, 6, and 7). The visible bands at the origin were also observed in the previous report (by Maruyama *et al.*) that the soluble complexes between DNA and polycation are available by conjugating water-soluble polymer chains such as dextran to polycations (*30*). In this study, the PVIm backbone solubilized the DNA complexes with PLL·Lac-*grafts* or PLL-*grafts*, since PVIm backbone did not interact with DNA at pH 7.5 (Figure 3.3). At pH 6.0, the migrating DNA disappeared owing to the protonation of the PVIm backbone (lanes 2' and 5'). Furthermore, it should be noted that no visible band was observed in case of the DNA/PVIm-*graft*-PLL·Lac complexes at lower mixing ratios (lanes 2' and 3'). In this case, the pH-dependent property of the DNA/PVIm-*graft*-PLL·Lac complexes was similar to that of the DNA/PVIm complexes (Figure 3.3, lanes 3' and 4'). These results suggest that the DNA preferentially formed complexes with the PVIm backbone as against the PLL·Lac-*grafts*.

3.4.3 Solubility Change of the Polyelectrolyte Complex between DNA and the PVIm-*graft*-PLL·Lac Comb-Type Copolymer in Response to pH

To examine further the pH-dependent behavior of the DNA complex with the comb-type copolymer, we studied the solubility change of the DNA complex solution. Figure 3.5A shows the pH-dependent turbidity change of the DNA solution mixed with the PVIm-*graft*-PLL·Lac at a 2/1 (+/-) charge ratio calculated as pH 7.5. Neither the DNA solution nor the PVIm-*graft*-PLL·Lac solution exhibited any turbidity. The DNA/PVIm-*graft*-PLL·Lac mixture was the same sample used in the gel retardation assay (Figure 3.4, lanes 4 and 4'), so

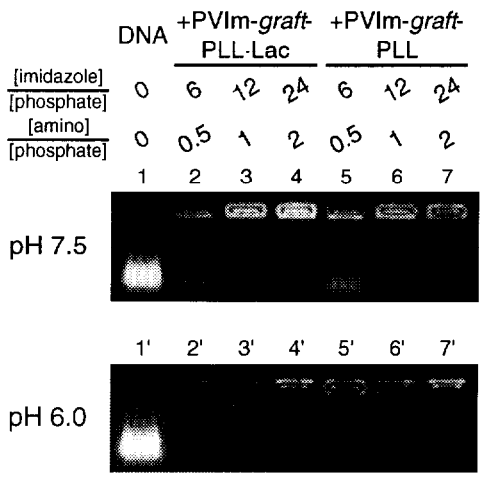


Figure 3.4. Analysis of the pH-dependent formation of the complex between DNA and comb-type copolymers by agarose gel electrophoresis. Interaction of PVIm-*graft*-PLL·Lac (lanes 2–4 and 2'–4') or PVIm-*graft*-PLL (lanes 5–7 and 5'–7') with DNA at pH 7.5 (lanes 1–7) or pH 6.0 (lanes 1'–7'): Lanes 1 and 1', DNA alone; lanes 2–7 and 2'–7', DNA/copolymer mixtures at different unit ratios relative to amino groups of graft chains per phosphate group of DNA ([amino]/[phosphate]). Lanes 2, 2', 5 and 5', 0.5×; lanes 3, 3', 6 and 6', 1×; lanes 4, 4', 7 and 7', 2×. Corresponding unit ratios relative to imidazole groups of PVIm backbone per phosphate group of DNA are also indicated ([imidazole]/[phosphate]). Lanes 2, 2', 5 and 5', 6×; lanes 3, 3', 6 and 6', 12×; lanes 4, 4', 7 and 7', 24×.

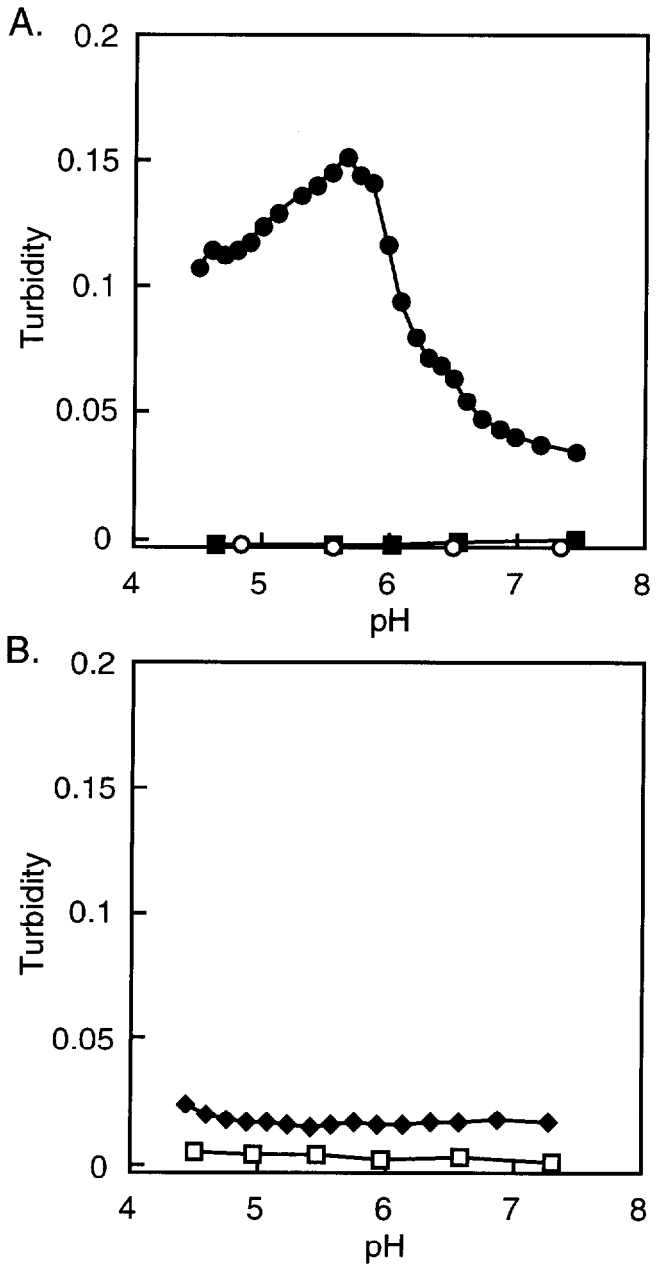


Figure 3.5. Effect of pH on the solubility of the DNA complex with (A) PVIm-*graft*-PLL·Lac or (B) PVIm-*graft*-PLL: (●) DNA/PVIm-*graft*-PLL·Lac mixture ([amino group]$_{PLL\cdot Lac\text{-}graft}$/[phosphate group]$_{DNA}$ = 2), (○) DNA alone, (■) PVIm-*graft*-PLL·Lac alone, (◆) DNA/PVIm-*graft*-PLL mixture ([amino group]$_{PLL\text{-}graft}$/[phosphate group]$_{DNA}$ = 2), and (□) PVIm-*graft*-PLL alone in 0.15 M NaCl. The pH was varied by adding 0.1 M HCl. The turbidity was measured by monitoring the absorbance of the mixture at 500 nm.

that the DNA and PVIm-*graft*-PLL·Lac must form complexes between pH 6.0 and pH 7.5. Little turbidity at pH 7.5 therefore revealed the DNA/PVIm-*graft*-PLL·Lac complex to be soluble. When the pH of the medium was gradually decreased up to 6.0, significant turbidity appeared. The turbidity was maintained even at pH 4.5. These results suggest that the protonation of the PVIm backbone induced the insoluble complex between the PVIm and DNA. Under this experimental conditions, the existence of relatively many PLL·Lac-*grafts* presumably protected the PVIm/DNA complex from the bulk precipitation (see Figure 3.4; compare lane 4' with lanes 2' and 3').

In case of the PVIm-*graft*-PLL, the formation of the DNA complex below pH 7.5 was also confirmed by the gel retardation assay (Figure 3.4, lanes 7 and 7'). Furthermore, the pH dependency of the retardation was observed; namely, the DNA migrated slightly into the cathode side of the gel. However, no significant turbidity was observed between pH 4.5 and pH 7.5, as shown in Figure 3.5B. These results suggest that the protonation of the PVIm backbone did not influence the interaction between the DNA and the PLL-*graft*.

Taking these results into account, the introduction of lactose groups to the PLL-*grafts*, as well as the protonation of the PVIm backbone, played an important role in the change of the assembling structure of the DNA complexes. It is worth noting that the solubility of the DNA/polycation complex in water decreased in spite of the increase of the net positive charges of DNA/polycation mixture. The interaction between DNA and PLL·Lac ought to be "weaker" than that between DNA and PLL.

3.4.4 Hemolytic Activity of the Polyelectrolyte Complex between DNA and the PVIm-*graft*-PLL·Lac Comb-Type Copolymer

I have observed the pH-dependent change of the properties of the DNA/PVIm-*graft*-

PLL·Lac complex between physiological pH and endosomal pH, so that the pH-dependent change is desired to enhance the interaction of the DNA complex with endosomal membranes. To examine the interaction of the DNA/PVIm-*graft*-PLL·Lac complexes with real cell membranes, I investigated the effects of the DNA complexes on erythrocyte membranes. As shown in Figure 3.6, the DNA/PVIm-*graft*-PLL·Lac complex which was used in Figure 3.5A caused a little hemolysis at pH 7.5 (approximately 10%). However, hemolysis increased significantly at pH 6.0 (approximately 25%). The charge ratios (+/-) of the DNA complex at pH 7.5 and pH 6.0 were 2 and 8, respectively. Furthermore, the hydrophobicity of the DNA complex also increased at pH 6.0 (Figure 3.5A). These factors seem to increase the hemolytic activity of the DNA complexes when the pH of the medium was lowered. It is expected that the DNA/PVIm-*graft*-PLL·Lac complexes will enhance the ability to escape from acidic endosomal vesicles.

3.4.5 Transfection of HepG2 Cells with the PVIm-*graft*-PLL·Lac Comb-Type Copolymer

Finally, I checked whether the DNA/PVIm-*graft*-PLL·Lac complexes led to gene expression. For this, the plasmid DNA pGL2 encoded for the firefly luciferase reporter gene was used. HepG2 cells seeded in a 96-well plate at 5, 000 cells/well were transfected with pGL2/PVIm-*graft*-PLL·Lac complexes at a 0.25/1 charge ratio (+/-) under physiological pH conditions (Figure 3.7). The pGL2/PVIm-*graft*-PLL·Lac complexes showed the significant luciferase activity, while no luciferase activity was observed for the cells incubated with DNA alone. Furthermore, from my preliminary study, almost no gene expression was observed when the pGL2/PVIm-*graft*-PLL·Lac complexes were formed with an excess of positive charge at physiological pH (results not shown). Although the reason for no gene expression

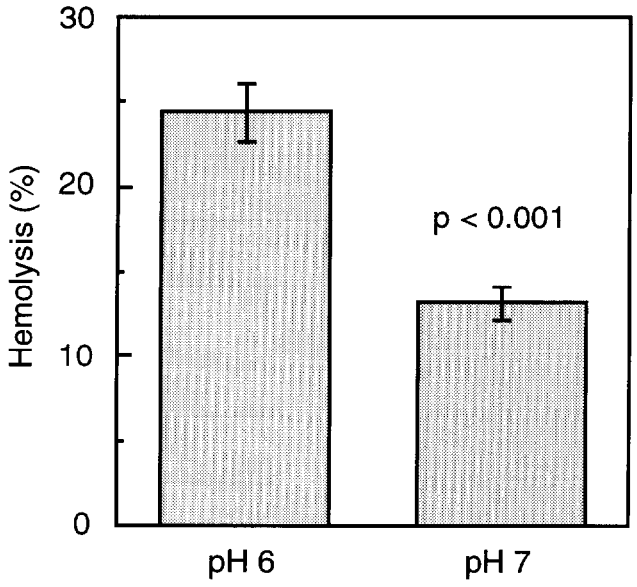


Figure 3.6. Effect of pH on the hemolytic activity of the DNA complex with PVIm-*graft*-PLL-Lac. Hemolytic activity was determined as described under Experimental Procedures. Erythrocytes were incubated with the DNA/PVIm-*graft*-PLL-Lac complex used in Figure 3.5A at 37 °C for 13 h at pH 6.0 or 7.5. Symbols and error bars represent the mean and standard deviation of the measurements made in paired samples (n = 3).

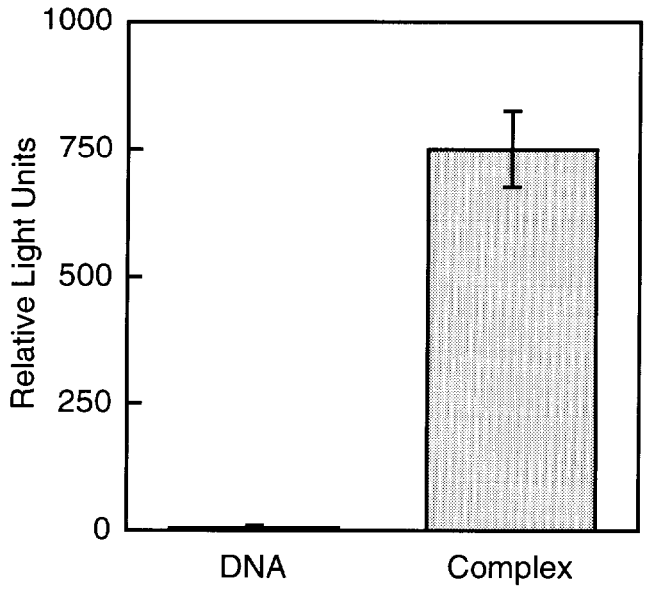


Figure 3.7. PVIm-*graft*-PLL·Lac-mediated transfection of HepG2 cells. Cells (5000 cells/well) were transfected as described under Experimental Procedures with total 6 μg of plasmid DNA complexed to PVIm-*graft*-PLL·Lac at a 0.25/1 charge ratio (+/-) at pH 7.5. Luciferase activity was measured 23 h post-transfection. Symbols and error bars represent the mean and standard deviation of the measurements made in triplicate wells.

is unclear, the significant gene expression proves that the DNA complex was taken up by the cell. The DNA is considered to be translocated to the nucleus through cytoplasmic space. Taking the study in Chapter 2 (*23*) into account, the pGL2/PVIm-*graft*-PLL·Lac complex leading to the gene expression was formed under the DNA excess condition, so that the PVIm-*graft*-PLL·Lac may vary DNA distortion and condensation in endosome for the translocation to the cytoplasmic space.

3.5 CONCLUSION

I have prepared the PVIm-*graft*-PLL·Lac comb-type copolymer with the pH sensitivity at endosomal pH. The PVIm-*graft*-PLL·Lac is a bi-phasic pH-sensitive polyelectrolyte. The weaker basic segment PVIm was water-soluble and inert to the interaction with DNA at physiological pH, so that the DNA complex with the stronger basic segment PLL·Lac-*graft* was solubilized by the PVIm backbone. Not PLL-*graft* but PLL·Lac-*graft* formed the DNA complex which exhibited the pH-dependent change of the solubility. The solubility change was not correlated with the pH sensitivity (protonation–deprotonation) of PVIm backbone; namely, in spite of the increase of the net positive charges of DNA/polycation mixture, the solubility of the DNA/polycation complex in water decreased. In addition, the nonlinear change of the DNA complex solubility was achieved by increasing the content of PVIm, which exhibited linear pH sensitivity, in the PVIm-*graft*-PLL·Lac copolymer.

From a practical point of view, the DNA/PVIm-*graft*-PLL·Lac complex showed higher hemolytic activity at pH 6.0 than at pH 7.5. Since endocytozed DNA complexes are subjected to the pH change from pH 7 to pH 5 (*22*), the increased activity of cell membrane disruption at pH 6 is the promising property for the escape from endosome. In this chapter, I have demonstrated that the PVIm-*graft*-PLL·Lac comb-type copolymer is capable of sensing endosomal pH and outputting the nonlinear unique change of the physicochemical properties of DNA polyelectrolyte complexes. The unique change has created "stimulus-responsive intramolecular exchange of polyelectrolyte complexes". The pH-induced output influenced the biological properties of DNA complexes, so that I have achieved the prime carrier made of the artificial polymer mimicking the intelligent virus infection pathway.

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Chapter 4

Synthesis of Novel Polyampholyte Comb-Type Copolymers Consisting of a Poly(L-lysine) Backbone and Hyaluronic Acid Side Chains for a DNA Carrier

4.1 ABSTRACT

The polyampholyte comb-type copolymers consisting of a poly(L-lysine) (PLL) main chain, a DNA binding site, and hyaluronic acid (HA) side chains, cell-specific ligands, have been prepared as the DNA carrier targeting sinusoidal endothelial cells of liver. The reducing end of HA and ε-amino groups of PLL were covalently coupled by reductive amination to obtain the resulting comb-type copolymers (PLL-*graft*-HA). The chain length of HA was controlled by the enzymatic hydrolysis of high-molecular weight HA. Since HA formed polyelectrolyte complexes with PLL, the coupling reaction was carried out with high-ionic strength media to suppress polyelectrolyte complex formation. The reaction proceeded in a homogeneous system, leading to a high efficiency of coupling (>70%) of HA onto the PLL backbone. By using the enzymatic hydrolysis of HA and the reductive amination reaction between HA and PLL with high-ionic strength media, it is possible to prepare the various comb-type copolymers with a defined density and a defined length of HA side chains. Furthermore, I also find that these polyampholyte comb-type copolymers vary their assembling structure in water in response to two kinds of environmental factors, i.e., ionic strength and pH. Finally, a ^{1}H NMR study reveals that the PLL backbone efficiently interacts with DNA molecules despite the presence of HA side chains having negative charges.

4.2 INTRODUCTION

Polycations have been used for the nonviral gene carriers because the polycations and DNAs form stable complexes in a noncovalent manner (*1–3*). The polycations, e.g., poly(L-lysine) (PLL), are reported to be conjugated with several ligands for targeted gene delivery (*4–8*). The physicochemical properties of the DNA complexes have been described as factors which influence transfection activity (*9–11*). However, the general method controlling and optimizing the complex properties has not been established yet.

Maruyama *et al.* have reported on several comb-type copolymers consisting of a PLL backbone and hydrophilic dextran side chains for controlling the assembling structure of DNA–copolymer complexes (*12, 13*). The dextran chains grafted onto PLL are found to reduce the aggregation of the resulting complexes and increase the solubility of the complexes. Furthermore, the grafting degree of the copolymer affects the DNA conformation in the complex, enabling us to regulate DNA compaction. The comb-type copolymers with a higher degree of grafting induce little compaction of DNA and stabilize DNA duplexes and triplexes by shielding the repulsion between phosphate anions of DNA. Moreover, the grafted chains reduce the nonspecific interaction of the PLL backbone with proteins (*14*). The comb-type copolymers therefore fulfill several requirements for the cell-specific carrier of DNA, if the copolymers are provided with cell-specific ligands.

Hyaluronic acid (HA) is an unbranched high-molecular weight polysaccharide consisting of alternating *N*-acetyl-β-D-glucosamine and β-D-glucuronic acid residues linked at the 1–3 and 1–4 positions, respectively (*15*). Liver sinusoidal endothelial cells (SECs) possess the receptors that recognize and internalize HA (*16, 17*). More than 90% of HAs in the blood stream are known to be taken up and metabolized by SECs. I have therefore chosen HA as the ligand for delivering the DNA to the SEC.

The binding affinity of the HA receptor is reported to increase as the chain length of HA is increased (*18*). In addition, the density and length of HA chains grafted onto PLL are expected to be the factors which affect the assembling structure of PLL–DNA complexes in the context of

the efficiency of both targeting and transfection *in vivo*. The various comb-type copolymers consisting of a PLL backbone and HA side chains would be useful in investigating the structure–function relationship and optimizing the complex structure.

In this chapter, I have prepared the PLL-*graft*-HA comb-type copolymers with various chain lengths of both PLL and HA segments and the densities of HA-*grafts*. For the synthesis of the comb-type copolymers, high-molecular weight HA was hydrolyzed, and then the HAs were covalently coupled with ε-amino groups of PLL at their reducing end by reductive amination. This chapter describes the synthesis and characterization of the PLL-*graft*-HA comb-type copolymers in the context of their physicochemical properties and complex formation with DNA.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Materials

PLL, obtained as its chloride or bromide salt, was purchased from Peptide Institute, Inc. (Osaka, Japan). High-molecular weight HA (5.9×10^5), obtained as its sodium salt (sodium hyaluronate), was kindly provided by Denki Kagaku Kogyo Co., Ltd. (Tokyo, Japan). Bovine testicular hyaluronidase (EC 3.2.1.35, type I-S) was purchased from Sigma Chemical Co. (St. Louis, MO), and sodium cyanoborohydride ($NaBH_3CN$) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Salmon sperm DNA (approximately 300 base pairs) was kindly provided by Nissan Chemical Industries, Ltd. (Tokyo, Japan). All other chemicals of a special grade were used without further purification.

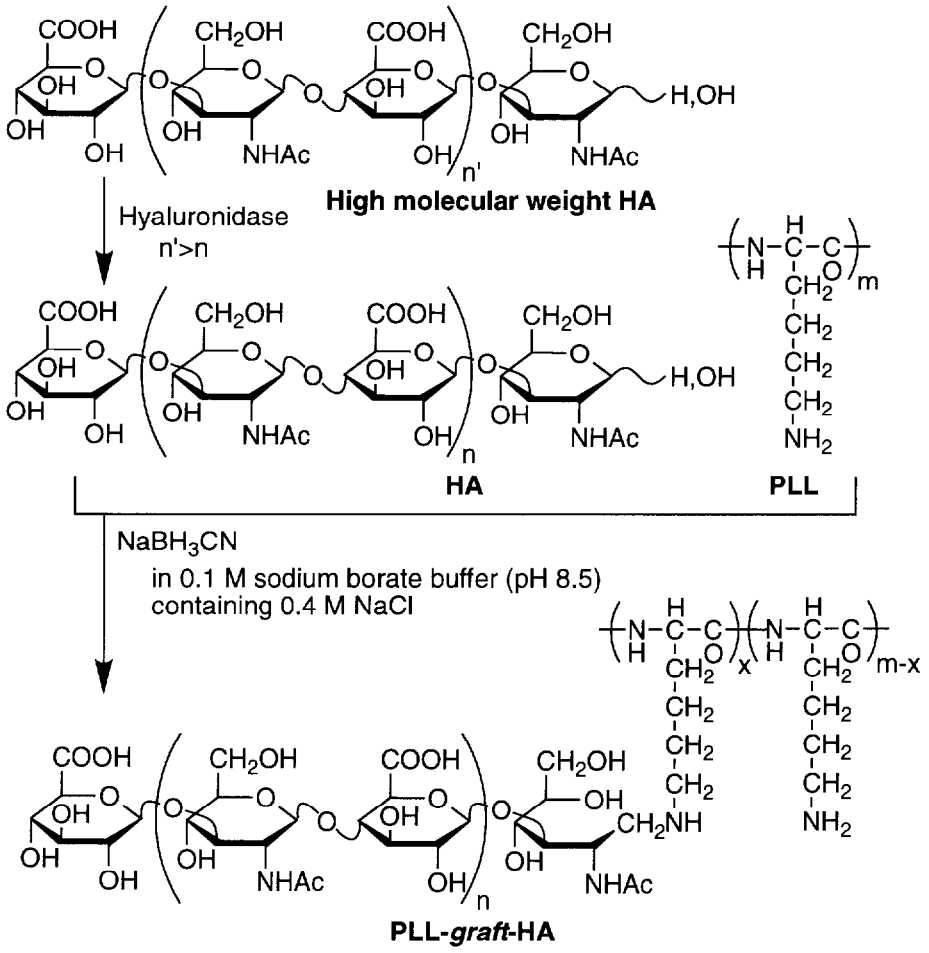
4.3.2 Enzymatic Hydrolysis of HA

High-molecular weight HA was partially degraded by hyaluronidase to low-molecular weight fragments (*19*). Hyaluronidase (20 mg) and HA (1 g) were added together in 120 mL of water. The solution was stirred at 50 ℃ for a desired time (1–20 h). Then, the solution was boiled for 5 min to terminate the reaction and allowed to cool to room temperature. After the filtration of the cooled mixture through a 0.45 μm filter (New Steradisc 25, KURABO, Osaka, Japan), the resulting HA fragments were obtained by freeze-drying.

4.3.3 Synthesis of PLL-*graft*-HA Comb-Type Copolymers

The obtained HA fragments were conjugated to PLL by reductive amination using $NaBH_3CN$ as a reducing agent (Scheme 4.1). The $NaBH_3CN$ was added to the mixture of HA (100–300 mg) and PLL (60–120 mg) in 15 mL of sodium borate buffer (0.1 M, pH 8.5) containing 0.4–1 M NaCl at a final concentration of 0.3 M. The reaction mixture was incubated at 40 ℃ for 3 days while it was stirred constantly, followed by dialysis against a 0.5 M NaCl aqueous solution using a Spectra/Por 7 membrane (molecular weight cutoff of 25 000) to remove

Scheme 4.1. Synthesis of PLL-*graft*-HA Comb-Type Copolymers



unreacted HA. Then, the solution was desalted by dialysis against distilled water, and the resulting copolymer was obtained by freeze-drying.

4.3.4 Gel Permeation Chromatography (GPC)

GPC was carried out using a JASCO 880-PU pumping system (Tokyo, Japan) at the flow rate of 1.0 mL/min at 25 ℃ with Ultrahydrogel 500 and Ultrahydrogel 250 columns (Japan Waters Ltd., Tokyo, Japan). The aqueous solution containing 0.2 M Na_2SO_4 and 5 mM sodium phosphate buffer (pH 8.0) was used as a mobile phase. Two hundred microliters of each sample was picked up from the reaction mixture and was injected into the columns. Eluate was detected by a refractive index (RI) detector (830-RI, JASCO) and a multiangle light scattering (LS) detector (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA). RI and LS signals were transferred to the computer to calculate the number-average (M_n) and weight-average (M_w) molecular weight according to the instruction manual (Wyatt Technology Co.) for Dawn-DSP.

4.3.5 ^{1}H NMR Spectroscopy

Each copolymer was dissolved in D_2O (99.95 at. % D; Merck, Darmstadt, Germany) containing 0.35 M NaCl. ^{1}H NMR spectra (400 MHz) were obtained by a Varian Unity 400plus spectrometer (Palo Alto, CA), at a probe temperature of 298 K. The chemical shifts are expressed as parts per million using internal HDO molecules (δ = 4.7 ppm in D_2O) as a reference.

4.3.6 Turbidity Measurement of PLL-*graft*-HA Solutions

The copolymer was dissolved in 3 mL of distilled water at a concentration of 1.0 mg/mL. The ionic strength of the solution was increased by the stepwise addition of a 5 M NaCl solution. The pH of the solution was varied by adding a 0.1 M solution of HCl or NaOH. The turbidity change of the solution at different ionic strengths or pHs was monitored as the absorbance at 500 nm with a Beckman DU-640 spectrophotometer (Fullerton, CA).

4.3.7 Estimation of the DNA/PLL-*graft*-HA Interaction by ^{1}H NMR Spectroscopy

The stock solution of DNA (1 mg/mL) in 10 mM sodium phosphate buffer (pH 7.2) containing 0.5 mM EDTA and 1 M NaCl was mixed with the PLL-*graft*-HA solution (1 mg/mL) in 1 M NaCl at a copolymer/DNA charge ratio ($[\text{amino group}]_{\text{copolymer}}/[\text{phosphate group}]_{\text{DNA}}$) of 1.0. The mixture was then desalted by the step-down dialysis using a Spectra/Por 7 membrane (molecular weight cutoff of 1 000), followed by freeze-drying. The resulting sample was dissolved in D_2O. The ^{1}H NMR spectrum was obtained as described under Section 4.3.5.

4.4 RESULTS AND DISCUSSION

4.4.1 Synthesis of PLL-*graft*-HA Comb-Type Copolymers

Most polysaccharides are useful for the synthesis of comb-type copolymers using the coupling reaction because they have one reducing end per molecule; namely, they are semi-telechelic polymers (*20*, *21*). The polysaccharides whose glycosyl bonds are hydrolyzed enzymatically or chemically are also semitelechelic. Therefore, the comb-type copolymers with the different densities and lengths of polysaccharide side chains would be available by combining enzymatic hydrolysis and the conjugation of the reducing end to the main chain polymer (Scheme 4.1).

To regulate the molecular weight of HA, we hydrolyzed high-molecular weight HA with hyaluronidase for the indicated time period (Figure 4.1). The molecular weight of HA decreased and the molecular weight distribution narrowed with the increase in digestion time. When the time for enzyme reaction was controlled, the HAs with the desired molecular weight, i.e., chain length, were isolated.

The hydrolyzed HA fragment was then grafted onto PLL by reductive amination between the reducing end of HA and ε-amino groups of PLL using $NaBH_3CN$ as a reducing agent. The HA polyanion can form the polyelectrolyte complex, which prevents the conjugating reaction, with the PLL polycation. To suppress this polyelectrolyte complex formation, the ionic strength of the medium was increased (0.4–1 M NaCl). The increase in the NaCl concentration resulted in the reaction proceeding in a homogeneous system (see Figure 4.4A).

Figure 4.2 shows the representative GPC profile of the reaction mixture and the resulting copolymer. After the reaction, the peak area of PLL increased while that of HA decreased. Although an obvious elution volume shift was not observed in the chromatogram owing to some interaction between the polymers and column packing, the molecular weight determined by the LS detector increased drastically after the reaction, being 2.3×10^5 for the resulting copolymer in comparison with 9.7×10^4 for the original PLL. The incubation of HA or PLL alone with

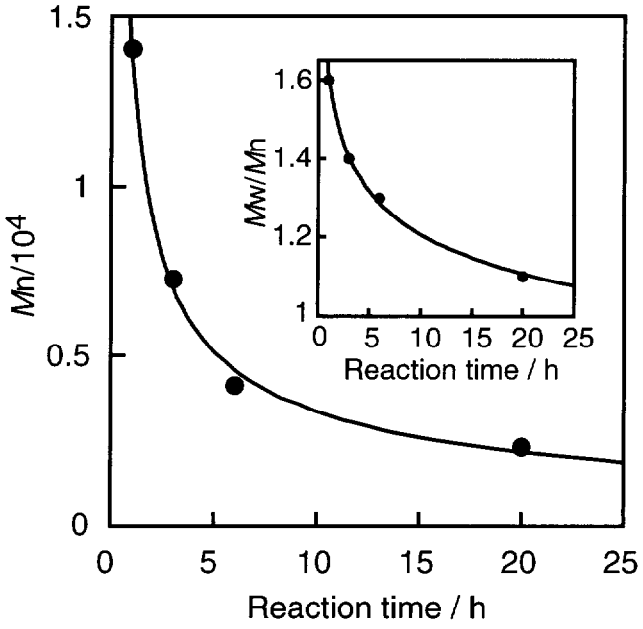


Figure 4.1. Time course of the hydrolysis of HA by hyaluronidase. HA (5.9×10^5; 1 g) was hydrolyzed by hyaluronidase (20 mg) at 50 °C. The M_n of HA was determined by GPC. The inset shows the change in the molecular weight distribution (M_w/M_n).

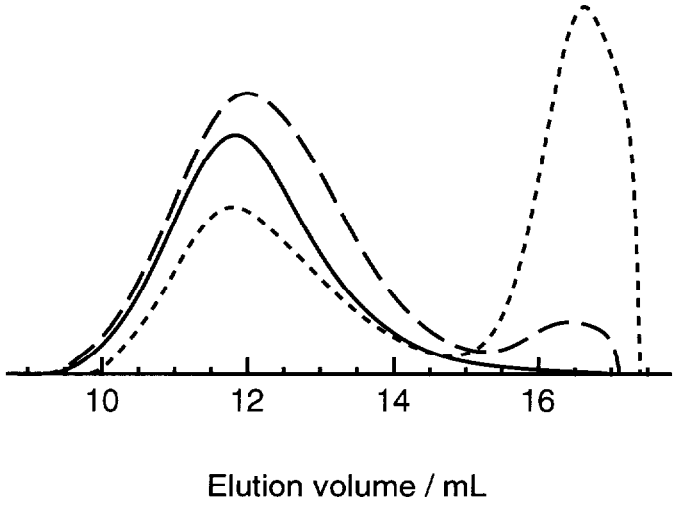


Figure 4.2. Gel permeation chromatograms of the isolated PLL-*graft*-HA copolymer (solid line) and the reaction mixture of PLL ($M_n = 9.7 \times 10^4$; 39 mg) and HA ($M_n = 2.2 \times 10^3$; 94 mg) at 0 h (dotted line) or 9 days (dashed line): column, Ultrahydrogel 500 and 250; eluent, 0.2 M Na_2SO_4 and 5 mM sodium phosphate buffer (pH 8.0); flow rate, 1.0 mL/min; temperature, 25 °C; and detection, RI.

$NaBH_3CN$ under the same conditions caused no change in GPC profiles, which suggested that neither degradation nor intermolecular side reaction was occurred (results not shown). When HA was incubated with PLL in the presence of a 10-fold molar excess of lactose or sucrose with respect to the reducing end of HA, not sucrose, the nonreducing sugar, but lactose, the reducing one, inhibited HA consumption (results not shown). Therefore, it can be concluded that the HA was conjugated with PLL at its reducing end through the reductive amination reaction (*22*). Figure 4.2 also shows that the comb-type copolymer was isolated from unreacted HA by dialysis.

As shown in Figure 4.3, the ^1H NMR spectra of the comb-type copolymer showed the characteristic signals of both PLL and HA moieties: PLL, δ 1.4–1.8 (β, γ, δ-CH_2), 3.0 (ε-CH_2), 4.3 (α-CH); HA, δ 2.0 (NAc-CH_3), 3.3–3.9 (H-2,3,4,5,6), 4.4–4.6 (H-1). From the signal ratio of methyl protons (2.0 ppm) of the *N*-acetyl groups of the HA-*grafts* to ε-methylene protons (3.0 ppm) of the PLL backbone, the content (wt % and grafting %) of HA in the copolymer was determined. The results of the synthesis of PLL-*graft*-HA comb-type copolymers are summarized in Table 4.1. The coupling efficiency was more than about 70%. Consequently, I have easily prepared the various PLL–HA conjugates with a well-defined comb-type structure by combining enzymatic hydrolysis and the reductive amination.

4.4.2 Polyampholyte Properties of the PLL-*graft*-HA Comb-Type Copolymer

The resulting PLL-*graft*-HA comb-type copolymer consists of a polycation backbone and polyanion side chains. The polyampholyte property of the comb-type copolymer was examined by measuring the turbidity of the copolymer solution. Figure 4.4A shows that the turbidity of the PLL-*graft*-HA solution varied with the change of NaCl concentration at neutral pH; namely, the solubility of the PLL-*graft*-HA markedly varied in water in response to the ionic strength of the medium. The PLL-*graft*-HA suddenly precipitated out of the aqueous medium at a particular ionic strength but became soluble again at higher ionic strengths.

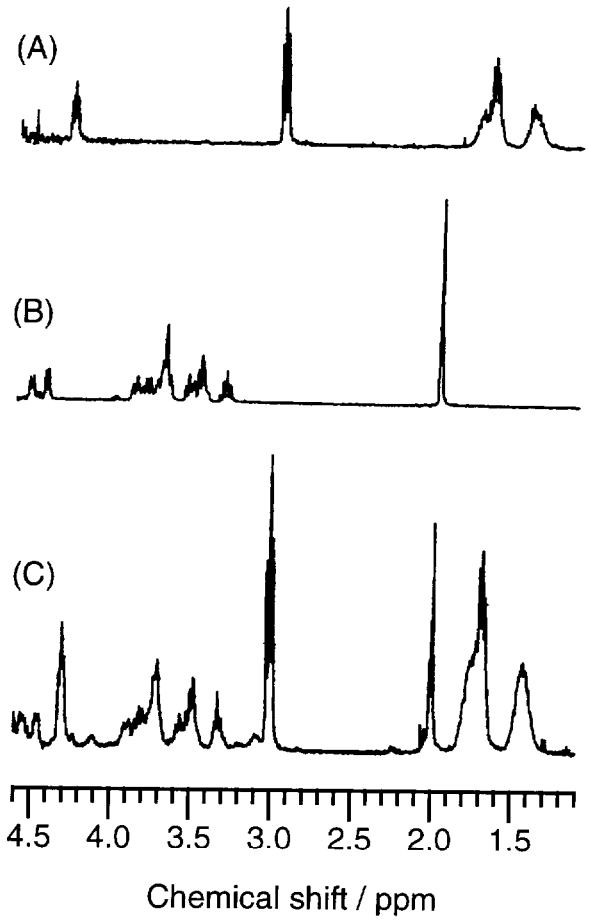


Figure 4.3. ^1H NMR spectra of PLL (A), HA (B), and PLL-*graft*-HA (C) in D_2O. For the PLL-*graft*-HA, D_2O containing 0.35 M NaCl was used.

Table 4.1. Synthesis of PLL-*graft*-HA Comb-Type Copolymers[a]

sample	in feed					copolymer					coupling efficiency[d] (%)	yield (%)
	PLL		HA			molecular weight[b]		HA content[c]				
	$M_n^b/10^4$	mg	$M_n^b/10^3$	mg	wt %	$M_n/10^4$	M_w/M_n	wt %	grafting %	charge ratio		
1	4.2	61.2	2.3	94.3	61	8.5	1.5	50	5.7	0.34	66	67
2	4.2	61.2	2.3	189	76	11	1.4	63	9.4	0.56	55	57
3	7.2	122	3.8	189	61	15	1.4	53	3.8	0.40	73	55
4	7.2	61.2	3.8	189	76	23	1.6	69	7.6	0.83	73	83
5	7.2	61.2	3.8	283	82	31	1.5	77	11	1.3	71	57
6	4.2	61.2	1.6	94.3	61	9.3	1.4	55	9.8	0.49	80	38
7	7.2	122	1.6	94.3	44	12	1.5	38	4.9	0.22	80	51
8	7.2	61.2	1.6	189	76	24	1.4	71	19	1.0	77	85

[a] Reducing reagent, 0.3 M $NaBH_3CN$; reaction temperature, 40 °C; reaction time, 56 h (samples 1 and 2), 80 h (samples 3–5), and 75 h (samples 6–8); solvent, 0.1 M sodium borate buffer (pH 8.5) containing 0.4 M NaCl (samples 1 and 2) or 1 M NaCl (samples 3–8).

[b] The molecular weight and its distribution (M_w/M_n) were determined by GPC.

[c] Determined by ^1H NMR; grafting % = (the mole fraction of the lysine residues grafted with HA) × 100%; charge ratio = $[\text{carboxyl group}]_{HA}/[\text{amino group}]_{PLL}$ in the copolymer.

[d] $[HA]_{copolymer}/[HA]_{in\ feed} \times 100\%$.

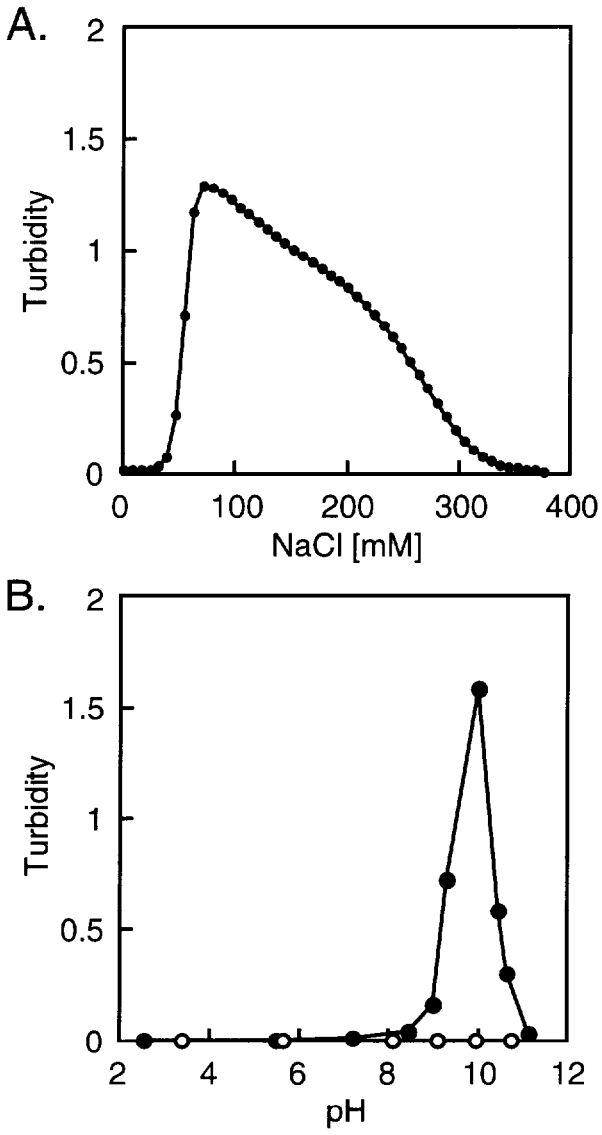


Figure 4.4. Polyampholyte properties of the PLL-*graft*-HA comb-type copolymer (sample 1). (A) Effect of ionic strength on the solubility of the copolymer at neutral pH. (B) Effect of pH on the solubility of the copolymer in the absence (●) or presence (○) of 400 mM NaCl. The turbidity was monitored as the absorbance at 500 nm.

It should be noted that the PLL-*graft*-HA seems to be soluble at lower ionic strengths where the comb-type copolymers should form polyelectrolyte complexes. Since the net charge of the PLL-*graft*-HA used in the experiment is positive at neutral pH, the copolymers were probably dispersed in the medium as colloidal particles without undergoing particle aggregation. The increase in the ionic strength of the medium presumably reduced the electrostatic repulsion among the colloidal particles, inducing particle aggregation (salting out) to exhibit turbidity. However, the further increase in the ionic strength of the medium over 350 mM NaCl probably dissociated the polyelectrolyte complex between PLL and HA chains, resulting in the complete disappearance of turbidity. To assess the solution behavior at the lower ionic strength, I varied the pH of the solution (*23*). As seen in Figure 4.4B, the sudden increase in turbidity was observed when the pH of the medium was increased to 9.0, where the deprotonation of the ε-amino groups of PLL was initiated (*24*). The partial deprotonation of the PLL moiety presumably decreased the repulsive force among colloidal particles and increased their hydrophobicity, triggering interparticle aggregation. Finally, the solution became transparent again above pH 11, owing to the complete dissociation of the polyelectrolyte complex, due to the thorough deprotonation of PLL, between PLL and HA. These results suggest that the PLL-*graft*-HA formed its intramolecular polyelectrolyte complex under low-ionic strength conditions (in 0 mM NaCl) and dissociated its polyelectrolyte complex under high-ionic strength conditions (>350 mM NaCl).

4.4.3 DNA/PLL-*graft*-HA Polyelectrolyte Complex

The DNA was mixed with the PLL-*graft*-HA ($[\text{amino group}]_{\text{copolymer}}/[\text{phosphate group}]_{\text{DNA}}$ = 1), and the ionic strength of the medium was gradually decreased from 1 to 0 M NaCl by step-down dialysis (*25*). The transparent solution obtained after dialysis was then lyophilized, dissolved in D_2O, and analyzed by ^1H NMR. The ^1H NMR spectra of the copolymer with or without DNA are shown in Figure 4.5. It is worth noting that the signals of ε-methylene protons of the PLL moiety in the PLL-*graft*-HA broadened in the presence of DNA (Figure 4.5C). This is probably caused by polyelectrolyte complex formation between the PLL moiety

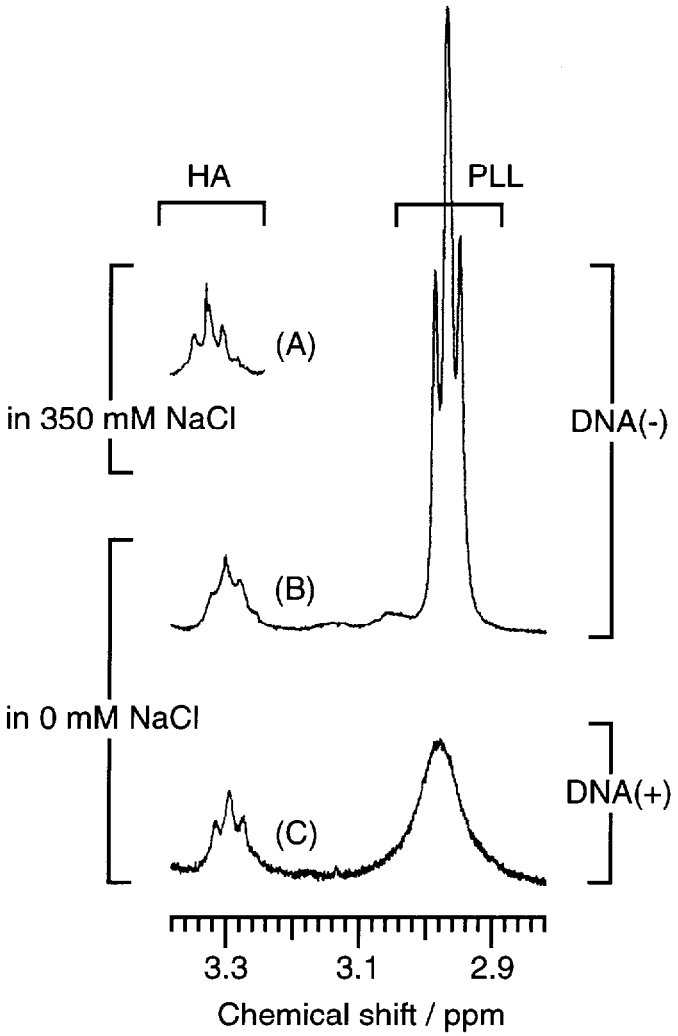


Figure 4.5. Effect of DNA on the ^1H NMR spectra of the PLL-*graft*-HA comb-type copolymer: PLL-*graft*-HA alone in D_2O containing 350 mM NaCl (A) and PLL-*graft*-HA in the absence (B) or presence (C) of DNA in D_2O without NaCl. The signal of ε-methylene protons of PLL (3.0 ppm) and the H-2 signal of glucuronic acid of HA (3.3 ppm) are represented.

and DNA. Although the PLL signals broadened, the HA signals remained unchanged, or rather became sharper. The sharp signals of HA in the presence of DNA under low-ionic strength conditions (Figure 4.5C) seem to be similar to those of PLL-*graft*-HA alone at high ionic strengths (Figures 4.3C and 4.5A), where the PLL-*graft*-HA did not form polyelectrolyte complex; i.e., HA chains were free. These results suggest that the PLL backbone selectively formed the polyelectrolyte complex with DNA even in the presence of the HA side chains. The preferential complex formation was further demonstrated by the acrylamide gel electrophoresis analysis of a PLL-*graft*-HA–oligoDNA mixture, as will be reported elsewhere. In addition, the PLL-*graft*-HA–DNA complex may form the multiphase structure in which the hydrophobic PLL–DNA complex is surrounded by the hydrated shell of free HA (*26*, *27*). Complex formation with free HA chains is considered to be essential for directing the complex to target cells.

4.5 CONCLUSION

In this chapter, I have prepared the various PLL-*graft*-HA comb-type copolymers with a defined density and a defined length of HA side chains. The synthesis of the comb-type copolymers has been achieved by (1) the control of the HA chain length by enzymatic hydrolysis, (2) the suppression of formation of a polyelectrolyte complex between HA and PLL in high-ionic strength media during the coupling reaction, and (3) the relatively high coupling efficiency of the reductive amination between the reducing end of HA and ε-amino groups of PLL.

By using the various PLL-*graft*-HA comb-type copolymers, it would be possible to control the properties of the DNA complex such as complex size, solubility, and the degree of DNA compaction (*13*, *28*). The resulting comb-type copolymers are, therefore, useful in investigating the relationship between DNA complex properties and the efficiency of both targeting and transfection. Furthermore, the efficient conjugation of the HA chains to other materials (*29*—*38*) can offer unique tools for studying the biological and biochemical properties of HA.

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4.7 ADDITIONAL SECTION: BIOLOGICAL PROPERTIES OF THE DNA/PLL-*GRAFT*-HA COMPLEX (Directed by Dr. Yoshiyuki Takei, who is my co-worker, of the Juntendo University School of Medicine)

4.7.1 Experimental Procedures

4.7.1.1 Plasmid. The expression plasmid encoding *lac*Z (pSV β-galactosidase control vector [pSV β-Gal]) obtained from Promega (Madison, WI) was grown in *Escherichia coli*, isolated, and purified by a standard method (*1'*). For the *in vivo* uptake study, the plasmid pSV β-Gal was labeled with ^{32}P by the multiprime method (*1'*) with a commercial kit (Random Primer DNA Labeling Kit version 2, TaKaRa, Kyoto, Japan).

For the detection of *lac*Z gene expression, a fluorogenic β-galactosidase substrate, 5-dodecanoylamino-fluorescein di-β-D-galactopyranoside (C_{12}FDG, Molecular Probes, Eugene, OR) was used (*2'*).

4.7.1.2. Distribution of the PLL-*graft*-HA/DNA complex *in vivo*. To ascertain that the PLL-*graft*-HA/DNA complexes were delivered to the SECs *in vivo*, the following experiments were carried out. First, the groups of Wistar rats weighing 200–250 g were injected intravenously via the tail vein with the ^{32}P-labeled pSV β-Gal (90 μg) complexed with PLL-*graft*-HA (For PLL, $M_n = 4.6 \times 10^4$; for HA, $M_n = 1.5 \times 10^4$; grafting %, 0.7%) in 500 μl saline. After 1 h, the animals were killed and the samples of blood, liver, spleen, gut, heart, lung, kidney, and thymus were obtained. The tissue samples were weighed, homogenized in saline, solubilized with a tissue solubilizer (Soluene-350, Packard Instrument, Meriden, CT), mixed with aqueous scintillant (High-Ionic Fluor, Packard), and counted on a liquid scintillation analyzer (TRI-CARB 1500, Packard).

4.7.1.3 *lacZ* gene expression after *in vivo* transfection. Seventy-two hours after the injection of either 90 μg of pSV β-Gal, or an equal amount of pSV β-Gal in a PLL-*graft*-HA-complexed form, rats were sacrificed and their livers were harvested, frozen and cut into 8-μm sections. Sections were fixed with 1% glutaraldehyde, washed with PBS, and incubated at 37 ℃ for 1 h in PBS containing 33 μM C_{12}FDG (2') and viewed under an epifluorescent microscope with an appropriate filter set.

4.7.2 Results

4.7.2.1 Organ distribution of the PLL-*graft*-HA/DNA complex after *in vivo* transfection. Organ distribution of the radiolabeled pSV β-Gal that had been injected in equal quantities (7.5×10^6 counts) is shown in Figure 4.6. When the plasmid was complexed with PLL-*graft*-HA, the PLL-*graft*-HA/DNA complex was almost exclusively delivered to the liver: 1 h after injection, more than 90% of the injected counts were detected in the liver. The residual counts distributed in other organs were as follows: 2.5% in intestine, 2% in spleen, 1.5% in kidney, and << 1% in heart, thymus, lung, and blood (Figure 4.6). Similar results were seen 3 h postinjection (results not shown).

4.7.2.2 *lacZ* gene expression in the liver transfected with the PLL-*graft*-HA/pSV β-Gal. Figure 4.7 shows the histological detection of the cells expressing β-galactosidase in the liver lobule 72 h after *in vivo* transfection. In the control in which pSV β-Gal alone was administered, no cells expressing β-galactosidase were observed (Figure 4.7A). In marked conrast, a large number of cells expressing β-galactosidase were detected along the sinusoidal walls (SECs) when transfected with an equal amount of pSV β-Gal in a PLL-*graft*-HA-complexes form (Figure 4.7B).

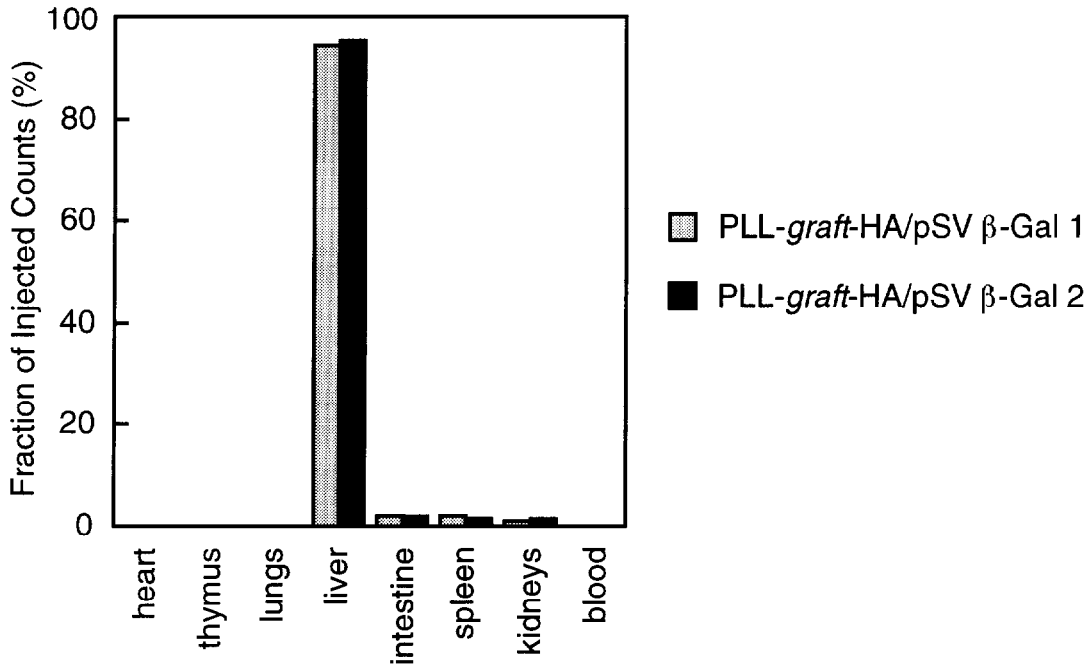

Figure 4.6. Organ distribution of the PLL-*graft*-HA/DNA complex injected intravenously. Wistar rats were injected intravenously via the tail vein with the PLL-*graft*-HA complexed with the pSV β-Gal labeled with ^{32}P. After 1 h, organ samples were homogenized, mixed with aqueous scintillant, and counted using a liquid scintillation analyzer. The results of two separate experiments (PLL-*graft*-HA/pSV β-Gal 1 and 2) were shown.

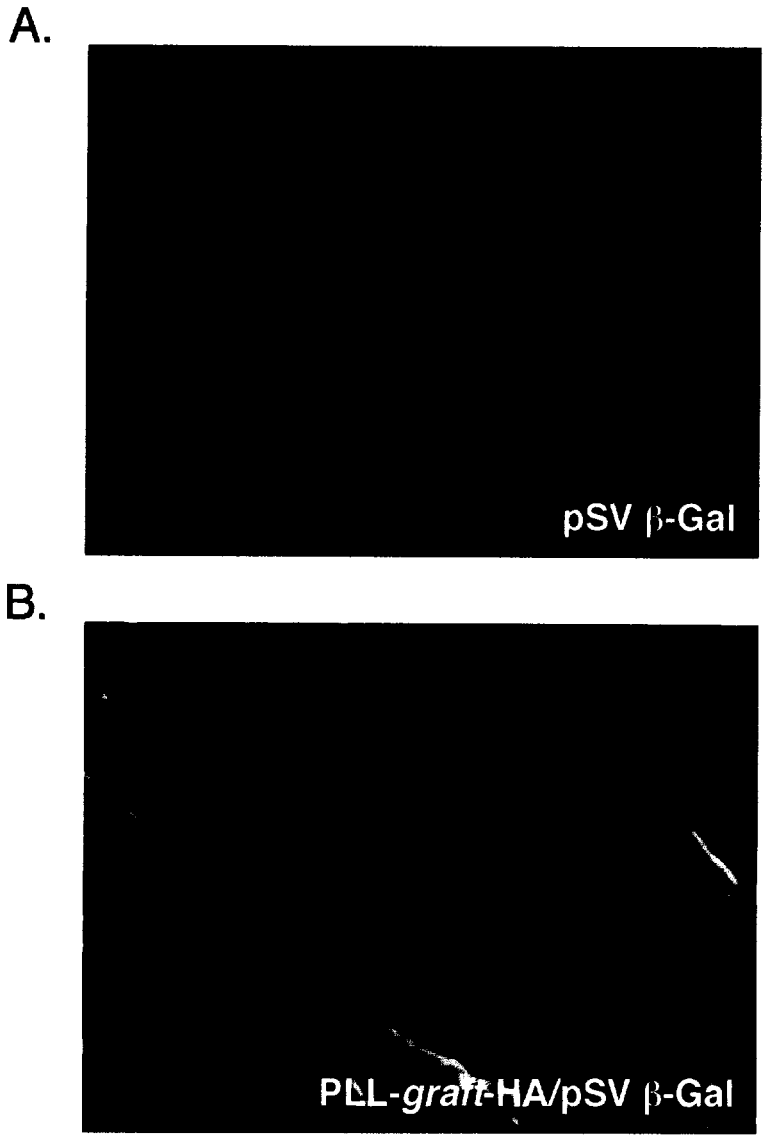

Figure 4.7. *lacZ* gene expression in the liver after *in vivo* transfection. Seventy-two hours after the *in vivo* transfection either (A) with free pSV β-Gal alone or (B) with the pSV β-Gal complexed with PLL-*graft*-HA, livers were harvested, and 8-μm sections were prepared. Sections were incubated at 37 °C for 1 h in PBS containing 33 μM C_{12}FDG, and viewed under the epifluorescent microscope with an appropriate filter set. Original magnification, ×200.

4.7.3 References

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Chapter 5

Comb-Type Prepolymers Consisting of a Polyacrylamide Backbone and Poly(L-lysine) Graft Chains for Multivalent Ligands

5.1 ABSTRACT

The comb-type copolymers consisting of a polyacrylamide (PAAm) backbone and poly(L-lysine) (PLL) graft chains have been prepared as the "prepolymer" for designing multivalent ligands. To regulate the length and density of the clusters of primary amino groups, the N^{α}-carboxyanhydride of N^{ε}-carbobenzoxy (CBZ)-L-lysine was first polymerized using *p*-vinylbenzylamine as an initiator. The resulting poly(CBZ-L-lysine) macromonomer was then radically copolymerized with AAm, followed by the deprotection of amino groups. For the model study, the reactive clusters of primary amino groups were completely converted into anion clusters by the reaction with succinic anhydride. The model multivalent ligands having the biotin label on the PAAm backbone were prepared by the terpolymerization of the macromonomer, AAm, and the biotin derivative having a vinyl group. The enzyme-linked immunosorbent assay showed that the biotin with no spacer on the PAAm backbone was recognized by the avidin–peroxidase conjugate specifically. Therefore, the highly sensitive detection of the interaction between cells and various model multivalent ligands was possible. The selective labeling onto the PAAm backbone revealed that the converted anion clusters of graft chains interacted exclusively with the cell and that the backbone was inert to the interaction with the cell. These results indicate that the various PAAm-*graft*-PLL comb-type copolymers with the defined length and density of the PLL-*grafts* are the potential prepolymers to investigate and to optimize the affinity of the multivalent ligands for receptors.

5.2 INTRODUCTION

Cell-surface receptors interact with their specific ligands such as growth factors, hormones, neurotransmitters, cell adhesion molecules, and extracellular matrices (*1*). These receptor–ligand interactions are mainly driven through the combination of electrostatic attraction, hydrogen bonds, and van der Waals attractive force (*2*). Interestingly, in the case of an asialoglycoprotein receptor (ASGP-R), the affinity of the galactosylated protein for the ASGP-R on hepatocytes is enhanced by increasing the number of the galactose residues on the protein (*3–6*). Furthermore, the particular geometry of a few galactose residues considerably increases the affinity of the galactosylated ligand for the ASGP-R on the hepatocytes (*7*, *8*).

Akaike *et al.* reported previously that the multivalent β-galactose ligands of a polystyrene derivative, poly[*N*-(*p*-vinylbenzyl)-4-*O*-β-D-galactopyranosyl-D-gluconamide] [PVLA, poly(vinylbenzyl-β-D-lactonamide)], are recognized by the ASGP-R on the surface of the hepatocytes; namely, the PVLA is found to be an artificial cellular matrix as the useful substratum for hepatocyte culture (*9–12*). When a culture dish is coated with a high density of PVLA, the hepatocytes attached on the dish remain spherical and show low DNA synthesis activity. In contrast, the hepatocytes on the coated dish with a low density of PVLA show spread morphology but high DNA synthesis activity (*13*). The particles covered with the PVLA are also recognized by the hepatocytes (*14*, *15*). The particle covered with the high density of β-galactose residues is promptly internalized by ASGP-R-mediated endocytosis, whereas the low density of β-galactose residues on the particle permits the binding to the hepatocyte without internalization (*16*). In these cases, the surface density of β-galactose residues is shown to be influential in the cellular responses and ligand binding. However, the influence of the size and distribution of the galactose cluster is not precisely estimated. The multivalent effect may occur in a variety of receptor–ligand interactions of cell adhesion molecules (*17–20*) such as P-selectin recognizing anionic glycoproteins (*21–24*).

In this chapter, I have designed the "prepolymer" for the multivalent ligands which have the well-controlled clusters of ligand molecules. The prepolymer consists of a polyacrylamide (PAAm) (*25*) backbone and poly(L-lysine) (PLL) graft chains. The clusters of primary amino groups on the PLL-*grafts* offer chemical modification sites, so that the amino groups can be converted into galactose clusters by the reaction with lactonolactone (*12*). The length and density of these clusters are properly characterized and controlled by varying the length of the PLL macromonomer or by varying the feed ratio for the copolymerization with AAm. The various PAAm-*graft*-PLL comb-type copolymers are, therefore, expected to be useful in investigating and optimizing the affinity of the multivalent ligands for receptors.

5.3 EXPERIMENTAL PROCEDURES

5.3.1 Materials

Acrylamide (AAm) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and was crystallized from benzene. Hexane was from Kanto Chemical Co., Inc. (Tokyo, Japan) and was washed with concentrated sulfuric acid to remove olefin, followed by water, drying, and distilling over sodium under nitrogen. Ethyl acetate (from Kanto) was dried with K_2CO_3, followed by distilling and redistilling over acetic anhydride to convert ethanol into ethyl acetate under nitrogen. Tetrahydrofuran (THF) was from Kanto and was dried with KOH, followed by distilling over *n*-butyllithium under nitrogen. *N*,*N*-Dimethylformamide (DMF) was from Kanto and was distilled under reduced pressure over $MgSO_4$. (+)-Biotin was purchased from Wako. Streptavidin–horseradish peroxidase conjugate and *o*-phenylene-diamine dihydrochloride (OPD) were purchased from Gibco Bethesda Research Laboratories (Grand Island, NY). All other chemicals of a special grade were used without further purification.

5.3.2 Synthesis of Poly[N^{ε}-carbobenzoxy (CBZ)-L-lysine] Macro-monomers

The N^{α}-carboxyanhydride (NCA) of CBZ-L-lysine (**2**) was prepared in THF, followed by crystallization from THF/hexane and recrystallization from ethyl acetate/hexane according to the method proposed by Daly and Poché (*26*). The NCA (**2**) was dissolved in dehydrated DMF. *p*-Vinylbenzylamine (**1**; VBA) (*27*) was dissolved in dehydrated DMF and added to the solution of **2**. The polymerization reaction was performed with 2.5 g of CBZ-L-lysine NCA (**2**) in 25 mL of DMF at the monomer (**2**)/initiator (**1**) molar ratios of 3, 5, 10, or 20 (for 36 h at 48 ℃). The reaction mixture was then poured into a 20-fold volume of diethyl ether. Precipitate was collected by centrifugation and washed with diethyl ether, followed by drying *in vacuo*.

5.3.3 Synthesis of the Biotin Derivative Having a Polymerizable Group

First, biotinyl-*N*-hydroxysuccinimide ester was prepared from biotin (**7**) and *N*-hydroxysuccinimide (NHS) by coupling with dicyclohexylcarbodiimide (DCC) (*28*). Each reactant was dissolved at 0.07 M concentration in DMF and incubated for 16 h at 50 ℃. The mixture was cooled to room temperature, the dicyclohexylurea was filtered off, and the filtrate was dried on a rotary evaporator. The residue was crystallized from 2-propanol. Then, the resulting active ester of biotin and VBA (**1**) were dissolved at 0.06 M concentration in DMF and incubated for 22 h at 25 ℃. The mixture was diluted with a 19-fold volume of H_2O, and the precipitate was collected by filtration, followed by washing with H_2O (to remove resulting NHS and unreacted **7**) and diethyl ether (to remove unreacted **1**). The residue was crystallized from DMF/H_2O mixture.

5.3.4 Synthesis of PAAm-*graft*-PLL Comb-Type Copolymers

The resulting macromonomer (**3**) and AAm (**4**) were dissolved in 2.5 mL of DMF at the total monomer concentration of 100 mg/mL. In the case of biotinylation, 5 mg of the resulting biotin monomer (**8**) was added to the above mixture. The radical copolymerization reaction was carried out for 2–4 h at 47 ℃ in a sealed glass ampule using 10 mM 2,2'-azobis(2,4-dimethylvaleronitrile) (V-65) as an initiator. After the reaction, the content was poured into a large excess of ethanol, and precipitate was dried *in vacuo*. The crude polymers were dissolved in trifluoroacetic acid (TFA), followed by the addition of thioanisole to deprotect CBZ-amino groups. The final concentrations of thioanisole and the polymer were 0.3–1 M and 20–100 mM (based on the CBZ groups), respectively. The deprotection reaction was carried out for 2.5–3.0 h at 25 ℃. The reaction mixture was poured again into a large excess of ethanol. Precipitate was redissolved in TFA and poured into ethanol repeatedly. The precipitate was dried *in vacuo*, dissolved in water, and obtained by freeze-drying.

5.3.5 Succinylation of PAAm-*graft*-PLL Comb-Type Copolymers

The resulting copolymer (**5**) or biotinylated **5** was dissolved in 1 mL of H_2O at the concentration of 50 mg/mL, the pH of which was increased to 10 by the addition of triethylamine (TEA). The DMF solution (150 μL) of succinic anhydride (60 mg) was subsequently added to the copolymer aqueous solution. The mixture was incubated for 3 h at 25 ℃. The above DMF solution of succinic anhydride was added again to the mixture, the pH of which was adjusted to 10 with TEA. The resulting mixture was further incubated for 1 day at 25 °C. The degree of amino group modification was examined by the spectrophotometric amino group determination with trinitrobenzenesulfonic acid (TNBS) (*29*). The reaction mixture was dialyzed against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10^3), followed by freeze-drying.

5.3.6 Gel Permeation Chromatography (GPC)

GPC was carried out using a JASCO 880-PU pumping system (Tokyo, Japan) at a flow rate of 1.0 mL/min at 25 ℃ with Ultrahydrogel 500 and Ultrahydrogel 250 columns (Japan Waters Ltd., Tokyo, Japan). The aqueous solution containing 0.5 M CH_3COOH and 0.3 M Na_2SO_4 was used as a mobile phase. Three hundred microliters of 1 mg/mL samples was injected into the columns. Eluate was detected by a UV (wavelength = 274 nm) detector (875-UV, JASCO), a refractive index (RI) detector (830-RI, JASCO), and a multiangle light scattering detector (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA).

5.3.7 ^{1}H NMR Spectroscopy

Each polymer was dissolved in $(CD_3)_2SO$ (DMSO-d_6) or D_2O (99.8 at. % D; Merck, Darmstadt, Germany). ^{1}H NMR spectra (400 MHz) were obtained by a Varian Unity 400plus spectrometer (Palo Alto, CA) at a probe temperature of 298 K. The chemical shifts are expressed as parts per million using internal DMSO (δ = 2.5 ppm in DMSO-d_6) or HDO (δ = 4.7 ppm in D_2O) molecules as a reference.

5.3.8 Enzyme-Linked Immunosorbent Assay (ELISA) Measuring Polymer–Cell Interaction

Endothelial cells (ECV304, ATCC CRL1998) were seeded in tissue culture wells and allowed to grow to confluence in M199 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum. The binding assays between the polymers and the cells were performed in a Sumilon multiwell plate (No. MS-8096F, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) as below. First, the cells were washed once with an isotonic and low-ionic incubation buffer (285 mM sucrose, 10 mM Hepes, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$). The confluent cells were incubated with 100 μL/well of the buffer containing each polymer (1 mg/mL) for 2 h at 4 °C. The wells were then washed once with the buffer, followed by the addition of 100 μL of the peroxidase-conjugated streptavidin in the buffer (1 μg/mL). After 1 h of incubation at 4 ℃, the wells were washed three times with the buffer. Finally, 100 μL of 1 mg/mL OPD solution (in 50 mM citric acid, 100 mM Na_2HPO_4 buffer, pH 5.0) containing 0.02% H_2O_2 was added to the well as a substrate. Fifty microliters per well of 1.5 M H_2SO_4 was added to stop the enzyme reaction after the incubation at room temperature. The absorbance at 492 nm was measured with an MTP-120 microplate reader (Corona Electric Co., Ltd., Ibaragi, Japan).

5.3.9 Estimation of the Dependence of the Succinylated PAAm-*graft*-PLL (PAAm-*graft*-PLLS) Cell Binding on the Ionic Strength of the Medium

As stated above, the endothelial cells were seeded and washed once with the isotonic and low-ionic incubation buffer. The PAAm-*graft*-PLLS was dissolved in the isotonic buffer containing 10 mM Hepes, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and various sucrose/NaCl mixtures (285 mM/0 mM, 230 mM/27.5 mM, 155 mM/65 mM, 80 mM/102.5 mM, or 5 mM/140 mM) being added to the cells. These cells were incubated with 100 μL/well of each buffer containing PAAm-*graft*-PLLS (10 μg/mL) for 2 h at 4 ℃. The wells were washed once with the isotonic

and low-ionic incubation buffer. One hundred microliters of the peroxidase-conjugated streptavidin in the low-ionic buffer (1 μg/mL) containing 1 mg/mL bovine serum albumin was added to the wells. After the incubation for 1 h at 4 °C, the wells were washed three times with the low-ionic buffer. One hundred microliters of a 1 mg/mL OPD solution containing 0.012% H_2O_2 was then added to the well, followed by the measurement of the absorbance at 492 nm.

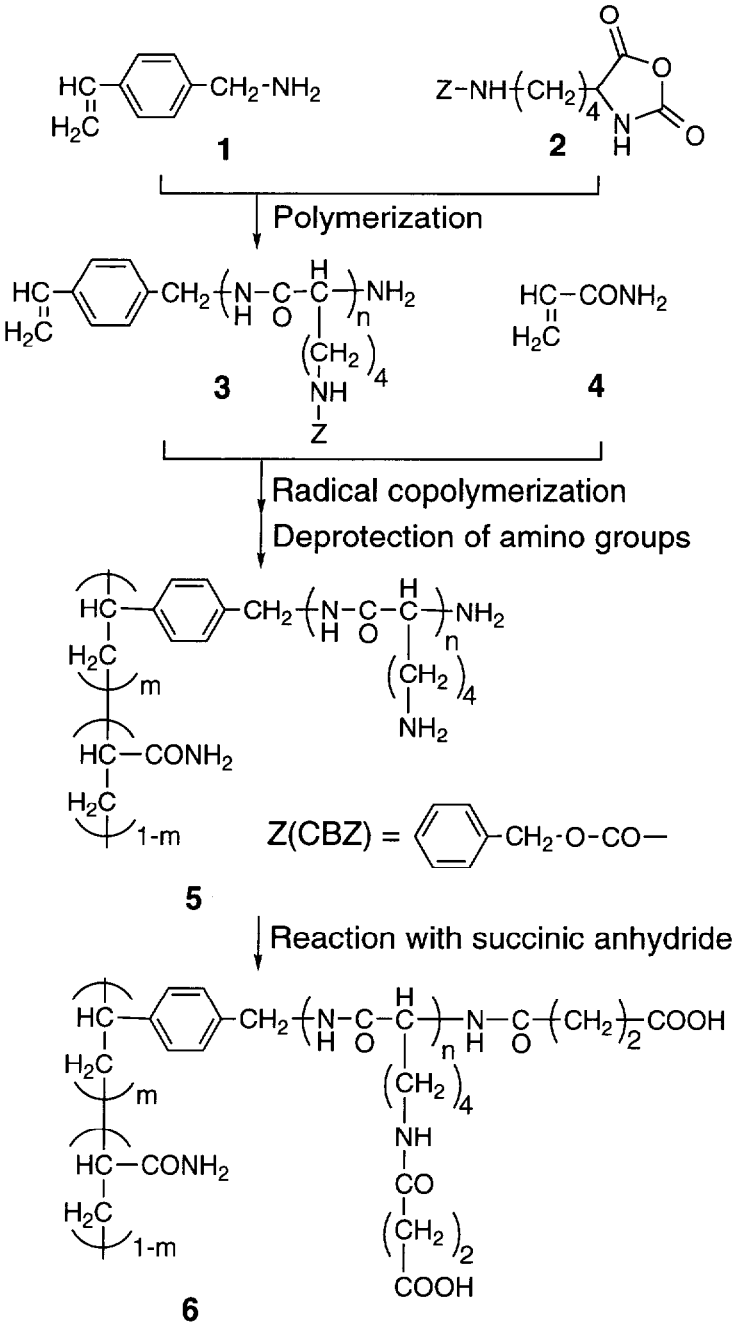
5.4 RESULTS AND DISCUSSION

5.4.1 Synthesis of Poly(CBZ-L-lysine) Macromonomers

To synthesize the graft copolymers (Scheme 5.1), I chose the macromonomer method (*30*) because it has several advantages over other methods. The length (molecular weight) of the polyamino acid macromonomer is easy to control by varying the feed ratio of monomers to the initiator having a polymerizable functional group. The density of graft chains is also controllable by varying the feed ratio of the macromonomer to comonomers. Therefore, the structure of graft chains would be well-defined and characterized. Furthermore, I am able to vary the properties of backbone polymers by replacing comonomers (*31*) (cf. Chapters 2 and 3).

Poly(CBZ-L-lysine) macromonomers (**3**) were prepared by the polymerization of CBZ-L-lysine NCA (**2**) using VBA (**1**) as an initiator. The polymerization progressed homogeneously in the solvent of dehydrated DMF. The polymers were purified by precipitating into diethyl ether. The vinyl groups were preserved in the resulting polymer (**3**), which was confirmed by ^{1}H NMR spectroscopy (Figure 5.1) in DMSO-d_6: δ 5.2 (doublet), 5.8 (doublet), and 6.7 (quartet). The signals of CBZ-L-lysine were also identified: δ 1.2–1.8 (β-, γ-, and δ-methylene protons), 3.0 (ε-methylene protons), 4.3 (α-methine protons), 5.0 (CBZ-methylene protons), and 7.2–7.4 (phenyl protons). From the signal ratio of vinyl protons and lysine protons, the number-average degree of polymerization (P_n) of **3** was determined. The result is shown in Table 5.1. The P_n of **3** almost corresponded with the feed ratio of monomer (**2**) to initiator (**1**). Therefore, it is possible to vary the average length of the poly(CBZ-L-lysine) macromonomer (**3**) as the graft chain of PAAm-*graft*-PLL comb-type copolymer (**5**).

Scheme 5.1. Synthesis of PAAm-*graft*-PLL Comb-Type Copolymers



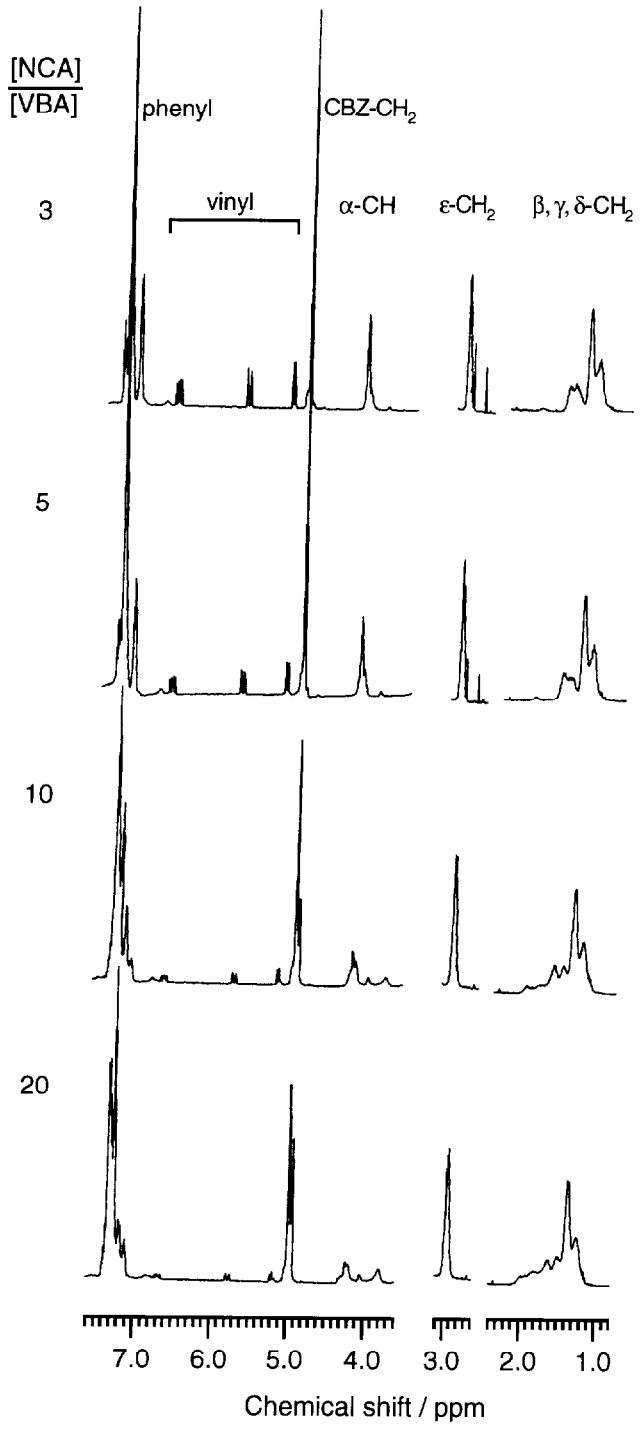


Figure 5.1. ^{1}H NMR spectra of poly(CBZ-L-lysine) macromonomers in DMSO-d_6. The feed ratio of monomer (NCA) to initiator (VBA) is represented.

Table 5.1. Polymerization of CBZ-L-lysine NCA Using VBA as an Initiator[a]

sample	infeed			polymer
	NCA (mmol)	VBA (mmol)	[NCA]/[VBA]	P_n [b]
1	8.2	2.7	3	2.6
2	8.2	1.6	5	4.7
3	8.2	0.82	10	8.4
4	8.2	0.41	20	16

[a] Reaction temperature, 48 °C; reaction time, 36 h; solvent, DMF.
[b] Determined by ^{1}H NMR.

5.4.2 Synthesis of PAAm-*graft*-PLL Comb-Type Copolymers

At first, I attempted to deprotect CBZ-amino groups of the macromonomer (**3**) before the copolymerization reaction. However, the undesirable side reaction of vinyl groups occurred when the deprotection was carried out in the TFA/thioanisole mixture. Accordingly, I deprotected CBZ-amino groups after the copolymerization. The radical copolymerization of **3** with AAm (**4**) using the initiator of an azo compound, V-65, was carried out in DMF at 46 ℃. During the reaction, a precipitate gradually appeared. After the copolymerization, the mixture was poured into a large excess of ethanol to precipitate the resulting polymer (**5**), together with unreacted **3**, and to remove unreacted **4**. The removal of the unreacted macromonomers (**3**) from the copolymers (**5**) in this step was difficult because the solubility of **3** was similar to that of **5**. Eventually, I was unable to find any selective solvents for the purification. Therefore, I isolated the copolymers after the deprotection of CBZ-amino groups. Because deprotected **3** was soluble in ethanol, the copolymers were isolated by the precipitation into ethanol after the deprotection. Figure 5.2 shows the representative GPC profiles of the resulting copolymer (**5**) detected by either absorbance at 274 nm (A_{274}) or RI. The A_{274} was mainly attributed to the terminal phenyl groups of the graft chains. The GPC profiles therefore validated the incorporation of the macromonomer (**3**) in the copolymer (**5**) and the removal of unreacted **3**. The number-average molecular weight of the each copolymer determined by GPC was $\sim 3 \times 10^4$. The ^{1}H NMR spectra (Figure 5.3) of the copolymer showed the characteristic signals of both PLL-*graft* and PAAm backbone: δ 1.4–1.8 (methylene protons of PAAm), 2.1–2.4 (methine protons of PAAm), 3.0 (ε-methylene protons of PLL), and 4.3 (α-methine protons of PLL). No residual CBZ groups were detected in the final polymer samples, indicating the successful deprotection of amino groups. From the signal ratio of the PLL-*grafts* and the PAAm backbone, the content (mole fraction) of **3** in the copolymer was determined. As the mole fraction of **3** in feed increased, the mole fraction in the copolymer increased (Table 5.2). Furthermore, various macromonomers (**3**) with different P_n were copolymerized. Thus, it is possible to obtain the various PAAm-*graft*-PLL comb-type copolymers (**5**) with the well-controlled length and density of graft chains by using the macromonomer method.

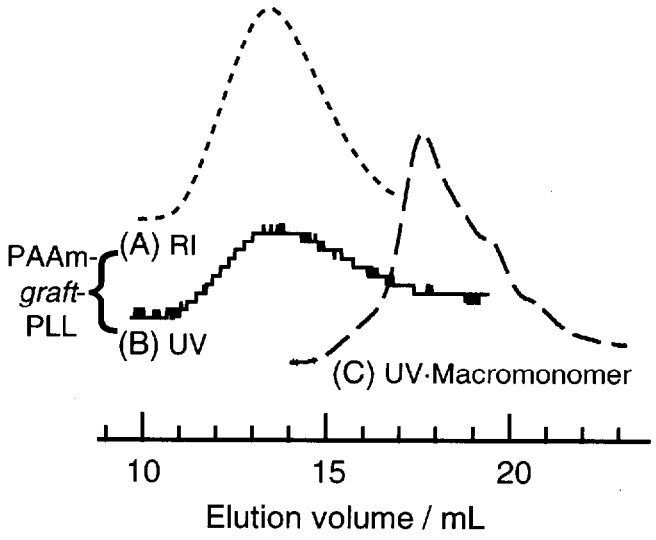


Figure 5.2. Gel permeation chromatograms of the PAAm-*graft*-PLL comb-type copolymer (sample 4 in Table 5.2) detected by (A) RI and (B) A_{274}, and (C) the deprotected CBZ-L-lysine macromonomer (sample 3 in Table 5.1) detected by A_{274}: column, Ultrahydrogel 500 + 250; eluent, 0.5 M CH_3COOH + 0.3 M Na_2SO_4; flow rate, 1.0 mL/min; temperature, 25 °C.

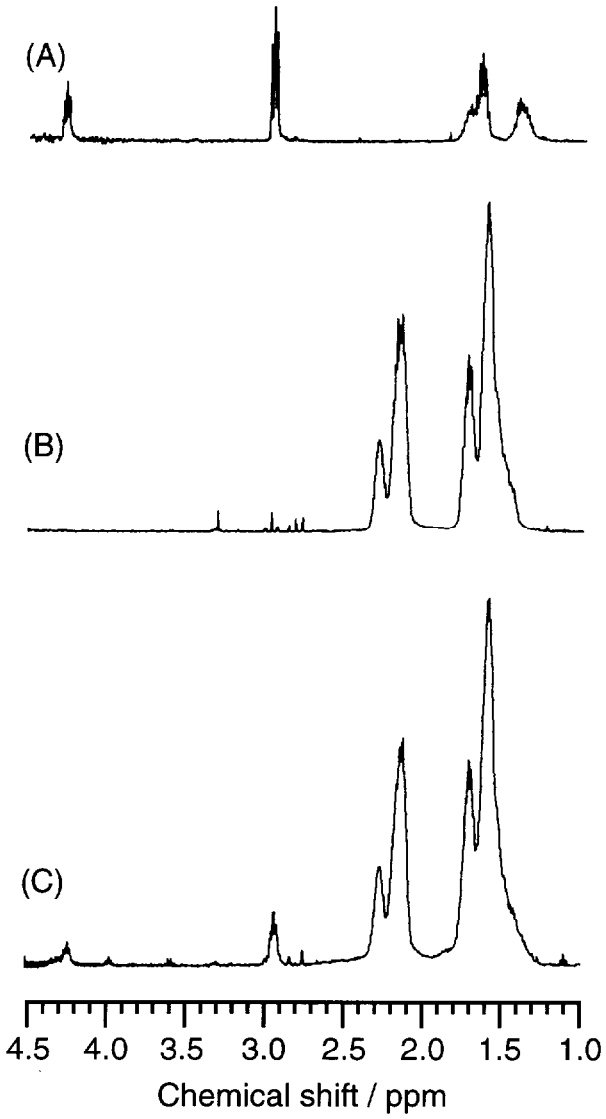


Figure 5.3. ^{1}H NMR spectra of (A) PLL, (B) PAAm, and (C) PAAm-*graft*-PLL in D_2O.

Table 5.2. Synthesis of PAAm-*graft*-PLL Comb-Type Copolymers[a]

sample	monomers				copolymer [b]	
	4	**3**			**3** (deprotected)	yield
	mg	mg	P_n	10^3 mole fraction	10^3 mole fraction	(%)
1	242.5	7.5	4.7	1.6	3.6	45
2	232.5	17.5	4.7	3.8	5.7	40
3	212.5	37.5	4.7	8.9	11	39
4	242.5	7.5	8.4	0.95	0.9	56
5	232.5	17.5	8.4	2.3	2.5	68
6	212.5	37.5	8.4	5.4	3.9	70
7	242.5	7.5	16	0.51	0.5	50
8	232.5	17.5	16	1.2	1.3	60
9	212.5	37.5	16	2.9	2.6	57

[a] Initiator, 10 mM V-65; reaction temperature, 46 °C; solvent, 2.5 mL of DMF; reaction time, 2 h (samples 1, 4, and 7), 3 h (samples 2, 5, and 8), 4 h (sample 3), and 4.5 h (samples 6 and 9).

[b] The deprotection reaction of CBZ-amino groups was carried out in TFA/thioanisole mixture at 25 °C: 0.27 M thioanisole/19 mM CBZ, 2.5 h reaction (samples 1, 4, and 7); 0.54 M thioanisole/45 mM CBZ , 2.75 h reaction (samples 2, 5, and 8); and 1.1 M thioanisole/95 mM CBZ, 3.0 h reaction (samples 3, 6, and 9).

5.4.3 Modification of the Amino Groups of the PAAm-*graft*-PLL Comb-Type Copolymers

I examined the reactivity of the amino groups of the graft chains. Here the acylation of the graft chains by succinic anhydride was examined. The PAAm-*graft*-PLL (**5**) was dissolved in water, and the pH of the solution was increased to 10 by TEA. The succinic anhydride dissolved in DMF was added to the aqueous solution of the PAAm-*graft*-PLL, which was insoluble in DMF. Thus, the succinylation reaction was carried out in the mixture of water and DMF. The complete succinylation of the amino groups of the graft chains was confirmed by the spectrophotometric amino group determination with TNBS (*29*). Figure 5.4 shows the ^{1}H NMR spectra of the resulting polymer (**6**) purified by dialysis. After the succinylation (Figure 5.4B), the signal of ε-methylene protons of the PLL-*grafts* shifted downfield (from δ 3.0 to 3.2), and the signals of the methylene protons of succinic acid appeared (δ 2.4–2.6). These results proved the complete succinylation of the amino groups again. The amino groups of the graft chains retained high reactivity, so that the graft chains are expected to be modified with ligand molecules such as β- galactose (cf. Chapter 3).

5.4.4 PAAm-*graft*-PLL Comb-Type Copolymers Having the Biotin Label on the Backbone

To analyze the interaction between cells and the comb-type copolymers, I intended to label the PAAm-*graft*-PLL with biotin, which is detected by ELISA using avidin (biotin-binding protein) (*32*). The comb-type copolymer PAAm-*graft*-PLL has two segments, that is, the nonionic backbone of PAAm and the PLL-*graft*. I prefer to incorporate biotin molecules into the PAAm backbone rather than into the PLL-*graft* to minimize the influence on the avidin–biotin binding and the interaction between the PLL-*graft* and the receptor. For the biotin-labeling, the biotin derivative (**8**) having a polymerizable group was prepared and terpolymerized with the poly(CBZ-L-lysine) macromonomer (**3**) and AAm (**4**) (Scheme 5.2).

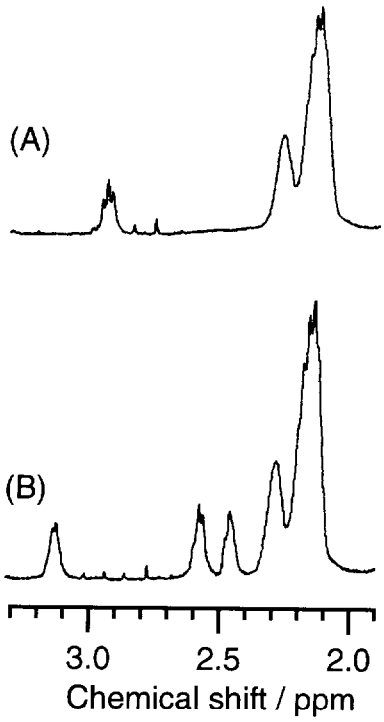
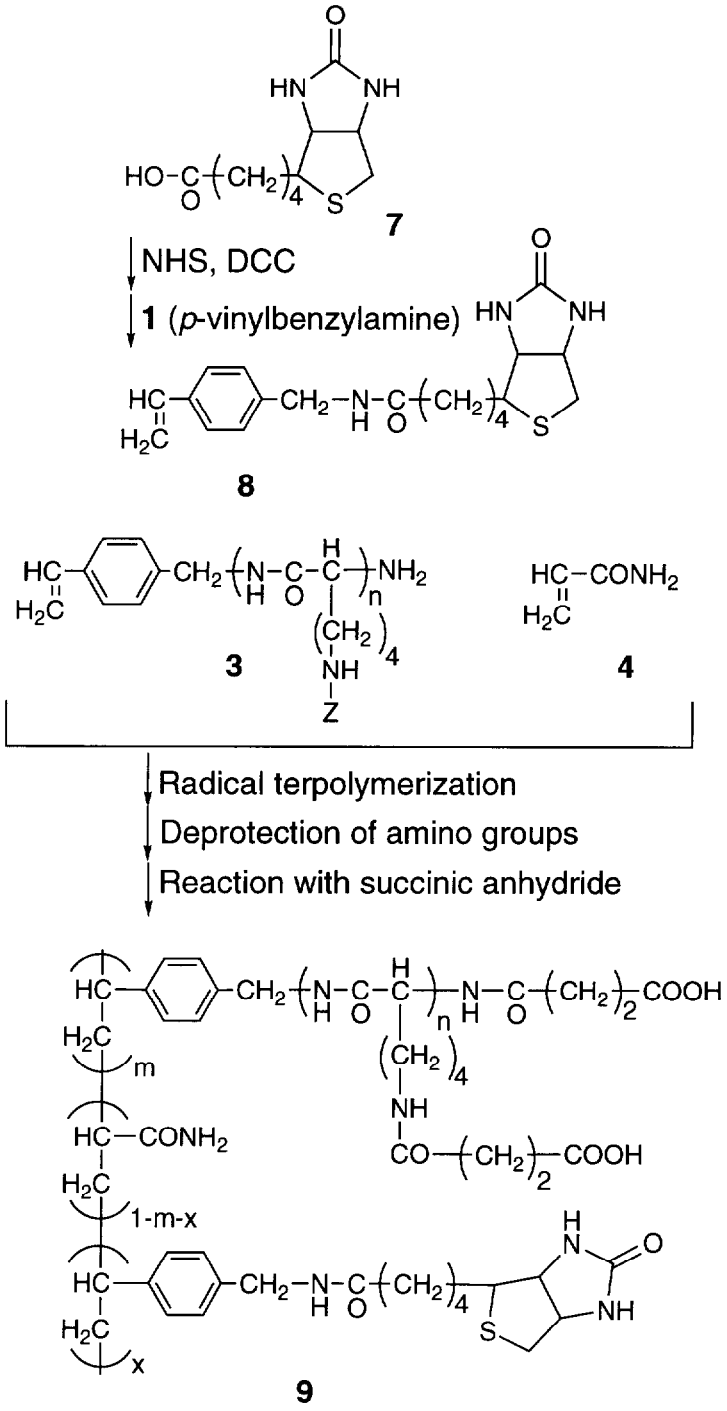


Figure 5.4. Change in the ^{1}H NMR spectra of the PAAm-*graft*-PLL comb-type copolymer (sample 6 in Table 5.2) by the reaction with succinic anhydride: (A) before and (B) after the reaction.

Scheme 5.2. Biotinylation of PAAm-*graft*-PLL Comb-Type Copolymers



First, the carboxyl group of biotin (**7**) was activated in DMF using NHS and DCC. The active ester of biotin was then reacted with VBA (**1**), resulting in the monomer (**8**) having a vinyl group. The biotin monomer (**8**) was terpolymerized with the macromonomer (**3**) and AAm (**4**). After the deprotection of amino groups, the ^{1}H NMR spectra of the resulting terpolymer were obtained (results not shown). From the signal ratio of methine protons (2.1–2.4 ppm) of the PAAm backbone and ε-methylene protons (3.0 ppm) of the PLL-*grafts*, the content (mole fraction) of **3** in the terpolymer was determined (Table 5.3). However, the ^{1}H NMR signal of **8** in the terpolymer was not detected, presumably because of considerably low feed and thereby low content in the terpolymer. The complete succinylation of the biotinylated PAAm-*graft*-PLL was also carried out in the same way as succinylating the unlabeled PAAm-*graft*-PLL.

5.4.5 Detection of the Interaction between Cells and the Biotinylated PAAm-*graft*-PLLS Comb-Type Copolymers by ELISA

I examined whether the interaction between cells and the PAAm-*graft*-PLLS comb-type copolymers was detected by ELISA. Figure 5.5 shows the detection of the biotinylated PAAm-*graft*-PLLS (**9**) bound to adherent cells by ELISA using peroxidase-conjugated streptavidin (*33*). The biotinylated PAAm-*graft*-PLLS (**9**) and not unlabeled copolymer (**6**) showed significant absorbance over the background level. The PAAm-*graft*-PLLS bound to the cells was, therefore, detectable by ELISA using the peroxidase-conjugated streptavidin.

To evaluate the role of graft chains in the binding to the cell, I carried out a set of the ELISA experiments with the PAAm-*graft*-PLLS comb-type copolymer and PAAm homopolymer. As shown in Figure 5.6, the biotinylated PAAm was not detected. Therefore, the graft chains played a major role in the binding to the cell, whereas the PAAm backbone presumably had no interaction with the cell surface.

The ionic interaction between the graft chains and the cell surface was considered the major driving force for the binding to the cell, so that we estimated the effect of the ionic strength of the medium on the interaction of the PAAm-*graft*-PLLS with the cell (Figure 5.7). The PAAm-*graft*-PLLS was highly bound to the cell under low-ionic strength conditions (0 mM NaCl and

Table 5.3. Biotinylation of PAAm-*graft*-PLL Comb-Type Copolymers[a]

sample	monomers						copolymer [b]	
	4	**3**			**8**		**3** (deprotected)	yield
	mg	mg	P_n	10^3 mole fraction	mg	10^3 mole fraction	10^3 mole fraction	(%)
1	242.5	7.5	8.4	0.95	5	4.1	2.2	26
2	232.5	17.5	8.4	2.3	5	4.2	2.6	39
3	212.5	37.5	8.4	5.4	5	4.6	4.4	45
4	242.5	7.5	16	0.51	5	4.1	1.4	24
5	232.5	17.5	16	1.2	5	4.2	1.7	36
6	212.5	37.5	16	2.9	5	4.6	2.2	46
7	250	0			5	3.9		16

[a] Initiator, 10 mM V-65; reaction temperature, 46 °C; solvent, 2.5 mL of DMF; reaction time, 2 h (samples 1 and 4), 3 h (samples 2 and 5), 4 h (samples 3 and 6), and 1 h (sample 7).

[b] The deprotection reaction of CBZ-amino groups was carried out in TFA/thioanisole mixture at 25 °C for 3 h: 0.91 M thioanisole/58 mM CBZ (sample 1); 1.1 M/67 mM, 1.0 M/95 mM, 0.27 M/19 mM, 0.54 M/45 mM, and 1.1 M/95 mM (samples 2–6, respectively).

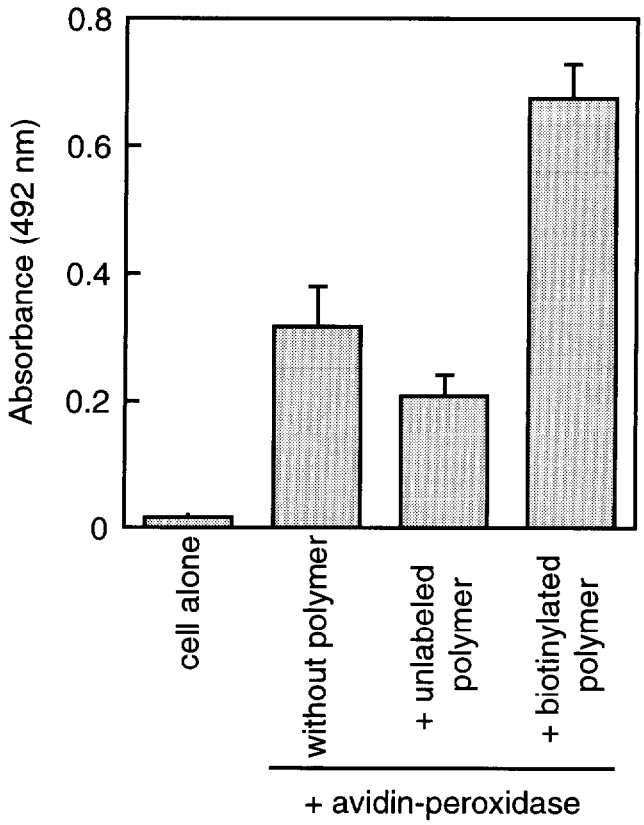


Figure 5.5. Detection of the interaction between adherent cells and the biotinylated PAAm-*graft*-PLLS comb-type copolymers by ELISA using the peroxidase-conjugated streptavidin. The biotin-labeled (sample 3 in Table 5.3) or unlabeled (sample 6 in Table 5.2) PAAm-*graft*-PLLS was added to confluent endothelial cells at 4 °C, as described under Experimental Procedures, followed by the addition of the peroxidase-conjugated streptavidin. After washing, the substrate (OPD + H_2O_2) was added and the color development was read as the absorbance at 492 nm. Symbols and error bars represent the mean and standard deviation of the measurements made in quadruplicate wells.

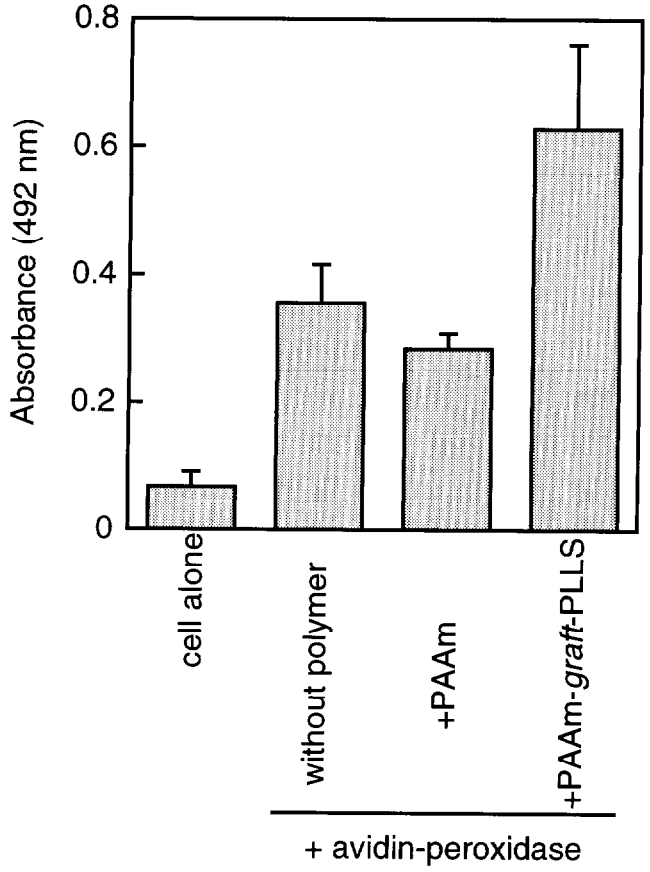


Figure 5.6. Binding assay between endothelial cells and polymers by ELISA. The binding of the PAAm-*graft*-PLLS (sample 1 in Table 5.3) or PAAm (sample 7 in Table 5.3) was determined by the color development of the substrate as described in Figure 5.5. Symbols and error bars represent the mean and standard deviation of the measurements made in quadruplicate wells.

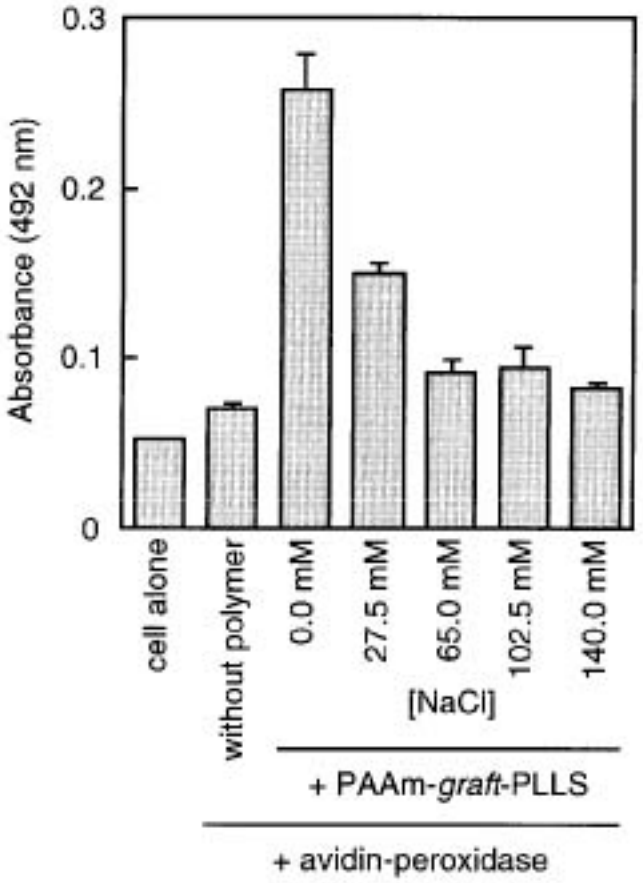


Figure 5.7. Effect of the ionic strength of the medium on the interaction between endothelial cells and the PAAm-*graft*-PLLS comb-type copolymers. The PAAm-*graft*-PLLS (sample 3 in Table 5.3) was added to confluent endothelial cells at 4 °C in the isotonic solution containing the indicated concentration of NaCl, as described under Experimental Procedures. The binding of the PAAm-*graft*-PLLS was determined by the color development of the substrate (the absorbance at 492 nm). Symbols and error bars represent the mean and standard deviation of the measurements made in quadruplicate wells.

285 mM sucrose). However, the increase in the ionic strength of the medium drastically reduced the binding of the PAAm-*graft*-PLLS, leading to almost no binding at physiological ionic strength (140 mM NaCl and 5 mM sucrose). These results proved that the electrostatic attraction between PLLS-*grafts* and the cell surface mainly mediated the binding of the PLLS-*graft* to the cell. In other words, the nonspecific interaction of the PAAm-*graft*-PLLS with the cell was negligible under physiological salt conditions.

5.5 CONCLUSION

I have synthesized the various PAAm-*graft*-PLL comb-type copolymers with the defined length and density of graft chains by using the macromonomer method. The PAAm-*graft*-PLL was synthesized as the "prepolymer" for multivalent ligands. I can introduce the various types of ligand molecules into the graft chains, via the primary amino groups, with certain chemicals such as lactonolactone (*12*) to form ligand clusters. In this chapter, the complete modification of the graft chains with succinic anhydride was achieved for the model study to explore the interaction between a cell surface and the resulting polymer having anion clusters (PAAm-*graft*-PLLS). To detect the interaction between the cell surface and the model multivalent ligands having the anion clusters, I prepared the biotin monomer, labeling the prepolymer on the PAAm backbone. By ELISA, I confirmed that the anion clusters of the graft chains worked exclusively for the interaction with the cell and that the PAAm backbone was inert to the interaction with the cell. The anion clusters of the model multivalent ligand may interact with cation cluster sites [e.g., P-selectin (*21–24*, *34*)] or anionic sites [via Ca^{2+} bridges (*35*)] on the cell surface under low ionic strength conditions. From a practical point of view, the nonspecific interaction of the model multivalent ligand PAAm-*graft*-PLLS with the cell was negligible under physiological salt conditions, so that I expect the multivalent effect of ligands to be properly extracted by incorporating ligands of interest into the PLL-*grafts*.

The multivalent effect has been studied by conjugating ligand molecules to soluble linear polymers or insoluble polymer matrices. In these cases, however, it is difficult to control the density and distribution of ligands. Indeed, the increase of ligand density must result in the formation of the clustered domain of ligands, so that I am unable to distinguish between cluster effect and density effect. The various PAAm-*graft*-PLL comb-type copolymers with the defined length and density of the PLL-*grafts* are the potential prepolymers to investigate and to optimize the affinity of the multivalent ligands for receptors.

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Chapter 6

Concluding Remarks

In this thesis, various polyelectrolyte graft copolymers have been synthesized (Figure 6.1). Especially, these graft copolymers are applied for the field of gene delivery, i.e., DNA carrier.

In Chapter 2, I have synthesized the PDEAEMA-*graft*-PLL comb-type copolymer and demonstrated that the PDEAEMA-*graft*-PLL is capable of sensing a pH signal and outputting the nonlinear change of the physicochemical properties of DNA polyelectrolyte complexes. The pH-induced nonlinear change of DNA complex properties have been established as the pH-responsive device considered useful in delivering DNA into cells. For the purpose of the practice of the device, I have synthesized the PVIm-*graft*-PLL·Lac comb-type copolymer in Chapter 3. The PVIm-*graft*-PLL·Lac is capable of sensing endosomal pH (= ~6) and outputting the unique change of the physicochemical properties of DNA complexes. The unique change is considered "stimulus-responsive intramolecular exchange of polyelectrolyte complexes", increasing the interaction between DNA complexes and cell membrane at endosomal pH. From a general point of view, a novel temporal control of the delivery of DNA polyelectrolyte complexes have been achieved in Chapters 2 and 3.

To use the above pH-responsive device efficiently inside cells, I have to carry out the site-specific control of DNA polyelectrolyte complexes. In Chapter 4, the PLL-*graft*-HA comb-type copolymers have been synthesized by the convenient method of coupling. The resulting DNA–polycation complexes were directed to liver sinusoidal endothelial cells, owing to the HA as a homing device. To control the recognition of homing devices (ligands) strictly, I have established the comb-type prepolymer PAAm-*graft*-PLL for multivalent ligands in Chapter 5. The PAAm-*graft*-PLL has the segment which reduces nonspecific interaction and the segments where ligands molecules are introduced. The prepolymers present the possibilities of the control of both density and distribution of ligands.

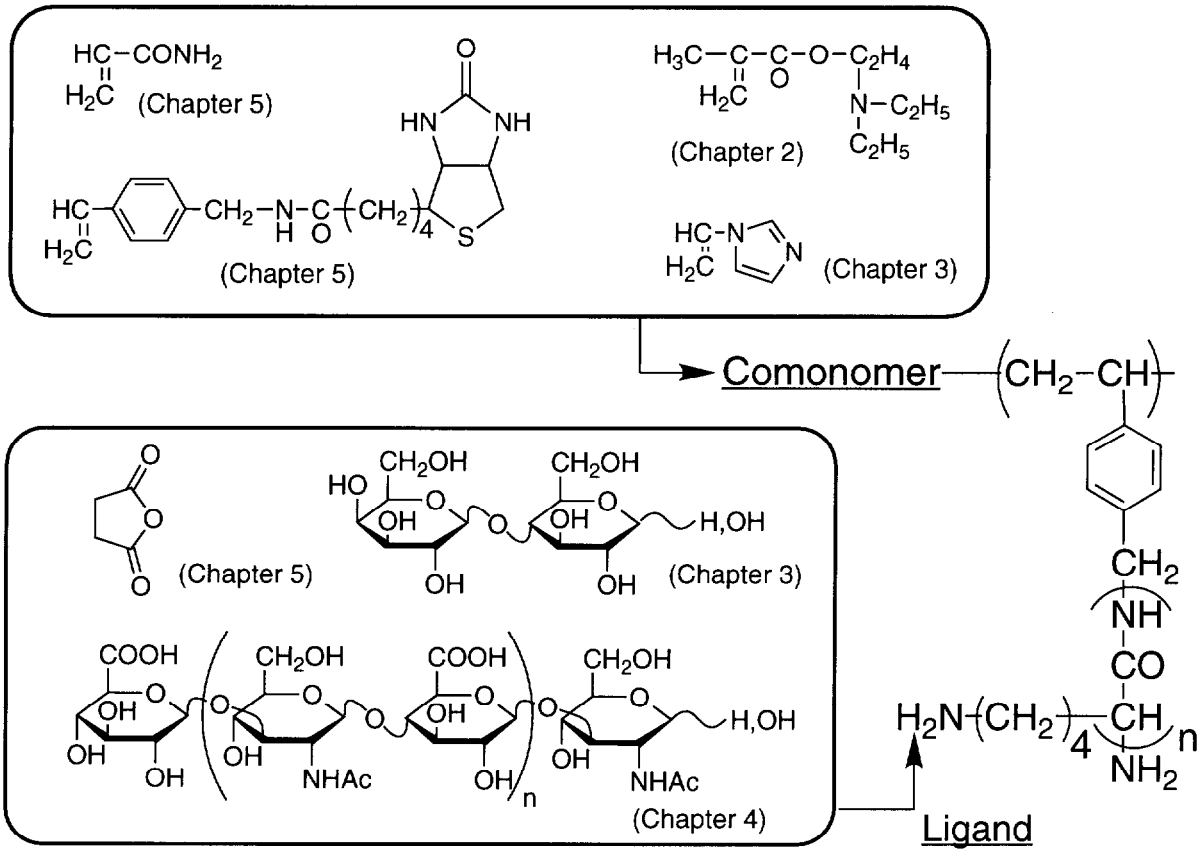

Figure 6.1. Polyelectrolyte graft copolymers in this thesis.

As above, each chapter creates an important component of the strategy of the efficient gene delivery with artificial polymer carriers. To inject a gene into a specific cell, some viruses, I think, exhibit both temporal and site-specific control of their infection skillfully. Therefore, I would like to expect the DNA carriers, made of artificial polymers, with the viruslike infection pathway by combining the polyelectrolyte graft copolymers synthesized by the biomolecular engineering of this thesis.

Achievements

LIST OF PUBLICATIONS

Chapter 2:

Design of Comb-Type Polyamine Copolymers for a Novel pH-Sensitive DNA Carrier.

Shoichiro Asayama, Atsushi Maruyama, Chong-Su Cho, and Toshihiro Akaike.

Bioconjugate Chemistry, Volume 8, Number 6, Pages 833–838 (1997).

Chapter 2:

Design of Bi-Phasic Polyamine Graft Copolymers for the pH-Dependent Control of DNA Complex Properties.

Shoichiro Asayama, Atsushi Maruyama, and Toshihiro Akaike.

Intelligent Materials, Volume 8, Number 2, Pages 11–15 (1998) (in Japanese).

Chapter 3:

Design of Bi-Phasic Polyelectrolytes Consisting of a Poly(1-vinylimidazole) Backbone and "Lactosylated" Poly(L-lysine) Graft Chains for the DNA Carrier with the pH Sensitivity at Endosomal pH.

Shoichiro Asayama, Toshihiro Akaike, and Atsushi Maruyama.

In preparation.

Chapter 4:

Synthesis of Novel Polyampholyte Comb-Type Copolymers Consisting of a Poly(L-lysine) Backbone and Hyaluronic Acid Side Chains for a DNA Carrier.

Shoichiro Asayama, Masayuki Nogawa, Yoshiyuki Takei, Toshihiro Akaike, and Atsushi Maruyama.

Bioconjugate Chemistry, Volume 9, Number 4, Pages 476–481 (1998).

Chapter 4 (Additional section):

DNA Nanoassociate Bearing Hyaluronan-Glycocalyx for Targeted Gene Delivery to Sinusoidal Endothelial Cells and Expression *in vivo*.

Yoshiyuki Takei, Atsushi Maruyama, Sunao Kawano, Kenichi Ikejima, Shigetoshi Okumura, **Shoichiro Asayama**, Masayuki Nogawa, Masao Hashimoto, Yoko Makino, Masahiko Kinoshita, Masatsugu Hori, Toshihiro Akaike, Sumio Watanabe, and Nobuhiro Sato.

Nature Biotechnology, submitted.

Chapter 5:

Comb-Type Prepolymers Consisting of a Polyacrylamide Backbone and Poly(L-lysine) Graft Chains for Multivalent Ligands.

Shoichiro Asayama, Atsushi Maruyama, and Toshihiro Akaike.

Bioconjugate Chemistry, Volume 10 (1999), in press.

LIST OF SUPPLEMENTARY PUBLICATIONS

Polylysine Graft Copolymers as a DNA Triplex Stabilizer and Cell-Specific DNA Carrier.
Atsushi Maruyama, Hiromitsu Watanabe, Masayuki Nogawa, **Shoichiro Asayama**, Anwarul Ferdous, Yoshiyuki Takei, and Toshihiro Akaike.
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Atsushi Maruyama, Anwarul Ferdous, Tsutomu Ishihara, **Shoichiro Asayama**, Jeong-Ung Park, Msayuki Nogawa, Hiromitsu Watanabe, Yoshiyuki Takei, Toshihiro Akaike.
Nucleosides & Nucleotides, accepted.

Conformational Transition of Nanoparticles Composed of Poly(γ-benzyl L-glutamate) as the Core and Poly(ethylene oxide) as the Shell.
Chong-Su Cho, Jae-Woon Nah, Young-Il Jeong, Jae-Bok Cheon, **Shoichiro Asayama**, Hirohiko Ise, and Toshihiro Akaike.
Polymer, accepted.

LIST OF INTERNATIONAL PRESENTATIONS

(1) Design of Polyanion Model Ligands for Cell Adhesion Molecules.

International Union of Pure and Applied Chemistry (IUPAC) International Symposium –Functional and High Performance Polymers–, Taipei International Convention Center, Taiwan, November 14–16 (1994).

(2) Design of pH-Responsive Polyamine Graft Copolymers for Application to Novel Polynucleotide Carriers.

Fifth World Biomaterials Congress, Metro Toronto Convention Centre, Canada, May 29–June 2 (1996).

(3) Design of Comb-Type Polyamine Copolymers for a Novel pH-Sensitive DNA Carrier.

International Symposium on Smart Polymers in Industry and Medicine, Kobe, Japan, June 5–6 (1998).

(4) Bi-Phasic Polyamine Copolymers as DNA Carrier.

The 26th International Symposium on Controlled Release of Bioactive Materials/Consumer and Diversified Products Conference, Marriott Copley Place, Boston, MA, U.S.A., June 20–25 (1999), submitted.

LIST OF PRESENTATIONS IN JAPANESE

(1) 細胞接着分子に対するポリアニオンモデルリガンドの設計
第43回高分子討論会、九州大学、福岡、10月12－14日（1994）

(2) ポリアミングラフト共重合体を用いたDNA・ポリカチオン複合体のpHによる構造制御
第25回医用高分子シンポジウム、上智大学、東京、7月25－26日（1996）

(3) 弱塩基性基を配したグラフト共重合体によるDNA複合体の構造制御
日本化学会第72春季年会、立教大学、東京、3月27－30日（1997）

(4) 細胞特異的DNAキャリアーとしてのポリリジン／ヒアルロン酸グラフト共重合体の合成とキャラクタリゼーション
第46回高分子討論会、名古屋工業大学、愛知、10月1－3日（1997）

(5) 異種塩基性セグメントを持つポリアミングラフト共重合体を用いたDNA複合体の構造制御
第7回インテリジェント材料シンポジウム、青山学院大学、東京、3月19日（1998）

(6) 細胞特異的DNAキャリアーとしてのポリリジン／ヒアルロン酸グラフト共重合体の合成
バイオアクティブポリマーシンポジウム、北海道大学、北海道、4月10日（1998）

(7) 新規pH応答性DNAキャリアーとしてのポリカチオングラフト共重合体の設計
第8回バイオ・高分子シンポジウム、上智大学、東京、7月23－24日（1998）

(8) イミダゾール基及びガラクトースを有する新規グラフト共重合体・DNA複合体のpH応答性
第47回高分子討論会、名古屋国際会議場、愛知、9月30日－10月2日（1998）

以下、講演申込受理済

（9）イミダゾール基及び糖鎖を有するポリカチオングラフト共重合体・DNA複合体のpH応答的物性変化
第8回インテリジェント材料シンポジウム、青山学院大学、東京、3月16日（1999）

（10）イミダゾール基を有する新規グラフト共重合体によるpH応答型DNA複合体
日本化学会第76春季年会、神奈川大学、神奈川、3月28－31日（1999）

（11）イミダゾール基及び糖鎖を有するエンドソーム内pH応答性グラフト共重合体の設計とDNAキャリアーとしての応用
第48回高分子学会年次大会、国立京都国際会館、京都、5月27日－5月29日（1999）

AWARD

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