 DOI: 10.1002/masy.200750351

Preparation of Cationic Comb-Type Copolymers Having High Density of PEG Graft Chains for Gene Carriers

Sung Won Choi,[1a] *Asako Yamayoshi,*[1a] *Miwa Hirai,*[2] *Takeshi Yamano,*[1] *Motoki Takagi,*[2] *Ayumi Sato,*[2] *Arihiro Kano,*[1] *Akira Shimamoto,*[2] *Atsushi Maruyama**[1,3]

Summary: A series of poly(L-lysine)-*graft*-polyethylene glycol (PLL-*g*-PEG) was prepared by a reductive amination reaction of PLL with aldehyde modified PEG using $NaBH_3CN$ as a reductant. The high coupling efficacy observed in the 80% acetonitrile aqueous solution led to the grafting polymers with abundant PEG chains (more than 90 wt % and 30 mol %). By the method, we can retain high cationic charge density of the polymer backbone, because the resulting secondary amino groups are still protonated. By controlling the primary structure of the copolymer, we can also regulate the ionic interaction with nucleic acids and other biological components for gene delivery purpose.

Keywords: cationic comb-type copolymer; drug delivery systems; graft copolymers; polymer brushes; water-soluble polymers

Introduction

Oligonucleotides and their analogs have been employed to suppress gene expression in a sequence-specific manner.[1] In particular, the RNA interfering (RNAi) strategy, where small interfering RNA (siRNA) reduces the expression of a specific gene, is now a realistic option for gene therapy.[1,2] A large number of reports have demonstrated the potency of this strategy, and some siRNA drugs are now under clinical trials.[3] However, siRNA therapeutics is hampered by poor membrane permeability and limited stability of siRNA *in vivo*.[4,5] When siRNA is administrated in blood, it is also readily digested by nuclease and eliminated from renal glomeruli before reaching target tissues.

Recently, surface modification of drug delivery systems with polyethylene glycol (PEG) has attracted increasing interests to enhance biocompatibility,[6] extend systemic circulation time,[7] and alter their biodistribution[8] due to the high resistance of PEG to protein adsorption.[9] For example, nanoparticles, liposomes, proteins, and nucleic acid drugs have been ''PEGylated'' to obtain mainly long-circulating particulate delivery systems, based on so-called ''stealth'' effects.[10] Thus, these non-viral stealth career systems are expected to open the applicability of gene delivery systems *in vivo*.

We have studied the copolymers consisting of a polycationic backbone and water-soluble graft chains of polysaccharide. These copolymers were prepared by a reductive amination reaction between the

[1] Institute for Materials Chemistry and Engineering, Kyushu University, 6-10-1, Hakozaki, Higashi, Fukuoka 812-8581, Japan
E-mail: maruyama@ms.ifoc.kyushu-u.ac.jp
[2] GeneCare Research Institute Co., Ltd., 200 Kajiwara, Kamakura, Kanagawa 247-0063, Japan
[3] CREST, Japan Science and Technology Agency, 4-1-8, Honcho, Kawaguchi, Saitama 332-0012, Japan
[a] These authors contributed equally to this work.

primary amino-group of a cationic polymer and the reductive end of polysaccharides.[11] By this reaction method, we can retain high cationic charge density of the polymer backbone, because the resulting secondary amino groups are still protonated. In our previous papers the copolymers, i.e. comb-type copolymers (CCCs) which have a small fraction (less than 30 wt%) of a polycationic backbone and abundant polysaccharide graft chains (more than 70 wt %, 36 mol %), showed unique properties in its interaction with polynucleotides.[12–15] Unlike cationic homopolymers, such as poly(L-lysine) and poly(ethylene imine), the CCCs form totally soluble complexes with DNA without condensing polynucleotides.[12] The CCCs are also found not to retard but accelerate hybridization of DNAs[13] and increase considerably thermodynamic stability of DNA duplexes and triplexes.[14] These observations indicate that the CCC strongly interacts with oligonucleotides regardless of the existence of abundant hydrophilic graft chains.

Considering the characteristics of the CCCs, they should be suitable as a long-blood circulating carrier, because abundant graft chains sterically protect the complexes between DNA (or RNA) and the CCC backbone from undesirable interactions with blood components. In this report, we describe the preparation of a new class of the CCCs, PLL-*g*-PEG, that have the high densities of cationic charges and PEG graft chains as a long circulating siRNA carrier.

Materials and Methods

Materials

Aldehyde-modified [α-methyl-ω-(3oxo-propoxy), polyoxethylene] (PEG- aldehyde), SUNBRIGHT HO-020AL ($M_n = 2\,000$) and ME-050AL ($M_n = 5\,000$) was obtained from NOF Co. (Tokyo, Japan). Poly(L-lysine) hydrobromide (PLL · HBr, $M_n =$ 7000 and 28 000) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Other solvents and chemicals of reagent grade were obtained from Wako Pure Chemical (Osaka, Japan) and were used without further purification.

Preparation of Comb-Typed Copolymers

The comb-type copolymers, poly(L-lysine)-*graft*-polyethylene glycol (PLL-*g*-PEG), were prepared by a reductive amination reaction of PLL · HBr with PEG-aldehyde. Briefly, the end aldehyde groups of PEG were covalently coupled with ε-amino groups of PLL in sodium borate buffer (pH 8.5) or 80% acetonitrile aqueous solution using sodium cyanoborohydride as a reductant. The conditions for the synthesis are given in Table 1. The copolymers were separated by ultrafiltration and dialysis from unreacted PEG and the reductant, and then lyophilized. The copolymers were characterized by ^{1}H-NMR (JNM-EX-270, JEOL, Tokyo, Japan), elementary analysis (CHN-corder MT-6, Yanako, Kyoto, Japan) and size-exclusion chromatography-multiangle light scattering (SEC-MALS).

Size-Exclusion Chromatography-Multiangle Light Scattering (SEC-MALS)

The resulting copolymers were analyzed using a size-exclusion chromatography (SEC) system (model 800, JASCO, Tokyo, Japan) on Shodex OHpak SB-804 and 806M columns connected with a multiangle light scattering (MALS) detector (Dawn-EOS, Wyatt Technology, Santa Barbara, CA, USA) and a differential refractive index (RI) detector (JASCO, model 830-RI). An aqueous solution of 0.5 M acetic acid and 0.2 M Na2SO4 was used as a mobile phase at a flow rate of 0.8 ml/min at 25 °C. An aliquot (100 μl) of graft copolymer solution (10 mg/ml) was injected into the columns. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were calculated from RI and MALS signals using Astra for Windows Version 4.90 (Wyatt Technology). The dn/dc value was determined by assuming 100% recovery of samples from the columns.

Table 1.
Preparation and characterization of the comb-type copolymers having dense graft chains.

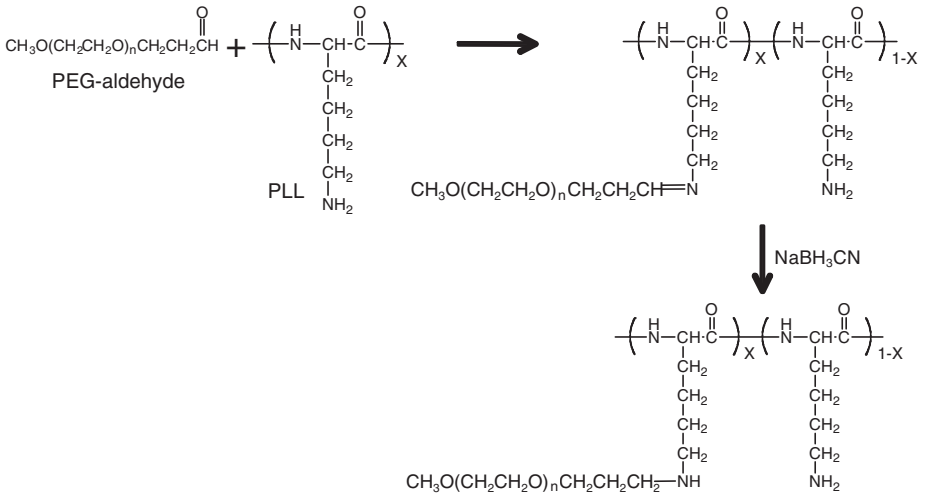
Run	In feed						Copolymer			
	PLL		PEG		$NaBH_3CN$	Solvent	Molecular weight		Grafting ratio	PEG content
	M_n[d]/10^3	(g)	M_n/10^3	(g)	mmol	ML	M_n[d]/10^4	M_w[d]/Mn[d]	(mol %)[e]	(wt %)[e]
1	7	0.12	2	1.39	3.68	90[b)]	4.0	1.2	56	89
2	28	0.12	2	1.39	3.68	91[b)]	30	2.0	72	91
3	7	0.19	5	2.41	2.09	18[a)]	3.3	1.6	17	87
4	28	0.12	5	1.28	1.32	88[b)]	31	2.5	36	93
5	7	0.28	5	1.28	1.00	27[a)]	2.0	1.3	6	71
6	28	0.15	5	0.71	0.62	15[a)]	9.6	1.8	5	67

[a)] Soudium borate buffer (pH 8.5).
[b)] ca. 80% acetonitrile solution.
[c)] H_2O (pH 9.5).
[d)] M_n = the number-average molecular weight; M_w = the weight-average molecular weight. M_n and M_w were determined by MALLS.
[e)] Determined by ^1H NMR.

Results and Discussions

Prepolymers, which have a reactive group at their one chain end, are required in the preparation of graft copolymers *via* the polymer coupling method. We have prepared the graft copolymers with polysaccharides, because most polysaccharides possess one reactive end. Actually, various graft copolymers with different backbones and side chains have been obtained by the reductive amination reaction between the polysaccharide end and amino-containing polymers, while keeping cationic properties of the amino groups.[11,15–17] Regarding systemic gene delivery using PEGylated polymers, both chain length and graft density of PEG obviously affect pharmacokinetic properties.[6–8,10]

In this study, we prepared the cationic comb-type copolymers, PLL-*g*-PEG, by the reductive amination reaction method (Scheme 1). The results of the coupling reactions are summarized in Table1. The reactions in sodium borate buffer (pH 8.5) resulted in low coupling efficacy



Scheme 1.
Synthetic scheme of the poly(L-lysine)-*graft*-polyethylene glycol (PLL-*g*-PEG).

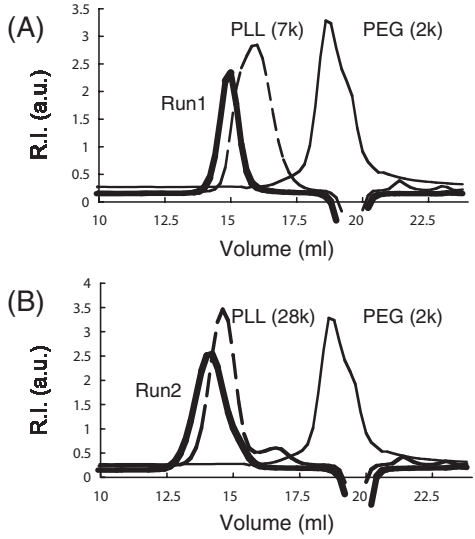


Figure 1.
The GPC profiles of PLL, PEG, and the resulting copolymers: Run1 (A) and Run2 (B). An aqueous solution containing 0.5 M acetic acid and 0.2 M Na_2SO_4 was used as a mobile phase at a rate of 1.0 ml/min.

presumably owing to insufficient Schiff's base formation between PEG-aldehyde and PLL (Table1; Run3, 5, and 6).

To promote Schiff's base formation, the reaction in 80% acetonitrile aqueous solution was examined. The high coupling efficacy observed in the 80% acetonitrile aqueous solution led to the graft copolymers with abundant PEG chains (more than 90 wt % and 30 mol %) (Table1: Run1, 2, and 4). These results suggest that an aprotic solvent is suitable for this coupling reaction. Figure 1 shows the GPC profiles of PLL, PEG, and the resulting copolymers (Table1: Run1 and 2). These reactions resulted in an expected increase in molecular weight. Furthermore, no unreacted PEG peak in the GPC profile of the isolated copolymer was observed, indicating the successful isolation of the copolymers. As summarized in Table 1, the graft copolymers having various molecular weights and grafting ratios of PEG side chains were obtained. The copolymer composition calculated from the molecular weight was in good agreement with that determined from ^{1}H NMR measurements and elementary analysis. These results indicate that no detectable side reactions such as cross-linking or degradation of the copolymers occurred under the reaction conditions in this study.

Conclusions

In this study, we obtained the various PLL-*g*-PEG comb-type copolymers with a well-defined comb-type structure by the reductive amination method between the aldehyde group of PEG and the ε-amino groups of PLL. The high coupling efficacy observed in the 80% acetnitrile aqueous solution enable us to obtain the graft polymers with abundant PEG chains (more than 30 mol % grafting ratio), building a bottlebrush-type structure on the copolymers without a serious decrease in the cationic charge density of the copolymer backbone. By controlling the primary structure of the copolymer, we could regulate the interactions with nucleic acids and other biological components for gene delivery purpose.

[1] W. Lv, C. Zhang, J. Hao, *World J Gastroenterol.* **2006**, *7*, 4636.
[2] C. Chetty, P. Bhoopathi, P. Joseph, S. Chittivelu, J. S. Rao, S. Lakka, *Mol. Cancer Ther.* **2006**, *5*, 2289.

[3] D. W. Sah, *Life Sci.* **2006**, *4*, 1773.
[4] Y. Dorsett, T. Tuschl, *Nat. Rev. Drug Discov.* **2004**, *3*, 318.
[5] T. C. Karagiannis, A. El-Osta, *Cancer Gene. Ther.* **2005**, *12*, 787.
[6] A. Kidane, G. C. Lantz, S. Jo, K. Park, *J. Biomat. Sci. Polym. Ed.* **1999**, *10*, 1089.
[7] C. Monfardini, F. M. Veronese, *Bioconjugate Chem.* **1998**, *9*, 418.
[8] E. T. Dams, W. J. Oyen, O. C. Boerman, G. Storm, P. Laverman, P. J. Kok, W. C. Buijs, H. Bakker, J. W. van der Meer, F. H. Corstens, *J. Nucl. Med.* **2000**, *40*, 2066.
[9] W. R. Gombotz, W. Guanghui, T. A. Horbett, A. S. Hoffman, in: ''*Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications*'', J. M. Harris, Eds., Plenum Press, New York **1992**, p. 247.
[10] F. M. Veronese, G. Pasut, *Drug Discov. Today.* **2005**, *10*, 1451.
[11] A. Maruyam, M. Katoh, T. Ishihara, T. Akaike, *Bioconjugate Chem.* **1997**, *8*, 3.
[12] Y. Sato, Y. Kobayashi, T. Kamiya, H. Watanabe, T. Akaike, K. Yoshikawa, A. Maruyama, *Biomaterials* **2005**, *26*, 703.
[13] W. J. Kim, T. Akaike, A. Maruyama, *J. Am. Chem. Soc.* **2002**, *124*, 12676.
[14] A. Maruyama, Y. Ohnishi, H. Watanabe, H. Torigoe, A. Ferdous, T. Akaike, *Colloids Surf. B.* **1999**, *16*, 27.
[15] S. Asayama, M. Nogawa, Y. Takei, T. Akaike, A. Maruyama, *Bioconjugate Chem.* **1998**, *9*, 476.
[16] J. U. Park, T. Ishihara, A. Kano, T. Akaike, A. Maruyama, *Prep. Biochem.Biotech.* **1999**, *29*, 353.
[17] A. Maruyama, T. Ishihara, T. Akaike, J.-S. Kim, S. W. Kim, *Bioconjugate Chem.* **1997**, *8*, 735.

