Enhancement of Transcriptional Activity of DNA Complexes by Amphoteric PEG Derivative

Yoshiyuki Koyama,*,† Misa Yamashita,† Naoko Iida-Tanaka,‡ and Tomoko Ito†

Department of Textile Science and Department of Food Science, Otsuma Women's University, Sanbancho, Chiyoda-ku, Tokyo, 102-8357 Japan

Received July 2, 2005; Revised Manuscript Received January 25, 2006

A water-soluble PEG derivative having both amino and carboxylic acid side chains (PEG-AC) was synthesized and explored for its transcription- and transfection-enhancing activity. PEG-AC could be deposited onto the surface of DNA/polycation complexes to form a ternary complex with slightly negative surface potential. PEG-AC-coating on the plasmid/PEI complexes obviously enhanced their transcriptional activity, and 31-fold higher consumption of UTP was observed. Amphoteric PEG-AC would loosen the tightly compacted DNA/PEI complex and facilitate the approach of transcriptional factors. PEG-AC also evidently improved the transgene expression level on the cultured CHO cells.

Introduction

Many kinds of nonviral vectors have been designed and developed with the aim of an efficient gene delivery and sufficient transgene expression in a target tissue. Several polycations and cationic lipids have already realized effective gene transfer in cell culture.[1,2] A human clinical trial with polyethyleneimine (PEI)-mediated gene therapy was also performed, and a high expression level of the therapeutic gene was detected in the carcinoma tissues.[3] However, there still remain major obstacles to therapeutic applications of these synthetic gene delivery systems. In comparison with viral vectors, nonviral vectors show much lower efficiency in gene transport and expression, though they have attractive features such as low acute toxicity, low immunogenecity, and feasibility to be produced on a large scale.

To attain the efficient gene transfer, nonviral vectors must overcome many barriers in delivery to the target tissue, uptake by the cells, escaping the endosome, and transporting to the nucleus.[4] After these delivery barriers are overcome, the transported gene in the nucleus must face other problems with transcriptional activity. Stable complex formation of the DNA molecules with polycationic vectors is required for their delivery and escape from the enzymatic degradation. The tightly compacted conformation of the DNA molecule in the complex with polycations, however, seems to interfere with the access of transcription factors, and diminishes the production of mRNA transcripts. It was reported that several polycations have a very low transgene enhancing effect due to the lack in the transcriptional activity of their complex with plasmid.[5] The plasmid/polycation complex should have a moderately loosened structure to allow the access of transcription factors, at least under the conditions in the nucleus.

We have developed a new type of polyanion, PEG-Cs, which are water-soluble polymers having PEG-like ether-linked main chain and carboxylic pendants.[6] PEG-Cs are deposited onto the positively charged DNA/polycation complex and form a ternary complex having negative surface charge, while most other polyanions would disrupt the DNA/polycation complex, releasing free DNA molecules.[7] PEG-C does not completely disassemble the DNA−polycation electrostatic binding, but would loosen it to some extent by interacting with the polycation. PEG-C was, thus, expected to relax the complex and improve its transcriptional activity.

In mammalian cell nuclei, the higher-order chromatin structure is, when needed to be transcribed, deformed and loosened by high mobility group (HMG) proteins to enhance the accessibility of transcriptional factors.[8] HMG proteins have a characteristic structure comprising both cationic DNA-binding domains and an anionic C-tail.[9] This acidic C-tail is known to be essential to transcriptional stimulation.[10] Specific interactions between the acidic region of HMGB1 and the histone H3 N-tail[11] or p53[12] have been reported, but such an amphoteric macromolecule is thought to also have a nonspecific physical property to relax the tight binding between DNA and the polycation through the electrostatic interactions.

An amphoteric derivative of PEG-C was, thus, expected to be a superior transcription activator to PEG-C. A PEG derivative having both carboxyl and amino pendants, PEG-AC, was then synthesized and explored for its transcription- and transfection-enhancing activity.

Materials and Methods

Materials. Uridine 5′-triphosphate P[3]-(5-sulfo-1-naphthylamide) tetra(triethylammonium) salt (γ-AmNS−UTP) and rhodamine active ester were purchased from Molecular Probes, Inc. Fluoresceineisothiocyanate isomer-I (FITC), protamine sulfate from salmon (PRT), and 4′6-diamidinophenylindol (DAPI) were purchased from Wako Pure Chemical Industries, Ltd. Plasmid DNA containing firefly luciferase gene under control of cytomegalovirus promoter was a kind gift from Professor T. Niidome (Kyushu University, Fukuoka, Japan); it was prepared by removing the Bgl II and Hind III insert from the plasmid PGV−C (PicaGene control vector, Tokyo Ink, Tokyo, Japan), followed by the ligation with the Bgl II and Hind III fragment from the pRc/CMV (Invitrogen) containing cytomegalovirus promoter. It was amplified in *Escherichia coli* and purified with a QIAGEN plasmid mega kit. *E. coli* RNA polymerase holoenzyme was purchased from EPI-

* To whom correspondence should be addressed. Tel.: +81−3−5275−6017. Fax: +81−3−5275−6932. E-mail address: koyama@otsuma.ac.jp.
† Department of Textile Science.
‡ Department of Food Science.

10.1021/bm0504633 CCC: $33.50 © 2006 American Chemical Society
Published on Web 03/24/2006

CENTRE Technologies. ATP, CTP, and GTP were obtained as lithium salt from Roche Diagnostics Co. Linear polyethyleneimine (PEI; Mw = 25 000) was purchased from Polyscience, Inc. Poly(ally glycidyl ether-*co*-ethylene oxide) synthesized as described before[13] was kindly given by NOF Corporation.

Synthesis of PEG-C and PEG-AC. PEG-C having 17.7 carboxyl pendants was prepared as previously reported.[6] Calculated molecular weight is 8940. PEG-AC having 5.6 amino and 12.1 carboxyl groups was synthesized as follows: To the mixed solution of aminoethanethiol hydrochloride (5.19 g) and mercaptopropionic acid (16.2 g) in methanol (40 mL) was added 10.0 g of poly(allyl glycidyl ether-*co*-ethylene oxide) (17.7 allyl pendants per polymer; Mn = 7060) dissolved in 30 mL of methanol. After standing at 30 °C for 50 h, the solution was diluted with water and dialyzed against running water for 1 day, then 2 more days against distilled water. A yellowish syrup (8.9 g) was obtained after freeze−thawing. It was further purified by gel filtration and ion exchange by a sulfonate-type resin before use. The ratio of amine to carboxyl groups in the polymer was determined from ^{1}H NMR spectrum of its acetylated derivative.[7]

Fluorescence Labeling of the Polymer. PEI, PRT, and PEG-AC were labeled by fluoresceine or rhodamine by adding their aqueous solutions to the dimethylformamide solution of FITC or rhodamine active ester, respectively. Rhodamine-labeled PEG-C was prepared by the addition of rhodamine active ester to PEG derivative having 0.7 amino and 17.0 carboxyl groups, which had been synthesized similarly to the PEG-AC synthesis described above by the reaction of poly(allyl glycidyl ether-*co*-ethylene oxide) with a mixture of aminoethanethiol hydrochloride and mercaptopropionic acid at 1:50 in weight. Fluorescence-labeled polymers were purified by repeated gel filtration and ion exchange. The amount of the fluorophore in the polymers was 7−200 μmol per 1 g of polymer as was estimated from their fluorescence intensity.

Composition Analysis of DNA/Polycation Binary Complex. The mixing charge ratio of DNA and polyions in the composition study is expressed as follows: The negative charge in DNA or PEG-C expresses the whole amount of the phosphoric acid or carboxylic acid in the molecule, respectively. All the nitrogen atoms in PEI were calculated as positive charge. Whole arginine residues and a terminal amino group were counted as positive charge in protamine (PRT). A negative charge of PEG-AC was calculated as excess carboxylic acid groups over amines.

DNA from salmon sperm (26.4 μg) in 100 μL of water was mixed with the same volume of the aqueous fluorescent polycations. It was incubated for 15 min and then centrifuged at 15 000 rpm for 1 h. The precipitated complex was redissolved in 30% NaCl, and the concentration of the polycation was determined from fluorescence intensity. The content of DNA was measured using DAPI, a fluorescent dye, which binds to the DNA molecule.

Composition Anaysis of DNA/Polycation/Polyanion Ternary Complex. DNA (from salmon sperm)/polycation (nonlabeled) complex suspension was prepared as above and mixed with rhodamine-labeld PEG-C or PEG-AC. After incubating for 15 min, it was gently put on the top of 30% sucrose solution. Allowing the material to stand overnight in the dark at room temperature let only a DNA-containing complex precipitate, leaving free polyions and polycation/polyanion complex without DNA suspended (except in the case with a great excess of PEG-C at PEG-C/DNA = 20 in charge). The supernatant was carefully suctioned, and the content of PEG-C or PEG-AC was analyzed by fluorescence analysis of the redissolved ternary complex in 30% NaCl. Content of the polycations in the ternary complex was determined by analyzing DNA/rhodamine-polycation/nonlabeled PEG-C (or PEG-AC) complex by the similar method.

Fluorescence Microscopic Observation. The fluorescence microscopic observation was performed using an IX70 microscope (Olympus) equipped with a 100× oil-immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera. The final concentration of DNA was 4.5 μM (in base). DNA was visualized with fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI) (final 0.7 μM). Mercaptoethanol was added as an antioxidant (final 0.7% v/v).

Measurement of ζ-Potential and Size of the Complex. In the ζ-potential, transcription, and transfection study, the DNA/PEI/PEG-C (or PEG-AC) mixing ratio was expressed as P/N/COOH, which represents the molar ratio of phosphoric acid in DNA, nitrogen atoms in PEI, and whole COOH in the PEG derivative. Samples were prepared by mixing the aqueous solutions of plasmid (1.25 μg in 12.5 μL) and linear PEI (1.30 μg in 12.5 μL) followed by addition of aqueous PEG-C or PEG-AC solution (25 μL). The suspension was diluted with PBS (950 μL) and analyzed for ζ-potential and diameter by a MALVERN Zetasizer Nano ZS.

Transcription Procedure. The plasmid complex suspension was prepared by mixing the aqueous solutions of plasmid (0.33 μg in 3.3 μL) with PEG-C or PEG-AC (2.5−14.5 μg in 6.6 μL) and then with linear PEI (0.34 μg in 3.3 μL) and 15 μL of water. A mixture of 100 mM Tris-HCl (pH 7.5; 5 μL), 500 mM KCl (10 μL), 1 M $MgCl_2$ (0.25 μL), 10 mM ATP, CTP, and GTP (each 0.5 μL), 5 mM γ-AmNS−UTP (0.1 μL), and 100 mM DTT (1 μL) was added to the plasmid complex suspension prepared above. After incubation for ca. 30 min at 37 °C, *E. coli* RNA polymerase (0.2 U/μL, 5 μL) was added, and the samples were kept at 37 °C for a given time. The reaction was discontinued by adding 50 mM EDTA (950 μL). The amount of the produced mRNA transcripts was estimated by analyzing the released AmNS-pyrophosphoric acid from the increase in the fluorescence intensity at 465 nm (Ex = 330 nm).

YOYO-1/DNA Fluorescence Restoration by PEG Derivatives. The loosening effect of the PEG derivatives on the DNA/PEI complex was evaluated by the YOYO-1/DNA fluorescence restoration. To an aqueous solution of plasmid (5 μg in 12.5 μL (1212 μM in base)) were added the solutions of YOYO-1 (240 μM in 12.5 μL) and 25 μL of 50% 2-mercaptoethanol (ME). The solution was diluted with 800 μL of PBS, and the fluorescence intensity of the solution was measured by a JASCO Spectrofluorometer FP-777 at 512 nm (Ex = 492 nm). PEI (5.2 μg in 50 μL PBS) was then added to the plasmid solution at N/P = 8, and the fluorescence intensity was again measured. After 2 min, PEG-C or PEG-AC in 100 μL PBS was added at P/COOH = 1:15, and recovery of the fluorescence intensity was monitored. The mixed solution of YOYO-1/plasmid and PEG derivatives without PEI was also measured to examine the influence of PEG derivatives themselves on the fluorescence efficiency.

Transfection Procedure. The ternary plasmid complex suspension for transfection was prepared as follows: Plasmid (1.25 μg in 12.5 μL in pure water) was mixed with PEG-C or PEG-AC solution (25 μL), and then, linear PEI (1.30 μg in 12.5 μL in quadruple-condensed PBS) was added. CHO cells, a Chinese hamster ovary cell line, were seeded into 24-well multiplates at about 2.5 × 10^4 cells per well and grown for 2 days in F12 with 10% fetal bovine serum (FBS) and 1% penicillin. The primary growth medium was removed and replaced with 450 μL of F12 with 1% penicillin (without FBS). The plasmid complex suspension (50 μL) was then added and incubated for 4 h at 37 °C. After removal of the transfection medium, the cells were incubated in the fresh medium containing 15% FBS for further 24 h at 37 °C and assessed for transgene expression by a luciferase assay kit (Pica Gene). Protein content in the lysate was also analyzed by protein assay kit (Bio-Rad).

Results

Synthesis of PEG-C and PEG-AC. PEG-C and PEG-AC were synthesized by an addition reaction of mercaptans to the double bond of poly(allyl glycidyl ether-*co*-ethylene oxide) (Figure 1). Completion of the reaction was confirmed by ^{1}HNMR spectra, where no residual allyl group was detected.

Composition of the Complex. DNA was mixed with PRT or PEI at various ±charge ratios and centrifuged. Free DNA or polycation did not precipitate by the centrifugation, and so, the

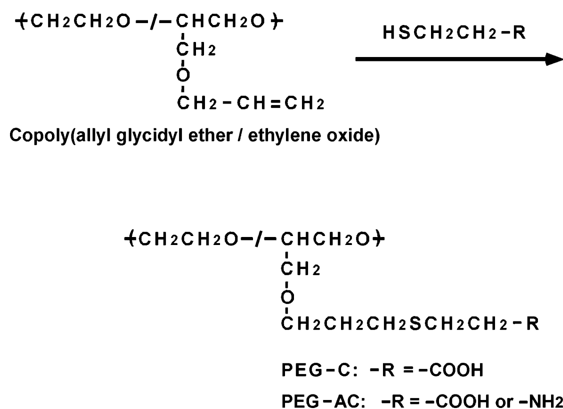

Figure 1. Synthesis of PEG-C and PEG-AC.

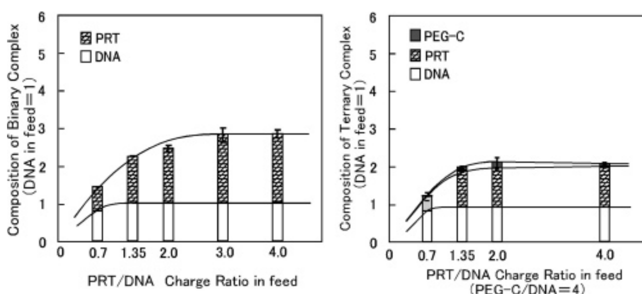

Figure 2. Composition of DNA/protamine (PRT) complexes. The binary or ternary DNA complex was isolated by centrifugation or precipitation in 30% sucrose, respectively, and redissolved in 30% NaCl. The concentrations of the DNA and polycation in the solution were then determined by the fluorescence methods, as described in the text. The concentrations of the polycation and PEGs in the ternary complex were determined in the separate experiments. Contents are expressed as equivalent values to the DNA in feed.

complex composition could be determined by analyzing the precipitation. The ternary complex could also be isolated by 30% sucrose, in which polycation/PEG-C (or PEG-AC) binary complex was suspended close to the surface, and the composition of the precipitate was analyzed.

When PRT was added to DNA at ±charge ratio > 1, no DNA was detected in the centrifuged supernatant by fluorescence spectroscopy or fluorescence microscopy. It indicates that all the DNA molecules formed complexes with the polycations and were precipitated under these conditions. Analysis of the precipitate showed that the PRT/DNA content ratio in the complex increased with the feed ratio, and as shown in Figure 2, it reached a plateau at PRT/DNA = 1.8 (in charge). Unlike other common polyanions, PEG-C did not decompose the DNA/PRT complex, but removed some of the polycation molecules from DNA, and DNA/PRT/PEG-C ternary complexes with PRT/DNA = 1.2 and PEG-C/DNA = 0.1−0.2 (in charge) were obtained.

When PEI was mixed with DNA at ±charge ratio > 2, no DNA molecules were left in the supernatant. Addition of a great excess of PEI resulted in the complex containing more than a fourfold equivalent of PEI to DNA (Figure 3). A high PEI content would be probably due to the apparently low pK_b of the polycation.[14] The PEI content in the complex did not change obviously by PEG-C. Much more PEG-C could be deposited onto the DNA/PEI complex than in the case with DNA/PRT, and 2−4× charge equivalent (3−6 times in weight) of PEG-C to DNA was detected in the DNA/PEI/PEG-C ternary complexes. The content of the ternary complex prepared at DNA/PEI/PEG-C = 1:20:10 in charge could not be clearly determined

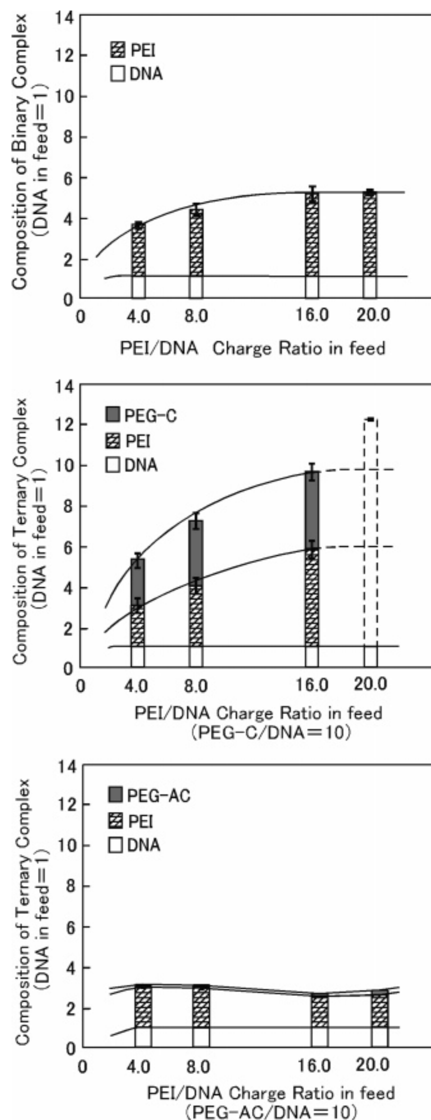

Figure 3. Composition of DNA/polyethyleneimine (PEI) complexes. Each concentration was determined as in Figure 2. Contents are expressed as equivalent values to DNA in feed.

because of the precipitation of PEI/PEG-C complex in 30% sucrose.

Addition of PEG-AC evidently diminished the PEI content in the complex, and the PEI/DNA charge ratio in the ternary complex became 1.6−2. The PEG-AC/DNA ratio in the complexes was in the range 0.1−0.2 in charge (0.4−0.9 in weight), which was lower than the PEG-C content in DNA/PEI/PEG-C ternary complexes.

ζ-Potential and Size of the Plasmid Complexes. ζ-Potential of the plasmid/PEI binary complex prepared at P/N = 1:8 was +16.3 mV. Addition of PEG-C effectively recharged the complex, and the complexes with negative ζ-potential (−1.8 to −3.6 mV) were obtained at DNA/PEI/PEG-C (P/N/COOH) = 1:8:10−20 (Figure 4). PEG-AC could also recharged the plasmid/PEI complexes, and the plasmid/PEI/PEG-AC ternary complexes having negative ζ-potential (−3.8 to −4.4 mV) were obtained at the same mixing ratio.

The complexes were about 150−250 nm in diameter. After addition of PEG-C, larger aggregated particles with diameters of 350−500 nm appeared, but an apparent change was not seen in the size of the unaggregated complexes. On the other hand, PEG-AC did not make them aggregate, but rather decreased

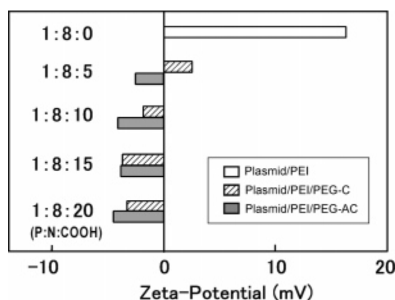

Figure 4. ζ-Potential of the plasmid complexes. Complexes were prepared in pure water, then diluted by 20× volume of PBS and subjected to the measurement.

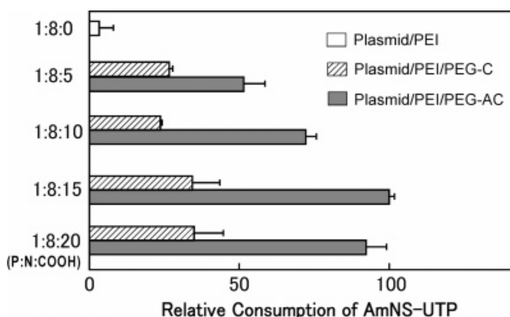

Figure 5. Relative transcriptional activity of the plasmid and its complexes. The plasmid or its complex was incubated with ATP, CTP, GTP, γ-AmNS−UTP, and *E. coli* RNA polymerase at 37 °C for 90 min. The consumption of γ-AmNS−UTP was estimated from the fluorescence increase. Results are expressed as a relative consumption of γ-AmNS−UTP (the value with plasmid/PEI/PEG-AC (1:8:15) = 100). $n = 2$.

their size to 85% of the original. It should be owing to the partial shelling off of the loosely bound PEI as described above.

Transcription Study. Enhancing effect of PEG-C and PEG-AC on the transcriptional activity was examined with RNA polymerase (from *E. coli*) by in vitro system without cells. γ-AmNS−UTP was used for simple estimation of the conversion. After incubation at 37 °C for 90 min, the transcription reaction was stopped by EDTA, and the fluorescence intensity was measured. The naked plasmid showed a high consumption of the UTP derivative and production of AmNS−pyrophosphoric acid, while no UTP derivative consumption was detected in the cases without RNA polymerase or without plasmid. The UTP consumption with plasmid DNA was strongly reduced by PEI, and plasmid/PEI binary complex at P/N = 1:8 showed only 1−2% of fluorescence increase compared to that with naked plasmid. As shown in Figure 5, PEG-C- and PEG-AC-containing ternary complexes induced much higher consumption of the UTP derivative than plasmid/PEI binary complex. On the other hand, neither PEG-C nor PEG-AC enhanced the transcription of naked DNA without PEI. It indicates that those polyanions could obviously activate the transcription of the plasmid complexes, presumably owing to their loosening effect on the DNA/PEI complexes. PEG-AC which was synthesized mimicking HMG protein structure, as expected, showed a higher enhancing effect than PEG-C on the transcriptional activity, and the DNA/PEI/PEG-AC complex prepared at P/N/COOH = 1:8:15 showed a 31-fold higher transcription activity than the plasmid/PEI binary complex. The time dependence of the consumption of UTP with the DNA/PEI/PEG-C (or PEG-AC) complex at 1:8:15 was then examined. An almost linear time−conversion relationship was observed, and high transcriptional activity of the DNA/PEI/PEG-AC terplex was again confirmed (Figure 6).

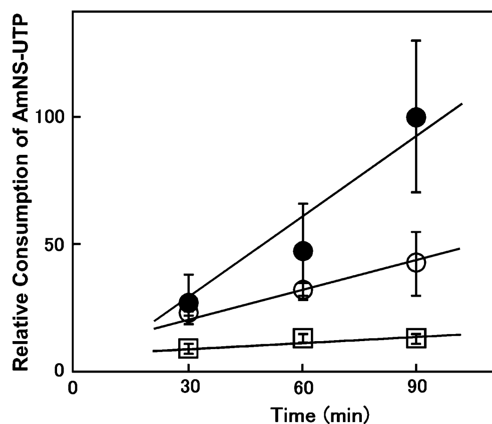

Figure 6. Transcriptional rates of the plasmid and its complexes. The consumption of γ-AmNS−UTP was measured as described in Figure 5. Reaction was discontinued after incubation for 30, 60, or 90 min, and the relative consumption of AmNS−UTP was measured. Results are expressed as a relative consumption of γ-AmNS−UTP (the value with plasmid/PEI/PEG-AC (1:8:15) at 90 min = 100). $n = 4-6$. □, plasmid/PEI (1:8); ○, plasmid/PEI/PEG-C (1:8:15); ●, plasmid/PEI/PEG-AC (1:8:15).

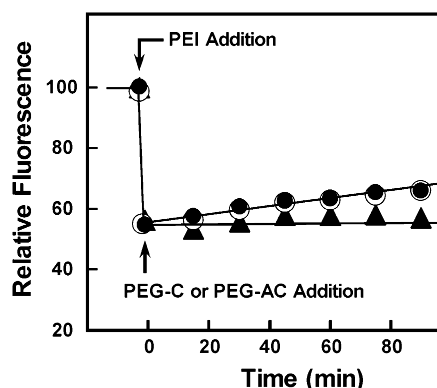

Figure 7. YOYO-1/DNA fluorescence restoration by PEG-C or PEG-AC. YOYO-1/DNA was mixed with PEI at N/P = 8 in PBS. After 2 min, PEG-C or PEG-AC was added at P/COOH = 1:15, and fluorescence restoration was monitored. ○, PEG-C; ●, PEG-AC; ▲, control (PBS).

YOYO-1/DNA Fluorescence Restoration by PEG Derivatives. Loosening of the DNA complex by PEG-C and PEG-AC was examined by fluorescence restoration. When the DNA was compacted by PEI into a globular state, the fluorescence of YOYO-1 intercalated in the DNA molecule was reduced to 56−58%. Both PEG derivatives showed fluorescence restoration, and the fluorescence intensity was recovered up to 66−67% in 90 min (Figure 7), probably due to their loosening effect on the compacted DNA complexes. PEG-AC did not show so much higher fluorescence restoration than PEG-C as expected from the transcription result. The influence of the PEG derivatives on the fluorescence efficiency was then examined. PEG-AC was revealed to reduce the fluorescence intensity of YOYO-1/DNA to 80%, while PEG-C did not affect it (data not shown). With the quenching effect of PEG-AC taken into account, the amphoteric polymer would have a greater loosening effect than PEG-C on the DNA/PEI complex.

Transfection Study. Ternary complexes with PEG-C or PEG-AC were shown to have the properly relaxed structure favorable toward transcription. They were, thus, expected to have high gene-expressing activity. The transfection efficiency of the plasmid/PEI/PEG-C (or PEG-AC) ternary complexes prepared at P/N/COOH = 1:8:15−20, which showed highly enhanced transcriptional activity, was examined on cultured

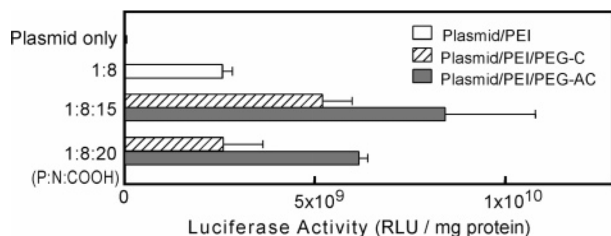

Figure 8. Transgene expression efficiency of PEG-C- or PEG-AC-coated plasmid/PEI complexes on CHO cells. Plasmid encoding firefly luciferase was mixed with PEG-C or PEG-AC in pure water and then with PEI in PBS. The complex suspension was then added to CHO cells and incubated at 37 °C for 4 h. After incubation in the fresh medium for further 24 h, the cells were assessed for luciferase activity, as described in the text. Results are expressed as relative light units per mg protein. $n = 3$.

CHO cells. Figure 8 shows the transgene expression efficiency observed with the binary or ternary complexes. PEI itself mediated quite high gene expression on CHO cells. PEG-C could further enhance the efficiency of PEI-mediated transfection, and the ternary complex prepared at plasmid/PEI/PEG-C = 1:8:15 showed twofold higher gene expression than the plasmid/PEI binary complex. As was expected from the results of the transcription study, PEG-AC showed a still higher enhancing effect than PEG-C. Addition of PEG-AC at P/COOH = 1:15 or 1:20 increased the transgene expression 3.3- or 2.4-fold, respectively, compared to the plasmid/PEI binary complex. Judging from the total protein content in the cell lysate, obvious cell toxicity was not observed in any case.

Discussion

Fluorescence labeling of the components and selective precipitation of their ternary complexes with 30% sucrose solution allowed us to analyze the complex composition. DNA/PEI/PEG-C ternary complexes contain a much greater amount of PEG-C than the DNA/PRT/PEG-C complex. The PEG-C content in the ternary complex was always close to the excess amount of cation over phosphate. The difference in the PEG-C content between the complexes with PRT and PEI should be attributed to the difference in the polycation content in the complexes.

The effect of the order of addition of PEI and PEG-C to DNA on the complex composition was examined, but no significant difference was observed between pre-addition and post-addition of PEG-C (data not shown). PEI seems to preferentially bind to the DNA molecule regardless of the presence of PEG-C. The DNA/polycation complex in a high-salt solution tends to stick together and often make a large aggregate, but the aggregation could be suppressed by PEG-C addition.[15] Thus, to prepare the suspension of the small complexes, PEG-C should be added to DNA prior to polycation addition.

PEG-AC did not completely decompose the DNA/PEI complex nor release free DNA molecules, but it obviously diminished the PEI content in the complex, while PEG-C did not decrease it. PEG-AC should have peeled a considerable amount of PEI from the preformed DNA/PEI complex. Godbey et al. reported that the ζ-potential of the DNA/PEI complex changed after centrifugation.[16] There should be some removable PEI loosely associated to the complex, and PEG-AC seems to remove it.

Both PEG-C and PEG-AC recharged the ζ-potential of the DNA/PEI complex. It indicates that PEG-C and PEG-AC were deposited onto the plasmid/PEI complexes, and the DNA//PEI/PEG-C (or PEG-AC) ternary complexes with negative surface charge were formed. As described before,[6] fluorescence microscopic observation revealed that PEG-C coating on the DNA/polycation complexes effectively protects them from unfavorable interactions with serum proteins. The slightly negative charge of the ternary complex would account for the protective effect. PEG-AC was also expected to show the protecting effect and was examined in the similar way.

DNA visualized by DAPI was mixed with PEI (P/N = 1:8). Binary complexes were observed as fluctuating small particles by a fluorescence microscopy with UV excitation (wavelength range: 330−385 nm). Rhodamine-labeled PEG-AC (Rh-PEG-AC) was then added at P/N/COOH = 1:8:15. Complexes became detectable also with green excitation for rhodamine (wavelength range: 510−550 nm), and red luminous particles appear at the same spot with the UV excitation. Addition of PEG-AC made no visible change in the size or movement of the complexes. When BSA was added to plasmid/PEI binary complexes without PEG-AC at a final concentration of 10 mg/mL, the complexes soon aggregated and precipitated. On the other hand, BSA did not induce aggregation of the DNA/PEI/Rh-PEG-AC ternary complexes. After 30 min incubation with BSA, the ternary complexes still kept Brownian motion as small particles, which could be observed with both UV and green excitation. Similar results were also obtained with rhodamine-labeled PEG-C. It indicates that the DNA complexes were stably covered with PEG-C or PEG-AC under the existence of the serum protein.

PEG-C and PEG-AC evidently enhanced the transcriptional activity of the plasmid/PEI complex. PEG-AC has a characteristic amphoteric structure like HMG proteins and showed higher enhancing effect than PEG-C. HMGs were reported to stimulate the transcription of genes through the specific interactions with histones or p53. However, PEG-AC should not have such specific interactions with those proteins. A possible mechanism of such a synthetic water-soluble polyampholyte to stimulate the transcription would be the loosening of the DNA complex through the nonspecific physical interactions.

PEG-AC has more carboxyls than amines and is thus overall negatively charged. It will deposit onto the positively charged DNA/polycation complex surfaces. Amphoteric PEG-AC could then electrostatically interact with both polycation and DNA and relax the binding between them. Double-stranded DNA is a semiflexible polymer and has a relatively long persistent length on the order of 50 nm (corresponding to ca. 170 base pairs), which is larger than the diameter of the single molecular complex of the plasmid. The DNA molecules in the complex should have high structural distortion in the folded or looping conformation. When the binding between DNA and PEI is relaxed by the polyampholyte, the DNA molecule would extend its segments to reduce the distortion and form a loosened structure. The charge imbalance of PEG-AC would also contribute to the loosening effect. Then, the tightly compacted DNA/PEI complex would be relaxed, and the approach of transcriptional factors would be facilitated. PEG-AC is the first synthetic transcription cofactor that stimulates the transcription of DNA complexes and would also be expected as an artificial model for HMG proteins to clarify their mechanisms of transcriptional stimulation.

The loosening effect of the PEG derivatives was then examined by fluorescence restoration. The YOYO-1 molecule intercalated into the DNA double-helix showed much higher fluorescence efficiency than that of free YOYO-1. The fluorescence intensity was strongly reduced when DNA formed a

CDV

small compacted complex with PEI and then restored by the addition of PEG derivatives (Figure 7), indicating that the plasmid/PEI complexes were effectively loosened by the PEGs. PEG-C and PEG-AC showed almost the same degree of fluorescence restoration, though PEG-AC has a quenching effect on the fluorescence of YOYO-1/DNA. The high loosening effect of the water-soluble polyampholyte was confirmed.

PEG-C and PEG-AC could obviously enhance the transfection efficiency. The higher luciferase activity obtained by the ternary complexes would be brought about by the finely dispersed suspension of the PEGs-coated complexes and the increased transcribing activity of the loosened complexes. Both PEG-C and PEG-AC have similar surface-recharging and suspension-stabilizing effects. PEG-AC exhibited a higher enhancing effect on the extragene expression than PEG-C. The superior improving effect of PEG-AC would, thus, be attributed to its higher transcription-enhancing ability. The magnitude of the enhancement in the luciferase expression was not so high as in the transcription study, probably due to the diminished interaction of the recharged anionic complex with negative cell surfaces. At high doses of PEG-C or PEG-AC, they would partly remain in the medium as uncomplexed polyions, which would competitively prevent the complex adhesion to the cells and result in the relatively reduced expression (Figure 8). The loss in the cell adhesion could be compensated by introducing the cell-adhering ligand into the polycation[17] or the polyaninon.[7]

A water-soluble polyampholyte, PEG-AC, would thus be expected as novel multifunctional gene transfection improver, which makes plasmid complexes stably suspended, protects them against the unfavorable interaction with serum components, and enhances their transcriptional and transfectional efficiency. It may introduce a new way to enhance the efficiency of polycation-mediated gene transfection simply by the addition of such an activating agent to the vectors.

Acknowledgment. The authors wish to thank Prof. Kenichi Yoshikawa (Kyoto University), Prof. Tatsuo Akitaya (Meijo University), and Dr. Kanta Tsumoto (Mie University) for their helpful suggestion in the transcription study. Prof. Kawakami, Dr. Okuda, and Prof. Hashida (Kyoto University) are acknowledged for their great help in ζ-potential measurements. NOF Corporation is appreciated for the synthesis and supply of poly(allyl glycidyl ether-*co*-ethylene oxide). This work was supported by Japan Society for the Promotion of Science (no. 00162090).

References and Notes

(1) Kabanov, A. V.; Kabanov, V. A. *Bioconjugate Chem.* **1995**, *1*, 7−20.
(2) Niidome, T.; Huang, L. *Gene Ther.* **2002**, *9*, 1647−1652.
(3) Ohana1, P.; Gofrit, O.; Ayesh1, S.; Al-Sharef, W.; Mizrahi, A.; Birman, T.; Schneider, T.; Matouk, I.; de Groot, N.; Tavdy, E.; Sidi, A. A.; Hochberg, A. *Gene Ther. Mol. Biol*. **2004**, *8*, 181−192.
(4) Nishikawa, M.; Huang, L. *Gene Ther*. **2001**, *8*, 861−870.
(5) Kimura, T.; Yamaoka, T.; Iwase, R.; Murakami, A. *Macromol. Biosci*. **2002**, *2*, 437−446.
(6) Koyama, Y.; Ito, T.; Matsumoto, H.; Tanioka, A.; Okuda, T.; Yamaura, N.; Aoyagi, H.; Niidome, T. *J. Biomater. Sci. Polym. Ed.* **2003**, *6*, 515−531.
(7) Koyama, Y.; Yamada, E.; Ito, T.; Mizutani, Y.; Yamaoka, T. *Macromol. Biosci*. **2002**, *2*, 251−256.
(8) Tremethick, D. J.; Molloy, P. L. *J. Biol. Chem.* **1986**, *261*, 6986−6992.
(9) Tsuda, K.; Kikuchi, M.; Mori, K.; Waga, S.; Yoshida, M. *Biochemistry* **1988**, *27*, 6159−6163.
(10) Aizawa, S.; Nishino, H.; Saito, K.; Kimura, K.; Shirakawa, H.; Yoshida, M. *Biochemistry* **1944**, *33*, 14690−14695.
(11) Ueda, T.; Chou, H.; Kawase, T.; Shirakawa, H.; Yoshida, M. *Biochemistry* **2004**, *43*, 9901−9908.
(12) Banerjee, S.; Kundu, T. K. *Nucleic Acids Res.* **2003**, *31*, 3236−3247.
(13) Koyama, Y.; Umehara, M.; Mizuno, A.; Itaba, M.; Yasukouchi, T.; Natsume, K.; Suginaka, A. *Bioconjugate Chem.* **1996**, *7*, 298−301.
(14) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297−7301.
(15) Maruyama, K.; Iwasaki, F.; Takizawa, T.; Yanagie, H.; Niidome, T.; Yamada, E.; Ito, T.; Koyama, Y. *Biomaterials* **2004**, *25*, 3267−3273.
(16) Godbey, W. T.; Wu, K. K.; Hirasaki, G. J.; Mikos, A. G. *Gene Ther.* **1999**, *6*, 1380−1388.
(17) Finsinger, D.; Remy, J. S.; Erbacher, P.; Koch, C.; Plank, C. *Gene Ther.* **2000**, *7*, 1183−1192.

BM0504633