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Review

Polyethylene Glycol-Conjugated Copolymers for Plasmid DNA Delivery

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Polymeric gene delivery systems have been developed as an alternative for viral gene delivery systems to overcome the problems in the use of viral gene carriers. Polymeric carriers have many advantages as gene carriers such as low cytotoxicity, low immunogenicity, moderate transfection efficiency, no size-limit, low cost, and reproducibility. In the efforts to develop safe and efficient polymeric gene carriers, polyethylene glycol (PEG) has widely been used because of its excellent characteristics. PEG-conjugated copolymers have advantages for gene delivery: 1) The PEG-conjugated copolymers show low cytotoxicity to cells *in vitro* and *in vivo*, 2) PEG increases water-solubility of the polymer/DNA complex, 3) PEG reduces the interaction of the polymer/DNA complex with serum proteins and increases circulation time of the complex, 4) PEG can be used as a spacer between a targeting ligand and a cationic polymer. A targeting ligand at the end of a PEG chain is not disturbed by the interaction of a cationic polymer with plasmid DNA, and the PEG spacer increases the accessibility of the ligand to its receptor. In this review, PEG copolymers as gene carriers are introduced, and their characteristics are discussed.

KEY WORDS: cationic polymer; gene delivery; gene therapy; polyethylene glycol; polymeric gene carriers.

INTRODUCTION

Gene therapy is a method to introduce genetic materials into cells for the production of therapeutic proteins or blocking the expression of harmful proteins. During the past decade, gene therapy technology progressed remarkably, and many clinical trials have been reported (1). Gene therapy research is divided into two major research fields. One is to develop a therapeutic gene. This research includes development of an effective therapeutic gene to a specific disease and a tissue specific or regulated gene expression system. The other is to develop an efficient and safe delivery system. Gene delivery systems include viral vectors and nonviral vectors. Nonviral vectors include liposomes, polymers, and naked DNA. Nonviral vectors have many advantages over viral gene vectors, although viral vectors are currently the most efficient way to deliver genes to cells. Unlike viral vectors, nonviral vectors have low cytotoxicity, low immunogenecity, no size limit, low cost, and reproducibility (2).

The polymeric gene carrier was first introduced in late 1980s, when poly-L-lysine (PLL)-asialoorosomucoid was synthesized for liver-targeting gene delivery (3–5). The main requirements of polymeric gene carriers are 1) safety with low cytotoxicity and low immunogenecity, 2) high gene delivery efficiency, and 3) specificity for delivery of genes to target organs. Until now, many polymeric gene carriers have been developed to meet the requirements of gene delivery to humans. These polymeric carriers were able to condense and protect plasmid DNA, resulting in the enhancement of gene delivery efficiency. However, there still remain drawbacks such as low biocompatibility and transfection efficiency. In the efforts to overcome these drawbacks, cationic polymers such as PLL and poly(ethylenimine) (PEI) have been modified with polyethylene glycol (PEG). PEG is one of the most widely used biocompatible polymers in drug delivery (6). PEG has also widely been used in the polymeric gene carriers because of its excellent characteristics. First, it reduces the cytotoxicity of the polymer/DNA complex. Second, PEG increases the water-solubility of the DNA/polymer complex. Third, PEG shields excess positive charges of polymer/DNA complex, resulting in the reduction of interaction between the polymer/DNA complex and blood components. Fourth, PEG can be used as a spacer between a targeting ligand and a polymeric carrier, which facilitates the access of the ligand to its receptor. In this review, we explore the current progress of PEG-conjugated copolymers as gene carriers. PEG-PLL and PEG-PEI copolymers are introduced, and their characteristics as gene carriers are discussed. In addition, targeting gene carriers are introduced as examples of gene carriers with PEG spacers.

CHARACTERISTICS OF POLYMERIC GENE CARRIERS

Most polymeric gene carriers have positive charges at their amine groups. The positive charges of the carriers interact with negative charges of phosphate groups in plasmid DNA, resulting in condensation of plasmid DNA. The poly-

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mer/plasmid DNA complexes are usually prepared in the presence of an excess amount of cationic polymer, and the complex has a net positive charge. The positively charged complexes interact with negatively charged cell membranes, facilitating cellular uptake of the polymer/plasmid DNA complexes via endocytosis. The polymer/plasmid DNA complexes have small particle size of around 100 nm. Although there is no size limit for the transfection, it was suggested that polymer/plasmid DNA complex with diameters of around 100 nm corresponds to the diameter of the coated pits in endocytosis (7). The size of the complex is dependent on the ratio and concentration of the polymer/plasmid DNA complex. When the ratio of polymer and plasmid DNA is not enough for condensation, the complex size is usually more than 300 nm. The increase of the polymer/plasmid DNA ratio reduces the size of the complex. The concentration is another factor for the complex size. The complex has a tendency to aggregate at a high concentration (8).

Degradation of DNA by nucleases is a problem for gene therapy. Degradation of plasmid DNA by nucleases results in loss of gene expression. Therefore, polymer carriers must protect plasmid DNA from nucleases. Condensation of plasmid DNA by polymeric carriers prevents the access of nucleases to plasmid DNA, improving resistance of plasmid DNA against enzymatic degradation.

PLL and PEI have widely been investigated for polymeric gene delivery. PLL has positive charges at ε-amine groups. PEI has 25% primary, 50% secondary, and 25% tertiary amine groups. PEI has higher transfection efficiency than PLL, due to the proton buffering effect. The high charge density of PLL and PEI contributes to complex formation and high transfection efficiency. However, the high charge density is closely related to cytotoxicity (9). Therefore, shielding of the surface charge is required to reduce cytotoxicity. Many kinds of PLL and PEI copolymers have been investigated for high transfection efficiency and low cytotoxicity.

PEG-PLL COPOLYMERS

It is generally accepted that the positive charge of the polymer/DNA complex facilitates cellular uptake of the complex, as the positive charge of the complex interacts with negatively charged cell membranes. However, the positive charge may also induce cytotoxicity depending on the charge density and shape of the complex. In addition, the polymer/DNA complex is rapidly cleared after intravenous injection due to the positive surface charge of the delivery system (10). Positively charged complexes bind to proteins in the blood and form aggregates, resulting in rapid clearance and reduction of delivery efficiency (11,12). Water solubility is another obstacle in polymeric gene delivery, as the polymer/DNA complex has poor water-solubility at charge neutralized condition (13). To overcome these drawbacks of cationic polymer carriers, PEG has been conjugated to the polymers. PEG-PLL copolymers for gene delivery are summarized in Table I.

PEG-PLL Block Copolymer

An A-B type cationic-hydrophilic block copolymer was synthesized to introduce a protective surface for hydrophilic PEG shielding of the complex (14). The block copolymer of PEG-PLL was synthesized by polymerization of the *N*-carboxyanhydride (NCA) of the z-protected L-lysine, using α-methoxy-ω-amino-PEG as an initiator. The size of the PEG-PLL/plasmid DNA complex was more than 100 nm, showing an extended shape compared to the PLL/plasmid DNA complex. In gel retardation assay, the PEG-PLL/plasmid DNA complex was completely retarded at a 2:1 charge ratio (±). The surface charge of the PEG-PLL/plasmid DNA complex measured by zeta potential is decreased compared to that of the PLL/plasmid DNA complex. The cytotoxicity of the PEG-PLL/plasmid DNA complex was decreased significantly due to the reduced surface charge. In addition, the PEG-PLL showed significant levels of transfection into human embryonic kidney 293 cells.

PEG-PLL block copolymer was further studied by Kataoka *et al.* (15–18). In the study, plasmid DNA and PEG-PLL formed polyion complex (PIC) micelles, in which PLL and plasmid DNA formed a hydrophobic core with the PEG shell. Dynamic light scattering showed that the PIC micelle had a size of 48.5 nm in diameter with a small fraction of secondary aggregates in the 140-nm region. The smaller size of the complex compared to the previous report may be due to differences in chain length of PLL. It was suggested that PEG-PLL, which had a longer PLL chain, had a tendency to form a larger complex with DNA (15). This PIC micelle stabilized plasmid DNA in the hydrophobic core preventing the access of nuclease or solvent. Therefore, PEG-PLL protected plasmid DNA efficiently from DNase I more than 60 min (15–17). In addition, the PIC micelles with PEG-PLL did not precipitate at various salt concentrations. The PIC micelles showed the highest transfection efficiency to human hepatoma HepG2 cells at a 4:1 charge ratio, which was higher than that of PLL of the same molecular weight (18). *In vivo* delivery of a reporter plasmid complexed with PEG-PLL was also evaluated after intravenous injection (18). Southern blotting

Table I. PEG-PLL Copolymers

Gene carrier	Size of PEG (Da)	Optimized polymer/DNA ratio	Cytotoxicity	Reference
PEG-PLL block copolymer				
PEG-PLL diblock copolymer	4300	4:1 charge ratio	Lower than PLL	14–18
PEG-PLL dendrimer	5757	N/A	N/A	19
PLL dendrimer-PEG-PLL dendrimer	3350	N/A	Nontoxic	20
PEG-PLL multiblock copolymer	1450	15:1 N/P ratio	Nontoxic	21
PEG grafted PLL				
PEG-*g*-PLL	550	3:1 weight ratio	Nontoxic	22, 23
	5000	N/A	Similar to PLL	24

N/A, not available.

assay showed that naked plasmid DNA was degraded in the blood within 5 min after intravenous injection. On the contrary, when plasmid DNA was transferred in the form of PIC micelles with PEG-PLL, the supercoiled DNA was detected in the blood for 30 min, suggesting longer circulation time than naked DNA. The highest gene expression in the liver was obtained at a 4:1 charge ratio, and the gene expression persisted for more than 10 days (18).

Another type of PEG-PLL block copolymer was synthesized by Choi *et al.* (19). Methoxy PEG-*block*-PLL dendrimer (PEG-PLLD) was synthesized by the liquid phase peptide synthesis method. Physical characterization of the block copolymer showed that the PEG-PLLD formed a stable spherical polymer/DNA complex at a 2:1 charge ratio. The water-solubility of the PEG-PLLD/DNA complex was higher than the standard PLL/DNA complex due to the effect of PEG. The PEG-PLLD was further developed as a barbell-like PLL dendrimer-*block*-PEG-*block*-PLL dendrimer (PLLD-PEG-PLLD) (20). The fourth generation of PLLD-PEG-PLLD triblock copolymer formed a spherical complex with a particle size of 50–150 nm. The transfection efficiency of the diblock or triblock copolymer is not available. However, MTT assay showed that the cytotoxicity of the PLLD-PEG-PLLD triblock copolymer was negligible.

Recently, PLL-PEG multiblock copolymer with ester bonds between PEG and PLL was synthesized (21). The multiblock copolymer was composed of low-molecular-weight PLL (3000 Da) and PEG (1450 Da). After degradation, the multiblock copolymer produced nontoxic low-molecular-weight PLL and PEG. The multiblock copolymer showed exponential degradation with a half-life of approximately 5 h in PBS. However, in the complex with plasmid DNA, the polymer showed complete stability up to 6 days despite the short polymer half-life. To increase the endosomal escape rate, histidine was grafted into the multiblock copolymer. The histidine conjugated-multiblock copolymer showed a significant buffering effect, which facilitates endosomal escape of the complex. Due to the low cytotoxicity of the degradable PEG-PLL and the proton buffering effect of the histidine moiety, the histidine conjugated-multiblock copolymer showed higher transfection efficiency and lower cytotoxicity than PLL. This strategy is effective in that it has characteristics of a high-molecular-weight PLL in transfection and characteristics of a low-molecular-weight PLL in cytotoxicity. In addition, PEG moiety stabilizes the polymer/plasmid DNA complex and increases solubility and circulation time in *in vivo* application.

PEG Grafted PLL

Comb-shaped PEG grafted PLL (PEG-*g*-PLL) was first synthesized with different PEG-grafted ratios (PEG: 550; PLL: 25,000) by Choi *et al.* (22) (Fig. 1). PEG-*g*-PLL formed a complex with plasmid DNA at or above a 1:1 weight ratio. Above a 1:3 weight ratio, the PEG-*g*-PLL/plasmid DNA complex showed maximum condensation with little difference from the PLL/plasmid DNA complex. DNase I protection assay showed that 10 mol% PEG-*g*-PLL protected plasmid DNA completely for more than 60 min, whereas the plasmid DNA in the complexes with 15 or 20 mol% PEG-*g*-PLL was degraded partially (23). This suggests that lower mol% PEG-*g*-PLL forms a tighter complex than higher mol% PEG-*g*-PLL. PEG-*g*-PLL with 10 mol% PEG showed the highest

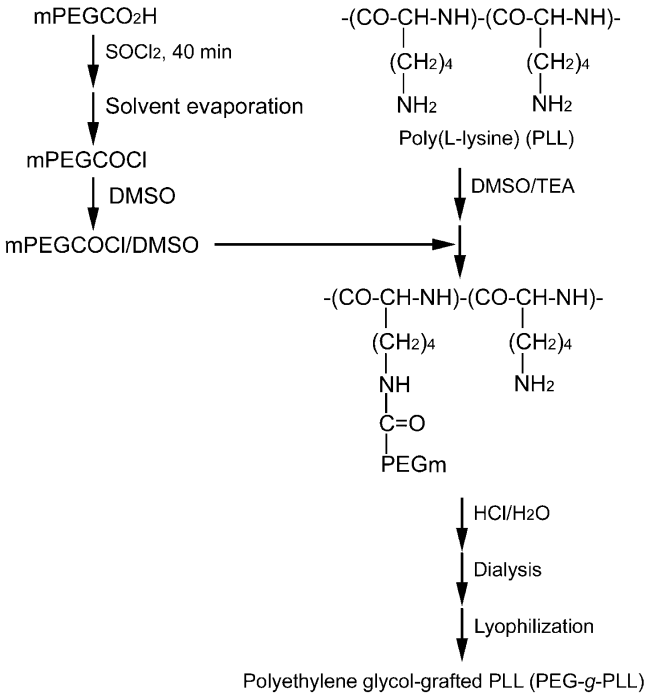

Fig. 1. Synthesis of PEG-*g*-PLL. (Reprinted with permission from Ref. 22.)

transfection efficiency (Fig. 2). In addition, PEG-*g*-PLL had negligible toxicity to HepG2 cells (Fig. 3). PEG-*g*-PLL was further studied with a different molecular weight of PLL and PEG (24). PEG-*g*-PLLs were synthesized with two molecular PLL weights (9.6 and 22.4 kDa) and two molecular PEG weights (5 and 12 kDa) by Toncheva *et al.* (25). The degrees of substitution were 5 and 10 mol%. In this research, the size of PEG was higher than the previous research, but the same results were obtained in *in vitro* characterization and transfection studies. PEG-*g*-PLL formed a complex with a diam-

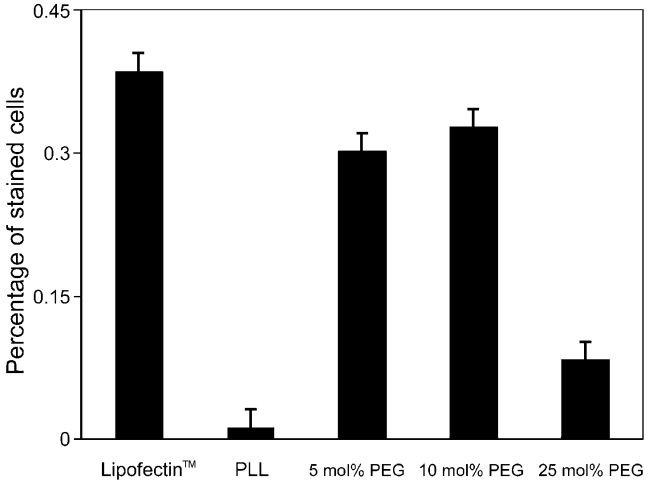

Fig. 2. Effect of PEG contents in PLL on transfection efficiency to HepG2 cells. Various polymer/DNA complexes were formulated at a 3:1 weight ratio (polymer/pSV-β-gal). The complexes were transfected into HepG2 cells. The transfected cells were stained with X-gal, and the number of stained cells was counted. Among tested PEG-*g*-PLL, 10 mol% PEG-*g*-PLL showed the highest transfection efficiency. (Reprinted with permission from Ref. 22.)

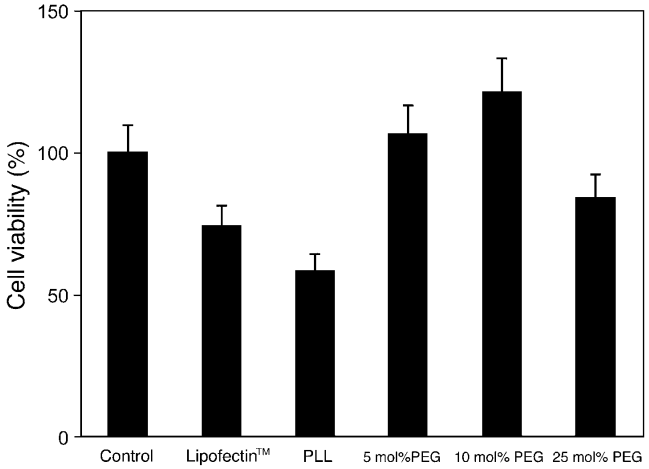

Fig. 3. Cytotoxicity of PEG-*g*-PLL to HepG2 cells. Polymer (30 μg/ml in MEM medium) was incubated on HepG2 cells for 4 h. After incubation, cell viability was measured by MTT assay. PEG-*g*-PLL had negligible toxicity to HepG2 cells, whereas other control carriers showed significant toxicity. (Reprinted with permission from Ref. 22.)

eter ranging from 55 to 140 nm. Zeta potential showed that the positive surface charge of the complex was decreased, suggesting shielding effects of PEG. Furthermore, the solubility of the complex with PEG-*g*-PLL increased compared to the same molecular PLL weight. The transfection efficiency of 10 mol% PEG-*g*-PLL was higher than that of PLL, irrespective of the size of PEG.

PEG-*g*-PLL was evaluated *in vivo* as a carrier of the anti-sense glutamic acid decarboxylase (GAD) plasmid to the pancreas for the prevention of type 1 diabetes (23). GAD is an autoantigen, and it was previously reported that complete suppression of GAD expression prevented development of type 1 diabetes in the transgenic mice (25). To suppress the GAD expression in pancreas, the PEG-*g*-PLL/anti-sense GAD plasmid was injected intravenously via tail vein. Reverse transcription-polymerase chain reaction (RT-PCR) with the RNA from the pancreas proved that the anti-sense GAD plasmid was delivered to the pancreas and expressed the anti-sense mRNA, although this PEG-*g*-PLL did not have any targeting ligand to the pancreas. This suggests that pancreas targeting delivery may be further improved using a targeting ligand conjugated PEG-*g*-PLL.

PEG-PEI COPOLYMERS

PEI has been known as an efficient gene carrier due to its high charge density and endosomal disruption function. PEI has been used for plasmid DNA delivery into a variety of cells *in vitro* and *in vivo* (26–29). However, the application of PEI to clinical settings is limited because of its high cytotoxicity (26). PEI has linear and branched forms, depending on the molecular structure. PEG-PEI copolymers for gene delivery are summarized in Table II. The effect of PEGylation to branched PEI (BPEI) was investigated by Petersen *et al.* (30). PEG-grafted-BPEI (PEG-*g*-BPEI) was synthesized with various molecular weight PEGs (550 Da, 5 kDa, and 20 kDa) and a BPEI (25 kDa). The PEG-*g*-BPEI formed a complex with plasmid DNA with a 100-nm diameter. The diameter of the complex had a tendency to increase with incubation time when BPEI or PEG550-*g*-BPEI was used. However, PEG5k-*g*-BPEI and PEG20k-*g*-BPEI formed stable complexes with plasmid DNA, and the size did not change with time. In addition, PEG20k-*g*-BPEI formed a small complex 50 nm in size, but PEG550-*g*-BPEI resulted in large and diffuse structures with a 130-nm diameter. The PEG content in PEG-*g*-BPEI also has a significant influence to the size and morphology of the polymer/plasmid DNA complex. As the PEG grafting ratios increase, the ability to make the complex was decreased. In a gel retardation assay, the plasmid DNA was completely retarded by all the PEG-*g*-BPEI at a 2:1 N/P (nitrogen atom in polymer)/(phosphate group in DNA) ratio, whereas BPEI retarded the plasmid DNA at a 1.6:1 N/P ratio. More amounts of PEG-*g*-BPEI are required for complete condensation with plasmid DNA than BPEI because of the shielding effect of PEG. Transfection efficiency of PEG-*g*-BPEI was comparable to that of BPEI. However, the cytotoxicity of the PEG-*g*-BPEI/DNA complex was significantly reduced. PEG-*g*-BPEI was evaluated *in vivo* for gene delivery to the spinal cord (31). In the study, prolonged gene expression in the spinal cord was achieved by repeated intrathecal administration of the PEG-*g*-BPEI/DNA complex. The repeated administration of the BPEI/DNA complex showed 70% attenuation of gene expression after the second injection at a 2-week interval. However, the PEG-*g*-BPEI/DNA complex did not have an attenuation effect of gene expression. It was suggested that direct toxic effects of the BPEI/DNA complexes may be responsible for apoptosis and gene expression attenuation.

Star-shaped PEG-block-BPEI (star-shaped PEG-BPEI) was synthesized by Petersen *et al.* (32). Low-molecular-weight (LMW) BPEI formed a large complex with a particle size of more than 500 nm. In addition, LMW BPEI did not protect plasmid DNA completely from nucleases, due to the insufficient condensation. Interestingly, the star-shaped PEG10k-BPEI800/plasmid DNA complex had a smaller size than the BPEI800/plasmid DNA complex, which was comparable to the size of the BPEI25k/plasmid DNA complex. Due to the effect of PEG, the star-shaped PEG-BPEI/plasmid DNA complex had a lower surface charge. Therefore, with the enhanced condensation ability, the star-shaped PEG-BPEI may

Table II. PEG-PEI Copolymers

Polymer	Size of PEG (Da)	Optimized polymer/DNA ratio	Cytotoxicity	Reference
PEG-*g*-BPEI	550/5000	50/1 N/P ratio	Lower than BPEI	30, 31
Star-shaped PEG-BPEI diblock copolymer	10,000/15,000	N/A	N/A	32
PEG-LPEI diblock copolymer	2000	12/1 N/P ratio	Lower than LPEI	33
PEG-BPEI biodegradable copolymer	2000	4/1 N/P ratio	Lower than BPEI	34

N/A, not available.

have improved transfection efficiency and lower cytotoxicity compared to LMW BPEI.

PEG-*block*-LPEI (PEG-LPEI) was also synthesized with PEG2k and LPEI22k and evaluated as a gene carrier (33). PEG-LPEI formed a stable complex and completely retarded plasmid DNA at a 3/1 N/P ratio. However, PEG-LPEI did not protect plasmid DNA from DNase I. It is notable that LPEI itself has a poor ability to protect plasmid DNA from nuclease. The solubility of the complex in water was much improved. The LPEI/DNA complex precipitates at 0.5 mg/ml DNA concentration. However, the PEG-LPEI/DNA complex did not show any precipitation even at a 1.5 mg/ml solution. Transfection efficiency of PEG-LPEI was lower than LPEI. However, PEG-LPEI had much lower cytotoxicity than LPEI. This PEG-LPEI system was used to deliver DNA to the nasal tissue (33). The maximum gene expression was detected at 24 h after delivery, and the expression level was decreased thereafter.

Recently, biodegradable PEG-BPEI copolymer was synthesized using difunctional PEG (SS-PEG) and low-molecular-weight BPEI (1.8 kDa) (34). The reaction between difunctional PEG and BPEI generally produces a water insoluble cross-linked copolymer. However, careful controls of reaction conditions made it a water-soluble copolymer. This copolymer formed a complex with DNA at a 4:1 N/P ratio, and the particle size was around 150 nm. The transfection efficiency of the copolymer was higher than BPEI1.8k. The cytotoxicity of the copolymer was lower than BPEI25k, as it was degraded into nontoxic low-molecular-weight BPEI and PEG. Degradation rate of the biodegradable PEG-BPEI is not available. However, this study suggests that the degradation rate of the copolymer may be controlled by the synthesis of the copolymers with different biodegradable linkage groups. Degradation rate of the degradable copolymer may be optimized by this approach.

PEG-COATED CATIONIC POLYMER/ DNA COMPLEX

Another strategy to shield positive surface charge is coating the polymer/plasmid DNA complex with PEG after complex formation. Kircheis *et al.* designed the PEG-coated transferrin (Tf)-BPEI/DNA complex (35,36). PEG coating of the complex was performed via the primary amino groups in the PEI molecules by the reaction with methoxy-succimidyl-propionat-PEG. This strategy allows more efficient condensation of plasmid DNA with cationic polymer than PEG-conjugated polymers. In addition, the complexes have advantages of PEG by PEG coating after complexation. The PEG-coated Tf-BPEI/DNA complex was injected intratumorally, and gene expression in neuroblastoma Neuro2a tumor was evaluated. The gene expression in the tumor by the PEG-coated complex was substantially higher than the Tf-BPEI/plasmid DNA complex. In addition, systemic application by intravenous injection showed that most of the gene expression was localized in the tumor with low levels of expression in the kidney, while the BPEI/plasmid DNA complex showed major gene expression in the lung. This suggests that the surface coated complex has long circulation time and was not trapped in the lung. Similar results were confirmed by Rudolph *et al.* (37). In this research, the PEG-coated BPEI/plasmid DNA complex did not accumulate in the lung, unlike the BPEI/DNA complex. Ogris *et al.* demonstrated that the PEGylated Tf-BPEI/DNA complex reduced interaction with blood components and extended circulation in the blood (11). With PEG coating, the mean complex size did not increase in the presence of serum, whereas the size of the complex without PEG increased up to 700 nm. The PEG-coated polycation/plasmid DNA complex was recently systematically characterized (38). The PEG-coated PLL (29k or 205k) or PEI (25k or 800k)/DNA complexes showed around 100 nm of particle size in hepes buffer or 0.15 M NaCl solution. Without PEG coating, all the polymers aggregated and had much larger complex sizes in 0.15 M NaCl. The *in vivo* administration showed that the PEG-coated polycation/plasmid DNA complexes were localized in the liver after intravenous injection. In addition, the PEG-coated PLL (205 kDa)/plasmid DNA circulates significantly better at higher doses, suggesting that the elimination mechanism may be saturable.

PEG AS A SPACER IN TARGETING GENE CARRIERS

PEG has widely been used as a spacer between a targeting ligand and a cationic polymer (Table III). The shielding effect of PEG is able to decrease nonspecific interaction with negatively charged cellular membranes, which results in the reduction of nonspecific cellular uptake. In addition, hydrophilic PEG presents the targeting moiety on the surface of the complex, and the targeting ligand may behave as a free molecule due to the highly flexible PEG chain. Therefore, using PEG as a spacer in targeting gene carriers is a useful technique for the enhanced efficiency and specificity of targeting gene delivery. The size or conjugation ratio of PEG in a targeting carrier has significant effect on the transfection and targeting efficiency. Therefore, the size and conjugation ratio of PEG in a targeting carrier with a PEG spacer are optimized as in the following examples.

BPEI-*g*-PEG-RGD

An angiogenic endothelial cell targeted gene carrier was synthesized by conjugating RGD peptide to BPEI via a PEG spacer (Fig. 4) (39). RGD peptide is a αvβ3/ αvβ5 integrin-binding peptide, which has a sequence ACDCRGDCFC. The αvβ3 and αvβ5 integrins are overexpressed on angiogenic endothelial cells within tumors (40–43). Therefore, the localized expression of the αvβ3 and αvβ5 integrins enables targeting gene delivery by using a RGD peptide. The binding affinity of BPEI-*g*-PEG-RGD to the αvβ3/αvβ5 integrin was evaluated by a cell attachment inhibition study. The results showed that BPEI-*g*-1PEG-RGD had binding affinity comparable to the RGD peptide. As PEI was more substituted

Table III. PEG-Conjugated Copolymers for Targeting Gene Delivery

Gene carrier	Target cells	Reference
BPEI-*g*-PEG-RGD	Angiogenic endothelial cells	39
Lactose-PEG-*g*-PLL	Hepatocytes	49, 50
Galactose-PEG-*g*-BPEI	Hepatocytes	52
AWBP-PEG-*g*-PLL	Artery wall cells	56
Folate-PEG-*g*-PLL	Cancer cells	61
Folate-PEG-folate-*g*-BPEI	Cancer cells	62, 63

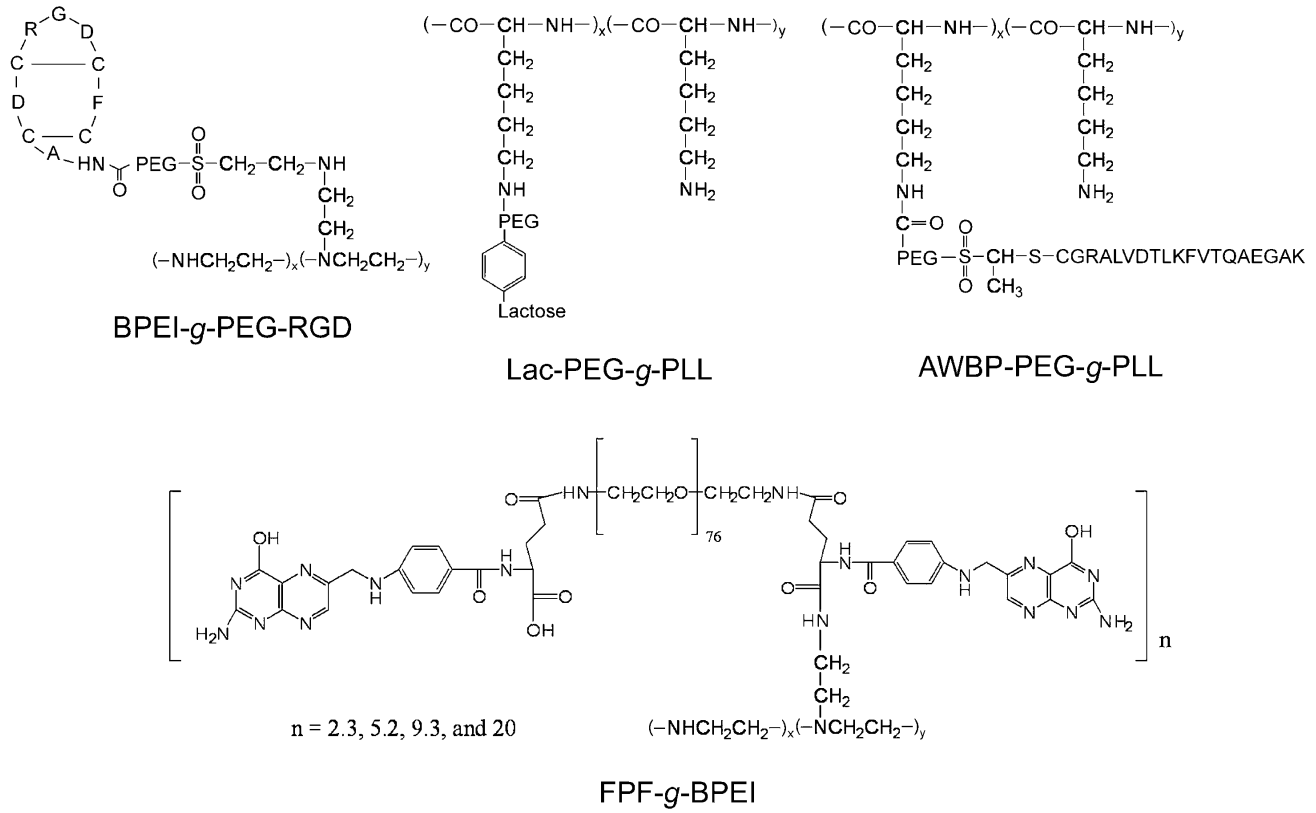

Fig. 4. Examples of targeting gene carriers with a PEG spacer.

with PEG-RGD, the binding affinity decreased. For example, BPEI-*g*-20PEG-RGD had no affinity for the integrins. It was suggested that the reduced affinity for the integrins may be due to the micelle-like structure of BPEI-*g*-20PEG-RGD in aqueous solution. The hydrophobic RGD peptides may be buried inside and surrounded by hydrophilic BPEI-PEG. The BPEI-*g*-1PEG-RGD/plasmid DNA complex had lower cytotoxicity and higher water-solubility than the BPEI/plasmid DNA complex. In addition, transfection efficiency to the vascular endothelial growth factor (VEGF)-induced angiogenic human dermal microvascular endothelial cells (HDMEC) was approximately five times higher than that of BPEI (Fig. 5). On the contrary, the transfection efficiency to the angiostatic HDMEC was much lower than BPEI. Therefore, BPEI-*g*-PEG-RGD is a highly specific carrier to angiogenic cells and may be a useful antiangiogenic gene therapy. For the synthesis of BPEI-*g*-PEG-RGD, various molecular weight PEGs (1, 5, 9, and 20 kDa) were used. It is also interesting to note that PEG1k showed the highest targeting efficiency over other molecular weight PEG.

The effect of PEG as a spacer is different, depending on a targeting ligand. For αvβ3 and αvβ5 integrins' targeting gene delivery, the smaller RGD peptide, RGDC, was coupled to BPEI with or without a PEG spacer (44). In the case of the smaller RGD peptide, RGD-BPEI showed 50 times higher transgene expression than PEI to Mewo cells, which expressed high level of RGD-sensitive receptors. However, the PEG spacer with the short RGDC peptide was not effective for targeting gene delivery, and RGDC-PEG-*g*-BPEI did not show any targeting effect. It suggests that the short RGDC peptide may be hidden by flexible PEG corona.

Lactose-PEG-*g*-PLL or Galactose-PEG-*g*-BPEI

Galactose or lactose is a ligand for the asialoglycoprotein receptor, and therefore it has been used for hepatocytes targeting gene delivery (45–48). The asialoglycoprotein receptor

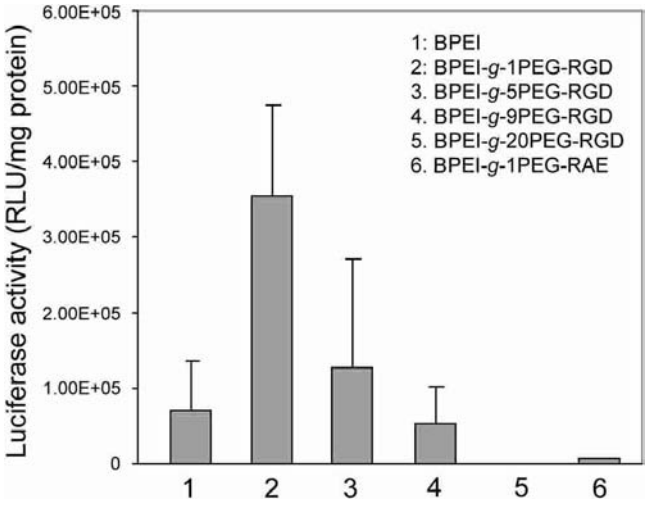

Fig. 5. BPEI-*g*-PEG-RGD mediated gene transfer to angiogenic HDMEC. Various BPEI-*g*-PEG-RGD/plasmid DNA complexes were prepared and transfected into angiogenic HDMEC. The transgene expression was measured by luciferase assay. BPEI-*g*-1PEG-RGD conjugates increased the luciferase expression in angiogenic HDMEC, compared to unmodified BPEI. The replacement of the essential integrin-binding motif, RGD, into RAE decreased the transfection efficiency. (Reprinted with permission from Ref. 39.)

is abundant in the membrane of hepatocytes. To use galactose or lactose as a targeting ligand, lactose-PEG-*g*-PLL (Lac-PEG-*g*-PLL) (Fig. 4) was synthesized with various lactose conjugation ratios: 6, 12, 22, and 30 mol% (49,50). The direct conjugation of saccharides to cationic polymers was unable to prevent aggregation of the complex in physiologic conditions (51). However, the Lac-PEG-*g*-PLL/plasmid DNA complex was highly water-soluble due to the hydrophilicity of PEG. The transfection efficiency of 30 mol% Lac-PEG-*g*-PLL was higher than the other mol% Lac-PEG-*g*-PLLs. In addition, the transfection efficiency of Lac-PEG-*g*-PLL to hepatocytes was 10 times higher than that of PLL (Fig. 6). Also, cytotoxicity of the complex was lower compared to the PLL/plasmid DNA complex. In a similar strategy, galactose was conjugated to BPEI using PEG as a spacer (52). In the case of direct conjugation of galactose to BPEI, increasing the number of galactose units of cationic polymers resulted in reduced binding to plasmid DNA. It suggests that sugar moieties may interfere with condensation of plasmid with a cationic polymer (47). However, in galactose-PEG-*g*-BPEI (Gal-PEG-*g*-BPEI) with a PEG spacer, the distances between sugar moieties increased, resulting in enhancement of the affinity for asialoglycoprotein receptors. In addition, the condensation ability was also improved in Gal-PEG-*g*-BPEI. Gal-PEG-*g*-BPEI had higher transfection efficiency than BPEI in HepG2 cells, whereas it had lower transfection efficiency in mouse fibroblast NIH3T3 cells, due to the lack of receptors in NIH3T3 cells. *In vivo* administration with the Gal-PEG-*g*-BPEI/plasmid DNA complex showed that the reporter gene expression was localized mainly in the liver, suggesting the targeting effect of the carrier.

Artery Wall Binding Peptide (AWBP)-PEG-*g*-PLL

Low-density lipoprotein (LDL) binds to various types of cells such as vascular endothelial cells, vascular smooth muscle cells, hepatocytes, and macrophages and can be taken up by the cells via receptor mediated endocytosis (53–55). Apolipoprotein B-100 (apo B-100), a major protein component of LDL, contains receptor-binding domains, including LDL receptor-binding domains, artery wall-binding domains, and heparin-binding domains. It was previously reported that a peptide containing 1000–1016 amino acids of apo B-100 is the artery wall-binding domain (54). For targeting the polymer/plasmid DNA complex to the arterial wall cells, the artery wall-binding domain of apo B-100 was chemically synthesized and introduced to the end of PEG-*g*-PLL (Fig. 4) (56). Artery wall-binding peptide (AWBP)-PEG-*g*-PLL condensed plasmid DNA and formed a spherical shape complex with a size of 100 nm. In gene expression studies, the transfection efficiency of the AWBP-PEG-*g*-PLL/plasmid DNA complex to bovine aorta endothelial cells and smooth muscle cells was 150–180 times higher than that of PLL or PEG-*g*-PLL (Fig. 7). In addition, free AWBP decreased the transfection efficiency of the AWBP-PEG-*g*-PLL/DNA complex in the competition study. These results suggest that AWBP-PEG-*g*-PLL functions as a targeted gene carrier to arterial wall cells via receptor-mediated endocytosis.

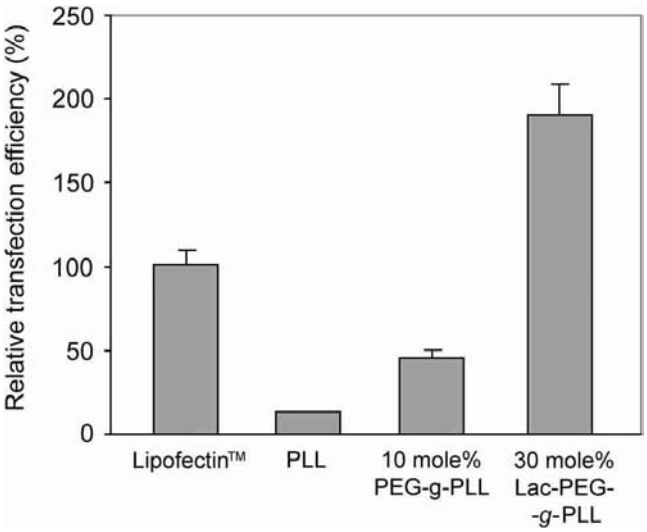

Fig. 6. Transfection efficiency of Lac-PEG-*g*-PLL in HepG2 cells. Various polymer/DNA complexes were prepared at a 3:1 weight ratio (polymer/pSV-β-gal). The complexes were transfected into HepG2 cells. The transgene expression was measured by β-galactosidase assay. Lac-PEG-*g*-PLL showed higher efficiency in HepG2 cells than in lipofectin, PLL, and PEG-*g*-PLL. (Reprinted with permission from Ref. 49.)

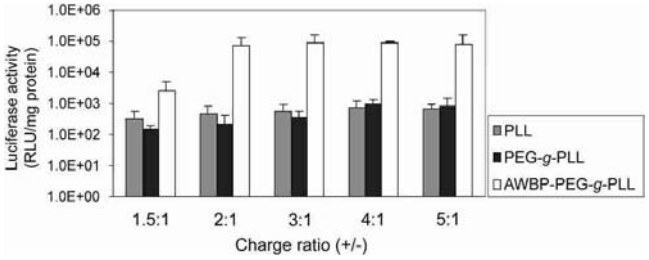

Fig. 7. AWBP-PEG-*g*-PLL mediated gene transfer to bovine aorta endothelial cells. Polymer/DNA complexes were prepared at various weight ratios. The complexes were transfected into bovine aorta endothelial cells. The transgene expression was measured by luciferase assay. AWBP-PEG-*g*-PLL increased transgene expression in bovine aorta endothelial cells. (Reprinted with permission from Ref. 56.)

Folate-PEG-*g*-PLL and Folate-PEG-Folate-*g*-BPEI

Folate receptors are overexpressed in a range of cancers. Previously, it was shown that folate-protein conjugates were delivered into various cancer cells (57). Therefore, antineoplastic drugs and nucleic acids were delivered to cancer cells using folate-mediated targeting (58). For cancer targeting gene delivery, the folate-PLL conjugate was evaluated as a gene carrier by *in vitro* transfection into cancer cells (59). The results showed that the folate-PLL/plasmid DNA complex was internalized into cells using a specific receptor and that folate-PLL had higher transfection efficiency in cancer cells than PLL. However, *in vivo* evaluation of folate-PLL showed that the folate-PLL/plasmid DNA complex interacted with serum protein and aggregated in the blood stream (60). To improve the physicochemical characteristics, folate-PEG-*g*-PLL was synthesized with different sized PEGs (61). In a transgene expression study, PEG 3400 was the most favorable spacer. Transfection efficiency of folate-PEG-*g*-PLL did not change in 10% serum-supplemented media and cell viability remained higher than 85%. *In vivo* evaluation showed that blood circulation time of the folate-PEG-*g*-PLL/plasmid DNA complex increased. Interestingly, folate-PEG-PLL in-

creased the gene expression level compared to folate-PLL, although the amount of the folate-PEG-*g*-PLL/plasmid DNA complex internalized into cells was almost the same as that of the folate-PLL/plasmid DNA complex. It was previously suggested that PEG has a dehydrating fusogenic effect when applied at a high local concentration (14). Therefore, this enhanced gene expression may be due to an ability of the folate-targeted vector to escape from endosomal/lysosomal compartments.

In another study, folate-PEG-folate grafted BPEI (FPF-*g*-BPEI) was synthesized (Fig. 4) (62,63). Folic acid was linked to both ends of PEG, and then folate-PEG-folate (FPF) was grafted into BPEI. FPF-*g*-BPEI was synthesized with various FPF conjugation ratios (FPF-2.3*g*-BPEI, FPF-5.2*g*-BPEI, FPF-9.3*g*-BPEI, and FPF-20*g*-BPEI). The size of the complex increased as the FPF conjugation ratio became higher. This result may be due to the steric hindrance and the charge shielding effect of PEG, suggesting that the conjugation ratio should be optimized for the complex stability. Transfection assay of CT26 colon cancer cells showed that FPF-5.2*g*-BPEI had the highest transfection activity (Fig. 8). Also, the cytotoxicity of the polymer was much improved, and FPF-5.2*g*-BPEI had the lowest toxicity than other FPF-*g*-BPEI.

CONCLUSIONS

The polymeric carriers have many advantages as gene carriers. However, the drawbacks such as cytotoxicity, low water solubility and rapid clearance of the polymer/DNA complex in systemic circulation have limited the application of the polymeric carriers to clinical settings. As an effort to overcome these problems, PEG-copolymers have extensively been studied. As described above, the advantages of PEG conjugation have been proved in *in vitro* and *in vivo* studies. Many obstacles of the polymeric carriers could be overcome with PEG technology. In addition to biocompatibility and high water solubility, PEG has been used as a linker in targeting carriers. The hydrophilicity and flexibility of PEG promotes the availability of the ligand on the surface of the polymer/DNA complex. With all these advantages of PEG, PEG copolymers are promising gene carriers in the field of nonviral gene delivery.

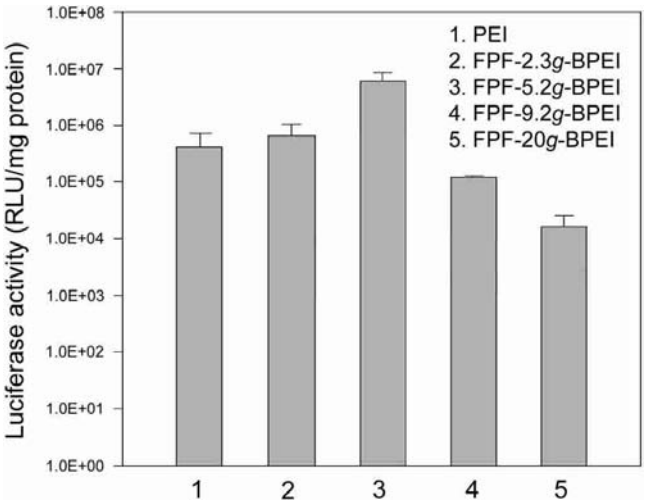

Fig. 8. Transfection efficiency of FPF-*g*-BPEI to CT-26 colon adenocarcinoma cells. FPF-*g*-BPEI/plasmid DNA complexes were prepared at various weight ratios. The complexes were transfected into CT-26 cells. The transgene expression was measured by luciferase assay. The transfection efficiency of FPF-5.2*g*-BPEI was higher than other FPF-*g*-BPEI and BPEI at a 5:1 weight ratio (polymer/DNA).

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