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REVIEW

Impacts of PEGylation on the Gene and Oligonucleotide Delivery System

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ABSTRACT: Poly(ethylene glycol) (PEG) is the most widely used polymer and also the gold standard in the field of drug delivery. Therapeutic oligonucleotides, for example, are modified with PEG at the terminus to increases nuclease resistance and the circulating half-lives. The surface of nanoparticle such as micelle and liposome has been also modified with PEG. At present, one PEGylated therapeutic oligonucleotide has been approved for the market and several more PEGylated products including oligonucleotide and liposome are being tested in clinical settings. This review summarizes the methods and effects of PEGylation on gene delivery. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40293.

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INTRODUCTION

PEGylation refers to the covalent attachment of poly(ethylene glycol) (PEG) to molecules to reduce their immunogenicity and prolong its blood-circulation time. In 1977, Abuchowski et al. reported that the covalent attachment of PEG to albumin reduced its immunogenicity.[1] Subsequently, they also found that PEGylated biomolecules had a longer blood-circulation time than the corresponding normal biomolecules.[2] On the basis of these findings, PEGylation has been widely recognized one of the more promising methods for exploring the use of therapeutic drugs as well as to increase the therapeutic efficacy of medicines in clinical settings. The main advantages of PEGylation are: (1) an increase in the size of the drug molecule, resulting in reduced filtration by the kidneys; (2) an increase in solubility; and (3) protection from enzymatic digestion and recognition by antibodies.[3–6] A variety of molecules, such as small molecules,[7–12] peptides,[13,14] proteins,[15–20] antibodies, and their fragments,[21,22] oligonucleotides,[23,24] and nanoparticles[25] have been PEGylated. Several PEGylated drugs have already been approved by the U.S. Food and Drug Administration (FDA), and several more are being tested in clinical settings. At present, the most frequently used methods for PEGylation are chemical conjugation. A variety of functionalized PEG have been developed for the PEGylation.[25] Suitable conjugation method should be adopted because linker structure has effect on the activity of the PEGylated product.[26]

PEGylation technology is also considered promising method in the development of a gene-delivery system, including oligonucleotide delivery. In recent years, a variety of nanoparticles such as micelles and liposomes have been used for gene delivery. PEGylation of these nanoparticles is an essential strategy for reducing nonspecific interactions with serum proteins and endothelial cells in the bloodstream, as well as avoiding recognition by immune system components such as the reticuloendothelial system; therefore, PEGylated nanocarriers tend to have an extended blood-circulation time and facilitate accumulation in tumor tissue, which is mediated by enhanced permeability and retention. This review summarizes and highlights the methods and effects of PEGylation on gene delivery, including therapeutic oligonucleotide delivery.

PEGYLATION OF NUCLEIC ACID

Oligonucleotide PEGylation

Oligonucleotide-based drugs such as antisense drugs, aptamers, and siRNA have attracted considerable attention as promising therapeutic agents for the treatment of various human diseases[27]; however, several issues must be overcome in the development of

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such oligonucleotide-based drugs.[28] These issues include the instability of oligonucleotides against enzymatic degradation and rapid renal clearance. For example, unmodified single-stranded DNA and single-stranded RNA are quite unstable, especially *in vivo*. Double-stranded RNA such as siRNA, in contrast, is stable under cell culture media, which contains low concentration of serum. For example, siRNA without any chemical modification was stable even after 72 h incubation in 5% serum.[29] *In vivo*, however, duplex RNA is unstable because of a higher concentration of serum.[30] Rapid renal clearance presents another problem. Short oligonucleotides drain from the kidneys because their molecular weights are far less than the molecular weight threshold in renal filtration, which is about 65 kDa.[31,32] In an attempt to stabilize oligonucleotides, many modifications on their sugar motif and/or the phosphate backbone have been reported[33]; however, these modifications often cause unwanted bioactivities such as toxicity.[34] Considering toxicities, PEG is very useful because it is categorized as "Generally Regarded As Safe (GRAS)" by the FDA. PEGylation increases nuclease resistance and the circulating half-lives of oligonucleotides. Accordingly, stabilization of oligonucleotides with PEG is a promising method for use in the development of therapeutic oligonucleotides. In fact, one of the therapeutic oligonucleotides, "Macugen," consists of PEGylated oligonucleotides.[35] In this aptamer drug, the oligonucleotide is modified with branched PEG (40 kDa) at the 5′ terminus. This modification increases the nuclease resistance of PEG-aptamer conjugates. The extent of stabilization is dependent on the length of PEG. High-molecular-weight PEG stabilizes molecules better than low-molecular-weight PEG.[35]

Several methods for the synthesis of PEGylated oligonucleotides have been reported.[36–40] Most of these are based on a conjugation between end-reactive PEG and oligonucleotides in the liquid phase. In these cases, functionalized PEG molecules such as PEG possessing N-hydroxysuccinimide (NHS)-activated ester are reacted with premodified oligonucleotides such as those that are amine modified. Other conjugation reactions, including click chemistry,[41] disulfide bond formation,[42] and the Michael addition reaction,[43] have also been reported (Table I). PEGylation of oligonucleotides in the liquid phase requires the preactivation of both PEG molecules and oligonucleotides and several purification steps, resulting in a high cost for mass production. Accordingly, the development of a simple alternative method that can produce PEGylated oligonucleotides at a lower cost is in high demand.

Compared with liquid-phase synthesis, solid-phase synthesis of the modified oligonucleotide is convenient if a conventional solid-phase system for oligonucleotide synthesis can be applied. Several methods of solid-phase synthesis of PEG-oligonucleotide conjugate have also been reported. In most cases, a low-molecular-weight PEG (i.e., <1000 Da) has been used for oligonucleotide modification; however, high-molecular-weight PEG is crucial to improving the stability of the conjugate, as stated above. Recently, our group developed a new solid phase method that enables solid-phase synthesis of a PEG-oligonucleotide conjugate (Figure 1).[44] A prepared solid phase method was preinstalled with linear PEG (5 kDa) to provide oligonucleotides modified with PEG at the 3′ terminus. Compared with the conventional liquid-phase synthesis method in which PEG containing a NHS-activated ester was reacted with the amine-modified

Table I. Chemistry of Conjugation of Oligonucleotide with PEG

PEG derivative	Oligonucleotide derivative	Product
PEG–C(=O)–O–N (NHS ester)	NH_2–	PEG–C(=O)–NH–
PEG–N (maleimide)	HS–	PEG–N (succinimide)–S–
$PEG–N_3$	≡–Linker–	PEG–N (triazole)–Linker–
PEG–O–C(=O)–CH=CH_2	HS–	PEG–O–C(=O)–CH_2CH_2–S–

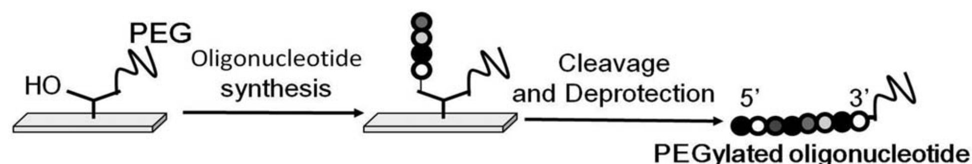

Figure 1. Solid phase synthesis of PEG-oligonucleotide conjugate. A prepared solid phase was preinstalled with PEG to provide oligonucleotides modified with PEG at the 3′ terminus.

oligonucleotide, the developed solid-phase method is simple and reproducible. Surprisingly, the modification of oligonucleotide with PEG using developed method stabilized not only DNA but also RNA more than the modification at the 5′ terminus, which has been widely used thus far (Figure 2). The half-lives of DNA modified at 5′ terminus and 3′ terminus were 11.6 and 31.8 h, respectively, and RNA modifies at 5′ terminus and 3′ terminus were 5.3 and 13.1 min, respectively. The obtained data are consistent with previous report. because in the plasma, the hydrolysis of DNA results from exonucleolytic activity and occurs exclusively in the 3′ to 5′ direction.[45] In the case of RNA, endonuclease is the main factor for the degradation[45]; therefore, the increased stability may be attributed to the rigid cyclopentyl structure joining PEG and RNA in the novel conjugate, which might enable more effective shielding of the RNA by PEG.

PEGylation Effects on the Activity of Therapeutic Oligonucleotides

PEGylation Site Effect. The activity of siRNA strongly depends on the modification sites in the sequence. siRNA possesses 4 terminal ends—a 3′- and a 5′-terminus of sense and antisense strands. Previous investigations indicated that the gene-silencing activity was substantially impaired by modification at the 5′ terminus of the antisense strand because the interaction between the 5′ phosphate and the PIWI domain, which is a component of argonaute 2, is critical for efficient gene silencing.[46,47] Accordingly, 3′- and 5′-end modifications of the sense strand and 3′-end modification of the antisense strand are considered to be the candidate modification sites.[48–50] Park et al. investigated the effect of the PEGylation site on gene-silencing efficiency of the siRNA-PEG conjugate in detail; however, in their study, they showed that PEGylation at the 5′ end of the antisense strand was also tolerable in gene silencing, as in the other 3′-terminus modifications.[51] This result indicates that, in PEGylation of siRNA, all 4 terminal ends can be a potential site for conjugation.

Another concern associated with PEGylation of oligonucleotides is the ability to hybridize to the target sequence. A steric PEG chain might inhibit the binding of therapeutic oligonucleotides to the target sequence. Govan et al. investigated the effect of PEGylation on hybridization ability by measuring the DNA melting temperature (T_m), the temperature at which a double-stranded oligonucleotide dissociates into a single strand.[52] The T_m of the PEGylated oligonucleotide decreased by 6°C when 5-kDa PEG was introduced at the 3′ terminus of the oligonucleotide. In contrast, T_m decreased by 13 and 19°C by modification with 20 and 40 kDa PEG, respectively; however, hybridization was detected.

Effect of Chemical Bond Properties: Irreversible Versus Reversible. Although covalent attachments of PEG to therapeutic oligonucleotides prolongs the lifetime of the oligonucleotide *in vivo*, they often have the opposite effect on biological and pharmacological properties because the therapeutic oligonucleotide is inactivated as a result of shielding by massive PEG chains. The target binding activity of Macugen, for example, is reduced to 25% of the original activity. New emerging technologies such as releasable PEGylation have been developed.[53–56] Specific biodegradable linkages between the oligonucleotide and PEG chains are introduced to allow de-PEGylation from

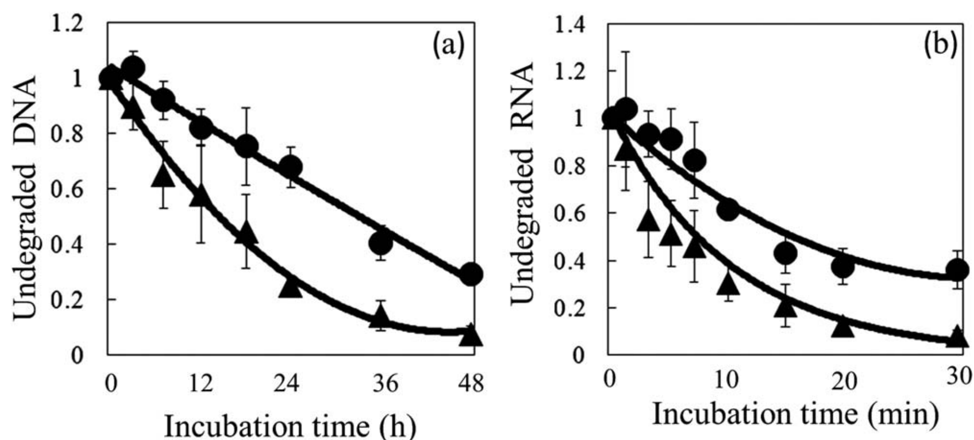

Figure 2. Stability analysis of PEG-oligonucleotide conjugate in the 50% serum-containing medium. Time course of the degradation of the DNA-PEG (a) and RNA-PEG (b). Closed circle: PEG-oligonucleotide conjugate modified at the 3′ terminus by newly developed method, Closed triangle: PEG-oligonucleotide conjugate modified at the 5′ terminus by conventional method.[44] Reproduced from Ref 44, with permission from The Royal Society of Chemistry.

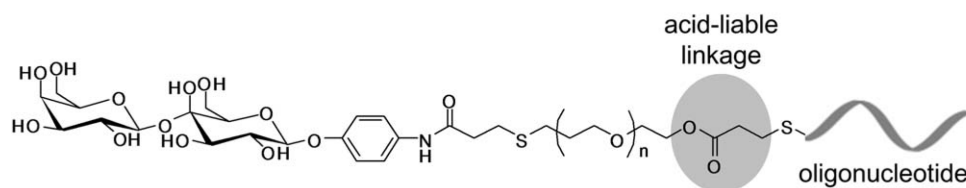

Figure 3. PEG-oligonucleotide conjugate developed by Oishi et al. Oligonucleotide was conjugated through $\beta-$thiopropionate linkage, enabling efficient release of therapeutic oligonucleotide under acidic conditions.

therapeutic oligonucleotides. Therapeutic activities of the oligonucleotide recover after the release of PEG chains. Several biodegradable linkages have been developed for de-PEGylation of oligonucleotides, including acid-liable and disulfide linkages. Acid liable linkages are expected to be cleaved under acidic conditions such as in endosomal compartments and acidosis in cancer.[43] Disulfide linkages are cleaved under a reductive environment inside the cells.[42]

Oishi et al. conjugated PEG-possessing lactose as a targeting ligand for the asialoglycoprotein receptors with siRNA at the 5′ terminus of the sense strands (Lac-PEG-siRNA) through acid-labile $\beta-$thiopropionate linkage (Figure 3).[43] This linkage is readily cleaved at pH 5.5, which corresponds to the pH in the intracellular endosomal compartment. They prepared polyion complex (PIC) micelles by complexation between Lac-PEG-siRNA and poly(L-lysine) at a charge ratio of 1 (N/P = 1). Efficient suppression of the targeted gene expression was achieved even in the presence of the serum.

De-PEGylation can also be controlled by external stimuli. Govan et al. used light-removal protecting groups for DNA PEGylation.[52] In this system, PEG was conjugated at the antisense terminus of oligo-DNA through the photolabile ortho-nitrobenzyl group. Once irradiated with UV light at 365 nm, PEG was released from the antisense reagent, enabling photocontrollable gene expression.

PEGylation of Nucleic Acid Other Than Oligonucleotides

To the best of our knowledge, no method has been reported on the PEGylation of nucleic acid other than oligonucleotides, including plasmid DNA and dumbbell-type nucleic acid. One obstacle to PEGylation of plasmid DNA is its instability and low reactivity. Plasmid DNA is unstable at high temperatures and unreactive toward general activated PEG such as PEG possessing NHS-activated ester. Accordingly, other PEG derivatives that can react with plasmid DNA in mild aqueous solution need to be developed. Dumbbell-type nucleic acid has previously been constructed by several groups.[57–59] Although the procedures of its construction are generally tedious, this end-free shape results in strong stability against nucleases. The unique architecture of the dumbbell shape also enables modification at the single-stranded loop with peptide and other molecules; PEGylation might be possible at this point.

PEGYLATED NANOPARTICLES

PEGylation of Nanoparticle

Recent progress in the development of nanoparticles for an efficient drug-delivery system is remarkable. Various types of nanoparticles have also been developed in the gene-delivery system to improve the therapeutic efficiency of nucleic acids, including plasmid DNA and oligonucleotides.[60–62] The pharmacokinetics of nanoparticles is largely influenced by the surface physicochemical properties. Accordingly, the precise design of the surface of nanoparticles is very important for the efficient and specific delivery of nucleic acids by the nanocarrier. Surface modification of nanoparticles, such as micelles and liposomes, with PEG represents an essential strategy by which nonspecific interactions with serum proteins and endothelial cells in the bloodstream can be reduced. Several factors have effects on the pharmacokinetics properties of PEGylated nanoparticle. PEG with short chain length are frequently used for the modification of nanoparticles, which are 50–100 nm in diameter because further increase in hydrodynamic radius increases uptake by liver.[63] Terminus structure of PEG and type of PEG (linear or branch) also influence behavior of nanoparticles *in vivo*.[64,65]

PEG density on the surface of nanoparticle is an essential factor for reducing nonspecific protein absorption. We have developed a mixed-PEG layer in which different molecular weights of PEG (e.g., PEG[2k] and PEG[5k]) were used to form a densely packed PEG layer.[66,67] A highly dense mixed-PEG layer nearly completely prevented nonspecific protein absorption. Note that this mixed-PEG system completely inhibited the nonspecific absorption of high-molecular-weight proteins as well as low-molecular-weight peptides. Another advantage of this system is the enhancement of biospecific interactions among biomolecules such as antibody and antibody fragments. These biomolecules have been widely used in the fields of biosensing, bioseparation systems, and targeted drug-delivery systems[68]; however, when they are introduced onto the surface of materials, these biomolecules are often inactivated because the interaction between interactive residues of biomolecules and the material surface changes their conformation and orientation. The mixed-PEG layer inhibits the interaction between the biomolecules and the materials, facilitating specific interaction among the biomolecules.[68] This technique has already been applied in biosensing[69,70] and can be applied to the construction of targeted drug-delivery systems, in which ligands are needed on the surface of nanoparticles to bind the target molecule with high specificity and efficiency.

PEGylated Micelle

Polymeric micelles containing anticancer drugs were originally independently developed by both Kataoka and Kabanov.[71,72] Anticancer drugs are incorporated into micelles through physical entrapment or chemical conjugation. A number of micelles are being assessed in clinical trials, and development of the polymer-micelle system is progressing.[73,74] Micelles have also been used in the gene-delivery system. A cationic polymer can self-assemble

with DNA to form a PIC. A block or graft copolymer composed of hydrophilic-cationic polymers such as PEG-poly(L-lysine) and PEG-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-PAsp(DET)) is generally used to form a complex with DNA.[75,76] The effect of PEG shielding has been widely demonstrated in *in vitro* and *in vivo* experiments. PEG shielding, for example, attenuates the positive charges of the polyplex and significantly suppresses the access and absorption of biomacromolecules, including serum proteins, resulting in the inhibition of the aggregation. Development of micelles for gene-delivery systems has been extensively investigated by the Kataoka group, who constructed a variety of block copolymers such as PEG-PLL and PEG-PAsp(DET).[77] Among them, PEG-PAsp(DET) is primarily considered as a potential carrier of genes because its cationic part has been found to degrade more rapidly than PEG-PLL under physiological conditions.[78] Biodegradability of the cationic polymer is very important in minimizing toxicity because the cytotoxicity of the cationic polymer increases according to the molecular weight. The degradation of cationic polymers facilitates the release of plasmid DNA by reduced electrostatic interaction. The potency of PEG-PAsp(DET) as a gene carrier has been evaluated *in vivo*.

PEGylated Poly(ethylene imine)

Poly(ethylene imine) (PEI) has been widely used in non-viral gene-delivery systems *in vitro* and *in vivo*.[79,80] Transfection efficiency of PEI and its cytotoxicity depend on its molecular weight. PEI with a molecular weight >25 kDa shows high transfection efficiency but also high cytotoxicity. In contrast, PEI with a low molecular weight (i.e., 1.8 kDa) shows less cytotoxicity; however, it also shows less transfection efficiency. One of the approaches for overcoming this problem is to modify PEI with hydrophobic molecules such as cholesterol, palmitic acid, oleic acid, and phosphatidylcholine. These hydrophobic molecules facilitate the interaction between the polyplex and cell membranes, resulting in improved gene-delivery efficiency. PEG is used extensively to shield the positive charge of PEI. Recently, Sawant et al. modified low-molecular-weight PEI with dioleoylphosphatidylethanolamine (PE).[81] The PEI-PE conjugate enhanced the transfection efficiency of low-molecular-weight PEI. To increase the biocompatibility and stability, PEI-PE/DNA complexes were mixed with degradable PEG-hydrazone-PE. Degradable linkage of hydrazine enables the tunable transfection activity, which is sensitive to changes in pH such as tumor acidosis.

Another approach for reducing cytotoxicity is to suppress inflammatory reactions.[82] Recently, PIC was found to cause inflammation, which also reduces its transfection efficiency. Reactive oxygen species (ROS) are reported to be generated in the inflammation area, and the polymeric carrier might play an important role in the generation of ROS. Yang et al., for example, have reported that the extent of ROS production increased with the increase in the molecular weight of PEI.[83] Accordingly, the development of ROS-scavenging mechanisms is important to reduce the damage caused by PIC used in gene delivery systems. Several groups have developed gene delivery systems, which possess the ability not only to deliver genes but also to scavenge the ROS generated in the cell to reduce the toxicity and achieve efficient transfection.[84–86]

PEGylated Liposomes

Several liposome-based drugs have been approved for clinical application.[87] PEG is widely used to protect the liposome from recognition by opsonins, thereby reducing liposome clearance. A number of PEGylation reactions with liposomes have been developed.[88] One of the methods uses lipophilic compounds that possess reactive amino and carboxyl groups. By incorporating these components into the bilayer membrane, 500–2000 functional groups can be introduced onto the liposome surface. This functionalized liposome can then be used for the preparation of PEGylated liposomes.

Another method for the preparation of PEGylated liposomes uses PEG, which possesses a lipid moiety at one end, in conjunction with low-molecular-weight lipid molecules during the preparation of the liposome. This method was originally reported by Kilbanov et al. in 1990,[89] who conjugated phosphatidylethanolamine with PEG possessing activated carboxyl groups. For liposome preparation, 7.4 mol % PEG lipid was incubated. The amount of PEG lipid is a critical factor for the efficient delivery system because very little PEG results in low stealth effects, whereas a high amount might lead to the formation of micelles instead of liposomes. Accordingly, there is an upper limit of the amount of PEG lipid that can be incorporated into liposomes without disrupting the liposome structure.

Gene delivery by cationic liposomes is considered the most potent method to examine under clinical settings, and several liposomes have been used in clinical trials. Harashima et al. developed a multifunctional envelope-type nano device (MEND) composed of a nucleic acid core complex with a polycation and a lipid envelope possessing various functional properties, such as PEG and targeting ligands. MEND could be applicable for the delivery of various kinds of nucleic acids, including plasmid DNA, antisense, and siRNA.[90,91] Tekmira Pharmaceuticals optimized the structure of cationic lipids by using combinatorial chemistry.[92] They prepared a library of head groups, linkers, and hydrocarbon chains. This library was screened for the development of an efficient carrier for siRNA. Optimized cationic lipid showed *in vivo* activity at siRNA doses of 0.01 mg/kg in rodents and 0.1 mg/kg in nonhuman primates. Several clinical trials using optimized cationic lipids are in the early stages of clinical settings.

The surface of the liposome is generally modified with PEG; however, one of the concerns of the delivery system using liposomes is the low density of PEG, resulting in its uptake by macrophages and the liver. Another concern of the cationic liposome system is the stimulation of the immune system. Cationic liposome stimulates the immune system by interacting with innate immune receptors in the endosomal compartment.[93] These interactions might be hampered by PEGylation, although there is a PEGylation threshold because PEGylation destroys the formation of liposomes, as mentioned above.

PEGylated Nanogels

Nanogels refer to nanoparticles that are composed of a polymer network and that are chemically or physically cross-linked. We have developed PEGylated nanogel particles that are composed of a chemically cross-linked polyamine core and PEG-modified

surface.[94] Because of their chemically cross-linked polyamine gel core, the PEGylated nanogels show stronger stability against extremely dilute and high salt conditions than self-assembled nanocarriers such as liposomes and micelles. Nucleic acids could be stabilized and were effectively delivered to cells *in vitro*[95]; however, when nanogel was intravenously administered into the bloodstream, cationic nanogel tended to interact with anionic serum proteins to form large aggregates. To use nanogels as a gene-delivery vehicle for systemic application, the non-fouling character of the nanogel surface needed to be improved. The loosely cross-linked gel structure of nanogels might expose amino groups, which interact with serum proteins and cells, to the outside of the particle to some extent. Actually, the nanogel causes strong hemolysis at a cross-linking density of 1%; however, the PEGylated nanogels with a cross-linking density of 5% showed the lowest toxicity ($LD_{50} > 200$ mg/kg), which is sufficient for *in vivo* applications.[96]

To further improve, the bioinert character of nanogels, the PEG corona density needed to be further increased. This stable nanoparticle is suitable for studying the influence of physicochemical changes of the nanoparticle surface by PEGylation on pharmacokinetics of nanoparticles *in vivo*. For contriving long-circulation nanogels, high PEG-density nanogels were developed using the new post-PEGylation reaction, which is a quaternized reaction between the amine in the nanogel core and the bromobenzyl-terminated PEG (Figure 4).[96] The blood-circulation time of post-PEGylated nanogels was significantly prolonged compared with that of those without post-PEGylation. This study clearly indicates the impact of nanoparticle PEGylation on the biodistribution of nanoparticles.

PEGylated Inorganic Nanoparticles

Inorganic nanoparticles have also attracted interest in the field of drug delivery.[97] These inorganic nanoparticles, including calcium phosphate, gold, silicon oxide, and iron oxide, can be easily prepared with a controllable size and can be easily functionalized. In particular, gold nanoparticles have been widely used in gene-delivery systems because of their low toxicity; however, inorganic nanoparticles are generally unstable and might be toxic in biological systems. Accordingly, surface modification is expected to improve the biological stability and biocompatibility.

Surface modification of inorganic nanoparticles with PEG is very useful to overcome the issues of instability and toxicity. For example, thiol groups are suitable anchors on gold nanoparticles. Kawano et al. combined the PEG-modified cationic gold nanoparticles with electroporation to achieve gene delivery *in vivo*.[98] They prepared cationic gold nanoparticle by sodium borohydride ($NaBH_4$) reduction of chloroauric acid ($HAuCl_4$) in the presence of 2-aminoethanol and PEG-SH. The resulting PEG-modified cationic gold nanoparticle can bind plasmid DNA. DNA complexes with PEG-modified nanoparticles were intravenously injected into mice and gene expression was confirmed by electroporation.

One concern is that PEGylation of inorganic nanoparticles by mono-end-functionalized PEG often causes de-PEGylation under physiological conditions, resulting in aggregation of the particles.[99] Alternative stabilization of gold nanoparticles, for example, by multiple anchoring between the gold nanoparticle and functionalized PEG such as pentaethylenehexamine-ended

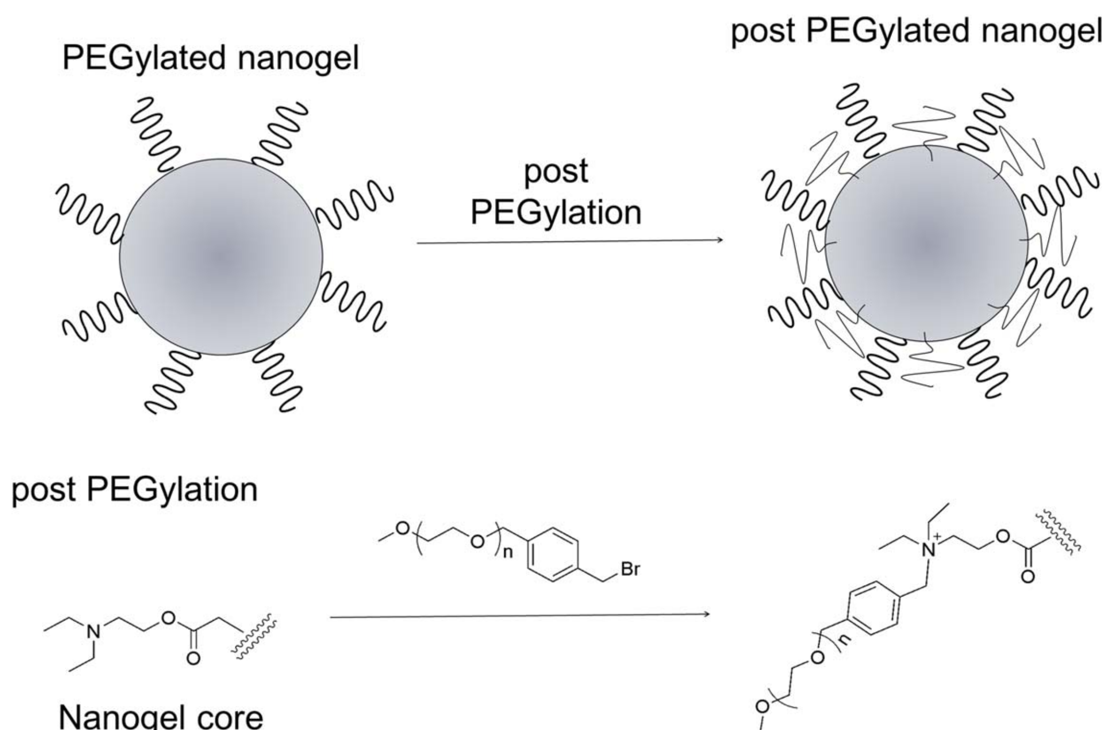

Figure 4. Post PEGylation reaction on the surface of nanogel. Bromobenzyl-terminated PEG reacted with the amine of the nanogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PEG (N6-PEG) might overcome this problem.[100] Multiple anchoring technology can also be applied to stabilize inorganic nanoparticles other than gold nanoparticle.[101,102]

PEG Dilemma

PEG on the surface of a nanoparticle prolongs the blood-circulation time of the nanoparticle; however, PEG also prevents cellular uptake because the outer PEG layer inhibits the interaction between a nanoparticle and the cell surface membrane, as well as the membrane fusion process during endocytosis. These inhibitions resulted in a decrease in therapeutic efficiency. Harashima et al. investigated the effect of the PEG density on the blood concentration *in vivo* and *in vitro* gene expression.[90] PEGylated liposome at 15 mol % modification exhibited more than 10% of injected dose (ID/mL) in blood concentration at 6 h after intravenous administration, however, PEGylated liposome showed much lower gene expression compared to the unmodified liposome *in vitro*. Moreover, they also reported that the efficiency of endosomal escape via membrane fusion was significantly decreased because of the steric hindrance of PEG.[90] To overcome this problem, several approaches have been reported. First approach is to enhance cellular uptake by active targeting. A variety of ligands such as peptides, proteins, vitamins, aptamar, antibodies, and antibody fragments have been employed.

Second approach is the de-PEGylation system in which PEG is cleaved from the nanoparticle after the nanoparticle reaches the target site to enhance endosomal escape of carriers (Figure 5). Hatakeyama et al. developed a de-PEGylation system in which PEGs on the surface of liposomes were designed to be cleaved by enzymes such as matrix metalloproteinases that are specifically expressed at tumor sites. Removing PEGs resulted in an improvement of cellular uptake and the subsequent escape of the liposome from the endosomal compartment.[103] Other enzymes also employed as stimulating triggers for the de-PEGylation system.[104] Suma et al. constructed multilayered PICs possessing detachable PEG through disulfide bonds.[105] In this system, a core PIC structure that was prepared by complexation of siRNAs with a polycation was stabilized by a silica-interlayer and an endosome-disrupting block copolymer possessing detachable PEG through disulfide bonds. Disulfide bonds are expected to be cleaved by reducing enzymes on the cellular membrane and endosomal/lysosomal compartment.

Another strategy to resolve the PEG dilemma is to use pH-sensitive liposomes in which the surface charge of the liposome changes in response to the pH of the environment. In this system, the surface charge changes from a neutral to cationic nature according to the acid environment, such as in the endocytosis process. Because the positive charges on the surface are reduced at a neutral nature, the amounts of PEG required for the effective shielding of the positive charge can be minimized. For example, the cationic lipid YSK05 developed by Sato et al.[106] possesses a pKa of ~6.6. This lipid becomes a cationic species in response to an acidic environment during endocytosis, facilitating the interaction with anionic charges of the endosomal membrane.

CONCLUSIONS

Because of the biocompatibility and inert character of PEG, it is widely used in the development of drugs. In the field of nucleic-acid delivery, PEG is used for the modification of oligonucleotides such as antisense oligonucleotide, aptamer, and siRNA. In oligonucleotide modification, PEG stabilizes the oligonucleotide and inhibits rapid clearance from the kidney. PEG is also used extensively in the modification of a nanocarrier for gene delivery. To achieve prolonged blood-circulation time, the density of PEG on the nanocarrier surface is critical because low PEG density results in nonspecific interaction between the nanocarrier and serum proteins and endothelial cells in the bloodstream; however, in most cases, the PEG layer on the nanoparticle surface strongly reduces transfection efficiency. This PEG problem should be resolved to achieve an efficient gene-delivery system.

Various de-PEGylation systems that facilitate cellular uptake and endosomal escape have been reported. Ligand modification is another approach to improve the cellular uptake of nanoparticles. Many ligands, including proteins, peptides, antibodies,

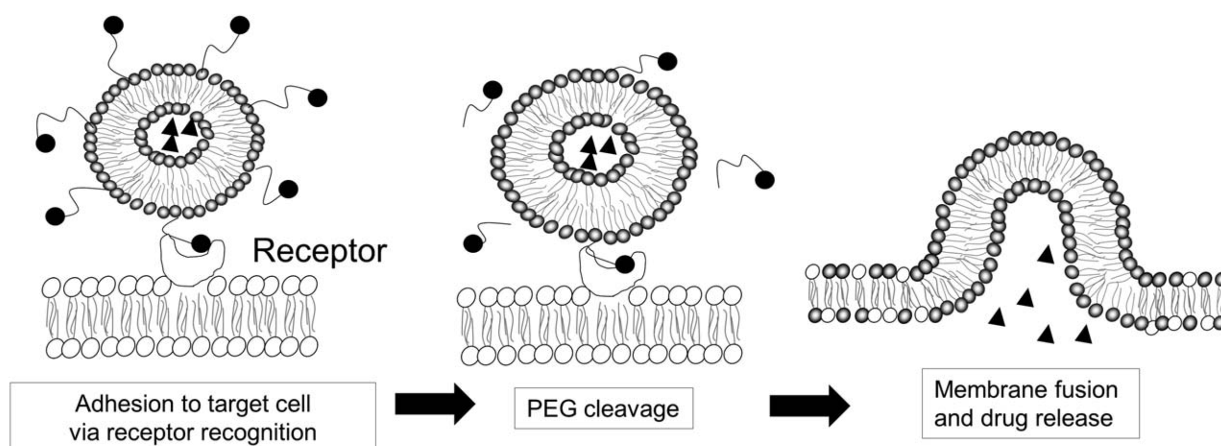

Figure 5. De-PEGylation technology in liposomes. After binding the target cell via specific recognition of the receptor by the ligand, PEG molecules on the surface of the liposome are cleaved. The release of PEG facilitates membrane fusion of the liposome and liposome decomposition, resulting in efficient drug delivery.

antibody fragments, aptamers, and carbohydrates, have been reported and their useful properties have been demonstrated. Some success was achieved using these ligands; however, modifications using ligands might alter the physicochemical properties of the original nanoparticle, resulting in changes of pharmacokinetics *in vivo*.

PEG is considered the first choice of material in gene-delivery systems. Although there are several drawbacks of PEG,[63] such as interaction with the immune system, non-biodegradability, and accumulation in the body, PEG is considered as the gold standard in the field of polymeric drug delivery. Alternative polymers to PEG have been reported; these include poly(glycerol), poly(2-oxazoline), poly(amino acid), and poly[N-(2-hydroxypropyl)methacrylamide]. These polymers show several promising results; however, further investigation is necessary for their use in clinical settings.

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