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Review article

Recent development of nonviral gene delivery systems with virus-like structures and mechanisms

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ABSTRACT

The concept of gene therapy includes not only the addition of normal genes to genetically deficient cells, but also the use of transgenes encoding several peptides that function to enhance the capacity of normal cells or to regulate cell differentiation. The application of gene therapy has been widely considered for various diseases, as well as for the field of tissue engineering. To overcome the problems with viral vectors, a broad range of nonviral systems for gene delivery have been developed, including systems composed of cationic lipids (lipoplexes) and cationic polymers (polyplexes). However, most of these systems are still much less efficient than viral vectors, especially for *in vivo* gene delivery. Paradoxically, to achieve a maximum transgene expression in the targeted cells, there is no question that natural viruses are the most effective nanocarriers. In this article, we highlight the approaches currently being taken to improve nonviral gene delivery systems so that they better replicate the typical structures and mechanisms of viruses, such as DNA (RNA) condensation in the core, surrounding structures with targeting molecules for specific receptors, as well as the toxic and immunogenic problems which should be avoided, with the ultimate goal of bringing these systems into a clinical setting.

1. Introduction

As our knowledge of the molecular background of various diseases continues to grow, gene therapy is becoming an increasingly attractive possibility in a wide range of diseases, as well as one of the great scientific challenges in modern medicine. The concept of gene therapy includes not only the addition of normal genes to genetically deficient cells, but also the use of transgenes encoding several peptides that function to enhance the capacity of normal cells or to regulate cell differentiation. The advantages of using transgenes for therapeutic purposes compared with delivery of exogenous proteins or bioactive molecules are (1) the transgene can express bioactive factors in the native form at the targeted site [1]; (2) the sustained synthesis of proteins from the transgene can facilitate synchronization between the kinetics of signaling receptor expression and bioactive factor availability [2]; and (3) transgenes are more flexible in terms of their potential applications. The sequence-specific inhibition of gene expression using antisense DNA and siRNA (and its expression vector) is also considered to be a type of gene therapy. Gene therapy has widely been considered not only for lethal diseases such as congenital hereditary disorders, end-stage malignant tumors, and severe infectious diseases, but also cardiovascular diseases [3], age-related degenerative diseases [4], wound healing [5] and tissue engineering [1,6].

Despite this great promise, however, there have been few successful outcomes of gene therapy so far. Early clinical trials using recombinant viral vectors have reported significant problems, such as short-term transgene expression, an inability to persist in host cells and toxicity. In 1999, direct injection of an adenovirus vector into the hepatic artery caused the death of a patient [7]. Analysis of the inflammatory cytokine profile indicated that the vector had caused systemic inflammatory response syndrome, resulting in intravascular coagulation, acute respiratory disorder and multiorgan failure [8,9]. Viral vectors were reported to be somewhat more successful in the treatment of children suffering from the fatal form of X-linked severe combined immunodeficiency disease (SCID-X1 syndrome) in 2000 [10]. The immune systems of these children were rendered functional by stem cell gene therapy using a retrovirus vector, but unfortunately, five patients so far developed a leukemia-type disease owing to insertional mutagenesis [11,12].

These results highlight the need for nonviral systems for gene delivery. In response to the observed problems with viral vectors, a broad range of nonviral systems for gene delivery have been developed, including systems composed of cationic lipids (lipoplexes) and cationic polymers (polyplexes). Such nonviral gene

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carriers may also trigger an inflammatory response but are not likely to induce specific recognition, making the carriers less hazardous in terms of antigen-specific immune response. However, most carriers are still much less efficient than viral vectors, especially for *in vivo* gene delivery. Paradoxically, to achieve a maximum transgene expression in the targeted cells, it is clear that the natural viruses are the most effective nanocarriers. For development of improved nonviral carriers for human gene therapy, an improved understanding of the mechanisms in natural viruses and its application to the molecular design of nonviral carriers would be beneficial.

In this article, we highlight the approaches currently being taken to improve nonviral gene delivery systems so that they better replicate the typical structures and mechanisms of viruses, such as DNA (RNA) condensation in the core, surrounding structure with targeting molecules for specific receptors, and the toxic and immunogenic issues which should be avoided. This review includes a discussion of our own recent efforts to improve nonviral gene delivery using polyplex micelles composed of poly(ethyleneglycol) (PEG)-block-polycations.

2. DNA condensation

Although there are many structural variations among virus species, most viruses are characterized by the presence of condensed DNA (or RNA) in the core and a multitiered structure surrounding the core. Apparently, the condensation of DNA – with one or more shells of protein wrapped around the DNA in an often helical or icosahedral structure called the capsid – is beneficial to protect the DNA or RNA genome from physical or enzymatic degradation [13,14]. In the case of enveloped viruses, there is also a lipid bilayer membrane that serves to protect the genome and the capsid itself.

Recently, the mechanisms underlying DNA packaging into the capsid have begun to be clarified [15,16]. Bacteriophages, herpes viruses and other large double-stranded DNA viruses possess sophisticated molecular mechanisms that pump DNA into preassembled procapsids. The packaging is initiated by recognition and cleavage of the specific *pac* sequence (*pac* cleavage), which generates the first DNA end to be encapsidated. A sequence-independent cleavage (heedful cleavage) terminates packaging, creating a new starting point for another round of packaging. These 'headful packaging' processes are ATP-dependent and, surprisingly, the internal capsid pressure exceeds, by 10-fold, that of bottled champagne. The structure of the tight dsDNA spooling has been shown to activate the switch that signals the headful chromosome packing density to the particle exterior [16]. Although the detailed mechanism of the packaging of circular DNA is unknown, the viruses clearly have active and sophisticated mechanisms to condense the DNA inside the core.

In contrast, the DNA condensation in nonviral gene carriers is achieved via electrostatic interactions between the anionic phosphate groups of DNA and the cationic molecules of the carriers [17]. Generally, polymer-based gene carriers possess positively charged groups that are known to condense DNA coils into 10^{-3}–10^{-4} of the original volume by forming a polyion complex (PIC) [18]. The condensed state of DNA significantly increases the tolerance from degradation by DNases existing in the body [19]. However, when a single cationic homopolymer is used, the condensation of DNA is likely to be a random process dependent on the order and rate of mixing DNA and polymer solutions, causing considerable polydispersity of the carriers. Overstabilization of DNA may also occur when there is an excess of cationic polymers, which inhibit the smooth release of DNA through an interexchange reaction with counter polyanions inside the cells [17,20,21].

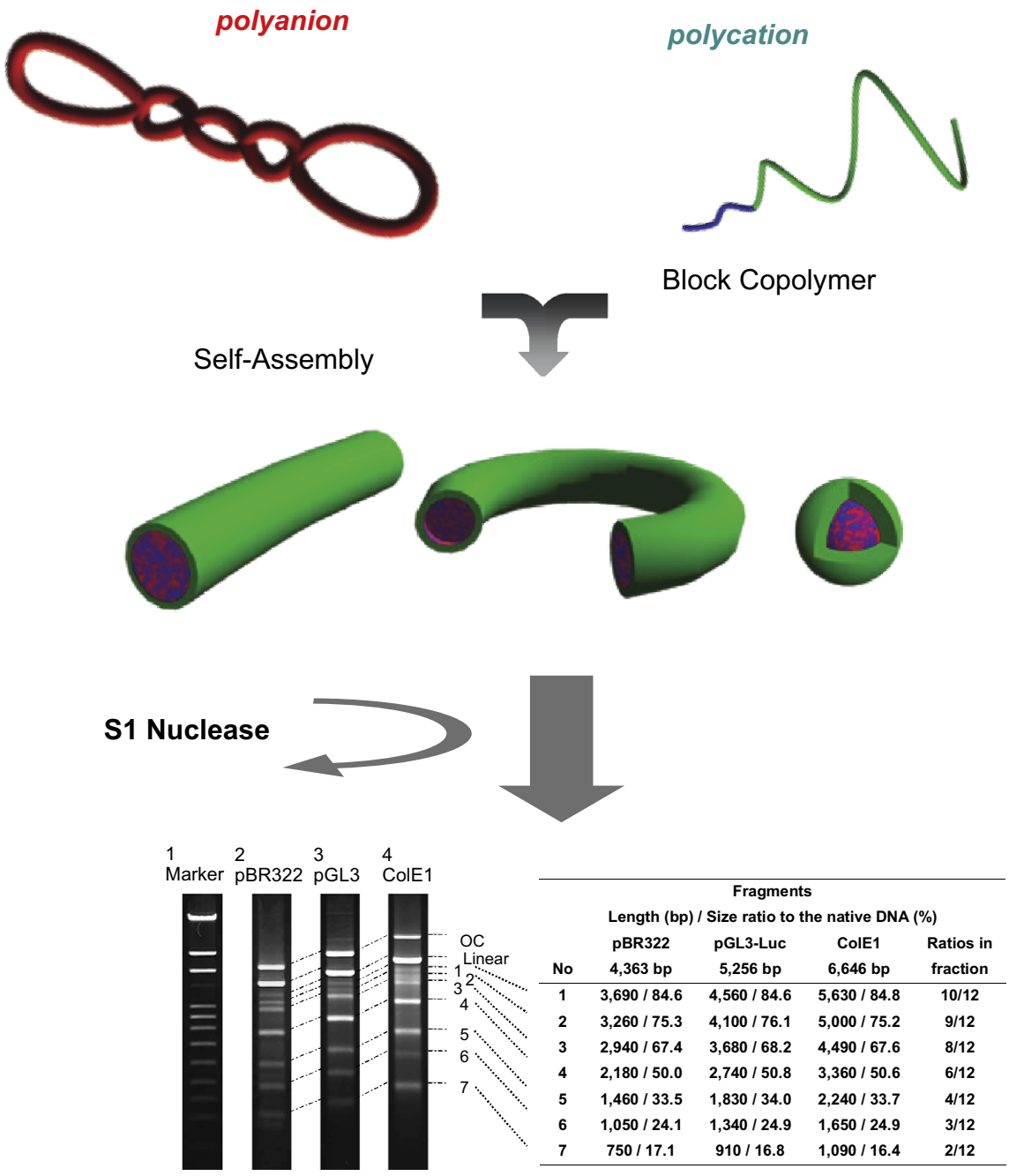
In order to regulate the status on DNA condensation, the use of cationic block copolymers with a hydrophilic segment is an interesting possibility. Due to the formation of polyplex micelles in which the PIC is surrounded by a hydrophilic poly(ethyleneglycol) (PEG) palisade, a water-soluble structure with condensed DNA can be obtained with narrow dispersity [22,23]. Using polyplex micelles of PEG-Polylysine (PLL) block copolymers and plasmid DNA, the condensation behavior of DNA was investigated by adding an S1 nuclease (single-strand specific cleavage enzyme) that cleaves the looped DNA strand, with the fragmentation of DNA expected to reflect the destabilization of DNA double strands. When the micelles were formed in a stoichiometric ratio of nitrogen in cationic segment to DNA phosphates, a surprising digestion behavior by the S1 nuclease was observed. The condensed DNA was separated into seven distinct fragments, which were 10/12, 9/12, 8/12, 6/12, 4/12, 3/12, and 2/12 the length of the original plasmid DNA, respectively [24,25] (Fig. 1). Moreover, the ordered fragmentation occurred in the full series of plasmid DNAs, which ranged in size from 2200 to 12,000 base pairs, suggesting that it was related to the inherent propensity of plasmid DNA. Note that no such ordered fragmentation was observed using polyplexes formed of PLL homopolymer, but the DNA was degraded in a nonspecific manner. These results suggest that the condensation of DNA in the polyplex micelles occurred under a regulated mechanism of DNA folding.

The important point is that the high-order structure of DNA may be strongly related to its biological activity. When the condensation is complete and the DNA molecules are fully packed, there is complete inhibition of enzymatic action, such as transcription [26]. However, when the folded structure is a swollen globule, enzymes can access DNA molecules. Indeed, based on studies of chromatin structure, it has been established that tightly packed and loosened parts coexist except during mitosis, presumably reflecting the on/off switching activation of a large number of genes [27]. It is reasonable to speculate that regulated condensation of DNA inside the carriers is crucial to smooth intracellular processes for efficient transcription. Our preliminary investigations have revealed that, just by changing the condensation state of DNA in the polyplex micelles, the transcription activity was significantly affected in both a cell-free examination and *in vivo* experiments. In nonviral systems, it may not be possible to actively package DNA in the manner of natural viruses in the near future. Nevertheless, studies on the regulation of DNA condensation should receive greater emphasis over the long-term in order to design sophisticated molecular structures for the nonviral carriers.

3. Poly(ethyleneglycol) (PEG)

Natural viruses have a multitiered structure surrounding the core that serves to protect the genome and operate as a 'transport vesicle' during cell-to-cell transmission. In nonviral systems, poly(ethyleneglycol) (PEG) has often been used for a similar purpose to protect polyplex and lipoplex-type gene carriers. Attachment of hydrophilic polymers such as PEG to the surface of liposomes, to create what are known as stealth liposomes [28], shields them from undesired binding activity in the blood, resulting in prolonged circulation times [29–31]. Many researchers have applied the same concept to shield targeted DNA polyplexes, and several strategies have been developed for the attachment of PEG to polyplexes [32]. The hydrophilic polymer was covalently coupled to the DNA-binding polycation either before polyplex formation (pre-PEGylation) [33,34] or after the polyplex formation (post-PEGylation) [35,36]. Self-organization into micellar structures using amphiphilic block copolymers composed of PEG and cationic segment has also been reported [22,23,36–39]. Shielding by PEG increased solubility, provided stability, reduced toxicity and extended the circulation time of polyplexes in the blood.

Although the PEG shield may increase systemic delivery to the target cells, it reduces gene expression activity within the target



Fragments				
	Length (bp) / Size ratio to the native DNA (%)			
	pBR322	pGL3-Luc	ColE1	Ratios in
No	4,363 bp	5,256 bp	6,646 bp	fraction
1	3,690 / 84.6	4,560 / 84.6	5,630 / 84.8	10/12
2	3,260 / 75.3	4,100 / 76.1	5,000 / 75.2	9/12
3	2,940 / 67.4	3,680 / 68.2	4,490 / 67.6	8/12
4	2,180 / 50.0	2,740 / 50.8	3,360 / 50.6	6/12
5	1,460 / 33.5	1,830 / 34.0	2,240 / 33.7	4/12
6	1,050 / 24.1	1,340 / 24.9	1,650 / 24.9	3/12
7	750 / 17.1	910 / 16.8	1,090 / 16.4	2/12

Fig. 1. Ordered fragmentation of condensed pDNA inside the polyplex micelles of PEG-Polylysine (PLL) block copolymers and pDNA. When the micelles were formed in a stoichiometric ratio of nitrogen in cationic segment to DNA phosphates, the condensed pDNA was separated into seven distinct fragments, which were 10/12, 9/12, 8/12, 6/12, 4/12, 3/12, and 2/12 the length of the original pDNA, respectively.

cells. Apparently, if the PEG shield is irreversible, it will hamper the intracellular processing of carriers. To avoid the overstabilization of the PEG shielding, bioresponsive PEG-polycation conjugates with pH-labile linkages, which may break in the acidic milieu of the endosomes, were introduced [41]. Polycation and PEG were linked via acylhydrazides or pyridylhydrazines. The pyridylhydrazone prepared from polylysine and propionaldehyde-PEG showed the greatest acid-dependent hydrolysis. Using this polyplex shielded with bioreversible PEG conjugates, up to 100-fold higher *in vitro* gene expression was achieved than in polyplexes with the analogous stable PEG shields.

From another viewpoint with respect to intracellular processing, the PEGylation of polyplexes was found to modulate the time-dependent expression profiles of the transgenes. We have observed that polyplex micelles composed of PEG-polycation block copolymers induced delayed onset and, subsequently, prolonged profiles of transgene expressions compared to the polyplexes from cationic homopolymers or lipoplexes [42–44]. The difficulty in the evaluation of gene expression for a prolonged time period is that continuous observation of a conventional monolayer cell-culture is limited to not more than several days while maintaining the normal cellular conditions. We thus used the multicellular tumor spheroid (MCTS) model, in which the cells have prolonged viable spans with actively proliferating outer cell layers [45]. In comparing the polyplexes from cationic homopolymer (P[Asp(DET)]: poly(*N*-substituted asparagine) copolymers having the *N*-(2-aminoethyl)-2-aminoethyl group in the side chain) and the polyplex micelles from the PEG-based cationic block copolymer with the

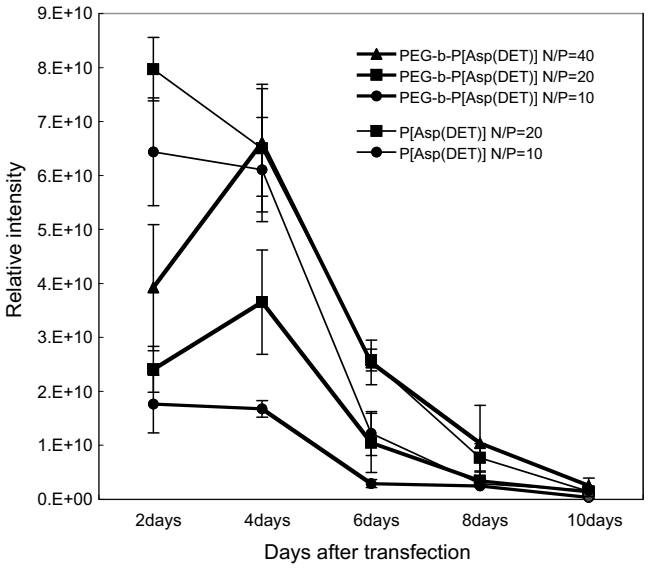


Fig. 2. Transgene expressions in the multicellular tumor spheroid (MCTS) model. A YFP gene was transfected using P[Asp(DET)] polyplexes or PEG-b-P[Asp(DET)] polyplex micelles. The gene expression was indicated as relative intensity (= total intensity/volume of spheroid) of YFP fluorescence.

same polycation segment (PEG-P[Asp(DET)]), the latter showed a peak in transgene expression at 4 days after transfection, while the former exhibited the highest expression at the beginning, followed by a continuous time-dependent decrease [44] (Fig. 2). Such delayed gene expression from the polyplex micelles might be due to their increased stability and, eventually, the delayed unpacking of pDNA inside the cells. This aspect of the time-dependent profiles of transgene expression is important for certain applications related directly to the clinical gene therapy; for example, the sustained gene expression of transcription factors that were transfected by polyplex micelles efficiently promoted the cell differentiation [43] (discussed later).

As previously described, PEGylation of PLL allows controlled packing of DNA into the polyplexes [23,24]. In addition to these characteristics, PEG apparently reduces the cytotoxicity, which will be discussed later. Thus, PEG possesses a wide range of properties relevant for composing synthetic gene carriers.

4. Incorporation of functional molecules

In order to infect, viruses must first bind to the cell surface. As for the mechanism of virus internalization, there are two general types of surface structures that viruses bind. The first consists of attachment molecules that bind the viruses and thereby help to concentrate them on the cell surface. Such interactions are relatively nonspecific, often involving interactions with heparin sulfate or other carbohydrate structures on the cell surface [46,47]. The other type of structure participates in active interactions that promote viral entry. The receptors on the cell surface trigger conformational changes in the virus particle, by activating signaling pathways, and by promoting endocytic internalization. The receptors often accompany the virus into the cell during endocytosis and may play a role in the intracellular transport of the virus particles [13]. The endocytotic internalization of virus particles involves many different mechanisms, such as macropinocytosis, clathrin-independent endocytosis, clathrin-mediated endocytosis, caveolar endocytosis, cholesterol-dependent and dynamin-2-dependent endocytosis – that feature an interaction between the viral surface and a particular receptor. Such interactions are usually highly specific, providing viral tropism to specific cells, tissues or organs. In recent years, hundreds of attachment molecules and receptors for different viruses have been identified, and their structures and functions have been made available as a large body of valuable information.

In the development of nonviral gene delivery systems, the first report to target a unique receptor on the cell surface employed a system possessing galactose-terminal asialo-glycoproteins, which targeted hepatocytes, to facilitate the internalization of gene carriers through receptor-mediated endocytosis. The asialo-orosomucoid-PLL carriers successfully realized specific gene expression in HepG2 hepatoma cells, but yielded no expressions in other cells without asialo-glycoprotein receptors [48–50]. Since then, site-specific gene delivery by nonviral carrier systems has received much attention, especially for *in vivo* direct gene transfer using various biological molecules as ligands, including sugar [51–53], transferrin [54–56], folate [57,58], and LDL [59,60]. Other candidates for receptor-mediated gene delivery include the targeting to integrin receptors [61] and growth factors [62].

The increase in the gene expression using ligands is considered principally due to the improved uptake of the carriers through receptor-mediated endocytosis [63,64]. However, these ligands may serve not only to increase the uptake of carriers by promoting specific cellular binding, but also to control the intracellular trafficking of the carriers. There have been only a few reports about the modulated intracellular trafficking of gene carriers using ligands; e.g., incorporation of bFGF, but not VEGF, has been shown to facilitate gene delivery, while both bFGF and VEGF installations result in a considerable increase in the cellular uptake of the gene [62]. Lactose modification of a gene carrier composed of chitosan resulted in altered intracellular trafficking compared to the carriers without lactosylation, resulting in the rapid escape of the former from the late endosome/lysosome [65]. A study on the inhibition of specific endocytic pathways, such as clathrin-dependent and lipid-raft-dependent internalizations, revealed that the optimized internalization played a substantial role in the enhanced transgene expressions [66].

In a related study, the control of cellular uptake by transferrin was shown to have a relevant effect on siRNA delivery *in vivo* [67]. Transferrin-conjugated siRNA nanoparticles showed more significant silencing of the target gene (luciferase) at the tumor compared to nontargeted siRNA nanoparticles, although both nanoparticles exhibited similar biodistribution and tumor localization one day after injection. This result indicates that the primary advantage of ligand conjugation was associated with the modulated intracellular trafficking in tumor cells rather than an increase in the overall tumor localization.

Recently, we realized the modulated intracellular trafficking using cyclic RGD peptide that recognizes $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors as the ligand for gene carriers [68]. A confocal laser scanning microscopic observation revealed that the cRGD (+) polyplex micelles preferentially accumulated in the perinuclear region of the transfected cells within 3 h, while no such fast and directed accumulation was observed by the cRGD (−) micelles, indicating that the increase in the gene expression by cRGD (+) micelles was not only due to the increase in cellular uptake, but also due to the facilitated intracellular trafficking toward the perinuclear region via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptor-mediated endocytosis (Fig. 3). Interestingly, the effect of cRGD on the transgene expression by the polyplex micelles was more prominent when the cRGD was attached to cross-linked polyplex micelles, which had the cross-linking core with disulfide bonds (submitted). Similarly, gene transfer via intra-articular injection of RGD-attached cross-linked polyplex micelles resulted in considerable gene expression on the synovium inside the joint, while no expression was observed using micelles without cross-linking (Fig. 4). Thus, a proper disposition of molecules on the polyplex surfaces is considered to be crucial to control the biological events such as cellular uptake and intracellular trafficking. In this con-

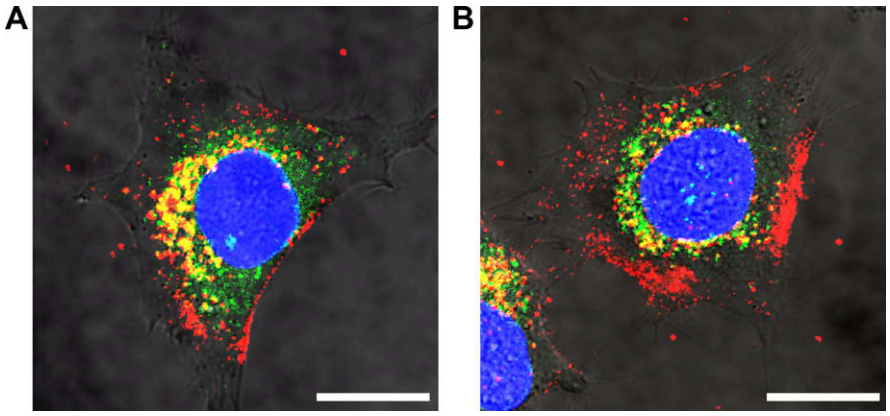


Fig. 3. Intracellular distribution of pDNA after 3 h of transfection using cRGD (+) polyplex micelles (A) or cRGD (−) micelles (B). The pDNA was labeled by Cy5 (red), and the acidic endosome and lysosome were stained by LysoTracker Green (green). The cell nuclei were stained with Hoechst 33342 (blue). The scale bar represents 20 μm (For interpretation of colour mentioned in this figure, the reader is referred to the web version of this article.).

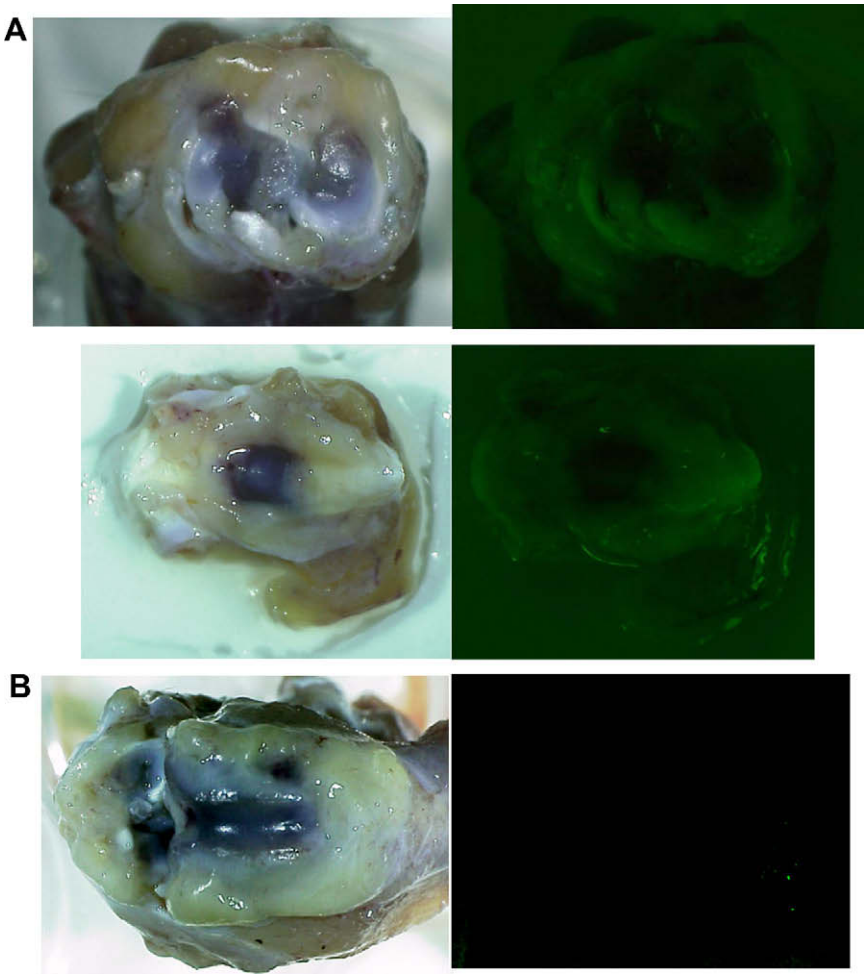


Fig. 4. Transgene expression on the synovium inside the joint. (A) RGD-attached cross-linked polyplex micelles containing YFP-encoding pDNA were injected into the knee joint of mouse. Two weeks after injection, the mouse was sacrificed and the inside of the knee joint was observed. (B) Knee joint after transfection using micelles without cross-linking.

text, the multifunctional envelope-type nano-devices (MENDs) show the potentials to realize multifunctionalities with adequate arrangement of molecules on the surface and inside of the carriers [69–71]. In this system, such molecules as the ligand-installed PEGs for cellular entry, a pH-sensitive fusogenic peptide for endosomal escape, protein transduction domains (PTDs) for efficient uptake, and a nuclear localization signal for efficient nuclear translocation were organized in the carriers, and a comparable gene expression to the adenovirus vector was achieved in *in vitro* transfection.

5. Toxicity and immunogenicity

As described in the Introduction, viral vectors may induce adverse side effects, including severe immunogenicity and toxicological responses. Recent clinical trials using viral vectors have been halted due to unprecedented toxicity, including the death of patients [7–11,72]. Moreover, even via local injection, the immune response may cause a systemic detrimental effect; a local injection of BMP-2-expressing adenoviral vector to a bone defect area in sheep unexpectedly reduced bone formation even at the untreated contralateral defect area, indicating that only a single injection of adenovirus induced a systemic inhibitory effect on bone formation [73].

However, due to 'innate immunity' mechanisms via toll-like receptors (TLRs), which recognize a variety of biomolecules, including DNA and RNA [74], the introduction of foreign DNA or RNA would inevitably elicit a range of acute physiological responses and elevation in serum transaminase and proinflammatory cytokines [75–78]. Such cytokines may become toxic in order to limit transgene expressions. The use of a bacterially derived plasmid DNA containing many unmethylated cytosine-phosphate-guanine (CpG) motifs, which are recognized as foreign materials by TLR9 [79], may be a major reason for the immune responses induced by gene carriers. The use of CpG-free plasmid DNA was demonstrated to reduce the inflammation, with fewer hematological changes and decreased liver toxicity [80]. However, the inflammatory responses induced by the plasmid DNA/cationic lipid complexes were not completely diminished in TLR9-KO mice, indicating that other mechanisms might be involved in their inflammatory responses [81]. Thus, further study is needed to clarify the mechanism of inflammatory responses so as to find a way to reduce any side effects accompanying gene introduction. It should be noted that, in some cases, the controlled induction of proinflammatory cytokines might increase a therapeutic index of cancer gene therapy through the nonspecific activation of the immune system [82,83].

Pharmacologic or toxicologic effects of carrier materials that might alter responses to delivered genes or drugs have recently attracted increasing attention. In this regard, a pharmacogenomic analysis of the global gene expression in the transfected cells is of particular interest. This approach has recently been advocated as polymer (material) genomics, and has been applied to evaluate the biocompatibility and bioactivity of nonviral gene carriers. Pluronics, the A–B–A type amphiphilic block copolymers of poly(ethylene oxide) (A) and poly(propylene oxide) (B), were reported to upregulate the expression of selected genes in cells and alter genetic responses to antineoplastic agents in cancer [84]. From the standpoint of the toxicology of gene carriers, lipid and polycationic dendrimer-based complexes were analyzed using cDNA microarrays, revealing that the carrier components induced a change in the expression of many endogenous genes without apparent cytotoxicity [85,86]. This type of toxicologic effect was also demonstrated in the delivery of siRNA and its expression vector, because the change in the endogenous gene expressions induced by delivery systems would significantly affect the gene silencing efficacy of RNA interference (RNAi) [87].

This aspect of toxicity may also induce time-dependent cytotoxicity, which could perturb cellular homeostasis. Although the use of an Exgen 500 (linear polyethylenimine) system has been shown to result in good transgene expression to various cell lines, it was also reported to result in a gradual decrease in the expression level of stably expressing luciferase in a bioluminescent cell line [88]. Evaluation of the expression of house-keeping genes as well as the profiles of plasmid DNA uptake after transfection suggested that the harmful effect of polyethylenimine appeared in the time-dependent manner (Fig. 5). Indeed, cell differentiation,

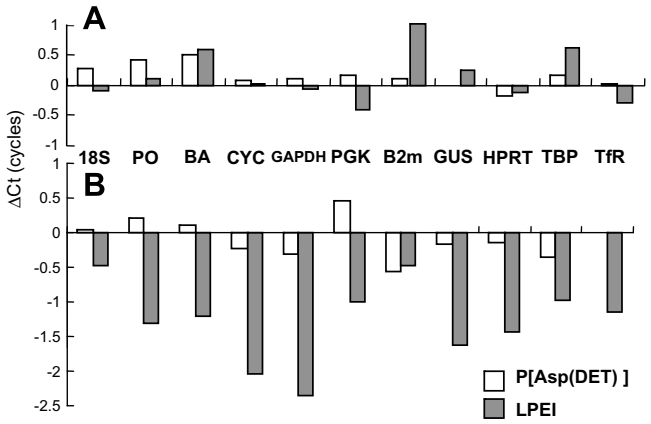


Fig. 5. Expressions of house-keeping genes after transfection. The mRNA expressions of various house-keeping genes were evaluated by a quantitative PCR at (A) 24 h and (B) 72 h after transfection using P[Asp(DET)] (open bar) or LPEI (filled bar).

which requires complex intracellular processes over an extended period, was revealed to be strongly perturbed by the toxicity of gene carriers; the induction of osteogenic differentiation by the functional gene introduction was achieved only by the use of a cationic polymer, P[Asp(DET)], with minimal cytotoxicity [40]. There was no induction of osteogenic differentiation by polyethylenimine, although an appreciable expression of the transgene was observed. A similar tendency was observed in a comparable study of polyplex micelles composed of the PEG-P[Asp(DET)] block copolymer with FuGENE6, a lipid-based reagent with considerably high transfection efficiency [89–93]. After transfection of genes encoding osteogenic factors to mouse primary osteoblasts by the use of these gene carriers, an identical increase in the mRNA expression of osteocalcin, an osteoblast-differentiation marker, was observed on day 5. On day 10, the micelle-transfected cells showed the more remarkable increase in osteocalcin induction, whereas no significant increase occurred by FuGENE6, although both carriers showed comparable transgene expression of osteogenic factors without apparent cytotoxicity [43] (Fig. 6). This difference in osteocalcin induction may be partly attributable to the sustained transgene expression profiles by the micelle system, as described in the previous section on Poly(ethyleneglycol). In addition, FuGENE6 may cause some adverse effects on cell differentiation that are difficult to detect by a nonspecific viability evaluation such as the MTT assay [94].

It is important to take this aspect of safety into account, especially when the transgene introduction is applied to primary cells to regulate such cell functions as differentiation. The nano-structured materials with a scale of 100 nanometers or less, such as gene carriers, may interact differently with biological systems from bulk materials of the same composition [95]. Thus, the unique principles and test procedures to ensure the safety of nanomaterials should be established as nanotoxicology for clinical application.

6. Summary

Despite their small size of only several-tens nanometers, natural viruses have a sufficiently sophisticated structure to accomplish complex biofunctions and realize effective gene introduction. Since the nonviral gene delivery systems pursue the effective gene delivery as well, it is reasonable that the approaches, so that the carriers better replicate the typical structures and mechanisms of viruses, such as DNA (RNA) condensation in the core, surrounding structures with targeting molecules for specific receptors, attract much attention. The most important goal in developing efficient and safe

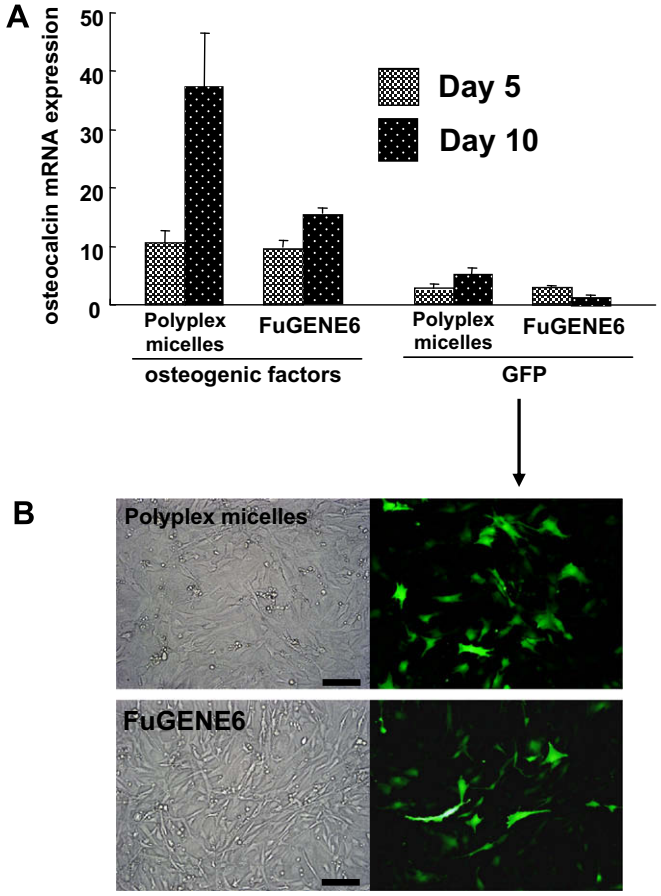


Fig. 6. (A) Evaluation of osteocalcin mRNA expression by a quantitative PCR. Osteogenic differentiation was induced on the mouse calvarial cells by transfection of osteogenic factors (caALK6 and Runx2) expressing pDNAs. As a negative control, a GFP gene was also used (The GFP expressions were shown in (B). Scale bar: 100 μm). After 5 and 10 days, the total RNA was collected and the osteocalcin expression was quantified by a quantitative PCR.

gene carriers is to control the profile of the transgene expression while reducing adverse effects on the targeted cells.

Although not described herein, one unique and valuable strategy to increase the gene transfection efficiency in nonviral systems is to use a physical trigger such as light [96], ultrasound [97] or local pressure (hydrodynamic delivery) [98,99]. These methods are highly controllable, and with an appropriate design of carriers, such a system would be expected to have much greater potential than natural viruses for achieving substantial therapeutic outcomes in a variety of clinical fields. Integration of the principles of physics, chemistry, and biology with the development of carriers will be essential for achieving urgently needed breakthroughs, and for bringing these nonviral delivery systems into a clinical setting.

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