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Polyamine – Lipid Conjugates as Effective Gene Carriers: Chemical Structure, Morphology, and Gene Transfer Activity

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1. Introduction

Development of more efficient and safer gene carriers using nonviral compounds is one of the most challenging aspects of gene therapy (Kay, 1997; Lasic, 1997). Compared to viral carrier systems, nonviral gene carrier systems have advantages in simplicity of use, lack of specific immune response, and ease of mass production due to the low cost of preparation; however, they have the disadvantage of low transfection efficiency, which needs to be overcome (Miller, 1998; Li & Huang, 2000). To improve the efficiency of nonviral carriers, many synthetic organic compounds, including cationic lipids (Felgner & Ringold, 1989; MacDonald et al., 1999; Felgner et al., 1987; Behr et al., 1989; Meyer et al., 1998), polycations (Boussif et al., 1995; Petersen et al., 2002; Koide et al., 2006; Russ et al., 2008; Haensler & Szoka, 1993; Shim & Kwon, 2009), and combinations thereof (Guillot-Nieckowski et al., 2007; Wu et al., 2001; Ewert et al., 2006; Takahashi et al., 2007; Matsui et al., 2006; Mustapa et al., 2009; Kogure et al., 2008), have been developed as nonviral gene carriers (Mintzer, M. A. & Simanek, E. E., 2009; also references cited therein). Substantial research has been reported on structure–activity relationships for cationic amphiphiles concerning the cationic and hydrophobic portions (Remy et al., 1994; Geall et al., 1999; Ewert et al., 2002; Byk et al., 1998; McGregor et al., 2001). Such amphiphiles form self-assembling micelles and liposomes in an aqueous phase, the structures of which have been investigated using small-angle X-ray scattering (SAXS), transmission electron microscopy (TEM), and atomic force microscopy (AFM) to gain knowledge about structure–activity relationship, particularly those involving ordered structures (lamellar, inverted hexagonal, and cubic phases) and their morphological changes (Koltover et al., 1998; Koynova, Wang & MacDonald, 2006) as well as about the size of complexes (Aoyama et al., 2003).

The mechanism of gene delivery by such cationic carriers probably involves an endosomal pathway (Wrobel & Collins (1995)): (i) cellular uptake via endocytosis, (ii) DNA release from endosome, and (iii) entry into the nucleus. Many researchers have devised cationic compounds that facilitate the process, for example, ligand-conjugated molecules targeting a receptor such as integrin (Mustapa et al., 2007; Varga, Wickham & Lauffenburger, 2000), pH-responsive or cleavable molecules that enable escape of DNA from endosome (Russ et al., 2008; Oupicky, Parker & Seymour, 2002; Dauty et al., 2001; Miyake et al., 2004; Anderson,

Lynn & Lange, 2003), and conjugation of nuclear localization signal peptides (NLS) (Zanta et al., 1999; Manickam & Oupicky, 2006) for steps (i)-(iii), respectively. For cationic lipids/DNA complexes (lipoplexes), it has been proposed that a morphological change from lamellar to inverted hexagonal phase in the acidic endosomal environment facilitates the endosomal release and escape of DNA (Bell et al., 2003; Xu & Szoka, 1996). In addition to investigation of intracellular trafficking of polycation-DNA complexes (polyplexes and lipoplexes), observation of morphology and metamorphosis of the complexes is very important to shed light on the mechanism of gene transfer and provide information for development of novel synthetic carriers (Koynova, Wang & MacDonald, 2006; Wan et al., 2008; Tarahovsky, Koynova & MacDonald, 2004).

We have reported that polycationic liposomes (PCL) containing cetylated polyethylenimine (cetyl-PEI) possess high gene transfer activity (Yamazaki et al., 2000; Oku et al., 2001; Matsuura et al., 2003). The cetyl-PEI molecule is anchored by the hydrophobic cetyl portion and is distributed over the liposomal surface. In our previous report, we proposed a possible mechanism of PCL-mediated gene transfer wherein PCL/DNA complexes are uptaken by endosomal pathway; this was based on tracking of fluorescence-labeled components, PCL lipid, cetyl-PEI, and DNA, which the release and transfer of cetyl PEI / DNA complex into the nucleus via the cytosol (Sugiyama et al., 2004). Compaction of DNA is therefore crucial, and both electrostatic and hydrophobic interactions in the cetyl PEI / DNA complex are responsible for its effective compaction.

PEI is used as a gene transfer vector by itself, however, it has inherent disadvantages, *i.e.*, cytotoxicity and polydispersity. We have previously reported successful syntheses of a series of polyamine-dicetyl phosphate (DCP) conjugates via reaction of a novel synthetic intermediate, dimerized DCP anhydride (compound 1 in Scheme 1), with various polyamines, spermidine, spermine, and PEI (Scheme 1) (Dewa et al., 2004a). Since spermidine and spermine are naturally occurring polyamines, we expected low cytotoxicity. When suspended in aqueous solution, they form micellar aggregates and exhibit moderate gene-transfer activity, the magnitude of which is relatively insensitive to the modification of the polyamine portion. We also observed the morphology of the conjugate / DNA complex by using atomic force microscopy (AFM). We discuss briefly the relation between the assembling structure of the conjugate/DNA and their transfection efficiency.

In this report, we describe (1) facile synthetic strategy of polyamine-lipid conjugates in brief and (2) evidence demonstrating that the transgene activity is dramatically enhanced when the conjugates are assembled into liposomes containing cholesterol and phospholipid and that the activity is susceptible to the chemical modification of the conjugate both in the polyamine and in the hydrophobic chain portions (Scheme 1). We show further that (3) gene transfer activity of the corresponding PCLs strongly depends on the type of polyamine in the conjugate, with notable differences between the lower molecular weight polyamines (spermidine and spermine) on one hand and the polymer type (PEI(1800)) on the other.

We also examined (4) the morphology of the lipoplexes by AFM and discuss the relationship between the structure of lipoplexes and their transfection efficiency. AFM analysis has a considerable advantage for observation of lipoplex morphology, especially for less ordered structures (Oberle et al., 2000), however, until now little clear evidence has been reported on the relationship between morphological change and DNA release. In this research, DNA release as a result of disassociation of the complex was revealed by AFM (Dewa et al., 2010). We discuss morphology-activity relationships on the basis of electrophoresis analysis,

dynamic light scattering (DLS) and AFM observation. Furthermore we introduce (5) our recent effort for synthesis of cleavable polyamine-lipid conjugates under a reductive cytosolic condition.

Scheme 1. Synthetic Strategy for Polyamine-lipid Conjugates.

2. Synthetic strategy of various polyamine-lipid conjugates via facile synthetic routes

In many cases, polycationic compounds have been synthesized through multi step reactions including protection/deprotection reactions on polyamine moieties. Our polyamine-lipid

compounds can be prepared via two-step reactions without such protection/deprotection reactions. The synthetic schemes are shown in Scheme 1, where two types of polyamine-lipid conjugate; dialkyl phosphate- and diacylphosphatidic acid-based compounds, are described. In this section, we showed a synthetic strategy for polyamine-lipid conjugates via facile routes (Dewa et al., 2004a,b, 2010).

2.1 Polyamine-dialkyl phosphate conjugates via a synthetic intermediate 1

Our preliminary idea for the syntheses of polyamine-dicetyl phosphate (DCP) conjugates was to use the bromoethylated compound, **2**, as a synthetic intermediate via condensation of DCP and 2-bromoethanol (Scheme 1) by using 1,3,5-triisopropylbenzenesulfonyl chloride, TPSCl (Uragami, Miyake & Regen, 2000; Tanaka, et al., 1987). Instead of the expected adduct, **2**, however, we obtained another product having high R_f value, 0.97 ($R_f = 0.56$ for DCP by eluting $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O} = 13/6/1$, v/v/v). The resulting product is stable enough to be isolated by column chromatography. SIMS indicates almost double molecular mass, 1075.4 (546.9 for DCP). The observed IR absorption band at 952 cm^{-1} is assignable to the P-O-P stretching mode. The ^{31}P NMR spectrum of the product, whose signals appear at $\delta = -12.27$ and -0.13 ppm, is clearly distinguishable from that of DCP having a signal at $\delta = 2.15$ ppm. This evidence, taken together, leaves no doubt that the product is dimerized DCP in anhydrous form, **1**, $[(\text{C}_{64}\text{H}_{133}\text{O}_7\text{P}_2)^+ \text{ calcd } 1075.9]$ connected via P-O-P bonding. There are some prior examples in the synthesis of pyrophosphate derivatives bearing small alkyl moieties, methyl, ethyl, propyl, and butyl (Cullis, Kaye & Trippett, 1987), however, the dimerized anhydride, **1**, bearing phospholipid-like long alkyl chains is an unprecedented compound. This compound is a convenient synthetic intermediate for forming phosphoramidate bonds, as described below.

The anhydride **1** readily react with amines, *e.g.*, spermidine, spermine, and even polymer, PEI(1800), to form the phosphoramidate, P-N bond, providing the corresponding adducts shown in Scheme 1. The reactivity toward these nucleophiles indicates that the anhydrous compound **1** is potentially a good synthetic intermediate for making polyamine-dialkyl phosphate conjugates via the P-N bond. When reacted with spermine, for example, the adduct **DCP-spm** readily forms, concomitantly with the loss of DCP. The ^{31}P NMR signal of compound **DCP-spm** is shifted downfield, to 9.76 ppm compared with that of DCP at 2.15 ppm, indicating the formation of the P-N bond in the compound. The down-field-shifted signal is attributed to the lower electronegativity of the nitrogen atom in spermine relative to the oxygen atom in DCP. Anhydride **1** is a useful synthetic intermediate because it is (1) stable yet reactive with amino groups, (2) very simple and easy to prepare, and (3) produced in high yield (~90 %). Furthermore, modification on the hydrophobic chain is easy when various dialkyl phosphates were used. These compounds could hence provide new category of polycationic lipids. Gall et al. have developed cationic lipid derivatives bearing P-N linkage, which possess gene transfer activity (Gall et al., 2010).

2.2 Polyamine-diacylphosphatidic acid conjugates via a synthetic intermediate 3

To diacylphosphatidic acid can be attached 2-bromoethanol with TPSCl, giving an intermediate **3**. When this reacted with an amino group of polyamine compounds via nucleophilic substitution reaction, a corresponding polyamine conjugate was formed as shown in Scheme 1 (Dewa et al., 2004b). Purified products can be afforded by column chromatography using an amino group-modified silicagel.

Fig. 1. Transfection efficacy of polyamine-DCP (A and B) and polyamine-DPPA conjugates (C) on VSMC, in the absence (A and C) and the presence of 20% of FBS (B). The ratios of compound/ β -galactosidase plasmid DNA (w/w) were 1.5/1, 3/1, and 6/1, respectively.

3. Transfection efficacy of various polyamine-lipid conjugates as micellar carriers

First, we describe the transfection efficacy of these polyamine-lipid conjugates. Figure 1A shows the efficacy of these carriers using β -galactosidase activity (milli-unit/well), in the absence of FBS. Compared with the commercially available transfection reagent, *O*-ethyl DOPC (E-DOPC) (MacDonald et al., 1999), which produced 0.1 milli-unit/well transgene expression, the compounds, **DCP-sp**, **DCP-spm**, and **DCP-PEI**, exhibit moderate efficiency, ~30 to 50 % of the efficiency of E-DOPC. The transfection efficiencies of the components themselves, *i.e.*, DCP, spermidine, spermine, and PEI(1800) were almost negligible. Thus, the transfection activity results from the conjugation of two moieties, a hydrophilic polyamine and a hydrophobic DCP. Compound **DCP-PEI** shows the highest efficiency at 3/1 (w/w) of **DCP-PEI** /DNA, whereas the efficiency of the other compounds are comparable and insensitive to the compound/DNA ratio within the range of error. In

Figure 1B is shown the transfection efficiency in the presence of 20% FBS. The efficiency of these compounds was not influenced by the presence of 20% FBS, retaining 80–100 % of the activity (except compound **DCP-PEI** at 3/1 (w/w)). Such serum-resistant activity was also observed for the PCL gene transfection system previously reported (Matsuura et al., 2003). It is well known that serum often inhibits transfection; such inhibition is due to binding of negatively charged serum proteins to the cationic transfection reagents resulting in forming aggregates ineffective to the transfection. Although it is not clear why the polyamine-DCP conjugates are not influenced by the presence of the serum, the polyamine part may be assumed to efficiently interact with DNA via electrostatic interactions. Polyamine-DPPA derivatives also exhibited transfection activity, whose extent is almost comparable to the polyamine-DCP derivatives (Figure 1C). The tendency of the activity is **DPPA-spd** ≥ **DPPA-spm** > **DPPA-PEI**. In the following section, we will discuss on the relationship between gene transfer activity and morphology of polyamine-lipid complexes.

4. Morphology of the micellar and complexes with DNA

It was found that DNA (ColE1 plasmid DNA) complex with various polyamines and polyamine-lipid conjugates by monitoring decrease of fluorescence from EtBr initially intercalated into DNA. With an increase in the cation (N) /anion (P) ratio, defined as N/P, the relative fluorescence intensity decreased as a result of complexation of DNA and polyamine. The polymeric molecule, PEI(1800) (N ~ 42 per molecule) most efficiently forms a complex with DNA; the complexation was almost complete at N/P = 3. Spermidine (N = 3 per molecule) and spermine (N = 4 per molecule) are less effective than PEI(1800), however, with complexation being complete at around N/P~7. For polyamine-DCP conjugates, the tendency for the fluorescence intensity to decrease is due to the complexation with DNA in the same way as that of the free amines, that is, **DCP-spd** < **DCP-spm** < **DCP-PEI**. DPPA-polyamine conjugates exhibited similar DNA condensation manner to DCP-polyamines, however, the tendency of DNA condensation was opposite way. The EtBr replacement experiment suggested that the tendency of DNA condensation is **DPPA-spd** > **DPPA-spm** > **DPPA-PEI**; the order is opposite to that of DCP-conjugates with regard to the polyamine portions.

In Table 1, the size of the micellar and complex with DNA was summarized. The mean particle diameters of various polyamine-DCP conjugates in an aqueous suspension, as given by dynamic light scattering is summarized in Table 1: 155 ± 54 nm for **DCP-spd**, 173 ± 46 nm for **DCP-spm**, 128 ± 38 nm for **DCP-PEI**, 90 ± 8 nm for **DPPA-spd**, 106 ± 41 nm for **DPPA-spm**, and 218 ± 35 nm for **DPPA-PEI**. AFM images for the suspension of these compounds exhibited spherical or ellipsoidal particles, whose sizes, defined according to diameter for the spheres and major × minor axes for the ellipsoid, were 132 nm for **DCP-spd**, 156 nm for **DCP-spm**, 209 × 145 nm for **DCP-PEI**, 96 nm for **DPPA-spd**, 111 nm for **DPPA-spm**, and 201 nm for **DPPA-PEI** (Figures 2A, B, C, D, E, and F, respectively). The particle sizes for these compounds as observed by AFM show good agreement with those obtained by DLS. The molecular shapes of these compounds are regarded to be the “cone” type, due to the attachment of the large polyamine moiety. We have endeavored to make liposome from these compounds, however, entrapment of a fluorescence probe, calcein, was impossible. Therefore, we assume that the particle consisting of polyamine-DCP conjugates is a micelle-like aggregate.

Compound	DLS (nm)		AFM / shape (size (nm)) and height (nm) ^{b)}			
	Micellar aggregate	Complex with DNA	Micelle aggregate		Complex with DNA	
DCP-spd	155 ± 54	409 ± 115	sphere (132)	21	aggregate (~1000 × 437)	16
DCP-spm	173 ± 46	237 ± 127	sphere (156)	24	aggregate (569 × 317)	20
DCP-PEI	128 ± 38	115 ± 40	ellipsoid (209 × 145)	12	sphere (120)	20
DPPA-spd	90 ± 8	140 ± 40	sphere (96)	24	sphere (129)	21
DPPA-spm	106 ± 41	109 ± 40	sphere (112)	16	sphere (172)	37
DPPA-PEI	218 ± 35	205 ± 39	aggregate (201)	12	aggregate (201)	17

a) The ratio of polyamine conjugate / DNA was 3/1 (w/w). Complexation of polyamine conjugate with DNA was carried out in water.

b) Sample solution was spread on a mica surface and dried. AFM images were obtained under dry condition. Width and height of the complexes were estimated from the AFM images in Figs 2 and 3.

Table 1. Estimated size and shape of polyamine-lipid micelles and complexes with Cole1 DNA suspended in aqueous solution ^{a)}

Fig. 2. AFM images of arrays of compounds, **DCP-spd** (A), **DCP-spm** (B), **DCP-PEI** (C), **DPPA-spd** (D), **DPPA-spm** (E), and **DPPA-PEI** (F). The compound was suspended in distilled water then dropped onto a mica surface by spin coating. All scale bars represent 200 nm. The object indicated by the arrow is discussed in the text.

Particle sizes of the conjugate/DNA (3/1: w/w) complex evaluated by DLS is 409 ± 115 nm for **DCP-spd**, 237 ± 127 nm for **DCP-spm**, 115 ± 40 nm for **DCP-PEI**, 140 ± 40 nm for **DPPA-spd**, 109 ± 40 nm for **DPPA-spm**, and 205 ± 39 nm for **DPPA-PEI**, respectively (Table 1). With increase in the size of polyamine portion in DCP-conjugates, the particle size significantly decreases, whereas opposite tendency was observed in DPPA-conjugates. AFM images support the tendency. Figure 3 shows an AFM image of DNA (A) and of the complexes it forms with various polyamines (B – I). Figure 3A reveals a clear image of partially-coiled Cole1 plasmid DNA (6646 bp), whose size is estimated to be 300~ 400 nm.

The height is ~ 2 nm, corresponding to the diameter of B-form DNA, ca. 2.4 nm. When DNA was complexed with compound **DCP-spd**, a “spider nest”-like structure was observed (Figure 3D). The size of the “quasi-ellipsoidal core region” is $\sim 1000 \times 437$ nm and the height is ~ 16 nm. The height of the radiating peripheral “nest” region is ~ 2 nm, suggesting the nest region consists of free DNA. The height of the core region (~ 16 nm) is clearly larger than that of the DNA part, therefore, the core region of the structure must consist of the **DCP-spd**/DNA complex. Thus, complexation of compound **DCP-spd** and DNA gives rise to a segregated array, having complex and free DNA portions. The compound **DCP-spm** forms a similar but distinguishably different complex structure with DNA (Figure 3E), which resembles a “pearl necklace-like” aggregate (Yoshikawa et al., 1996), 569×317 nm in the plane of the substrate and 4–20 nm in height. The size of the pearl parts is 120–170 nm in diameter and 11–20 nm in height. These parts are connected by a region that is 4–5 nm in height. It appears from the image that the assembly consists of a tightly packed **DCP-spm**/DNA complex and regions of partially compacted DNA parts. Pronounced compaction of the complex was observed for the **DCP-PEI**/DNA, which formed a spherical cluster (120 nm in width and ~ 20 nm in height) (Figure 3F). The order of increasing compaction of DNA, **DCP-spd** < **DCP-spm** < **DCP-PEI**, is consistent with the extent of intercalation of EtBr as mentioned above, that is, the more tightly packed was the DNA complex, the lower was the extent of intercalation of EtBr. The tendency of size of the complex and the DNA condensation for DPPA-polyamine conjugates, that is, **DPPA-spd** (G) > **DPPA-spm** (H) > **DPPA-PEI** (I). One may wonder why the morphologies of the complexes, DCP- and DPPA-based conjugates, as well as **DCP-spd** and **DCP-spm** with DNA are so different despite the structural difference in cationic portion seems very little. It might come from the difference in delicate balance of hydrophilic/hydrophobic factor and/or molecular shapes which reflect a packing parameter.

Upon comparison, of the observed DNA complex with free polyamines, spermine (Figure 3B) and PEI (Figure 3C), the complex size and shape are obviously distinguishable from the corresponding conjugate forms; much larger complexes form with these free polyamines. The dimension of the spermine/DNA complex (Figure 3B) is ~ 3 μ m; the compaction of DNA is obviously incomplete, judging from the presence of the “nest” portion of DNA that are similar to peripheral part of the **DCP-spd**/DNA complex (Figure 3D). The PEI/DNA complex is smaller (416×218 nm by 22 nm high) than the former complex, suggesting that the greater cationic charge makes the complex smaller. When one considers the effect of the hydrophobic portion in the polyamine compound on the size of the complex, it is clear that hydrophobic alkyl parts in the conjugates play an important role in the compaction of DNA, which is most prominently observed in conjugate **DCP-PEI** (Figure 3C vs 3F). From these results, the prominent transfection efficiency of **DCP-PEI** likely results from the more efficient compaction of DNA in the complex **DCP-PEI**/DNA, whereas the lower activity of **DCP-spd** and **DCP-spm** is likely due to their much weaker compaction.

Although the precise mechanism remains to be clarified, the compaction by these polyamine compounds probably plays an important role because entry into the nucleus is thought to be a key step and a smaller complex is likely to be associated with more efficient transfection. The conjugates in this study possibly decompose into separate polyamine and DCP portions in endosome. We assume that two critically important factors are involved in the present transfection system; compaction of DNA by polyamine part and with hydrophobic dialkyl portion and decomposition of the complex in endosome so as to liberate the

DNA/polyamine complex to be transported into nucleus. As previous report, DNA accompanied with cetyl-PEI enters into nucleus.

Fig. 3. AFM images of polyamine-Cole1 plasmid DNA complexes: (A), Cole1 plasmid DNA alone on polylysine-treated mica; (B), spermine/DNA; (C), PEI(1800)/DNA; (D), **DCP-spd**/DNA; (E), **DCP-spm**/DNA; (F), **DCP-PEI**/DNA; (G), **DPPA-spd**/DNA; (H), **DPPA-spm**/DNA; (I), **DPPA-PEI**/DNA. The polyamine/DNA ratio was 3/1 (w/w). Scale bars inserted in these images represent (A) 200, (B) 1000, (C) 200, (D) 500, (E) 500, (F) 200, (G) 500, (H) 500, and (I) 200 nm, respectively. The objects indicated by arrow(s) are discussed in the text.

In this section, we described novel types of polyamine-dialkyl phosphate conjugates that have moderate gene transfection activity for β -galactosidase assay. These conjugates are easy to prepare via a novel synthetic intermediate, dimerized DCP anhydride, **1**. The synthetic approaches described herein are flexible and possess potential for the rationale design of highly efficient gene carriers with single or narrow ranges of molecular weight.

5. Morphological effect of polyamine-lipid/DNA complexes on their transfection activity

As described above, when the polyamine-lipid conjugates were suspended in aqueous solution, they form micellar aggregates and exhibit moderate gene-transfer activity, the magnitude of which is relatively insensitive to the modification of the polyamine portion. Here we describe that preformed bilayer structure (as polycation liposomes, PCLs) significantly improves transfection efficacy compared with micellar aggregate form.

5.1 Preparation of Micellar Aggregates, PCL Vectors, and their complexes with DNA

Polycation liposome suspensions were typically prepared as follows: polyamine conjugate, phospholipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and cholesterol (1/1/1 as a molar ratio) were dissolved in t-butyl alcohol. After removal of the solvent under reduced pressure, the residual solvent was removed by freeze-drying overnight. The lyophilized powder was hydrated with Tris-HCl buffer (20 mM, pH 8.0) followed by three freeze-thaw cycles and the resultant suspension was then subsequently extruded through polycarbonate membranes of 0.4, 0.2, and 0.1- μ m pore diameter at room temperature. A suspension of the polyamine conjugate alone was prepared in the Tris-HCl buffer (20 mM, pH 8.0) by ultrasonication for 3 min. For convenience, particles so prepared are termed “micellar aggregates”. Hereafter, polycationic liposomes (PCL) composed of the conjugates are described as “**conjugate(PCL)**”, such as **DCP-spd(PCL)**. Otherwise, micellar aggregates are described as just the name of conjugate, such as **DCP-spd**.

A plasmid encoding luciferase gene, pCAG-luc3 (6480 bp, a gift of DNAVEC Institute, Tsukuba, Japan), was amplified in *E. coli* JM109 (Nippon Gene, Toyama, Japan) and purified as described before (Matsuura et al., 2003). One microgram of the plasmid DNA in a TE buffer was added to a suspension of PCL containing 1 mM of polyamine conjugate so as to give the desired nitrogen/phosphate ratio, N/P. The mixture was incubated for 20 min at room temperature when used for transfection.

Fig. 4. Transfection efficacy of polyamine-lipid conjugates, **DCP-spd** (A), **DCP-spm** (B), and **DCP-PEI** (C), and their constituents, DCP (A) and polyamines, spermidine (spd, A), spermine (spm, B) and PEI1800 (PEI, C), on COS-1 cells. Efficacy was evaluated with the luciferase activity. The observed values for spermidine and spermine indicated by the asterisks (*) were apparently negligible on the activity scale shown. The conjugates **DCP-spd**, **DCP-spm**, and **DCP-PEI** represent their micellar aggregate forms, and **DCP-spd(PCL)**, **DCP-spm(PCL)**, and **DCP-PEI(PCL)** represent the conjugate-based PCLs (conjugate/DOPE/cholesterol = 1/1/1 (mol/mol/mol)). The nitrogen/phosphate (N/P) ratio was 16/1 for the polyamine conjugate/DNA complexes. For the monoanionic DCP, the molar ratio, DCP/nucleotide = 16/1, was applied as a negative control experiment. Transfection was conducted in the presence of 10% FBS.

5.2 Transfection procedure

COS-1 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Japan Bioserum Co. Ltd.) under a humidified atmosphere

of 5% CO₂ in air. One day before a transfection experiment, 1×10⁵ COS-1 cells were seeded onto each of several 35-mm dishes and incubated overnight in a CO₂ incubator. Then, the cells were washed twice with DMEM, and a suspension of lipoplex (1 μg DNA) was added to them in the presence of 10% FBS-DMEM. After 3 h incubation (37°C, 5% CO₂), the cells were washed twice with DMEM and cultured for another 48 h in 10% FBS-DMEM. The cells in the 35-mm dishes were washed twice with phosphate-buffered saline at 37°C, and 200 μL of cell lysis buffer (LC-β, TOYO B-Net Co. Ltd., Tokyo) were added. After 15 min incubation, the cells were collected with a cell scraper, frozen at -80 °C, and then thawed at room temperature. The lysate was centrifuged at 15,000 rpm for 5 min at 4 °C. The supernatant was subjected to the luciferase assay (Pica Gene, TOYO B-Net Co. Ltd., Tokyo) using a luminophotometer (Luminescencer-PSN AB-2200, ATTO). The observed intensity in instrument light units was normalized to the amount of protein determined by BCA protein assay Kit (PIERCE) to give a relative light unit (RLU/mg protein).

5.3 Transfection efficacy of micellar aggregates and PCL vectors: Comparison of the dicetyl phosphate derivatives of spermidine, spermine, and polyamine

Figure 4A shows the transfection efficacy of the polyamine conjugate, **DCP-spd**, and its constituent molecules, DCP and spermidine (spd). The conjugate (in this case an aqueous micellar suspension) shows greatly increased efficacy relative to the constituent molecules, DCP and spermidine. For these polyamine/DNA complexes, the nitrogen/phosphorous ratio (N/P) was 16. The data of the figure indicate that coupling the lipophilic and cationic portions is essential to obtain gene transfer. Such an effect of conjugating these two moieties was also observed for the spermine conjugate, **DCP-spm** (Figure 4B). When the conjugates were further formulated with DOPE and cholesterol (conjugate/DOPE/cholesterol = 1/1/1 (mol/mol/mol)) to generate polycationic liposomes (PCL) (**DCP-spd**(PCL) and **DCP-spm**(PCL)), efficacies were further enhanced by a factor of 2–3 relative to the micellar aggregate suspensions (**DCP-spd** and **DCP-spm**). The polycation PEI(1800) itself showed moderate activity and the conjugate, **DCP-PEI**, exhibited even greater activity. However, in contrast to the other conjugates, the activity of the liposomal form, **DCP-PEI**(PCL), was comparable to that of the micellar version, **DCP-PEI**. The cytotoxicity of the conjugates, both as micellar aggregates and PCLs, was low; the latter vectors, which exhibited higher activity, also had slightly higher toxicity than the former.

It was found that the DOPE-based PCL exhibits significantly greater activity than the DPPC-based compound for both of the **DCP-spd**(PCL) and the **DCP-spm**(PCL). This is indicative of a lipid-mediated gene transfer mechanism; DOPE is well-known as a “helper” lipid, which is believed to facilitate membrane fusion and endosomal escape of the DNA (Felgner et al., 1994). DOPE in the present PCL systems presumably also plays a role in the mechanism to expedite membrane fusion and destabilization of the endosomal membrane. DPPC, whose T_m is 41.5 °C, renders the PCL more stable and more rigid than does DOPE. Thus, it is likely that the fusogenic property of DOPE is responsible for the enhanced transfection activity of its complexes relative to those containing DPPC.

5.4 Optimal structure of the conjugate molecules for gene transfer

The facile synthetic route provides a variety of polycationic compounds that can be exploited to examine the effect of the polycationic and hydrophobic portions on transfection efficiency. We hence examined the effect on transfection activity of different polyamine

Fig. 5. (A) Effect of polyamine and hydrophobic portions on PCL-mediated gene transfer efficiency. PCL were composed of polyamine conjugate/DOPE/Cholesterol (1/1/1 mol/mol/mol). N/P ratio was 24. Transfection was done in the presence of 10% FBS. LF represents Lipofectamine™ 2000 as a positive control experiment. (B) Effect of the N/P ratio on the transfection efficacy of **DCP-spm(PCL)** (**DCP-spm**/DOPE/cholesterol = 1/1/1 (mol/mol/mol)). Transfection was in the presence of 10% FBS.

conjugates incorporated into PCLs: C12, C14, or C16 alkyl chain in the lipophilic portion and spermidine, spermine, or PEI(1800) as the polycationic head group of the conjugate. The data on these compounds are shown in Figure 5A. This result reveals clear tendencies of longer length of the alkyl group and the lower molecular weight of the polyamines (spermidine, spermine) to enhance transfection. When compared with a commercial product, Lipofectamine™ 2000, the **DCP-spd(PCL)** possessed 3.6-fold higher activity.

5.5 N/P-dependent efficacy and complexation of PCL with DNA

Figure 5B shows the dependence of **DCP-spm(PCL)** efficacy on the ratio of the number of nitrogen atoms in the conjugate to that of phosphate in the DNA (N/P). The efficacy increases with the N/P ratio essentially linearly up to 24. A similar N/P-dependence has been also observed for **DCP-spd(PCL)** (data not shown for clarity), indicating that excess polyamine relative to DNA is needed for effective gene transfer by PCLs. In contrast, the transfection activity of micellar aggregates **DCP-spd** and **DCP-spm** reaches plateau values in the N/P range of 11–16 ($1\sim 2 \times 10^9$ of RLU/mg protein). This tendency is consistent with our previous data obtained with the β -galactosidase expression system (Dewa et al., 2004a).

5.6 Chemical structure of the polyamine conjugates

The polyamine-dialkyl phosphate conjugates can be readily synthesized via a two-step reaction: (i) formation of dimerized dialkyl phosphate anhydride and (ii) its nucleophilic substitution with polyamines. The synthetic strategy gives access to a wide variety of polyamine-dialkyl phosphate derivatives. Conjugation of the polyamine and hydrophobic portions is required for an effective gene carrier (Figure 4). Such amphiphilicity is essential

to condense DNA molecules, which requires both electrostatic and hydrophobic interactions (Yamazaki, 2000; Dewa et al., 2004a,b). We tested a number of combinations of dialkyl and polyamine portions for their activity in gene transfection. The longer alkyl chain exhibited higher efficiency (Figure 5A). The ζ -potential of the DOPE-based PCL increased with alkyl chain length, for example, 27.6, 33.4, and 37.1 mV for **DLP-spd**(PCL), **DMP-spd**(PCL), and **DCP-spd**(PCL), respectively, showing that the conjugate with the longer chain length provides the higher positive potential. The hydrophobic interaction results in stable incorporation of the conjugate into the PCL, consistent with transfection activity in the order of C16 $\geq$ C14 > C12. We found that the micellar aggregate of **DCP-PEI** conjugate exhibited slightly higher transfection activity than the low-molecular weight amine conjugates, **DCP-spd** and **DCP-spm**, however, the activity of **DCP-PEI**(PCL) was marginal. The size of **DCP-PEI**(PCL)/DNA lipoplex is significantly greater than that of the other lipoplexes (Table 2, entry 14). The **DCP-PEI** conjugate is composed of DCP/PEI(1800) = $\sim 1/1$, judged by ^1H -NMR (Dewa et al., 2004a). As previously reported, the cetyl-PEI, whose PCL possesses high transfection activity, consists of 10 cetyl portions in the polymer (Matsuura et al., 2003). The cetyl-PEI can attach to the PCL surface via the anchoring of cetyl portions in the lipid bilayer. However, that is not the case for **DCP-PEI**(PCL); the single hydrophobic portion in the conjugate is not enough to provide adequate covering of PEI over the PCL surface. This may cause “PEI-protrusion” from the surface, which gives rise to the large and heterogeneous aggregation seen upon combination with DNA molecules. This is likely the reason for the lower activity of the **DCP-PEI**(PCL). Taken together, these considerations suggest that a homogeneous positive charge distribution on the PCL surface is important to the transfection activity.

5.7 Morphological analysis of the complexes with DNA

5.7.1 Formation of complex with DNA

To elucidate the characteristics of N/P-dependence, formation of **DCP-spd**(PCL)/DNA and **DCP-spm**(PCL)/DNA lipoplexes was analyzed by agarose gel electrophoreses and DLS analysis. Electrophoretic analysis showed N/P ratio-dependent complexation; in the lower N/P range of 5–16, the open circular DNA band vanished and the supercoiled DNA band gradually faded for both of **DCP-spd**(PCL) and **DCP-spm**(PCL) and in the higher N/P range, 20–30, the latter DNA band totally disappeared. These observations indicate that the DNA molecules are completely entrapped within the lipoplex. Ethidium bromide (EtBr) replacement experiments also reveal condensation of DNA in the N/P range of 5–30 (Dewa et al., 2010).

The particle size of lipoplexes estimated by DLS analysis is summarized in Table 2. The diameters of the **DCP-spd**(PCL) and **DCP-spm**(PCL) alone are 158 ± 56 , and 159 ± 30 nm, respectively (entries 1 and 7). Lipoplexes were larger and their size increased with increasing N/P up to 5 (entries 2 and 8 at N/P = 2 and entries 3 and 9 at N/P = 5). At N/P = 5, the PCL/DNA lipoplexes became larger with a broad distribution from 650 nm to over 1 μm (entries 3 and 9). In the higher N/P range (N/P = 16–24), sizes were reduced, converging at 261 ± 114 nm (**DCP-spd**(PCL), entry 5) and 256 ± 116 nm (**DCP-spm**(PCL), entry 11). ζ -potential measurements indicated the polarity of surface charge of lipoplexes inverts from negative in the lower N/P (5–11) to positive in the higher N/P (>16) regions.

AFM images revealed characteristic morphologies of lipoplexes in the both low and high N/P ranges (Figures 6A–D). When the **DCP-spd**(PCL)/DNA and **DCP-spm**(PCL)/DNA lipoplexes at N/P = 5 were put on PLL-treated mica (positively charged surface), large

aggregates in the sub-~micrometer size range (600–1200 nm, Figures 6A and B) were observed. These structures resembled by bead-like aggregates (Yoshikawa et al., 1996) composed of small particles (80–120 nm in diameter, 8–20 nm in height) connected to one another. Such aggregates were not observed on a negatively-charged bare mica. This is understandable since the lipoplex at N/P = 5 is negatively charged, and the lipoplex must be adsorbed on the PLL-mica surface through electrostatic interaction to be imaged. The outer periphery of the large aggregates is rich in DNA molecules, presumably those were so loosely attached that they were liberated from the aggregates during electrophoresis.

entry	compound	N/P ratio	pH	size (nm)	
				PCL	Micellar aggregate
1	DCP-spd	—	8	158 ± 56	249 ± 106
2		2	8	231 ± 89	
3		5	8	651 ± 501	1181 ± 1057
4		16	8	190 ± 83	
5		24	8	261 ± 114	1791 ± 1630
6	DCP-spm	24	4	985 ± 1437	1624 ± 1566
7		—	8	159 ± 30	225 ± 102
8		2	8	293 ± 112	
9		5	8	764 ± 378	321 ± 181
10		16	8	296 ± 168	
11	DCP-PEI	24	8	256 ± 116	1767 ± 1284
12		24	4	1033 ± 1060	1209 ± 949
13		—	8	214 ± 90	275 ± 184
14		24	8	1062 ± 783	317 ± 243

Table 2. DLS analysis of various polyamine-dicetyl phosphate conjugate/DNA complexes

At the high N/P = 24, spherical complexes were observed for **DCP-spd**(PCL) and **DCP-spm**(PCL) complexes; their diameters were 200–400 nm for **DCP-spd**(PCL)/DNA (C) and 150–250 nm for **DCP-spm**(PCL)/DNA lipoplexes (D), and their heights were 12–30 and 27–60 nm, respectively. The size of the complexes is in good agreement with the values observed by DLS. The detailed topography of these **DCP-spd**(PCL)/DNA and **DCP-spm**(PCL)/DNA lipoplexes showed flat-topped spheres and spherical structures (line profiles (i)–(iii) for (C) and (iv)–(vi) for (D)). The profiles (ii) and (iii) are characteristically “step-like” (arrows). The heights indicated in (ii) are 6, 10, and 14 nm. Considering the thickness of lipid bilayer (4 nm) and the diameter of DNA (2 nm), these three values correspond to one bilayer (4 nm) + DNA (2 nm) = 6 nm, a double bilayer (8 nm) + DNA = 10 nm, and a triple bilayer (12 nm) + DNA = 14 nm, respectively. Thus, the step-like structure is reasonably indicative of a smectic lamellar assembly, where DNA molecules are laminated between bilayers.

Compared with **DCP-spd**(PCL) and **DCP-spm**(PCL) lipoplexes, the size of the **DCP-PEI**(PCL)/DNA lipoplex (N/P = 24) is large and has a broad distribution (1062 ± 783 nm, Table 2, entry 14). AFM images of the **DCP-PEI**(PCL)/DNA lipoplex show aggregates with heterogeneous and featureless shapes (Figure 6E and F). EtBr replacement experiments revealed DNA condensation in a similar manner to that of **DCP-spd**(PCL) and **DCP-spm**(PCL).

Fig. 6. AFM images of PCL/plasmid DNA (ColE1; 6646 bp) lipoplexes: (A), **DCP-sp_d**(PCL)/DNA (N/P = 5); (B), **DCP-sp_m**(PCL)/DNA (N/P = 5); (C), **DCP-sp_d**(PCL)/DNA (N/P = 24); (D), **DCP-sp_m**(PCL)/DNA (N/P = 24); (E and F), **DCP-PEI**(PCL)/DNA complex (N/P = 24). Scale bars shown in the images are 500 nm (A, B, and D) and 1000 nm (C, E, and F). The PCL/DNA complexes were applied to PLL-mica (A and B) and bare mica (C, D, E, F). All images were taken under an ambient air conditions. Height profiles of the objects (i)-(iii) in C and (iv)-(v) in D are shown below these images. Arrows in (ii) and (iii) indicate step-like profiles discussed in the text. Images E and F for **DCP-PEI**(PCL)/DNA were taken from a different area of the bare mica surface.

5.7.2 Effect of bilayer structure on the transfection activity

In the series of polyamines tested in this study, the low molecular weight polyamines were found to be more effective gene carriers when these conjugates were assembled into PCLs. The transfection activity of the PCL was ~3 times larger than as the corresponding micellar aggregate (Figure 4). The effect of the helper lipid, DOPE, was clearly substantial when compared with DPPC used instead of DOPE. DOPE, a predominantly non-lamellar lipid, is thought to facilitate fusion and destabilization of the endosomal membrane after uptake of cationic lipid/DNA complexes into a cell. In our previous report, we described intercellular trafficking of PCL (composed of cetyl-PEI and DOPE)-DNA complexes, which were taken up into cells by endosomal pathway (Sugiyama et al., 2004), followed by endosomal escape. In fact, transfection activity of the present PCLs was inhibited by nigericin, which is able to dissipate the pH gradient across the endosomal membranes, by 30-50%, suggesting that endosomal pathway is likely involved in the mechanism in the present lipoplex system. It appears therefore, that the mechanism of the lipofection by the compounds in this study may be similar to that of this and other agents known to be enhanced by DOPE. The lipoplexes made from the bilayer-structured PCLs evidently involve lamellar assemblies, given that AFM images reveal the presence of step-like profiles ((ii) and (iii) in Figure 6). The step-like profiles imply a lamellar complex, in which DNA rods (2 nm in diameter) are laminated between bilayers (4 nm thickness). Such an intrinsic bilayer structure may predispose lipoplexes to interact with cell and endosomal membranes. This is not the case for micellar aggregates, whose morphology is large spheres, in which polyamine conjugate

and DNA molecules likely aggregate randomly (Table 2 entry 5). This may be one of the reasons for the higher activity of PCL-based lipoplex, whose size is more favorable to transfection.

The linear dependence of activity on N/P (Figure 5B) is related to the morphology of the lipoplex. The electrophoresis experiment and AFM images (Figures 6A-D) suggest a reasonable explanation of the dependence, namely, the following: In the low N/P range (~ 5), the PCLs inadequately condense DNA molecules, giving the bead-like structures (Figures 6A and B). The DNA molecules loosely packed in the complex are readily released during electrophoresis. Such a complex, whose ζ -potential is negative, is too large to be introduced into the cell membrane via endocytosis; therefore the transfection level is low. With increasing N/P ratio, the morphology of the lipoplex transforms from large bead-like structure into smaller particles, wherein DNA molecules are condensed more tightly (Figures 6C and D). The size of the lipoplexes, whose ζ -potential is positive, is 150–400 nm, is more favorable for cellular uptake via endocytosis (Koynova, Wang & MacDonald, 2006). Given that the lamellar assembly in the lipoplex is responsible for its effectiveness as a gene carrier (Koltover et al., 1998; Koynova, Wang & MacDonald, 2006), the population of active species for gene transfer would increase with increasing in the N/P. Although highly positive-charged carriers are generally toxic, the PCL described here exhibit low cytotoxicity, an advantage for in vitro and in vivo applications.

5.7.3 Disassembly of the lipoplexes and DNA release

When the **DCP-spd(PCL)/DNA** and **DCP-spm(PCL)/DNA** lipoplexes (N/P = 24) were incubated in acidic solution (down to pH 4), the particle sizes measured by DLS became significantly larger and exhibited broad distributions (Table 2, entries 6 and 12). AFM imaging revealed morphological transformation of the PCL/DNA complexes upon acidification. When the dispersion of **DCP-spd(PCL)/DNA** lipoplexes (N/P = 24) was acidified at pH 4 for 1 h by addition of acetic acid, deformed structures were observed on bare mica (Figure 7A). Relative to the original structure (Figure 6C), the complex is decisively deformed by the acid treatment. Although some of flat-topped sphere complexes remain, the predominant morphology is particles connected with strings. The height of the clusters is 25–53 nm and they are connected with string portions that are 6–10 nm high. When the acid-treated complex solution was put on PLL-mica, additional deformed objects appeared on the surface (Figure 7B). A “beads on a string” deformed structure is composed of very small particles (50–100 nm in diameter) and string parts (~ 70 nm in width and 2–5 nm in height). The beads on a string structure observed on the positively charged surface must consist of DNA-rich fragments associated with some lipid components. The **DCP-spm(PCL)/DNA** lipoplex maintains its spherical structure on bare mica (Figure 7C). On the PLL-mica, on the other hand, deformed structures were observed as in the case of **DCP-spd(PCL)/DNA** lipoplex (Figure 7D). Such morphological changes upon acidification result from disassembly of the lipoplexes and the accompanying DNA release. Gel electrophoretic analysis provided evidence for the DNA release; the released plasmid band increased with the acidification from pH 8 to pH 4. In sharp contrast, such a morphological change was not observed for the micellar aggregate **DCP-spd**. DLS analysis indicates an insensitivity of the micellar aggregates to acidification (Table 2, entries 6 and 12).

Facile escape from the acidic endosomal compartment is necessary for efficient gene transfer. Disassembly of the lipoplex associated with DNA release has been clearly

observed by AFM imaging (Figure 7) and by electrophoretic analyses. Upon acidification (down to pH4), the extent of protonation of the polyamine portion is increased. Assuming that the lipoplex forms smectic lamella, electrostatic repulsion between layers must be increased with such protonation, resulting in the disruption of the lipoplex. For lipoplexes composed of **DCP-sp_d**(PCL) and **DCP-sp_m**(PCL), disruption accompanying DNA release has been confirmed. In sharp contrast, the size and shape of micellar aggregate/DNA complexes composed of **DCP-sp_d** and **DCP-sp_m** are insensitive to acidification (Table 2, entries 6 and 12). This would be due to their amorphous structure, which does not respond to pH change. Thus, the disassembly of the lipoplex composed of the bilayer-structured PCL is essential in effective gene transfer, especially in the process of endosomal escape, where lipid exchange and flip-flop are involved in disrupting the membrane and leading to DNA release (Xu & Szoka, 1996). Although the acidity of this experimental condition (pH4) seems higher than that in endosome (pH5.5), such a protonation process on the polyamines should be involved in the endosomal environment, because the protonation on the polyamines whose pK_a values are > 8 may proceed rather gradually in the acidic region, especially in the self-assembled lamellar structure, where polyamines are densely packed. This finding suggests a strategy for molecular design—especially in the polyamine portion—in which a morphological transformation of lipoplexes is taken advantage of for new nonviral transfection strategies.

Fig. 7. AFM images of disassembled lipoplexes, **DCP-sp_d**(PCL)/DNA (A and B) and **DCP-sp_m**(PCL)/DNA (C and D) by acidification at pH 4. The acid-treated suspensions of complexes were put on bare mica (A, C) and PLL-mica (B and D). After removal of the solution the images were acquired. Scale bars: 2000 nm (A), 1000 nm (B), 500 nm (C), 1000 nm (D).

6. Cleavable peptide-phospholipid conjugates under physiological conditions

Detachable conjugates between cationic and hydrophobic lipid portions may facilitate the DNA release from complexes in intracellular reductive environment. This is a promising strategy for improvement of efficacy of non-viral gene delivery. Such approaches have been reported using disulfide-linked polymer (Oba et al., 2008) and gemini lipids (Behr). Recently, we have successfully synthesized oligo-arginine bearing phospholipids via disulfide linkage, DPPE-SS-R_n (Scheme 2). Oligo-arginines, e.g., TAT peptide, are well known for effective carriers across cell membranes (Futaki et al., 2001). The reaction scheme is shown in Scheme 2. In brief, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-ethanolamine (DPPE) was reacted with a heterobifunctional coupling agent, *N*-succinimidyl-3-(2-pyridyldithio)-propionate

Scheme 2. Synthetic strategy for peptide-phospholipid conjugates

(SPDP) to form compound **4**, followed by coupling with oligopeptides, $(Arg)_nCys$ (R_nC ; $n = 5, 8$). Undetachable type of analogous conjugates (DPPE-S- R_n) were also synthesized with a coupling agent, *N*-(4-maleimidobutyroxy)succinimide (GMBS) and DPPE to form compound **5**, followed by coupling with $(Arg)_nCys$. When the disulfide-type conjugates were applied to gene delivery assay in vitro, 3-5 times higher transfection activity was observed compared with the corresponding undetachable conjugates. This result suggests that such a cleavable type of peptide-phospholipid conjugate is one of promising components to assemble effective non-viral vectors.

7. Conclusions

We described herein that PCL composed of the low-molecular-weight polyamine conjugates, DCP-spermidine (**DCP-spd**) and DCP-spermine (**DCP-spm**), exhibit much higher gene transfer activity than PEI(1800) conjugate-based **DCP-PEI(PCL)**. The former compounds generate 150–400 nm diameter lipoplexes whereas the latter gives rise to large aggregates. In the case of the former compounds, AFM images clearly reveal a morphological change upon acidification, indicating DNA release from the lipoplexes, whereas, in contrast, the morphology of micellar aggregates is insensitive to pH change. A

pH-dependent transformation is crucial in gene transfer; and the chemical structure of the polyamine portion may therefore play an important role in the acidification-induced transformation. We have also described the relation between the N/P-dependence of transfection activity and the morphology of the lipoplexes as revealed by AFM. Morphological study with AFM provided useful information for understanding the basis of lipoplexes with superior activity and for design strategies leading to optimally efficient gene carriers. Our recent efforts have demonstrated that such a DCP-polyamine-based liposomes are effective for systemic siRNA delivery (Asai et al., 2001).

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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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