Polymeric Gene Carriers

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1 Introduction

The concept of polymeric drugs for use as pharmaceuticals was first proposed around 1970 by Ringsdorf (1975). In this system, a water-soluble polymer is selected as a carrier material, to which organ-targeting moieties are bound along with the parent pharmaceuticals. The targeting moieties lead the drugs to the site of action by active targeting, hence the name, missile drugs. In this case, the main role of the carrier polymers is to prolong the half-life of the drug in the blood stream. The effects of molecular weight and electric charge of various water-soluble polymers, such as dextrans, pullulan, poly(ethylene glycol) (PEG), and poly(vinyl alcohol) (PVA), on the biological fate of the drugs have been reported (Yamaoka et al. 1995, 1994). Cationic macromolecules are known to affect on cellular interactions in vitro. Another role of polymeric carriers is to protect unstable drugs from various enzymes in the body. Protein and peptide drugs are susceptible to hydrolysis and would thus be digested by proteolytic enzymes in vivo.

Very recently, the genes coding for several peptide drugs have been attracting great attention with respect to curing genetic diseases, since protein drugs are very unstable in the body. In delivering genetic materials into cells or tissues, various water-soluble polymeric carriers have been selected as non-viral gene carriers because they are safer than viral vectors. However, the properties required for a gene carrier are quite different from those for conventional drug carries. Generally, polymeric gene carriers possess positively charged groups, which are known to condense DNA coils into 10^{-3}–10^{-4} of the original volume (De Smedt et al. 2000) by forming a polyion complex (PIC). PICs are formed via electrostatic interactions between the anionic phosphate groups of DNA and the cationic amino groups of polymeric gene carriers (Maes et al. 1967; Saymour 1999). These complexes of DNA and polycations are called "polyplexes". The electrostatic interaction of polyplexes is of increasing importance

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not only in DNA condensing but also in maintaining the relaxed state of the polyplexes, which allows them to release DNA, as described later (Arigita et al. 1999; Kunath et al. 2003; Ruponen et al. 1999).

The other role of cationic polymers is to protect DNA from degradation by DNases existing in the body, which is one of the biggest objectives of the gene delivery system. Polyplexes of PLL, PEI, and polyamidoamine (PAMAM) dendrimer showed significant resistance against nuclease actions compared with free DNA. Protective interactive noncondensing (PINC) polymers, such as poly(*N*-vinyl pyrrolidone) (PVP) and PVA, form flexible polyplexes with DNA via hydrogen bonds (Mumper et al. 1998). Molecular modeling confirmed that PVP was located in the major groove of DNA. These PINC polymers protect DNA from nucleases and enhance intracellular uptake by interactions between the hydrophobic surface of polyplexes and the cell membrane. However, the strong protecting ability of the carriers does not necessarily lead to high levels of gene expression. Indeed, inverse effects between nuclease resistance and the rate of interexchange reactions of polyplexes (described in detail later) has been pointed out (Seymour 1997).

In the case of conventional polymeric drugs, the parent drug would simply be released from the carrier polymer by hydrolysis of the chemical bonds followed by diffusion into cells or to the site of action (Kopeek and Pohl 1988). In contrast, in the case of the gene delivery system, DNA molecules are diffuse with difficulty because of their huge molecular weights. Thus, carrier polymers continue to play an important role in the interaction with cell surfaces, in intracellular trafficking, and in transcription of transgene in the nucleus. In this chapter, the chemical structure, function, and mechanisms of various polymeric non-viral carriers for use in gene delivery systems in mammalian cells, in vitro and in vivo, are reviewed.

2 Various Polymeric Carriers

About 70% of the gene-therapeutic protocols available to date are based on viral vectors (http://www.wiley.co.uk/genetherapy/clinical/). Despite their high efficiency in vitro, clinical trials are often limited by several concerns, e.g. toxicity, immunogenicity, inflammatory properties, the limited size of the DNA, production and packing problems, and the high cost. In addition, an overwhelming immune reaction against adenovirus occurred in a patient at Pennsylvania University in 1999 (Marshall 2000, 1999) and a leukemia-like disease were reported in a French patient in 2002 (Marshall 2002; Kaiser 2003). As a result, non-viral vector-mediated systems have become of interest, because they are much safer, more cost-effective, and easier to manufacture than viral vector systems.

Some of the reasons for the few clinical trials using non-viral gene vectors are their toxicity (Choi et al. 1998), low transgene expression, and the tendency of the carrier/DNA complexes to aggregate in the blood (Dash et al. 1999). In addition, the detailed mechanisms of transgene expression following non-viral carrier-based gene transfer are not yet clear. The efficacy of transgene expression has been found to be clearly affected by the chemical structure of the cationic carriers, which indicates that the characteristics of the non-viral vectors would alter the efficacy of some process, such as internalization in cells and transgene expression.

2.1 Chemical Structure

The synthetic vectors described above are depicted in Fig. 1. All of them possess cationic charges and form PICs based on electrostatic interactions, but their transfection efficacies are quite different and depend on the chemical structure, charge density, and molecular weight of the polycation. For example, poly(L-arginine) and poly(L-lysine) are known to form complexes with quite different features (Liquier et al. 1975), and recently the former were found to lead to much higher levels of gene expression.

We have reported that polycations having abundant side-chain hydroxyl groups (or amide groups), such as poly(vinyl alcohol) dimethylaminoacetal (PVA3), are effective carriers with low cytotoxicity. Although the role of the hydroxyl or amide groups is still uncertain, they seem to strongly and effectively maintain the hydrophilic nature of the formed complexes thus preventing compaction of the complexes and ultimately allowing their dissociation. The methylene group length of the polyburene (PB) main chain (Fig. 1) also change the hydrophilic/hydrophobic balance (Aubin et al. 1994; Mita et al. 1977). The charge density of the polycations is the other important factors. In a comparison of DEAE-dextrans with different cationic group densities ranging from 20 to 55%, both transfection efficiency and cytotoxicity greatly decreased with decreased cationic group densities (Fig. 2). Other polycations, such as chitosan (Lee et al. 1998), polyethyleneimine (Godbey et al. 1999b; Pollard et al. 1998; Demeneix et al. 1998), and cationic polymethacrylate derivatives (van de Wetering et al. 1998;

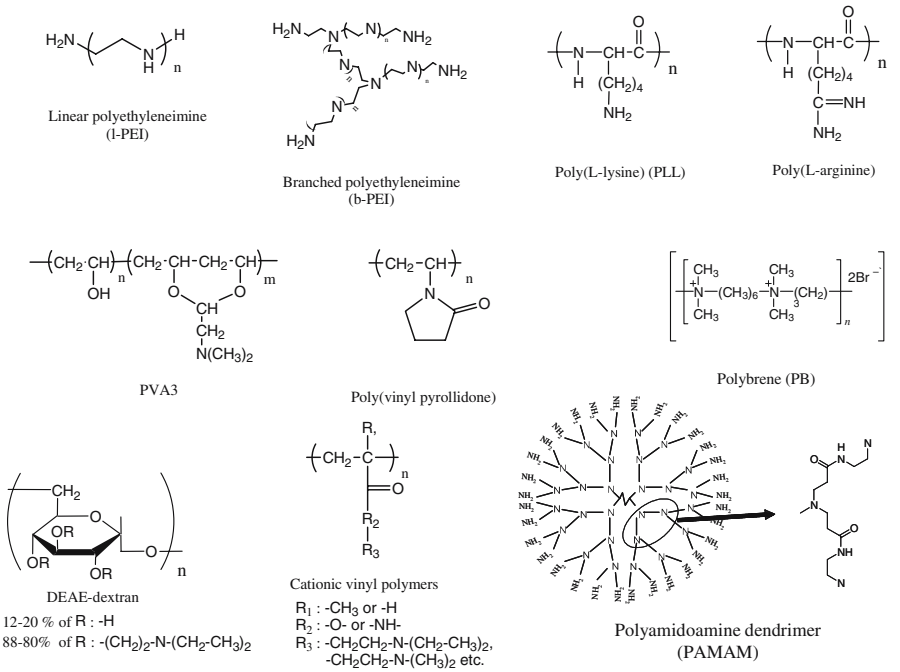

Fig. 1. Chemical structures of polycation-type non-viral gene carriers

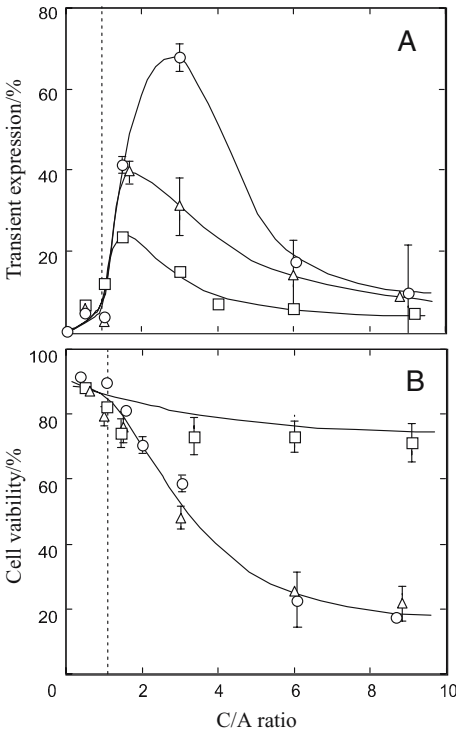

FIG. 2. **A** Transient expressio of *lacZ* introduced into COS-1 cells; **B** cytotoxicity after osmotic shock using DEAE-dextran with the substitution ratio for DEAE groups of: ○ 55, △ 31, and □ 22 %

Cherng et al. 1996) have also been proposed but there is little information on the correlation between their chemical structure and transfection efficacy. In 1995, Boussif et al. reported that polyethyleneimine (PEI) can be effectively used as a non-viral gene vector for the purpose of gene therapy (Boussif et al. 1995). Godbey et al. (1999a) reported the effect of molecular weight of PEI. Low-molecular-weight (1,800) PEI resulted in no gene expression but transfection efficacy increased with increasing molecular weight, with the most effective results obtained at 70,000.

2.2 Molecular Shape

When linear PEI and branched PEI were compared with respect to their transfection efficacy, the results were dependent on the adopted transfection procedure (Fig. 1) (Plank et al. 1999). They showed the efficiency of the branched structure, while Ohashi et al. reported more efficient gene transfer using linear PEI than branched PEI (Ohashi et al. 2001). Thus, further experiments are necessary to investigate the relationship between the molecular shape of polymers and gene expression.

In 1993, Haensler and Szoka reported on the effectiveness of hyper-branched polyamidoamine (PAMAM) dendrimers, a well-defined class of cascade polymers from methyl acrylate and ethylenediamine (Fig. 1) . They achieved excellent gene expression using heat-treated dendrimers (fractured dendrimers), which are a degraded form of the intact dendrimers, at the amide linkage (Tang et al. 1996) (see Chaps. 1–5).

Various amphiphilic block polymers, such as PEG ylated PLL, which self-organize into micelles in aqueous solution, have been used as carriers. DNA can form PICs with hydrophilic chains of block polymers. Micelles are formed from the core of these PICs surrounded by hydrophobic chains of block polymers (Erbacher et al. 1999).

3 Transfection Protocol Using Polyplexes

3.1 Various Inhibitors in In Vitro Gene Delivery

The roles of endosomes and lysosomes in gene transefer have been discussed in the literature (Wattiaux et al. 2000). Polyplexes are taken up via endocytosis and then travel through various intracellular pathway via endosomes and lysosomes. In order to avoid degradation of internalized polyplexes in these structures, the activity of the lysosomal enzymes must be suppressed by adding various endosomal or lysosomal inhibitors as shown in Table 1.

Weak bases, such as chloroquine and ammonium chloride, inhibit the acidication of endosomal or lysosomal environments as well as the degradation of polyplexes in endosomes and lysosomes (Maxfield 1982; Cotten et al. 1990; Choi et al. 1998). In a study comparing chloroquine and several other weak bases, it was found that only chloroquine enhances transgene expression, which may well be related to dissociation of the complexes (Erbacher et al. 1996). Bafilomycin A_1 and concanamycin A act as inhibitors of vacuolar ATPases known to block the endosomal proton pump. Photosensitizing compounds, such as AlPcS2a and TPPS2a, destabilize endosomes following photochemical reactions of these agents with visible light. Endosomal and lysosomal inhibitors thus improve the release of polyplexes from these organelles.

3.2 Mechanical Methods

Mechanical transfection, such as electroporation (Magin-Lachmann et al. 2004), microinjection (Zauner et al. 1999), and osmotic shock (Takai and Ohmori 1990; Okada and Rechsteiner 1982), are useful methods especially for studying transgene expression following endosomal escape. We use osmotic shock because, compared to microinjection, a larger number of cells can be easily treated and the polyplexes are directly delivered into the cytosolic compartment (Kimura et al. 2002). The method is applicable even for lymphoid cells. Briefly, cell suspensions are incubated with polyplexes for 30 min–1 h at 37°C, and a highly osmotic solution containing 1 M sucrose,

Table 1. Endosomal inhibitors

Inhibitors	Action	References
Chloroquine	Raises endosomal pH	(Erbacher et al. 1996; Cotten et al. 1990; Murphy et al. 1984)
Ammonium chloride		(Erbacher et al. 1996; Fredericksen et al. 2002;
Monensin, FCCP		Maxfield 1982)
Bafilomycin	ATPase inhibition	(Fredericksen et al. 2002; Drose and Altendorf 1997)
Fusogenic peptides	Membrane fusion	(Collins and Fabre 2004)

20% PEG 4000, 210 mM NaCl, and 70 mM Tris-HCl buffer (pH 7.3) is then added. After incubating the cells for a given period of time, they are rinsed twice with serum-free culture medium and exposed to hypotonic culture medium. After a 48-h incubation, transgene expression can be evaluated.

4 Biological Barriers

The mechanism of polyplex-mediated gene transfer is thought to follow the general endocytotic process. In order to lead to successfully high transgene expression, various biological barriers must be crossed: (1) interaction with the plasma membrane, (2) internalization, (3) escape from the endosome, (4) trafficking into the nucleus, and (5) dissociation of the complexes and/or DNA recognition by transcription factors (Fig. 3).

4.1 Internalization

Since positively charged complexes interact with the cell surface by an electrostatic interaction with anionic substances on the cell surface, such as sialic acid and proteoglycan, the zeta potential of polyplexes is important. Polyplexes composed of polycations with molecular weights of several thousands exhibit significantly higher zeta potentials (Ruponen et al. 1999; Jeong et al. 2001; Ahn et al. 2002; Wolfert et al. 1996; Toncheva et al. 1998; Cherng et al. 1996; Wolfert et al. 1999; Putnam et al. 2001; Howard et al. 2000), irrespective of the polycations used .

Internalization events also seem to be affected by polyplex size and zeta potential. The size of the polyplexes has been assessed by transmission electron microscopy

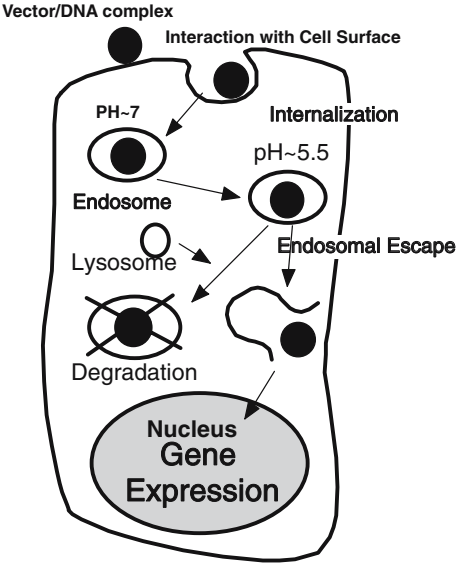

Fig. 3. Intracellular trafficking of polyplexes

(TEM) (Mannisto et al. 2002), atomic force microscopy (AFM) (Wolfert et al. 1996; Toncheva et al. 1998; Choi et al. 1999a), and dynamic light scattering (DLS) (Jeong et al. 2001; Cherng et al. 1996; Ogris et al. 1999). Generally, aggregation of the complexes makes their internalization difficult because of their large size. Tang and Szoka (1997) studied the aggregation properties of linear PLL, intact PAMAM dendrimers, fractured dendrimers, and branched PEI in forming complexes with DNA. These polycations formed similar complexes in terms of size and zeta potential but high-level gene expression was induced only by fractured dendrimers and branched PEI, due to the stability of the complexes and absence of aggregation (Tang and Szoka 1997). Despite these investigation, the correlation between transfection efficiency and size of the polyplexes remains unclear. Aggregation of the polyplexes in serum-containing medium or in blood is also a problem. In order to decrease both aggregation and the toxicity of polyplexes, conjugation with PEG has been proposed (Mannisto et al. 2002; Choi et al. 1999b). For example, the solubility of PEG ylated PEI and DNA polyplexes at higher therapeutic concentration was not only improved without aggregation but the in vivo toxicity was also reduced (Ogris et al. 1999; Kursa et al. 2003).

4.2 Receptor-Mediated Gene Delivery

In 1987, Wu et al. developed a system for targeting foreign genes to hepatocytes, which possess a unique receptor that binds and internalizes galactose-terminal asialoglycoproteins, through receptor-mediated endocytosis. It was shown that asialoorosomucoid-PLL carriers delivered pSV2-CAT plasmid DNA specifically to HepG2 hepatoma cells but not to other receptor (–) cell lines (Wu and Wu 1987, 1988a, b). Consequently, site-specific gene delivery has received much attention, especially in vivo direct gene transfer using various biologically active moieties, such as sugar (Midoux et al. 1993; Erbacher et al. 1996, 1997, 1995; Wu and Wu 1988a, b), transferrin (Wagner et al. 1991a,b), and LDL (Table 2).

Some types of cell, such as nonadherent primary hematopoietic cells, are well known to be difficult or almost impossible to transfect with foreign genes linked to conventional carriers because the endocytotic activity of these cells is quite low. Birnstiel and coworkers developed a system in which transferrin served as ligand and named the system "transferrinfection" (Wagner et al. 1991). The authors synthesized tranferrin-PLL conjugates using various molecular weights of PLL and different modification ratios of transferrin. A strong correlation was found between DNA condensation, evaluated using electron microscopy, and cellular DNA uptake.

Other candidates for receptor-mediated gene delivery are the receptors for integrin (Erbacher et al. 1999), insulin (Rosenkranz et al. 1992), and some growth factors (Fisher et al. 2000). Interestingly, polycations bound to VEGF (vascular endothelial growth factor) could not deliver DNA into nucleus but bFGF (basic fibroblast growth factor) could. The PEI derivatives conjugated to the integrin-binding peptide CYGGRGDTP via a disulfide bridge led to transgene expression in integrin-expressing epithelial cells (Hela) and fibroblasts (MRC5) at an expression level 10- to 100-fold higher than obtained with PEI. The advantage of receptor-mediated endocytosis is not just the cell-type specificity of the gene transfer but also the controlled intracellular trafficking of the complexes (Erbacher et al. 1999).

Table 2. Biological signal-mediated gene therapy

Barriers	Ligand	Base polymer	References
Plasma membrane	Galactose	PLL	(Wu and Wu 1987, 1988a, b; Nishikawa et al. 1998; Zanta et al. 1997)
		Vinyl polymer	(Lim et al. 2000)
	Lactose	PLL / PLL-PEG	(Erbacher et al. 1997; Midoux et al. 1993; Klink et al. 2001) / (Choi et al. 1999b)
	Folate	PLL / PLL-PEG	(Mislick et al. 1995; Leamon et al. 1999)
		PEI	(Guo and Lee 1999)
	Transfferin	PLL / PLL-PEG / PEI-PEG	(Wagner et al. 1991a,b 1990; Cotten et al. 1990;) / (Ogris et al. 1999; Vinogradov et al. 1999; Kursa et al. 2003)
	RGD	PLL	(Harbottle et al. 1998)
		PEI	(Erbacher et al. 1999)
	LDL	PLL	(Kim et al. 1998)
		PEI	(Furgeson et al. 2003)
Nuclear membrane	NLS	PLL	(Chan and Jans 1999; Chan et al. 1999)
		None	(Fritz et al. 1996; Balicki et al. 2002)

RGD; Arg-Gly-Asp tripeptide
LDL; low density lipoprotein
NLS; nuclear localization signal
PLL; poly(L-lysine)
PEG; poly(ethylene glycol)
PEI; polyethyleneimine

4.3 Endosomal Escape

The internal pH of endosomes containing polyplexes gradually decreases to about 5.5. Then, the endosomes fuse with lysosomes, resulting in the formation of secondary lysosomes, in which the incorporated DNA is normally hydrolyzed by lysosomal enzymes. DNA digestion at this step is one of the biggest barriers to effective gene transfer. When a foreign gene is transferred by microinjection or osmotic shock (Takai and Ohmori 1990), by which the transgene is compulsorily delivered into the cytosol, gene expression is generally much higher than obtained using the coculture method because there is no lysosomal digestion. If a non-viral vector has the ability to disrupt or fuse with the endosomal membranes, transferred foreign gene can escape from the endosome into the cytosol, and effective transgene expression should occur.

One promising strategy to release internalized complexes from the endosome is osmotic endosomal disruption. In 1995, Boussif et al. pointed out a novel mechanism, the "proton sponge hypothesis", which resulted in high-level expression of a transgene introduced into the cell using PEI as vector (Boussif et al. 1995). At pH 5.5.–7, PEI has a greater buffering capacity than PLL and other polycations. When PEI is used as carrier and is internalized into the endosome with DNA, a larger amount of H^+ should influx into the endosome, thus reducing the pH and raising the internal osmotic pressure, resulting in osmotic rupture of the endosome. In fact, fluorescence imaging showed that there was no overlap of PEI and labeled lysosomes while PLL was found together with lysosome when labeled PEI/DNA or PLL/DNA polyplexes

were transfected into cells (Godbey et al. 2000; Remy-Kristensen et al. 2001). Recently, it was reported that DNA with PEI (linear or branched) showed rapid emdosomal escape (Itaka 2003). Hennink studied the effect of pKa of the cationic groups on their transfection efficacy using various cationic vinyl polymers (Zuidam et al. 2000; van de Wetering et al. 1999). The pKa ranges from 7.5 (for poly(2-(dimethylamino)ethyl methacrylate)) to 8.8 [for poly(3-(dimethylamino)propyl methacrylamide)], and the lower the pKa the higher the amount of gene expression. The pKa of the cationic groups is also influenced by their arrangement based on the polymer effect of the adjacent charged groups (van de Wetering et al. 1999).

4.4 Nuclear Transport

Transgenes must be transported to nucleus by some means. When lipoplexes are injected into the nucleus, gene expression is strongly suppressed by the cationic lipids, while polyplexes lead to strong gene expression after nuclear microinjection (Pollard et al. 1998). These results indicate that the intracellular trafficking and gene expression mechanisms for polyplexes and lipoplexes differ from each other.

One possible mechanism is the transportation through the nuclear membrane pore but this seems unlikely because the sizes of the complexes are too large, being usually around 100 nm. Another possibility is the accumulation of the complexes during the mitotic event accompanying nuclear membrane disappearance. Zauner et al. compared the role of mitosis in the transfection of confluent, contact-inhibited primary human cells using polyplexes and lipoplexes. It was shown that lipoplexes cannot lead to high-level gene expression at the confluent stage but that polyplexes can (Zauner et al. 1999). Godbey et al. (1999b) reported another mechanism of nuclear transport for PEI/DNA complexes. They suggested a mechanism in which the polyplexes come into contact with phospholipids of the endosome; the membrane then becomes permeabilized and bursts due to osmotic swelling, resulting in the polyplexes becoming coated with the phospholipids. The coated complexes could then enter the nucleus via fusion with the nuclear envelope.

In order to enhance trafficking through the nuclear pore, several nuclear localization signals (NLS) were utilized (Garcia-Bustos et al. 1991; Yoneda 1997). NLSs are oligopeptides mainly composed of cationic residues; they are 5–20 amino acids long and different sequences have been found in many species. NLS bound to PLL has been evaluated by many researchers as a gene carrier and was shown to be effective (Table 2) (Chan and Jans 1999).

4.5 Transcription of the Transgene

The delivered polyplexes may require disassembly in order to be transcribed but electrostatic polyplexes dissociate with difficulty under normal physiological conditions. In contrast, it is also possible that the DNA in the polyplexes is recognized without prior disassembly. In any case, the polyplex should possess adequate characteristics allowing these events. The tendency towards polyplex dissociation can be estimated by adding another polyanion, such as heparin sulfate, polyvinyl sulfate, and heparin, into the polyplex suspension (Erbacher et al. 1999; Wolfert et al. 1996; Ruponen et al. 1999). Kabanov et al. reported that under these conditions a polyion interexchange

reaction occurs, resulting in free DNA release when an adequate amount of polyanion is added to polyplex suspensions (Vinogradov et al. 1998). The interexchange reaction of the complexes depends on the kind of polyanions added, such as poly(vinyl sulfonate) (Katayose and Kataoka 1998), poly(aspartic acid), or glycosaminoglycans (GAGs) (Mannisto et al. 2002). DNA was more easily released from pDMAEMA than from poly[(trimethylamino)ethyl methacrylate chloride)] (pTMAEMA) and the former showed high transfection efficiency than the latter (Arigita et al. 1999).

We reported that only cationic polymers containing nonionic hydrophilic groups lead to higher amounts of gene expression (Yamaoka et al. 1994). These groups seem to impart a hydrophilic nature to the complexes since they are not involved in complex formation. Gene expression in these complexes was higher even in an in vitro transcription/translation system using rabbit reticulocyte lysate. Based on this observation, we have attempted to improve the carrier ability of PLL (Yamaoka et al. 2000; Kimura et al. 2002). As shown in Fig. 4, PtmLS, which is a PLL derivative containing 25 mol % serine residues and quaternary ammonium groups, greatly enhanced transgene expression. PtmLS complexes were easily disassembled and were also transcribed in an in vitro translation system. Recently, low-molecular-weight PEI (LMW-PEI) was reported to be a promising carrier for gene transfer under in vitro and in vivo conditions compared with high-molecular-weight PEI (HMW-PEI). Poly-

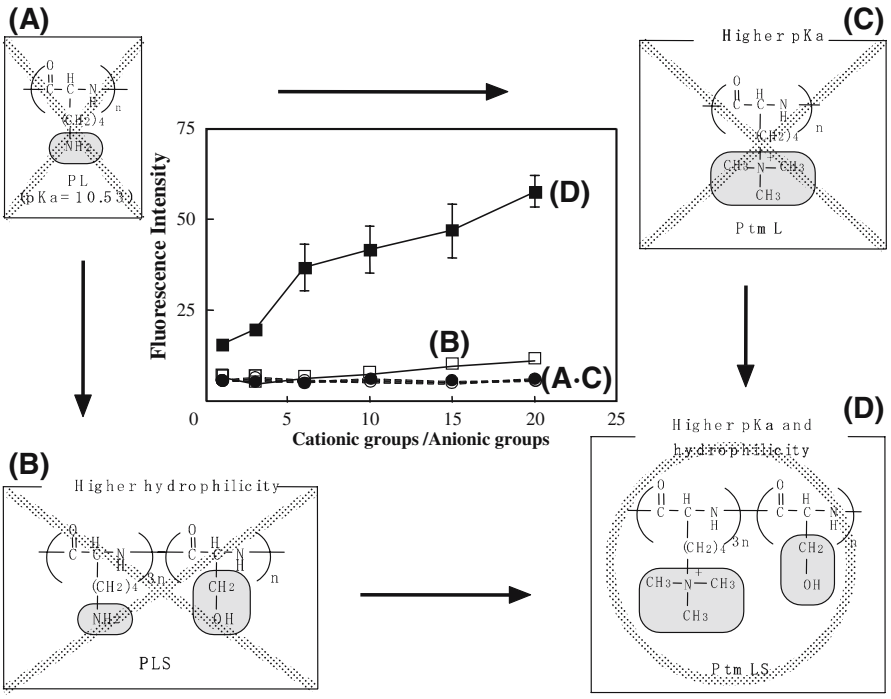

FIG. 4. Fluorescence intensity of EGFP expressed by COS-1 cells 40 h after osmotic shock of pEGFP using ○ PL, • PLS, □ PtmL, and ■ PtmLS

plexes formed from LMW-PEI also showed significantly reduced condensation and were reported to induce an higher transfection efficiency (Kunath et al. 2003).

5 Conclusions

The physicochemical features of the polyplexes are extremely important for designing effective non-viral carriers. Recently, several new systems have been described but are not well tested with respect to intracellular trafficking and transcription event. For example, non-condensing polyplexes are advantageous for transcription but, at the same time, are disadvantageous for nuclease digestion of DNA. In order to design and develop, new effective carriers, each step of intracellular trafficking route should be analyzed quantitatively.

References

Ahn CH, Chae SY, Bae YH, Kim SW (2002) Biodegradable poly(ethylenimine) for plasmid DNA delivery. J Control Release 80:273–282

Arigita C, Zuidam NJ, Crommelin DJ, Hennink WE (1999) Association and dissociation characteristics of polymer/DNA complexes used for gene delivery. Pharm Res 16: 1534–1541

Aubin RA, Weinfeld M, Mirzayans R, Paterson MC (1994) Polybrene/DMSO-assisted gene transfer. Generating stable transfectants with nanogram amounts of DNA. Mol Biotechnol 1:29–48

Balicki D, Putnam CD, Scaria PV, Beutler E (2002) Structure and function correlation in histone H2A peptide-mediated gene transfer. Proc Natl Acad Sci USA 99:7467–7471

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc Natl Acad Sci USA 92:7297–7301

Chan CK, Jans DA (1999) Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. Hum Gene Ther 10:1695–702

Cherng JY, van de Wetering P, Talsma H, Crommelin DJ, Hennink WE (1996) Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. Pharm Res 13:1038–1042

Choi JS, Lee EJ, Choi YH, Jeong YJ, Park JS (1999a) Poly(ethylene glycol)-block-poly(L-lysine) dendrimer: novel linear polymer/dendrimer block copolymer forming a spherical water-soluble polyionic complex with DNA. Bioconjug Chem 10:62–65

Choi YH, Liu F, Choi JS, Kim SW, Park JS (1999b) Characterization of a targeted gene carrier, lactose-polyethylene glycol-grafted poly-L-lysine and its complex with plasmid DNA. Hum Gene Ther 10:2657–2665

Choi YH, Liu F, Kim JS, Choi YK, Park JS, Kim SW (1998) Polyethylene glycol-grafted poly-L-lysine as polymeric gene carrier. J Control Release 54:39–48

Collins L, Fabre JW (2004) A synthetic peptide vector system for optimal gene delivery to corneal endothelium. J Gene Med 6:185–194

Cotten M, Langle-Rouault F, Kirlappos H, Wagner E, Mechtler K, Zenke M, Beug H, Birnstiel ML (1990) Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. Proc Natl Acad Sci USA 87:4033–4037

Dash PR, Read ML, Barrett LB, Wolfert MA, Seymour LW (1999) Factors affecting blood clearance and *in vivo* distribution of polyelectrolyte complexes for gene delivery. Gene Ther 6:643–650

De Smedt SC, Demeester J, Hennink WE (2000) Cationic polymer based gene delivery systems. Pharm Res 17:113–126

Demeneix B, Behr J, Boussif O, Zanta MA, Abdallah B, Remy J (1998) Gene transfer with lipospermines and polyethylenimines. Adv Drug Deliv Rev 30:85–95

Drose S, Altendorf K (1997) Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. J Exp Biol 200 (Pt 1):1–8

Erbacher P, Roche AC, Monsigny M, Midoux P (1995) Glycosylated polylysine/DNA complexes: gene transfer efficiency in relation with the size and the sugar substitution level of glycosylated polylysines and with the plasmid size. Bioconjug Chem 6:401–410

Erbacher P, Roche AC, Monsigny M, Midoux P (1996) Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. Exp Cell Res 225:186–194

Erbacher P, Roche AC, Monsigny M, Midoux P (1997) The reduction of the positive charges of polylysine by partial gluconoylation increases the transfection efficiency of polylysine/DNA complexes. Biochim Biophys Acta 1324:27–36

Erbacher P, Remy JS, Behr JP (1999) Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. Gene Ther 6:138–145

Fisher KD, Ulbrich K, Subr V, Ward CM, Mautner V, Blakey D, Seymour LW (2000) A versatile system for receptor-mediated gene delivery permits increased entry of DNA into target cells, enhanced delivery to the nucleus and elevated rates of transgene expression. Gene Ther 7:1337–1343

Fredericksen BL, Wei BL, Yao J, Luo T, Garcia JV (2002) Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus. J Virol 76: 11440–11446

Fritz JD, Herweijer H, Zhang G, Wolff JA (1996) Gene transfer into mammalian cells using histone-condensed plasmid DNA. Hum Gene Ther 7:1395–1404

Furgeson DY CW, Yockman JW, Kim SW (2003) Modified Linear Polyethylenimine-Cholesterol Comjugates for DNA Complexation. Bioconjug Chem 14:840–847

Garcia-Bustos J, Heitman J, Hall MN (1991) Nuclear protein localization. Biochim Biophys Acta 1071:83–101

Godbey WT, Wu KK, Mikos AG (1999a) Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 45:268–275

Godbey WT, Wu KK, Mikos AG (1999b) Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci USA 96:5177–5181

Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG (2000) Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J Biomed Mater Res 51:321–328

Guo W, Lee RL (1999) Receptor-targeted gene delivery via folate-conjugated polyethylenimine. AAPS PharmSci 1:E19

Haensler J, Szoka Jr FC (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjug Chem 4:372–379

Harbottle RP, Cooper RG, Hart SL, Ladhoff A, McKay T, Knight AM, Wagner E, Miller AD, Coutelle C (1998) An RGD-oligolysine peptide: a prototype construct for integrin-mediated gene delivery. Hum Gene Ther 9:1037–1047

Howard KA, Dash PR, Read ML, Ward K, Tomkins LM, Nazarova O, Ulbrich K, Seymour LW (2000) Influence of hydrophilicity of cationic polymers on the biophysical properties of polyelectrolyte complexes formed by self-assembly with DNA. Biochim Biophys Acta 1475:245–255

Itaka (2003) In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. J gene medicine 6:76–84

Jeong JH, Song SH, Lim DW, Lee H, Park TG (2001) DNA transfection using linear poly (ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline). J Control Release 73:391–399
Kaiser J (2003) Gene therapy. Seeking the cause of induced leukemias in X-SCID trial. Science 299:495
Katayose S, Kataoka K (1998) Remarkable increase in nuclease resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)-poly(L-lysine) block copolymer. J Pharm Sci 87:160–163
Kim JS, Kim BI, Maruyama A, Akaike T, Kim SW (1998) A new non-viral DNA delivery vector: the terplex system. J Control Release 53:175–182
Kimura T, Yamaoka Y, Iwase R, Murakami A (2002) Effect of physicochemical properties of polyplexes composed od chemically modified PL derivatives on transfection efficiency *in vitro*. Macromolecular Bioscience 2:437–446
Klink DT, Chao S, Glick MC, Scanlin TF (2001) Nuclear translocation of lactosylated poly-L-lysine/cDNA complex in cystic fibrosis airway epithelial cells. Mol Ther 3:831–841
Kopeek VuaJ, Pohl MBaVK J (1988) Cleavage of oligopeptide side-chains in N-(2-hydroxypropyl)meth-acrylamide copolymers by mixtures of lysosomal enzymes. J Control Release 8:133–140
Kunath K vHA, Fischer D, Petersen H, Bickel U, Voigt K, Kissel T (2003) Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and *in vivo* distribution with high-molecular-weight polyethylenimine. J Control Release 89:113–125
Kursa M, Walker GF, Roessler V, Ogris M, Roedl W, Kircheis R, Wagner E (2003) Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. Bioconjug Chem 14:222–231
Leamon CP, Weigl D, Hendren RW (1999) Folate copolymer-mediated transfection of cultured cells. Bioconjug Chem 10:947–957
Lee KY, Kwon IC, Kim YH, Jo WH, Jeong SY (1998) Preparation of chitosan self-aggregates as a gene delivery system. J Control Release 51:213–220
Lim DW, Yeom YI, Park TG (2000) Poly(DMAEMA-NVP)-β-PEG-galactose as gene delivery vector for hepatocytes. Bioconjug Chem 11:688–695
Liquier J, Pinot-Lafaix M, Taillandier E, Brahms J (1975) Infrared linear dichroism investigations of deoxyribonucleic acid complexes with poly(L-arginine) and poly(L-lysine). Biochemistry 14:4191–4197
Maes R, Sedwick W, Vaheri A (1967) Interaction between DEAE-dextran and nucleic acids. Biochim Biophys Acta 134:269–276
Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Cooke H, Huxley C, Wagner E (2004) In vitro and in vivo delivery of intact BAC DNA-comparison of different methods. J Gene Med 6:195–209
Mannisto M, Vanderkerken S, Toncheva V, Elomaa M, Ruponen M, Schacht E, Urtti A (2002) Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. J Control Release 83:169
Marshall E (1999) Gene therapy death prompts review of adenovirus vector. Science 286: 2244–2245
Marshall E (2000) FDA halts all gene therapy trials at Penn. Science 287:565, 567
Marshall E (2002) Clinical research. Gene therapy a suspect in leukemia-like disease. Science 298:34–35
Maxfield FR (1982) Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J Cell Biol 95:676–681
Midoux P, Mendes C, Legrand A, Raimond J, Mayer R, Monsigny M, Roche AC (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. Nucleic Acids Res 21:871–878

Mislick KA, Baldeschwieler JD, Kayyem JF, Meade TJ (1995) Transfection of folate-polylysine DNA complexes: evidence for lysosomal delivery. Bioconjug Chem 6:512–515
Mita K, Zama M, Ichimura S (1977) Effect of charge density of cationic polyelectrolytes on complex formation with DNA. Biopolymers 16:1993–2004
Mumper RJ, Wang J, Klakamp SL, Nitta H, Anwer K, Tagliaferri F, Rolland AP (1998) Protective interactive noncondensing (PINC) polymers for enhanced plasmid distribution and expression in rat skeletal muscle. J Control Release 52:191–203
Murphy RF, Powers S, Cantor CR (1984) Endosome pH measured in single cells by dual fluorescence flow cytometry: rapid acidification of insulin to pH 6. J Cell Biol 98: 1757–1762
Nishikawa M, Takemura S, Takakura Y, Hashida M (1998) Targeted delivery of plasmid DNA to hepatocytes *in vivo*: optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physicochemical properties. J Pharmacol Exp Ther 287:408–415
Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther 6:595–605
Ohashi S, Kubo T, Ikeda T, Arai Y, Takahashi K, Hirasawa Y, Takigawa M, Satoh E, Imanishi J, Mazda O (2001) Cationic polymer-mediated genetic transduction into cultured human chondrosarcoma-derived HCS-2/8 cells. J Orthop Sci 6:75–81
Okada CY, Rechsteiner M (1982) Introduction of macromolecules into cultured mammalian cells by osmotic lysis of pinocytic vesicles. Cell 29:33–41
Plank C, Tang MX, Wolfe AR, Szoka Jr FC (1999) Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and *in vitro* activity of DNA polyplexes. Hum Gene Ther 10:319–332
Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D (1998) Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J Biol Chem 273:7507–7511
Prasmickaite L, Hogset A, Berg K (2002) The role of the cell cycle on the efficiency of photochemical gene transfection. Biochim Biophys Acta 1570:210–218
Putnam D, Gentry CA, Pack DW, Langer R (2001) Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc Natl Acad Sci USA 98: 1200–1205
Remy-Kristensen A, Clamme JP, Vuilleumier C, Kuhry JG, Mely Y (2001) Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes. Biochim Biophys Acta 1514:21–32
Ringsdorf H (1975) Structure and properties of pharmacologically active polymers. J Poly Sci Symp 51:135–153
Rosenkranz AA, Yachmenev SV, Jans DA, Serebryakova NV, Murav'ev VI, Peters R, Sobolev AS (1992) Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. Exp Cell Res 199:323–329
Ruponen M, Yla-Herttuala S, Urtti A (1999) Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. Biochim Biophys Acta 1415:331–341
Seymour LW (1997) Synthetic polymers for vectorial delivery of DNA: characterisation of polymer-DNA complexes by photon correlation spectroscopy and stability to nuclease degradation and disruption by polyanions *in vitro*. Journal of Controlled Release 48: 269–276
Seymour LW (1999) Turbidometric analysis of polyelectrolyte complexes formed between poly(L-lysine) and DNA. Colloids and Surfaces B 16:253–260
Takai T, Ohmori H (1990) DNA transfection of mouse lymphoid cells by the combination of DEAE-dextran-mediated DNA uptake and osmotic shock procedure. Biochim Biophys Acta 1048:105–109

Tang MX, Rademann CT, Szoka FC (1996) In vitro gene delivery by degraded polyamidoamine dendrimers. Bioconj Chem 6:703–714

Tang MX, Szoka FC (1997) The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Ther 4:823–832

Toncheva V, Wolfert MA, Dash PR, Oupicky D, Ulbrich K, Seymour LW, Schacht EH (1998) Novel vectors for gene delivery formed by self-assembly of DNA with poly(L-lysine) grafted with hydrophilic polymers. Biochim Biophys Acta 1380:354–368

van de Wetering P, Cherng JY, Talsma H, Crommelin DJ, Hennink WE (1998) 2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. J Control Release 53:145–153

van de Wetering P, Moret EE, Schuurmans-Nieuwenbroek NM, van Steenbergen MJ, Hennink WE (1999) Structure-activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for non-viral gene delivery. Bioconjug Chem 10:589–597

Vinogradov S, Batrakova E, Li S, Kabanov A (1999) Polyion complex micelles with protein-modified corona for receptor-mediated delivery of oligonucleotides into cells. Bioconjug Chem 10:851–860

Vinogradov SV, Bronich TK, Kabanov AV (1998) Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. Bioconjug Chem 9: 805–812

Wagner E, Cotton M, Foisner R, Birnstiel ML (1991) Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci USA 88:4255–4259

Wagner E, Cotton M, Mechtler K, Kirlappos H, Birnstiel ML (1991) DNA-binding transferrin conjugates as functional gene-delivery agents: synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. Bioconjug Chem 2:226–231

Wagner E, Zenke M, Cotton M, Beug H, Birnstiel ML (1990) Transferrin-polycation conjugates as carriers for DNA uptake into cells. Proc Natl Acad Sci USA 87:3410–3414

Wall DA, Wilson G, Hubbard AL (1980) The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. Cell 21:79–93

Wattiaux R, Laurent N, Wattiaux-De Coninck S, Jadot M (2000) Endosomes, lysosomes: their implication in gene transfer. Adv Drug Deliv Rev 41:201–208

Wink T, Beer J, Hennink WE, Bult A, van Bennekom WP(1999) Interaction between plasmid DNA and cationic polymers studied by surface plasmon resonance spectrometry. Anal Chem 71:801–805

Wolfert MA, Dash PR, Nazarova O, Oupicky D, Seymour LW, Smart S, Strohalm J, Ulbrich K (1999) Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. Bioconjug Chem 10:993–1004

Wolfert MA, Schacht EH, Toncheva V, Ulbrich K, Nazarova O, Seymour LW (1996) Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. Hum Gene Ther 7:2123–2133

Wu GY, Wu CH (1987) Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system. J Biol Chem 262:4429–4432

Wu GY, Wu CH (1988a) Evidence for targeted gene delivery to Hep G2 hepatoma cells *in vitro*. Biochemistry 27:887–892

Wu GY, Wu CH (1988b) Receptor-mediated gene delivery and expression *in vivo*. J Biol Chem 263:14621–14624

Yamaoka T, Iwata H, Hamada N, Ide H, Kimura Y (1994) Transfection of foreign gene to mammalian cells using low-toxic cationic polymers. Nucleic Acids Symposium Series 31:229–230

Yamaoka T, Kimura T, Iwase R, Murakami A (2000) Enhanced expression of foreign gene transferred to mammalian cells *in vitro* using chemically modified poly(L-lysine)s as gene carriers. Chemistry Letters:118–119

Yamaoka T, Tabata Y, Ikada Y (1994) Distribution and tissue uptake of poly(ethylene glycol) with different molecular weights after intravenous administration to mice. J Pharm Sci 83:601–606

Yamaoka T, Tabata Y, Ikada Y (1995) Comparison of body distribution of poly(vinyl alcohol) with other water-soluble polymers after intravenous administration. J Pharm Pharmacol 47:479–486

Yoneda Y (1997) How proteins are transported from cytoplasm to the nucleus. J Biochem (Tokyo) 121:811–817

Zanta MA, Boussif O, Adib A, Behr JP (1997) *In vitro* gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjug Chem 8:839–844

Zauner W, Brunner S, Buschle M, Ogris M, Wagner E (1999) Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: a potential role of polylysine in nuclear transport. Biochim Biophys Acta 1428:57–67

Zenke M, Steinlein P, Wagner E, Cotton M, Beug H, Birnstiel ML (1990) Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. Proc Natl Acad Sci USA 87:3655–3659

Zuidam NJ, Posthuma G, de Vries ET, Crommelin DJ, Hennink WE, Storm G (2000) Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization. J Drug Target 8:51–66