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Review Article

Polycation gene delivery systems: escape from endosomes to cytosol

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Abstract

Clinical success of gene therapy based on oligonucleotides (ODNs), ribozymes, RNA and DNA will be greatly dependent on the availability of effective delivery systems. Polycations have gained increasing attention as a non-viral gene delivery vector in the past decades. Significant progress has been made in understanding complex formation between polycations and nucleic acids, entry of the complex into the cells and subsequent entry into the nucleus. Sophisticated molecular architectures of cationic polymers have made the vectors more stable and less susceptible to binding by enzymes or proteins. Incorporation of specific ligands to polycations has resulted in more cell-specific uptake by receptor-mediated mechanisms. However, there are still other barriers limiting the transfection efficiency of polycation gene delivery systems. There is a consensus that polycation–DNA complexes (polyplexes) enter cells via the endocytotic pathway. It is not clearly understood, however, how the polyplexes escape (if they do) from endosomes, how DNA is released from the polyplexes or how the released DNA is expressed. The primary focus of this article is to review various polycation gene delivery systems, which are designed to translocate DNA from endosomes into cytosol. Many polycation gene delivery systems have tried to mimic the mechanisms that viruses use for the endosomal escape. Polycation gene delivery systems are usually coupled with synthetic amphipathic peptides mimicking viral fusogenic peptides, histidine-based gene delivery systems for pH-responsive endosomal escape, polycations with intrinsic endosomolytic activity by the proton sponge mechanism and polyanions to mimic the anionic amphiphilic peptides.

Introduction

Although the field of non-viral gene delivery began with cationic lipid–DNA complexes (lipoplexes), polycation–DNA complexes (polyplexes) have recently gained more attention. Polycations have greater versatility in terms of the molecular weight, polymer type, polymer–DNA ratio, molecular architecture (linear, block copolymer, graft copolymer or dendrimer) and the ability to introduce target-specific moieties.

Of the many polycation gene delivery systems developed in the past decades, poly(L-lysine) (PLL) was the first polycation used for non-viral gene delivery (Wu & Wu 1987). Other synthetic and natural polycations developed as non-viral vectors are polyethyleneimine (PEI) (Pollard et al 1998), polyamidoamine dendrimers (Haensler & Szoka 1993), poly((2-dimethylamino)ethyl methacrylate) (Cherng et al 1996) and chitosan (Lee et al 1998). Complex nanoparticles can be formed spontaneously by the electrostatic interaction between negatively charged nucleic acids and polycations. To improve the stability of the polyplexes and to prevent their interaction with serum proteins, polycations have been modified with nonionic water-soluble polymers, such as poly(ethylene oxide) (PEO)–block–PLL (Kataoka et al 1996), PEO–graft–PLL (Choi et al 1998), and PEO–graft–PEI (Vinogradov et al 1998). For specificity in cell recognition and effective endocytosis, polycations were linked with various ligands, such as asialoorosomucoid (Wu & Wu 1987), transferrin (Wagner et al 1990), galactose (Plank et al 1992), antibody (Trubetskoy et al 1992), immunoglobulin (Rojanasakul et al 1994) and growth factor (Blessing et al 2001).

Current non-viral gene delivery systems are much less effective when compared with viral vectors. The gap between the transfection efficiency of viral and non-viral systems is still seemingly insurmountable. There are many extracellular and intracellular barriers for genes to arrive at the target cell, enter the cell and then be expressed.

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Degradation of DNA before it reaches the target cell must be avoided. Polycation vectors have to protect DNA from enzymatic digestion by nucleases in serum and extracellular fluids. The vectors must avoid the uptake by non-target tissues (e.g., elimination by cells of the reticuloendothelial system (RES)). The delivery systems should also overcome intracellular barriers, such as plasma membranes, endosomes and nuclear membranes. It is generally accepted that polyplexes are internalized into a cell by an endocytotic pathway. The efficacy of endocytosis is dependent upon the presence of specific ligands, such as transferrin, the size of polyplex particles, ionic characters of the vectors and hydrophobicity of the vectors. The primary focus of this review is on the polycation gene delivery systems designed to overcome the major hurdles after endocytosis (i.e., escape from endosomes). Nuclear uptake is not necessary for antisense oilgonucleotides (ODNs) and ribozymes, which down-regulate or inhibit gene expression at the RNA level. For this reason, escape from endosomes is probably the most important factor to be considered for the design of gene delivery vehicles.

Learning from nature: endosomal escape by viruses

Understanding of the mechanisms of viral escape from endosomes is important for improving non-viral gene delivery systems. The naturally evolved strategies by viruses are incomparably more efficient than those of non-viral delivery systems. It is beneficial for the development of non-viral vectors to review the endosomal escape mechanisms by two well characterized viruses — influenza virus (enveloped) and adenovirus (non-enveloped).

Influenza virus

The haemagglutinin (HA) protein of the influenza virus shows several steps of the molecular events for cell entry and endosomal escape. HA is a trimer, a bundle of 3 identical protein chains tightly bound together (Figure 1). This trimer consists of two parts: three HA1 subunits, where cell binding occurs, and three HA2 subunits, which perform actual events leading to membrane fusion and endosomal escape. The HA2 subunit contains a short chain of an N-terminal amphiphilic anionic peptide, which is termed the fusion peptide. The conformational change of HA releases the N-terminal amphiphilic anionic peptide residues, allowing them to interact with endosomal membranes. At neutral pH, the HA1 and HA2 domains of HA are held tightly together, and the fusion peptide is buried in the HA1 domain. This is the normal HA conformation when an influenza virus particle encounters the plasma membrane of a host cell. In response to low pH within endosomes, HA undergoes a rapid change of geometric structure (Figure 1). HA1 segments dissociate from the HA2 units removing the constraints from the HA2 and generating the fusion peptides of HA2. The fusion peptides spring open, piercing the lipid bilayer of the endosomal membrane and causing the fusion of viral and endosomal membrane (Carr & Kim 1993, 1994; Bullough et al 1994; Carr et al 1997). The cleavage step of HA triggered by the pH change in endosomes is crucial for the endosomal escape of influenza virus and its release into cytosol, resulting in infection and replication. Non-cleaved HA is not fusion-competent. The influenza virus takes advantage of the natural process of the endosome acidification to escape to the cytosol.

Adenovirus

Adenovirus is a non-enveloped DNA-containing virus that is internalized by receptor-mediated endocytosis. It has three external proteins known as penton, hexon and fibre proteins. After the virus attaches to a cell surface receptor via its fibre protein, it is transferred to clathrin-coated pits, confined inside the endosomes, and then escapes into the cytosol by disrupting the endosomal membrane. The acidic environment of the endosome acts as a trigger for its endosomal escape (Seth et al 1984, 1985; Blumenthal et al 1986). Adenovirus behaves like a pH-sensitive amphiphilic molecule within endosomes. The low pH induces hydrophobicity in adenovirus on its external surface. Among the three external proteins of adenovirus, the penton base protein gains the most hydrophobic properties under acidic environments. However, the other two external proteins (hexon and fibre) are also affected by the acidic environment in the endosome. All of these external proteins of adenovirus, which acquire hydrophobic characteristics, interact with the lipid bilayer and disrupt the endosomal membrane. Weak bases, such as chloroquine, elevate the pH of the endosome, preventing the adenovirus-mediated endosome disruption (Seth et al 1985). This implies that adenovirus could not disrupt the endosomal membrane at neutral pH. Just like the influenza virus, adenovirus utilizes the slightly acidic environment of the endosome to escape into the cytosol. The pH-induced hydrophobic property of adenovirus is reversible, which allows it to traverse toward the nucleus after it escapes from the acidic endosome to the neutral cytosol.

Changes in physicochemical properties of viruses induced by small pH variations have been observed in many types of viruses. Semliki Forest virus, an enveloped virus, gains access to cytosol through dissociation and reorganization of its subunits induced by low pH within endosomes (White et al 1981). Poliovirus, a non-enveloped virus, releases its RNA into cytoplasm at acidic pH (Madshus et al 1984).

Polycation gene delivery systems combined with adenovirus

To enhance DNA escape from endosomes, polycation gene delivery systems were applied in the presence of adenovirus. When a polycation gene delivery system and a human replication-defective adenovirus were co-incubated with cells, the level of gene expression was significantly increased (Curiel et al 1991). These findings suggest that without a specific mechanism for escaping endosomes, the gene transfer by polycations is limited.

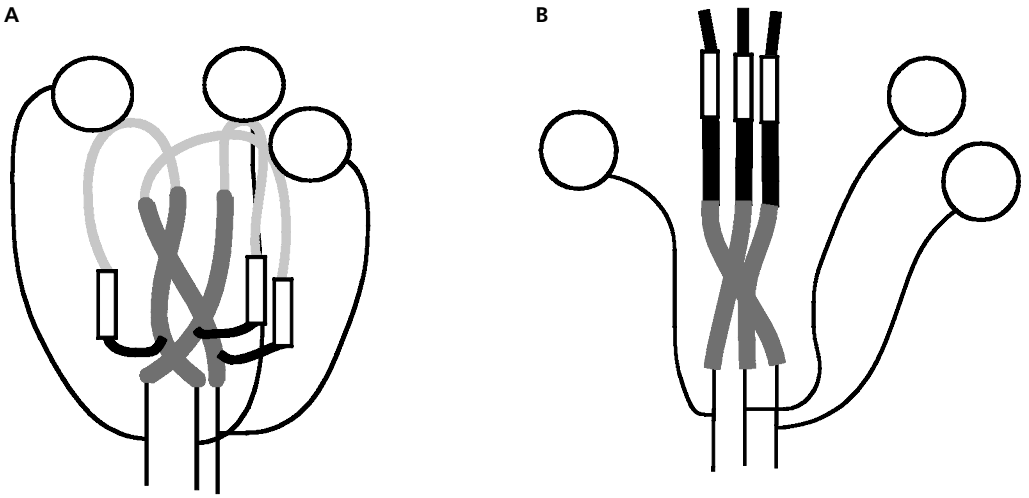


Figure 1 Schematic representation of the trimeric structures of haemagglutinin (HA) at neutral pH (A) and at low pH (B). The HA1 polypeptide (white ball and string) is connected to the HA2 polypeptide by a disulfide bond. At neutral pH, HA has the so-called spring-loaded metastable conformation where the fusion peptides (black) are buried by HA1. In the acidic environment of the endosome, HA1 dissociates from the top of HA2 and the loop region (gray) forms α-helix. As a result, the fusion peptide is exposed at the top of HA2.

In the co-incubation method of adenovirus with the polycation, endosomal escape requires co-internalization of adenovirus particles and the polyplexes into the same endosome. Thus, a large amount of adenovirus particles (no less than 1000 viral particles per cell) must be used, and this may result in toxic side effects. To reduce this problem, adenovirus particles were conjugated to PLL by an enzymatic method (e.g., using transglutaminase) or a biochemical method (e.g., biotinylation of adenovirus and streptavidinylation of PLL) (Wagner et al 1992b). The transfection efficiency was further increased by direct coupling of adenovirus to the polyplexes. This is probably due to an increase in the chance of endosomal escape by the polyplex-linked adenovirus. The adenovirus–conjugated PLL gene delivery systems have been tested in-vivo for suppression of tumours (Nguyen et al 1997a, b). Despite promise and potential, the virus-conjugated polycation system still presents many basic problems, such as the increased toxicity by non-specific uptake through virus receptors and a decrease of vector–DNA complex stability caused by the increased size of the polyplexes. Application of these types of gene delivery systems appears to be of limited use due to safety problems associated with the presence of a live virus. Although viral vectors have established the feasibility of the gene therapy, the clinical realization of the concept requires fully synthetic gene delivery vehicles with high safety, low immunogenicity and ease of manufacture.

Endosome-disrupting peptide

N-terminal amphiphilic anionic peptide of influenza virus haemagglutinin HA2

Polycation gene delivery systems have tried to mimic the endosomal escape mechanism evolved by viruses. The relationship between the structure of viral proteins and their function has been extensively studied. In many cases, small peptide domains of viral proteins were found to have a crucial function responsible for endosomal escape. Polycation gene delivery systems have been equipped with the endosomal escaping property derived from the small endosome-disrupting peptides, such as N-terminal amphiphilic anionic peptides of influenza virus haemagglutinin subunit HA2 (Wagner et al 1992a). The conformational change in HA proteins release the N-terminal amphiphilic anionic peptide residues of HA2, allowing them to interact with endosomal membranes. The N-terminus peptide, itself, undergoes a conformational change induced by the pH change (Lear & DeGrado 1987). At low pH, the protonation of the glutamic acid at positions 11 and 15 and the aspartic acid at position 19 causes its transformation to a helical structure. At neutral pH, they exert charge repulsions and the peptide folds into non-helical conformation. Thus, the membrane-disrupting activity of the peptide significantly depends on pH. The peptide containing the N-terminal sequence of influenza virus HA2 showed a 10-fold increase in the liposome leakage activity at pH 5 as compared with at pH 7 (Plank et al 1994).

The membrane destabilizing peptides from the influenza virus were chemically conjugated to PLL by reacting cysteine mercapto groups with pyridyldithio-modified PLL to yield disulfide-conjugated peptide–PLL derivatives by Wagner et al (1992a). In a liposome assay, the influenza peptide–PLL conjugate showed higher activity than the unconjugated, free peptide. The conjugate still had the pH-responsive liposome leakage activity, but it showed relatively high activity at neutral pH, while the free peptide had no leakage activity at neutral pH. Combination complexes for gene transfer to K562 (human erythroleukaemia) cells were made of plasmid DNA encoding the *Photinus pyralis* luciferase

(pCMVLuc) as a reporter gene, the trasferrin–PLL conjugate and the influenza peptide–PLL conjugate. In the absence of the influenza peptide–PLL conjugate, the DNA–transferrin–PLL complexes did not result in gene expression. The combination complexes containing the influenza peptide conjugate showed high luciferase expression. Similar gene transfer experiments were carried out with HeLa cells. Incorporation of the influenza peptide–PLL conjugate into the complexes resulted in more than 100-fold improvement over the DNA–transferrin–PLL complexes. The presence of the endosome-disruption peptides clearly improves the membrane-disrupting activity, and the key is the structural transformation from non-helical to helical structures by the small decrease in pH of the environment.

Synthetic amphipathic peptides

pH-dependent membrane destabilization by GALA (glutamic acid-alanine-leucine-alanine). The endosomal escape mechanism of the influenza virus shows that the membrane fusion is triggered by the pH-induced conformational change, leading to the exposure of the amphipathic helix. The hydrophobic residues of the amphipathic helix may interact with lipid bilayers to destabilize them. Several types of synthetic amphipathic peptides that mimic the viral peptides were designed to undergo a pH-dependent conformational change and induce leakage of the contents from lipid vesicles. Such synthetic peptides have some advantages over those isolated from biological sources. Synthetic peptides can be obtained in relatively large quantities. The amino-acid sequence can be carefully controlled. The important properties, such as the conformation, hydrophobicity, and peptide length, can be modulated according to the peptide sequence.

GALA is a synthetic amphipathic peptide that contains 30 amino acids with a major repeat sequence of glutamic acid-alanine-leucine-alanine and an N-terminal tryptophan (Figure 2). It undergoes a transition from a random coil at pH 7.5 to an amphipathic α-helix at pH 5.0 (Subbarao et al 1987). The conformational change is correlated with protonation of the glutamic acid side chains. The aligned negative charges on the peptide at neutral pH prevent a stable secondary structure. Protonation of the glutamic acid below its pK_a relieves repulsion, leading to the α-helical conformation and exposing a hydrophobic face that can interact with lipid bilayers. Leakage from lipid vesicles is initiated within 2 s after pH is lowered below 6, and is rapidly terminated when pH is raised to 7.5 (Parente et al 1990a). The pH-dependent leakage activity was greatly influenced by the positioning of the amino acids (Parente et al 1990b). Figure 2 compares the helical grids of GALA and LAGA. These peptides have the same amino-acid composition. The difference between the two peptides is that Glu and Leu residues are aligned on the opposite faces of the helix, making GALA amphipathic, while these residues have a more uniform distribution in LAGA. Both peptides undergo a similar coil to helix transition when pH is lowered from 7.5 to 5.0. However, GALA can induce leakage of the vesicle contents and membrane fusion while LAGA can not. The difference in activity arises from the rearrangement of Glu and Leu residues. GALA forms the amphipathic α-helix, whereas LAGA does not because of the random positioning of Glu and Leu residues. The pH-dependent leakage activity of GALA can be attributed to the amphipathic nature of its α-helical conformation at low pH.

KALA (lysine-alanine-leucine-alanine) for compacting DNA and disrupting endosomes. KALA, a cationic amphipathic peptide, was designed to achieve binding to DNA as well as for destabilizing endosomal membranes (Wyman et al 1997). Its major repeating sequence is lysine-alanine-leucine-alanine (Figure 2). The lysine residues with primary amino groups allows binding to DNA, and the glutamic acid residues at both ends of the peptide provide water solubility when KALA is associated with nucleic acids. KALA undergoes a pH-dependent conformational change from a random coil to an amphipathic α-helix as pH is increased from 5.0 to 7.5, while GALA shows a conformational change to α-helix as pH is decreased. Thus, KALA results in the liposome leakage upon pH increase from 5.0 to 7.5. In the α-helical conformation, one face displays hydrophobic leucine residues, while the opposite face displays hydrophilic lysine residues. At pH 7–9, the peptide has 43–45% of the α-helical content, as measured by circular dichroism. At pH 5, the α-helical content decreases to 24%. Further lowering of pH does not affect the α-helical content. At physiological pH, KALA exists in the α-helical conformation despite the protonated lysine side chains.

The pH dependency of KALA on the liposome leakage activity is not the same for different liposomes. The liposome leakage by KALA increased as pH was raised from 5.0 to 7.5, indicating that KALA destabilizes neutral membranes more efficiently when pH is increased. This result coincided with the increase in the α-helical content when pH was raised. However, with negatively charged liposomes, a more accurate model of cell membrane, KALA induced nearly 100% leakage of entrapped contents over the pH range 4.0–8.5. This might be attributed to the attractive electrostatic interaction between the positively charged peptides and negatively charged liposomes. This result suggests that KALA maintains the membrane-destabilizing activity in the acidic environment of endosomes.

KALA had a large effect on the intracellular localization of ODNs. Fluorescein-labelled ODNs, when added to CV-1 (monkey kidney fibroblast) cells, were poorly internalized and localized into punctuate cytoplasmic regions, corresponding to endosomes or lysosomes (or both). Irrespective of the concentration of ODN, the ODN was not detected in the nucleus of CV-1 cells. In contrast, the nuclear uptake was found in approximately 35% of the cells with KALA/ODN at a 10:1 ($\pm$) charge ratio. KALA modified the intracellular distribution of ODN and increased its nuclear accumulation.

CV-1 cells were transfected with pCMVLuc/KALA complexes at various charge ratios. KALA/DNA com-

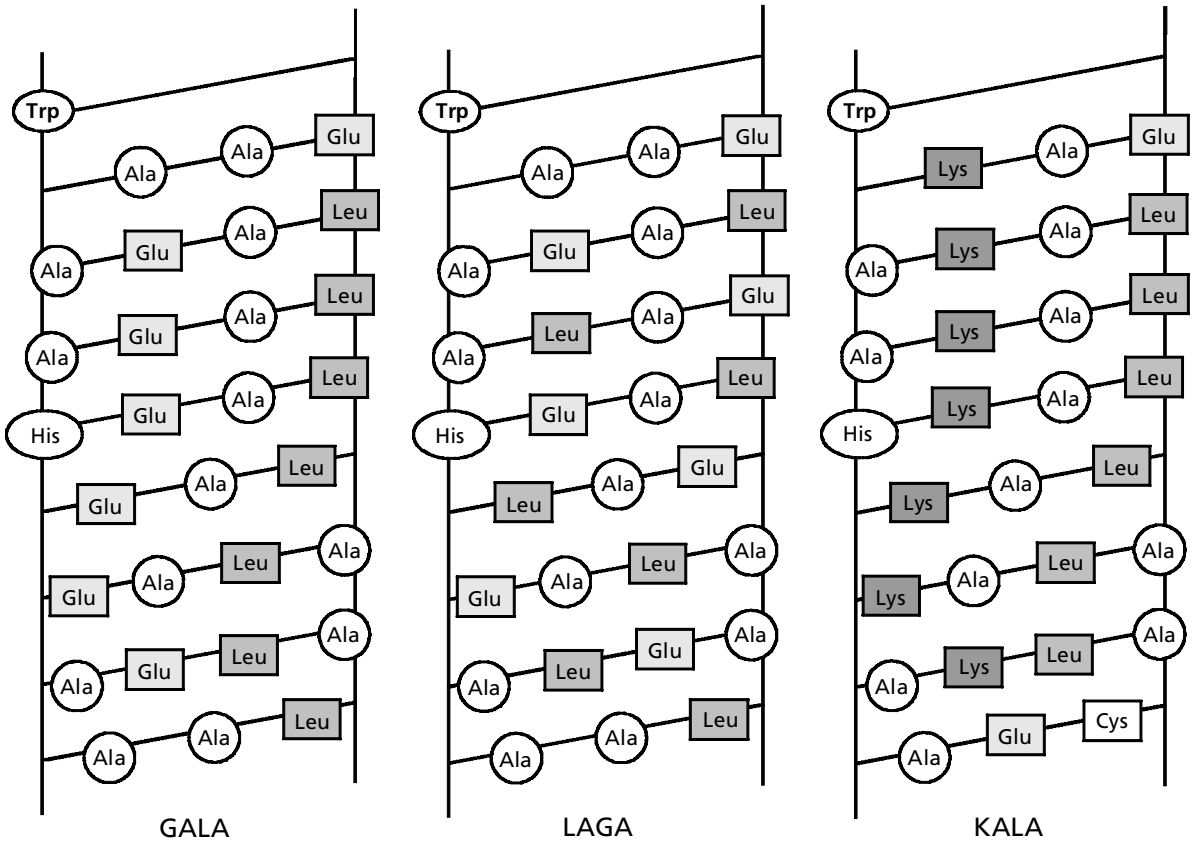


Figure 2 Helical grid representations of GALA, LAGA and KALA.

plexes showed the maximum luciferase activity (10^8 RLU (mg cell protein)$^{-1}$) at a 10:1 (±) charge ratio, which was 100-fold greater than that found in the optimal PLL–DNA complexes (10^6 RLU (mg cell protein)$^{-1}$). No luciferase activity was observed in cells incubated with DNA alone. High transfection required a large excess of KALA, implying that some of the KALA molecules might not be involved in the complexes with DNA but act more as a membrane-disrupting agent.

Polycations combined with amphipathic peptides. Recently, polycation gene delivery systems combined with an amphipathic peptide, KALA, were prepared (Lim et al 2000; Lee et al 2001, 2002). They provided polycation gene delivery systems with the endosomal escaping property by coating of polymer–DNA complex particles with KALA through electrostatic interactions. Figure 3 shows the complex formation among the polymer, DNA and KALA. The copolymer comprising 2-dimethylaminoethyl methacrylate (DMAEMA) and N-vinyl-2-pyrrolidone (NVP) with a terminal carboxylic acid was synthesized and conjugated with PEG-bis(amine) through a coupling reaction using a carbodiimide. Galactose was introduced at the PEG end of the conjugate for specific targeting to asialoglycoprotein receptors of hepatocytes (Lim et al 2000). Poly(DMAEMA-NVP)-b-PEG-galactose compacted DNA and formed complex nanoparticles with a diameter of around 200 nm. The complex particles showed negative surface charge up to a polymer:DNA weight ratio of 6 while maintaining their compacted structures around 200 nm in size. The negatively charged polymer–DNA complex particles were coated with the positive amphipathic peptide KALA to produce polymer–DNA–KALA complex nanoparticles with net positive surface charge. The gene transfer of the polymer–DNA complexes against HepG2 cells without KALA was greatly impaired by the presence of serum proteins. However, irrespective of the presence of serum proteins, the transfection efficiency of polymer–DNA–KALA ternary complex systems was greatly enhanced as the KALA:DNA weight ratio increased, due to the pH-dependent endosomal disruption by KALA (Lim et al 2000).

Histidine

Poly(L-histidine) (PLH) has been used in studies of membrane fusion and has served as a pH-responsive fusogen. Liposome fusion in the presence of PLH was undetectable at pH 7.4, but was greatly enhanced when pH was reduced below 6.5 (Wang & Huang 1984; Uster & Deamer 1985). The pH-dependent liposome fusion by PLH correlates with the protonation of imidazole groups of histidine. The pK_a of the imidazole group of histidine is around 6.0, and thus the imidazole group is protonated

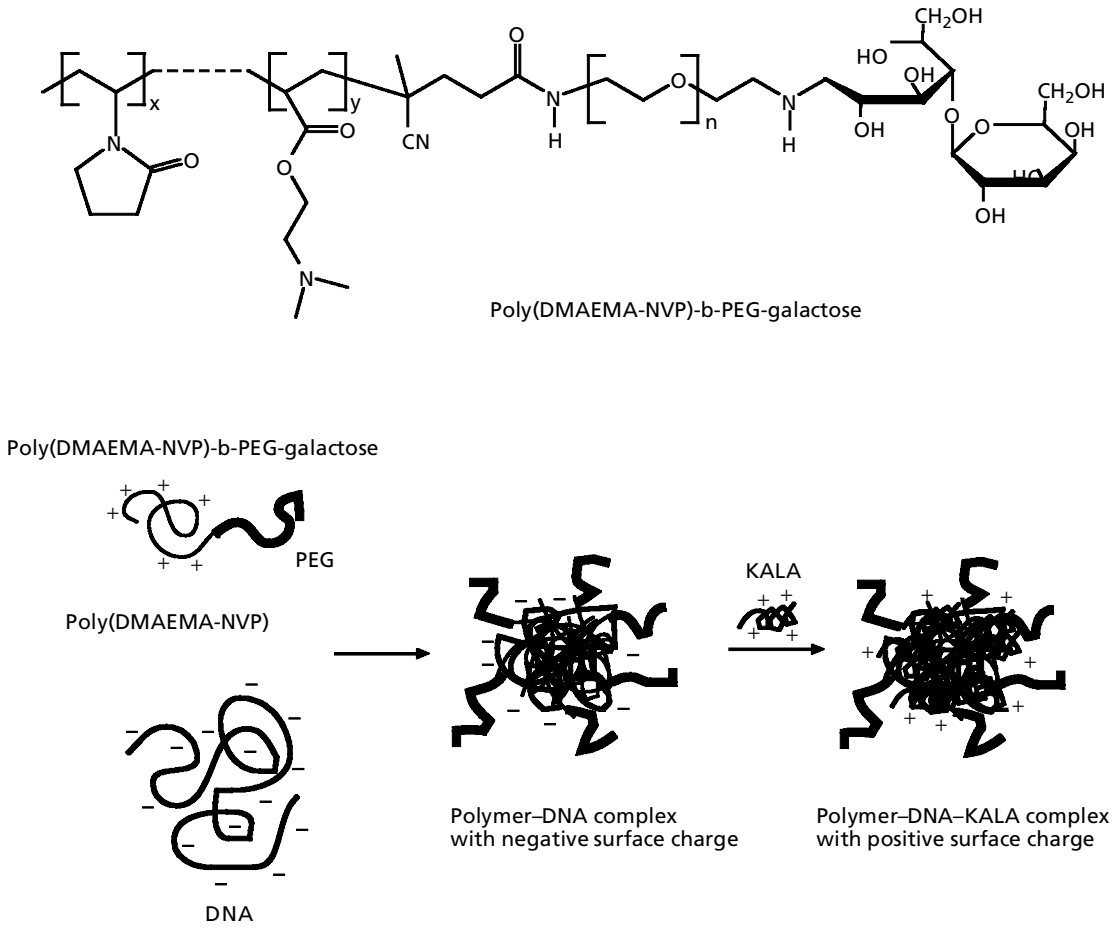


Figure 3 Schematic representation of poly(DMAEMA-NVP)-b-PEG-galactose–DNA–KALA complex formation. Poly(DMAEMA-NVP) = Poly(2-dimethylaminoethyl methacrylate-*N*-vinyl-2-pyrrolidone).

under a slightly acidic milieu (Figure 4). Since PLH becomes a strong polycation at acidic pH, it interacts with negatively charged lipid bilayers, induces bilayer phase separation, and eventually brings about liposome fusion. PLL is also fusogenic at neutral pH because its primary amino groups are protonated even at neutral pH. However, in acidic medium, PLH is more fusogenic than PLL. Maximum fusion of the negatively charged liposomes by PLL occurred at a charge ratio of around 1. However, PLH induced the corresponding liposome fusion at a charge ratio of 0.2 or less (Uster & Deamer 1985).

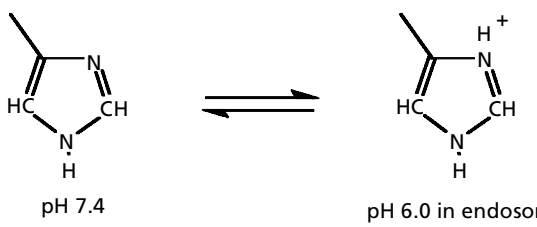


Figure 4 Protonation of imidazole groups of histidine according to pH change.

Histidine-rich peptide

The fusogenic peptide from influenza virus HA2 contains several acid residues. Its fusogenic activity is attributed to the protonation of the acid residues of the peptides and the following conformational change from random to α-helix. Midoux and coworkers synthesized a cationic amphiphilic peptide, GLFGAIAGFIEGGWTGMIDGYWG (H5WYG), which is analogous to the peptide from influenza virus HA2 in which G-4, G-8, E-11, T-15 and D-19 were replaced by histidyl residues and M-17 was replaced by a leucyl residue (Midoux et al 1998). H5WYG undergoes a conformational change between neutral pH and slightly acidic pH. At neutral pH, the peptides are slightly associated or aggregated. Between pH 6.5 and 5.0, the peptide aggregates dissociate. Below pH 5.0, the peptides are not associated or aggregated. This conformational change in water is closely related with the protonation of histidine. H5WYG containing five histidyl residues did not adopt a helical structure in acidic medium in contrast to the HA peptides, but displayed pH-sensitive permeabiliza-

tion of plasma membranes. Within 10 min of being in the presence of 36 μM H5WYG, 97% of U937 cells were permeabilized at pH 6.2, 50% at pH 6.8 and $< 5\%$ at pH 7.1. The efficiency of gene transfer by lactosylated PLL–DNA complexes was remarkably improved in the presence of the cationic amphiphilic peptide. Human hepatoma (HepG2) cells, murine melanoma (B16) cells and rabbit vascular smooth muscle (Rb-1) cells were transfected with lactosylated PLL–DNA complexes. In the presence of H5WYG, the luciferase activity increased 93, 2150 and 630 fold in HepG2, B16, and Rb-1 cells, respectively. The histidine-rich peptide is supposed to disrupt the endosomal membrane. The transfection was drastically inhibited in the presence of bafilomycin A_1, an inhibitor of vacuolar ATPase endosomal proton pump, which is known to block the acidification of the lumen of the endosome. The lack of acidification prevents the protonation of the imidazole groups and abolishes the membrane-disrupting activity of the peptide. H5WYG showed a higher permeabilization activity in slightly acidic medium than did the anionic amphiphilic peptide from influenza virus HA2. In addition, it is notable that the transfection efficiency of the polyplexes combined with the anionic amphiphilic peptides was dramatically reduced in the presence of serum, while the transfection efficiency with the cationic amphiphilic peptides was not significantly affected.

Polylysine coupled with histidine

Histidylated oligolysines were designed to improve the cytosolic delivery of antisense ODNs by using the endosomolytic activity of histidyl residues (Pichon et al 2000). Oligolysines with a degree of polymerization of 19 were reacted with protected histidine (Boc)His(Boc)-OH. Before deprotection of both α-amino and imidazole groups of the histidyl residues, the remaining ϵ-amino groups of the lysyl residues were blocked by either acetylation or gluconoylation. Gluconoyl residues were introduced to increase the water solubility of the histidylated oligolysine (Figure 5).

The oligolysine partially substituted with histidyl residues and ODN were labelled with rhodamine and fluorescein for observing intracellular trafficking, respectively. In the absence of the histidylated oligolysine, ODN was located inside intracellular vesicles and was rarely detected in the cytosol or in the nucleus. In contrast, in the presence of the histidylated oligolysine, ODN was present in the cytosol and in the nucleus, as well as inside vesicles scattered throughout the cytoplasm. In addition, the size of the vesicles containing ODN was larger in the presence of the histidylated oligolysine. The presence of the larger vesicles implies that there was fusion of several vesicles. The increase in ODN delivery to the cytosol resulted in significant improvement of its biological activity. These histidylated oligolysines were effective in different cell lines, but their efficiency varied from one cell type to another, possibly due to the differences in the pH of the lumen of endosomes as well as in the uptake efficiency and intracellular trafficking.

PLH was combined with PLL-based gene delivery systems for pH-responsive endosomal escape (Pack et al 2000). Since PLH is not soluble in aqueous solutions at neutral pH, it was modified with gluconic acid to give solubility at physiological pH. The gluconic acid-conjugated PLH (G-PLH) was added to transferrin-conjugated PLL (Tf-PLL)–DNA complexes. The DNA–G-PLH–Tf-PLL ternary complex system formed well-condensed nanoparticles with a size of 100 nm due to PLL, displayed a targeting and receptor-mediated endocytosis due to transferrin and exhibited endosomolytic activity due to PLH. COS-7 cells were transfected in-vitro with plasmid DNA encoding for the reporter gene β-galactosidase using a ternary complex system, which resulted in β-galactosidase activity equivalent to that observed for gene delivery with DNA–Tf-PLL in the presence of chloroquine, which is a well-known transfection helper.

PLH mediates an acid-dependent membrane fusion and leakage of negatively charged liposome, but does not form complexes with DNA at biological pH, because it is not positively charged at neutral pH. The ϵ-amino groups of PLL were partially substituted with histidine both for complex formation with DNA by lysine residues and for endosomal membrane disruption by histidine residues (Midoux & Monsigny 1999; Roufaï & Midoux 2001). PLL with histyl residues (His-PLL) forms complexes with DNA at pH 7.4. The complexes actively transfected HepG2 cells in culture in the absence of any helper compounds, such as chloroquine, glycerol or fusogenic peptides. The luciferase activity with His-PLL–DNA complexes was 4.5 orders of magnitude higher (10^7 RLU/10^6 cells) than with PLL–DNA complexes (200 RLU/10^6 cells). It was 3.5 orders of magnitude higher than with PLL–DNA complexes in the presence of chloroquine (2×10^3 RLU/10^6 cells). The transfection efficiency was optimal with PLL having 72 ± 9 histidyl residues, corresponding to about 38% of the ϵ-amino groups substituted with histidyl residues.

A comb-shaped polymer, consisting of PLL as a backbone and PLH as branches, was developed (Benns et al 2000) (Figure 6A). First, poly(imidazole-benzyl-L-histidine), an 18-mer linear polymer without a free amine group, was synthesized by solid-phase peptide synthesis using Fmoc-chemistry. Second, the protected PLH was grafted on PLL through coupling reaction using dicyclohexacarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS). Finally, the protecting groups were removed. One-fourth of amino groups of PLL were conjugated to PLH, forming a comb-shaped polymer, *N*-acetyl-poly(L-histidine)–graft–poly(L-lysine) (PLH–g–PLL). The graft polymer efficiently condensed DNA into small polyplex particles, suggesting that the grafted PLH did not significantly deteriorate the ability of PLL to condense DNA. The PLH–g–PLL showed higher transfection efficiency in 293T cells than PLL. However, its transfection efficiency was further increased by addition of chloroquine, indicating that there is still room for improvement in the polycation gene carrier.

Another type of comb-shaped polymers, composed of a poly(1-vinylimidazole) (PVIm) backbone and lactosylated PLL side chains, were prepared with the purpose of using the pH-responsive imidazole protonation (Asayama et al

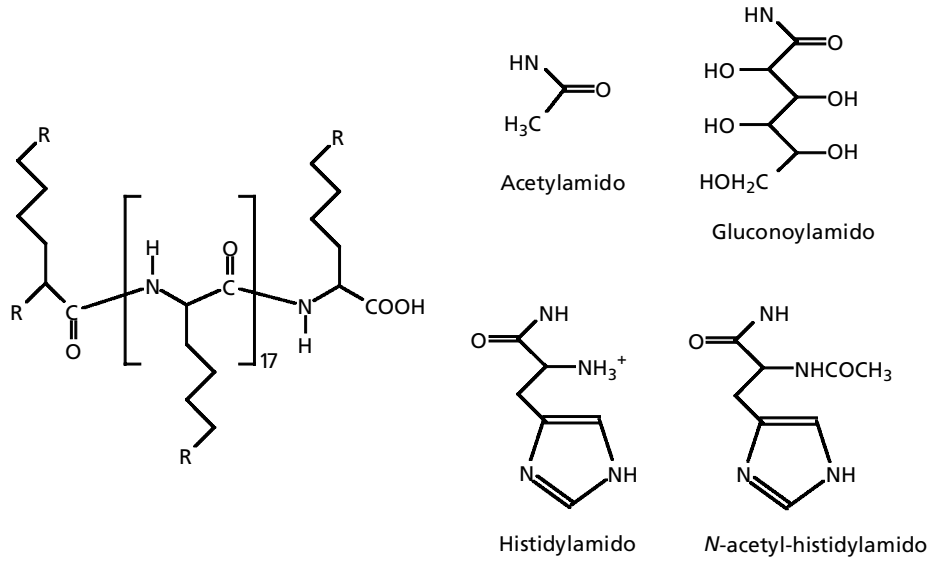


Figure 5 Schematic structures of histidylated oligolysines. R = NH_3^+, acetylamido, histidylamido, gluconoylamido or *N*-acetyl-histidylamido.

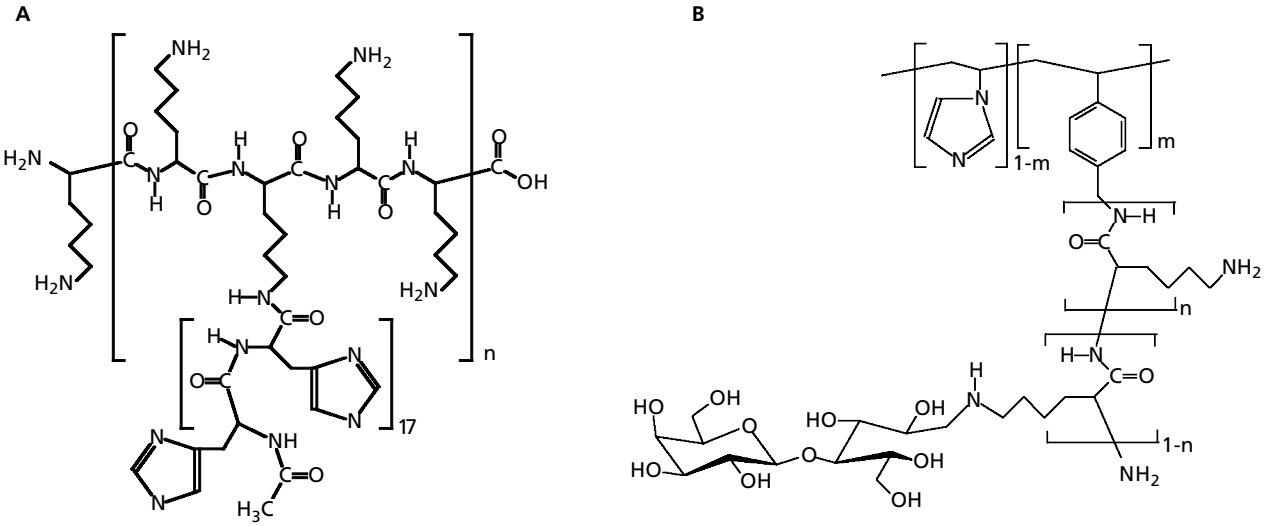


Figure 6 Chemical structure of comb-shaped polymers consisting of lysine and histidine residues. A. *N*-Acetyl-poly(L-histidine)–graft–poly (L-lysine). B. Lactosylated polylysine–graft–poly(1-vinylimidazole).

2001) (Figure 6B). Poly(N^{ϵ}-carbobenzoxy (CBZ)-L-lysine) macromonomers were prepared by polymerization of N^{α}-carboxyanhydride (NCA) of CBZ-L-lysine with *p*-aminomethyl-styrene as an initiator. The macromonomer was radically copolymerized with vinylimidazole (VIm). After deprotection, the ϵ-amino groups of the intermediate polymer and the reducing end of lactose were covalently coupled by reductive amination to produce the lactosylated PVIm-graft–PLL comb-type copolymer. At pH 7.5, PVIm, the backbone polymer, has a slight tendency to form complexes with DNA. At pH 6.0, no free DNA was observed when DNA was mixed with an excess of PVIm. In the case of lactosylated PLL, a side chain polymer formed the stoichiometric complex with DNA and completely compacted DNA at pH 7.5. The graft copolymer containing both polymers formed the stoichiometric complex with DNA at pH 7.5 through PLL side chains. Protonation of the PVIm backbone at low pH significantly changed the assembling structure of the DNA–polymer complexes.

Although it is evident that the imidazole protonation induced by the acidic environment of endosomes mediates destabilization of the endosomal membranes, its detailed mechanism is not yet fully understood. Two major

mechanisms were suggested: an osmotic gradient leading to endosome rupture (Pack et al 2000) and the interaction of histidine-rich molecules with endosomal membranes (Midoux & Monsigny 1999). The former is based on a buffer effect of imidazole groups of histidine residues. The hypothesis states that histidine-rich molecules exhibit a buffer effect upon imidazole protonation, resulting in disruption of the endosomal bilayer membrane. This mechanism is basically the same as that of PEI, the so-called proton sponge (see Polyethyleneimine, below). However, double staining experiments using the rhodamine-labelled histidylated oligolysines and the fluorescein-labelled ODNs showed that the histidylated oligolysines remained inside the endosomes while the ODNs were delivered to the cytosol and the nucleus, which strongly suggested the latter mechanism (i.e., the interaction of histidine-rich molecules with endosomal membranes) (Pichon et al 2000). In addition, PLH is more fusogenic than PLL in acidic medium. Fusion of liposomes occurs at a positive:negative charge (PLH–liposomes) ratio of 0.2, while it occurs at a ratio of 1 in the case of PLL (Wang & Huang 1984). This implies that the mechanism involved in the membrane destabilization by the histidine residues could be different from that by PEI, which is supposed to act as a buffer.

Polycations with intrinsic endosomolytic activity

Starburst polyamidoamine dendrimers

Starburst polyamidoamine dendrimers are highly branched spherical polymers that can be synthesized with well-defined diameters and a surface of primary amino groups. Starburst polyamidoamine dendrimers can be synthesized from ammonia or ethylenediamine initiator cores and step-wise polymerization with methyl acrylate and ethylenediamine (Figure 7) (Haensler & Szoka 1993; Tang et al 1996). Controlled step-growth propagation produces increasingly higher generations of polymers with a precise number of terminal amino groups. With each new generation, the number of surface amino groups doubles.

The transfection efficiency of dendrimer–DNA complexes was greatly affected by its generation number. The transfection efficiency was dramatically increased when the generation increased from 3 to 5, which may be related to the increase in diameter and the change in polymer structure (Haensler & Szoka 1993). Through generation 3, the dendrimers resemble a starfish and by generation 5 they have a spheroidal form about 54 Å in diameter.

Starburst polyamidoamine dendrimers are believed to have intrinsic endosomolytic activity. The transfection efficiency was particularly high when a large excess of terminal amines against nucleotides were used, whereas an excess of PLL could not increase the transfection efficiency. The transfection activity was not enhanced by the addition of chloroquine, whereas the transfection efficiency of the PLL–DNA complex system was dramatically enhanced in the presence of chloroquine (Cotten et al 1990). The dendrimers have two kinds of amino groups: a terminal primary amino group with pK_a of 6.9 and an internal tertiary amino group with pK_a of 3.9. At physiological pH, the dendrimers are only partially protonated as weak bases. At low pH within endosomes, most of the terminal primary amino groups are thought to be protonated and contribute to its endosomolytic activity. In addition, the interior tertiary amino groups might also contribute to membrane disruption in the more acidic environment of lysosomes. The dendrimer is thought to buffer endosomal acidification after endocytosis of the complex. Accumulation of proton and chloride ions is followed by the influx of water, resulting in the disruption of endosomes. The endosomolytic activity of the dendrimers can explain why a large excess of dendrimers are required for high transfection efficiency and why its transfection efficiency is not enhanced by chloroquine.

GALA, a membrane-destabilizing amphipathic peptide, was covalently attached to polyamidoamine dendrimers via a disulfide linkage (Figure 7). When 50% of the dendrimer in the complex was replaced with its GALA conjugate, the transfection efficiency was increased by 2–3 orders of magnitude. However, the conjugate can only function at a low dendrimer–plasmid ratio. The transfection efficiency was not enhanced further by GALA at a high dendrimer–plasmid ratio.

Polyethyleneimine

PEI with a high cationic charge density is known to be an efficient gene carrier. PEI is available in linear and branched forms. The unique feature of PEI is its high cationic charge density, because every third atom is a potentially protonable amino nitrogen (Figure 8A). Branched PEI comprises 25, 50 and 25% of primary, secondary and tertiary amines. PEI has a high buffer capacity over a broad pH range (Suh et al 1994). The complex with DNA is formed by ionic interactions between primary amines of PEI and phosphate groups of DNA. It is reported that secondary and tertiary amines provide the endosomolytic activity due to its buffering effect, which supports the high transfection efficiency of PEI.

Boussif and coworkers proposed the proton sponge hypothesis (Figure 8B) to explain the high efficiency of PEI–DNA and polyamidoamine dendrimer–DNA polyplexes (Boussif et al 1995). The hypothesis asserts that polymers with high cationic charge potential, such as PEI, act as a proton sponge upon acidification within endosomes or lysosomes. At physiological pH, every sixth nitrogen atom of PEI is protonated. Under the highly acidic environment within lysosomes, every third nitrogen atom is protonated. The estimated fraction of protonated nitrogens increases 15% to 45%. The highly branched network of PEI absorbs a large amount of proton ions, like a sponge, upon the lowering of pH. Protonation of amine groups of PEI in the acidic environment of endosomes or lysosomes brings a charge gradient, which induces influx of counter ions, Cl^-, to compensate this charge gradient. The increased Cl^- concentration increases endosomal or lysosomal osmolarity, leading to a water influx into endosomes or lysosomes to relieve the

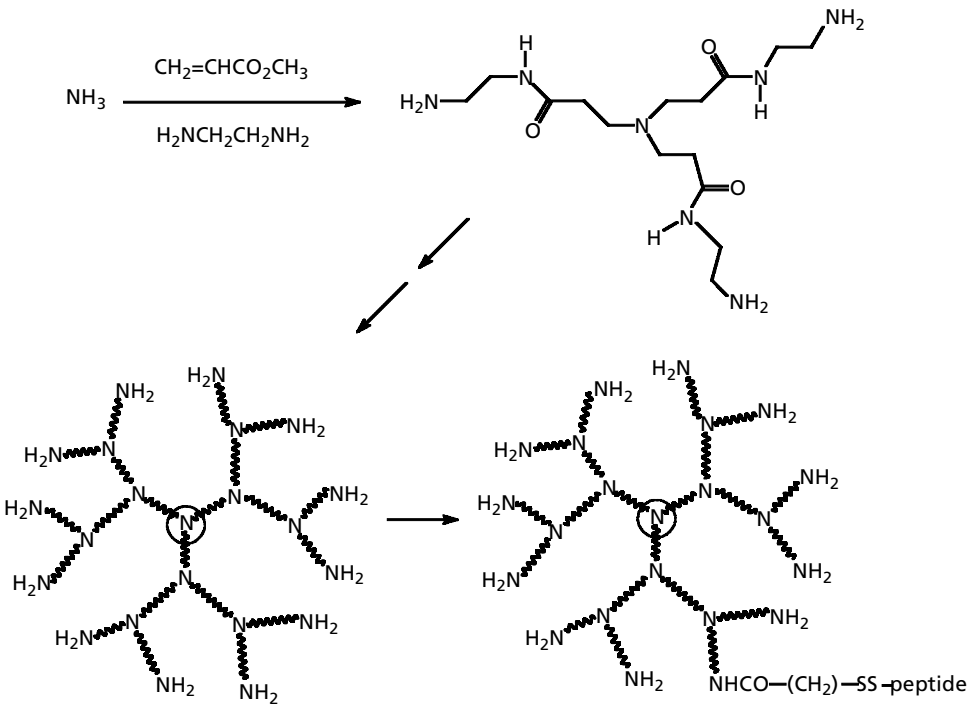


Figure 7 Synthesis of polyamidoamine starburst dendrimers and the attachment of membrane-destabilizing peptides.

gradient, resulting in endosomal or lysosomal swelling and subsequent bursting, and thus allowing escape of the contents in vesicles into cytosol.

Polyanions inducing membrane disruption

Synthetic anionic polymers that induce pH-responsive membrane disruption have been developed (Lackey et al 1999; Murthy et al 1999; Cheung et al 2001; Kyriakides et al 2002). These polymers were also designed to mimic anionic amphiphilic peptides that induce the endosomal membrane disruption. The typical endosome-disruptive amphiphilic anionic peptides, like the HA peptides of the influenza viruses, have the composition of anionic amino acids having carboxylic side chains intermixed with hydrophobic amino acids with alkyl chains. Poly(ethyl acrylic acid) (PEAAc) and poly(propyl acrylic acid) (PPAAc) have basically similar compositions including carboxylic groups and hydrophobic alkyl side chains (Figure 9).

The potential of these anionic polymers to disrupt endosomes was evaluated by a haemolysis assay. Both PEAAc and PPAAc showed pH-responsive haemolytic activity. Although they were not haemolytic at pH 7.4, PPAAc and PEAAc showed the maximum haemolysis at pH 6.0 or lower and at pH 5.0 or lower, respectively. At pH 6.1, PPAAc was approximately 15 times more effective than PEAAc in haemolytic activity. The difference in chemical structure between the two polymers is in the alkyl side chain: a propyl group for PPAAc and an ethyl group for PEAAc. The significant difference in haemolytic activity is most likely related to their hydrophobic characters when the carboxylate ions become protonated at low pH. A 1:1 random copolymer of ethyl acrylate and acrylic acid (EA–AAc), which combines the aliphatic and carboxylic groups in a random fashion rather than as repeating groups as in PEAAc, showed a similar pH-responsive haemolytic activity to PEAAc. These studies suggest that synthetic, anionic polymers with carboxylic and hydrophobic groups could effectively induce the membrane disruption within defined and narrow pH ranges.

Addition of PPAAc to DOTAP–DNA lipoplexes greatly enhanced transfection efficiency in NIH3T3 mouse fibroblast cells (Cheung et al 2001). Complex particles were formed by electrostatic interaction between a positively charged DOTAP liposome and negatively charged DNA and PPAAc. The level of β-galactosidase gene expression with the ternary system was increased 20 fold over the control (DOTAP–DNA only). In addition, PPAAc also significantly improved the stability of lipoplexes in serum.

Dissociation of polyplexes and transport into nucleus

Despite extensive studies on the complex formation between nucleic acids and polycations, dissociation of the complex has not been studied enough. Although dissociation of the complexes is one of the essential steps for efficient gene transfer, the dissociation mechanisms of most polycation gene delivery systems are still unknown. The complexes may be dissociated in endosomes, in the cytosol or in the nucleus. It has been reported that the transfection efficiency of polylysine gene delivery systems

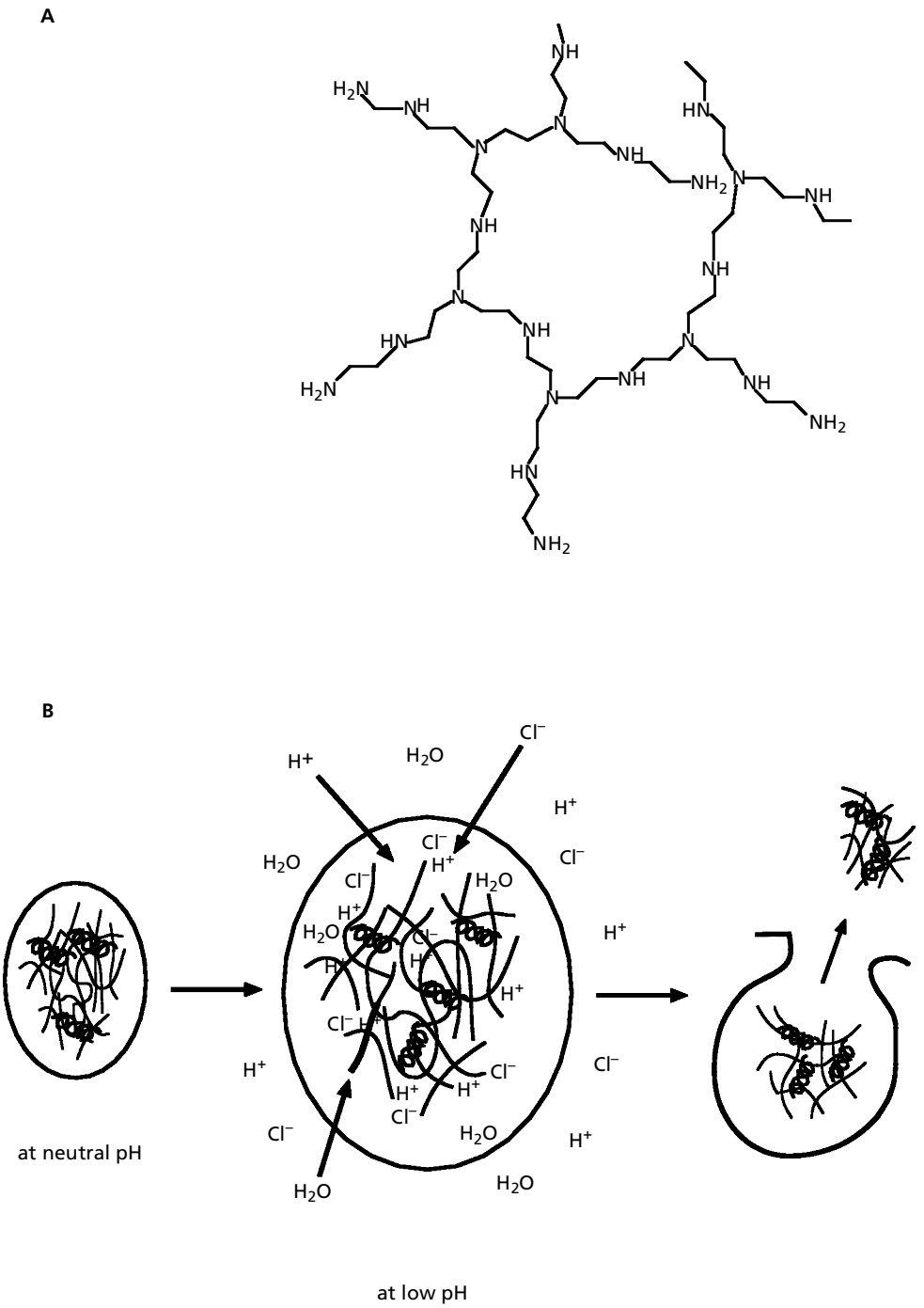
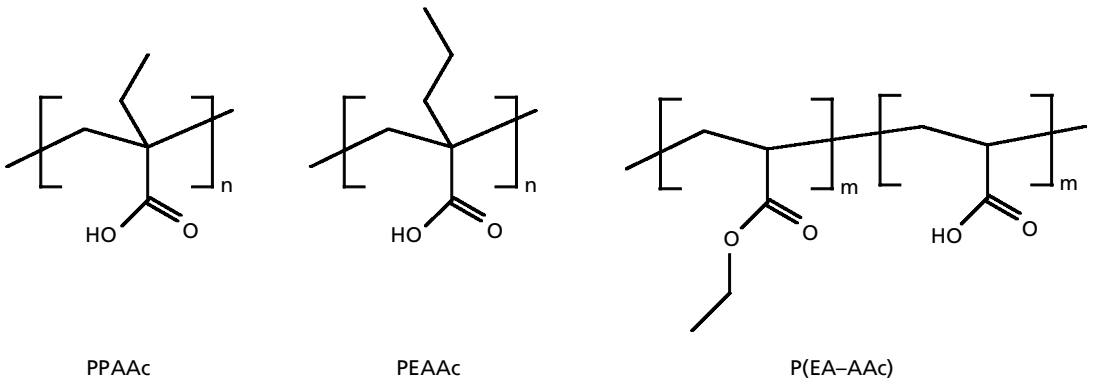


Figure 8 Chemical structure of polyethylenimine (A) and the proton sponge hypothesis (B).

was significantly enhanced by using lower-molecular-weight PLL which can dissociate from DNA more rapidly than higher-molecular-weight PLL. Clearly, release of free nucleic acids from vectors is one of the most critical steps determining the efficiency of non-viral gene delivery systems (Schaffer et al 2000). From this point of view, polycations were designed to release nucleic acids in a reductive medium containing a thiol (e.g. 1,4-dithiol-L-threitol) or an enzyme (e.g. thioredoxin reductase) (Balakirev et al 2000). The lipoic acid-derived polycations can reversibly bind and release nucleic acids. In the oxidized state, the polycations condensed DNA into spherical complex particles with a diameter of around 32 nm. Upon reduction, the spherical particles swelled



PPAAc PEAAc P(EA–AAc)

Figure 9 Chemical structures of poly(propyl acrylic acid) (PPAAc), poly(ethyl acrylic acid) (PEAAc) and a 1:1 random copolymer of ethyl acrylate and acrylic acid (P(EA–AAc)).

and transformed into a population of toroids with a diameter of about 100 nm, then disappeared. These studies suggest that the unpackaging property can be added to non-viral vectors.

After polyplexes are released from endosomes to cytosol, they still have to move into the nucleus through the cytoplasm and the nuclear membrane or the polyplexes have to release DNA before the released DNA can move into the nucleus. The precise mechanisms for these processes are still poorly understood but transport into the nucleus is certainly one of the major limitations for efficient gene delivery. Studies using microinjection of DNA demonstrated the importance of transport into the nucleus. Most of the cytoplasmically microinjected DNA remained at the site of injection. DNA injected far from the nuclei (60–90 μm) showed much less protein expression than DNA injected near the nuclei (Dowty et al 1995). Furthermore, when 10 000 naked DNA copies were injected into the cytoplasm, only 13% of the cells expressed the transgene. A corresponding expression efficiency was obtained when less than 10 copies of naked DNA were directly injected into the nucleus (Pollard et al 1998).

Small molecules permeate into nucleus membranes through nuclear pores. However, the transport of large macromolecules through nuclear pores is mediated by their own nuclear localization signals (NLSs). The first NLS sequence (PKKKRKV) was found and derived from the simian virus 40 large tumour antigen. Most NLSs were generally characterized by one short basic peptide sequence containing several lysine and arginine residues (Adam & Gerace 1991; Robbins et al 1991). To escort DNA into the nucleus, NLS sequences have been incorporated into non-viral gene delivery systems (Brandén et al 1999; Singh et al 1999; Zanta et al 1999).

Concluding remarks

Although polycation gene delivery systems are attractive due to high safety, low immunogenicity and an easy manufacturing process, their gene transfection efficacy in all of the in-vitro experiments is quite low when compared with that of viral vectors. Their in-vivo transfection efficiency is even lower. Endosomal escape to cytosol, transport to the nucleus and dissociation of complexes have been major limitations for efficient gene transfer by polycations. Polycation gene delivery systems have adapted the mechanisms evolved by viruses that exploit the natural acidification process occurring within endosomes or lysosomes. Membrane-destabilizing peptides derived from viruses were incorporated into polycation gene delivery systems. Histidine-rich polymers displaying pH-responsive endosomolytic activity have also been developed. Polycations with a high charge density and a highly branched network structure showed endosomolytic activity based on a buffering effect. In addition to the ability to escape from endosomes, the ideal gene delivery vectors should be able to escort genes to the nucleus and release (or dissociate) the genes for their expression. Understanding these cellular events is the key to the development of ideal vectors.

Recent gene therapies include antisense and ribozyme strategies to modulate gene expression at the RNA level. Over the past decade, antisense drugs, including antisense ODNs and ribozymes, have gained increasing attention. Since they are short oligonulceotides, it is relatively easy to manipulate them chemically. They have great specificity for their target, as antibodies do. Their clinical success will also be greatly dependent on the development of appropriate delivery vehicles. Considering that antisense drugs do not require uptake into the nucleus, one could conclude that endosomal escape may be the most critical factor, and many polycation gene delivery systems are specifically designed to achieve such a process, as described in this review.

References

Adam, S. A., Gerace, L. (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell* **66**: 837–847

Asayama, S., Akaike, T., Maruyama, A. (2001) Bi-phasic polycation for the DNA carrier responding to endosomal pH. *Colloids Surf. B: Biointerf.* **22**: 183–191

Balakirev, M., Schoehn, G., Chroboczek, J. (2000) Lipoic acid-derived amphiphiles for redox-controlled DNA delivery. *Chem. Biol.* **7**: 813–819

Benns, J. M., Choi, J.-S., Mahato, R. I., Park, J.-S., Kim, S. W. (2000) pH-Sensitive cationic polymer gene delivery vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer. *Bioconj. Chem.* **11**: 637–645

Blessing, T., Kursa, M., Holzhauser, R., Kircheis, R., Wagner, E. (2001) Different strategies for formation of PEGylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. *Bioconj. Chem.* **12**: 529–537

Blumenthal, R., Seth, P., Willingham, M. C., Pastan, I. (1986) pH-dependent lysis of liposomes by adenovirus. *Biochemistry* **25**: 2231–2237

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., Behr, J.-P. (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

Brandén, L. J., Mohamed, A. L., Smith, C. I. E. (1999) A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. *Nature Biotech.* **17**: 784–787

Bullough, P. A., Hughson, F. M., Skehel, J. J., Wiley, D. C. (1994) Structure of influenza hemagglutinin at the pH of membrane fusion. *Nature* **371**: 37–43

Carr, C. M., Kim, P. S. (1993) A spring-loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* **73**: 823–832

Carr, C. M., Kim, P. S. (1994) Flu virus invasion: Halfway there. *Science* **266**: 234–236

Carr, C. M., Chaudhry, C., Kim, P. S. (1997) Influenza hemagglutinin is spring-loaded by a metastable native conformation. *Proc. Natl Acad. Sci. USA* **94**: 14306–14313

Cherng, J.-Y., Wetering, P. V. D., Talsma, H., Crommelin, D. J. A., Hennink, W. E. (1996) Effect of size and serum proteins on transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. *Pharm. Res.* **13**: 1038–1042

Cheung, C. Y., Murthy, N., Stayton, P. S., Hoffman, A. S. (2001) A pH-sensitive polymer that enhances cationic lipid-mediated gene transfer. *Bioconj. Chem.* **12**: 906–910

Choi, Y. H., Liu, F., Kim, J.-S., Choi, Y. K., Park, J. S., Kim, S. W. (1998) Polyethylene glycol-grafted poly-L-lysine as polymeric gene carrier. *J. Control. Release* **54**: 39–48

Cotten, M., Langle-Rouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., Birnstiel, M. L. (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

Curiel, D. T., Agarwal, S., Wagner, E., Cotten, M. (1991) Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc. Natl Acad. Sci. USA* **88**: 8850–8854

Dowty, M. E., Williams, P., Zhang, G., Hagstrom, J. E., Wolff, J. A. (1995) Plasmid DNA entry into postmitotic nuclei of primary rat myotubes. *Proc. Natl Acad. Sci. USA* **92**: 4572–4576

Haensler, J., Szoka, F. C. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconj. Chem.* **4**: 372–379

Kataoka, K., Togawa, H., Harada, A., Yasugi, K., Matsumoto, T., Katayose, S. (1996) Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block colpolymer in physiological saline. *Macromolecules* **29**: 8556–8557

Kyriakides, T. R., Cheung, C. Y., Murthy, N., Bornstein, P., Stayton, P. S., Hoffman, A. S. (2002) pH-Sensitive polymers that enhance intracellular drug delivery in vivo. *J. Control. Release* **78**: 295–303

Lackey, C. A., Murthy, N., Press, O. W., Tirrell, D. A., Hoffman, A. S., Stayton, P. S. (1999) Hemolytic activity of pH-responsive polymer-streptavidin bioconjugates. *Bioconj. Chem.* **10**: 401–405

Lear, J. D., DeGrado, W. F. (1987) Membrane binding and conformational properties of peptides representing the NH_2 terminus of influenza HA-2. *J. Biol. Chem.* **262**: 6500–6505

Lee, H., Jeong, J. H., Park, T. G. (2001) A new gene delivery formulation of polyethyleneimine/DNA complexes coated with PEG conjugated fusogenic peptide. *J. Control. Release* **76**: 183–192

Lee, H., Jeong, J. H., Park, T. G. (2002) PEG grafted polylysine with fusogenic peptide for gene delivery: high transfection efficiency with low cytotoxicity. *J. Control. Release* **79**: 283–291

Lee, K. Y., Kwon, I. C., Kim, Y.-H., Jo, W. H., Jeong, S. Y. (1998) Preparation of chitosan self-aggregates as a gene delivery system. *J. Control. Release* **51**: 213–220

Lim, D. W., Yeom, Y. I., Park, T. G. (2000) Poly(DMAEMA-NVP)-b-PEG-galactose as gene delivery vector for hepatocytes. *Bioconj. Chem.* **11**: 688–695

Madshus, I. H., Olsnes, S., Sandvig, K. (1984) Mechanism of entry into the cytosol of poliovirus type 1: requirement for low pH. *J. Cell Biol.* **98**: 1194–1200

Midoux, P., Monsigny, M. (1999) Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconj. Chem.* **10**: 406–411

Midoux, P., Kichler, A., Boutin, V., Maurizot, J.-C., Monsigny, M. (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconj. Chem.* **9**: 260–267

Murthy, N., Robichaud, J. R., Tirrell, D. A., Stayton, P. S., Hoffman, A. S. (1999) The design and synthesis of polymers for eukaryotic membrane disruption. *J. Control. Release* **61**: 137–143

Nguyen, D. M., Wiehle, S. A., Roth, J. A., Cristiano, R. J. (1997a) Gene delivery into malignant cells in vivo by a conjugated adenovirus/DNA complex. *Cancer Gene Ther.* **4**: 183–190

Nguyen, D. M., Wiehle, S. A., Koch, P. E., Branch, C., Yen, N., Roth, J. A., Cristiano, R. J. (1997b) Delivery of the p53 tumor suppressor gene into lung cancer cells by an adenovirus/DNA complex. *Cancer Gene Ther.* **4**: 191–198

Pack, D. W., Putnam, D., Langer, R. (2000) Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnol. Bioeng.* **67**: 217–223

Parente, R. A., Nir, S., Szoka, F. C. (1990a) Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry* **29**: 8720–8728

Parente, R. A., Nadasdi, L., Subbarao, N. K., Szoka, F. C. (1990b) Association of a pH-sensitive peptide with membrane vesicles: Role of amino acid sequence. *Biochemistry* **29**: 8713–8719

Pichon, C., Roufaï, M. B., Monsigny, M., Midoux, P. (2000) Histidylated oligolysines increase the transmembrane passage and the biological activity of antisense oligonucleotides. *Nuleic Acids Res.* **28**: 504–512

Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., Wagner, E. (1992) Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconj. Chem.* **3**: 533–539

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem.* **269**: 12918–12924

Pollard, H., Remy, J.-S., Loussouarn, G., Demolombe, S., Behr, J.-P., Escande, D. (1998) Polyethyleneimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J. Biol. Chem.* **273**: 7507–7511

Robbins, J., Dilworth, S. M., Laskey, R. A., Dingwall, C. (1991) Two interdependent basic domains in nucleplasmin nuclear targeting sequence: Identification of a class of bipartite nuclear targeting sequence. *Cell* **64**: 615–623

Rojanasakul, Y., Wang, L. Y., Malanga, C. J., Ma, J. K. H., Liaw, J. (1994) Targeted gene delivery to alveolar macrophages via Fc receptor-mediated endocytosis. *Pharm. Res.* **11**: 1731–1736

Roufaï, M. B., Midoux, P. (2001) Histidylated polylysine as DNA vector: Elevation of the imidazole protonation and reduced cellular uptake without change in the polyfection efficiency of serum stabilized negative polyplexes. *Bioconj. Chem.* **12**: 92–99

Schaffer, D. V., Fidelman, N. A., Dan, N., Lauffenburger, D. A. (2000) Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol. Bioeng.* **67**: 598–606

Seth, P., Willingham, M. C., Pastan, I. (1984) Adenovirus-dependent release of ^{51}Cr from KB cells at an acidic pH. *J. Biol. Chem.* **259**: 14350–14353

Seth, P., Willingham, M. C., Pastan, I. (1985) Binding of adenovirus and its external proteins to Triton X-114. *J. Biol. Chem.* **260**: 14431–14434

Singh, D., Bisland, S. K., Kawamura, K., Gariepy, J. (1999) Peptide-based intracellular shuttle able to facilitate gene transfer in mammalian cells. *Bioconj. Chem.* **10**: 745–754

Subbarao, N. K., Parente, R. A., Szoka, F. C., Nadasdi, L., Pongracz, K. (1987) pH-dependent bilayer destabilization by an amphipathic peptide. *Biochemistry* **26**: 2964–2972

Suh, J., Paik, H.-J., Whang, B. K. (1994) Ionization of poly (ethyleneimine) and poly(allylamine) at various pH's. *Bioorg. Chem.* **22**: 318–327

Tang, M. X., Redemann, C. T., Szoka Jr., F. C. (1996) In vitro gene delivery by degraded polyamindoamine dendrimers. *Bioconj. Chem.* **7**: 703–714

Trubetskoy, V. S., Torchilin, V. P., Kennel, S. J., Huang, L. (1992) Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. *Bioconj. Chem.* **3**: 323–327

Uster, P. S., Deamer, D. W. (1985) pH-dependent fusion of liposomes using titratable polycations. *Biochemistry* **24**: 1–8

Vinogradov, S. V., Bronich, T. K., Kabanov, A. V. (1998) Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonulceotides. *Bioconj. Chem.* **9**: 805–812

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

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., Birnstiel, M. L. (1992a) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl Acad. Sci. USA* **89**: 7934–7938

Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D. T., Birnstiel, M. L. (1992b) Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc. Natl Acad. Sci. USA* **89**: 6099–6103

Wang, C.-Y., Huang, L. (1984) Polyhistidine mediates an acid-dependent fusion of negatively charged liposomes. *Biochemistry* **23**: 4409–4416

White, J., Matlin, K., Helenius, A. (1981) Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. *J. Cell Biol.* **89**: 674–679

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

Wyman, T. B., Nicol, F., Zelphati, O., Scaria, P. V., Plank, C., Szoka, F. C. (1997) Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry* **36**: 3008–3017

Zanta, M. A., Belguise-Valladier, P., Behr, J.-P. (1999) Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl Acad. Sci. USA* **96**: 91–96