Pharmaceutical Research, Vol. 21, No. 1, January 2004 (© 2004)

Review

Strategies to Improve DNA Polyplexes for *in Vivo* Gene Transfer: Will "Artificial Viruses" Be the Answer?

Ernst Wagner[1,2]

Received September 29, 2003; accepted September 29, 2003

For the purpose of introducing nucleic acids into cells, cationic polymers have been steadily improved as gene carriers. This has resulted in improved polymer-based gene transfer formulations, termed polyplexes, which efficiently transfect cell cultures and also have shown encouraging gene transfer potential in *in vivo* administration. Targeted delivery to liver, lung, tumor, or other tissues has been achieved in experimental animals by localized or systemic application. Therapeutic effect has been demonstrated, although efficiencies are still too low to justify clinical use. The limitations of first-generation polymeric carriers (modest activity and significant toxicity) have been addressed by developments of new biodegradable polycations, incorporation of targeting and intracellular transport functions, and polyplex formulations that avoid unspecific adverse interactions with the host. A key future step will be the development of polyplexes into artificial viruses, with virus-like entry functions presented by smart polymers and polymer conjugates. These polymers have to sense their biologic microenvironment, respond in a more dynamic manner to alterations in pH, ionic or redox environment, undergoing programmed structural changes compatible with the different gene delivery steps.

KEY WORDS: nonviral vectors; cationic polymers; gene delivery; targeted gene therapy.

INTRODUCTION

Forty years ago it was reported that basic proteins could enhance the cellular uptake of viral RNA (1). Only few years later, a synthetic cationic polymer, diethylaminoethyl-modified dextran, was used for nucleic acid delivery (2). Since then, numerous polycations have been used for formulating DNA and other nucleic acids into complexes now termed "polyplexes" (3). Polycations include natural DNA binding proteins such as histones; the synthetic amino acid polymers such as polylysine; PEI, cationic dendrimers, and other polymers such as pDMAEM; or carbohydrate-based polymers such as chitosan. The characteristics of these polymers and their use in transfections have been reported extensively (see Refs. 4–6). The most potent polyplex formulations have reached efficiencies of viral vectors, although far more particles per cell are required for successful transfection.

Besides their use as cell culture reagent, polyplexes might eventually metamorphose into potent pharmaceutical drugs, where DNA is the payload and prodrug that has to be specifically delivered by the formulation to the target site in the body and converted by the transcription/translation machinery into a drug. This requires polyplexes that in addition to the intracellular delivery fulfill a series of drug delivery functions. This is evident by the common observation that gene transfer activity in cell culture does not necessarily correlate with the activity in animals. The focus of the current review is the use of DNA/polymer complexes for *in vivo* applications. The article summarizes on the current achievements and limitations with polyplex systems; it intends to define the major critical points and bottlenecks where refinement of polyplexes is required and also describes recent strategies that might be very useful for further optimization.

POLYMER CHARACTERISTICS, FORMULATION, AND DELIVERY FUNCTIONS

Ideally, the cationic polymer will perform multiple tasks, including compacting DNA into particles of virus-like dimensions that can migrate to and into target cells, protecting DNA from degradation, shielding the DNA particles against undesired interactions, and enhancing cell binding and intracellular delivery into cytoplasm and nucleus. In reality, the polymer is unable to carry out all the tasks; in some cases its primary role is to bind and protect DNA from the environment. In most cases additional functional domains have to be included into the DNA formulation. For example, unmodified polylysine was found to protect DNA, condense it into toroids of virus-like dimensions, and deliver DNA into cells through its positive charge interactions with the cell surface. However, particles were found to accumulate in intracellular vesicles, and endosomolytic agents had to be added or linked to the polymer for obtaining efficient gene transfer (7). In addition, cell-binding ligands had to be conjugated to the polymer to render cellular binding of polyplexes target-specific.

Apart from the individual components used for the DNA

[1] Department of Pharmacy, Pharmaceutical Biology-Biotechnology, Ludwig-Maximilians-Universitaet, Butenandtstr. 5-13, D-81377 Munich, Germany.

[2] To whom correspondence should be addressed. (e-mail: ernst.wagner@cup.uni-muenchen.de)

0724-8741/04/0100-0008/0

formulation, additional parameters influence the biophysical characteristics of the polyplex. Usually polyplex formation is kinetically controlled; it is performed at ionic strength where the polycation/polyanion association is rapid and almost irreversible. Hence, the order and sequence of mixing of components in ternary complexes is one strategy to design the characteristics. The sizes of polyplex particles usually increase with increasing DNA concentration and with increasing ionic strength of the formulation buffer, e.g., from <30 mM to 150 mM salt; polyplexes may increase their size by aggregate formation. With increasing charge ratio (positive charge of cationic polymer: negative charge of nucleotide phosphodiester) to >1, compaction of DNA improves and the surface charge of polyplexes increases. Usually at a charge ratio of 1 (polymer cation residue per DNA phosphate) the DNA is fully bound and, in many cases, also compacted. Around electroneutrality, however, polyplexes often aggregate and show low solubility; this strongly depends on the hydrophilicity of the polymer. At high charge ratios, a large fraction of polymer is present in the free state, i.e., not bound to DNA. Some polymers like PEI can change their degree of protonation depending on the surrounding pH. At neutral pH, in the absence of DNA only one of seven nitrogens within the PEI chain is protonated. In the presence of DNA, a (N/P = PEI nitrogen/DNA phosphate) charge ratio of at least 2 is required for complete complexation. In transfections N/P ratios of 5 or higher are usually applied, generating polyplexes with considerable buffering capacity at lower (endosomal) pH. The "proton sponge" character of PEI probably contributes to its intrinsic ability to facilitate endosomal release, which is assumed to be triggered by an osmotic imbalance of the endosome upon PEI protonation. As a consequence, PEI is one of the polymers with the highest transfection efficiency (8).

IN VIVO ADMINISTRATION

Polyplexes have been found very useful in cell culture transfections, but achieving *in vivo* gene transfer has proven far more difficult, especially when the goal is targeted delivery into a distant organ upon intravenous application. For improved gene transfer, 1) DNA polyplexes have to be stable and inert in blood, 2) they reach their target tissue by crossing different biologicals barriers, including vascular endothelium and extracellular matrix, 3) once reaching the target cell they internalize, 4) disassemble and release the DNA into the nuclear compartment, but protect the DNA against intracellular degradation during the uptake process, 5) they must elicit minimal inflammatory response, and 6) be nonimmunogenic. Despite these multiple requirements, encouraging results of *in vivo* gene transfer into various target organs have been achieved with existing polyplex formulations (see following sections and Tables I–III).

GENE TRANSFER TO THE LIVER

Wu and Wu (9) first reported *in vivo* gene expression after intravenous injection of polyplexes in rats. For targeted delivery to the hepatocyte-specific asialoglycoprotein receptor, DNA/asialoorosomucoid-polylysine complexes were administered, resulting in marker gene expression in rat liver. Expression was transient but was shown to be significantly prolonged by partial hepatectomy (10); this procedure induced hepatocyte proliferation and also increased persistence of plasmid DNA in hepatocytes of the treated animals. In another study, Nagase analbuminemic rats were treated by this concept with a human albumin expression construct (11). Circulating human albumin was observed after 2 days and increased to a maximum concentration by 2 weeks postinjection, remaining stable for further 2 weeks. Subsequent work applied the system for hepatocyte-specific gene transfer of the low-density lipoprotein receptor in a rabbit animal model for familial hypercholesterolemia, which resulted in a temporary amelioration of the disease phenotype (12).

Perales and colleagues (13) reported long term (up to 140 days) gene expression of factor IX in the liver after application of DNA/galactose–polylysine complexes into the caudal vena cava of rats. The assembly of the polyplexes into small particles (of below 20 nm, by a special protocol) was considered as a key prerequisite for success.

A synthetic multifunctional polyplex system was applied by Hashida and colleagues (14) consisting of polyornithine that was modified first with galactose (to serve as ASGR ligand), then with a fusogenic peptide derived from the influenza virus HA2 (as endosomal release domain), and complexed with a luciferase gene. Upon intravenous injection in mice, a large amount of transgene product was detected in the liver, with the hepatocytes contributing more than 95% of the total activity in all tissues examined.

More recently, the same research group also evaluated

Table I. Systemic Gene Delivery of Polyplexes to the Liver

System		Gene	Results
Wu, 1988 Chowdhury, 1993	Asialoorosomucoid–polylysine conjugate	Reporter gene	Expression in liver after i.v. injection; prolonged by partial hepatectomy
Wu, 1991		Albumin	Circulating albumin in Nagase rats
Wilson, 1992		Low-density lipoprotein receptor	Temporary amelioration of hypercholesterolemia
Perales, 1994	Galactose–polylysine conjugate	Factor IX gene	Long-term expression from liver (up to 140 days)
Nishikawa, 2000	Galactose–polyornithine-HA2 peptide conjugate	Reporter gene	Hepatocyte-targeted expression
Morimoto, 2003	Galactose–PEI conjugates	Reporter gene	Portal vein injection; evaluates effect of MW of PEI
Nguyen, 2000	Pluronic (P123)–PEI conjugate	Reporter gene	Highest gene expression in liver, followed by spleen, lung, and heart

Table II. Systemic Gene Delivery of Polyplexes to the Lung

System		Gene	Results
Ferkol, 1995	Polylysine conjugated to anti pIg receptor Fc	Reporter gene	Highest expression in lung; also expression in liver
Ziady, 1999	Polylysine conjugate with peptide ligand for serpine-enzyme-complex receptor	Reporter gene	Evaluates effect of length of polylysine chain on gene expression
Zou, 2000 Goula, 2000	Linear PEI	Reporter gene	High gene expression in the lung after intravenous delivery
Rudolph, 2000 Kichler, 2002	Branched PEI PEG-PEI	Reporter gene	Intratracheal instillation intranasal application

galactosylated (Gal) or unmodified PEIs for gene transfer to mouse liver (15). They showed that different MWs (1.8, 10, or 70 kDa) of PEI greatly influence the polyplex activity. The smallest polymer (PEI-1.8K) showed the highest but unspecific activity, Gal-PEI-10K showed the highest receptor-specific activity in cell culture. Upon portal vein injection of mice, Gal-PEI-70K showed the highest liver expression levels. Different polyplex sizes (particle size of >1000 nm for the low MW PEI-1.8K, about 100 nm for the high MW PEI-70K) may contribute to the differences. Unfortunately, tail vein injection did not result in efficient liver expression; possible reasons are discussed in the following sections.

Among other PEI-polyether conjugates, a conjugate of 2-kDa low MW PEI with the Pluronic 123 (a polyethyleneoxide-polypropyleneoxide block copolymer) was synthesized by Kabanov and colleagues (16). In combination with free P123 and DNA the conjugate forms small (110 nm), stable complexes, which after i.v. injection into mice exhibit highest gene expression in liver, followed by spleen > lung > heart.

GENE TRANSFER TO THE LUNG

Several strategies for gene transfer to the lung have been evaluated (Table II). Davis and colleagues developed several strategies for polyplex-mediated gene transfer to the lung. For targeting the polymeric immunoglobulin receptor, they generated small-sized anti-pIg Fab-polylysine polyplexes (13). Systemic delivery of the anti-pIg polyplexes in rats resulted in reporter gene expression in cells of the airway epithelium and submucosal glands (17). In an alternative approach, using a synthetic peptide ligand (derived from alpha 1-antitrypsin) for the serpin-enzyme complex receptor, polyplexes were optimized for ligand: polylysine ratio and MW of polylysine (18). They observed that shorter chain polylysines (10 kDa, 36 amino acids) generate 40-nm polyplexes whereas the longer chain polylysines (54 kDa, 256 amino acids) gave smaller (25 nm) polyplexes, which in turn resulted in significantly higher and longer duration of expression *in vivo*. Subsequent work of the same researchers showed that the polyplexes upon nasal administration were able to transiently correct the chloride transport defect in nasal epithelium of CF mice.

Recently, a 22-kDa MW linear PEI (L-PEI) was successfully used for systemic gene delivery, resulting in very high gene expression in the lung (8). Crossing of the pulmonary endothelial barrier resulted in location of expression in alveolar cells (19). In this application L-PEI mediates much higher transfection activity than branched PEI of similar MW (20). However, a positive charge ratio (polycation nitrogen to DNA phosphate ratio) is required, resulting in a narrow window between efficiency and severe toxicity. PEI/DNA activates the lung endothelium and forms small aggregates, a side-effect that is linked to the transfection efficiency (21). Therefore, gene transfer of PEI polyplexes or PEG-modified

Table III. In Vivo Delivery of Polyplexes to Tumors or Other Tissues

	System	Gene	Results
Tumor			
Coll, 1999	Linear PEI	Reporter gene	Infusion into tumor by micropump
Gautam, 2002	PEI	p53	Aerosol delivery; reduction of lung tumor growth
Ogris, 1999	PEI conjugates, Tf-targeted, PEG shielded	Reporter gene	High gene expression in distant tumors in mice
Kursa, 2003		TNF-α	Tumor necrosis and inhibition of tumor growth
Kircheis, 2002	PEI conjugates, Tf-targeted and Tf-shielded	Reporter gene, TNF-α	Hemorrhagic tumor necrosis and inhibition of tumor growth in mice
Wolschek, 2002	PEI, conjugates, EGF-targeted, PEG shielded	Reporter gene	Expression was predominantly found in the tumor (human hepatoma in SCID mice)
Other tissues			
Lemkine, 1999	PEI	Marker genes	Intracranial injection, brain delivery
Boletta, 1997	PEI		Delivery to kidney via artery
Lemieux, 2000	Pluronics L61 and F127	Marker genes, erythropoietin	10-fold enhanced gene expression in muscle compared with naked DNA
Prokop, 2002	Tetronic block copolymers	Marker genes	Injection into subcutaneous tissue

PEI polyplexes (22) is being explored by direct instillation (23) or nebulization (24).

GENE TRANSFER TO TUMORS

PEI polyplexes have been found to very effectively transfect tumor cell cultures, and efforts have been made to apply them to *in vivo* cancer therapy (see Table III, top). Gautam and colleagues (24) successfully delivered PEI polyplexes expressing the p53 gene as an aerosol to established B16-F10 lung metastases that, in combination with 9-nitrocamptothecin therapy, result in tumor growth inhibition.

Direct intratumoral delivery of PEI polyplexes has also been investigated; however, expression levels are far lower than in cell culture. Special forms of administration, such as local infusion of PEI polyplexes into the tumor mass by micropump (25) have to be applied to obtain satisfactory results.

Targeting tumors through the intravenous route might present a unique opportunity to reach and attack multiple spread metastatic tumor nodules, but displays also an even more difficult challenge. Firstly, interactions with blood components and healthy tissues have to be avoided. Upon i.v. injection of positively charged polyplexes or lipoplexes into mice, gene expression localizes in the lung (see above) and is associated with adverse side effects. For example, work by Verbaan and colleagues (26) indicates that aggregate formation of positively charged pDMAEM polyplexes with blood components followed by entrapment in the lung capillaries is responsible for a preferential lung uptake and transfection. Plank and colleagues (27) observed that positively charged polyplexes activate the alternative pathway of the plasma complement system, which is part of the innate immune system. It is apparent that for specific targeting *in vivo* polyplexes must not be positively charged. Otherwise, another undesired side effect is binding to erythrocytes (28).

Besides the DNA/polymer charge ratio, polymer characteristics may contribute to these unspecific interactions with blood. Seymour *et al.* (29) showed that the MW of polylysine influences the circulation of polyplexes in mice. Polyplexes of medium MW polylysine (200 amino acids) displayed up to 20 times greater blood levels compared with polyplexes of low MW polylysine (20 amino acids). While both types of polyplexes bind to erythrocytes and associate with Kupffer cells, only the low MW polylysine polyplexes consumed mouse complement factor C3.

Nonspecific interactions in the blood have been minimized by hydrophilic agents like polyethylene glycol (PEG), hydroxypropyl methacrylate, or the serum protein transferrin, which have been attached to the polyplex surface. For the PEGylation of transferrin (Tf)–PEI polyplexes, two strategies have been developed, attaching ligand and PEG molecules to PEI either before (30) or after (28) DNA complex formation. Shielding the particle surface by PEG not only improves circulation times but also reduces toxicity, increases solubility and provides stability for freeze-thawing. Intravenous injection of shielded, Tf- or Tf/PEG- coated polyplexes resulted in gene transfer into distant subcutaneous neuroblastoma tumors of syngeneic A/J mice (28,30,31). Similarly, EGF–PEG-coated polyplexes were successful in systemic targeting human hepatocellular carcinoma xenografts in SCID mice (32). Luciferase marker gene expression levels in tumor tissues were 10- to 100-fold higher than in other organ tissues.

Using the therapeutic gene encoding tumor necrosis factor alpha (TNF-α), repeated systemic application of Tf polyplexes into tumor-bearing mice induced tumor necrosis and inhibition of tumor growth in four murine tumor models of different tissue origin (30,33). As gene expression of TNF was localized within the tumor, no systemic TNF-related toxicities were observed.

The type of delivered nucleic acid influences the efficiency. Delivery of RNA by standard 25 kDa PEI has been found to be unsuccessful. Low-MW PEI, however, which was found effective for DNA delivery *in vitro* (34) but not *in vivo* (15), showed encouraging activity for RNA delivery *in vitro* (35) and *in vivo* (36). RNA ribozymes (directed against the growth factor pleiotrophin) complexed by low-MW PEI and injected intraperitoneally were able to reach a distant subcutaneous tumor and caused a marked tumor growth retardation (36).

GENE TRANSFER TO OTHER TISSUES

Examples of relevant investigations are listed in Table III (bottom). Encouraging *in vivo* results with PEI polyplexes were obtained in the brain of mice after intracranial injection (37) and after renal artery injection for expression in rat kidney (38). Local applications to muscle, skin, or subcutaneous tissues however appear to follow different rules for DNA formulation: the negatively charged naked DNA can mediate significant although moderate expression, whereas positively charged polyplex formulations block the gene transfer activity. PVP, a non-ionic, noncondensing DNA carrier previously established for muscle transfection, also was found to enhance gene delivery into subcutaneous tissue, similarly as tetronic block copolymers (39). Another non-ionic carrier composed of two amphiphilic block copolymers, pluronics L61 and F127, increases intramuscular expression of plasmid DNA about 10-fold (40). Comparison of these poloxamers with PVP showed a high efficacy at a lower DNA dose.

NEXT GENERATION OF CATIONIC POLYMERS

Current developments of polycationic carriers have two major aims: to generate backbones that mediate higher transfection efficiency than existing carriers and to make carriers less toxic, more biocompatible, and biodegradable.

OPTIMIZATION OF EXISTING POLYMERS

PEI is a nonbiodegradable polymer with endosomal escape characteristics as a result of its buffering capacity. Poly(L)lysine lacks such an endosomolytic domain but can easily be metabolized because of the natural amino acid backbone. Putnam *et al.* (41) conjugated up to 87 mol% imidazole groups to the epsilon-amines of polylysine. The imidazole groups served as protonable endosomal escape moieties. The transfection efficiency increased with increasing imidazole content of the polymers. The polymer with the highest imidazole content mediated gene expression levels similar to those mediated by PEI, but with less cytotoxicity. Similarly, Midoux *et al.* (42) reported that polylysine substituted with histidyl residues mediated high *in vitro* transfection.

In the case of PEI, the average MW, backbone structure (linear or branched) or residual protective groups from the synthesis (e.g., propionamide residues deriving from poly-2-

ethyl-2-oxazoline) have been found to strongly influence the transfection characteristics. Thomas and Klibanov (43) have systematically introduced chemical modifications of the nitrogen atoms, such as acetylation or dodecylation, which led to PEI derivatives with markedly enhanced performance. For example, dodecylation of 2-kDa PEI yields a nontoxic polycation whose transfection efficiency in cell culture is 400-fold enhanced.

BIODEGRADABLE POLYMERS

Toxicity is an inherent property of polycationic carriers that is associated with their ability to nonspecifically bind to negatively charged DNA and other biological materials. Toxicity increases with the degree of polymerization of the carrier molecule. Neutralization of the positive polymer charge by polyplex formation reduces toxicity both at the cellular level and in the host. In many applications, however, polyplex formulations are positively charged and, in addition, also contain free polymer. Better purification schemes for polyplexes will at least partly overcome this problem.

In addition to acute toxicity aspects, the long-term fate of the polymeric carrier has to be considered in an organism, after release from the delivered DNA. Therefore more biocompatible polymers, which can be easily degraded by the host would be advantageous. Alternatively, low MW polymers with low toxicity can be used, if the polymers are crosslinked by bioreversible linkers to enhance their capacity for binding DNA and stabilizing polyplexes.

Lim *et al.* (44) synthesized a biodegradable ester analog of polylysine, poly[alpha-(4-aminobutyl)-L-glycolic acid] (PAGA). The polymer displayed no cytotoxicity, but only modest transfection activity. Poor transfection may be due to a too fast hydrolysis and, similarly to polylysine, lack of efficient endosomal escape functionality. Another biodegradable cationic polyester, poly (4-hydroxy-L-proline ester) showed similar characteristics (45).

Instead of ester linkages bioreducible disulfide bonds have been introduced into polycationic polymers. Rice and colleagues (46) developed low MW disulfide cross-linking DNA carrier peptides consisting of oligolysine with terminal cysteine residues that polymerizes through disulfide bond formation when bound to DNA. This results in small, stable polyplexes that mediate efficient *in vitro* gene transfer. Substitution of histidine for lysine residues resulted in an optimal peptide of Cys-His-$(Lys)_6$-His-Cys that also provided buffering capacity to enhance gene expression in the absence of chloroquine. In a similar manner, Oupicky *et al.* (47) demonstrated that cross-linking polylysine with a bioreducible cross-linking agent increased the stability of polyplexes, and after masking the surface with PEG, a 10-fold increased *in vivo* plasma circulation following intravenous administration to mice. This and other work would indicate the break-up of the disulfide bonds after cellular uptake, upon reaching the reducing environment of the cytosol, releasing the reduced polymers and DNA. Bioreversible disulfide cross-linking has also been investigated using low MW PEI (48).

Several additional examples of biodegradable polycation gene carriers can be found in the literature, including for example block-copolymers with noncationic, hydrophilic polymers. However, often they suffer from poor transfection efficiencies. Recently, Lim *et al.* (49) described a biodegradable polymer of a branched network of amino esters (n-PAE), which has transfection efficiencies similar to PEI 25 kDa but lower cytotoxicity. The high transfection efficiency was attributed to the proton sponge effect in the endosome, similarly as described for PEI. The network structure of the polymer is based on polycondensation of TRIS molecules N-disubstituted with methyl acrylate, and terminal amino groups were attached to the polyester condensate in form of 6-amino hexanoic acid esters. This structure provides multiple ternary and primary amines for DNA binding and endosomal buffering. The network structure is also important to control the rate of polyester degradation. n- PAE displays medium stability, while linear amino-modified polyester appear to show too fast hydrolysis rates. Polymers such as n-PAE fulfill the two main requirements, efficient transfection and low toxicity, and should be useful in the future.

STRATEGIES TO DEVELOP POLYPLEXES INTO "ARTIFICIAL VIRUSES"

One might predict that any polyplex that has a "static" make-up will have difficulties to successfully overcome all biological delivery steps; for the simple reason that they will have to mediate different functions upon the delivery pathway. A key future step will be the development of "smart" polyplexes, based on polymers that "sense" their biological environment, enabling them to undertake programmable changes triggering specific actions (see Fig. 1): to sense at which time point polymers have to stabilize and protect the DNA from unspecific interactions (e.g., in the blood circulation), when they have to trigger target interaction (with the cell surface), trigger destabilization of vesicular membranes, intracellular transport, and when they have to release the DNA within the nucleus for gene expression. In other words, polymers have to be less static, they should respond in a more dynamic manner to their microenvironment. Viruses present natural examples for such characteristics; the "assembly-disassembly paradox" is fascinating and shows us that in a biological environment virus particles are assembled in a very controlled fashion in one cell, but undergo controlled disassembly when infecting the neighboring cells. Obviously, one biochemical structure (a virus particle) can display very different functions as a consequence of small changes triggered by the environment (the biological pathway).

To enhance transfection efficiencies of nonviral vectors, investigators have incorporated viral cell entry functions into the particles. These so-called "artificial viruses" do have several features common to viruses, for instance cell targeting domains, or endosomal release agents. However, unlike viruses the current versions of virus-like gene transfer systems do not necessarily undergo programmed structural changes that would make them more compatible at each step toward cell transfection; in general, polyplexes still have a too static make-up.

To obtain a more "chameleon"-like capability, polymers have to be provided with elements that enable structural and functional changes triggered by the micro-environment. These elements could undergo conformational changes, or contain linkages that are rapidly cleaved under specific conditions. Biological triggers can be changes in the pH, ionic or

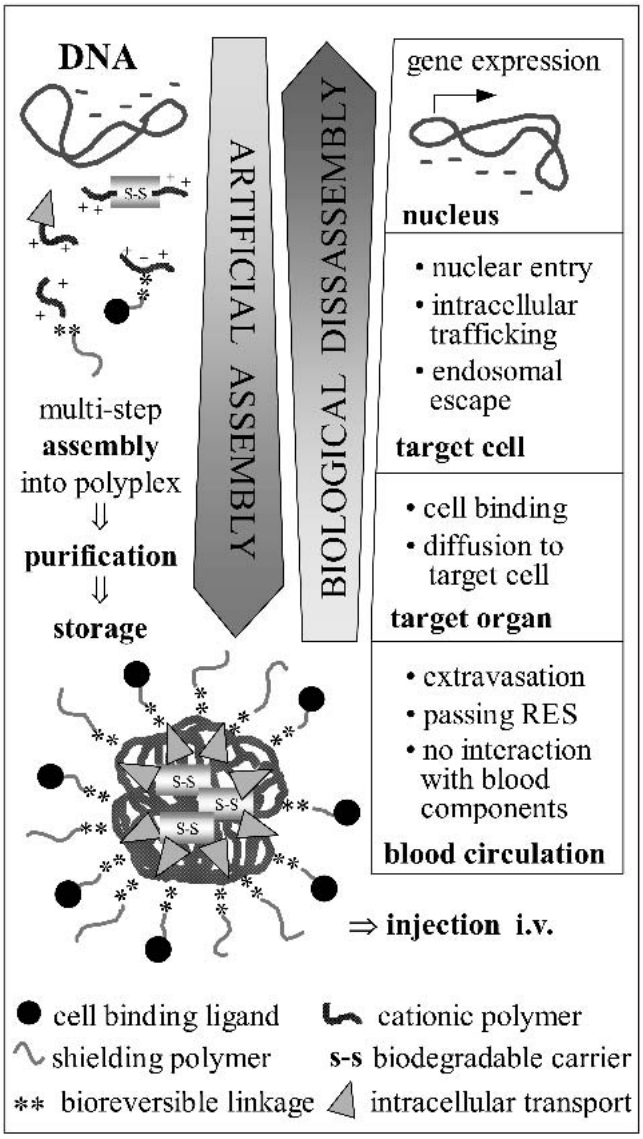

Fig. 1. Assembly and dissassembly of polyplexes: toward artificial viruses.

redox environment, presence of specific peptidases, or binding to specific ligands. For instance, shielding and targeting molecules like PEG and receptor ligands that are required only in the early extracellular steps of the delivery process, might be released upon entry into the cell. This, for instance, may result in exposure of membrane disrupting functions required within the endosome (but not earlier). Endosomal acidification may activate such functions by pH-specific conformational changes in peptides or hydrolysis (for example, see Ref. 50). The intracellular reducing environment may contribute to polyplex disassembly by cleaving disulfide-bridged cationic carriers.

In essence, polyplexes of the future will be multifunctional systems assembled from biodegradable, non-toxic components. They will be, on the one hand, formulations with high stability upon storage, and, on the other hand, designed to act in a very dynamic fashion in the delivery process; undergoing environment-specific structural changes to activate the required delivery functions, similarly as natural viruses do.

ACKNOWLEDGMENTS

Funding by the German Science Foundation (DFG), research grant SFB 486, is gratefully acknowledged. The author would like to thank Dr. Greg Walker for the critical review of the manuscript and Olga Brück for skillful assistance in preparing text and figures.

REFERENCES

1. C. E. Smull and E. H. Ludwig. Enhancement of the plaque forming capacity of poliovirus ribonucleic acid with basic proteins. *J. Bacteriol.* **84**:1035–1040 (1962).
2. A. Vaheri and J. S. Pagano. Infectious poliovirus RNA: a sensitive method of assay. *Virology* **27**:434–436 (1965).
3. P. L. Felgner, Y. Barenholz, J. P. Behr, S. H. Cheng, P. Cullis, L. Huang, J. A. Jessee, L. Seymour, F. Szoka, A. R. Thierry, E. Wagner, and G. Wu. Nomenclature for synthetic gene delivery systems. *Hum. Gene Ther.* **8**:511–512 (1997).
4. S. C. De Smedt, J. Demeester, and W. E. Hennink. Cationic polymer based gene delivery systems. *Pharm. Res.* **17**:113–126 (2000).
5. M. D. Brown, A. G. Schatzlein, and I. F. Uchegbu. Gene delivery with synthetic (non viral) carriers. *Int. J. Pharm.* **229**:1–21 (2001).
6. S. Han, R. I. Mahato, Y. K. Sung, and S. W. Kim. Development of biomaterials for gene therapy. *Mol Ther.* **2**:302–317 (2000).
7. E. Wagner, C. Plank, K. Zatloukal, M. Cotten, and M. L. Birnstiel. 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 (1992).
8. S. M. Zou, P. Erbacher, J. S. Remy, and J. P. Behr. Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* **2**:128–134 (2000).
9. G. Y. Wu and C. H. Wu. Receptor-mediated gene delivery and expression in vivo. *J. Biol. Chem.* **262**:14621–14624 (1988).
10. N. R. Chowdhury, C. H. Wu, G. Y. Wu, P. C. Yerneni, V. R. Bommineni, and J. R. Chowdhury. Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis in vivo. Prolonged persistence in cytoplasmic vesicles after partial hepatectomy. *J. Biol. Chem.* **268**:11265–11271 (1993).
11. G. Y. Wu, J. M. Wilson, F. Shalaby, M. Grossman, D. A. Shafritz, and C. H. Wu. Receptor-mediated gene delivery in vivo. Partial correction of genetic analbuminemia in Nagase rats. *J. Biol. Chem.* **266**:14338–14342 (1991).
12. J. M. Wilson, M. Grossman, C. H. Wu, N. R. Chowdhury, G. Y. Wu, and J. R. Chowdhury. Hepatocyte-directed gene transfer in vivo leads to transient improvement of hypercholesterolemia in low density lipoprotein receptor-deficient rabbits. *J. Biol. Chem.* **267**:963–967 (1992).
13. J. C. Perales, T. Ferkol, H. Beegen, O. D. Ratnoff, and R. W. Hanson. Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc. Natl. Acad. Sci. USA* **91**:4086–4090 (1994).
14. M. Nishikawa, M. Yamauchi, K. Morimoto, E. Ishida, Y. Takakura, and M. Hashida. Hepatocyte-targeted in vivo gene expression by intravenous injection of plasmid DNA complexed with synthetic multi- functional gene delivery system. *Gene Ther.* **7**:548–555 (2000).
15. K. Morimoto, M. Nishikawa, S. Kawakami, T. Nakano, Y. Hattori, S. Fumoto, F. Yamashita, and M. Hashida. Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver. *Mol Ther.* **7**:254–261 (2003).
16. H. K. Nguyen, P. Lemieux, S. V. Vinogradov, C. L. Gebhart, N. Guérin, G. Paradis, T. K. Bronich, V. Y. Alakhov, and A. V. Kabanov. Evaluation of polyether-polyethyleneimine graft copolymers as gene transfer agents. *Gene Ther.* **7**:126–138 (2000).
17. T. Ferkol, J. C. Perales, E. Eckman, C. S. Kaetzel, R. W. Hanson, and P. B. Davis. Gene transfer into the airway epithelium of animals by targeting the polymeric immunoglobulin receptor. *J. Clin. Invest.* **95**:493–502 (1995).
18. A. G. Ziady, T. Ferkol, D. V. Dawson, D. H. Perlmutter, and P. B. Davis. Chain length of the polylysine in receptor-targeted

gene transfer complexes affects duration of reporter gene expression both in vitro and in vivo. *J. Biol. Chem.* **274**:4908–4916 (1999).
19. D. Goula, N. Becker, G. F. Lemkine, P. Normandie, J. Rodrigues, S. Mantero, G. Levi, and B. A. Demeneix. Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. *Gene Ther.* **7**:499–504 (2000).
20. L. Wightman, R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa, and E. Wagner. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **3**:362–372 (2001).
21. P. Chollet, M. C. Favrot, A. Hurbin, and J. L. Coll. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J. Gene Med.* **4**:84–91 (2002).
22. A. Kichler, M. Chillon, C. Leborgne, O. Danos, and B. Frisch. Intranasal gene delivery with a polyethylenimine-PEG conjugate. *J. Control Release* **81**:379–388 (2002).
23. C. Rudolph, J. Lausier, S. Naundorf, and R. H. Muller. and J. Rosenecker. In vivo gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. *J. Gene Med.* **2**:269–278 (2000).
24. A. Gautam, J. C. Waldrep, C. L. Densmore, N. Koshkina, S. Melton, L. Roberts, B. Gilbert, and V. Knight. Growth inhibition of established B16-F10 lung metastases by sequential aerosol delivery of p53 gene and 9-nitrocamptothecin. *Gene Ther.* **9**:353–357 (2002).
25. J. L. Coll, P. Chollet, E. Brambilla, D. Desplanques, J. P. Behr, and M. Favrot. In vivo delivery to tumors of DNA complexed with linear polyethylenimine. *Hum. Gene Ther.* **10**:1659–1666 (1999).
26. F. J. Verbaan, C. Oussoren, I. M. van Dam, Y. Takakura, M. Hashida, D. J. Crommelin, W. E. Hennink, and G. Storm. The fate of poly(2-dimethyl amino ethyl)methacrylate-based polyplexes after intravenous administration. *Int. J. Pharm.* **214**:99–101 (2001).
27. C. Plank, K. Mechtler, F. J. Szoka, and E. Wagner. Activation of the complement system by synthetic DNA complexes: A potential barrier for intravenous gene delivery. *Hum. Gene Ther.* **7**: 1437–1446 (1996).
28. M. Ogris, S. Brunner, S. Schuller, R. Kircheis, and E. Wagner. 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 (1999).
29. C. M. Ward, M. L. Read, and L. W. Seymour. Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. *Blood* **97**:2221–2229 (2001).
30. M. Kursa, G. F. Walker, V. Roessler, M. Ogris, W. Roedl, R. Kircheis, and E. Wagner. Novel Shielded Transferrin-Polyethylene Glycol-Polyethylenimine/DNA Complexes for Systemic Tumor-Targeted Gene Transfer. *Bioconjug. Chem.* **14**:222–231 (2003).
31. R. Kircheis, L. Wightman, A. Schreiber, B. Robitza, V. Rossler, M. Kursa, and E. Wagner. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther.* **8**:28–40 (2001).
32. M. F. Wolschek, C. Thallinger, M. Kursa, V. Rossler, M. Allen, C. Lichtenberger, R. Kircheis, T. Lucas, M. Willheim, W. Reinisch, A. Gangl, E. Wagner, and B. Jansen. Specific systemic nonviral gene delivery to human hepatocellular carcinoma xenografts in SCID mice. *Hepatology* **36**:1106–1114 (2002).
33. R. Kircheis, E. Ostermann, M. F. Wolschek, C. Lichtenberger, C. Magin-Lachmann, L. Wightman, M. Kursa, and E. Wagner. Tumor-targeted gene delivery of tumor necrosis factor-alpha induces tumor necrosis and tumor regression without systemic toxicity. *Cancer Gene Ther.* **9**:673–680 (2002).
34. D. Fischer, T. Bieber, Y. Li, H. P. Elsasser, and T. Kissel. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* **16**:1273–1279 (1999).
35. T. Bettinger, R. C. Carlisle, M. L. Read, M. Ogris, and L. W. Seymour. Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. *Nucleic Acids Res.* **29**:3882–3891 (2001).
36. A. Aigner, D. Fischer, T. Merdan, C. Brus, T. Kissel, and F. Czubayko. Delivery of unmodified bioactive ribozymes by an RNA-stabilizing polyethylenimine (LMW-PEI) efficiently downregulates gene expression. *Gene Ther.* **9**:1700–1707 (2002).
37. G. F. Lemkine, D. Goula, N. Becker, L. Paleari, G. Levi, and B. A. Demeneix. Optimisation of polyethylenimine-based gene delivery to mouse brain. *J. Drug Target.* **7**:305–312 (1999).
38. A. Boletta, A. Benigni, J. Lutz, G. Remuzzi, M. R. Soria, and L. Monaco. Nonviral gene delivery to the rat kidney with polyethylenimine. *Hum. Gene Ther.* **8**:1243–1251 (1997).
39. A. Prokop, E. Kozlov, W. Moore, and J. M. Davidson. Maximizing the in vivo efficiency of gene transfer by means of nonviral polymeric gene delivery vehicles. *J. Pharm. Sci.* **91**:67–76 (2002).
40. P. Lemieux, N. Guerin, G. Paradis, R. Proulx, L. Chistyakova, A. Kabanov, and V. Alakhov. A combination of poloxamers increases gene expression of plasmid DNA in skeletal muscle. *Gene Ther.* **7**:986–991 (2000).
41. D. Putnam, C. A. Gentry, D. W. Pack, and R. Langer. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc. Natl. Acad. Sci. USA* **98**:1200–1205 (2001).
42. M. Bello Roufai and P. Midoux. 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. *Bioconjug. Chem.* **12**:92–99 (2001).
43. M. Thomas and A. M. Klibanov. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**:14640–14645 (2002).
44. Y. B. Lim, S. O. Han, H. U. Kong, Y. Lee, J. S. Park, B. Jeong, and S. W. Kim. Biodegradable polyester, poly[alpha-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharm. Res.* **17**:811–816 (2000).
45. Y. Lim, Y. H. Choi, and J. Park. A self-destroying polycationic polymer: biodegradable poly(4-hydroxy-L-proline ester). *J. Am. Chem. Soc.* **121**:5633–5639 (1999).
46. D. L. McKenzie, E. Smiley, K. Y. Kwok, and K. G. Rice. Low molecular weight disulfide cross-linking peptides as nonviral gene delivery carriers. *Bioconjug. Chem.* **11**:901–909 (2000).
47. D. Oupicky, R. C. Carlisle, and L. W. Seymour. Triggered intracellular activation of disulfide crosslinked polyelectrolyte gene delivery complexes with extended systemic circulation in vivo. *Gene Ther.* **8**:713–724 (2001).
48. M. A. Gosselin, W. Guo, and R. J. Lee. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjug. Chem.* **12**:989–994 (2001).
49. Y. B. Lim, S. M. Kim, H. Suh, and J. S. Park. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjug. Chem.* **13**:952–957 (2002).
50. D. B. Rozema, K. Ekena, D. L. Lewis, A. G. Loomis, and J. A. Wolff. Endosomolysis by Masking of a Membrane-Active Agent (EMMA) for Cytoplasmic Release of Macromolecules. *Bioconjug. Chem.* **14**:51–57 (2003).