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Review

Cationic Polymer Based Gene Delivery Systems

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Gene transfer to humans requires carriers for the plasmid DNA which can efficiently and safely carry the gene into the nucleus of the desired cells. A series of chemically different cationic polymers are currently being investigated for these purposes. Although many cationic polymers indeed condense DNA spontaneously, which is a requirement for gene transfer in most types of cells, the physicochemical and biopharmaceutical behavior of the current generation of polyplexes severely limits an efficient gene transfer *in vitro* and especially *in vivo*. This paper summarizes recent physicochemical and biological information on polyplexes and aims to provide new insights with respect to this type of gene delivery system. Firstly, the chemical structure of frequently studied cationic polymers is represented. Secondly, the parameters influencing condensation of DNA by cationic polymers are described. Thirdly, the surface properties, solubility, aggregration behavior, degradation and dissociation of polyplexes are considered. The review ends by describing the *in vitro* and *in vivo* gene transfection behavior of polyplexes.

KEY WORDS: cationic polymers; polycations; DNA plasmid; non-viral gene therapy; gene carriers.

INTRODUCTION

Drug delivery research currently evaluates the potentials and benefits of synthetic gene carriers, including liposomes and polymers, for gene therapy. With regard to polymers, major attention is paid to cationic polymers (CPs) which are able both to condense large genes into smaller structures and to mask the negative DNA charges, necessities for transfecting most types of cells. Also neutral polymers like pVA; which do not condense DNA, are evaluated to protect "naked" genes from extracellular nuclease degradation and to retain them better at the site of injection after intramuscular administration (1). Moreover, polymeric nano(micro)particles (based on e.g. polycyanoacrylate, poly(D,L-lactic acid) (2), gelatin, alginate, chitosan); which adsorb or encapsulate oligonucleotides or genes, are under investigation as sustained release matrices for genetic drugs.

This review focuses on polyplexes which are defined as cationic polymer-nucleic acid complexes (3). Although non-viral and cationic lipid-based gene carriers ("lipoplexes" (3)) are currently being clinically evaluated further than polyplexes, arguments remain considering polyplexes as valuable candidates for gene carriers. First, depending on specific therapeutic applications and locations, it is very likely that several types of gene carriers may be ultimately applied to humans. Second, while for some therapeutic applications lipoplexes are sufficiently active *in vivo*, they may fail in other applications. To illustrate, Duncan *et al.* (4) and Ernst *et al.* (5) warns that pulmonary surfactants may inhibit cationic liposome-mediated gene delivery to respiratory epithelial cells. Third, using viral carriers there remains the risk of an immune response to the viral particle, not allowing repeated *in vivo* transfection using the same carrie (6). Although no random recombination has been observed for all clinical trials conducted with viruses today, random integration mediated by (retro)viruses and recombination with wild-type viruses is theoretically possible. Moreover, the size of the DNA that can be transported by viral carriers is also limited.

As many excellent papers (7–10) present the step-by-step requirements of a of successful gene carrier, this is beyond the scope of this review. Neither do we intend to describe in detail the pros and cons of non-viral versus viral gene cariers. Our purpose is to gather specific, recent information on polyplexes and to provide new insights with respect to this type of gene delivery system. The chemical structure of frequently studied CPs is represented in the first section. The second part describes what influences condensation of DNA by CPs. The third section focuses on the surface properties, solubility, aggregration

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ABBREVIATIONS: ζ, zeta-potential φ, charge ratio; the ratio of the positive charge equivalents of the cationic component to the negative charge equivalents of the nucleic acid component; bp, base pairs; k_d, dissociation constant; R_h, hydrodynamic radius; AFM, atomic force microscopy; CP(s), cationic polymer(s); DEAE, diethylaminoethyl dextran; DLS, dynamic light scattering; EM, electron microscopy; HA, hyaluronic acid; HBS, hepes buffered saline; pAA, poly(acrylic acid); pAMAM, poly(amidoamine); pHPMA, poly(*N*-(2-hydroxypropyl) methylacrylamide); pDMAEMA, poly(dimethylaminoethyl methylacrylate); pDEAEMA, poly(diethylaminoethyl methylacrylate); pEG, poly(ethylene glycol); pEI, poly(ethyleneimine); pEVP, poly-(*N*-ethyl-4-vinyl pyridinium bromide); pLL, poly(L-lysine); pTMAEMA, poly(trimethylammonioethyl methacrylate chloride); pVA, poly(vinylalcohol); pVP, poly(*N*-ethyl-4-vinylpyridinium bromide); pVS, poly(vinylsulfonate); MW, (average) molecular weight; SPR, surface plasmon resonance.

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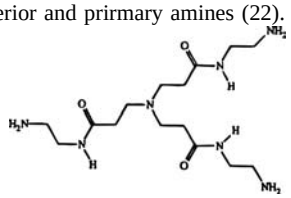
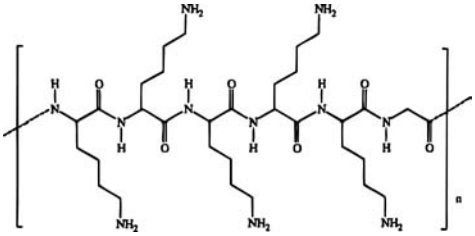
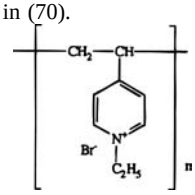
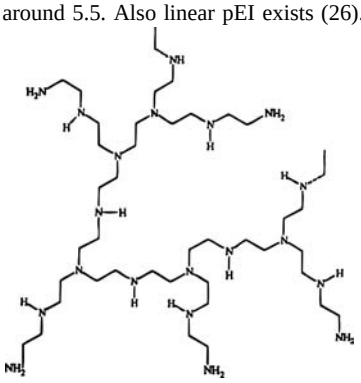
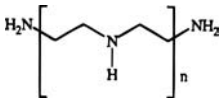
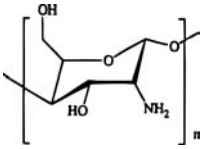
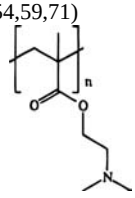
Table 1A. Cationic Homopolymers Studied as Gene Carriers

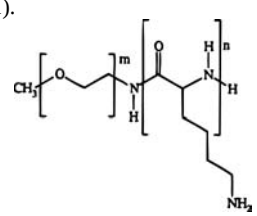
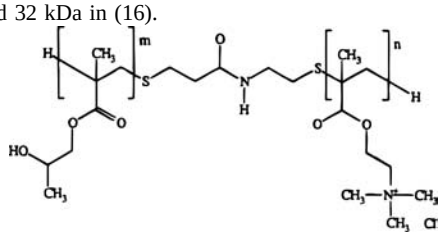
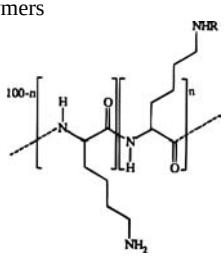
Linear backbone	Branched backbone
1. DEAE-dextran MW 500 kDa and around 40 mol DEAE per 100 mol glucose in (11).	1. pAMAM dendrimer (generation 1) A wide MW range is studied; pKa's are 3.9 and 6.9 for respectively the interior and prirmary amines (22). 
2. Pll MW 9.6 kDa in (16), between 4 kDa and 224 kDa in (58), between 2.7 kDa and 180 kDa in (35); pKa between 9 and 10. 	2. Fractured dendrimers Are partially degraded pAMAM dendrimers (by solvolysis (67)).
3. PVP A small amount of the *N*-ethyl groups on pVP were Replaced by *N*-cetyl groups in (70). 	3. pEI MW between 0.7 kDA en 800 kDa in (9); pKa of the primary amines is around 5.5. Also linear pEI exists (26). 
4. Linear pEI MW between 22 kDa and 220 kDa in (9,26); similar pKa's as in branched pEI. 	
5. Chitosan MW between 108 kDa and 540 kDa in (29,45), 1000 kDa in (9); pKa 6.5. 	
6. pDMAEMA (27,28,42,54,59,71) 	

behavior, degradation and dissociation of polyplexes. While the fourth and fifth section continue with the *in vitro* and *in vivo* gene transfection behavior of polyplexes.

CATIONIC POLYMERS AS GENE CARRIERS

Table 1 represents the chemical structures and some molecular characteristics of CPs which are frequently studied as gene carriers. CPs generally bear protonable amines. The relative number and pKa of the protonable amines differs between CPs. Some CPs, such as pLL, are linear polymers, while other ones, like pEI and dendrimers, are highly branched chains. Furthermore, some CPs have the positive charges on the backbone (as in pEI) while they are on side groups in e.g. pLL. Moreover, both block copolymers (like the pEG-pLL block copolymer) as well as comb-type copolymers (like pLL-*gr*-dextran) with

Table 1B. Cationic Copolymers Studied as Gene Carriers

Block copolymers	Grafted copolymers
1. pEG-pLL MW of the pEG and pLL segments were 10 kDa in (16), MW pEG was 12 kDa and MW pLL varied between 1 and 6 kDa in (21).  2. pHPMA-pTMAEMA MW of the pHPMA and pTMAEMA segments were 18 kDa and 32 kDa in (16). 	1. pLL grafted copolymers  - R = pEG In (17) MW pLL segments are 20 kDa, MW pEG segments are 5 kDa and 12 kDa; between 5 and 10 mol% of the lysine monomers are substituted with pEG. Shorter pEG segments (0.5 kDa) were studied in (72). - R = dextran In (17) MW pLL segments are 20 kDa and 11 kDa, MW dextran segments are 1 kDa; between 5 and 7 mol% of the lysine monomers are substituted with dextrain in (17) while 20 mol% of the lysine monomers are substituted in (18). - R = HA In (96) MW pLL segments are 4.2 kDa and 7.2 kDa, MW HA segments are 1.6, 2.3 and 3.8 kDa; between 5 and 19 mol% of the lysine monomers are substituted with HA. - R = pHPMA In (17) MW pLL segments are 19 kDa, MW pHPMA 4,4 kDa; 8 mol% of the lysine monomers are substituted with pHPMA. - R = pDEAEMA (53)

polycation backbones and grafted hydrophilic side chains have been investigated.

Diethylaminoethyl-dextran (DEAE-dextran) can be considered a main predecessor of the CPs for gene transfection (11). Its relatively low transfection efficiency, toxicity and nonbiodegradibility discouraged its exploitation with regard to gene therapy. For more than a decade now, the linear pLL has been widely investigated for gene delivery (10,12). It was the chain length heterogeneity of commercially available PLL, and the resulting major variabilities in size distribution of the polyplexes, which was the major reason for the development of polyplexes based on oligolysines and synthetic polypeptides (13,14). To improve solubility and stability of polyplexes and to reduce aspecific interactions with biomolecules, cationic copolymers bearing hydrophilic segments (pEG) were developed (15–21). Major attention was paid to block copolymers and comb-type copolymers based on pLL. A new class of cationic polymers as candidates for gene carriers appeared with the description of the transfection properties of pAMAM dendrimers (22). Major differences with pLL was the spheroidal structure and also their ionization properties. While at physiological pH the *N*-atoms of pLL are nearly fully protonated, not all the amine groups on pAMAM dendrimers are protonated. Consequently, CPs such as branched pEI (9,23–25) and linear pEI (26) were considered, which, like pAMAM dendrimers, are not fully protonated at physiological pH. More recently, methacrylate based CPs (27,28) and cationic polysaccharides like chitosan (29) have been introduced in studies on gene carriers.

The targeting of gene complexes to a desired cell population is an important subject in the field of gene therapy. Most studies focus on the effect of targeting ligands that are covalently attached to the DNA complex and allow the uptake of DNA into cells via receptor-mediated endocytosis. Many CPs can be easily conjugated to targeting ligands. Among them, pLL has been the most widely used for attaching targeting ligands. The ligand-pLL system was pioneered by Wu and Wu (12,30). Since recent publications have reviewed ligand-pLL systems in detail (10,31,32), Table 2 considers only the principal ligands used to target pLL polyplexes and updates ligands studied in combination with other CPs. The effects on gene expression observed by linking targeting moleules to CPs are explained further.

CONDENSATION OF DNA BY CATIONIC POLYMERS

Under a wide variety of conditions, plasmid DNA undergoes a spectacular compaction in the presence of condensing agents such as multivalent cations and CPs (15,31,33,34). Naked DNA coils, typically with a hydrodynamic size (R_h) of hundreds of nanometers, after condensation R_h may become only tens of nanometers which means that condensed DNA coils occupy only 10^{-3}–10^{-4} of the volume of naked DNA coils (34). Contrary to proteins which show a unique tertiary

Table 2. Ligands Used in Combination with CPs for Targeting of Genes

CP	Target cell	Ligand	Ref.
1. pLL	Hepatocytes	Asialoorosomucoid[a]	(12,30,97,98)
	Hepatocytes	Lactose, galactose	(46,91,99,100)
	Hepatocytes	Insulin based ligand	(101)
	Macrophages	Mannose	(102)
	Liver SE cells[b]	Hyaluronic acid	(96)
	Lung epithelial cells	Fab fragments of IgG	(92,103)
	Lung epithelial cells	Antibody	(104)
	Cancer cells	B4G7 antibody[c]	(105)
	(Smooth muscle cells)	Low density lipoprotein	(47)
	Various cell types	Transferrin[d]	(106,107)
	Various cell types	Multiantennary galactose derivatives	(108)
	Various cell types	Insulin based ligand[d]	(101)
2. PEI	Hepatocytes	Galactose[e]	(24)
3. Trimethyl-chitosan	Hepatocytes	Galactose[f]	(109)

[a] Asialoroosomucoid is a galactose terminal asialoglycoprotein which has receptors uniquely on hepatocytes and hepatoma cells.
[b] Liver sinuoidal endothelial (SE) cells possess the receptors that recognize and internalize most of the endogeneous hyaluronic acid.
[c] B4G7 antibodies bind to the human epidermal growth factor (EGF) receptor. It allows targeting to EGF-receptor overproducing cancer cells.
[d] As transferrin transports iron into cells, it is found in most cell types. Also insulin receptors are present on various types of cells (110).
[e] A 5% galactose bearing pEI was studied: 5% of the number of *N*-atoms on pEI were covalently linked to galactose via imine formation with lactose which results in the presence of a four-carbon hydrophilic spacer between the pEI backbone and the galactose residues.
[f] 5 mol % and 20 mol % (mol galactose/100 mol sugar units in chitosan) were studied, respectively.

structure, DNA coils do not condense into unique compact structures.

Many reports (7,31,35) using different types of CPs, have shown that R_h of polyplexes upon increasing the charge ratio generally look those like represented in Fig. 1A. The charge ratio (φ) being the ratio of the positive charge equivalents of the cationic component to the negative charge equivalents of the nucleic acid component (3). At low values of φ (<1), water soluble polyplexes with a net negative charge exist. Upon increasing the concentration of CP, the polyplexes become larger. Strongly polydisperse aggregates of polyplexes are formed as a result of the lowered negative charge (Fig. 1B). The largest aggregates exist at a value of φ close to 1, while a further increase in the polycation concentration reduces the size of the polyplexes due to electric repulsion.

Over a wide range of DNA lengths, DNA coils form toroidal structures upon condensation. However, short DNA molecules ($<$400 bp) do not form toroids, while giant DNA chains (166 000 bp) form spherical globules (36). From EM and AFM, DNA toroids are indeed usually observed when CPs are used as a condensing agent (16,37–39). However, Toncheva *et al.* detected spherical structures and toroids when respectively pEG-pLL graft copolymers and pEG-pLL block copolymers were used (17). The reason for this difference in morphology remained unclear.

It is often assumed that the helix in DNA toroids has the B-conformation while e.g. spermidine and spermine condense short DNA molecules into a liquid crystalline phase (34,40). As the structural DNA properties may influence the transfection efficiency of polyplexes, alterations in the tertiary structure of DNA by CPs were cause for investigation, mostly by circular dichroism. While some CPs seemed to change the tertiary structure, other prevented structural changes in DNA. Kim and colleagues observed a tertiary structure similar to that of the noncondensed plasmid DNA when it was complexed with low amounts of hydrophobized (stearyl)-pLL (41). As more stearyl-pLL was used, CD spectra differed from the B-conformation. Maruyama *et al.* showed that the structure of noncondensed calf thymus DNA was significantly altered by pLL while the copolymer pLL-*gr*-dextran prevented serious structural changes (18,19). They suggested that, compared with pLL, the dextran grafts on the copolymer may inhibit a close contact of DNA to the pLL backbone, thereby preventing dehydratation and compaction, and may weaken the interactions.

EM and light scattering measurements suprisingly revealed that over a wide range of DNA lengths (400 bp—50 000 bp) the mean particle size of the condensed particles appears to be largely independent of both the molecular weight (MW) and the sequence of base pairs of the DNA (33,34,36). The independence of the size of the condensed particles on the MW of the DNA was indeed observed using pLL (43). Using lysine containing peptides, Adami *et al.* showed that the mean particle size of condensed DNA also did not differ significantly when linear, supercoiled and circular DNA were condensed (44).

Different observations exist regarding the influence of the type and properties of the CPs on the size of the DNA condensates. On one hand, AFM revealed that increasing the MW of pLL from 3900 Da to 244 000 Da enlarges the mean particle size and polydispersity of the polyplexes from 20–30 nm in diameter (MW 3900 Da) to 120–300 nm (MW 244 000 Da) (38). Also Mumper *et al.* showed that the mean hydrodynamic size of chitosan-polyplexes increases from 150 nm (MW 7000 Da) up to 500 nm (MW 540 000 Da) (45). On the other hand, even using different types of CPs (pEI, dendrimers and pLL) Szoka and colleagues observed from EM that DNA toroids all having a size of 40–60 nm in diameter were formed (37). Also Wagner's group observed toroidal condensates between 50 and 100 nm in diameter for pLL polyplexes (43), while from AFM 100 nm polyplexes were observed when pLL (9600 Da), pEG-pLL block copolymers, p(HPMA)-*co*-p(TMAEMA) (16) and

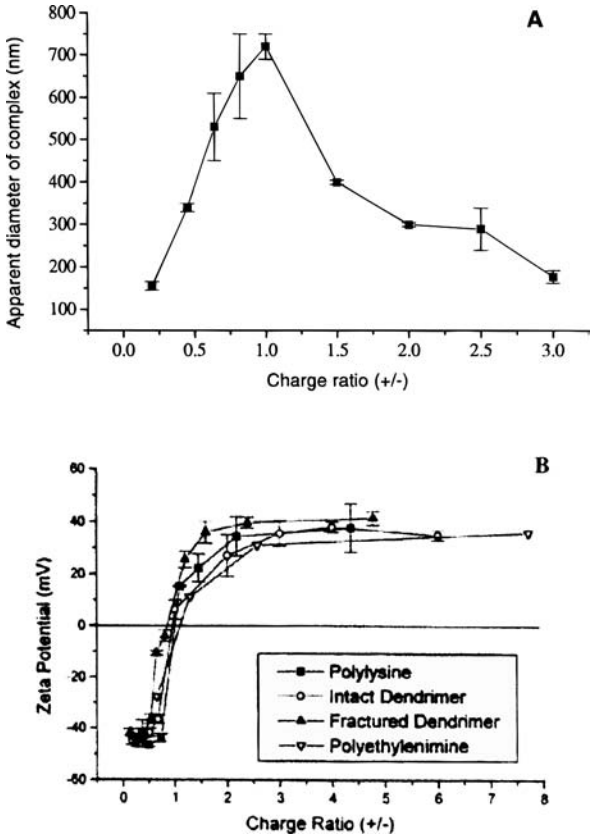


Fig. 1. (A) The influence of the charge ratio on the hydrodynamic dimensions of (pLL) polyplexes (35). Polydisperse aggregates exist at a charge ratio value close to one. Further increase of the polycation concentration reduces the size of the polyplexes. (B) The influence of the charge ratio on the zeta-potential of polyplexes (37). For many types of CPs the cross-over from a negative to a positive zeta-potential of the polyplexes occurs at or very near a charge ratio value of one (Reproduced with permission from reference 37. Copyright 1997 Stockton Press).

hydrophobized pLL (stearyl-pLL) were used (41). Wolfert and Seymour suggested that large DNA condensates arise from the entrapment of more than one plasmid in one polyplex during condensation (38). Tang and Szoka (37) questioned this and proposed that large condensates may arise from clustering of smaller DNA toroidal units after condensation as has been noted also by Bloomfield and colleagues (46).

Attaching hydrophilic segments like pEG (17,20), dextran (17,18) and even the negatively charged hyaluronic acid (HA;(18)) or hydrophobic chains and stearyl chains (41,47) to CPs like pLL (Table 1B) still allow DNA condensation. Although, one wonders why such segments would not inhibit DNA condensation e.g. by preventing a close contact between the cationic backbone and the DNA. Toncheva *et al.* indeed reported that DNA condensation was inhibited with polycation copolymers synthesized by random copolymerisation of cationic and hydrophilic monomers (17). They also showed that in pLL-*gr*-pEG, pLL-*gr*-dextran and pLL-*gr*-pHPMA, pLL was slightly hampered in its ability to condense DNA as, in comparison to pLL homopolymer, higher concentrations of the grafted copolymers were required to quench the fluorescence of DNA etidium bromide complexes.

PHYSICOCHEMICAL PROPERTIES OF POLYPLEXES

Surface Properties of Polyplexes

It is well known that φ determines the charge on the surface of polyplexes (Fig. 1B). For many polyplexes the cross-over from a negative to a positive zeta-potential (ζ) occurs at or very near a value of $\varphi = 1$ (37). In contrast to "small" multivalent cations like e.g. spermine and spermidine which condense DNA but cannot associate in a complex with a positive ζ, CPs allow constructing DNA complexes with ζ up to the 20–40 mV range which favors their solubility (35,37). It should be noted however that ζ of polyplexes is usually measured at an ionic strength much lower than the ionic strength which exists *in vivo*. Grafting hydrophilic chains like pEG, dextran and pHPMA on pLL only moderately lowers ζ which still exhibits a positive value: e.g. from 14 mV to 5 mV upon increasing the dextran content in the pLL-*gr*-dextran copolymers (17,18). Also ζ of polyplexes based on pEG-pLL block copolymers was decreased compared with ζ of the corresponding pLL homopolymer polyplexes (16).

Solubility, Aggregation, and Interactions with Biomolecules

In spite of the presence of a strong positive surface charge, many polyplexes do aggregate in aqueous media *in vitro*. According to Tang and Szoka, the clustering behavior seems to depend upon the type of CP (37). From EM they showed that unit pLL polyplexes (40–60 nm) tend to cluster while the unit DNA complexes of pEI and fractionated dendrimers, which are morphologically similar to the unit pLL polyplexes having a similar ζ, do not. DLS did indeed confirm the existence of large aggregates in the case of pLL polyplexes while these were absent (or less present) when pEI and fractionated dendrimers were used. They concluded that the type of CP plays a role in the aggregation and that simple electrostatic stabilization models are inadequate for describing the general aggregation behavior of polyplexes.

The preparation conditions of polyplexes strongly influence their aggregation behavior. The way of adding the CP solution to the DNA solution (or vice versa), the DNA and salt concentration upon mixing, diluting the polyplexes after their preparation, all influence the aggregation. Wagner and colleagues reviewed this matter with regard to the preparation of pLL polyplexes (10). Perales *et al.* showed that small pLL polyplexes can be prepared by slowly adding pLL solution to a DNA solution followed by stepwise addition of a NaCl solution (48). This protocol allows a gradual accretion of pLL to the DNA backbone and the formation of condensation nuclei along the length of each single DNA molecule which prevents intermolecular DNA aggregation. Also Duguid *et al.* showed that keeping φ constant (=3), the hydrodynamic diameter of polyplexes based on pLL composed of synthetic polypeptides ranges from 30–60 nm at a DNA concentration of 20 μg/mL to the 80–160 nm region at 400 μg/mL, accompanied by a large increase in the polydipersity index which indicates that the polyplexes become increasingly unstable and aggregate stronger at higher DNA concentrations (14). A strong particle growth was observed when pLL polyplexes, which were prepared in water, were diluted in an electrolyte solution (35).

This was propbably attributed to aggregation as a result of the lowered effective ζ by a change in the electrical double layer.

While the stability of the unit DNA complexes under physiological conditions, for example in serum and in the extracellular matrix, is an extremely important property, relatively few studies have examined the potentials of CPs in preventing DNA aggregation. It is well known that CPs exhibit serious precipitation problems when in contact with serum proteins. Plank *et al.* showed that pLL, dendrimers and pEI, especially the polymers with high molecular weight, activate the complement system (49). An important finding was that upon complexation with DNA, the complement activation by CPs was reduced (49). This suggests that the most important factor regulating complement activation and the interactions between proteins and CPs is the number of accessible cationic charges, while type and geometry of the CP are of minor importance. Further studies are required to investigate whether the cationic charges on the uncomplexed cationic polymers or those on the polyplexes are responsible for complement activation.

Strategies to increase the solubility of polyplexes or to reduce polyplex aggregation and interactions with biomolecules were tackled with the development of cationic copolymers bearing hydrophilic segments (Table 1B) (15–21). Significant progress is expected by these copolymers as they allow the construction of non-aggregating, soluble, charge-neutralized polyplexes that show fewer interactions with biomolecules. The hydrophilic shell at the exterior of the polyplexes not only prevents aggregation by sterical repulsion but also enhances the aqueous solubility of charge-neutralized polyplexes. Maruyama et al. showed that dextran grafted to pLL forms soluble polyplexes in a 40 μg/mL DNA solution, while non-soluble aggregated polyplexes arose when pLL homopolymer was used (18). In the study of Toncheva *et al.* (17), charge-neutralized polyplexes based on pLL comb-type copolymers (pLL-*gr*-dextran, pLL-*gr*-pEG and pLL-*gr*-pHPMA) were up to three times more soluble than pLL homopolymer polyplexes, while Kataoka and colleagues (20) observed an enhanced solubility when pEG-pLL block copolymers were used. Erbacher *et al.* studied pLL substituted with (short) hydrophilic groups like glucunoyl and lactosyl which do not form a polymer brush (50,51). Up to 74 glucunoyl residues were attached per pLL chain of 190 lysines. Compared with pLL homopolymer, the hydroxyalkanoyl residues favored the aggregation of the polyplexes. The higher the number of the lactosyl residues per pLL chain, the stronger the aggregation occured. It is generally thought that hydrophilic segments like pEG may prevent aggregation of polyplexes with serum components, as similarly occurs in Stealth liposomes or block copolymer micelles. However, experimental studies which show this effect, even in serum *in vitro*, remain very scarce.

CPs able to construct polyplexes with a temperature dependent solubility and aggregation behavior were recently studied. Bromberg *et al.* showed that *N*,*N*-diethylacrylamide oligomers attached to the ε-*N*-terminus of pLL form polyplexes with a low critical solution temperature of around 29°C (52). Maruyama's group suggested that pDEAEMA-*gr*-pLL polyplexes show a pH dependent aggregation and solubility behavior (53). Such polyplexes show a dual ionic character owing to the cationic pLL segments and the cationic DEAEMA groups. They exhibit turbidity above pH 7.5 while the turbidity discontinuously decreases at pH 7.5 which is probably attributed to protonation of the pDEAEMA segments with pKa around 7.5 (54).

Enzymatic Degradation of Polyplexes

Nucleases easily degrade unprotected DNA. Naked DNA is fragmented within a few minutes *in vivo* after intravenous injection. Condensing DNA with CPs generally improves the resistance of DNA against enzymatic breakdown (39). It is assumed that this is attributed to an altering of the accessibility of the DNA to the enzymes (39). The pEG palisade surrounding pEG-pLL polyplexes results in a stronger resistance against nuclease attack compared to the DNA degradation in pLL homopolymer polyplexes (21).

Dissociation of Polyplexes

The dissociation of polyplexes both *in vitro* as well as *in vivo* are critically important. If the affinity between the DNA and the CP is too low, the polyplex will dissociate prematurely, e.g. in the blood stream, while a strong affinity might prevent the release of DNA intracellularly. The multiple binding sites on oppositely charged macromolecules, as found in polyplexes, result in an integrated stabilization. There exists a cooperativity of the ionic bonds between the CP and DNA (20,31,55). Therefore, "simple dissociation" by small anions may be prevented. However, when other polyions are present an exchange with the DNA or the CP may occur. It is generally questioned whether DNA is released from polyplexes *in vivo* by exchanging with anionic biological macromolecules like sulfated glycosaminoglycans (56), hyaluronan (56) and mRNA. Kabanov's group studied polyion exchange in detail (15,31,57). They showed that polyion exchange is a complicated process which is influenced by the ionic strength of the medium, the nature of the low molecular weight counterions and the charge density and molecular parameters of the polyions. To illustrate this, in the system containing pVP (Table 1A) as polycation and DNA and polymethacrylate as polyanions, at a NaCl concentration of 0.125 M, pVP is mainly bound to the polymethacrylate anions, while at 0.25 M NaCl pVP becomes almost completely complexed to DNA (55). Even more spectacular, when LiCl or KCl instead of NaCl is used, the equilibrium shifts from pVP-DNA to pVP-polymethacrylate. Recent studies investigated the interexchange of DNA with polyanions in condensates based on pEG-pLL block copolymers. Katayose and Kataoka showed that by addition of an equi-unit-molar ratio of pVS to pEG-pLL polyplexes ($\varphi = 1$), all the DNA was dissociated from pEG-pLL (21). Dissociation was also observed when pAA was added (20,58). Polyion exchange by pVS and pAA seems to be influenced by the MW of the pLL segment in pEG-pLL block copolymers (21,58). Cooperative effects were indeed observed in the dissociation of DNA from pEG-pLL polyplexes: pLL polyplexes proved much more stable to disruption by small anions like EDTA and sulfate ions, compared with the disruption by the polymerized negative charges as in pAA (58).

Electrostatic forces play by far the dominant role in the affinity and dissociation of DNA to/from CPs. To illustrate, based on DNA/ethidium bromide complexation measurements, a linear relation was observed between the affinity of DNA to pLL, dendrimers and pEI and the NaCl concentration which highly suggests that the interactions are predominantly electrostatic (37). As another example, by partial substitution of the

N-atoms on pLL with glucunoyl groups (lowering the amount of positive charges), the affinity of DNA to pLL seems to decrease as the polyplexes more easily dissociate by NaCl (51). Besides the dominant electrostatic interactions, other factors may influence the DNA affinity to CPs. Wink *et al.* studied the interaction of pLL and pDMAEMA with plasmid DNA using SPR (59). It was shown that k_d between pDMAEMA and plasmid DNA was higher than k_d between pLL and plasmid DNA. The relatively easy dissociation of pDMAEMA based polyplexes might be one of the reasons for their higher transfection potential as compared to pLL based polyplexes (28).

IN VITRO GENE EXPRESSION BY POLYPLEXES

Studies on gene expression in cultered cells currently remain the first choice for evaluation of the transfection efficiency of polyplexes, although many questions may arise concerning the correlation with transfection efficiency *in vivo* (60). A comparison of *in vitro* gene expression of all polyplexes studied would be a huge attempt as different preparation conditions, various transfection conditions (incubation time, with or without serum, plasmid concentration, the absence or presence of endosome disrupting agents), distinct reporter plasmids (luciferase gene, galactosidase gene, chloramphenicol acetyl transferase gene) and a variety of cell types (either suspended cells or confluent cells) were used. To illustrate, using transfectam and pEI, Boussif *et al.* showed that by "optimized galenics", with regard to the cell transfection protocol and the way of mixing the DNA and cationic vector, one can already improve *in vitro* gene transfer up to 1000-fold (23). The cell type dependence of transfection efficiency *in vitro* was clearly demonstrated for pEI and polypeptide polyplexes (23,35). Pouton *et al.* suggested that the resistance of cells to transfection may be determined by the nature of their plasma membranes and the resistence of their endosomes to disruption (35). A general *in vitro* transfection protocol and making use of standard DNA condensing agents, could be recommended. Moreover, finding out which DNA complexes mediate gene transfection is further complicated by the heteregeneous properties (with respect to e.g. size and charge density) of the polyplex populations which in part arises from the heterogeneous and polydisperse features of the CPs. The continuation of this section focuses on the main similarities and disimilarities which are observed in gene transfection by polyplexes *in vitro*.

Influence of the Size of Polyplexes on *In Vitro* Gene Transfection

One would expect that the size of polyplexes plays a role in the endosomal uptake, the cytoplasmatic transport and the migration through the nucleoporecomplexes which mediate the bidirectional transport between cytoplasm and nucleus.

With regard to endosomal uptake, both for ligand-polyplexes as ligand-free polyplexes, it is absolutely unclear whether it occurs more efficiently for smaller polyplexes. Studying transferrin-pLL polyplexes, Wagner *et al.* observed higher *in vitro* gene transfection with smaller particles (43). They suggested that the polyplexes with diameters of 100 nm or less correspond to the diameter of the coated pit in receptor mediated endocytosis. However, more recently it was shown that also large transferrin-pEI polyplexes of around 500 nm in diameter can also benefit from the mechanism of specific transferrin-receptor binding in transfecting cells (61). Uncertainties currently remain on whether clathrin-coated pits are involved in receptor mediated endocytosis of polyplexes (23,61). Many studies show that ligand-free polyplexes of hundreds of nanometers, even after aggregation by e.g. a lowering of ζ, are able to transfect cultured cells (35). Also large calcium phosphate/DNA or DEAE dextran/DNA precipitates allow gene transfection. This may suggest that uptake into endocytic vesicles of less than 100 nm in diameter may not be the important mechanism for cellular uptake of ligand-free polyplexes *in vitro*.

With regard to the nucleocytoplasmatic transport, many studies (62,63) revealed that upon microinjection of plasmids into the cytoplasm they become poorly expressed (e.g. in $<0.001\%$ of the cells (62)) while microinjection into the nucleus results in a much higher expression (between 50% and 100% of the cells (62)). This suggests that the filamentous network in the cytoplasm and the nuclear envelope may prevent migration of large gene complexes into the nucleus. Recently, Wilke *et al.* indeed showed that for peptide based polyplexes the nuclear membrane is an important barrier as cells that were allowed to perform a mitosis after exposure to polyplexes were transfected much more efficiently than cells arrested in the cell cycle (64). Luby-Phelps *et al.* showed that the diffusion of particles in the cytoplasm is indeed size dependent and expected that particles larger than 54 nm in diameter may be completely nondiffusable in the cytoplasmatic space (65). However, large particles may migrate in the cytoplasm not only by diffusion but also other mechanisms where cytoskeletal components like microtubules and actin filaments are involved, which may facilitate the transport (63). Also, by energy dependent mechanisms, nuclear localization sequences facilitate the transport of particles which are larger than the 9 nm aqueous central channels in the nucleoporecomplexes. By expanding the central channels, particles up to 28 nm in diameter may be transported into the nucleus (66).

On one hand, based upon the considerations described above, evidence exists to show that, ideally, the size of polyplexes should be as small as possible. On the other hand, if endocytosis of polyplexes did not involve clathrin-coated vesicles and as most polyplexes are too large anyway to cross the nuclear pores (>30 nm), one could expect larger polyplexes (>100 nm) to transfect cells as efficiently as smaller ones (between 30 nm and 100 nm). Studies which focus on the influence of the size of polyplexes on *in vitro* gene transfection are scarce and the results are conflicting. Kim *et al.* noted that the smaller sized stearyl-pLL polyplexes (about 250 nm in diameter) transfected better than the bigger ones (about 400 nm in diameter) (47). However, it is unclear whether the polyplexes only differed in size while possessing e.g. a similar φ. Szoka and colleagues suggested that fractured dendrimers and pEI polyplexes mediate higher transfection than pLL polyplexes probably due to minimal aggregation of the 40–60 nm pEI and fractionated dendrimer polyplexes in contrast to pLL polyplexes which strongly aggregate (37). However, they questioned this interpretation, as no clear correlation between polyplex size and *in vitro* gene transfection in an earlier study on fractured dendrimers was observed (67). Wagner's group found a strong correlation between the size of pEI and transferrin-pEI polyplexes and *in vitro* gene transfection (61). Small (30–60 nm) nonaggregating pEI and transferrin-pEI polyplexes were

obtained under salt free conditions whereas when pEI and transferrin-pEI polyplexes were formed in HBS they aggregated into larger particles (300–600 nm). Unexpectedly, in all cell types the transfection efficiency of the small, stable polyplexes was from 100 to 500 times lower compared with the larger, aggregated polyplexes. Similarly, a lower *in vitro* transfection efficiency was observed in cases where the polyplexes were prepared with a lower amount of DNA, which also resulted in smaller particles (Fig. 2) (61). They pointed out that the reduced *in vitro* transfection efficiency of smaller pEI and transferrin-pEI polyplexes was probably partially attributed to a limited contact with the cells. While larger polyplexes sedimented onto the cells, smaller ones stayed in solution and contact with cells was limited (23,61). Indeed, smaller polyplexes transfected more efficiently when either the transfection volume or the transfection time was increased. Since adding lysosomotropic compounds (like chloroquine) or endosomolytic influenza peptides to the transfection medium increased the transfection efficiency with the small (but not with the large) polyplexes, they concluded that the smaller particles were less able to destabilize the endosomes resulting in a lower transfection. As it is believed that pEI acts as a proton sponge (68) which destabilize the endosomes, Ogris *et al.* (61) assumed that a critical minimum amount of pEI has to be present in the endosome for successful disruption and questioned whether this critical concentration was provided by the small pEI polyplexes or not. Interestingly, it was also observed that the difference between small and large polyplexes in overall gene expression results from different expression levels per cell and less from a different percentage of expressing cells.

Interactions Between Polyplexes and Cell Surfaces which Mediate *In Vitro* Gene Transfection

It is generally observed that the *in vitro* transfection efficiency is enhanced by increasing the positive charge of polyplexes (22,35,37). Typically, an optimal φ-value exists at which maximal transfection occurs which depends on the type of CP. Upon increasing φ further, cell toxicity appears which is attributed to free CP. It is believed that the increased transfection efficiency at higher values of φ arises from enhanced electrostatic interactions between the anionic cell surface proteoglycans and the positive polyplexes. Mislick and Baldeschwieler showed that *in vitro* treatment of HeLa cells with heparinase and chondroitinase ABC, as well as the addition of anionic glycosaminoglycans to the transfection medium, dramatically inhibited transfection by pLL polyplexes (69). They also suggested that the variable expression of proteoglycans among tissues may explain why some cell types are more susceptible to transfection than others. A dramatically reduced transfecton by several types of polyplexes upon adding sulfated glycosaminoglycans, was also observed by Ruponen *et al.* (56). For polyplexes based on synthetic polypepetides, Duguid *et al.* showed that ζ of the polyplexes is essential for the prediction of the *in vitro* transfection efficiency (14). Compared with e.g. the concentration of the polypeptide, they argued that ζ is much more adequate in predicting transfection efficiency because it provides a real measure of the affinity of the gene delivery complex to charged cell surfaces.

As explained above, much attention has been paid to the development of soluble nonaggregating charge-neutralized polyplexes. As electrostatic interactions between charge-neutralized polyplexes and cell surfaces are absent, a way of establishing interactions is the use of ligands (Table 2). Fig. 3 shows how the *in vitro* transfection for charge-neutralized pEI polyplexes increased by galactose groups (24). Although, even without using targeting ligands, charge-neutralized linear pEI polyplexes were more efficient than cationic lipids in transfecting epithelial lung cells *in vitro*. The possibility of reaching high transfection efficiency by using complexes with a charge ratio close to neutral is a major advantage of pEI.

Hydrophobized CPs were suggested to enhance the affinity of polyplexes (70). Replacing a small amount of the *N*-ethyl groups on pVP (Table 1A) by *N*-cetyl groups allowed pVP polyplexes to penetrate the liposomal membranes, which were considered to mimic the cell surface, better (70). This is propably due to the incorporation of the hydrophobic domains into the hydrophobic part of the lipid bilayer. To facilitate cellular uptake by the use of hydrophobized CPs, polyplexes based on stearyl- bearing pLL are currently under investigation (41,47). Van de Wetering *et al.* reported that copolymers of DMAEMA

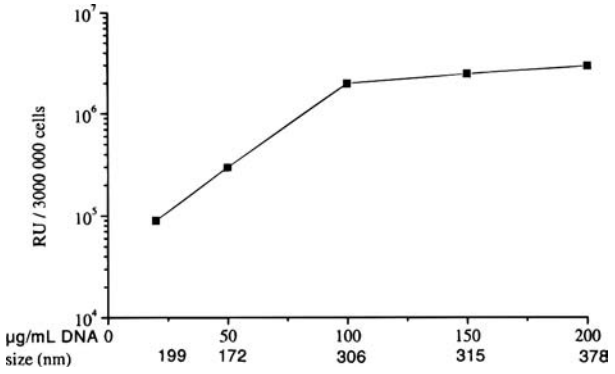


Fig. 2. Rather unexpected, larger transferin-PEI polyplexes, prepared with a higher amount of DNA, transfect neuro2A cells *in vitro* more efficiently than smaller transferin-PEI polyplexes which are obtained when a lower amount of DNA is used in the prepararion of the polyplexes (61). In all the transfection experiments 5 μg plasmid was applied to the cells. (Reproduced with permission from reference 61. Copyright 1998 Stockton Press).

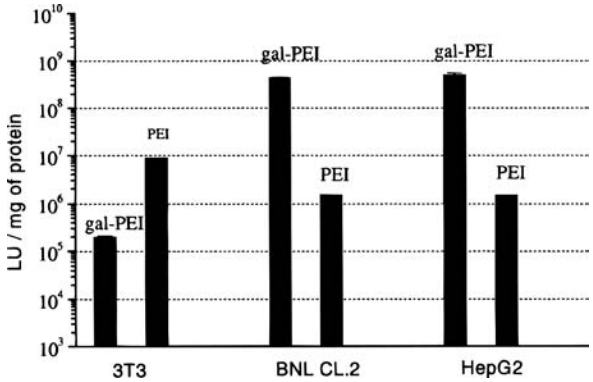


Fig. 3. *In vitro* transfection of hepatocytes (BNL CL.2 and HepG2) by pEI polyplexes increases when galactose groups are attached to the polyplexes (24). The hepatocytes were transfected 10^4–10^5 fold more efficiently than fibroblasts (3T3) which do not express the asialoglycoprotein receptor. (Reproduced with permission from reference 24. Copyright 1997 American Chemical Society).

and hydrophilic ethoxytriethylene glycol methacrylate and N-vinyl pyrrolidone had a better efficacy/toxicity ratio as compared to a homopolymer of DMAEMA (71). On the other hand, introduction of a limited amount of a hydrophobic comonomer (methyl methacrylate) substantially increased the cytotoxicity of the polymer.

One wonders whether hydrophilic segments on CPs, like e.g. pEG and dextran, which (moderately) lower the effective ζ, would inhibit *in vitro* gene transfection. Compared to pLL homopolymer polyplexes, several studies indicated that the pEG-pLL polyplexes show increased transfection efficiency *in vitro* (20,72). Toncheva *et al.* observed a higher transfection ability for polyplexes containing longer pEG chains (12 000 Da versus 5000 Da) or a larger amount of pEG (10 mol% (i.e. mol PEG/100 mol lysines) versus 5 mol%) (17). However, Choi *et al.* measured a significant lower transfection when the pEG substitution was further increased to 25 mol% (72). pEG is known to associate with the phospholipid headgroup of cell membranes which may facilitate penetration into cells (73). At higher pEG concentrations, Toncheva et al. (17) suggested that pEG may locally dehydrate the membranes thereby promoting entry into the cytoplasm while Choi et al. (72) suggested that the contact between the cell membranes and the polyplexes may become inhibited.

Promoting the Endosomal Release of Polyplexes to Enhance *In Vitro* Transfection

It is widely accepted that the release of gene complexes from cellular vesicles like endosomes is a great barrier in the process of gene transfer. To enhance the endosomal escape, different strategies have been developed, like the addition of lysosomotropic agents to the transfection medium and the inclusion of inactivated virus particles or membrane active peptides in the gene complexes.

The effect of the lysosomotropic drug chloroquine on gene transfer was studied for many polyplexes in a variety of cell lines. Most polyplexes mediate a relatively low degree of transfection *in vitro* which usually becomes significantly improved by chloroquine (17,35,47,51,61). However, some CP like pEI, (fractured) dendrimers and pDMAEMA (28) do not require lysosomotropic agents to show a substantial *in vitro* gene transfection. In the case of pEI and dendrimers the addition of chloroquine generally has little or no effect (22,61). This is explained by the proton-sponge hypothesis (68) which assumes that pEI and (fractured) dendrimers are able to act in the endosomes through osmotic swelling, just as chloroquine does. While at the physiological pH of the transfection medium the *N*-atoms of e.g. pLL are nearly fully protonated (pKa between 9 and 10), the *N*-atoms on pEI and (fractured) dendrimers are only partially protonated (pKa of pEI is 5.5 while it is 6.9 and 3.9 for respectively the primary and interior amino groups in (PAMAM) dendrimers). Consequently, after endocytosis of pEI or dendrimer polyplexes, pEI and dendrimers should buffer the endosomal acidification accompanied by an accumulation of protons in the endosomes which are coupled to a simultaneous influx of chloride anions (74). It is hypothesized that swelling and disruption of the endosomes finally occur due to water entry as a consequence of the net increase in ion concentration and expansion of the pEI and dendrimers by internal charge respulsion.

It is generally believed that chloroquine (pKa 8.1 and 10.2) enhances gene transfer by a similar swelling and destabilization of the endosomes and by inhibiting lysosomal enzymatic degradation of the gene complexes (75) due to the raised luminal pH of the endosomes. However, as it is well established that chloroquine binds to DNA, Erbacher *et al.* suggested that chloroquine may also contribute to a higher gene transfection by enhancing the dissociation of the polyplexes (76). As they observed that chloroquine dissociates lactosylated pLL polyplexes, they compared the transfection efficiency of lactosylated pLL polyplexes in the presence of respectively chloroquine, amonium chloride, and methylamine. Amonium chloride and methylamine are able to buffer the endosomes like chloroquine but do not dissociate lactosylated pLL polyplexes. The transfection efficiency seemed unenhanced in the presence of amonium chloride and methylamine. Moreover, while the neutralization of the endosomes was already effective at 20 μM chloroquine, the transfection efficiency was not. Conversely, the transfection efficiency did increase using 100 μM chloroquine which leads to endosomal concentrations high enough to dissociate the polyplexes.

The use of chloroquine is limited because of its toxicity and because it enhances transfection efficiency in only a limited number of cells. Promoting the endosomal release by the incorporation of viruses was initially studied by Wagner and colleagues (for a recent review see (10,32)). *In vitro* transfection efficiency of transferrin-pLL polyplexes could be significantly enhanced by the addition of replication-defective adenoviral particles to the transfection medium (77–79). However, by inclusion of adenoviral particles gene complexes may enter cells via the adenovirus receptor which is a clear disadvantage for ligand specific gene delivery. Therefore, and as it was shown that the membrane fusion capacity of the influenza virus resides in the *N*-terminus of the hemagglutinin 2 subunit (HA-2) (80), HA-2 was studied which revealed a beneficial effect on *in vitro* gene transfer (81). This was attributed to a peptide sequence in HA-2 which upon endosomal acidification forms an amphipathic helix, destabilizing the endosomal membranes. Besides HA-2, other peptides enhanced the *in vitro* transfection efficiency of pLL polyplexes (82). *In vitro* gene transfer of dendrimer polyplexes was increased by covalently binding a synthetic amphipathic peptide (GALA; 30 amino acids) (22). *In vitro* gene transfer by synthetic polypeptide polyplexes was strongly enhanced by GM225.1 (14). This is a synthetic amphipathic peptide which, like HA-2 and GALA, contains an α-helical structure and a *N*-terminal hydrophobic GLF sequence that is considered to enhance membrane insertion. Santos *et al.* suggested the use of hydrophobic polyelectrolytes such as poly(ethylacrylic acid) to increase the endosomal release (83). These polymers change conformation upon lowering pH, become more hydrophobic, increasing the affinity for phospholipid membranes and ultimately may solubilize the membranes (83,84). It was also observed that poly(ethylene oxide)-*block*-poly(propylene oxide) copolymer enhances *in vitro* gene transfer by pEVP polyplexes, possibly by promoting the escape from the endosomes.

Promoting the Intracellular Dissociation of Polyplexes to Enhance *In Vitro* Transfection

For gene expression to take place, it is generally assumed that the cationic carrier has to dissociate from the DNA. Although, to our knowledge, no experimental evidence exists for this hypothesis with regard to polyplexes. Evidence for

this hypothesis with regard to oligonucleotide/cationic lipid complexes was recently shown by Marcusson *et al.* (85). While the fluorescently labeled DNA appeared in the nucleus, the cationic lipid did not, suggesting that the complex has indeed dissociated before the oligonucleotide entered into the nucleus.

Since technical limitations exist to characterize intracellular dissociation of gene complexes, studies which focus on the influence of intracellular dissociation of polyplexes on *in vitro* gene transfection are scarce. Erbacher *et al.* observed that the *in vitro* (chloroquine mediated) transfection efficiency of pLL could be increased by partially substituting pLL with glucunoyl groups (50,51). This was attributed to a lowered affinity of DNA to the polymer because substituting the *N*-atoms of pLL with glucunoyl groups decreases the electrostatic interactions. This conclusion was based on dissociation measurements in an acellular system which indicated that the chloroquine induced dissociation of glucunoylated pLL polyplexes occurs more easily than for sugar-free pLL polyplexes. Pouton *et al.* compared the *in vitro* transfection efficiency of the heteropolyaminoacid (poly (alanine-*co*-lysine)) polyplexes with polyplexes based on homopolyamino acids (pLL, polyornithine, polyarginine) (35). In contrast to the homopolyamino polyplexes, the heteropolyaminoacid polyplexes showed a much lower gene transfection. As poly(alanine-*co*-lysine) is considerably more hydrophobic than pLL, it may limit the intracellular release of DNA from the CP, thereby preventing gene transfer.

IN VIVO GENE EXPRESSION BY POLYPLEXES

A rather limited amount of reports have been published on *in vivo* gene expression by polyplexes. Only the most simple type of polyplex, namely DNA-ligand-CP, has been investigated *in vivo*. Little information is available on the influence of e.g. the incorporation of inactivated virus particles or membrane active peptides on *in vivo* gene transfer by polyplexes. To our knowledge, there are only two ongoing clinical trials using pVP based polyplexes directly administered to head and neck tumors. An *ex vivo* phase I study using transferrin-pLL polyplexes in the presence of inactivated adenoviral particles is currently ongoing (86). The subcutaneously administred cancer vaccine consists of the individual patient's melanoma cells which are transfected *ex vivo* by transferrin-pLL polyplexes to produce human interleukin-2.

CP mediated *in vivo* gene transfection has had only limited success and reproducibility is a problem. Generally considered a great challenge for successful *in vivo* gene transfer is the reduction of the size of polyplexes to avoid uptake by Kupffer cells (before they can interact with the target in the case of receptor mediated gene delivery) and to cross the vascular endothelium fenestration. Moreover, the positive surface charge, which mostly promotes the *in vitro* effectiveness, may strongly reduce the effectiveness *in vivo*, as it leads to interactions between the polyplexes and serum proteins. *In vivo* studies on gene transfer and immunocompatibility by charge-neutralized polyplexes are limited and should receive more attention (87).

In vivo gene expression by polyplexes was first reported by Wu and Wu (88). Gene expression in liver cells after intravenous injection of asialo-orosomucoid pLL polyplexes in rats was highest after 24 hours, while expression was no longer observed after 96 hours. Partial hepatectomy, 30 minutes after the injection, drastically prolonged the gene expression up to 11 weeks (89). Interestingly, most of the DNA was found in the endosomes while little was detected in the nucleus which suggested that the polyplexes in the endosomes provided a constant supply of genes to the nucleus. After intravenous injection of small galactose-pLL polyplexes (around 15 nm in diameter as measured by EM), without liver surgery, Perales *et al.* observed prolonged gene expression up to 20 weeks (90). Hashida *et al.* observed that upon intravenous administration, negatively charged galactose-pLL based polyplexes (180 nm as measured by DLS) are eliminated from the circulation within minutes and preferentially taken up by the liver's parenchym cells (91). Plank *et al.* noticed that all the successful *in vivo* studies on pLL polyplexes used polyplexes that had a calculated net negative charge and showed that these polyplexes did not activate the complement system in their experiments (49). Work by Ferkol *et al.* has shown specific gene expresion in respiratory epithelium and submucosal glands after intravenous administration of polyplexes in mice (92). To target the lung endothelial surface, they complexed DNA to pLL that had been coupled to a Fab fragment with specificity for a receptor highly expressed in lung epithelium cells. The mechanism by which the polyplexes travel from the endothelium to the epithelium currently lacks any mechanistic explanation.

In vivo gene transfer by pEI polyplexes was recently reviewed by Remy *et al.* (9). Encouraging *in vivo* results were obtained after intracranial injection in mice (25,93). The *in vivo* transfection efficiency was similar to their transfection efficiency *in vitro* for the same amount of DNA applied to neuronal cells. The highest transfection efficiency *in vivo* was observed with pEI polyplexes bearing net charges around neutrality. pEI polyplexes prepared with 25 kDa pEI transfected better *in vivo* than those pepared with 50 kDa and 800 kDa pEI. Boletta *et al.* studied *in vivo* gene transfer to rat kidneys by pEI polyplexes (94). They were injected into renal arteries of rats and allowed to remain in contact with the kidney for 10 minutes. Besides lipoplexes, three different forms of pEI were evaluated, differing either in average molecular weight (800 kDa branched pEI versus 25 kDa) branched pEI) or in chemical structure (25 kDa branched pEI versus 22 kDa linear pEI). A maximum of transfection efficiency was obtained with moderately positively charged polyplexes ($1.5 < \varphi < 2.5$). Compared with lipoplexes, branched pEI 25 kDa polyplexes yielded significantly higher gene transfection. Despite the similar *in vitro* transfection behavior of all forms of pEI, *in vivo* branched 25 kDa pEI proved superior to its linear counterpart pEI 22 kDa and to the much larger branched pEI 800 kDa. The reason is unclear. Ferrari *et al.* showed that instillation of linear pEI 22 kDa polyplexes into the lungs of rabbits transfered the luciferase gene more efficiently than transfectam based gene complexes (26). Within 1 week after instillation, gene expression was decreased by two orders of magnitude. Again, the best levels of transfection were obtained using neutral polyplexes. Kircheis *et al.* showed that gene transfer after subcutaneous administration into tumors in mice was 10-100 fold more efficient with transferrin-pEI based polyplexes in comparison to naked DNA (87). Even after systemic application, gene delivery into subcutaneously growing tumors was achieved using charge neutralized pEGylated transferrin-pEI polyplexes, whereas

application of positively charged polyplexes resulted in predominant gene expression in the lungs and was associated with considerable toxicity.

Efficient gene transfer into a murine cardiac transplantation model by dendrimer polyplexes ($10 < \varphi < 100$) was reported by Qin *et al.* (95). They were directly injected into the grafts at the time of transplantation and were evaluated to deliver immunosupressive molecules in order to prolong graft survival. An improved expression of viral interleukin-10 was reported which prolonged the survival of the graft.

CONCLUSIONS

This review has focused on the condensation of DNA by CPs, the physicochemical characteristics of the resulting polyplexes and their *in vitro* and *in vivo* transfection behavior. Currently, a series of chemically different CPs, ranging from linear homopolymers to block and comb-type copolymers, has been reported to condense DNA and proposed as gene carriers. Although the physicochemical investigations on the association, dissociation, solubility and aggregation of polyplexes have received much attention, both theoretical and experimental research on the complicated physicochemical behavior of polyplexes is highly recommended. One wonders whether more research efforts should be applied towards the evaluation of new types of CP which are new from a chemical point of view, or, whether optimization of the physicochemical and pharmaceutical features of the already existing polyplexes needs more attention. With regard to this debate, Behr's group argued that optimization of the preparation of pEI polyplexes and the way used to transfect the cells, has been more fruitful than their synthesis of new CPs (23).

Compared with the high efficiency of viral gene transfection, the efficiency of gene transfection *in vitro* and especially *in vivo* by polyplexes is still relatively low. It is generally considered that in order to improve gene transfer, the first generation of polymer based transfection systems should now be followed by a second generation of polyplexes that lacks the drawbacks of the first generation. To rationally design this new generation, attention should be paid to all critical steps in the process of polyplex transport from the extracellular space into the nucleus which includes the improvement of the site specific delivery, the cellular uptake, the endosomal escape capacity and the efficiency of the transport of the plasmids to the nucleus. Considering the critical importance and the complexity of DNA dissociation from polyplexes, to obtain real breakthroughs in polyplex design and in understanding dissociation of polyplexes in biological environments like in serum and cells, there is currently an urgent need for advanced physicochemical methods which allow characterizing these critical steps in such media.

Currently missing from polyplex literature are reports dealing with pharmaceutical technological aspects and the toxicological and immunological behavior of polyplexes. Although, with respect to stability, plasmid DNA has some advantages over protein based pharmaceuticals, which are susceptible to a loss of biological activity by small changes in their tertiary and quaternary structure, pharmaceutical research on polyplex formulations which are stable for extended periods of time are recommended. Freeze drying and other drying strategies could be evaluated. In the fabrication of pharmaceutical formulations and in the application of some delivery systems like aerosols, often shear stress is involved. No information is currently available on whether the structure of DNA can be altered or whether dissociation of polyplexes may occur by such types of forces. With clinical studies in mind, sterilization and upscaling of polyplexes may also become interesting challenges to be dealt with in the future. Finally, fundamental research on the toxicological and immunological aspects of polyplexes is highly recommended as the information currently available is very limited.

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