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Polymeric Carriers in Non-viral Gene Delivery

A Study of the Physicochemical Properties and the Biological Activity in Human RPE Cell Line

Doctoral dissertation

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ABSTRACT

Gene therapy is a promising new tool to treat some diseases that currently are incurable such as, genetic disorders, cancer diseases and some retinal diseases, but it has still not become an established practice in medicine mainly because of either insufficient efficacy or safety problems. The basic idea in gene therapy is straightforward: the failure to produce some protein coded by a defective gene is overcome by delivering a new intact gene into the nucleus of the cells. Since naked DNA as such is not usually efficiently internalized by cells, a carrier system is needed for gene delivery. The first systems were based on modified viruses. However, the safety issues of viral gene delivery systems generated a new research field, non-viral gene delivery systems. A battery of different kinds of alternatives has been generated but none have achieved ultimate success: non-viral gene delivery systems are still less effective than viral systems.

The objective of the present study was to develop new kinds of gene carriers and to study their suitability for gene delivery purposes, and also to study cellular mechanisms/properties involved in gene delivery. Systematic physicochemical and biological characterization of plasmid DNA (pDNA) carriers and mechanistic studies can help in designing new more efficient non-viral carriers. We investigated the role of structural properties (shape, PEGylation, molecular weight (MW)) of poly-L-lysine (PLL) gene carriers, and the influence of biological processes and properties (cell cycle, intracellular kinetics, glycosaminoglycans, (GAGs)) on polymeric DNA delivery into a cultured human retinal pigment epithelium (hRPE) cell line, D407. This is important cell line, since RPE maintains the function of photoreceptors and eyesight, and therefore, is a potential target for gene delivery.
Physicochemical and biological structure-property relationships of PLLs (3–20 kDa) exhibited no clear correlations between the tested physicochemical properties (condensation, relaxation, zeta-potential, size and shape of the polyplexes) and biological activities (cell uptake, transgene expression and cytotoxicity of the polyplexes). Most PLLs (20 kDa) condense DNA (linear, grafted, branched > dendritic) and condensation is not decreased if the polyethylene glycol (PEG) content is about 60 % or less (fraction of MW). PEGylated PLLs (20 kDa) form sterically stabilized toroidal or rod-like complexes with diameters of 27–123 nm, but they are not totally protected from interacting with polyanionic chondroitin sulphate. Further studies with two carriers with different gene delivery properties, PLL 200 kDa and PEI 25 kDa (polyethyleneimine), demonstrated a relationship between cell cycle phase (G1, S, G2, M) and transfection efficiency. The transgene expression of the polyplexes is influenced by cellular uptake and transcription, and both processes are cell cycle-dependent. Cellular uptake of the polyplexes was at its highest during the S phase (80–90 %) and lowest during the G1 phase (5–30 %). PEI 25 kDa was a more efficient as a transfection agent than PLL 200 kDa. Furthermore, all promoters (CMV, SV40, tk, PDE-β) and reporter genes (β-galactosidase, luciferase) showed dependence on the cell cycle. However, as expected, only a small fraction of the pDNA was found in the nucleus, partly carrier-bound, but having been accumulated in a cell cycle-dependent manner. Interestingly, the gene expression after PEI25 kDa mediated transfection was 1–2 orders of magnitude higher than after PLL mediated delivery even though the gene transfer into the nuclei was approximately the same. This indicates that there is higher transcriptional efficacy after PEI transfection. Finally, since it is possible that interactions with endogenous polyanionic GAGs could interfere with cellular uptake or transgene expression, the GAG profile of synchronized D407 cells was determined. However, we found that the GAG contents alone do not explain the transfection efficiencies, since major changes occur simultaneously in the general rate of fluid-phase endocytosis, and nuclear access of the endocytosed pDNAs.

In conclusion, the present study indicates that the physicochemical properties of PLL polyplexes can differ to some extent without this having any great impact on biological activity. Also, a knowledge of cell cycle-dependent variation in gene transfer can hep promote targeting in gene therapy into uncontrollably dividing cells in diseases such as proliferative vitreoretinopathy (PVR) and retinal generations, or different types of cancer diseases. Nonetheless even greater understanding of the intracellular kinetics of polyplexes and underlying molecular mechanisms of the diseases is needed before of non-viral gene therapy can become a clinical reality.

National Library of Medicine Classification: QZ 52, QU 470, QU 475, WW 103, WW 270
Medical Subject Headings: Gene Therapy/methods; Gene Transfer Techniques; Gene Targeting; Transfection; Plasmids; Polylysine; Polymers; DNA; Molecular Structure; Glycosaminoglycans; Polyethylene Glycols; Pigment Epithelium of Eye; Retina; Cell Line; Structure-Activity Relationship; Cell Cycle; Gene Expression; Endocytosis; Kinetics

To My Family ✯

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Helsinki, May 2007

Marjo Männistö

ABBREVIATIONS

bp	base pairs
CHEMS	cholesteryl hemisuccinate, 3β-hydroxy-5-cholestene 3-hemisuccinate
CMV	cytomegalovirus
CPP	cell-permeable peptide
CS	chondroitin sulfate
D407 6-2	stably luciferase expressing retinal pigment epithelial cell line
DEAE	diethylaminoethyl/diethylaminoether
DNA	deoxyribonucleic acid
DO	diolein, glycerol-1,2-and-1,3-dioleate
DOGS	dioctadecylamidoglycylspermine
DOPE	1,2-dioleyl-3-phosphatidylethanolamine
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate
DOTMA	dioleyloxypropyl trimethylammonium bromide
DPPES	dipalmitoyl phosphatidylethanolamidospermine
DTAF	5-(4,6-dichlorotriazinyl)aminofluorescein
EDTA	ethylene diaminetetra acetic acid
EMA	ethidium monoazide
EtBr	ethidium bromide
FITC	fluorescein isothiocyanate
G1 phase	Gap 1 phase of cell cycle
G2 phase	Gap 2 phase of cell cycle
GAG	glycosaminoglycan
HA	hyaluronic acid, hyaluronan
HEPES	4-(2-hydroxyethyl)-1 piperazine ethane sulphonic acid
HS	heparan sulfate
i.v.	intravenous
kDa	kilodaltons
luc	luciferase
M phase	mitosis
MCS	multiple cloning site
MES	2-(N-morfolino)ethane sulfonic acid
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazole)-2-yl-2,5-diphenyl tetrazolium bromide, thiazolyl blue tetrazolium bromide
NE	nuclear envelope
NLS	nuclear localizing signal
NPC	nuclear pore complex
OA	oleic acid, cis-9-octadecanoic acid
ODN	oligonucleotide
ONPG	orthonitrophenyl-β-D-galactopyranoside
PAMAM	polyamidoamine

PBS	phosphate buffered saline
PCR	polymer chain reaction
PDE-β	phosphodiesterase-β
PDMAEG	poly[N-(2-N,N-dimethylaminoethyl)glutamine]
PDMAEMA	poly(2-(dimethylamino)ethyl methacrylate)
PEG	polyethylene glycol
PEI	polyethyleneimine
PG	proteoglycan
PLGA	poly(DL-lactide-co-glycolide)
PLL	poly-L-lysine
PMSF	phenylmethylsulfonyl fluoride
RISC	RNA induced silencing complex
RPE	retinal pigment epithelium
S phase	DNA synthesis phase
siRNA	short interfering RNA
SV40	simian virus 40
tk	thymidine kinase
TEM	transmission electron microscope

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals **I–III.** In addition, the summary of the thesis includes unpublished results.

I Marjo Männistö, Sylvie Vanderkerken, Veska Toncheva, Matti Elomaa, Marika Ruponen, Etienne Schacht, Arto Urtti: Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. J. Control. Rel. 83:169–182, 2002.

II Marjo Männistö, Seppo Rönkkö, Mikko Mättö, Paavo Honkakoski, Mika Hyttinen, Jukka Pelkonen, Arto Urtti: The role of cell cycle on polyplex-mediated gene transfer into retinal pigment epithelial cell line. J. Gene Med. 7: 466–476, 2005.

III Marjo Männistö, Mika Reinisalo, Marika Ruponen, Paavo Honkakoski, Markku Tammi, Arto Urtti: Polyplex-mediated gene transfer and cell cycle: effect of carrier on cellular uptake and intracellular kinetics, and significance of glycosaminoglycans. J. Gene Med. (in press)

CONTENTS

1 INTRODUCTION

Exogenous transgenes can replace or supplement the function of defective or malfunctioning genes. In this sense, they can be considered to be treating the underlying cause, i.e. the genetic defect, of the inherited or acquired disease. In contrast, the conventional drugs act at the protein level, usually providing only treatment of the symptoms. Proteins are the building blocks of life that regulate bodily functions, and are regulated by genes, therefore, the lack or overproduction of specific proteins, or production of defective proteins affect the onset of the symptoms of genetic diseases.

Gene medicines (or nucleic acid drugs) can be divided into different categories according to the method of application: gene inhibitors, gene vaccines and gene substitutes. Gene inhibitors are oligonucleotides (ODN) which silence defective genes, usually at the mRNA level, by binding (antisense, aptamer) to mRNA or by splicing it (ribozymes) in the cytoplasm, but also by interactions with mRNA precursor in the nucleus (Galloutzi and Steitz 2001). Gene vaccines are antigens of specific pathogens encoding either the genes or RNA that activate cell immune responses and production of antibodies (Srivastava and Liu, 2003). Gene substitutes are transcriptionally fully competent genes introduced into cells to compensate for the lack of a particular protein or its insufficient protein production.

The efficiency of gene transfer can be defined as the percentage of the cells containing the transgene or as the amount of transgene expression. It has been proposed that the replacement of a mere 5 % of the amount of protein present in normal individuals should be enough to achieve a therapeutic cure of hemophilia (Roth et al. 2001). As the efficiency of gene therapy is still being debated, it is important to know the level of gene expression that will be required to cure diseases. Subsequently, designing and adjustments of gene carrier systems can be carried out and the possible problem of overproduction of proteins could be avoided. Today gene therapy is still taking its first faltering steps in the field of medicine and the difficulty of targeting genes into cells *in vivo* represents the main barrier in progress. Also, many of the techniques available for in vitro transfections cannot be applied in vivo.

Gene delivery methods can be divided into viral and non-viral systems. Viral systems are still more useful in clinical use, since they are more efficient than non-viral systems, but the safety issues surrouding viral systems have not been solved and even fatal adverse reactions have taken place in the clinical studies. These adverse reactions can be attributed to the random integration of transgene into chromosomal DNA that may be manifested as cancer and immunological responses which may lead even to the death of the patient (Raper et al. 2003). In addition, the transgenes have size limitations and the preparation usually is more laborious with viral systems than non-viral systems. The control over the properties of non-viral carriers is important in designing new gene carriers, although the main problem currently in polymeric carriers is overcoming their poor efficacy. The advantage of non-viral gene carriers over the viral systems is that usually they do not evoke immunological reactions, they can be completely defined and since they are synthetic, this facilitates scale-up production. This is the ideal system and theoretically polymeric systems should be easier to apply than viral systems. Furthermore, if non-viral methods remain relatively simple carrier systems, it will be much easier to translate these techniques into practical pharmaceutical products.

2 REVIEW OF LITERATURE

2.1 Non-viral gene delivery methods

Non-viral gene medicines are based on either plasmids or oligonucleotides (Mönkkönen and Urtti 1998). The former is used in gene inhibitor medicines for silencing a defective gene with a piece of nucleic acid called an oligonucleotide (ODN) and the latter is used in gene substitute medicines for replacing (augmenting) a defective gene by a fully transcription competent gene delivered into the nucleus as inserted into a plasmid-DNA. Permanent gene transfer is not usually achieved with plasmid-based therapy, because of plasmid DNA´s inability to integrate into chromosomal DNA. Rather, it remains as episomal in the nucleus and is lost upon cell division, thereby causing only a transient effect, which is also usual case with ODNs.

Gene delivery can be further divided into *ex vivo*, *in vivo* and *in situ* methods. In *Ex vivo* gene delivery, the cells are removed from the recipient and transfection of the therapeutic gene occurs outside the body, this being followed by reinfusion or retransplantation of the transfected cells into the recipient. *Ex vivo* is the most widely used method and is well suited to skin, liver, tumor and hematopoietic cells which can be fairly easily isolated. *In vivo* delivery, instead, is better suited to lung, brain and heart cells, and it faces more challenges to successful therapy than the ex vivo method. The gene of interest is administered either locally or systemically into the body where it has to find its way to the target tissues. Systemic in vivo treatment requires that there is a small carrier/DNA complex size to allow the penetration of the complexes into target tissues across the relatively tight barrier of vascular endothelial cells (Seymour 1992), and with only a marginal cationic surface charge to prevent binding of the complexes to negatively charged blood components. The *In situ* – type of gene correction, in which an abnormal gene could be swapped for a normal gene through homologous recombination, is still difficult to carry out though there have been some successful experiments (Richardson et al. 2002).

The non-viral gene delivery techniques used with the above mentioned methods can be divided into two broad categories: physical and chemical techniques.

2.1.1 Physical techniques

Direct injection of naked DNA (i.e., uncomplexed DNA) into the nucleus by *microinjection* (del Vecchio et al. 2005) would appear to be conceptually the most simple and appealing technique for delivering genes but this could only be done one cell at a time. Since this technique is impractical and laborious, its use is limited (i.e., for producing transgenic organisms). *Electroporation* utilizes high-voltage electric current in gene transfer. It is one of the most effective gene transfer techniques (Wong and Neumann 1982; Neumann et al. 1982), but is limited because it evokes extensive cell mortality as well as the difficulties in optimization and in applying in clinical use. *"Gene-gun"* particle bombardment

utilizes a high-pressure –or electrical discharge device to accelerate DNA-coated microscopic gold –or tungsten particles to a high velocity, forcing the particles to penetrate cell membranes (Yang et al 1990). Since direct exposure of the target tissue is required, this technique is suitable for local expression in skin, muscle or mucosal tissue, or DNA vaccination where a limited local expression of delivered DNA is adequate to evoke an immune response (Qiu et al. 1996; Fynan et al. 1993). In *magnetofection*, magnetic beads are associated with DNA complexes that are transported to the nucleus when an external magnetic field is applied (Krötz et al. 2003). Therapeutic *ultrasound* in the frequency of 1–3 MHz can increase the transfection efficiency by increasing transiently the permeability of the cells (Duvashani and Machluf, 2005).

2.1.2 Chemical techniques

Basically, chemical carriers are the most straightforward techniques for practical use. The earliest chemical carriers were based on *diethylaminoether-dextran* (DEAE-dextran) (Takai and Ohmori 1990) and *calcium phosphate* (Chen and Okayama 1987) but their use is hampered by variations in the complex size, the difficulty of use in vivo studies as well as their cytotoxicity. The mechanism of cytotoxicity involved with chemical techniques has been attributed to increasing cell membrane permeability due to exposure to polycations, leading to collapse of the membrane potential and finally to loss of cytoplasmic proteins (Bashford et al. 1986). Release of endosomal contents into cytoplasm can also evoke cytotoxicity. Although many new techniques are being developed, such as, *human artificial chromosome* (called 47. chromosome) (Chromos Molecular Systems, Burnaby, British Columbia) and *sleeping beauty* –transposon (Horie et al 2001), the non-viral techniques employ mainly *cationic lipids, peptides* and *polymers*.

Lipids may be the most frequently used chemical carriers but they suffer from several drawbacks including lack of targeting, poorly understood structure of lipoplexes and variations arising during fabrication. The charges of cationic head groups are either monovalent or multivalent, having either tertiary or quaternary ammonium groups. The latter is more efficient in gene delivery but it is also more toxic. The most commonly used lipids include monovalent N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethyl ammonium methyl-sulfate (DOTAP) (Eibl and Wooley 1979) and dioleyloxypropyl trimethyl ammonium bromide (DOTMA) (Felgner et al. 1987), and the multivalent dioctadecylamidoglycyl-spermine (DOGS) (Behr et al 1989), and dipalmitoyl phosphatidylethanolamidospermine (DPPES) (Behr et al 1989). The role of the neutral “helper” lipid DOPE is to facilitate cytosolic release of DNA by fusion with and disruption of the endosomal membrane (Litzinger et al. 1992; Farhood et al 1995). Positively charged liposomes do not usually exist naturally in cells being either neutral or negatively charged. Encapsulation of pDNA into liposomes has proven to suffer form a lack of efficiency, therefore, its use has been limited (Nicolau and Cudd 1989).

Two types of peptides or proteins are used for gene delivery purposes. Poly-L-lysine (PLL), poly-L-ornithine and poly-L-arginine (Pouton et al. 1998) are able to condense DNA and mediate gene transfer as such, while membrane-destabilising and lytic *fusogenic peptides* (i.e. HA2, Gal4, JTS-1, GALA, KALA), and *nuclear localization signals*, NLS-peptides, (i.e TAT) are used as auxiliary agents. Arginine- and histidine-rich peptides can help to promote endosomal escape, e.g. the endosomolytic activity of arginine-rich protamine has been shown to enhance cationic liposome-mediated delivery in vitro (Sorgi et al. 1997) and in vivo (Schwartz et al 1999). Poly-L-histidine (Uster and Deamer 1985), JTS-1 (Gottschalk et al.1996) GALA and KALA (Nir et al. 1999; Parente et al. 1988; Wyman et al. 1997) mediate an acid-dependent fusion and leakage of negatively charged liposomes. GALA and KALA undergo a pH change-dependent conformational change before adopting an amphipathic structure. The cationic NLS moiety consists of basic amino acids and can mediate the transport of DNA to the nucleus by promoting its binding to a carrier protein called importin (but this also affects the rate of the nuclear transport) (Nakanishi et al. 2001; Zanta et al 1999; Svahn et a. 2004).

2.2 Polymeric gene delivery systems

Cationic DNA condensing polymers are used to overcome poor cellular uptake of naked DNA, due to the high molecular weight ($>10^6$) and highly negatively charged nature of DNA. Only in some dense tissues, i.e, muscle, can fairly high cellular uptake and transfection efficiency be achieved by a non-condensed DNA system. In this case, DNA is coated with protective, interactive and *neutral polymers* that allow DNA to retain its flexibility, and thus, promote high diffusivity within the tissue. Some natural and synthetic polymers (i.e., poly(DL-lactide-co-glycolide), PLGA) which are biodegradable and often negatively charged can be employed in substrate-mediated delivery. A polymeric scaffold assists in supporting cell adhesion and migration and transports the immobilized DNA directly into the cellular microenvironment, providing a controlled release systems for DNA delivery (Segura and Shea 2002).

One important and frequently used transfection parameter of non-viral gene delivery systems is the charge ratio, which means nucleotide equivalents, the ratio of the cationic carrier nitrogens to DNA phosphates (N:P) (Boussif et al. 1995). Successful transfection is dependent on many different factors including charge ratio, cell line, medium composition (especially salt concentration), the aggregation of complexes, the number of primary, secondary and tertiary amines in the carrier, the side-chain length, molecular weight, the hydrophilic components and the chemical structure of the carrier. Usually, polymeric gene delivery systems have linear, branched, dendritic or grafted structures. The structures of some non-viral polymeric carriers are presented in Figure 1.

2.2.1 Structures and properties

Structure of plasmid-DNA – Successful gene expression requires some crucial elements in the synthetic circular plasmid-DNA (pDNA) expression cassette. *Promoter* provides recognition sites for RNA polymerase to initiate the transcription process and it also controls the transgene expression, the transgene of interest is *inserted* into the MCS (multiple cloning site), a poly (A)-sequence is required for *termination of transcription* and the ORI (origin of replication) is the *initiation site for replication* which also defines the copy number of plasmid molecules for stable episomal transfections. Sometimes *introns* are also needed, although they are usually not present in cDNA, since it is prepared from mRNA by reverse transcription. The next chapters will focus mainly on the cationic polymeric carriers used for condensing of pDNA.

Poly-L-lysine – Histone, the condensing agent of genomic DNA contains a high proportion of the basic amino acid, lysine. Therefore, synthetic polylysine (PLL) has maintained the scientific interest and there have been supporters of its usability in gene delivery since the early ages of non-viral gene delivery. Linear PLL has only primary amine groups on its side chains (Fig. 1) but a high charge density, and thus, a high affinity for DNA at physiological pH leading to strong binding with DNA (Zama et al. 1971). This is why PLL fails to undergo a rapid release from complexes and this also impacts on its transfection ability. Some studies have shown that at low salt concentrations, the interaction of PLL with DNA is irreversible, i.e., PLL and DNA molecules do not exchange with free PLL and DNA in the medium (Tsuboi et al., 1966), but at high salt concentrations (~ 1 M NaCl) this interaction occurs readily due to the high degree of hydration of the complexes. Due to the fairly rigid structure of PLL, it usually forms large insoluble clusters with DNA (up to some microns in size) (Kabanov, 1998b). Unlike most non-viral gene carriers, PLL may have immunogenic and toxic properties due to its amino acid backbone, especially the high molecular weight PLLs (Wolfert and Seymour 1996). Furthermore, the L-form of polylysine is biodegradable though the D-form is not (Laurent et al. 1999). Since PLL as suchs seem to function with variable success in gene delivery, different modifications have been developed to improve its efficiency in transfections. One significant advantage is that it can be fairly easily modified, i.e., conjugated with ligands, for cell specific targeting.

Poly(ethyleneimine) – PEI has become a traditional polymer used in gene delivery because of its efficiency of transfer into a fairly broad range of cell lines. This *branched* polymer of ethylamine is a weak polybase with a unique structure containing primary (25 %, Kichler et al. 2001), secondary (50 %) and tertiary (25 %) amines (Fig. 1). Correspondingly, the *linear* PEI has only secondary amines, and consequently, it is a less efficient condensing agent than its branched counterpart (Dunlap et al., 1997). Somewhat polydispersed branched PEI is a flexible polymer which forms DNA complexes with a hydrophobic core and a positively charged surface. Along with increasing molecular weight, the cytotoxicity of PEI

is increased (Fisher et al. 1999). Every third atom in PEI has an amino nitrogen providing it with the highest possible cationic charge density of any molecule (Suh et al. 1994). However, only one out of six of all nitrogen atoms (~ 17 %) are protonated in 10 mM aqueous solution (pH 7.4) (Boussif 1995), and therefore, neutral polyplexes of PEI 25 kDa are not obtained until there is a N:P ratio of 3.5 (Erbacher et al. 1999). The remaining amine groups retain the ability to become charged at lower pHs, thereby providing PEI with a high buffer capacity. The mechanism of the function of PEI in gene delivery has been explained by the "proton sponge" hypothesis. Due to the acidification of endosome, positively charged protons enter the endosome, protonating the previously uncharged tertiary amines on the PEI. The high concentration of positive ions results in an inflow of negative ions (restoring the electrical gradient in the endosome), which then leads to endosome swelling and eventual bursting of the endosome, thus releasing the DNA into cytoplasm. However, this hypothesis has been challenged by the observation that PEI can escape from the endosomes prior to their acidification (Godbey et al. 2000).

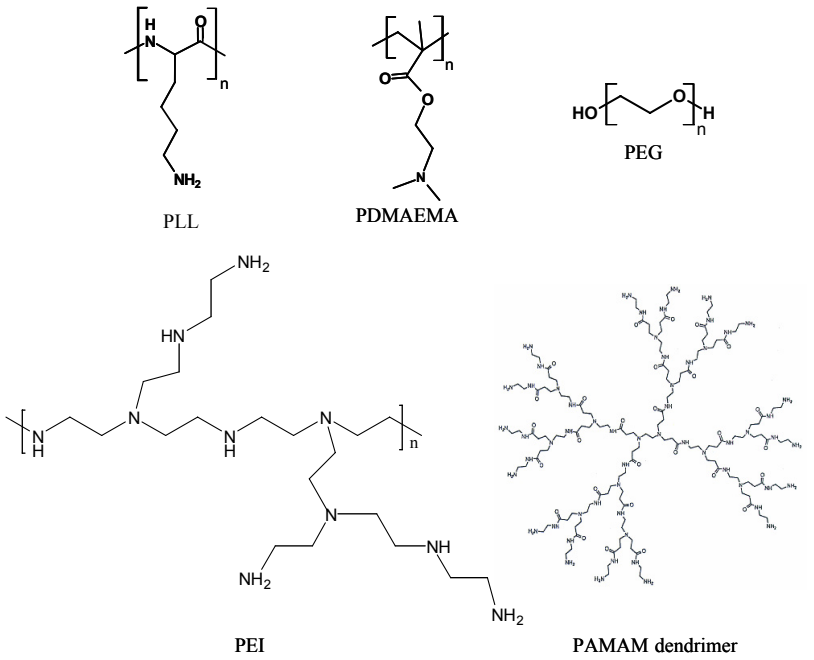


Figure 1. Chemical structures of some polymeric pDNA carriers.

Dendrimer – Polyamidoamine dendrimers (PAMAM, Starburst dendrimer) are synthesized stepwise by building up spherical shells around the core molecule (Fig. 1). Each new shell forms a generation and the molecule can continue growing until steric hindrance prevents addition of the next generation (Esfand and Tomalia 2001). Generation zero includes the core molecule and each new generation results in a two-fold increase in the number of available primary amino groups on the surface. The polydispersity of these

molecules is very small because of the strictly controlled surface charges. Intact dendrimers with an integrated structure have proven to be less efficient in gene delivery than partially degraded, so-called "fractured", dendrimers (Tang et al. 1996). The "fractured" dendrimer is a more flexible molecule than the intact form and it is able to expand due to an increase in the positive charge at lower pH (Tang et al. 1996), as is the case of branched PEI, which supports the idea that "fractured" dendrimers act as proton sponges. Although some generation-dependent cytotoxicity has been seen associated with dendrimers, in general, these compounds have exhibited relatively low levels of toxicity (Roberts et al. 1996). Linear polyamido amines have shown molecular weight-dependent and charge density-dependent cytotoxicity (Hill et al. 1999).

Methacrylate – The first amino methacrylate polymer to be evaluated for its ability to mediate gene transfer was the linear poly(2-(dimethyl-amino)ethyl methacrylate) (PDMAEMA) (Cherng et al. 1996). Cherng et al. (1997, 1999) showed that with freeze-drying, PDMAEMA/plasmid complexes formed in >2 % sucrose solution retained their transfection ability for a ten month period. A long shelf-life is a major advantage for a polymeric gene delivery vehicle in comparison with viral carrier systems. Unmodified PDMAEMA containing only tertiary amino groups has proven to be almost as efficient a transfection vehicle as PEI in some cell lines but some cytotoxicity was noted to be present (Dubruel et al. 2003). Other methacrylate-based polymers containing pyridine groups, acidic groups and imidazole groups have been generated and tested (Dubruel et al. 2003). Using monomers with varying pKa values, some of the polymers containing imidazole groups or acid functions provided a buffering capacity comparable to PEI at endosomal pH. However, due to a possible restriction in cellular uptake, it remains unclear whether a "proton sponge" occurs with these polymers.

Poly(ethylene glycol) – The apparent simplicity and lack of chemical activity has made polyethylene glycol (PEG) a widely used stabilizing surface coating for complexes in biological environments. PEG is an uncharged hydrophilic polymer possessing high water solubility, but due to its amphiphilic nature it is soluble also in organic solvents. Since it is a flexible molecule, PEG can adopt different states in aqueous solutions. PEG has been considered to be biologically inert, however, it can form directional bonds with water (Antonson and Hoffman 1992) and it can bind to proteins (Sheth and Leckband 1997). Also, certain molecular weights PEG were shown to induce membrane destabilization and fusion (Kuhl et al. 1996). Due to the lack of any immunogenic effect (Nguyen et al. 2000), the non-toxic nature and the extended lifetime in the body because of the reduced cationic surface charge, PEG has been widely studied, especially in vivo. However, the prolonged blood circulation time of PEGylated complexes is not undisputed (Mullen et al. 2000). The presence of PEG is expected to improve solubility, leading to less aggregated complexes which are stable also at high concentrations. PEGylated PLL (Katayose and Kataoka 1997)

and PDMAEMA (Rungsardthong et al. 2001) have been shown to form more stable DNA complexes, but in the case of PDMAEMA at the expense of a reduced level of transfection. In addition, PEG does not significantly reduce the buffering capacity of the PEGylated PDMAEMA (Rungsardthong et al. 2001).

Block-co-polymer – Block copolymers are heteropolymers consisting of two or more blocks, groups of repeating polymers, in the main chain. The block copolymers proposed for gene delivery usually contain polycationic and hydrophilic blocks (A-B type), for example, PEGylated PLLs belong to this type, but also, A-B-A type copolymers are used. The cationic block can associate with DNA, thereby, inducing complex formation and the hydrophilic block is likely to remain orientated towards the solvent forming nonionic hydrophilic corona on the surface of the complex.

2.2.2 Complex formation

The conformational change of DNA from an elongated into a compact state is called "DNA condensation."The condensation phenomenon was first discovered by Lerman (1971). Understanding of the processes involved in DNA condensation is essential for the optimization of gene delivery systems. In addition to cationic liposomes, polymers or peptides, DNA can be condensed with various chemical agents including neutral polymers, such as, PEG together with a salt (Laemmli). Intramolecular condensation occurs within milliseconds (Porschke 1984; Xu and Szoka 1996; Gershon et al 1993) leading to the formation of small self-assembled particles with an orderly morphology and finite size.

Mechanism for DNA collapse – Electrochemical theory and thermodynamics are the basis of DNA condensation, although electrostatic interactions are highly attenuated in water because of dielectric constant of the water. In aqueous solution, the negative charges of DNA molecule (one charge/1.7 Å) are neutralized by monovalent counterions. It has been proposed that DNA condensation is induced when ~ 90% of the charges are neutralized by multivalent cations (Wilson and Bloomfield 1979; Stevens 2001). Entropy gain is the driving force for complex formation (Manning 1978). Due to the negative net charge of two DNA chains and non-electrostatic repulsive contributions (i.e., steric repulsion), DNA chains repel each other, thus, entropy is low and DNA packaging is entropically disfavoured. The role of a polymer carrier in condensation is to reduce the electrostatic repulsions of adjacent negatively charged DNA segments. They also cause a heterogenous charge distribution along the DNA chain, leading to a counterion-mediated attraction, thereby increasing the entropy of the DNA and finally leading to condensation of DNA.

Polyvalent polymers introduced into a DNA solution will replace the monovalent counterions because of the polymers` higher affinity towards DNA, which readily undergoes a structure transition to its secondary or tertiary structure, allowing each molecule to collapse into a compact soluble colloidal particle (Fig. 2).

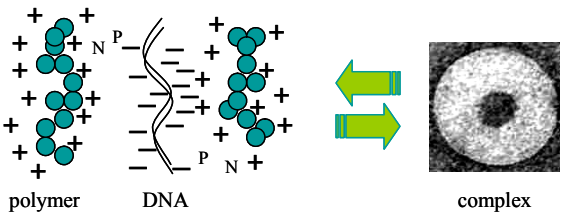


Figure 2. Polyplex formation. Nitrogen atoms of the polymer neutralize phosphates on the DNA, leading to collapse of DNA and complex formation, usually to the size of 50–300 nm. The complex should be stable under physiological conditions but be able to disassemble and release the DNA for transcription.

Condensation depends on the characteristics of the solvent, i.e., temperature, salt concentration, dielectric constant, etc. For example, salt has the effect to retard the folding of DNA. The collapse induced by multivalent cations is stronger than that achieved by monovalent cations. The critical cation concentration necessary to induce DNA compaction decreases as the valencey of the cation increases, being lowest for tetravalent and highest for monovalent cations. Unlike monovalent counterions, polyvalent counterions do not only reduce the DNA backbone charges, but they make the charge distribution very heterogeneous since they can neutralize more than one backbone charge.

Morphology of the complexes – Although heterogeneity in size and shape of self-assembled systems is a common feature for carrier/DNA complexes, certain regularities have been shown in polyplex formation. The most commonly seen morphology in compact DNAs is a toroid form. Sizes of about 45–200 nm have been described with 3 kbp plasmid and 166 kbp T4 DNA molecule, respectively (Shen et al. 2000; Yoshikawa et al 1999). The density of the DNA strands compacted into a toroidal form can be relatively high, thus, small particles as tiny as ~ 20 nm (theoretical minimum) can be formed. Some PLL-based polyplexes have been shown to have a volume of mainly about 30–35 nl and a height about 4–6 nm, indicating that most often one plasmid-DNA is condensed into one complex (Golan et al. 1999; Bloomfield 1998; Sergeyev et al.1999). There are atomic force microscopy (AFM) experiments revealing that DNA truly forms toroids also in the solution (Martina et al. 2000; Golan et al. 1999), these being somewhat larger than as dried form. Thus, toroids are not an artefact occurring in the dried form only, which sometimes has been considered, since most experimental imaging methods involve dehydration of the samples. The mechanism by which toroids are formed is explained as follows. Since DNA does not favor tight bending, its stiffness sets limits on tight curves, and therefore, to minimize the loss of energy associated with bending accompanied by folding under a poor solvent (due to charge neutralization, hydrophobicity increases) DNA wraps itself in a circle with a hole at the centre (see Fig. 3) (Marx et al. 1987). It has also been hypothesized that rod-like structures may open up, forming a toroid (Dunlap et al. 1997) leading to a conclusion that rod-like complexes might be meta-stable structures (Bloomfield 1991). The ultrastrucuture of the complexes is difficult to explore, and is still something of a mystery.

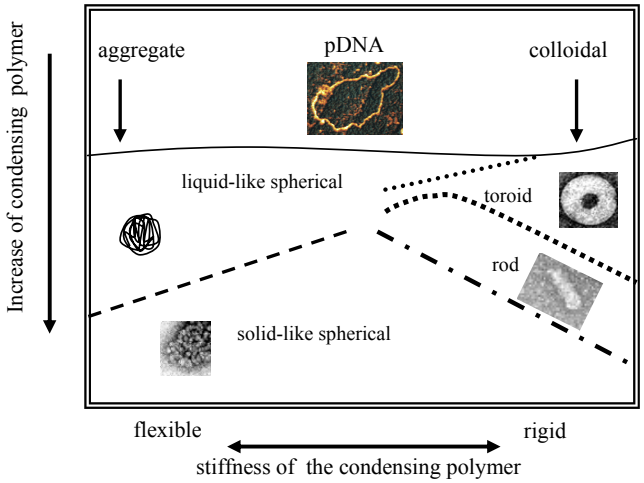


Figure 3. Phase diagram of the morphology of DNA complexes deduced from theoretical calculations (modified from Noguchi and Yoshikawa, 1998). The image of pDNA from K. Yoshida et al. (Biophys J., 1998).

A simulated phase diagram of compact state DNA, depicting the changes of the condensing polymer stiffness is shown in Figure 4. A rigid polymer forms a toroid or rod-like structures with DNA depending on the degree of attraction between DNA segments and the stiffness of the condensing polymer (Noguchi and Yoshikawa, 1998). A flexible polymer forms a spherical compact state with less dense liquid-like packing, and when attractive interactions become sufficiently strong, they form spherical solid-like particles.

Stability of the complexes – Aggregation of the complexes would prevent their use, and therefore, the surface charge of the complexes must be optimized. Increasing amount of polycations added to the non-stoichiometric, low concentration DNA solution decreases the negative complex charge and increases the proportion of the *hydrophobic sites* (Kabanov and Kabanov 1998) (Fig. 4). The residual electrical charges on the complexes prevent aggregation. At some point, all of the DNA charges are neutralized by the polycation and the hydrophobicity of the complex increases, leading to an increasing proportion of water insoluble stoichiometric complexes. Further addition of the polycation may lead to recharging of the complex and its solubilization. The complexes must be stable against not only aggregation but also extracellular and intracellular substances, which have a polyanionic nature (i.e. GAGs). This is because too rapid dissociation in the cytoplasm may lead to extensive degradation of plasmid DNA, and too slow dissociation may lead too weak transgene expression due to impaired accessibility of the transcription factors.

Experimental and computational data indicates that the binding energy and subsequent polycation-DNA complex stability is dependent on the quality of polycation amines, being most stable with primary amines followed by secondary, tertiary and quaternary amines (Reschel et al. 2002; Dybal et al 2004). In addition, ion-exchange between monovalent and multivalent cations plays a major role in stabilizing the complexes. It is noteworthy that a complex containing equal amounts of phosphate and amino groups may still carry a net positive or negative surface potential, if the complex do not allow the neutralization of all charged groups due to differences in amine pKa´s.

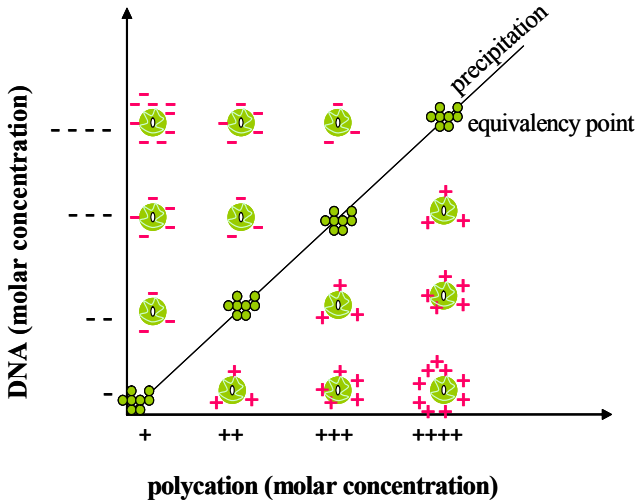


Figure 4. Effect of polycation/DNA ratio on the electrical charge of complexes. Binding of polycation to the DNA neutralizes the phosphate group charges. Small amounts of polycation on the DNA leads to negatively charged complexes due to the excess of DNA. With an increasing amount of the polycation, the net negative charge becomes the positive. At the equivalency point (equal amounts of DNA and polycation) soluble complexes precipitate but further addition of polycation may lead to the recharging of complexes and their solubilization. Near to neutrality, complexes usually have a strong tendency to precipitate and form aggregates, whereas complexes carrying a net negative or positive charge are relatively stable.

2.2.3 Mechanism of DNA release

Important as complex formation is for gene delivery, complex dissociation or relaxation is an equally crucial factor. It is generally thought that DNA must be released from the carrier before it is transcribed, although there is some evidence that DNA can be transcribed also from complexed DNA. At N:P ratios of 5 and up to 15, transcription of the PEI complexed plasmid was as efficient as that of the free plasmid (Honoré et al. 2005). Even if DNA release from the complxes is required, it is not known, however, at which point this release should happen, before or after the nuclear uptake. Premature release of DNA may lead to

degradation of unprotected DNA. Although the mechanism of release is not precisely known, it has been proposed that polyions exchange and substitution reactions (flip-flop), may play a significant role in release of DNA in its active form (Kabanov 1992; Xu and Szoka, 1996). This is based on the fact that the carrier incorporated into the polyelectrolyte complexes may be exchanged by the free carrier from the bulk solution, and this involves a dissociation mechanism. It has also been shown that an excess of anionic liposomes or anionic polysaccharides, such as heparin, can release a substantial amount of the DNA from the lipoplexes (Xu and Szoka, Jr 1996).

2.3 Intracellular distribution and kinetics of plasmid-based systems

A better understanding of the intracellular distribution and kinetics of plasmid-based systems would be useful in the optimization and design of new gene delivery systems. A kinetic model to analyze rate-limiting processes of intracellular kinetic and optimize intracellular trafficking of internalized plasmid DNA has been developed (Varga et al. 2001; Kamiya et al 2003). This poor quantitative knowledge of intracellular distribution of plasmid DNA in each subcellular compartment (endosomes, lysosomes, cytoplasm and nucleus) is a disadvantage for the modelling.

The steps involved in gene transfer are presented in Figure 5. Positively charged complexes attach to the negatively charged plasma membrane which forms a pit and pinches off, forming an endosome. The complexes must escape from the endosomes before they are attached by degradative lysosomal enzymes. The DNA, complexed or free, must diffuse through the cytoplasm into the nucleus where it can be transcribed into mRNA which is translated into the appropriate protein in the cytoplasm.

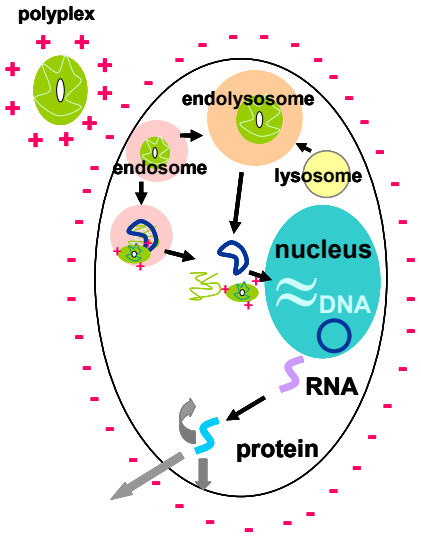


Figure 5. Steps in polycationic-mediated gene transfer. Positively charged cell-surface attaching complex is internalized into an endosome. Thereafter, the complex is released from the endosome or the endolysosome and transferred into the nucleus where the DNA will be transcribed into mRNA, and finally, translated into a protein in the cytoplasm. The newly synthesized protein can be used for the intracellular processes (e.g constituents of the cellular enzymes), transferred to the cell surface (e.g. cell-associated PGs) or secreted outside the cells (e.g. hormones).

2.3.1 Binding and internalization

Extracellular materials are taken up by cells via *phagocytosis* or *pinocytosis* (fluid-phase endocytosis). The latter process involves clathrin-dependent *receptor-mediated* cellular uptake and clathrin independent *adsorptive* cellular uptake (Alberts et al 1994a; Nichols et al. 2001). Possible alternatives to clathrin-mediated endocytosis include *caveolae* or *actin-based* mechanisms. Most often the clathrin-dependent endocytosis seem to be responsible for cellular uptake of complexes (Clark and Hersh 1999; Meyer et al 1997). The mechanism for uptake of the complexes is thought to be as follows: Positively charged complexes attach to negatively charged cell membrane or to the receptor at the plasma membrane. The stimulus results in a localized polymerization of actin at the site of particle attachment and subsequent invagination of endocytosed material and plasma membrane components, forming a vesicle which rapidly buds off (Greenberg et al. 1990). Clathrin coated vesicles rapidly lose their coats, facilitating their fusion with early endosomes. The acceptor of the polyplexes at the plasma membrane may be sulfated glycosaminoglycans (such as heparan sulfate or chondroitin sulfate) or phospholipids themselves (Farhood et al 1995). It is noteworthy that only a small fraction, less than 15 %, of cell-associated DNA is released into the cytoplasm (Legendre and Szoka 1992; Felgner et al. 1987).

The particle size is important in terms of selective internalization via receptor-mediated endocytosis and this might determine the mechanism of internalization. Large complexes (several hundreds of nm) may enter the cell either by phagocytosis or clathrin independent endosytosis, whereas small complexes (<200 nm) can be internalized by *non-specific clathrin dependent endocytosis* (Simões et al. 1999).

2.3.2 Endosomal escape

The endosomal membrane is the first barrier in the intracellular trafficking of plasmid DNA. Viruses have developed a sophisticated pH-dependent mechanism to escape from endosomes. This is mimicked by incorporating or adding fusogenic amphipathic peptides in the non-viral vectors (Plank et al 1994; Wagner 1999). Internalized complexes end up first in the early endosomes, which are slightly acidic vesicles (pH ~ 6.0–6.8) maintained by an ATP-driven proton pump (Al-Awqati 1986; Mellman et al 1986; Forgaq et al 1992) promoting dissociation of many ligand-receptor complexes. Early endosomes fuse with late endosomes which are responsible for accumulating and digesting exogenous and endogenous macromolecules in conjugation with the lysosomes (pH ~ 4–5, Mukherjee et al. 1997). DNA which is on its way into the nucleus has to escape from endosomes before they fuse with lysosomes. Thereby it avoids degradation by lysosomal enzymes. The mechanism for endosomal escape is based on the loss of membrane asymmetry (Fattal et a. 1994; Bevers et al. 1994). Then the mechanical or osmotic stress can rupture the endosomal bilayer, thus releasing the DNA into the cytoplasm. Loss of clathrin and the accessory proteins during the uncoating of the vesicles might make the uncoated vesicles more prone to rupture than the plasma membrane (Xu and Szoka 1996). A similar mechanism has been described with biodegradable PLGA and is based on selective reversal of the surface from being anionic to becoming cationic in the complexes in acidic surroundings. This then leads to an interaction between the complex with the membrane and its escape from the lysosomes (Panyam et al. 2002). Although there is no agreement on whether PEI polyplexes are able to destabilize endosomal membranes (Kichler et al. 2001; Klemm et al 1998; Godbey et al 1999), the “proton sponge” hypothesis is the most popular explanation to account for endosomal escape. PLL polyplexes escape rather inefficiently from internal vesicles, but when free or complexed PEI is used as an auxiliary agent, the ability to escape from endocytic vesicles is enhanced (Kichler et al. 2001).

Only a small fraction of the internalized complexes is able to escape from endosomes and reach the cytoplasm, the majority appears to be trapped and eventually degraded within the lysosomes (Plank et al. 1994; Zabner et al. 1995; El Ouahabi et al. 1997; Wattiaux; 2000).

2.3.3 Diffusion in cytoplasm

Cytoplasm is composed of a network of microfilaments and microtubule systems which is responsible not only for the mechanical resistance of the cells but also for the cytoplasmic transport of organelles and macromolecules (Luby-Phelps 2000). The cytoskeleton is embedded in fluidic cytoplasm which has approximately the same viscosity as water (Fushimi and Verkman 1991; Luby-Phelps et al. 1993), however, this does depend on the cell type and spatial location (Srivastava and Krishnamoorthy 1997). The diffusion of plasmid DNA is greatly impeded in cytoplasm (Dowty et al. 1995). DNA fragments sized of 2 kDa have very limited mobilities in the cytoplasm and diffuse >100 times more slowly in cytoplasm compared to water (Luckacs et al. 2000). This can due to many factors: i) the presence of organelles in cytoplasm, ii) the mesh-like structure of the cytoskeleton, acting as a sieve, and iii) the high protein concentration (up to 100 mg/ml) promoting binding to intracellular components (Luby-Phelps 2000). In this light, it appears that the constituents of cytoplasm are able to create a diffusional barrier to transport plasmid DNA near the nuclear envelope.

2.3.4 Nuclear uptake

The purpose of a nuclear envelope (NE) is to preserve the stability of chromosomal DNA and to protect it from the intrusion of exogenous substances, and therefore, the penetration of the membrane is not an easy task for DNA. NE is composed of a double lipid bilayer and transport between cytoplasm and nucleus is regulated by nuclear pore complexes (NPCs), ~ 10 nm width (central canal) and 50 nm length, which restrict the *passive diffusion* of globular molecules up to ~ 40–70 kDa (Paine et al.1975; Hicks and Raikhel 1995; Görlich and Mattaj 1996) this being especially difficult, as the size increases. *Facilitated diffusion* requires an interaction with NPC components before internalization. The outer diameter of NPC is estimated to be about 100 nm, and therefore, particles much larger than that should not even theoretically be able to pass through the nuclear pores. (Nakanishi et al 2001). NPC can convert from an acting simple sieve that separate two compartments into a smart barrier that adjusts its permeability according to the metabolic demands of the cell (Mazzanti et al. 2001). The diameter of NPCs can vary by up to several tens of nm according to cell type and cell cycle (Dworezky et al. 1988; Maul 1977; Miller et al. 1991; Feldherr et al 2001). Electrical voltage across nuclear envelope is attributable to the electrical charge separation due to selective membrane permeability and the unequal distribution of charged macromolecules across NE, and thus, nucleocytoplasmic transport may be driven by electrical gradients (Mazzanti et al. 2001).

Since polyplexes are polydisperse systems of various sizes, and plasmid DNA (3–11 kb) itself has a radius of gyration of about 90–130 nm (Sebestyen et al.1998), the mechanism of nuclear uptake through NPCs is not obvious, and therefore, it has been proposed that only when the nuclear membrane break downs during mitosis, is pDNA capable of penetrating

into the nucleus (Tseng et al 1999). However, it has been shown that plasmid DNA can also enter the nucleus of non-dividing cells (Ludke et al.). Many research groups have shown that cytoplasmic DNA, either complexed with a carrier or microinjected, is taken up poorly into the nucleus of cells and not more than 0.1–0.01 % of the cells become transcribed. (Capecchi 1980; Zabner et al.1995; Labat-Moleur et al. 1996; Pollard et al 2001). Moreover, naked DNA does not have a nuclear targeting component, thus its efficiency at traversing into the nucleus as a free molecule is low. S*ignal-mediated import* of exogenous DNA based on NLS-conjugated carrier systems (Sebestyen et al. 1998; Branden et al. 1999; Ludke et al. 1999; Wilson et al.1999; Zanta et al. 1999) or NLS containing transcription factors (Dean 1997; Dean et al. 1999; Wilson et al. 1999) have enhanced nuclear uptake and transgene expression. This, however, does not exclude the possibility that DNA can also be transported through NPCs by an NLS-independent mechanism. Furthermore, transport of macromolecules through NPCs can be inhibited by conformational changes of the NPCs due to the low concentration of Ca^{2+}in the NE. Therefore, cellular events altering the Ca^{2+} concentration in the NE can obstruct the pathway through the NPC being used by macromolecules (Pante and Aebi 1996) and this would be expected to be reflected at the level of gene activity and gene expression due to the impeded translocalization of mRNA or transcription factors (Mazzante et al. 2001; Burg et al.1996, Hardingham et al.1997).

Macromolecules, such as, nucleic acids, can also be excluded from the nucleus, thus decreasing the amount of nuclear exogenous DNA, leading to low transgene expression. Nucleic acids larger than 250 bp have been shown to be excluded from nuclei (Swanson et al. 1987; Luckacs et al. 2000).

2.3.5 Degradation of DNA

Since foreign DNA within cells is a sign of an intruder, naked exogenous DNA is rapidly eliminated both in the extracellular or intracellular environments by Ca^{2+}-sensitive *endo*- and *exonucleases*, leading to a low efficiency of gene transfer (Lechadeur et al.1999). Nucleases recognize the phosphodiester linkage in the DNA backbone and this leads to hydrolytic degradation of DNA. The half-life of plasmid DNA can be increased when it is stabilized by liposome encapsulation or incorporated into phospholipid vesicles (Lechardeur et al 1999; Wheeler et al. 1999) but also many chemical DNA carriers prolong the half-life. For example, PEI, can improve the pharmacokinetics of DNA by protecting it from degradation before it has reached the site of action. Plasmid DNA degradation is faster from complexes with poly-L-lysine than with poly-D-lysine, since poly-L-lysine is a biodegradable molecule (Laurent et al. 1999). A quantitative assessment of the decay kinetics revealed that 50 % of the microinjected DNA was eliminated within 1–2 hours in HeLa and COS cells (Lechardeur et al. 1999). Also, the rate of intracellular degradation of the plasmid DNA within 1–4 days was estimated to be 4.5–10 and 8–15 copies/cell/min for 293 and HepG2 cells, respectively (Kichler et al. 2001). It seems that the majority of the DNA does not participate in the expression of the transgene. Most of the DNA is trapped either inside vesicles or within the

cytoplasm and this is degraded by the cell more or less immediately, depending on the amount of delivered DNA, the cell type, and the capacity of the carrier to protect DNA from degradation (Kichler et al. 2001).

2.4 Control of transgene expression

Transcription requires the presence of *transcription factors* but the synthesis starts with the binding of *RNA II polymerase* (eukaryotes) at the promoter, the unit that directs the synthesis of mRNA, which directs the synthesis of proteins. Promoters vary in their efficiency to bind RNA polymerase and weak promoters may require transcription *enhancers*.

Uncontrolled expression of foreign proteins may disturb the physiology of the host cell, and therefore, control over transgene expression is essential. Furthermore, the foreign gene should preferably be expressed only in certain tissues in order to maintain the organism´s normal physiology. This can be achieved by selecting an appropriate tissue-specific promoter or targeting the therapy only to certain cells. If the moment of time and the level of transgene expression could be controlled, this would help to tune the efficiency and improve the safety of gene-based therapies to the level that gene therapy could become an established form of treatment. Delivery of gene transcription regulating proteins may have offer this kind of capability. Cell-permeable peptides (CPP) are able to transport other proteins (i.e. gene transcription regulating proteins) into the cytoplasm and nucleus where they can upregulate or downregulate targeted genes either by binding to DNA or mRNA or alternatively by perturbing specific protein-protein interactions (Järver and Langel 2004). A "Gene switch" is a regulatable gene expression system that can be switched on or off by an external inducer, e.g., by the antibiotic tetracycline, which activates transcription factors and their target promoters (Goverdhana et al. 2005). Ideally, these switches permit limited transgene expression at a defined level. siRNA (short interfering RNA) is a natural mechanism used by the body to transiently silence certain genes. The mechanism is activated when cytoplasmic RNase, a so-called Dicer, splices long dsRNA (double stranded) into oligonucleotides (~21–23 nucleotides), which then become attached to silencing complex called RISC (RNA Induced Silencing Complex) guiding this complex to the complementary site at target mRNA, which will be spliced by the RISC (Mello and Conte 2004).

2.5 Cellular properties affecting gene delivery

2.5.1 Cell division cycle

The cell division cycle can be divided into four phases, G1 (Gap1), S (synthesis), G2 (Gap 2) and M (mitosis) (Fig. 6). G1 and G2 phases are interphases in which cells mainly grow and monitor their size and the environment. During the S phase, the chromosomal

DNA and other organelles and cellular contents are replicated prior to their division into daughter cells. In the G2 phase, the cells also check whether the newly synthesized DNA is flawless and the environment is favorable for proceeding to the last phase, mitosis, in which the cells divide. Sometimes cells can pause in their progress and exit from the cell cycle to a phase called G0. Basically, cells can rest in this phase for an indefinite time before entering the cycle again. The transition from one cell cycle phase to another is regulated by *cyclin-dependent protein kinases* (Cdk) but other factors, such as, soluble growth factors, insoluble extracellular matrix molecules, mechanical force or cell distortion can all contribute to the control of cell cycle phase and each factor can be rate-limiting step for the others (Huang and Ingber 1999). Normally, cell proliferation is tightly controlled but sometimes when the control fails, the cells can be converted cancerous cells.

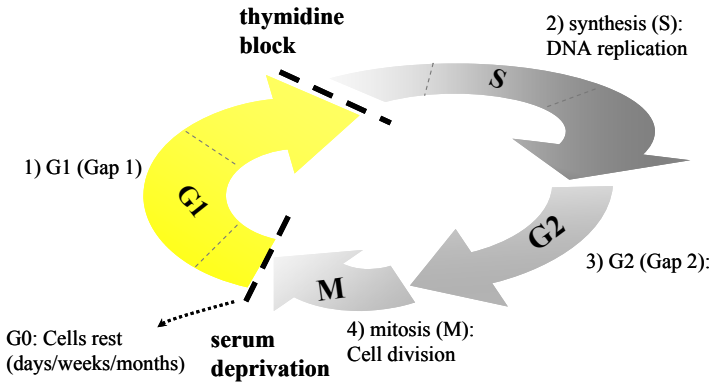


Figure 6. Cell division cycle. The lengths of the phases and cell cycle are dependent on the cell type but generally the length of one cycle is at least 12 h. The illustration includes the cell synchronization treatments. Serum deprivation arrests the cell cycle at the beginning of G1 phase and double thymidine blocking arrests the cells at the boundary of G1/S phases. Cells monitor their cell cycle during the G phases, any problem with the cell size, environment or DNA replication may prevent cell cycle progression from one phase to another.

All of the changes that cells undergo during cell division cycle are still not completely understood. The cell volume (Fidorra et al.1981) and intracellular pH (Musgrove et al. 1987) are both dependent on the cell cycle phase and both tend to increase toward mitosis. This may due to the proton pump activation during cell growth phase (Imai and Ohno 1995). Certain cell organelles, such as lysosomes, react to a change in the intracellular pH by changing their localization and shape (Heuser 1989). The nucleolus is a membraneless organelle within the nucleus and its size and morphology reflects the cell cycle phase and transcriptional activity. Cells prepare themselves to enter mitosis by increasing the tension of the plasma membrane, decreasing the rate of endocytosis and depolymerizing the microtubules – the structures responsible for trafficking of cellular organelles such as endosomes and lysosomes. Intranuclear negativity follows the charges of chromatin, being lowest at the end of mitosis, when chromosomes are in a condensed state and the charges of the DNA are partially neutralized by positively charged histones, and thereafter negativity

increases until G2 phase when it is at its highest, before the condensation takes place. The nuclear envelope disappears and reconstitutes at each cell division. Reassembly of the envelope is completed within 15 min after a cell division (Robbins and Gonatas 1964; Feldherr and Akin 1990). The number of nuclear pores increases throughout the cell cycle, but the rate of pore formation is at its highest soon after the cell division, decreasing continuously towards the end of the cycle (Maul et al. 1972).

Finally, it is not known how these changes in cells during the cell cycle affect the polyplexes and their ability to deliver genes.

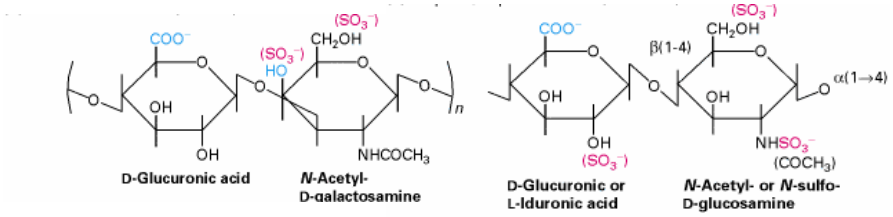
2.5.2 Glycosaminoglycans (GAGs)

GAGs, also called mucopolysaccharides, are highly negatively charged, fairly rigid and linear polysaccharides consisting of repeating disaccharides with amino sugars (N-acetylgalactos-amine/N-acetylglucosamine linked to glucuronic acid). There are several major classes of GAGs, including the heparan sulfate (HS), chondroitin sulfate (CS), hyaluronic acid (HA) and keratan sulfate (KS) families (Fig. 7). Sulfated GAGs (HS, CS, KS) are covalently linked to core proteins forming proteoglycans (PGs) while HA is not linked to proteins. HA is a high molecular weight (up to 10^4 kDa), viscose GAG that endows structure and flexibility to the tissue. HA also interacts with lipids and membranes, in water and also with itself, leading to aggregation (Scott and Heatly 1999). The size of sulfated GAGs is not usually more than 50 kDa. Depending on the cell type and their role in the body, variable amounts of GAGs are found within cells, bound to the plasma membrane and secreted to the medium.

The role of GAGs in gene delivery is not yet fully understood. GAGs were previously shown to influence nonviral gene delivery: high concentrations of sulfated GAGs in the *extracellular* space (Mislick and Baldeschwieler 1996; Belting and Pettersson 1999a; Ruponen et al. 2001) and on the *cell surface* (Belting and Pettersson 1999b; Ruponen et al. 2004) decrease or even completely block transgene delivery and expression. However, according to some reports, the presence of sulfated GAGs on the cell surface can be beneficial for gene delivery (Mislick and Baldeschwieler 1996; Mounkes et al. 1998). High concentrations of *exogenous* HA improve the cellular uptake and the transgene expression (Ruponen et al. 2001; Ruponen et al 2004). Some reports demonstrate that the presence of sulfated GAGs on the cell surface are beneficial for gene delivery (Mislick and Baldeschwieler 1996; Mounkes et al. 1998).

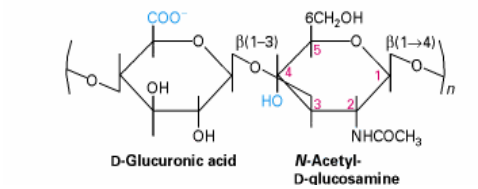
Changes in GAG synthesis and their secretion throughout the cell cycle have been only occasionally studied. It is known that the synthesis of CS, heparan sulfate HS and HA takes place during various phases of the cell cycle (Davidson and MacPherson 1975). Also, the synthesis and secretion of the sulfated GAGs have been reported to be reduced or terminated during S phase in pig SMC cells, murine melanoma cells and rabbit aorta endothelial cells (Blair and Sartorelli 1984; Breton et al.1986 Porcionatto et al. 1998). Furthermore, HS synthesis on the cell surface is reduced before mitosis in synchronized CHO cells (Kraemer

and Tobey 1972). However, it is not known how those cycle-dependent fluctuations in GAGs can influence the efficiency of gene transfer.



Chondroitin sulfate

Heparan sulfate/ Heparin



Hyaluronic acid

Figure 7. Chemical structures of disaccharide units in some GAGs (Lodish et al. 2000)

3 AIMS OF THE STUDY

The primary objective of this thesis was to study the factors affecting polyplex-mediated gene delivery. The following specific questions were posed:

1) Does a polymer structure-biological activity relationship exist for non-viral gene carriers?

2) Is PEGylation relevant for complex formation and biological activity?

3) What is the role of cell cycle phase and cell division in gene delivery?

4) How does the polymer type modify intracellular kinetics and biological activity of pDNA complexes during cell division cycle?

5) Are GAGs relevant during cell division cycle in gene delivery?

6) Are there new efficient gene carriers among the starch-, dendrimer-, glutamate- or methacrylate-based polymer families?

4 MATERIALS AND METHODS

4.1 Plasmids

The *luciferase* reporter gene encoding plasmids were driven under the control of CMV (pCLuc4; 6.4 kb), (**I–III**) PDE-β, (**II**) SV40 and (**II**) tk (**II**) promoters. The cloned genes were inserted into *Xho*I-*Hind*III (pCLuc4, PDE-β, SV40) and *Bam*HI-*Bg*lII (tk) sites of pGL3-Basic vectors (Reinisalo et al 2003). pCMVß (7.2 kb) (**I–III**) plasmid encoded *ß-galactosidase.* All plasmids were amplified in *Escherichia coli* (DH5α) and their integrity was confirmed by gel electrophoresis. The concentrations of plasmid DNA were determined in a UV- spectrophotometer at a wavelength of 260 nm. For the cell uptake study, pCMVß was labeled with ethidium monoazide (EMA) by photoactivation as described by Zabner et al. (1995) and Ruponen et al. (2001). Fluorescein-labelled DNA with the β-galactosidase reporter gene was a commercially available product (Gene Therapy Systems, Inc. San Diego, CA, USA).

4.2 Polymers

***Linear* poly-L-lysine** (**I–III**) (**PLL**): mean molecular weights of PLL 2.9 (**I**), 4 (**II**), 20 (**I–II**), 200 kDa (**II–III**), PEG20-PLL5 (note: MW of PEG was 20000 and MW of PLL was 5000), PEG12-PLL5, PEG5-PLL5, PEG20-PLL10, PEG12-PLL10, PEG5-PLL10, PEG20-PLL20, PEG12-PLL20, PEG5-PLL20 and PLL5-PEG10-PLL5; ***dendritic* PLLs** (**I**): PEGylated 3rd, 4th, 5th, 6th generation and unpegylated (gen.3 and 5) PLLs; ***grafted* PLLs** (**I**): PEG5-g-PLL20 (5% grafting density), PEG5-g-PLL20 (10% grafting density) and PEG12-g-PLL20 (5% grafting density); ***branched* PLL** (**I**): PLL10 was pegylated with PEG5; **polyethyleneimines (PEIs)** (**I-III**): mean MW of 25 kDa (**II-III**), 50 kDa (**II**), 750 kDa (**II**), and 800 kDa (**II**), fractured sixth generation; **polyamidoamine dendrimer** (PAMAM) (**II**). Non-PEGylated dendritic PLLs (gen.3 and 5), PEGylated PLLs, and Texas-Red-tagged PLL 20 kDa (**II**) and PEI 25 kDa (**II**) polymers were synthesized in the Department of Organic Chemistry, University of Ghent, Belgium.

4.3 Lipids and other materials

Lipids – [1,2-dioleoyloxy-3-(trimethylammoniopropane)] (DOTAP; **II**), dioleoyl-phosphatidylethanolamine (DOPE; **II**), dimethyloctadecylammonium bromide (DDAB; **II**) dipalmitoylphosphatidylethanolamylspermine (DPPES; **II**), dioctadecylamidoglycyl-spermine DOGS (**II).**

Glycoasaminoglycans (GAGs) (I, III) – Hyaluronic acid (HA) and chondroitin sulfate-C (CS) were used as 12 mM (**I**) and 6 mM (**I**) aqueous solutions, respectively. Heparan sulfate (HS)(**III)** was used as a 90 μM solution.

Other chemicals – were commercially available and were used without further modification.

4.4 Physicochemical studies

4.4.1 Complex condensation, stability and binding assays (I)

DNA *condensation and stability* of the polymer/DNA complexes against polyanions was assessed with the ethidium bromide displacement assay (Xu and Szoka 1996). Polymer/DNA complexes (0.6μg DNA/well) were prepared in high ionic strength MES-HEPES buffer solution at different N:P (+/-) charge ratios (0.25:1–32:1). DNA *binding* by polylysines was confirmed by gel electrophoresis. The complexes were prepared and loaded with bromophenol blue in glycerol into 1 % agarose gel in Tris-Borate-EDTA buffer (TBE) pH 8.0 and electrophoresed at 60 V, 3 hours. Gels stained with EtBr solution (0.5 mg/l) were transilluminated on a UV-light to localize the DNA.

4.4.2 Complex size and electrical properties (I)

For the *size distribution* measurements, polymer/DNA complexes were prepared in high ionic strength MES-HEPES buffer at charge ratio 2:1 by adding diluted polymer into DNA solution (20 μg/ml DNA) obtaining a final sample volume of 2 ml. The scattered light of the complexes were measured at the wavelength of 488 nm (DLS-700, Otsuka, Japan). For ζ-*potential* measurements, polymer/DNA complexes were prepared in water at charge ratios 0.5:1 - 4:1 obtaining a final sample volume of 2 ml (20 μg/ml DNA). ζ-potentials were measured at the wavelength of 632.8 nm (NICOMP™ zetapotential/particle sizer 380 ZLS, Santa Barbara, CA, USA).

4.4.3 Complex morphology (I)

Complexes of DNA with PLL G3 (dendrimer), PLL 20 (linear), PEG-PLL G5 (dendrimer), PEG5-PLL20 (linear) and PEG5-g-PLL20 (5% grafted) polymers were chosen for the morphological study. Polymer/DNA complexes of +/- charge ratio 4:1 were placed on carbon-coated grid and negatively stained with a droplet of 2 % uranyl acetate (aqueous solution, pH 4.5) for 2 min. The samples were analyzed under transmission electron microscope (TEM) (JEOL JEM-1200 EX, Japan).

4.5 Biological studies

4.5.1 Synchronization of cells (I–III)

Cells were arrested in the early G1 phase by incubation in the growth medium with a reduced serum concentration (0.1 % FBS) for 72 h. Synchronization of D407 cells to the G1/S phase boundary was performed with the double thymidine blocking procedure (Stein et al.). Cells were first cultured in growth medium containing 2 mM thymidine for 16 h,

followed by culturing for 9 h with growth medium containing 24 µM deoxycytidine and, then finally, for additional 16 h with 2 mM thymidine in the growth medium. In order to reach G2/M phase, the cells were grown in the culture medium for another 9 hours after the removal of the second thymidine block. Flow cytometric analysis of D407 cells showed that both G1 and S phases lasted for about 9 h, whereas the G2/M phase was 3 h.

4.5.2 Cellular uptake studies (I–III)

One day before transfection, the cells were seeded on plates for the *cellular uptake* study of non-synchronized cells (**I**). The complexes were prepared by adding polymer solution on the EMA-labelled DNA (6.7 µg) to obtain optimal polymer/DNA (+/-) charge ratios of 4:1 (PLL 20 kDa). Synchronized cells (**II**) were transfected with 4 µg of EMA-labelled DNA at optimal polymer/DNA (+/-) charge ratios of 4:1 (PEI25 kDa) and 2.4:1 (PLL 200 kDa). For *fluid-phase endocytosis* experiments (**III**), cells were arrested to early (G, S) and middle (G, S) subphases, and G2/M phase of the cell cycle and exposed to fluorescein-labeled (FITC) dextran (anionic, MW 10 kDa) with concentrations of 21.5 µg/ml and 50 µg/ml at different time points (20 min, 60 min, 180 min). After 5 hours exposure to the complexes or FITC-dextran, the fluorescent cells were analyzed. Cells were fixed in 1 ml of 1 % paraformaldehyde, and analyzed with a flow cytometer (Becton Dickinson FACScan, San Jose, CA, USA). From each sample, 10 000 events were collected and fluorescence detected using a 525 nm (FITC) or 630 nm (EMA) filter. The background autofluorescence of the cells and complexes was excluded by using unlabelled DNA polyplexes as controls.

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4.5.3 Transfection experiments (I-III) and cytotoxicity (I)

Non-synchronized (**I**) retinal pigment epithelial cells (D407), were seeded into wells one day before transfection. Polymer/DNA complexes were prepared in high ionic strength 50 mM MES-50 mM HEPES-75 mM NaCl buffer at different charge ratios by adding polymer solution on DNA (1.8 µg), incubated for 15–30 min and added on cells in serum-free media for 5 hours. The amount of reporter gene was analyzed 43 hours later. Reporter genes were delivered into *synchronized* (**II–III**) cells – stably and non-stably expressing luciferase – in plasmids with a variety of promoters controlling expression of either luciferase (pCLuc4, SV40-luc, tk-luc, PDE-β-luc) or ß-galactosidase (pCMVβ). Cells were seeded on 6-well plates 24–72 h before transfection, synchronized and grown in the culture medium for 3 or 9 hours until middle subphase (G1 and S phases) or G2/M phase was reached. Complexes with optimal charge ratios of +/- 4 (PEI 25 kDa:DNA) and +/- 2.4 (PLL 200 kDa:DNA) were formed in MES-HEPES buffered saline (4 µg DNA) and incubated with cells for 3 h. After removal of the complexes, the cells were washed with PBS buffer and incubated for an additional 20–43 h in complete medium. The cell lysate was assayed for the β-galactosidase or luciferase activity and protein content.

The cytotoxicity of the polymers was tested using colorimetric MTT assay. Cells were seeded on plates one day before transfection, and then 0.6 μg of DNA complexed with polyplexes were added on the cells in the serum-free medium for 5 hours, and cell viability was analyzed after 43 hours of incubation

4.5.4 Localization of pDNA (II-III)

For visualization, localization and quantification of the DNA and the complexes by *confocal microscopy* (**II**), D407 cells were plated on 8-coverglass chambers, synchronized as described earlier, and transiently transfected in the middle phases with fluorescently (FITC-labelled) DNA. Carriers PEI and PLL were labelled with Texas-Red. After 1 h transfection and 3 h in serum-containing medium living cells were studied under a confocal microscope (Perkin Elmer-Wallac-LSR, Oxford, UK). The images of green (FITC) and red (Texas-Red) fluorescence were collected separately and combined as a RGB image representing the middle section of 10 optical slices. IpLab software (Scanalytics, Fairfax, VA, USA) was used for image-analysis. Positive particles having the area of one pixel were omitted from the analysis. Finally, integrated positive signal area of red and green fluorescence, and their co-localization ratio were calculated for each cell and nucleus areas. *PCR experiments* (**III**) were performed for quantification of the free/loosely complexed cytoplasmic and nuclear pDNA. Nuclei of the cells, detached with trypsin/EDTA were isolated 20 h post-transfection by repeated centrifugations in lysis and wash buffers. Nuclear, cytoplasmic and PCR-mixture was set up in 96-well reaction plates and diluted samples in 5 μl of sterile water were added (total volume 15 μl). In order to quantitate the total amount of pDNA in the nucleus, the complexes were disrupted by HS treatment prior to PCR amplification.

4.5.5 Analysis of GAGs (III)

D407 cells were arrested at different phases of the cell cycle with reduced serum or thymidine block and double-labeled by incubating with [^{3}H]-glucosamine and [^{35}S]-sulfate. Medium, supernatant (trypsin) and the cell pellets were collected separately for *isolation* of the GAGs. Radiolabeled GAGs were *purified* with cysteine-EDTA or sodium acetate-cysteine-EDTA solutions followed by papain endopeptidase, cetylpyridinium and ethanol treatments. Samples were incubated overnight with 25 mU chondroitinase ABC, 1 mU hyaluronidase and 0.5 M ammonium acetate (pH 7.0) at 37 °C and *quantified* as disaccharides on the following day by gel filtration. HA control was used to monitor the recovery and to calculate corrections for any losses in purification process. Quantification of GAGs was based on the calculated specific activity of [^{3}H]-glucosamine (HS, HA) and the measured specific activity of [^{35}S]-sulfate (CS) (Yanagishita1989; Tammi 2000).

4.5.6 Statistical analysis (II)

The statistical significances of the transfection difference between cell cycle phases and between polyplexes were tested by unpaired t-test (**II**). Differences between cell cycle phases for transgene uptake, luciferase expression and GAGs were tested with Mann Whitney´s U-test (**II–III**).

4.6 Additional studies with various polyplexes

Transfection and cytotoxicity studies were carried out with various polymers to discover their efficiency in gene delivery. Modified starches (pure amylose, amylose-rich and pure amylopectin), starburst dendrimers (PEGylated, histidylated, oleylated, lysinated), poly-L-glutamic acids (PEGylated block-co-polymers, pyridine, carboxylic acid and imidazole as functional groups) and polymethacrylates (pyridine, carboxylic acid and imidazole as functional groups). pDNA binding and polyplex size were investigated with most of the polymeric carriers. The cellular uptake study evaluated by flow cytometer was carried out with some of the derivatives of the starch carrier. DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein) labelled starch was complexed with pDNA (0.5 µg), and thereafter 3 x 10^4 cells of the D407 cell line were exposed to the complexes for 1–24 h and analyzed for cellular uptake of polyplexes by confocal microscopy. Starch derivatives were synthesized by Raisio Chemicals Oy, Finland and VTT Processes, Finland. Starburst dendrimers, poly-L-glutamic acids and polymethacrylates were synthesized in the Department of Organic Chemistry, University of Ghent, Belgium.

Lipid-coating was prepared onto PEI 25 kDa/pDNA polyplexes in order to study the mechanism of cellular uptake and intracellular distribution of polyplexes. Diolein (DO), cholesteryl hemisuccinate (CHEMS) and oleic acid (OA) were purchased from Sigma and DOPE from Avanti Polar Lipids. Diolein/CHEMS or OA/DOPE in chloroform was used at a lipid ratio of 2:1. After evaporation of chloroform, the lipid films were dissolved in a surfactant octylglucoside (Sigma). The coating was formed when PEI/pDNA complexes were added step-wise into the lipid mixture and diluted drop-by-drop with 10 mM HEPES buffer below the solubility ratio of octylglycoside/lipid, 2.62 (molar ratio). Finally, the formed complexes were dialysed overnight against 10 mM HEPES buffer and the sizes of the complexes were measured.

The strength of the lipid-coating against anionic dextran sulfate was assessed with the EtBr binding assay with slight modifications to the protocol presented above. EtBr can bind and fluorescence only when intercalated between the strands of the DNA double helix, therefore, the recovery of fluorescence after addition of anionic dextran sulfate (0.5 mM) into the solution of complexes (0.6 µg/well) and EtBr (0.3 µg/well) reflects the degradation of lipid-coating. Triton-X 100 (10 %) was used to degrade the lipid-coating, thereby allowing dextran sulfate to relax the complexes in order to reveal the amount of pDNA encapsulated within the lipid-coat.

5 RESULTS

5.1 Effects of polymer structure on complex formation

5.1.1 Shape and size

At low +/- charge ratios, almost all *linear* 5-20 kDa PLLs condensed DNA more efficiently (1:1) than *dendritic* PLLs (2:1–8:1) (**I**: Fig. 2) but increasing the mount of primary amines at the surface of dendritic PLL improved DNA binding (non-PEGylated: 8 NH_2 G3 vs. 32 NH_2 G5, PEGylated: 8 NH_2 G3 vs. 64 NH_2 G6). Similarly, the 14 lysine residues in a linear PLL 2.9 kDa (non-PEGylated) molecule are not able to bind DNA efficiently at charge ratios near to neutrality, unlike the 95 lysine residues in a linear PLL 20 kDa (non-PEGylated). These experiments showed that also *grafted* and *branched* polymer shapes of PLL (PEGylated) bound DNA completely at a charge ratio of 1:1. Although DNA binding and condensation by the linear and dendritic PLLs were different, this was not usually seen as different complex size distributions. In the case of non-PEGylated PLLs, this is due to the aggregation of the complexes by both PLLs. There was one exception, the PEGylated dendritic PLL (G3) gave a large complex size compared with the other shapes due to its poor ability to condense DNA. In addition, there were no large differences in the complex surface charge (ζ-potential) among the various molecular shapes.

TEM studies were carried out to characterize the morphology of the complexes. The shape of the polymer itself had no effect on the morphology of the complexes. PEGylated PLLs exhibited a similar rod or toroid form irrespective of the polymer shape. Instead, non-PEGylated dendrimer and linear PLLs revealed different morphologies most probably arising from differences in their ability to condense DNA. Linear PLL produced aggregates of spherical complexes, thus, the actual morphology of the complexes was not evaluated. Third generation *dendritic* PLL that showed poor DNA binding and condensing, formed loose toroids (**I**: Fig.3A) and some rods, that appeared to be trapped in a net of DNA strands.

5.1.2 PEGylation

PLLs were modified in a variety of ways with PEG in this study. Linear PLL was converted to diblock (PEG-PLL), triblock (PLL-PEG-PLL) and grafted (5 or 10%) copolymers. Also, PEGylated dendritic and branched PLLs were generated and tested.

PEGylation improved binding and condensation of DNA only in the case of dendritic PLL G5 (**I**: Fig 2.). The PEGylated 3rd generation PLL did not have enough primary amines on its surface, leading to poor binding of DNA. Most of the PEGylated PLLs were as efficient with respect to DNA binding as linear non-PEGylated PLL, but DNA condensation was most efficient when the fraction of PEG was about 60 % or less of the PLL MW (**I**: Fig.7). PEGylation of PLLs had a clear effect on the size distribution of polyplexes. The size range of all PEGylated complexes was 27–123 nm. Despite the high ionic strength of

MES-HEPES with 75 mM NaCl, the PEGylated complexes retained low polydispersities and small complex sizes, in contrast to the non-PEGylated complexes. PEGylation decreased the electrical surface charge of the complexes at positive +/- ratios, but the ζ-potentials were still positive (+3 – +23 mV). There was one exception, samples of pegylated G3 PLL dendrimer/DNA showed negative ζ-potential even at a 4:1 ratio, due to the poor DNA binding by this polymer.

Microscopy results were in line with the size determinations. While complexes of non-PEGylated linear PLL showed highly condensed but aggregated particles, the PEGylated linear, grafted and dendritic (G5) polymers formed separate rods upon complexation with DNA (**I**: Fig.3 C-E). The size range of the toroidal and rod-like complexes varied from 50 to 250 nm. In addition, the dendritic non-PEGylated PLL (G3) revealed some loose toroid-like structures with diameters of about 150–200 nm and rods about 300–450 nm of length, although they were not separate from the net of the DNA. However, PEGylation of PLL seemed to have a favourable impact on the formation of toroid and rod-shape complexes.

5.2 Factors affecting biological activity of polyplexes

5.2.1 Polymer structure

Type – In general, the transfection efficiency of non-PEGylated PLLs is lower than some other transfection carriers, such as PEI 25 kDa and DOTAP (**II**: Fig. 2). However, the flow cytometric (FACS) study revealed a similar efficiency in cellular uptake of PEI 25 kDa and PLL 200 kDa polyplexes showing internalized pDNA in ~ 80 % of the cells. Despite the similar efficiency in cellular uptake, more pDNA was accumulated in the nucleus of the PLL treated (1.2 to 3.5-fold) than the PEI treated cells. This was not reflected in the luciferase transgene expression, however, since PEI-treated cells expressed 3–55 times higher levels of luciferase than PLL-treated cells (**III**: Fig. 2). PLL polyplexes also showed less variation in luciferase expression compared to PEI polyplexes throughout the cell cycle showing nearly constant expression, while PEI revealed an increasing trend (**III**: Fig. 2). *Expression efficiency* (i.e. ratio between luciferase expression and nuclear pDNA) of PEI polyplexes was ~10–100 times that of PLL polyplexes (**III**: Fig. 3).

Shape – At a charge ratio 4:1, *linear* unmodified PLL 20 kDa polyplexes were taken up by the cells efficiently (> 60% positive cells; **I**: Fig. 6). It is also a better transfection agent than *dendritic* PLL or *linear* PLL 2.9 kDa. PEGylated *linear* or *grafted* PLLs carried pDNA into the cells more efficiently than PEGylated *branched* and *dendritic* PLLs (>70 % vs. < 40 %; **I**: Fig. 6). However, in general, the transfection ability of PLL 20 kDa was very limited, therefore, linear PLL 200 kDa was selected for further studies.

PEGylation – PEGylation of PLL20 increased slightly (up to 90 %) the cellular uptake of linear PLL 20 polyplexes at the optimal charge ratio of 4:1 (**I**: Fig. 6), and also improved the transfection efficiency (Fig. 5). The most efficient carrier was linear PEG5-PLL20 and *triblock* (PLL5-PEG10-PLL5) was less efficient than most of the *diblock* polymers. All

PEGylated dendritic PLLs showed lower transfection activities than their linear, grafted or branched counterparts. PEGylated branched and grafted PLLs transfected to a similar extent as the linear PLLs (**I**: Fig. 5A-B).

5.2.2 Cell cycle phase

The results of the pinocytosis study (fluid-phase endocytosis) (**III**: Fig. 5) with dextran molecules and experiments with D407 6-2 cell line cells having stably integrated luciferase gene (**II**: Fig. 9) support the findings of the cellular uptake (**II**: Fig. 4) and transfection studies (**II**: Figs. 5-6; **III**: Fig.2) with the polyplexes. The cellular uptake of dextran was at its lowest in the cells in the G1 phase and the integrated transgene expressed about twice more luciferase in the cells in the G1 phase than cells in the S or G2/M phases. Therefore, the cell cycle phase is clearly a major determinant of gene delivery.

Cell cycle-related experiments can be performed only if the total lengths of the cell cycle phases G1, S, and G2/M phases are known. The determination of the lengths of these phases in D407 cells was carried out by treatments with *0.1% serum* (G1 phase) and *double thymidine block* (S and G2/M phases) and by analysis with the flow cytometer based on the amount of DNA in the cells. The lengths of the phases were about 10, 9, and 3 h, respectively.

Promoter – Different promoters may be differentially sensitive to the cell cycle possibly due to the presence of distinct binding sites for various transcription factors. This was assessed by studying transfections with several promoters driving the expression of luciferase. The results showed that strong viral promoters CMV and SV40, and also tk promoter were more effective in gene transfer than PDE-β (**II**: Figs. 6A-B) and the levels of gene expression were always cell cycle-dependent. PEI-mediated transfections exhibited high sensitivity to the cell cycle, especially between G1 and S phases. Furthermore, all promoters were affected by the cell cycle in a similar way with PLL complexes, but the differences were not always significant and the levels of tranfection were low. These results demonstrate that the effect of the cell cycle phase on transfection efficiency is more pronounced with PEI polyplexes than with PLL polyplexes, and it is not limited only to the CMV promoter.

Cellular uptake and intracellular distribution of DNA – The flow cytometric results demonstrated high cellular uptake of polyplexes (~ 80 %) during phases other than G1 phase (~ 5–30 %) (**II**: Fig. 4). In this experiment, only the free and/or loosely complexed pDNA which is presumably directly accessible to the transcription machinery were quantified. After polyplex treatments, G1 phase cells contained ~8–20 pDNA copies per nucleus (**III**: Fig. 2) being at S phase ~ 4 to 5-fold and G2/M phase ~ 10 to 20-fold more than in the G1 phase cells. PLL treated cells contained up to 3.5 times more nuclear pDNA available for PCR amplification than the corresponding PEI treated cells (G2/M<G1<S). The majority of the

internalized pDNA remained in the cytoplasm (**III**: Fig. 2), but the fraction that ended up in the nucleus increased when the cells went from G1 through S to the G2/M phase (from 0.3 % to >8 %, G1<S<G2/M). However, these figures overestimate to some extent the fraction, since most likely a part of the administered pDNA was degraded in the cytoplasm during the 20 h between transfection and isolation. HS was used to completely disrupt the polyplexes for determination of the total pDNA content in the nuclear fraction. This indicated a ~2–265 times increase (PEI>PLL) in the pDNA content (data not shown).

Visualization of the complexes in the confocal microscope revealed similar results as obtained in the PCR experiments: the plasmid-DNA appeared mostly in the cytoplasm and mainly in the complexed form (**II**: Fig. 7A–C). Typically, cells contained only small amounts of polyplexes, but some cells, especially at S phase, contained substantial amounts of complexes and the free carrier. A total of 180 cells transfected with PEI 25 kDa were analyzed but only the cells containing plasmid-DNA were included: 21 (G2/M), 16 (G1), and 25 (S) cells. The cells were divided into three groups mainly based on the *number of nucleoli* (**II**: Fig. 7A–C). The nucleolus is the most visible nuclear structure and it changes during the cell cycle, reflecting its transcriptional activity (Bloom and Fawcett 1975; Alberts et al 1994b). Before mitosis, the nucleolus decreases in size and then disappears. After mitosis, tiny nucleoli reappear and during G1, S and G2 phases they fuse to form one large nucleolus. However, not only changes in the nucleolus were evaluated, also the shape of a cell, the size of the nucleus and the synchronization method were taken into account when assessing the phase of a cell. Also, quantitation of pDNA by image-analysis revealed the same results as the PCR experiments (**II**: Figs. 8A–B), although quantification with PCR was more precise. The cells had accumulated up to 6.5 times less DNA into the nucleus than was present in the cytoplasm.

Interestingly, there was free DNA in the cytoplasm only in the G1 group cells. Furthermore, microscopic examination revealed a similar trend, with free DNA in the nucleus as was noted by PCR with respect to nuclear-pDNA: the amount of pDNA increased steadily throughout the cell cycle.

Transgene expression – The level of transgene expression depends strongly on the polyplex-type used but also the cell cycle phase. The highest expressions were seen either in the cells in the S (analyzed 43 h post-transfection, **II**: Figs. 6A–B) or G2/M (20 h post-transfection, **III**: Fig. 2) phases in comparison with the low expression levels at the G1 phase. Although the expression level of the luciferase reporter gene is modified by the cell cycle, it exhibits a non-linear relationship with the nuclear pDNA (**III**: Fig. 2). A poor correlation was particularly evident in the cells transfected with PLL, and a better, but still non-linear relationship with PEI transfection. We also used D407 6-2 cells that stably express tetracycline-repressible luciferase (Antopolsky et al. 1999) to examine whether the effects of the cell cycle effects on transgene expression were due to: i) cell cycle-depedent differences in transcription and translation or ii) DNA delivery into the nucleus in an active form. Synchronized cells were exposed to polymer/pCMVβ complexes and non-

synchronized cells were used as controls. Although the effect appeared to be mild, the expression levels of endogenous luciferase under CMV promoter both with and without polyplex treatment were decreased during the G1 phase (**II**: Fig. 9). Without administration of polyplexes, luciferase expression was about 1.3 times higher in the S phase compared to the G1 phase. After polyplex treatment, stable luciferase was expressed about 1.8–2.3 times more in the S and G2/M phases than in the G1 phase ($P<0.05$; Fig. 9).

5.3 GAGs in gene delivery

5.3.1 Exogenous GAGs

Lipoplexes and polyplexes may react with GAGs leading to DNA release from the complexes or relaxation of the complexes (Xu and Szoka 1996), thereby affecting transfection. When GAGs are added to the complexes, some of the fluorescence of the EtBr bound to DNA may recover due to the displacement of the carrier or to changes of the complex conformation. In our experiments, anionic HA did not relax the complexes of linear or dendritic PLL at any +/- charge ratio (data not shown), while CS-C could recover 10–15 % of the fluorescence at charge ratios above 1:1 (**I**: Fig. 4).

5.3.2 Endogenous GAGs

Differences in GAG profiles were investigated as one possible explanation for cell cycle-dependent gene transfer. The GAGs expressed by D407 cells on their cell surface, in intracellular compartments and secreted into the medium were predominantly sulfated GAGs (>85 %; **III**: Table1). HA was expressed to a lesser extent (<15 %). The cell surface (>56 %) and the medium fractions (> 59 %) were dominated by CS whereas HS was the main intracellular GAG (54–59 %). The largest quantities (~ 500–3400 amol/cell) and the greatest variations of GAG concentrations were seen in the secreted fraction (Table 1). Cells in the G1 phase secreted about 5–40 times larger quantities of each GAG than cells in the S and G2/M phases (**III**: Table 1). Relatively small differences were noted in the secretion of the GAGs between the cells in the S and G2/M phases. Assuming a typical chain size of 30 kDa (≈ 50 disaccharides, length 50 nm) (Yanagishita and Hascall, 1992) for HS and CS, the surface of these cells were covered by around 2-5 $\times 10^6$ sulfated GAG chains, while the higher average size of HA (2000 kDa) (≈ 5000 disaccharides, 5 μm) (Ruponen et al. 2004) suggested 1–4 $\times 10^3$ cell surface chains (**III**: Table 1).

5.4 Additional studies with various polyplexes

We also studied other polymers in addition to PEI 25 kDa and PLL 200 kDa for gene delivery purposes. We found that pure amylose or amylose-rich starch (~70 %) bound pDNA totally mainly at charge ratios of 1:1, but sometimes at 2:1, while pure amylopectin bound at a charge ratio of 8:1 or not at all. Pure amylose can produce polyplexes of ~ 30–150 nm size, but when combined together with amylopectin, the sizes can increase to ~ 45–400 nm. The degree of substitution (DS) varied between 0.17–1.1 in this study. The experiments to determine the effect of MW on the size distribution exhibited an inverse relationship between MW of the carrier and polyplex size at charge ratios above 2:1 (Fig. 8). The polyplex size is also dependent on the charge ratio. *Acetylated* amylose-rich starch (acetylation DS 0.3–2.8, cationization DS 0.07–0.3) bound pDNA totally at charge ratios of 1:1 but the complex size was mainly above 1µm. Cationic *inulines* (DS 0.37–1.23) bound pDNA totally at charge ratios of 2:1 and 1:1 depending on the DS, but they formed polyplexes with high dispersities (<100 nm to 1-2µm). *Diethyaminoethylethers* of starch bound pDNA efficiently, but *carboxy methyl cellulose* derivatives of starch were inefficient at binding pDNA.

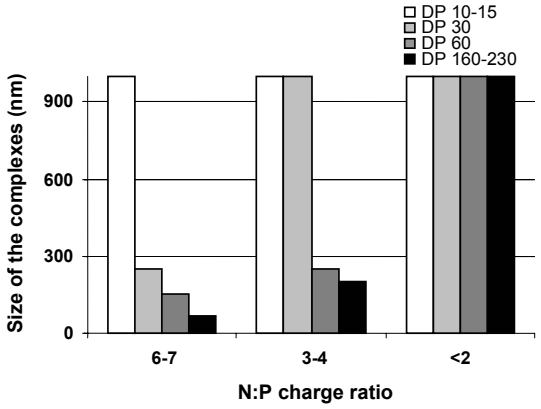

Figure 8. The effect of MW on size distribution with pure amylose carrier/pDNA complexes. Degree of polymeration (DP) varied between 10 and ~230 and DS varied between 0.1–0.55. The increase in MW was ~10 kDa being ~50 kDa between the smallest and largest molecules.

Polyplexes with pure amylose or amylose-rich carriers exhibited ~ 40–60 % cellular uptake even at a charge ratio of 1.5:1 when DS was > 0.5. The confocal microscopic pictures support the cellular uptake study, but they also suggest that although carrier, pDNA and the complexes are for the most part localized near to the cell membrane (Figs. 9B–D), they are also present in the nucleus, but mainly in a complexed form (Fig. 9E). Transfection studies with starch/pDNA polyplexes resulted in no transgene expression in D407 cell line cells in comparison with PEI 25 kDa or PLL 200 kDa, but also toxicity was low (< 25 %).

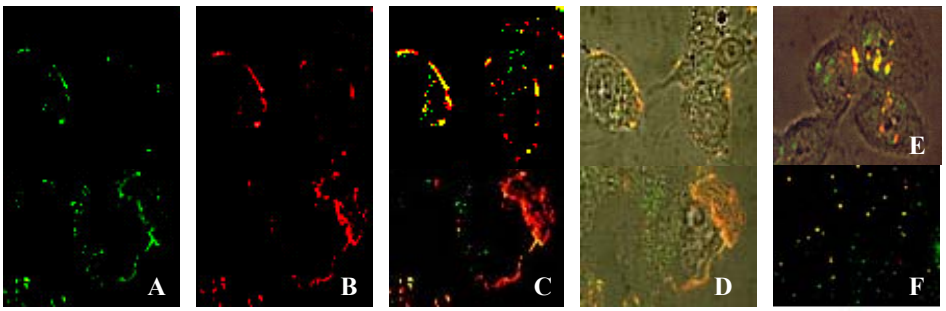

Figure 9. Confocal microscopy images of D407 cell line cells after 24 hour (A–D) and 3 h (E) of exposure to the amylose starch/pDNA polyplexes. (A) Localization of DTAF-labelled starch carrier (DS 0.52, Mw 185 kDa). (B) Localization of rhodamin-labelled pDNA. (C) Combination image of the images A and B, yellow color shows the localization of the carrier/pDNA complexes. (D–E) Combination of the confocal and light microcopic images showing the localization of polyplexes within the cells. (F) Image of the polyplexes without cells.

We also investigated structural-activity relationships of different modifications of *Starburst dendrimers* (SB) (Gen. 3-4). We found that all of the studied modifications bound totally pDNA at a charge ratio 1:1 but PEGylated SB formed smaller complexes (mainly <250 nm) than lysine and oleoyl modifications (nearly 1µm). The transfection efficiency remained low with carriers other than lysine coated SB in comparison with PEI 25 kDa (~3.5–10 times less), although the efficient charge ratios with SB lysine were relatively high (Fig. 10). Cytotoxicity was mainly found to be < 30 %.

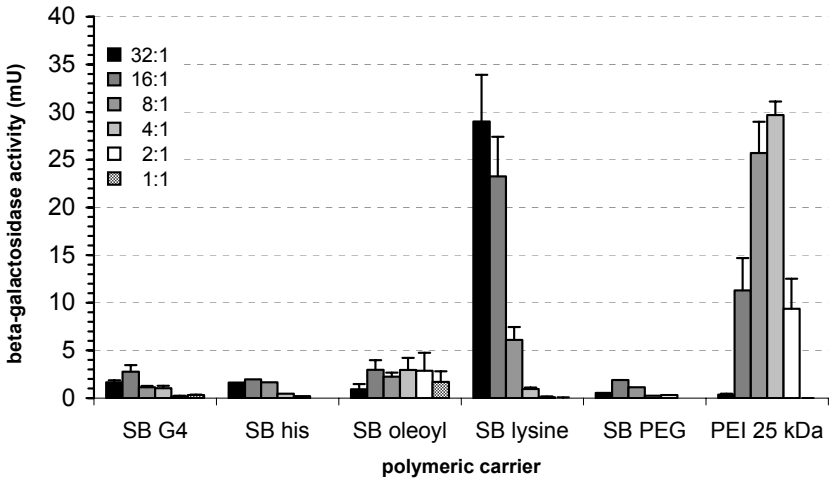

Figure 10. Transfection efficiencies of SB derivatives in D 407 cell line cells at different polymer/pDNA charge ratios (32:1–1:1). Charge ratios of PEI 25 kDa were between 36:1–1.1.

A variety of MW of *poly-L-glutamic acid* (PDMAEG) block-co-polymers with PEG or pyridine, carboxylic acid and histidine as functional groups were tested but they displayed no efficiency in comparison with PEI in gene delivery into D407 cell line cells. However, some differences between PDMAEG derivatives have been seen with cells of the 293 cell line (Dekie et al. 2000). The toxicity of these polymers was 10–15 % in D407 cells and 15–20 % with EA.hy 926 (Human-derived endothelial cell line) (Dubruel et al. 2003b). *Polymethacrylate* derivatives (PDMAEMA) bound totally pDNA at a charge ratio of 1:1. Although they were not as efficient in gene delivery into D407 cells as PEI 25 kDa, they did exhibit some efficiency, which was related to the functional group in the polymer structure. Derivatives with pyridine showed an efficiency which was nearly half of the efficiency of PEI, while carboxylic acid and imidazole derivatives displayed no efficiency at all. Similarly, carboxylic acid and imidazole derivatives have not been able to transfect other cell lines, e.g. COS-1 cells (Dubruel et al. 2003a). PDMAEMA derivatives showed cytotoxicity which was less than 30 %.

Finally, we prepared coated PEI 25 kDa/pDNA polyplexes with diolein/CHEMS (DO/CHEMS) or oleic acid/DOPE (OA/DOPE) at charge ratios of 4:1–16:1 in order to study the mechanism of cellular uptake and distribution of polyplexes within cells. The EtBr displacement –assay showed that coating resisted relatively well the addition of dextran sulfate, thereby protecting the complexes from the anionic polymer and preventing the release of pDNA (Fig.11). After 1 h, ~5–8 % and at 24h ~25–50 % of the fluorescence was recovered with the diolein/CHEMS-coated polyplexes. Correspondingly, 17 % and 43 % recoveries were observed after 1 h and 24 h exposure with OA/DOPE-coated polyplexes. The difference to uncoated PEI polyplexes was conspicuous (Fig. 11). The agarose gel – experiment supported the result of the EtBr assay showing that pDNA was not released from the complexes after addition of dextran sulfate. We also transfected different cell lines with the coated polyplexes but precipitation of the complexes in the wells remained a problem.

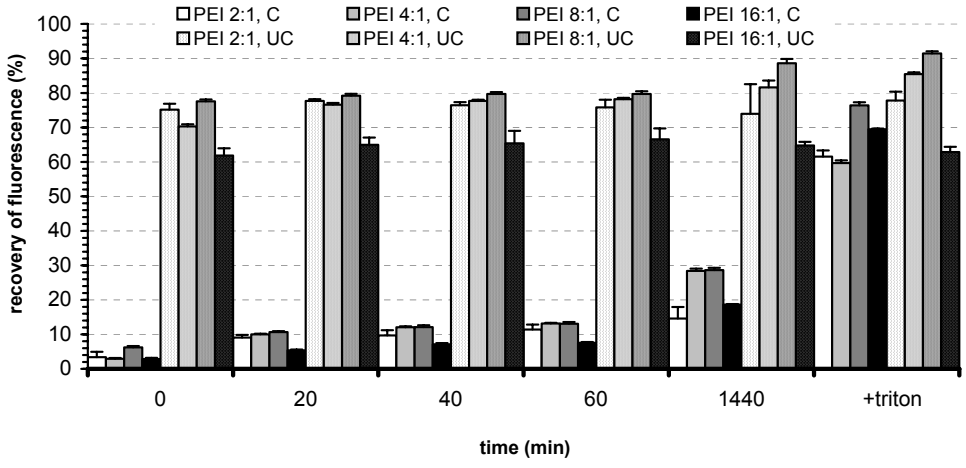

Figure 11. The effect of dextran sulfate on DO/CHEM-coated PEI 25 kDa/pDNA polyplexes at charge ratios of 4:1–16:1. C and UC stand for coated and uncoated complexes, respectively. Fluorescence of 100% represents the completely relaxed/free DNA in solution.

6 DISCUSSION

6.1 Polymeric gene delivery systems

Physicochemical properties affecting gene delivery – The dendritic shape and the small amount of primary amines on the surface of PLL G3 and PLL G5 molecules, 8 and 32, respectively, are not optimal for pDNA binding. In addition, the orientation of the amines may not be favourable, since pDNA is not flexible enough to wrap around the small PLL G3 molecules, and therefore, some positive charges are not reached by the phosphates of pDNA. Linear PLL 20 kDa contains about three times more primary amines than the G5 dendrimer and due to its long and more flexible structure, its amines are more readily accessible to the phosphates of DNA. The disadvantage of the dendritic shape has also been observed with geometrically differing lipopolyamines (DOGS as a starting material) on HeLa cells (Byk et al. 1998). A linear shape of lipopolyamines is the most efficient in terms of transgene expression, followed by a branched shape, whereas the globular shape results in the lowest activity. Previously, PAMAM dendrimers have been shown to be very active transfection agents (Haensler and Szoka 1993; Tang et al. 1996; Ruponen et al. 2001; Urtti et al. 2000), with fractured dendrimers being more effective than intact spherical dendrimers (Tang and Szoka 1997). Furthermore, branched and flexible PEI 25 kDa and linear PLL 200 kDa plasmid carriers are not only structurally very different, but they also have different buffering capacities at endosomal pH range (5.5–7.4) (Tang and Szoka 1997). PEI with its good buffering capacity, can destabilize endosomal and lysosomal membranes (Klemm et al 1998; Godbey et al 1999), thereby facilitating the release of the complex from endolysosomal vesicles. PEI is clearly a better transfection agent than linear unmodified PLL, which has a poor buffering capacity at pH < 8. Therefore, apparently, the shape of the polymer alone is not the crucial factor in DNA condensation and efficient gene delivery.

PEGylation provided sterical stabilization and a small size to the PLL polyplexes, but only partial shielding, which was not able to protect the complexes from interactions with CS-C. The amount of PEG may be insufficient or PEG may become poorly oriented (not always on the surface of polyplex). These results also suggest that PEGylated complexes may react with the cell surface proteoglycans (i.e. CS or HS), and in that way transfer the associated GAGs into the cells, as previously described for non-PEGylated PLL (Ruponen et al. 2001). The effect of PEGylation on transfection by other carriers is not straightforward. Ross and Hui (1999) reported enhanced lipoplex-cell association and lipofection with many different cell lines in the presence of PEG in the transfection media. Also, Toncheva et al. (1998) and Choi et al. (1999) reported enhanced polyfection after incorporation of PEG onto PLL, whereas transfection efficiency has been reported decline after PEGylation of amine methacrylates (Rungsardthong et al. 2001) and PEI (Nguyen et al. 2000; Erbacher et al. 1999).

Linear PLL 20 showed no rod-like or toroidal complexes, but rather aggregates of spherical complexes. This is in line with the more efficient DNA condensation by linear than

dendritic PLL (20 kDa). Clearly, also non-PEGylated PLLs (G3) are trying to form complexes with a defined structure, but it appears that the 8 primary amines on the polymer surface are not enough to condense DNA properly. It seems that the final particle size of PLL polyplexes, at least in high ionic strength solution, is determined by aggregation rather than condensation of pDNA in single particles. Even though non-PEGylated PLL condenses DNA efficiently, these complexes may aggregate, unlike PEGylated condensates which form toroidal complexes. This is line with earlier reports on PEGylation (Tonceva et al 1998; Seymour et al 1998; Kwoh 1999, Rungsardthong et al. 2001). Apparently the size distribution, ζ-potential and DNA binding results with dendritic PLL G3 polyplexes are a consequence of combinations of the polyplexes and the associated net of uncondensed DNA. Probably, linear and grafted PLL molecules are more flexible than their dendritic counterparts, and therefore, the negative charges of DNA can bind to the cationic charges in spite of PEG.

Effects on biological activity – The improved transgene expression of PLL 20 kDa upon PEGylation may due to several reasons: i) improved cellular uptake of the polyplexes provides more polyplexes that may be available for nuclear uptake. In addition, ii) PEG is known to induce association and fusion of phospholipid vesicles at high concentrations (Yamazaki et al. 1990). Therefore, surface-oriented PEG molecules on the polyplexes may induce leakage of endosomal membranes resulting in improved cytoplasmic release of DNA or complexes. On the other hand, iii) electrically neutral PEG molecules might improve the diffusivity of the polyplexes in the cytoplasm. However, the validity of these mechanisms still awaits verification. Despite the improvement of transgene expression by PEGylation, the efficiency with PLL 20 kDa was extremely limited. Also, the efficiency of PLL 200 kDa was restricted, but clearly higher than with PLL 20 kDa

The cellular uptake of PEI 25 kDa and unmodified PLL 200 kDa polyplexes differs only in the cells at the G1 phase, 30 and 5 %, respectively. Although cellular uptake of pDNA is relatively high (up to ~85 %), only 10^{-6} – 10^{-4} parts of the administered pDNA (~10–20 pg/cell) accumulated into the nucleus of D407 cells and was available for PCR amplification. Here, we quantified only the free or complexed pDNA, which was in a loose enough state to permit PCR amplification, and presumably also for transcription, thus, reflecting a "therapeutic" situation. However, there is a significantly higher amount of the transgene in the nucleus, but most of this pDNA is still bound and cannot be amplified by PCR. Bound nuclear pDNA may result from its delivery in a complexed form or from its re-complexation in the nucleus by the cationic carrier and/or by the histones. The fact that we observed more pDNA in the nuclei of cells exposed to PLL polyplexes (< 3.5-fold), but luciferase reporter gene was expressed more with cells exposed to PEI polyplexes (< 55-fold), suggests that the difference in transgene expression between PEI and PLL polyplexes may also arise from differences in the nuclear transcription efficiency. We found that PEI possesses ~ 10 to 100-fold better (G1<G2/M<S) *expression efficiencies* in comparison with PLL. The non-linear correlation between the amount of nuclear pDNA and luciferase expression with the

polyplexes is not clear but has also been noted previously with lipoplexes (Hama et al. 2005; Morguchi et al. 2006).

6.2 The relevance of cellular cycle phase in gene delivery

Cellular uptake of the complexes is low when most of the cells are in the G1 phase and higher in cells in the S and G2/M phases, thus, the cell cycle phase restricts the gene delivery. Similarly, nuclear uptake, transgene expression and expression efficiency are dependent on the cell cycle phase. The largest amount of nuclear pDNA was found in the G2/M phase and was smallest in the G1 phase irrespective of the polyplex-type used. Although the amount of transgene expression is cell cycle phase-dependent, it also depends on the polyplex-type. The transgene expression is strongly dependent on the cell cycle phase in the case of PEI but not with PLL, perhaps because of the different expression efficiencies but also because of the PLLs ability to bind DNA strongly, thereby preventing the release of the pDNA and/or other aspects of its intracellular kinetics. A similar effect of cell cycle phase on transgene expression has been reported by other groups (Brightwell et al. 1997; Brunner et al. 2000; Brisson et al.1999). The cell cycle phase restricted also the expression of the integrated endogenous luciferase gene to some extent in a stable cell line, suggesting that this effect of cell cycle on cellular uptake and transgene expression is real. These results suggest that transcription and translation machinery is affected by the cell cycle phase. In our case this effect may be contributed by competition of transcription factors due to simultaneous expression of CMV-driven stable endogenous (luciferase) and transient exogenous (β-galactosidase) proteins in the cells. It also appears that non-specific pinocytosis (fluid-phase endocytosis) is also cell cycle-dependent, being least efficient during the G1 phase, whereas receptor-mediated is not cell cycle-dependent. Brunner et al. (2000) reported a constant cellular uptake in all phases by the receptor-mediated transferring-coupled PEI, while our results with non-specific cellular uptake revealed clear dependency on the cell cycle phase. In addition, our results demonstrate that the effect of cell cycle is not promoter specific, since all of the promoters tested exhibited similar trends in cell cycle dependence. However, the efficiency in terms of absolute protein expression depends on the promoter. The cell cycle effect is also not protein specific, because both β-galactosidase and luciferase reporters revealed similar trends in transfection.

Very little is known about the compositional changes occurring in the cytoplasm during cell cycle and their potential impact on the intracellular kinetics of DNA. The less pronounced difference (PLL vs. PEI) in expression levels during the G1 phase compared to the substantial difference noted in the other phases may be a reflection of unfavorable circumstances (e.g. pH) for PEI within the cell or endosome resulting in weak transgene expression. Also, changes in membrane tension during the cell cycle (Raucher et al. 1999) may modify cellular uptake and transgene expression. The role of mitosis in nuclear uptake and efficient gene delivery is controversial (Zauner et al. 1999; Chan et al. 1999; Pollard et al. 1998). Our data indicates that though mitosis may promote nuclear uptake, its necessity

seems to have been overestimated. Also the study by Ludke et al. (2002) indicated that DNA can enter the nucleus in non-dividing cells after cytoplasmic microinjection, but at a lower efficiency than into the nucleus of dividing cells, and thus, nuclear membrane breakdown does not seem to be essential for penetration in to the nucleus. Although nuclear breakdown may assist in nuclear uptake and successful transgene expression, this nuclear reassembly may extrude large macromolecules from the nucleus (Swanson et al. 1987), resulting in lowered gene expression. Furthermore, the size of nuclear pores can change according to the activity of the cell, and therefore, it has been claimed that access to the nucleus might be better during S phase than during the G1 phase (Feldher et al. 2001). Finally, the intranuclear electrical potential changes during the cell cycle. The negativity increases when cells go through G1 phase towards the beginning of mitosis reaching the highest electrical potential during the G2 phase with the lowest potential occurring during mitosis (Giulian et al. 1977). This follows the charges of chromatin: during mitosis, when chromosomes are in a condensed state, the charges of the DNA are partially neutralized by positively charged histones, but after mitosis, DNA unravels and its negativity increases until the cells start to progress to mitosis again. Thus, our transfection results seem to follow the intranuclear potential present during the cell cycle.

6.3 The relevance of GAGs in gene delivery

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GAGs are abundant polyanions in the extracellular matrix and are present on the cell surface as part of the proteoglycans. Lipoplexes and polyplexes may react with GAGs, leading to DNA release or relaxation (Ruponen et al. 1999), and in that way modify transfection. The experiments with exogenous anionic HA, the least abundant endogenous GAG in D407 cells, did not relax the complexes of linear or dendritic PLL 20 kDa at any N:P charge ratio, whereas CS-C, the most abundant endogenous GAG in D407 cells, could recover 10–15 % of the fluorescence at charge ratios above 1:1. The sulfated CS interferes with positive PLL polyplexes more efficiently than HA due to its higher negative charge density. Despite their initially different DNA binding properties, dendritic and linear PLL 20 kDa showed similar sensitivity to interactions with GAGs. Therefore, GAG interactions on the cell surface probably do not account for their different transfection activities.

Our data on cells of D407 RPE cell line show that CS is the predominant endogenous GAG on the cell surface and in the growth medium, while HS, and particularly HA, are less abundant. Interestingly, GAGs analyzed from the culture medium, cytoplasm and nucleus during the cell cycle can be qualitatively very different (Fedarko et al.1986). For example, confluent cells (primarily in G1 phase) have a higher degree of sulfation than dividing cells; this being especially the case for the amounts of HS in the nuclear membrane and nucleus (Fedarko et al.1986). Consequently, structural differences in GAGs, together with the differences in the amounts of GAGs during cell cycle could have influenced the cellular uptake of polyplexes.

Taking into account the average molecular mass of one nucleotide (~325 g/mol), the size of pCLuc plasmid (6244 bp) and the estimation that usually a single plasmid (Golan et al.1999) is condensed into a single complex with PEI, we can roughly calculate that 20 pg/cell of pDNA is equivalent to about 3×10^6 complexes/cell. The number of total sulfated GAG chains on the surface of each cell was about $2.2–4.0 \times 10^6$. This implies that if the complexes interacted with the cell surface GAG chains at the same time, there would be approximately a stoichiometric ratio (0.8–1.3) between the GAG and the complex. If we take into account the cellular uptake results, showing clearly higher cellular polyplex uptake in the cells in the S and G2/M phases (>3-fold) than G1 phase cells (~30 % PEI 25 kDa, ~ 5 % PLL 200 kDa), it is unlikely that such small quantitative differences in GAG concentrations on the cell surface would have such dramatic impacts on PEI- or PLL-mediated cellular uptake and transgene expression.

6.4 New efficient gene carriers?

We found that starch derivatives can form polyplexes, but their transfection efficiency was weak and they are not applicable for gene delivery purposes. However, starch is a biodegradable polymer, which is an advantage in a gene carrier. We also studied modifications of the SB dendrimer, but none of the investigated polymers were more efficient transfection agent than PEI 25 kDa. We observed that PEGylation decreased the size of SB dendrimers, which is also supported by our observation with PEG-PLL: PEGylation sterically stabilizes PLL polyplexes and permits the determination of the size of individual complexes. We also found that inclusion of a carboxylic moiety as a functional group was not benefical in gene delivery with the polymers PDMAEG and PDMAEMA. Complexes with a pyridine moiety, however, displayed some efficiency of transfection into D407 cell line cells, but did not reach the level of PEI 25 kDa in our study. Thus, none of the carriers examined in this study presented an alternative for PEI carrier in gene delivery. However, these results do not mean that these polymeric carriers may not be useful in other cell lines (than D407) or perhaps novel modifications may solve these problems.

Liposomal coating can protect polyplexes from extracellular polyplex-binding proteins when they are administerd i.v., but they can also be beneficial in mechanistic studies of gene delivery (i.e. intracellular routing and examining the importance of GAGs in gene delivery). We found that coating can rather well protect PEI polyplexes from attack from anionic polymers, but the precipitation of the coated-complexes prevented further studies.

7 CONCLUSIONS

1. The physicochemical properties of PLLs can differ to some extent but this does not necessarily have a major impact on the biological activity – cellular uptake and transfection efficiency.

2. The dendritic polymer structure is less favourable than the linear structure in terms of cellular uptake and transfection efficiency of PLL polyplexes.

3. PEGylation sterically stabilizes the complexes in buffer, but does not protect them from sulfated GAG. In addition, PEGylation does not hamper the cellular uptake or the transfection efficiency of PLL polyplexes.

4. The cellular uptake and the efficiency of transcription machinery are dependent on the cell cycle phase. The cellular uptake, nuclear accumulation of pDNA, and transfection efficiency are less efficient in cells in the G1 phase than in cells in the S and G2/M phases. Only a part of the nuclear pDNA is available for transcription.

5. Efficiency of the transgene expression depends on the promoter: the viral promoters SV40, CMV and tk are more efficient in terms of absolute protein expression than human origin PDE-β. The efficiency of the promoter is dependent on the cell cycle phase in PEI-mediated gene delivery: transgene expression is at its highest in the S phase cells but almost negligible in the cells in the G1 phase.

6. Polyplex-type modifies the effect of cellular uptake, intracellular distribution and transfection efficiency during the cell cycle, but the main difference in PLL- and PEI-mediated transfections is detected at the nuclear level. PEI-mediated cellular uptake of pDNA is more efficient than PLL-mediated uptake only in cells in the G1 phase, and the levels of transgene expression and expression efficiency are higher with PEI than PLL polyplexes in all phases of the cell cycle.

7. Cell surface GAGs do not solely account for the differential pDNA uptake or transgene expression during the cell cycle.

8. Amylose-rich starch derivatives are able to condense pDNA efficiently, while pure amylopectin does not possess this property. Amylose-rich starch/pDNA polyplexes can be taken up by cells but they do not promote transgene expression.

9. Carboxylic acid as a functional group in polymeric PDMAEG and PDMAEMA carriers does not seem to confer any benefits in terms of transfection efficiency.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals **I–III.**

I Marjo Männistö, Sylvie Vanderkerken, Veska Toncheva, Matti Elomaa, Marika Ruponen, Etienne Schacht, Arto Urtti: Structure-activity relationships of poly(L-lysines):effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. J. Control. Rel. 83:169–182, 2002.

II Marjo Männistö, Seppo Rönkkö, Mikko Mättö, Paavo Honkakoski,Mika Hyttinen, Jukka Pelkonen, Arto Urtti: The role of cell cycle on polyplex-mediated gene transfer into retinal pigment epithelial cell line. J. Gene Med. 7: 466–476, 2005.

III Marjo Männistö, Mika Reinisalo, Marika Ruponen, Paavo Honkakoski, Markku Tammi, Arto Urtti: Polyplex-mediated gene transfer and cell cycle: effect of carrier on cellular uptake and intracellular kinetics, and significance of glycosaminoglycans. J. Gene Med. (in press)

Kuopio University Publications A. Pharmaceutical Sciences

A 85. Mönkkönen, Hannu. Intracellular metabolism of bisphosphonates: impact on the molecular mechanism of action and side-effects.
2005. 63 p. Acad. Diss.

A 86. Juntunen, Juha. Water-solube prodrugs of cannabinoids.
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A 87. Salo, Outi M.H. Molecular modeling of the endogenous cannabinoid system.
2006. 95 p. Acad. Diss.

A 88. Holappa, Jukka. Design, synthesis and characterization of novel water-solube chitosan derivates.
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A 89. Hämeen-Anttila, Katri. Education before medication. Empowering children as medicine users.
2006. 111 p. Acad. Diss.

A 90. Lääkepäivät. Lääkehoito on yhteistyötä. 24.-25.3.2006. Kuopio.
2006. 114 p. Abstracts.

A 91. Jäppinen, Anna Liisa. Stability of hospital pharmacy-prepared analgesic mixtures administered by a continuous infusion.
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A 92. Hyvönen, Zanna. Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery: structure-activity relationships and mechanisms.
2006. 95 p. Acad. Diss.

A 93. Lahnajärvi, Leena. Reseptien uusiminen - Miten pitkäaikaislääkitystä toteutetaan terveyskeskuksissa?.
2006. 168 p. Acad. Diss.

A 94. Saario, Susanna M. Enzymatic Hydrolysis of the Endocannabinoid 2-Arachidonoylglycerol - Characterization and Inhibition in Rat Brain Membranes and Homogenates.
2006. 96 p. Acad. Diss.

A 95. Palmgrén, Joni J. Development of Analytical Methods for the Characterization of Absorption Cell Models.
2006. 115 p. Acad. Diss.

A 96. Haapalinna, Antti. The Effects of Atipamezole on Brain Neurochemistry and Behaviour in Laboratory Rodents – Possible Implications for the Treatment of Neurodegenerative Diseases with an Alpha2-adrenoceptor Antagonist.
2006. 119 p. Acad. Diss.

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