CHAPTER 1

General Introduction and Outline of this Thesis

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Introduction

1. Gene delivery and therapy in general

The concept of gene therapy is a promising approach towards clinical treatment of pathological processes. The underlying principle of gene therapy is based on the introduction of genetic material into living cells in order to achieve a therapeutic biological effect [1]. Generally, this involves introducing DNA encoding a gene for a therapeutic protein. In somatic gene therapy the target cells are not part of the germ line and therefore the effects are restricted to the individual. In contrast, in germ line gene therapy the egg or sperm cells are manipulated and thus the potential future offspring of the individual are effected. For this reason, germ line gene therapy is not permitted under current legislation [2].

Gene therapy offers the potential of correcting the underlying cause of hereditary monogenetic diseases such as cystic fibrosis (CF) [3] and haemophilia B [4], for which the responsible gene is known. Therapeutic benefits of gene therapy can be expanded to a wide range of diseases that are not strictly hereditary, such as cancer [5] and cardiovascular diseases [6]. In addition, applications of gene therapy can reach much further: introducing disease-modifying genes into already dysfunctional organs may alter the course of disease [7]. When an infectious agent is involved, gene therapy can be directed towards elimination of the agent from the organism or towards prevention of infection in the form of vaccination [8]. Recent developments indicate that besides delivery of dsDNA, other applications such as delivery of RNA sequences, RNAi, and RNA decoys that bind regulatory proteins [9] offers potential. For example, infectious agents such as HIV [10] and Hepatitis C virus [11] are targets for the development of RNAi for virus inhibition.

A gene delivery system (GDS) or vector consists of a polynucleotide, encoding the therapeutic gene, and a carrier. The carrier has several important properties (Fig 1). First, it condenses the polynucleotide, protecting it from mechanical stress and

enzymatic attack. Second, the carrier should facilitate transport of the therapeutic gene from the extracellular space into the nuclear compartment, where transcription can take place [12].

Living organisms are generally well protected by intra- and extracellular barriers against invasion of foreign genetic material. This is required to ensure the genetic stability of the species. Depending on the method of administration, the vector needs to circumvent host defense mechanisms, i.e. extracellular barriers. For example, when injected into the bloodstream, circulating antibodies and complement factors may inactivate the vector [13-15]. Vectors delivered to the airways face a spectrum of host defense mechanisms: the vector can be entrapped in the mucus layer and subsequently removed by cilliary clearance. Scavenging macrophages and dendritic cells can degrade the vector by phagocytosis and induce an inflammation response. In addition, many potential receptors for GDSs are located on the basolateral membrane of airway epithelial cells, safely shielded from the *milieu exterieure* by tight junctions thus the receptors are inaccessible for GDSs [16].

Gene delivery systems can be can be divided into two classes. First, there are those based on viral vectors and second those based on synthetic self-assembling systems. In this chapter we will discuss the advantages, drawbacks and working mechanisms of each class. Subsequently, we describe DNA condensing components of non-viral gene delivery and the glycosylation of these components for cell targeting. Finally, an outline of this thesis is given.

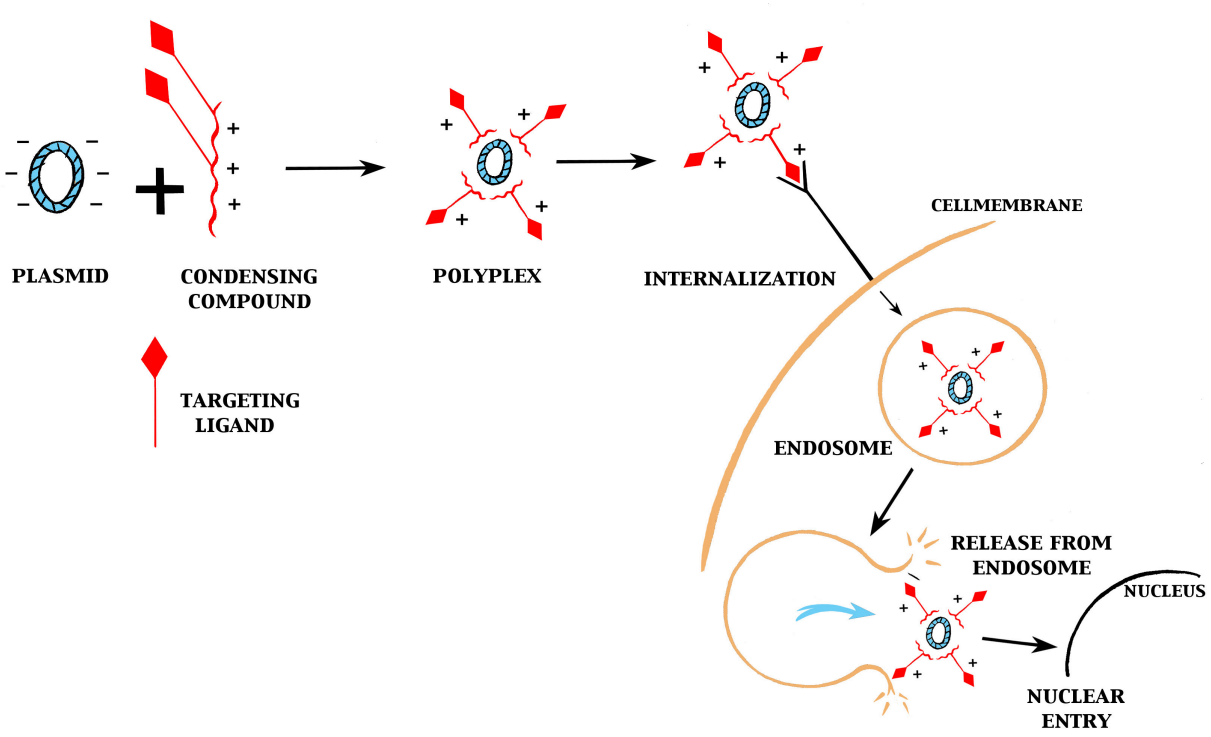

Figure 1. Scheme illustrating steps in non-viral gene transfer

2. Viral and non-viral vectors.

Gene delivery systems can be based on various viruses. They include adenoviral [17], adeno-associated viral (AAV)[18], lentiviral vectors [19, 20]. In addition, viral vectors based on poxvirus [21, 22], sendai [23] and herpes virus [24] are under investigation for gene transfer. Viral capsids and envelopes of these known viruses are well adapted to perform gene delivery. When applied *in vivo*, the main advantage of viral vectors is the relatively high transduction efficiency, which is either defined as the number of transgene expressing target cells per transduced genome, or as the percentage of transduced target cells. In addition, the use of integrating vectors such as AAV and HIV derived vectors results in stable transduction of a population of mitotically active target cells [1], which can not be achieved with the commonly used plasmid derived synthetic vectors.

On the down side, viral-based gene therapy poses serious safety concerns. Administration of viral vector evokes a host defense response to the viral vector [25, 26]

and transduced cells, containing viral components. This response can range from a relatively mild acute inflammation to a cytotoxic lymphocyte response. Indeed, one fatality was reported due to an escalated immune response to an administrated adenoviral vector [27]. The viral genome of the vector can recombine with or be activated by wild type viral genome, giving rise to potentially hazardous agents. When integrating vectors are used, there is a risk of insertional mutagenesis, as randomly integrating genes can alter the host genome. As recently reported, two patients treated with bone marrow cells transfected with a retroviral vector were diagnosed with leukemia caused by an insertional modification of a proto-oncogene by the therapeutic gene containing vector [28, 29].

In conclusion, viral vectors are highly efficient for gene transfer *in vivo,* but their clinical applications may be restricted due to their safety concerns

The restrictions imposed on viral vectors have led to the development of synthetic gene delivery systems such as cationic lipids and polymers. These vectors may overcome the current problems in safety, immunogenicity and mutagenesis associated with viral vectors [30]. In addition, the size of the therapeutic gene is not limited for self-assembling synthetic vectors and cost-effective large-scale production is feasible. The major challenge is that non-viral vectors lack the high *in vivo* transduction efficiency demonstrated by viral vectors. Viral based vectors have addressed the complexity of the gene delivery process with specialized mechanisms for intracellular transport and nuclear delivery [31] and non-viral vectors need to be designed to do so either.

3. Viral and non-viral vector approaches to barriers in gene transfer

Viruses are parasitic non-cellular organisms that require cells for their viability and growth. They have evolved over time to deliver their genome to the host cell nucleus with high efficiency. In many different shapes and molecular designs, viruses consist of a polynucleotide genome packaged in a protein complex called a capsid. The capsid proteins are well adapted to allow efficient binding to target cells,

penetration of the plasma- or endosomal membrane, facilitation of intracellular transport, and subsequent import of the genome into the nucleus [1]. Viruses have developed many different mechanisms to approach this trafficking problem. What they have in common is the use of innate cellular transport systems, through interactions of their capsids with intracellular transport systems of the host. The latest generation of non-viral vectors is designed to mimic the properties of viral capsids with target proteins.

In the following paragraphs we will describe and compare critical properties of viral and non-viral GDSs.

3.1. Packaging of genetic material

Viral vectors are generated by replacing part of the viral genome (either DNA or RNA) with a therapeutic gene. Most viral genes are removed from the genomic backbone of the virus with the exception of the genetic components involved in the genome packaging in the capsid and replication. The genes removed are required for capsid assembly and genome replication and these gene products are provided by packaging cells. Packaging cells transfected with the therapeutic viral vector can then produce infectious therapeutic virus. The size of the vector sequence and thus of the therapeutic gene insert is limited by the properties of the viral capsid [32].

Non-viral vectors generally comprise cationic polymers (Fig 1) or lipids that are able to condense the negatively charged DNA. For example, poly-L-lysine (PLL) is a widely used component of transfection agents. The primary amino group on the side chain of the monomer L-lysine allows for protonation and thus a positive charge. At physiological pH nearly all N-atoms of the polymer are protonated. Electrostatic interaction with the negatively charged phosphate groups of the DNA results in compact, ordered particles varying from 20 to 200 nm diameter in size [33]. The packaged genetic material, usually plasmid DNA isolated from bacteria, is protected from mechanic stress and enzymatic degradation [34].

3.2. Vector target cell binding and entry

A GDS has to target a specific cell to achieve its therapeutic goal and minimize unwanted side effects. Prior to cell entry, non-viral and viral GDSs attach to the surface of target cells. Subsequently the attached vehicle is endocytosed (Fig.1). Viral vectors generally target specific receptors on the plasma membrane and can enter the cell by fusion with the plasma membrane (HIV, influenza), pore formation (polio virus) or by acid induced membrane disruption after receptor-mediated endocytosis (adenovirus) [35].

Adenovirus and adenoviral vectors bind to the cell following two sequential receptor interactions. Firstly, the capsid fiber binds to a glycoprotein of the immunoglobulin family called CAR (Coxsackie, Adenovirus Receptor), which is expressed in many human cell types but is located on the basolateral membrane of airway epithelial cells [36]. Secondly, the fibronectin-binding integrin (integrin $\alpha v\beta 5$) binds to the penton base protein, an adenoviral capsid protein [37]. The binding is followed by receptor-mediated endocytosis on the basolateral membrane of airway epithelial cells for cell entry [38]. A receptor (heparan sulfate) for adeno associated virus derived vectors has been located on the basolateral membrane of airway epithelial cells [39] and it has been demonstrated that the apical membrane contains an abundant high affinity receptor for serotypes AAV5 and AAV4 [40, 41]. Vesicular stomatitis virus G protein (VSV-G) pseudotyped retro- and lentiviral vectors are not able to infect airway epithelia efficiently when administrated to the apical surface of polarized airway epithelial cells due to the lack of an apical receptor. To overcome this problem retroviral vectors were pseudotyped with apical membrane-binding envelope proteins [42], leading to the development of a filovirus-pseudotyped HIV vector that efficiently infects airway epithelia *in vivo* [43]. Also, a feline immunodeficiency virus (FIV)-based lentiviral vector was pseudotyped with the glycoproteins from Ross River Virus, resulting in a vector that displayed an increased tropism for the liver [44].

Non-viral gene delivery systems generally are completely dependent on endocytosis for cell entry. Polycations complexed to DNA result into positively charged particles named polyplexes. They interact electrostatically with negatively charged proteoglycans of the cell membrane, which leads to endocytosis [45, 46]. This non-specific mode of cell entry can be altered by the addition of a targeting ligand to the surface of the DNA delivery vehicle. The first targeting ligand used for non-viral gene delivery was asialorosomucoid which is specific for hepatocytes [47]. Numerous ligands are under investigation and are reviewed. They include transferrin, folate, specific monoclonal antibodies [31], [48], invasin [49] and several carbohydrates [50]. While such formulations were effective *in vitro,* specific cell targeted gene delivery *in vivo* is still problematic. The positive surface charge of the synthetic vector, causes non-specific electrostatic interaction with non-target cells [51]. This unwanted interaction of synthetic vectors with biomolecules of non-target cells can be reduced by addition of hydrophilic segments such as poly(ethylene glycol) (PEG). A PEGylated GDS has a hydrophilic shell at the exterior that masks the positive surface charge and prevents interaction by steric repulsion [52] resulting in less aggregation and reduced transfection of non-target cells.

3.3. Endosomal escape

After endocytosis, the normal endosomal transition into lysosomal vesicles results in acidification, which provides a milieu for the activity of hydrolytic enzymes. In order to avoid the degradation of the therapeutic DNA, a GDS must escape from endosomes [53].

To alleviate this degradation problem viral vectors take advantage of pathways that were selected during the evolution of natural viruses. Adenovirus, a non-enveloped virus, uses the acidification of endosomes to activate three capsid proteins, which then lyse the endosomal membrane [54] [55]. The enveloped influenza virus uses an amphiphilic protein, the haemagglutinin protein (HA) to escape from the endosome. When the pH in the endosome drops, the geometric structure of HA changes rapidly,

leading to a fusion of the viral and endosomal membrane and so endosomal escape of the virus particle[56].

Non-viral vectors must also have mechanisms to circumvent lysosomal degradation: some polycationic polymers such as polyethylenimine [57] and pDMAEMA [58] are able to escape the endosome due to intrinsic properties of the polymer. First, the high buffer capacity of the compounds results in swelling of the endosomes as the lysosomal proton pump attempts to reduce the luminal pH. Further, cationic polymers in high concentrations tend to destabilize membranes. However, since the bulk of the DNA delivered this way does not escape hydrolysis, the DNA-containing particles are largely retained in perinuclear endosomes/lysosomes [59]. This phenomena remains one of the bottlenecks in delivery by synthetic GDS.

Pharmacological agents such as chloroquine can be used to disrupt the normal internal routing of the GDS from the endosomes to the lysosomes [60]. Chloroquine is a weak base (pKa 8.1 and 10.2) that accumulates in the endosome when the luminal pH is lowered. It is thought this accumulation results in osmotic swelling, followed by the destabilization of the endosomal membrane and subsequent release of the contents into the cytosol [52]. The presence of chloroquine enhanced transfection efficiency of lactosylated PLL considerably [61], which is generally low unless endosomolytic or lysosomaltropic agents are present [62]

Another strategy to promote endosomal release is the addition of endosome-disrupting peptides such as N-terminal amphilic anionic peptide of HA2 [63], derived from the influenza virus HA [64]. Also the synthetic amphipathic peptides GALA (glutamatic acid-alanine-leucine-alanine) [65] has been shown to enhance the transfection efficiency of liposomes [66]. KALA (lysine-alanine-leucine-alanine) is a cationic amphipatic peptide and was designed to both condense DNA and destabilize the endosomal membrane. When compared to gene transfer using PLL, KALA was more efficient and yielded a 100-fold greater reporter gene expression [67]. Glycerol is

another agent that promotes endosomal escape. It was reported that PLL had endosomal membrane disrupting properties in the presence of glycerol [68].

Poly-L-histidine (PLH) has been used in studies of membrane fusion and has served as a pH-responsive fusogen [69]. PLH was used in combination with PLL-based GDS for pH responsive escape [70]. Also partial substitution of the ε-amino groups of PLL with histidine resulted in a gene transfer agent that was able to transfect HepG2 cells without the addition of helper agents such as chloroquine, glycerol and fusogenic peptide [71, 72].

A relatively new approach to facilitate endosomal release of the gene delivery vehicle is photochemical internalization (PCI), based on light-induced photochemical reactions [73]. A number of photosensitizers such as tetra(4-sulfonatophenyl)porphine ($TPPS_4$) and meso-tetraphenylporphine ($TPPS_{2a}$) localize primarily to endosomes and lysosomes [74]. When exposed to light, these photosensitizers induce the formation of reactive oxygen species. These are short-lived and have a short range of action (10-20 nm) [75]. Therefore, once light-activated, photosensitizers located in endo- or lysosomes may destroy the membrane but leave the organelle contents intact [76]. PCI enhanced the transfection efficiency of PLL by 10-fold and of adenovirus-mediated gene transfer by 6-fold [77].

3.4. Intracellular transport

Once released from endosomes the vector DNA must reach the nucleus to be effective. Extremely large molecular structures such as viral capsids and non-viral vector complexes do not diffuse easily through the viscous and crowded cytoplasm. Viral vectors exploit the intracellular transport system of the host cell, including the microtubule/motors or actin filaments for intracellular movement [78]. Baculovirus is thought to transport itself in mammalian cells *via* actin filaments [79]. Vaccinia virus is also reported to use actin filaments for transport. Herpes viruses employ microtubule-based transport to move within the host cell towards the nucleus, presumably mediated

by the attachment of viral capsid to the motor protein dynein [78]. It is generally thought that viral-based vectors employ the same strategies as their wild-type virus counterparts.

Little is known about how synthetic gene delivery vehicles move within the host cell. They may diffuse freely through the cell or "piggy back ride" with the host cell's own intracellular carriers. An attractive option to enhance intracellular transport of synthetic vectors is the addition of peptides, derived from viral proteins responsible for intracellular transport.

3.5. Nuclear translocation

Once in the perinuclear region, the foreign DNA must enter the nucleus in order to be trasnscribed. However, the nucleoplasm is separated from the cytosol by a hydrophobic barrier: the nuclear envelope that consists of an outer nuclear membrane and an inner nuclear membrane. These membranes are connected to each other by the nuclear pore complex (NPC), a protein structure [80]. NPCs form aqueous channels through the double membrane of the nucleus [81] and allow for transport of cargo. Small molecules (up to 9 nm in diameter, or proteins up to 50 kDa) can passively diffuse, but larger cargo (up to 25 nm in diameter, or ~1000 kDa) is transported in an active signal-mediated manner. Nuclear localization signals (NLS) on cargo, bind to cytoplasmatic proteins named α-importins, which in turn bind to β-importin. β-Importin is responsible for docking the import cargo on the NPC. The translocation into and across the NPC involves a key regulatory molecule, GTPase Ran [82].

In principle, there are two approaches for viral and non-viral vectors to deliver the genetic material into the nucleus. First, the vector resides in the cytosol until the nuclear envelope is disassembled during mitosis. The foreign genome can enter a newly assembling nucleus. Alternatively, the genomic material can be delivered through the envelope of the interase nucleus *via* the NPC [83].

Many viruses that are used as GDS are able to transport their genome into the cell nucleus. Retroviruses, with the exception of lentiviruses, cannot deliver their genome into an intact nucleus and can therefore only infect dividing cells. Most viruses have developed a mechanism to deliver their genome through the NPC. For example, influenza virus transports its genome through the NPC in small RNA segments packaged into viral ribonucleoprotein complexes [84]. Adenoviral DNA has a linear structure and is transported to the nucleus stored in a protective capsid. The capsid docks to the nucleus via nucleoporins, the building stones of the NPC. Subsequently, the viral DNA is transported through the NPC [85]. Baculovirus has a cigar-shaped plasmid that is able to penetrate the NPC of mammalian cells [79]. The nature of the interactions between capsid and NPC is yet unclear.

Lentiviruses such as HIV-1 have developed mechanisms to transport their genome into cell nucleus of non-dividing cells [86]. After the reverse transcription of genomic HIV-1 RNA into linear double stranded DNA, the DNA remains part of a nucleoprotein complex called the preintegration complex. Proteins involved in the nuclear import are matrix protein, Vpr and integrase. Their function remains unclear but they may act as a NLS. In addition, Vpr may facilitate nuclear docking of the complex and induce transient herniations of the nuclear envelope allowing nuclear translocation of the complex [83]. HIV-1 nuclear import has been reported to also depend on signals present in viral DNA: the reverse-transcribed HIV-1 genome contains a short triple-stranded overlap, the central DNA flap. When the central DNA flap is mutated, the virus cannot enter the nucleus in an integration-competent form [87].

Non-viral GDS do not have such sophisticated mechanisms as their viral counterparts and it is still unknown how DNA of therapeuticly relevant sizes (up to 10 kbp) can pass through the NPC [88]. It was postulated that nuclear uptake occurs preferentially in those cells entering mitosis, consequent to break down of the nuclear membrane [63, 89]. In contrast, there are also reports that suggest a nuclear import mechanism through the NPC. Since transferrin/polylysine complexes were found to gave high

transgene expression levels in growth arrested fibroblasts [90]. Moreover, Pollard *et al* [91] reported that some polycations facilitate the nuclear uptake of DNA complexes. The size-restrictions for NPC-mediated import as outlined in section 3.5 were also challenged by reports that plasmid DNA up to 14.4 kbp was found localized in the nucleus in the absence of cell division. The nuclear import required energy, cytoplasmic factors and was blocked by wheat germ agglutinin an inhibitor of NPC function. Furthermore plasmid DNA is thought to form a complex with NPC proteins prior to transit and translocation [92]. Likewise it has been shown that single-stranded DNA/protein complexes were efficiently imported in mammalian nuclei following the classical importin-dependent nuclear import pathway [93]. Polycations modified by the addition of a NLS have been reported to increase gene transfer [94].

Unfortunately, none of these studies appear completely conclusive. In experiments with supposedly 'non-mitotic cells', actual controls of mitotic activity after addition of the vector are often missing. It should be noted that non-viral GDS are generally cytotoxic. Application of such GDS can easily result in a burst of transient mitotic activity. With regards to transport studies using fluorescent-labelled DNA, fragmentation of the DNA prior to nuclear transport cannot be excluded. It is well known that small DNA fragments (<500 bp) readily accumulate in the nucleus, as opposed to larger molecules that are required for a therapeutic gene [95].

In conclusion, well-documented studies have shown that viruses have efficient strategies for nuclear import. How and if non-viral vectors can enter an intact nucleus still remains unclear despite reports suggestive for NPC-mediated nuclear import. Indisputable evidence of transport of synthetic vectors through the NPC has not yet been presented.

3.6. Transgene expression.

After entry in the nucleus, transcription of the therapeutic gene should take place. A prerequisite is the availability of the DNA for the host cell transcriptional

machinery. Transgene expression is generally transient [96]. Viral and non-viral GDS must address this problem.

Lentiviral vectors and recombinant AAV address the problem of poor persistence [97] by integration into the host DNA. However, the major drawback of integration is the possible risk of mutagenesis [29].

Several strategies are developed to resolve the problem of transient reporter gene expression in non-viral gene transfer [96]. So far, expression vectors are generally of relatively simple design, comprising a core promoter/enhancer, therapeutic cDNA, and a simple intron structure if any. Clearly, this does not match the intricate mosaic of regulatory and structural elements found in chromosomal genes, which is required for stable and cell-specific expression. The construction of minichromosomes that have full telomere and centromer functions, offers the possibility to introduce a large genomic element, complete with distal regulatory elements. [98]. Transfer of such a therapeutic minichromosome to a target cell population should lead to stable and perfectly controlled expression of the therapeutic gene. However, successful transfer of such a large construct into a sufficient number of relevant target cells remains a challenge [99, 100]. Another approach to extend reporter gene expression is the design of tissue-specific self-replicating plasmid. A replicating episomal plasmid mediated under the human beta locus control region sustained reporter gene expression in culture up to 60 generations [101]. Development of integrating vectors based on the Sleeping Beauty transposon system resulting in sustainable transgene expression have been reported [102-104]. In addition, in non-viral gene transfer using plasmid DNA, absence of the methylated sequences may contribute to prolonged longevity of transgene expression of bacterial plasmid DNA in the human host cell [105]

4. Non-viral vectors: DNA condensing components and their glycosylated derivatives.

As described above a GDS must be well designed to a) condense DNA, b) bind to a specific cell surface and promote uptake, c) escape from the endosomal compartment, and d) provide for nuclear translocation either by nuclear import or by entry during mitosis. While vectors based on viruses take advantage of the strategies that were developed by their natural counterparts during evolution, non-viral GDSs will have to be constructed *de novo* to meet the high demands of efficient gene transfer. Essentially, a synthetic GDS is an artificial virus, consisting of DNA, usually in the shape of a plasmid and a carrier. The carrier therefore needs to be equipped with tools that can fulfill the task of the viral capsid. In order to condense DNA, the carrier has to be positively charged. Carriers can be roughly divided in two groups: cationic lipids and cationic polymers. Both can be modified to mimic the actions and functions of the viral capsid. One specific modification is glycosylation. The presence of carbohydrates on the DNA condensing compound allows for interaction with lectins which are proteins that bind to specific carbohydrate structures [106].

4.1. Glycosylation to target cellular lectins

The natural ligands for most lectins are typically complex glycoconjugates that carry clustered arrays of the cognate carbohydrate, thus cooperating with clustered lectin-binding sites to generate high-avidity binding [107]. Some membrane-bound lectins are internalized upon ligand binding, followed by delivery to internal acidic compartments [108]. The covalent linkage of carbohydrates to non-viral GDS may enhance cell specific binding by targeting membrane-bound lectins and triggering receptor-mediated endocytosis. There are two well-known lectins that are carbohydrate-binding receptors. The mannose receptor recognizes glycoproteins with

mannose, glucose, fucose and *N*-acetylglucosamine residues in exposed positions [109] and is located on a variety of macrophage subtypes [110]. Macrophages are targets for gene therapy of diseases as Gaucher's disease [111] and HIV infection [112] but may also be a target cell for expressing an exogenous gene with therapeutical effects [113]. The asialoglycoprotein receptor is located on the hepatocyte membrane and specifically recognizes terminally linked β-galactose or Gal*NAc* (*N*-acetyl galactosamine) residues on circulating glycoproteins and cells [114]. A variety of DNA carriers are in development using galactose/lactose as a tageting ligand for the hepatocyte asialoglycoprotein receptor, taking advantage of receptor-mediated endocytosis to both enhance efficiency and specificity of gene transfer [50].

4.2. Glycosylation of cationic lipids

Cationic-lipid based gene delivery systems are composed of lipid:DNA complexes (liposomes). Such complexes have been successfully delivered to e.g. airway epithelium *in vivo* [115], although at generally less efficiency rates than viral vector gene delivery [116]. Initial clinical trials have indicated that liposomes have a relatively low toxicity profile when administrated to nasal epithelium [117-120]. However, liposome administration to human lungs caused mild influenza-like symptoms which may be related to the DNA component of the complex [121].

The composition of lipids in the liposome based gene delivery vehicles varies and is known to be critical to vector targeting and efficiency. Additional components such as carbohydrates can both increase efficiency and promote selective targeting [122]. Indeed, when the biodistribution of mannosylated liposomes in mice was studied, it was found that intravenously administered mannosylated liposomes were taken up mainly by non-parenchymal cells in the liver [123]. Moreover, mouse peritoneal macrophages were efficiently transfected *in vitro* [124]. However, *in vivo* expression of the reporter gene was detected in the non-parenchymal cells of the liver. This was reduced by predosing with mannosylated bovine serum albumin and was enhanced by the incorporation of PEI into the liposome complex [125]. Correspondently, galactosylated

liposome/DNA complex transfected the mouse liver through asialoglycoprotein receptor-mediated endocytosis [126]. The liver is reported to express two types of galactose receptors, one specific for parenchymal cells and one for Kupffer cells. The lactosyl residue of galactose provides the targeting ligand. Indeed, when bound to low-density lipoprotein the lactosyl residue can target both Kupffer cells and parenchymal cells of the liver. The specificity depends on the degree of lactosylation. At high substitution of lactose, lactosylated low-density lipoprotein is mainly taken up by Kupffer cells. At low substitution of lactose, parenchymal cells are the main site of uptake of lactosylated low-density lipoprotein [127].

4.3 Cationic polymers for DNA condensing

Polymers with primary and secondary amino groups that are protonated at physiological pH have the potential to interact with the negatively charged DNA through their positive charges. The electrostatic interaction results in the formation of a transfection complex [31] and that packages the DNA and protects it from degradation [34]. Numerous natural and synthetic polyations are under investigation for use in gene transfer. Natural polycations include histones [128] and chitosan, an aminopolysaccharide [129]. Among synthetic vectors are peptides such as poly-L-lysine and poly-L-ornithine, as well as polyamines such as polyethylenimine [57]. Also synthetically manufactured are poly(*ß*-aminoesters) both linear [130] and dendritic [131] and metacrylate-based polymers such as poly(2-dimethylamino) ethyl methacrylate (pDMAEMA) [132].

Currently most synthetic vectors are being modified so as to optimize important steps in the transfection process. The addition of a targeting ligand, e.g. carbohydrate, can enhance cell specificity. Also, adding a nuclear localization signal appears to improve nuclear delivery [133]. Despite all efforts, a satisfactory gene delivery system for clinical applications has not been developed. In the next paragraphs the polycations poly-L-lysine, pDMAEMA and polyethylenimine and glycosylated derivatives are discussed.

4.3.1. Poly-L-lysine (PLL)

PLL, a homopolymer of residues of L-lysine with its length ranging from 4 kDa to 224 kDa [134], was the first polycation used in polymer-based non-viral gene transfer. Asialoorosomucoid was covalently bound to polylysine and used as a ligand for the transfection of HepG2 cells [47] and *in vivo* rat liver [135]. Glycosylation of PLL was reported to diminish the toxicity and immunogenicity of PLL since it did not to evoke an inflammatory response in animal experiments [136, 137]. Mannosylated PLL was most efficient in gene transfer into human *in vitro* differentiated macrophages, when compared to other glycosylated poly-L-lysine based DNA carriers [113] and was used to transfect murine macrophages *in vivo* successfully [138, 139]. Also, galactosylated PLL was characterized [140] and used to deliver genes to the rat liver [141]

Lactosylated PLL, substituted with lactosyl residues on approximately 40% of the ε-amino groups of L-lysine [62] proved most effective in gene transfer into CF airway epithelial cells in primary culture [61] and tracheal serous glands *in vitro* [142], when compared to other glycosylated PLL. Lactosylated PLL was further characterized for gene transfer into CF and Non-CF airway epithelial cells and it was shown that the presence of combined endosomolytic agents enhanced transgene expression. The combination of chloroquine and a fusogenic peptide (E5CA) increased reporter gene expression in immortalized CF airway epithelial cells, compared to transfection with chloroquine alone, resulting in a reporter gene expression in 90% of the cells [62]. The combination of glycerol and E5CA peptide gave the highest reporter gene expression in CF airway epithelial cells in primary culture [143]. Moreover, when immortalized CF airway epithelial cells were transfected using lactosylated PLL complexed to a GFP-CFTR fusion gene, patch clamp analysis demonstrated the functional correction of Cl^-

channel activity [143]. These reports provided the incentive for further characterization of this delivery system as described in this thesis.

4.3.2. Polyethylenimine (PEI)

PEI is a polyamine with a high cationic charge density available in both branched and linear form. Every third atom is a potentially protonable amino nitrogen, rendering PEI with a high buffer capacity over a broad pH range [144]. One key feature of PEI is its capability of endosomal escape without the aid of endosome disruptive additives. The exact endosomal escape mechanism is unclear, although a “proton sponge” hypothesis has been proposed by Boussif *et al* [57]. To enhance the specificity of PEI in gene delivery targeting ligands have been added to this complex, including transferrin [60] and galactose [145]. Galactosylated PEI transfected hepatocytes *in vitro* with an efficiency of 50%. For a feasible *in vivo* application, size reduction of galactosylated PEI/DNA complexes was warranted [146]. For other clinical applications, lactosylated PEI was used to deliver RNA/DNA oligonucleotides in rat hepatocytes [147].

4.3.3. poly(2-dimethylamino) ethyl methacrylate (pDMAEMA)

Methacrylate polymers, such as pDMAEMA, have been applied to prevent tissue rejection to cell transplantation [148, 149]. In addition to biocompatibility with living tissue, methacrylates are synthesized relatively easily and can be derivatized to accommodate specific requirements. For example, the polymer can be PEGylated or a targeting ligand can be added.

Charaterization PDMAEMA/cDNA complexes showed that in Hepes buffered saline (HBS, pH 7.4) the polymer formed complexes with plasmid DNA, which were 100-200 nm in size and exhibited a zeta potential of 25 mV. These small positively charged particles efficiently transfected OVCAR-3 (human ovarian carcinoma cell line) [150] and

COS-7 (African green monkey kidney cell line) [151] as shown by van de Wetering *et al*. DNA condensed with pDMAEMA is protected from DNAseI digestion [152]. Data on the effects of the presence of endocytosis inhibitors and an endosome-disruptive peptide support endocytosis as the principle pathway for cell entry of pDMAEMA/plasmid complexes [153]. Assuming endocytosis is the mode of cell entry, the complex has advantageous properties to escape from the endosome. At physiological pH half of the tertiary amines are protonated, since their pKa is around 7.4. When the pH lowers in the endosome, the non-charged amines become protonated. According to the "proton sponge" hypothesis [154] this may lead to increased osmotic pressure in the endosome and eventually membrane disruption [58]. However, confocal microscopy and EM studies did not reveal complexes or plasmid outside the endocytic vesicle. Apparently, endosomal escape of these complexes is not an efficient process, in contrast to what is observed with viral capsids Complexes were also not observed within the cell nucleus in these studies. Therefore, intracellular trafficking of complexes from the endosome and nuclear translocation seems to be inefficient process as well. The high transfection efficiency,(expressed as percentage of transgene expressing cells) that can be achieved with these and similar transfection systems relies on a high loading of the endosomal compartment of the cells. Only a small fraction of the DNA is actually delivered to the nuclear compartment. This may be improved by modifications such as the addition of a nuclear targeting ligand [155]. To enhance cell specificity of uptake in the application in ovarian cancer gene therapy, folate-targeted pDMAEMA-based polyplexes are under investigation for [156]. For specific targeting of the asialoglycoprotein receptor on hepatocytes, pDMAEMA-co-N-vinyl-2-pyrrolidone-galactose is investigated [157].

5. Scope of this thesis.

The principle aim of this thesis is to study and characterize the interaction of polycation/DNA complexes with target cells. The effect of lactosylation of the carriers on the transfection process is studied, with emphasis on two crucial steps (1) binding and uptake, and (2) nuclear translocation. The second aim of this thesis is development of novel strategies to design polycationic polymer based gene delivery vehicles for targeted DNA delivery.

In **Chapter 2** the potential role of the lactose-residue in the cellular internalization of lactosylated PLL was investigated. Using fluorescent and phase/contrast microscopy, the binding of lactosylated PLL/DNA complex to CF and non-CF airway epithelial cells was characterized. To visualize the complex-to-cell membrane binding in detail, electron microscopy was applied. The vesicles that lactosylated PLL/DNA complex employed for internalization were further characterized with confocal microscopy using immunocytochemistry and cell organelle markers.

In **Chapter 3** the intracellular fate of the glycosylated PLL/DNA complex was studied in primary cultures of CF airway epithelial cells, using confocal microscopy. The fates of lactosylated PLL, mannosylated PLL or unsubstituted PLL complexed to DNA were compared. The advantageous role of lysolytic agents such as chloroquine, glycerol or fusogenic peptide was investigated. In addition, the effect of the presence of wheat germ agglutinin, which blocks the nuclear pore complex, was studied.

In **Chapter 4** the potential role of lactose in intracellular trafficking was further explored. The use of PLL is most likely limited in clinical applications due to the need of endosome disruptive agents. Therefore, we have opted for another cationic polymer to lactosylate, namely pDMAEMA. The confocal studies described in chapter 3 were performed on fixed cells. Consequently the images are one point of the transfection process of one particulate cell. It is impossible to determine the cell's history regarding cell cycle. Therefore, although the nuclear presence of complex was detected, it was

never established how the complex was translocated into the nucleus. The dynamics of the actual import process could not be observed. In addition, it could not be excluded that nuclear membrane has disassembled prior to the nuclear presence of complex. In a more sophisticated approach, fluorescence-labeled lactosylated pDMAEMA/DNA complex was followed within the living cell with confocal microscopy *in real time*. In addition, living cells and their mitotic activity from the start of the transfection were closely monitored with time-lapse confocal microscopy of cells.

In **Chapter 5** lactosylated pDMAEMA-*co*-AEMA was investigated for gene transfer in bladder malignancies. Two different degrees of lactosylation (10% and 20%) were studied. The lactosylated pDMAEMA-*co*-AEMA/cDNA complex was characterized for particle size and zeta potential. The effect of lactosylation on binding and cellular uptake, transfection efficiency (the percentage of transfected bladder tumor cells *in vitro*) and reporter gene expression per cell was studied. To more closely mimic the condition of bladder cancer *in vivo*, a co-culture model of normal murine urothelium and human bladder tumour cells (1207 cells) was used. Binding of labeled lactosylated pDMAEMA-*co*-AEMA/cDNA complex and transfection efficiency were investigated in the co-culture model.

In **Chapter 6** the construction of a GDS that selectively targets cells of interest was pursued. pDMAEMA was chosen to serve as the backbone for its DNA-binding capabilities, intrinsic endosomolytic properties and its relatively ease to synthesize and derivatize. To shield the cationic aspect of the complexes, and thus reduce non-specific interactions, polyethylene glycol (PEG) moieties were linked to the polymer. Addition of a targeting ligand is another requirement, and the high-affinity binding of avidin to biotin was opted for as a means to link a targeting ligand to the polymer. In this chapter we report the preparation of a pDMEAMA-*co*-AEMA-*graft*-avidin/poly(DMAEMA)-*graft*-PEG/DNA compound. The structural properties, biological activity and pharmacokinetics of the PEG-modified polyplexes are reported in comparison to those of the native pDMEAMA-*co*-AEMA-*graft*-avidin polyplex.

In **Chapter 7** a discussion of the results of this thesis is presented.

In **Chapter 8** a summary of the thesis is given in English and Dutch

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CHAPTER 2

Lactosylated Poly-L-Lysine Targets a Potential Lactose Receptor in Cystic Fibrosis and Non-Cystic Fibrosis Airway Epithelial Cells

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Abstract

Poly-L-lysine with 40% of the ε-amino-groups substituted with lactosyl residues facilitated the internalization of lactosylated poly-L-lysine/cDNA complexes into CF and non-CF airway epithelial cells. It was previously shown that lactosylated poly-L-lysine enhanced the transfer of cDNA into the cell nucleus resulting in transfection. However, the cell entry of lactosylated poly-L-lysine/cDNA complexes was not elucidated. It is shown here that binding of the vector/cDNA complexes to the cell membrane was inhibited by lactose but not *N*-acetyl glucosamine, an irrelevant sugar. Examination by electron microscopy revealed the complexes in clathrin-coated pits. Furthermore, the complexes co-localized with transferrin during cell entry and were shown in early endosomes. These results demonstrated that lactosylated poly-L-lysine/cDNA complexes enter airway epithelial cells via receptor-mediated endocytosis utilizing lactose binding receptors which employ the clathrin-coated pit for internalization. Taken together with the fact that nuclear translocation also is enhanced by lactose, these results demonstrated why lactosylated poly-L-lysine is an excellent vector for transfection of airway epithelial cells. Moreover, other carbohydrates covalently linked to poly-L-lysine for targeting other specific cell types, combined with lactosyl residues, can be designed for the development of other molecular conjugates for gene transfer.

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Introduction

Synthetic gene delivery systems can be designed to control the delivery of the gene of interest to the target cells. Means of control include 1) recognition by a cell-surface receptor 2) intracellular trafficking and 3) nuclear translocation [1]. In general, non-viral gene delivery systems have been proposed to rely on normal cellular uptake mechanisms [2] with the attachment of the delivery vehicle to the surface of target cells preceding endocytosis.

The specificity of gene expression can be increased by the addition of a targeting ligand to the surface of the DNA delivery vehicle [3]. For example, poly-L-lysines substituted with specific carbohydrates were complexed to cDNA and used to determine transfection efficiency in CF airway epithelial cells. It was found that lactosylated poly-L-lysine was the most effective vector for transfection to CF and non-CF airway epithelial cells in primary culture [4]. Several other carbohydrates have been used to design glycosylated gene delivery systems including galactose for hepatocytes and mannose for macrophages [5]. Lectins are the proteins that bind specific carbohydrates [6] and membrane-bound lectins are internalized upon ligand binding, followed by delivery to internal acidic compartments [7]. Therefore glycosylated gene delivery systems may take advantage of membrane bound lectins to enter a specific cell through receptor-mediated endocytosis.

In further studies the efficiency of lactosylated poly-L-lysine as an agent for gene transfer into airway epithelial cells was shown to be equivalent to that of viral vectors when agents that enhance endosomal escape such as chloroquine were present [8]. As described in Chapter 3, lactosylated poly-L-lysine/cDNA complexes were proven to be highly efficient in nuclear translocation of cDNA, when the internalization into to the nucleus was visualized using confocal microscopy [9]. Indeed, the lactosylated poly-L-lysine/cDNA complex was localized within the nucleus 1 hour post transfection. The lactosyl residue was shown to be a key feature in providing nuclear translocation of lactosylated poly-L-lysine/cDNA complexes since mannosylated poly-L-lysine/cDNA

complexes or unsubstituted poly-L-lysine/cDNA complexes were both less efficient in nuclear translocation [9].

Although the lactosyl residues on the lactosylated poly-L-lysine/cDNA complex enhanced transfection [4], it has not been heretofore unequivocally demonstrated how lactosylated poly-L-lysine/cDNA complexes are internalized by the cell. In this current study we investigated the binding and internalization of lactosylated poly-L-lysine/cDNA complexes into CF and non-CF airway epithelial cells and describe the specifity of lactose. It was found that lactose played a significant role in cell surface recognition followed by internalization. Thus the lactosyl residues of lactosylated poly-L-lysine provide a ligand with dual activity; namely targeted delivery of cDNA across the cell membrane and subsequent import into the cell nucleus of CF and non-CF airway epithelial cells.

Results

Binding of lactosylated poly-L-lysine/cDNA complexes to the cell membrane is lactose-dependent.

We investigated the binding characteristics of lactosylated poly-L-lysine/cDNA complexes were in CF/T43 cells and NHBE cells in primary culture. Airway epithelial cells were incubated with fluorescein-5-isothiocyanate (FITC)-labeled lactosylated poly-L-lysine/rhodamine-labeled cDNA complexes at 4°C to allow binding but not internalization. Using fluorescent microscopy we found that in the presence of lactose or lactosylated poly-L-lysine binding of the complex to the cells was inhibited. The binding properties of NHBE cells were comparable to those of immortalized CF/T43 cells.

We counted the fluorescent complexes and cells using a digital overlay of the fluorescent and phase contrast microscope image of the same field. When 100 cells were counted, fluorescence was noted in approximately 54 % of the CF/T43 cell population and in 57% of the NHBE population. In a field of 100 cells the total number of lactosylated poly-L-lysine/cDNA complexes bound to CF/T43 cells was found to be 98 (±12) (Fig. 1.). In the presence of 0.1 M lactose, the total number of complexes in a field of 100 cells dropped to 30 (±13), a 70% (±13) inhibition of the complex-to-cell binding. When 200 μg of lactosylated poly-L-lysine was present as an inhibitor the number of complexes was 24 (±13) corresponding to 76% (±14) inhibition. In contrast when 0.1 M GlcNAc, which served as an irrelevant sugar, was present, the number of complexes bound to CF/T43 cells was 94 (± 13) comparable to the binding without 0.1 M GlcNAc (Fig. 1).

We obtained similar results for the binding of FITC-lactosylated polylysine/rhodamine-cDNA complexes to NHBE cells (Fig 1). The total number of lactosylated poly-L-lysine/cDNA complexes that bound in a field of 100 cells was 102 (±18). In the presence of lactose and uncomplexed lactosylated poly-L-lysine this number was

reduced to 20 (±1) and 24 (±0), respectively, representing an inhibition of 80% (±2) and 76% (±4). In the presence of GlcNAc the number of complexes that bound was 101 (±24).

Inhibition by lactose was also accompanied by a change of distribution of complexes in both CF/T43 and NHBE cells. In the CF/T4 population the percentages of cells with one, two, and three to five complexes were 54, 27 and 19%, respectively. The presence of lactose increased the percentage of cells with one complex to 77% and reduced the percentage of cells with two or three to five complexes to 18% and 2%, respectively. In the NHBE cells the presence of lactose increased the percentage of cells containing 1 complex from 57% to 94% and reduced the percentage of cells with 2 complexes from 22% to 3% and for 3 complexes per cell from 22% to 3%.

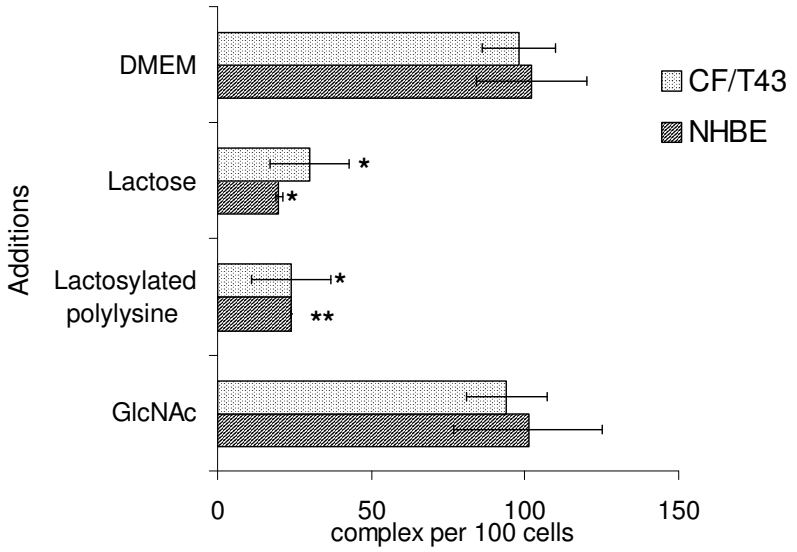

Figure 1. Binding of lactosylated poly-L-lysine/cDNA complex to airway epithelial cells. CF/T43 cells and airway epithelial cells in primary culture grown on coverslips were incubated with 1 μg rhodamine-pCMV*LacZ* complexed to 4 μg FITC-lactosylated poly-L-lysine at 4°C in either DMEM alone or DMEM with the addition of 0.1 M lactose, 0.1 M GlcNAc or lactosylated poly-L-lysine (200 μg). After fixation of the cells, the complex was visualized with fluorescent microscopy and digitally merged with the phase contrast image of the same field. 100 cells were counted in each field and the total of bound fluorescent-labeled complex was determined. Bar represents mean ± standard deviation (*n* = 5 experiments in duplicates for CF/T43 (dotted bars); *n*=2 experiments in triplicates for NHBE cells (striped bars). $^{*}p$ value < 0.001 and **p-value < 0.01 compared to DMEM.

To demonstrate further that the binding characteristics were attributed to the lactosyl residues of lactosylated poly-L-lysine, we incubated NHBE cell with either FITC-labeled mannosylated poly-L-lysine/rhodamine-cDNA complexes or unsubstituted FITC-poly-L-lysine/rhodamine-cDNA complexes for 1h at 4°C with or without 0.1 M lactose present. Lactose did not inhibit the binding of the fluorescence-labeled mannosylated poly-L-lysine/cDNA complexes or unsubstituted poly-L-lysine/cDNA complexes to the cells (Fig 2). The total number of mannosylated poly-L-lysine/cDNA complexes bound to the cells in a field of 100 cells was 65 (±1) and in the presence of lactose the number was 67 (±7). The total number of unsubstituted poly-L-lysine/cDNA complexes in a field of 100 cells was 19 (±3) and remained the same in the presence of lactose (19 ± 1). Furthermore in each case, fewer fluorescent complexes were seen per cell when compared to lactosylated poly-L-lysine.

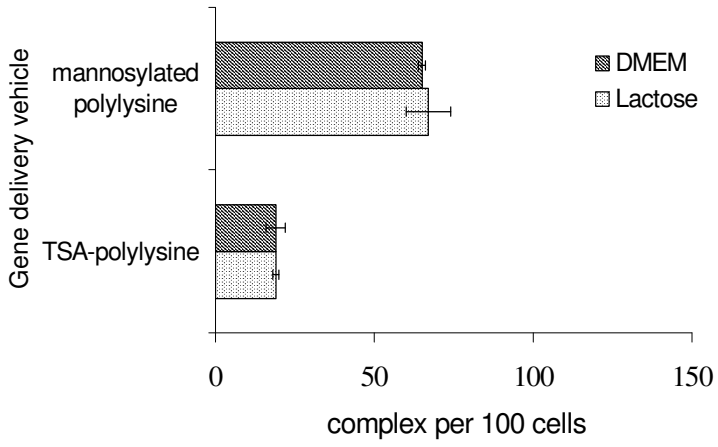

Figure 2. Lactose does not inhibit binding of mannosylated poly-L-lysine/cDNA complex and TSA-poly-L-lysine/cDNA complex to NHBE cells. NHBE cells were grown on coverslips and incubated with 1 µg rhodamine-pCMV*LacZ* complexed to either 4 µg FITC-mannosylated poly-L-lysine or FITC-TSA poly-L-lysine. Incubation at 4°C took place in DMEM alone (striped bars) or with the addition of 0.1 M lactose (dotted bars). See legend to Fig 1 for details. Lactose does not inhibit the binding of mannosylated poly-L-lysine/cDNA complex or TSA-poly-L-lysine/cDNA complex. Bar represents mean ± standard deviation (*n* = 2 experiments in triplicate).

These studies demonstrate that binding of lactosylated poly-L-lysine/cDNA complexes to airway epithelial cells was attributed to the lactosyl residues and that the recognition was carbohydrate specific. Therefore the binding may take place via a lactose binding lectin on the cell membrane of airway epithelial cells. As expected the binding could not be related to transfection efficiency [4] directly since these binding studies were performed under non-physiological temperature.

Lactosylated poly-L-lysine/cDNA complexes enter airway epithelial cells via clathrin-coated pits.

Lactosylated poly-L-lysine/cDNA complexes were detected intact in vesicles in CF airway epithelial cells [9]. To characterize this cell entry further, we used electron microscopy to detect whether internalization of lactosylated poly-L-lysine/cDNA complexes took place *via* clathrin-coated pits.

For the detection of lactosylated poly-L-lysine/cDNA complexes with electron microscopy we labeled the complexes with ferritin. CF/T43 cells were incubated with the ferritin-labeled complex for 30 minutes at 37°C. Subsequent examination by electron microscopy demonstrated the localization of the lactosylated poly-L-lysine/cDNA complex in clathrin-coated pits. The complex was visualized as electron dense material and was located in the cell membrane structure characteristic for clathrin-coated pits (Fig. 3). The clathrin-coated pit was detected by common ultrastructural features. The pits were coated, on the cytosolic face, with a fine and regular basket of electron dense lattice. At a right section plane, the coating lattices were apparently arranged in a radiated fashion. In the conventional preparation of TEM, ultrathin sections (~80 nm) are double stained with uranyl acetate and lead citrate. The ultrastructural features of the vector-containing vesicles and their distinctive subcellular location were characteristic of the clathrin-coated pits or vesicles often associated with receptor-mediated endocytosis [10] and recently reviewed [11].

To rule out the effect that ferritin could have on the transfection process, the ability of the ferritin-labeled lactosylated poly-L-lysine/pCMV*Lac*Z complex to transfect CF/T43 cells was examined as previously described [4]. The transfection efficacy of ferritin-labeled complex did not differ significantly from that of unlabeled complex (data not show).

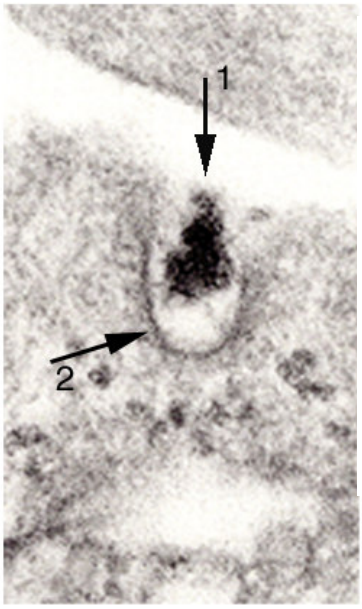

Figure 3. Localization of lactosylated polylysine/cDNA complex in clathrin-coated pits at the surface of CF/T43 cells. Cells were grown in a six-well plate for 24 hrs and incubated with ferritin-labeled lactosylated poly-L-lysine/cDNA complex for 30 minutes at 37°C. Cells were fixed and sectioned followed by electron microscope examination. Note the ferritin-containing, electron dense complex (arrow 1) inside the membrane pit at the cell surface, as well as the spike-like clathrin coat (arrow 2) on the cytosolic face of the membrane. See *Materials and Methods* for details. Original magnification 150,000 X

Support for internalization of the complex via clathrin coated pits shown by co-localization with transferrin

Transferrin is internalized by receptor-mediated endocytosis through clathrin-coated vesicles and is a marker for early endosomes [12-14]. For these experiments, we transfected CF/T43 cells, CF and non-CF airway epithelial cells in primary culture with FITC-labeled lactosylated poly-L-lysine/cDNA complexes in the presence of chloroquine with the addition of Alexa Fluor 594 labeled transferrin. All cell types showed similar results and data is shown with non-CF cells in primary culture (Fig. 4).

Ten minutes (Fig. 4A) after the addition of the transfection medium, confocal microscopy of the non-CF primary airway epithelial cells showed the presence of vesicles containing a yellow signal, representing co-localization of the FITC-labeled lactosylated polylysine/cDNA complex (green) and Alexa fluor 594 labeled transferrin (red). After 30 minutes optical sectioning by confocal microscopy showed an increase of yellow vesicles in the perinuclear region, as seen above the nucleus (Fig. 4B) and around the nucleus (Fig. 4C). After 4 h (Fig. 4D) the FITC-lactosylated polylysine/cDNA complex accumulated in the cell nucleus as observed by the presence of green fluorescence in the cell nucleus. After 6 hours (Fig. 4E) the nuclear accumulation of the

FITC-lactosylated polylysine/cDNA complex increased but Alexa Fluor 594-transferrin remained mainly in the perinuclear region of the non-CF primary airway epithelial cells.

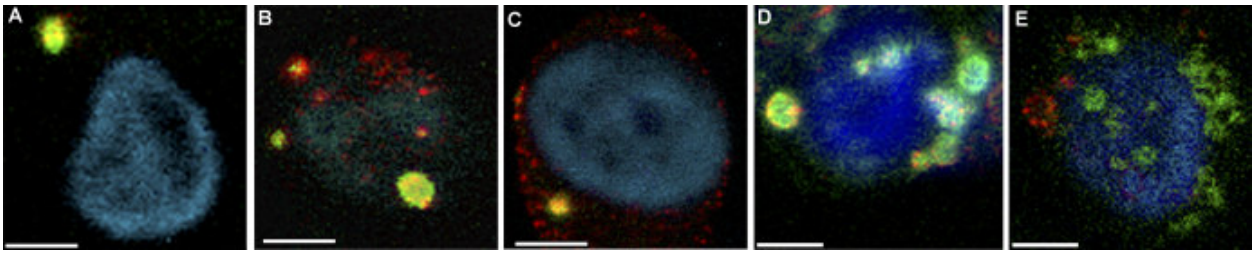

Figure 4. Co-localization of FITC-lactosylated polylysine/cDNA complex with Alexa Fluor-transferrin in non-CF airway epithelial cells in primary culture. Cells were transfected with FITC-labeled lactosylated polylysine/cDNA complex (green) in the presence of 50 µM chloroquine and 17 µg Alexa Fluor transferrin (red) (See *Materials and Methods*). Yellow fluorescence represents the co-localization of the Alexa Fluor and FITC signal. Times posttransfection: (A) 10 min; (B) 1-µm section, 30 min; (C) 3-µm section, 30 min around nucleus; (D) 4 h; (E) 6 h. *Bar, 10 µm*

These results demonstrated that internalization of lactosylated poly-L-lysine/cDNA complexes is a receptor-mediated event and does not occur by fluid-phase endocytosis.

Early endosome pathway.

To characterize further the organelle through which lactosylated poly-L-lysine/cDNA complexes internalized, CF and non-CF airway epithelial cells in primary culture were incubated with lactosylated poly-L-lysine/cDNA complexes, followed by immunofluorescence cytochemistry to detect EEA1, an early endosome marker, which co-localizes with early endosomes but not with late endosomes [15]. Experiments in both CF and non-CF primary cells gave the same results and data is shown for CF primary cells (Fig. 5).

The presence of lactosylated poly-L-lysine/cDNA complexes in early endosomes was visualized by fluorescent labeling. Unlabeled lactosylated poly-L-lysine was complexed to rhodamine-labeled plasmid and added to primary CF airway epithelial cells. After 30 minutes cells were fixed and unlabeled primary antibody to early endosome antigen 1

(EEA1) was applied and subsequently visualized with Alexa Fluor 488 goat anti mouse IgG. We found that the endosomal compartments used for internalization of lactosylated poly-L-lysine /rhodamine-labeled cDNA complexes (red fluorescence) contained EEA1 (green fluorescence) (Fig. 5A). In addition reversal of the fluorescent markers gave similar results: FITC-labeled lactosylated poly-L-lysine was complexed to unlabeled plasmid for transfection followed by red stain for EEA1. FITC-labeled lactosylated poly-L-lysine/cDNA complexes (green) were located in endosomes containing EEA1 (red) (Fig. 5B). As a control, transfection using FITC-labeled lactosylated poly-L-lysine/rhodamine-labeled cDNA complexes demonstrated, as previously shown [9], that the complex remained intact during cellular internalization (Fig. 5C).

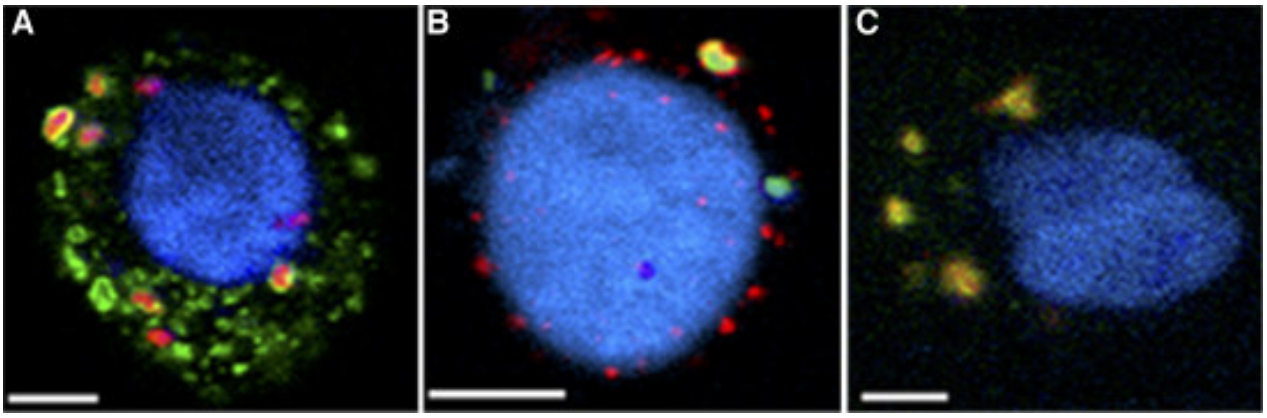

Figure 5. Localization of lactosylated polylysine/cDNA complex in early endosomes during internalization into CF airway epithelial cells in primary culture. Cells were grown on coverslips for 48 h and transfected with fluorescent–labeled complex in the presence of 50 µM chloroquine. After 30 min cells were fixed and primary antibody to EEA1 was applied and cells were subsequently incubated with fluorescent secondary antibody. Prior to mounting nuclei were stained with Hoechst reagent (blue). Yellow fluorescence represents the co-localization of the red and green signal. (A) lactosylated polylysine/rhodamine-cDNA complex (red) and EEA1 (green). (B) Reversing the fluorescent marker, FITC-lactosylated polylysine/cDNA complex (green) and EEA1 (red). (C) Double-labeled FITC-lactosylated polylysine/rhodamine-cDNA complex colocalized in unstained endosome. *Bar, 10 µm.*

Discussion

Lactosylated poly-L-lysine is an effective vector to transfer reporter genes into CF and non-CF airway epithelial cells *in vitro* with a 90-100 % efficiency [8], comparable to that of viral vectors. The vector transferred the CFTR gene into CF airway epithelial cells and corrected the chloride channel dysfunction [16] . Subsequently it was shown that the intact complex was translocated to the nucleus where the reporter gene was expressed. [9]. Lactose was found to be highly efficient in nuclear translocation in airway epithelial cells, however, the mode of cellular entry has heretofore not been described.

The binding of lactosylated poly-L-lysine/cDNA complexes to CF/T43 cells and NHBE cells was shown to be lactose specific since it was inhibited by lactose and lactosylated poly-L-lysine (Fig. 1). The fluorescence-labeled lactosylated poly-L-lysine/cDNA complexes bound to approximately 50 to 60% of the cells, of which 40% contained 2 or 3 complexes. This is in contrast to previous studies, in which the cells were transfected at 37°C. In the latter experiments 90% of the cells expressed the reporter gene after transfection for 6 h at 37°C and 48 h incubation at 37°C. The binding studies were performed at 4°C for 1 h. Therefore the expression of the complex was not examined. It is also interesting that the amount of bound lactosylated poly-L-lysine/cDNA complexes under inhibiting conditions corresponded to that of unsubstituted poly-L-lysine (Figs 1 and 2). Apparently there is a non-specific type of binding, possibly through the electrostatic properties of polycation/cDNA complexes [17].

Galectins are a family of mammalian β-galactoside-binding proteins that are differentially expressed in many cells and tissues [18]. The galectin carbohydrate recognition domain displays highly specific interactions with galactosyl residues [19] and X-ray crystallographic analyses of the carbohydrate recognition domain have demonstrated a lactose-binding site [20]. Therefore a galectin may provide a receptor for lactosylated poly-L-lysine. Indeed the galectin family is very extensive and genome searching has indicated that there are additional galectins not as yet reported [21].

Ligands co-valently attached to poly-L-lysine to target specific cell types include other carbohydrates such as mannose [22, 23] and galactose [24]. Using galactose as a ligand, several non-viral delivery systems are under investigation for gene transfer into hepatocytes. These systems appear to take advantage of asialoglycoprotein receptor-mediated endocytosis in both *in vivo* [25-27] and *in vitro* models [22, 28, 29]. Targeted cell entry is also employed by viruses under development for gene transfer [30]. Adeno-associated virus (AAV) serotype 4 and AAV5 both required sialic acid binding for transfection but differed in sialic acid. AAV4 binding required α-2,3sialic acid to *O*-linked glycans, whereas AAV5 required α-2,3 or α-2,6sialic acid to *N*-linked glycans [31]. AAV5 may use α 2,3sialic acid as a receptor or as a necessary component of a receptor complex [32].

Receptor-mediated endocytosis via clathrin-coated pits is a shared pathway used for the internalization of a variety of ligand-receptor complexes. We have observed for the first time lactosylated poly-L-lysine/cDNA complexes within clathrin-coated pits (Fig. 3). Clathrin-coated pits are ~150 nm invaginated structures on the plasma membrane in which some receptors are constitutively concentrated, but others become concentrated upon ligand binding. After internalization through clathrin-coated pits, the vesicles are uncoated and internalized molecules are delivered to early endosomes [33]. As expected during internalization some of the lactosylated poly-L-lysine/cDNA complexes co-localized with transferrin, (Figs 4A and 4B) which is known to enter the cell through many surface markers via clathrin coated pits [12, 34].

Transferrin under physiological conditions remains attached to the transferrin receptor, enters the recycling compartment and is recycled back to the cell surface [35]. It was shown that when chloroquine, which causes endosome destabilization and release of endosomal contents [36] was present, transferrin accumulated in the perinuclear region. At the same time, poly-L-lysine/cDNA complexes were translocated into the nucleus (Fig.4D and 4E). The intranuclear localization of the complex has been verified with optical sectioning of the nucleus after the nuclear envelope was stained with anti-lamin A and C [9]. Therefore when chloroquine was present, the endosome containing both

the lactosylated poly-L-lysine/cDNA complexes and transferrin destabilized and the complex and transferrin were released into the cytosol. Transferrin, which lacks a nuclear localization signal remained in the perinuclear region. These results demonstrated that lactosylated poly-L-lysine/cDNA complexes entered the cell through clathrin-coated pits *via* a lactose-binding moiety.

As pointed out above, different glycosylated gene delivery vehicles appear to be specific to selected cell types. However, in general, efficiency of transfection is low to intermediate [37]. It may be possible that apart from the carbohydrate required for binding to the cell surface, glycosylated gene delivery vehicles also require lactose as a more universal target for nuclear translocation. Lactosyl residues modulate both of these steps in airway epithelial cells resulting in the high efficiency of transfection. Therefore further development of lactosylated poly-L-lysine and dual glycosylated molecular conjugates is warranted for non-viral gene delivery.

Materials and Methods

Vector preparation.

Poly-L-lysine, HBr (average molecular weight 40,000) was purchased from Bachem Feinchemikalien, (King of Prussia, PA) and Fluorescein-5-isothiocyanate (FITC) isomer I was from Molecular Probes (Eugene, OR). Lactosylated or mannosylated poly-L-lysine was prepared as described [4]. Briefly, 4-isothiocyanatophenyl-derivatives of either β-D-lactose or α-D-mannose (Sigma, St Louis, MO) were added to poly-L-lysine *p*-toluenesulfonate salt in dimethylsulfoxide in the presence of diisopropylethylamine and reacted for 24 hours at ambient temperature. The polymer was precipitated by the addition of 10 volumes of isopropanol. After centrifugation the isopropanol was removed. Subsequently the glycosylated poly-L-lysines were lyophilized for storage at -40°C. Prior to use the glycosylated poly-L-lysines were dissolved in distilled water.

For fluorescent labeling, glycosylated or unsubstituted poly-L-lysine (10 mg) was dissolved in dimethylsulfoxide containing diisopropylethylamine (5.0 µl). FITC isomer (1.0 mg in 400 µl DMSO) was added and the mixture was incubated in the dark at ambient temperature for one hour. The polymer was precipitated by the addition of 10 volumes of isopropanol. After centrifugation the isopropanol was removed. Subsequently the FITC-labeled glycosylated poly-L-lysine was lyophilized for storage at -40°C. For experiments, a mixture of FITC-labeled glycosylated or unsubstituted poly-L-lysine was diluted prior to use with the corresponding unlabeled poly-L-lysine derivative in a 1:7 (wt:wt) ratio in distilled water.

Plasmid.

A rhodamine-labeled plasmid (pGeneGrip) with hCMV IE promotor/enhancer driving the *β*-galactosidase gene was obtained from Gene Therapy Systems (San Diego, CA). Unlabeled plasmid (pCMV*LacZ*) was obtained from the DNA Sequencing Core Facility (University of Pennsylvania, Philadelphia, PA).

Cell culture.

CF airway epithelial cells in primary culture were prepared from nasal polyps from CF patients at the time of polypectomy in accordance with the Institutional Review Board of The Children's Hospital of Philadelphia. Stripped and gently minced pieces were placed in 25 cm^2 flasks (Becton Dickson Labware, Franklin Lakes, NJ), coated with collagen and incubated in SAGM medium (Clonetics, Walkersville, MD) at 37˚C in a humidified atmosphere of 5% CO_2. Based on morphology, when epithelial cells grew out from the tissue, the pieces were removed to a new flask until additional epithelial cells were observed. This procedure was continued until the cells no longer grew [8]. In addition CF/T43, an immortalized nasal epithelial cell line from a CF patient homozygous for the ΔF508 mutation, was obtained from Dr. J. Yankaskas (University of North Carolina, Chapel Hill, NC). The immortalized cells were passaged once a week and cultured as described [38].

Non-CF airway epithelial cells in primary culture were obtained from two sources. Normal human bronchial epithelial cells in primary culture, designated as NHBE by the provider, were purchased from Clonetics (Walkersville, MD). The NHBE cells were grown according to the Clonetics instructions. In addition human tracheal tissue was obtained from Dr. R. Panettieri (University of Pennsylvania, Philadelphia, PA) and treated and cultured as described above for the CF airway epithelial cells in primary culture.

For experiments CF and non-CF airway epithelial cells from primary culture were seeded at 1.2 X 10^5 on 25-mm collagen coated glass coverslips and grown 48 hour in SAGM medium prior to transfection. CF/T43 cells were seeded at 1.2 X 10^5 on 25-mm glass cover slips and grown for 24 hours in KGM medium (Clonetics, Walkersville, MD) prior to transfection.

Preparation of vector/plasmid complex.

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FITC-labeled lactosylated, mannosylated or TSA-poly-L-lysine was added dropwise to the rhodamine–labeled plasmid in a 4:1 (wt/wt) ratio and held for 30 min at ambient temperature. In some cases unlabeled lactosylated poly-L-lysine was added to rhodamine-labeled plasmid or FITC-labeled lactosylated poly-L-lysine was added to unlabeled plasmid. The complex was suspended, 3.3 μg of DNA per 1 ml in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD). When CF/T43 cells were transfected the transfection medium contained 100 μM chloroquine (Sigma, St Louis, MO). The transfection medium for CF and non-CF primary cells contained 50 μM chloroquine [4].

Binding of glycosylated poly-L-lysine/cDNA complexes to airway epithelial cells.

Airway epithelial cells on coverslips were kept at 4°C to allow binding but not energy-dependent internalization [39] for 20 minutes. In some experiments the cells

were subsequently incubated with either 300 µl DMEM or 300 µl DMEM containing 0.1 M lactose or 0.1 M GlcNAc or 200 µg lactosylated poly-L-lysine at 4°C. After 30 minutes DMEM was removed and cells were incubated at 4°C for 60 minutes with 1 µg rhodamine-labeled cDNA complexed to 4 µg FITC-labeled lactosylated poly-L-lysine in 300 µl DMEM. When applicable the transfection medium contained 0.1 M lactose or 0.1 M GlcNAc or 200 µg lactosylated poly-L-lysine. Subsequently cells were washed with PBS three times and fixed in 1% paraformaldehyde in PBS. The coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

To detect the binding of complexes to the cells, microscopic images both fluorescent and phase-contrast were obtained with an Olympus IX70 microscope equipped with a Hamamatsu digital camera (HiTech Instruments, Edgemont, PA). The phase contrast and fluorescent image were digitaly merged and processed using Metamorph Imaging System software. From each coverslip 3 fields (30X or 60X) were taken and cells and complexes were counted visually. Statistical analyses were done with Excel software (Microsoft) using double-tailed paired Student's *t* test.

Detection of lactosylated poly-L-lysine/cDNA complexes in clathrin coated pits.

We used ferritin to mark lactosylated poly-L-lysine/cDNA complexes for electron microscopy detection ferritin was used. Lactosylated poly-L-lysine mixed with 10% ferritin was added dropwise to plasmid in a 4:1 (wt/wt) ratio and held for 30 min at ambient temperature. CF/T43 cells grown in 6-well plates were incubated with the lactosylated poly-L-lysine-ferritin/cDNA complex for 30 minutes at 37°C.

At the end of the incubation period, cells were washed briefly with serum-free buffer and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.2 M sodium cacodylate buffer for 1 hour. The cells were collected from the culture plate and centrifuged to generate a tight pellet, and post-fixed with 1% osmium tetroxide. The pellet was dehydrated with ascending grade of ethanol, embedded in LX-112 medium, and polymerized at 68^{o}C for 72 hours. Ultrathin sections were cut with a diamond knife,

mounted on 200-mesh thin-bar copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips CM-100 electron microscope operated at 60 kv accelerating voltage.

Intracellular trafficking experiments.

To follow the internalization of the vector/plasmid complexes with transferrin, Alexa-Fluor 594 conjugate of transferrin (Molecular Probes, Eugene, OR) (50 μg/ml) was added to 300 μl of transfection mixture and then added to cells grown on coverslips. The cells were incubated at 37ºC with 5% CO_2 for the specified times up to 6 hours. At the designated times the transfection medium was removed and cells were washed with PBS (pH 7.3) and fixed with ice-cold methanol at 4˚C for 8-10 minutes. Subsequently Hoechst 33342 stain (Molecular Probes, Eugene, OR) was applied to label the nucleus and the coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Confocal microscopic images of the cells were obtained using a computer-interfaced, laser-scanning microscope (Leica TCS4D), of the Confocal Core Facility, Children's Hospital of Philadelphia. Slides were sectioned optically at 1.0 -μm intervals through the cell monolayer to obtain the appropriate focal depth. An argon/krypton mixed gas laser with excitation lines at 351, 488, 560 nm was used to induce fluorescence. The blue fluorescence (nuclear stain) was induced by the 351 nm wavelength. Excitation of the green fluorophore (FITC-signal) was achieved by using the 488 nm excitation wavelength, with resulting fluorescent wavelengths observed by using an optical filter. Red fluorescence (rhodamine-signal) was induced by the 560 nm excitation line. Images were digitalized with Leica Scanware. Obtained images were processed, including digital enlargement, and embossed with a 10-μm bar using Adobe Photoshop.

To detect lactosylated poly-L-lysine/cDNA complexes in early endosomes, cells grown on coverslips were incubated with either FITC-lactosylated polylysine/unlabeled cDNA complex or unlabeled lactosylated poly-L-lysine/rhodamine-cDNA complex for 30

minutes. For subsequent immunofluorescence cytochemistry [40] cells were fixed in 1% paraformaldehyde in PBS, followed by washes in PBS with 5 mM NH_4Cl . To reduce autofluorescence cells were incubated in freshly prepared 0.1% sodium borohydride and was followed by incubation with 5% BSA, 10% normal goat serum in PBS for 30 minutes at ambient temperature to reduce nonspecific binding. Cells were permeabilized using 0.3% Triton X-100 in PBS. Specific antiserum was diluted in PBS containing 0.3% Triton X-100 in PBS with 5% BSA and 10% normal goat serum. The unlabeled primary antibody to early endosome antigen 1 (EEA1, Transduction Laboratories, Lexington KY) 1:100 was applied overnight at 4°C. After washing in PBS with 0.3% Triton X-100 in PBS, Alexa Fluor goat anti mouse IgG (Molecular Probes, Eugene, OR) was diluted at 2.5:1000 in the above described blocking solution and incubated for 2 hours at 4°C. When cells were transfected with FITC-labeled complex Alexa Fluor 594 goat anti mouse IgG was used and in the case of transfection using rhodamine-labeled complex Alexa Fluor 488 goat anti mouse IgG was applied. Cells were washed in PBS with 0.3% Triton X-100. DAPI (Molecular Probes, Eugene, OR) was applied to mark nuclei and coverslips were mounted using ProLong (Molecular Pobes, Eugene, OR). The cells were examined by confocal microscopy as described above.

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CHAPTER 3

Nuclear Translocation of Lactosylated Poly-L-lysine/cDNA Complex in Cystic Fibrosis Airway Epithelial Cells

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Abstract

Poly-L-lysine, with 40% of the amino-groups substituted with lactose, is an effective vector to transfer the CFTR gene into CF airway epithelial cells and correct the chloride channel dysfunction. The intracellular fate of the lactosylated poly-L-lysine/cDNA complex was studied using confocal microscopy. In the presence of chloroquine the complex remained intact during internalization, intracellular transport and, most importantly, transport into the nucleus. When cells were transfected in the presence of agents that enhance transfection efficiency such as E5CA peptide, a fusogenic peptide, or glycerol a similar fate of the lactosylated poly-L-lysine/cDNA complex was seen. However when these agents were omitted from the transfection medium, the complex remained in the perinuclear region. Uncomplexed lactosylated poly-L-lysine reached the nucleus efficiently. In contrast mannosylated poly-L-lysine or unsubstituted poly-L-lysine complexed to plasmid did not. Therefore the nuclear accumulation of the complex may be attributed to the substitution of poly-L-lysine with lactose. It is hypothesized that the lactose residues provide for nuclear localization by means of targeting a potential lectin-like protein with galactose/lactose specificity. This mechanism may be responsible for the nuclear internalization of the complex.

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Introduction

Glycosylated polylysines, in which 30 to 40% of the ε-amino groups of poly-L-lysine are substituted with a specific mono- or di-saccharide, represent a novel approach to gene transfer. Lactosylated polylysine has been employed successfully to transfect airway epithelial cells with a high efficiency using both reporter genes [1, 2] and CFTR cDNA [3]. In the latter studies, functional correction of the chloride conductance was obtained in cystic fibrosis (CF) airway epithelial cells grown in primary culture.

Poly-L-lysine has been used alone in gene transfer because it compacts DNA efficiently and it can be used as a crude transfecting agent without substitution. However, alone and unsubstituted, it is toxic to many cells, immunogenic, does not release bound DNA easily after internalization and does not provide specific targeting. In contrast, the glycosylated polylysines are neither immunogenic nor cytotoxic. The method for substituting polylysine with simple sugars was described by Monsigny *et al*. [4]. As an alternative approach to CF lung disease, this method was adapted to transfect airway epithelial cells and lactose was demonstrated to be the optimal sugar for targeting to the CF cells in primary culture [1].

CF is a disease for which a number of Phase I clinical trials of gene therapy were initiated [5]. The vectors employed in these Phase I trials have included recombinant adenoviruses, AAV, liposomes and cationic lipids. While there have been some positive results, the success of the vectors until now has been limited by either immunogenicity or low efficiency [6-8]. A fundamental obstacle to using the adenovirus, the absence of receptors for the adenovirus on the apical surface of airway epithelial cells, was emphasized in a review by Boucher [7].

Some have predicted that non-viral vectors will be the preferred choice for gene therapy in the future and they have described their advantages [5, 9]. However a major barrier to increasing the efficiency of non-viral vectors has been that DNA uptake into the

nucleus was a rate limiting factor [10]. In this report, it is shown that lactosylated polylysine, complexed with plasmid, trafficks to the nucleus and is internalized into the nucleus along with the complexed plasmid.

Results

Lactosylated poly-L-Lysine/cDNA complex was translocated into the cell nucleus intact and required chloroquine.

Lactosylated poly-L-lysine has proven to be an efficient gene delivery vehicle in the presence of chloroquine [1]. Poly-L-lysine is known to condense DNA [11] but further elucidation of the role of lactosylated poly-L-lysine in the transfection process was needed. Chloroquine is frequently used as an agent to enhance transfection for various gene delivery systems such as molecular conjugates [12] but its influence is still unclear. Therefore the intracellular fate of the lactosylated poly-L-lysine/cDNA complex in airway epithelial cells in primary culture was investigated during transfection in the presence and absence of 50 μM chloroquine (Fig. 1).

Early in the transfection process the intracellular fate of the complex was similar in both cases. Ten minutes after the administration of the transfection medium there was faint general fluorescence distributed fairly homogeneously throughout the cells on the coverslip but optical sectioning of the cells at this time showed the presence of the green fluorescent signal in and around the nucleus (Figs. 1A and 1B). Occasionally some complex was present around the nucleus. Thirty minutes post transfection a vesicular form containing both the FITC (lactosylated poly-L-lysine) and the rhodamine (plasmid) signal, thus appearing yellow, was detected in the cytoplasm and in the perinuclear region (Figs. 1C and 1D). The fluorescence was still present on the outside of the cells.

After 1 hour the double-labeled lactosylated poly-L-lysine/plasmid complex was present in the nucleus with chloroquine in the transfection medium (Fig. 1E). In addition the

vesicles increased in number throughout the cytoplasm and the green fluorescence could no longer be observed on the outside of the cell membrane one hour post transfection. When chloroquine was omitted nuclear localization of the complex could not be detected and vesicles started to accumulate in the perinuclear region (Fig. 1F).

The difference became more prominent as time progressed. In the presence of chloroquine there was an increase of complex in the nucleus and the perinuclear region after 2 hours (Fig. 1G). This increase continued for the next 2 hours and some dissociation of the complex was observed as seen by the separation of the green and red signal (Fig. 1I). After six hours, accumulation of the labeled lactosylated poly-L-lysine/cDNA complex in both the nucleus and the perinuclear region was seen. Also there was an increase of green fluorescence in the perinuclear region (Fig. 1K). When the cells were examined at a lower magnification (40X) at this time post transfection, it was shown that the complex was present in approximately 90% of the cells. Although it appeared that most complex was in and around and the nucleus (Fig. 2A), optical sectioning at 100X magnification (Fig 2B) showed that the complex was present in 30% of the cell nuclei.

When chloroquine was omitted from the transfection medium more vesicles containing the complex appeared but most were retained in the cytoplasm after two hours. Occasionally the complex could be detected in the nucleus (Fig. 1H). Four hours (Fig. 1J) and six hours (Fig. 1L) after the addition of the transfection medium, the accumulation of the complex in the perinuclear region progressed and little nuclear localization of the complex was observed.

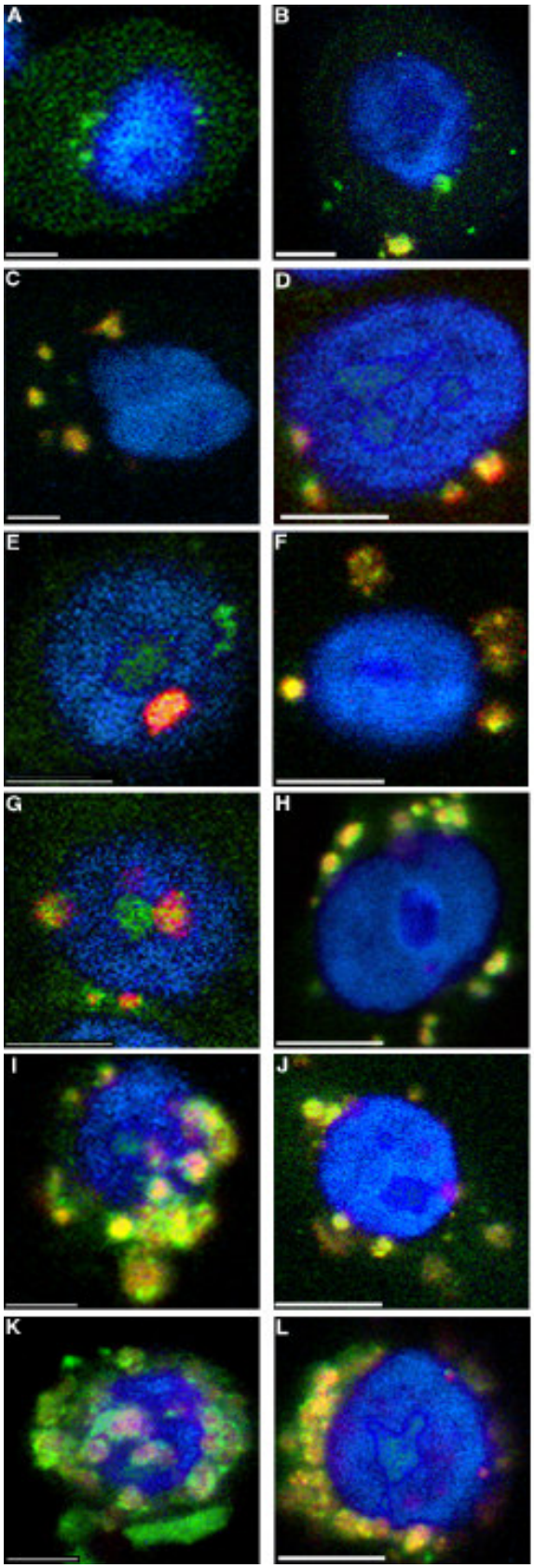

Figure 1. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in airway epithelial cells in primary culture in the presence and absence of chloroquine. Cells were grown on cover slips for 48 hrs. The cells were transfected with FITC-labeled lactosylated polylysine complexed to rhodamine labeled pCMV*Lac*Z in the presence or absence of 50 µM chloroquine. At designated times after addition, transfection medium was removed and cells were washed. Subsequently the cells were fixed in methanol and the nuclei were stained with Hoechst reagent. A Leica TCS4D confocal microscope was used for optical sectioning. Rhodamine was visualized in red, FITC was visualized in green and Hoechst was in blue. Yellow fluorescence represents the co-localization of the rhodamine and FITC signal. Left column represents the time course of the complex in the presence of chloroquine and the right column represents the time course without chloroquine present. Times post addition were as follows: A,B) 10 minutes; C,D) 30 minutes; E,F) 1 hour; G,H) 2 hours; I,J) 4 hours; K,L) 6 hours. Original magnification X100. *Size bar, 10 µm*.

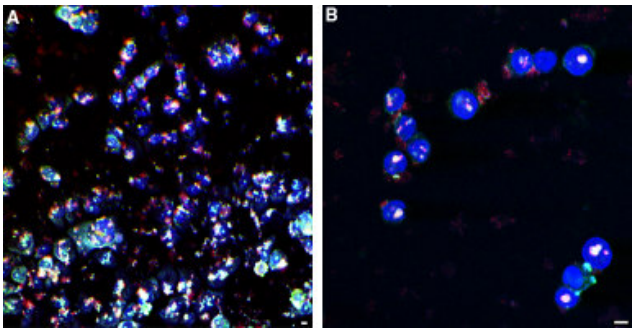

Figure 2. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in airway epithelial cells in primary culture in the presence of 50 µM chloroquine 6 hours post transfection. Original magnification A) 40X; B) 100X. See legend to Figure 1. *Size Bar, 10 µm*.

To investigate nuclear membrane integrity the nuclear envelope was visualized with anti lamin A&C. Fig 3 shows the nuclear accumulation of lactosylated poly-L-lysine/rhodamine–labeled cDNA complex (red fluorescence) in primary CF airway epithelial cells in the presence of 50 µM chloroquine. The green fluorescence represents anti lamin A&C. Co-localization of the green and red signal could not be detected in the nucleus. Therefore the complex present in the nucleus did not co-localize with the nuclear membrane. The possibility of nuclear invaginations of complex was ruled out. The complex observed in the nucleus must have passed the nuclear membrane and is located intranuclearly.

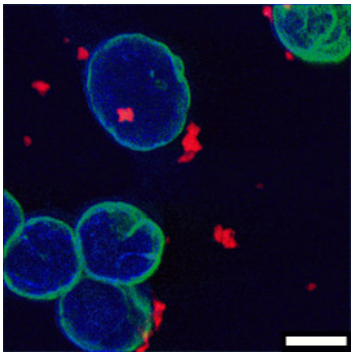

Figure 3. Photomicrograph of the nuclear membrane and lactosylated polylysine/rhodamine-labeled DNA complex in airway epithelial cells in primary culture in the presence of 50 µM chloroquine 6 hours post transfection. After cells were fixed, the nuclear envelope was stained with anti lamin AC and visualized in green fluorescence. See legend to Figure 1 and *Materials and Methods.* Original magnification X100. *Size bar, 10 µm*.

These results showed that when lactosylated poly-L-lysine and cDNA were complexed, the complex remained stable during cellular internalization and intracellular transportation. Most importantly the co-localization persisted during the transport into the nucleus (Fig. 1C). When the complex was in the nucleus, dissociation occurred after four hours at which time the exogenous DNA became available for transcription. After separation of the plasmid and the lactosylated poly-L-lysine, the vector may be transported or diffuse out of the nucleus, as detected by the increase of green fluorescent signal in the perinuclear region. However without chloroquine very little

complex entered the nucleus, in contrast to transfection with chloroquine as shown (Figs. 1K and L). Early in the transfection process the patterns as seen with (Figs. 1A and 1C) and without (Figs. 1B and 1D) chloroquine were similar. The omission of chloroquine did not appear to affect the cellular internalization of the complex. Therefore the presence of chloroquine was required for the nuclear accumulation of the lactosylated poly-L-lysine/cDNA complex. This is in concordance with previous reports that chloroquine effected the intracellular path of the complex [13].

Mannosylated Poly-L-Lysine and TSA Poly-L-Lysine were less efficient in nuclear translocation.

Mannosylated poly-L-lysine [1] and TSA-poly-L-lysine [2] were less efficient in gene transfer than lactosylated poly-L-lysine. This difference in gene transfer efficiency was more marked for airway epithelial cells in primary culture. To determine whether the intracellular fate of either poly-L-lysine/cDNA complex would differ from that of lactosylated poly-L-lysine/cDNA complex, airway epithelial cells in primary culture were transfected with either mannosylated poly-L-lysine/cDNA complex or TSA-poly-L-lysine/cDNA complex in the presence of 50 μM chloroquine. After six hours the cellular localization of the complex was visualized.

Mannosylated poly-L-lysine/cDNA complex was internalized and translocated into the nucleus (Fig. 4A). However the nuclear translocation of cDNA was less than when lactosylated poly-L-lysine was used for gene transfer. When cells were examined at 40X magnification it was found that mannosylated poly-L-lysine/cDNA complex was located in 80% of the cells (Fig 5A). Optical sectioning at 100X demonstrated that mannosylated poly-L-lysine/cDNA complex was located in only 10% of the nuclei (Fig. 5B). TSA poly-L-lysine was even less efficient for nuclear translocation of cDNA. Very little complex was detected in the nucleus and most of the complex was aggregated in the perinuclear region (Fig. 4B).

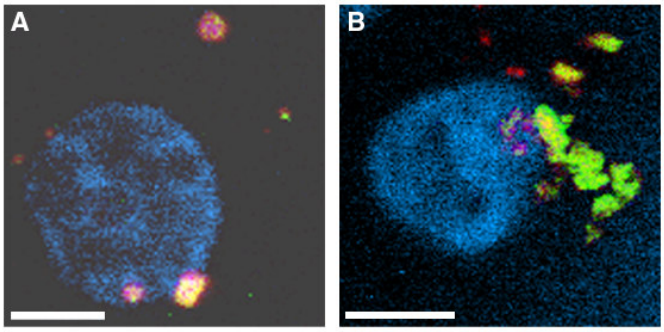

Figure 4. Photomicrographs of FITC-labeled mannosylated polylysine/rhodamine-labeled DNA complex and FITC-labeled TSA-polylysine/rhodamine-labeled DNA complex. Airway epithelial cells in primary culture were transfected in the presence of 50 µM chloroquine. After 6 hours the cellular localization of the complex was detected. A) Mannosylated polylysine/cDNA complex; B) TSA-polylysine/DNA complex. See legend to Figure 1. Original magnification X100. *Size bar, 10 µm*.

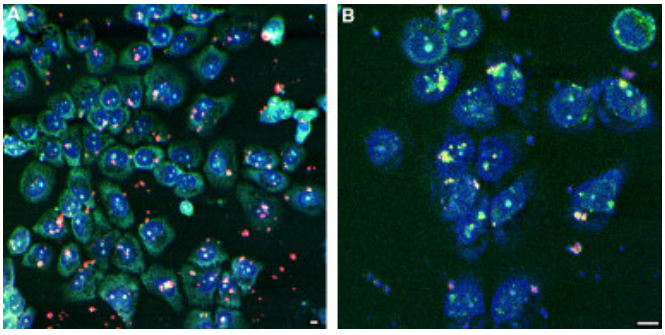

Figure 5. Photomicrographs of FITC-labeled mannosylated polylysine/rhodamine-labeled DNA complex in airway epithelial cells in primary culture. Cells were transfected in the presence of 50 µM chloroquine and fixed 6 hours post transfection. Original magnification A) 40X; B) 100X. See legend to Figure 1. *Size bar, 10 µm*.

Nuclear accumulation of lactosylated poly-L-Lysine/cDNA complex is inhibited by wheat germ agglutinin (WGA).

In order to determine whether the transfection process with lactosylated poly-L-lysine/cDNA complex was responsive to agents that block the nuclear pore complex (NPC), cells were pretreated with WGA and subsequently transfected. WGA binds to the N-acetylglucosamine residues present on a class of NPC proteins and is thought to block the channel [14]. Primary CF airway epithelial cells were incubated for 60 min with FITC-labeled WGA and subsequently transfected with lactosylated poly-L-lysine/ rhodamine-labeled cDNA complex in the presence of 50 µM chloroquine.

After one hour incubation with FITC-labeled WGA, the WGA was present around the nucleus (Fig. 6A) and was still present 6 hours later (Fig. 6B). When WGA treated cells were transfected, the complex could not be detected in the nucleus even after 6 hours incubation. Instead the complex accumulated in the perinuclear region (Fig. 6C). At the same time untreated cells were transfected and as usual the nuclear accumulation of

the complex could be detected (Fig. 6D). Optical sectioning of the cell nucleus clearly demonstrated the intranuclear localization of the complex (Fig. 7). Fig 7A represents the first optical section (1 µm in thickness) showing complex located in the perinuclear region. Fig 7B represents the second section, taken 1 µm past the nuclear membrane. The complex is located in the next two sections (Fig. 7C and Fig. 7D). Fig 7E represents the opposite perinuclear region.

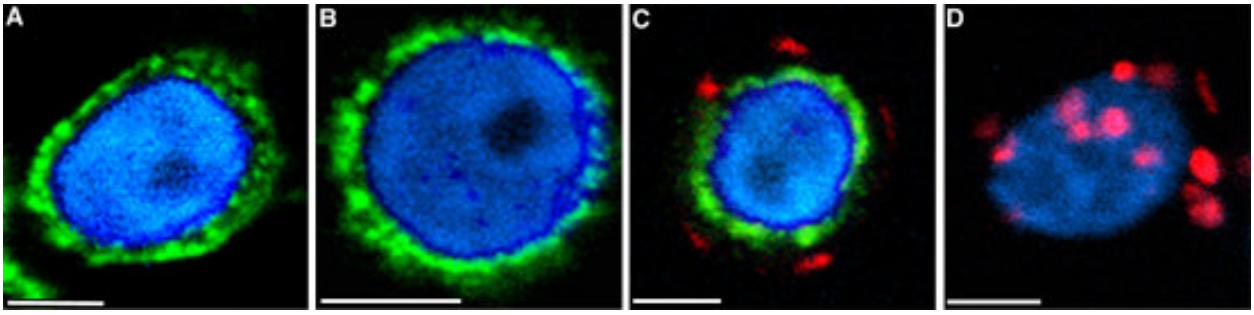

Figure 6. FITC-labeled WGA (green) inhibits nuclear accumulation of lactosylated polylysine/rhodamine-cDNA complex (red) in airway epithelial cells in primary culture. Cells were incubated with 300 µl DMEM containing 40 µg/ml FITC-WGA for A) 1 hour. After 1 hour the FITC-WGA was removed and cells were refed with growth medium for B) an additional 6 hours. After 1 hour incubation C) with FITC-WGA or D) DMEM cells were transfected with lactosylated polylysine/rhodamine-cDNA complex for 6 hours. See legend to Figure 1. Original magnification X100. *Size bar, 10 µm*.

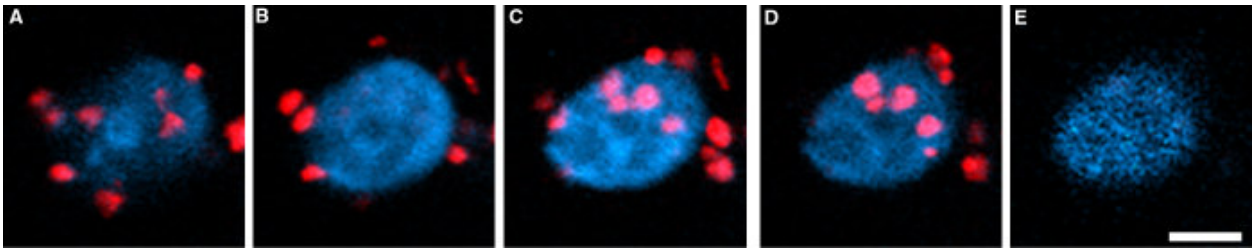

Figure 7. Photomicrographs representing optical sections through the nucleus which demonstrate the intranuclear localization of lactosylated polylysine/rhodamine-labeled DNA complex. Nucleus is sectioned down in 1-µm thick slices starting from nuclear envelope. A) 0-1 µm; B) 1-2 µm; C) 2-3 µm; D) 3-4 µm; E) 4-5 µm. See legend to Figure 1. Original magnification X100. *Size bar, 10 µm*.

These results showed that WGA, a known nuclear import inhibitor, prevented nuclear accumulation of complex in airway epithelial cells. This suggests that the NPC may be involved in the nuclear import of lactosylated poly-L-lysine/cDNA complex.

E5CA peptide and Glycerol enhance nuclear translocation of lactosylated poly-L-Lysine/cDNA complex.

It was previously shown [2] that E5CA peptide, a fusogenic peptide, and glycerol were both capable of enhancing transfection efficacy and efficiency of lactosylated poly-L-lysine *in vitro*. Fusogenic peptides [15] and glycerol [16] have been used by others for enhancement of gene transfer. To assess the influence on the transfection process, the intracellular fate of the double-labeled lactosylated poly-L-lysine/cDNA complex in CF airway epithelial cells was followed in the presence of either 25 μg E5CA peptide (Fig. 8) or 5% glycerol (Fig. 9).

In the presence of 25 μg E5CA peptide the complex was detected in the nucleus two hours after the addition of the transfection medium (Fig. 8B). Over the next four hours the amount of complex increased in the perinuclear region. Moreover, nuclear accumulation was also observed (Fig. 8C). Compared to transfection aided with E5CA peptide the nuclear translocation of the complex in the presence of glycerol occurred more rapidly. The complex was detectable in nuclei after 30 minutes (Fig. 9B) after which it accumulated in and around the nucleus over the next 4 to 6 hours (Fig. 9C).

These results demonstrated that the vector/DNA complex reached the nucleus intact in the presence of either enhancing agents. Cellular uptake did not appear to be affected by 25 μg E5CA peptide (Fig. 8A) or 5% glycerol (Fig. 9A). Thus glycerol and E5CA peptide may influence the intracellular path of the transfection process, therefore enhancing nuclear translocation of the complex.

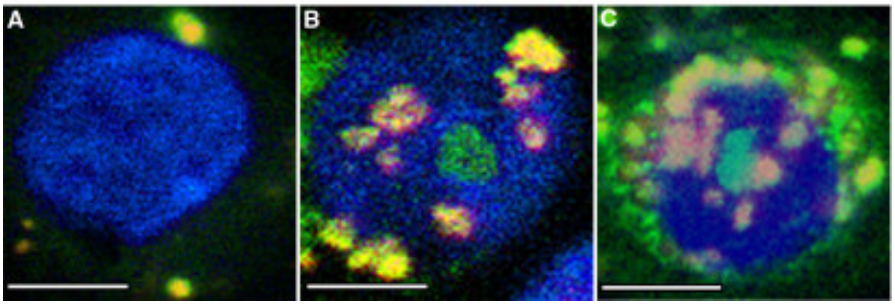

Figure 8. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in the presence of E5CA peptide. Airway epithelial cells in primary culture were transfected with FITC-labeled lactosylated polylysine complexed to rhodamine labeled pCMV*Lac*Z in the presence of 25 µg E5CA peptide. Times post transfection were as follows: A) 30 minutes; B) 2 hours; C) 6 hours. See legend to Figure 1. Original magnification X100. *Size bar, 10 µm*.

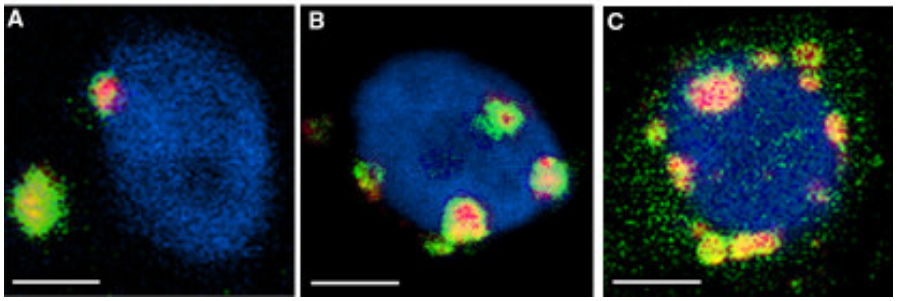

Figure 9. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in the presence of glycerol. Airway epithelial cells in primary culture were transfected with FITC-labeled lactosylated polylysine complexed to rhodamine labeled pCMV*Lac*Z in the presence of 5% glycerol. Times post transfection were as follows: A) 10 minutes; B) 30 minutes; C) 6 hours. See legend to Figure 1. Original magnification X100. *Size bar, 10 µm*.

Addition of second agent to E5CA peptide accelerated nuclear translocation

When airway epithelial cells were transfected with lactosylated poly-L-lysine/cDNA complex in a transfection medium that contained only chloroquine, a transfection efficiency of 20% was accomplished. However the transfection efficiency increased dramatically when a second enhancing agent was added [2]. It has also been shown that the combination of E5CA peptide and glycerol in the transfection medium gave an efficiency of 90% in CFTR gene transfer in CF airway epithelial cells in

primary culture [3]. To assess the effect of two enhancing agents on transfection in airway epithelial cells in primary culture the lactosylated poly-L-lysine/cDNA complex was followed in the presence of 50 μM chloroquine and 25 μg E5CA peptide (Fig. 10) or 5% glycerol and 25 μg E5CA peptide (Fig. 11).

In both cases the complex was detected in vesicles (Figs. 10A and 11A) immediately after internalization. One hour post transfection the complex was present in the nucleus (Fig. 10B and 11B). After the next five hours there was an increase in number of vesicles in the perinuclear region and the complex accumulated in the nucleus (Figs. 10C and 11C). When cells were transfected in the presence of only E5CA peptide the complex was detected in the nucleus after two hours (Fig. 8B).

These results show that two enhancing agents combined accelerated nuclear translocation. Combination of enhancing agents have been shown to increase reporter gene expression and to increase transfection efficiency [3].

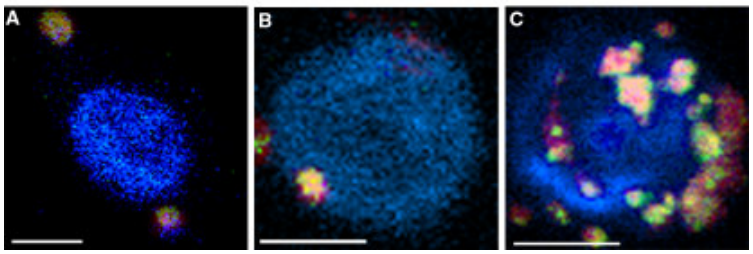

Figure 10. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in the presence of E5CA peptide and chloroquine. Airway epithelial cells in primary culture were transfected with FITC-labeled lactosylated polylysine complexed to rhodamine labeled pCMV*Lac*Z in the presence of 25 μg E5CA peptide and 50 μM chloroquine. Times post transfection were as follows: A) 30 minutes; B) 2 hours; C) 6 hours. See legend to Figure 1. Original magnification X100. *Size bar, 10 μm.*

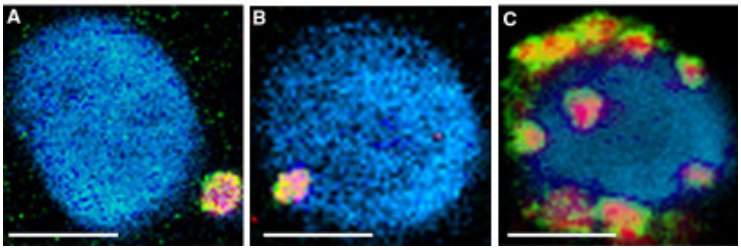

Figure 11. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in the presence of E5CA peptide and glycerol. Airway epithelial cells in primary culture were transfected with FITC-labeled lactosylated polylysine complexed to rhodamine labeled pCMV*Lac*Z in the presence of 25 μg E5CA peptide and 5% glycerol. Times post transfection were as follows: A) 30 minutes; B) 2 hours; C) 6 hours. See legend to Figure 1. Original magnification X100. *Size bar, 10 μm.*

Transfection of CF/T43 cells was similar to that in airway epithelial cells in primary culture.

The gene transfer efficiency of lactosylated poly-L-lysine in CF/T43 cells, an immortalized CF airway epithelial cell line, was investigated previously [1]. When the transfection was visualized the fate of lactosylated poly-L-lysine/cDNA complex in CF/T43 cells (Fig. 11) was found to be similar to that in cells in primary culture.

Thirty minutes post transfection of CF/T43 cells in the presence of 100 μM chloroquine, yellow vesicles, representing the co-localization of the FITC and the rhodamine signal, were detected in the cytoplasm and in the perinuclear region (Fig. 12A). After 1 hour the lactosylated poly-L-lysine/plasmid complex was present in the nucleus. The vesicles increased in number throughout the cytoplasm (Fig. 12B). The accumulation of the complex in the perinuclear region and in the nucleus was observed after six hours (Fig. 12C). The intracellular paths of the complex in CF/T43 cells (Fig. 12) and in airway epithelial cells in primary culture (Fig. 1) were similar. Only the nuclear accumulation of the complex after six hours in the primary cells (Fig. 1K) was more prominent when compared to CF/T43 cells (Fig. 12C).

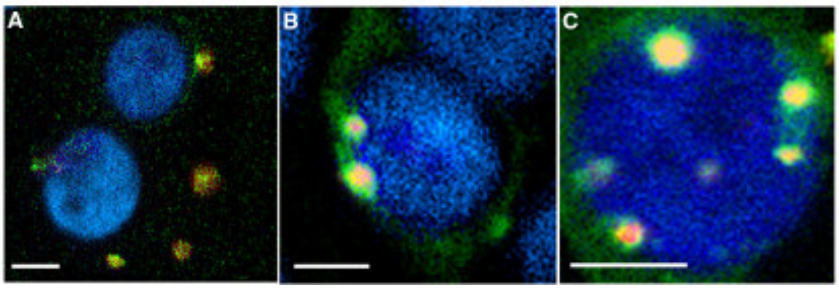

Figure 12. Photomicrographs of FITC-labeled lactosylated polylysine/rhodamine-labeled DNA complex in CF/T43 cells. The cells were transfected with FITC-labeled lactosylated polylysine complexed to rhodamine labeled pCMV*Lac*Z in the presence of 100 μM chloroquine. See legend to Figure 1. Times post transfection were as follows: A) 30 minutes; B) 1 hour; C) 6 hours. Original magnification X100. *Size bar, 10 μm*.

Lactosylated Poly-L-Lysine Entered the Nucleus Rapidly but Naked DNA did not

The transfection of airway epithelial cells with naked plasmid was very inefficient compared to transfection efficiency of cDNA complexed to lactosylated poly-L-lysine (2). To determine how transfection with naked plasmid proceeded, CF/T43 cells were transfected with medium containing 1 μg rhodamine-labeled pCMV*LacZ.* Little rhodamine signal could be detected in the cells after ten minutes (Fig. 13A) or even after one hour (Fig. 13B). Finally there was little rhodamine signal detectable in the nuclei after six hours (Fig. 13C).

In contrast, when the vector alone, 4 μg of FITC-labeled lactosylated poly-L-lysine, was added to the cells green fluorescence could be observed in the nucleus after 10 minutes (Fig. 13D). A similar image was observed when cells were incubated with complex (Fig. 1A). Therefore the green fluorescence observed in and around the nucleus early in the transfection process can be attributed to unbound lactosylated poly-L-lysine. In the next one (Fig.13E) to six hours (Fig. 13F) the FITC signal accumulated in the perinuclear region and in the nucleus.

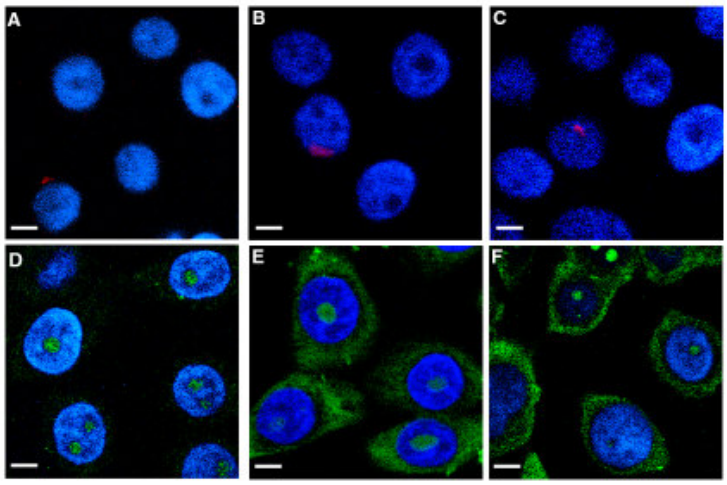

Figure 13. Photomicrographs of FITC-labeled lactosylated polylysine only and uncomplexed rhodamine-labeled DNA in CF/T43 cells. Cells were grown on cover slips for 24 hrs. The cells were incubated with either A-C) rhodamine-labeled pCMV*Lac*Z or D-E) FITC-labeled lactosylated polylysine in the presence of 100 μM chloroquine. See legend to Figure 1. Times post transfection were as follows: A and D) 10 minutes; B and E) 1 hour; C and F) 6 hours. Original magnification X100. *Size bar, 10 μm.*

The results showed that the internalization and nuclear translocation of uncomplexed plasmid was far less than that of plasmid complexed to lactosylated poly-L-lysine. In contrast, lactosylated poly-L-lysine itself was internalized and transported to the nucleus very efficiently. Therefore the high efficiency in gene transfer of airway epithelial cells using lactosylated poly-L-lysine/plasmid complex can be attributed to properties of lactosylated poly-L-lysine.

Expression of rhodamine-Labeled pCMVLacZ delivered by lactosylated poly-L-lysine in CF/T43 Cells

In the previous experiments the intracellular path of the complex was followed and the nuclear localization of the complex was clearly observed. For expression of the rhodamine-labeled pCMV*LacZ*, CF/T43 cells were transfected with 2.0 μg rhodamine-labeled plasmid, complexed to 8 μg FITC-labeled lactosylated poly-L-lysine for 4 hours in the presence of 100 μM chloroquine. For comparison cells were also transfected with naked DNA. After 48 hours the expression of β-galactosidase was measured. It was found that when cells were transfected with the complex the expression of the reporter gene was 100 fold higher then when cells were transfected with naked DNA (Fig. 14). Thus as expected, not only was the lactosylated poly-L-lysine/cDNA complex

transported into the nucleus but also transcription and translation of the reporter gene took place.

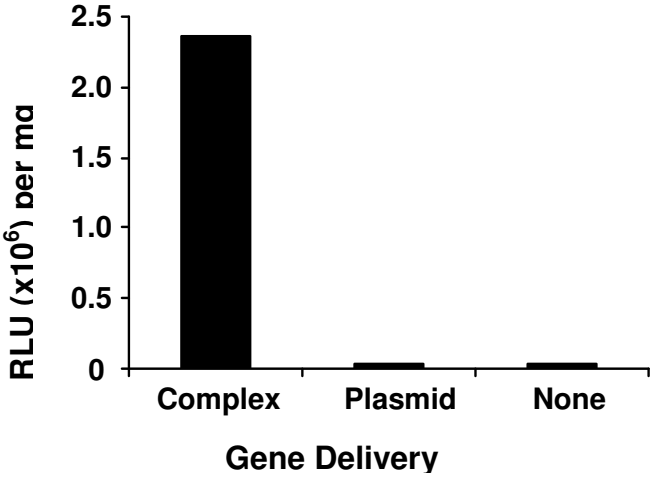

Figure 14. Expression of the rhodamine-labeled pCMV*LacZ.*
Complex) FITC-labeled lactosylated poly-L-lysine complexed to rhodamine-labeled plasmid;
Plasmid) rhodamine-labeled plasmid only
None) medium only
The expression of pCMV*LacZ* was detected with the Galacto-Star assay. See *Materials and Methods* for details.

Discussion

The intracellular trafficking of the lactosylated poly-L-lysine/cDNA complex in CF airway epithelial cells was tracked using confocal microscopy. Optical sectioning of the cell nucleus showed that under conditions, which are used to enhance expression, the lactosylated poly-L-lysine/cDNA complex entered the nucleus (Fig 1). In addition, the detection of the nuclear membrane with antibody to lamin A and C (Fig. 3) added further proof of the nuclear location, since invaginations were not found in conjunction with the complex.

The nuclear translocation of lactosylated poly-L-lysine/cDNA complex is attributed to presence of the lactose residues on poly-L-lysine. Nuclear localization of lactosylated poly-L-lysine was detected within 10 minutes, when cells were incubated with lactosylated poly-L-lysine without plasmid (Fig 13D). Moreover, when cells were transfected with mannosylated poly-L-lysine/cDNA (Fig. 4A and Fig 5) or unsubstituted poly-L-lysine/cDNA (Fig. 4B) there was little nuclear localization of the complex in airway epithelial cells in primary culture. Gene transfer using lactosylated poly-L-lysine is known to be more efficient than mannosylated poly-L-lysine [1] in spite of the higher

cellular uptake of mannosylated poly-L-lysine/cDNA complex in non-CF airway epithelial cells [17].

The lactosylated polylysine/cDNA complex must traverse the surface membrane, and intracellular compartments prior to entering the nucleus. The nucleus is bound by the nuclear envelope, which poses a considerable hydrophobic barrier to macromolecular transport. [10]. The nuclear envelope consists of an outer nuclear membrane and an inner nuclear membrane, connected to each other by pore membranes [18]. Nuclear pore complexes (NPC) form aqueous channels through the double membrane of the nucleus (21) and thus create passageways for proteins and genetic material [19]. It is still unknown how DNA of therapeutic relevant sizes (up to 10 kbp) can pass through the NPC [20]. It was postulated that nuclear uptake occurs preferentially in those cells entering mitosis, consequent to break down of the nuclear membrane [21]. However transferrin/polylysine complexes used for gene transfer in the presence of glycerol gave high transgene expression levels in both growing and growth arrested fibroblasts [22]. Pollard *et al* [23] reported that some polycations facilitate the nuclear uptake of DNA complexes. It was reported that plasmid DNA up to 14.4 kbp localized in the nucleus in the absence of cell division. Its nuclear import required energy, cytoplasmic factors and was blocked by wheat germ agglutinin. Furthermore plasmid DNA is thought to form complex with proteins prior to transit to the NPC and translocation [24]. Likewise it has been shown that single-stranded DNA/protein complexes were efficiently imported in mammalian nuclei following the classical importin-dependent nuclear import pathway [25].

WGA clearly inhibits the entry of lactosylated polylysine/cDNA complex into the nucleus (Fig. 6). Passage through the NPC is inhibited by WGA which binds to *N*-acetylglucosamine residues present on a class of NPC proteins [14]. Entry into the nucleus is clearly a multi-faceted and complex process ranging from conformational changes of the virus DNA to conformational changes of the NPC. These mechanisms are reviewed by Gerber, *et al*., [26]. The relatively high number of cells containing the lactosylated poly-L-lysine/cDNA complex (Fig. 2) and shown to be within the nucleus

(Fig. 2A) corresponds to the expression of the plasmid pCMV*LacZ* after 48 hrs (Fig. 14). Moreover, the entry of complex into the nucleus gradually increased with time. If nuclear entry were a result of a mitotic event, the increase into a nucleus would occur in a single increment unless that airway epithelial cell in primary culture divided several times during the 6 hr period of incubation.

The mechanisms of entry into the nucleus are varied and will be the subject of future studies. Currently, we favor the hypothesis of entry of the lactosylated polylysine/cDNA complex through a receptor-mediated event for which the lactosyl residues provide the ligand. The specific nuclear import of glycoconjugates could be related to the presence of lectins in the cell nucleus [27]. Galectin-3 a galactose/lactose-specific lectin was located in the nucleus [28, 29]. The nuclear import and retention of galectin-3 has been ascribed to the carbohydrate recognition domain [30]. Galectin-3 has been shown to facilitate transfer of spliceosomes out of the nucleus [31]. Although the entry of large molecules into the nucleus depends on a short stretch of basic amino acids called nuclear localization signal (NLS) [32], glycosylated proteins lacking a conventional NLS can enter the nucleus [33]. The nuclear import of glycoproteins from the cytosol has been reported to be sugar dependent and is distinct from the conventional NLS pathway since sugar residues may function as nuclear targeting signals and thus define a new nuclear import system [34]. It is possible that the recently described Chrp protein [35] may play a role in translocating glycoproteins which are bound to Galectin-3 to the perinuclear region.

In order to reach the nucleus the complex must avoid degradation. In the presence of chloroquine the lactosylated poly-L-lysine/cDNA complex accumulated in both the nucleus and perinuclear region. In contrast when the airway epithelial cells in primary culture were transfected in the absence of chloroquine the complex was largely retained in the perinuclear region and cytoplasm (Fig 1L). Chloroquine is a widely used enhancing agent in non-viral gene transfer, but the effects of chloroquine on the transfection process are not yet fully understood. Chloroquine is a weak base and has an affinity for lysosomes. It penetrates the cells where it accumulates in acidic

compartments of endocytosed material, raising the luminal pH, inducing a reduction of the delivery to lysosomes and of the intravesicular degradation of endocytosed material [13]. Therefore chloroquine, by preventing lysosomal degradation ensured that the lactosylated poly-L-lysine/plasmid complex reached the perinuclear region intact where it was then transported into the nucleus. When chloroquine was omitted from the transfection medium the complex arrived in the perinuclear region in a degraded form (Fig. 1L). The potential function of the localization signal of the lactosylated poly-L-lysine may be lost in the degradation process and therefore nuclear localization is not achieved.

Escape from endosomes is key for transport into the nucleus. Poly-L-lysine has been described to have membrane disruptive properties, which were enhanced in the presence of glycerol [16]. In the presence of 5% glycerol the lactosylated poly-L-lysine/cDNA complex does enter the nucleus intact (Fig. 4B). Glycerol therefore enabled the lactosylated poly-L-lysine/cDNA complex to avoid lysosomal degradation. In the presence of E5CA peptide the lactosylated poly-L-lysine/DNA complex was able to enter the nucleus (Fig 3B). E5CA peptide may disrupt the vesicle membrane and so allowing the complex to enter the cytoplasm and find a way to the nucleus. Alternatively, fusogenic peptides have been described to mediate membrane fusion under low-pH conditions [36]. E5CA peptide may contribute to fusion of the vesicle membrane with the cell organelle membrane, prior to lysosomal degradation of the complex and so enable the complex to enter the nucleus.

In summary, lactosylated poly-L-lysine/cDNA complex remains intact during cellular internalization and nuclear translocation. These processes are enhanced by the presence of lactose substitution of poly-L-lysine. The internalization appears to take place via a lactose binding surface receptor and lactose may modulate intracellular trafficking as well as nuclear transport. These two features make lactosylated poly-L-lysine an attractive gene delivery vehicle for the development of future therapy for CF and other airway diseases.

Materials and Methods

Fluoresceinylated glycosylated polylysine.

Poly-L-lysine, HBr (average molecular weight 40,000) was purchased from Bachem Feinchemikalien, (King of Prussia, PA) and Fluorescein-5-isothiocyanate (FITC) isomer I was from Molecular Probes (Eugene, OR). Glycosylated poly-L-lysine was prepared as described [3]. Briefly, 4-isothiocyanatophenyl-derivatives of either β-D-Lac or α-D-Man were added to poly-L-lysine *p*-toluenesulfonate salt (TSA-poly-L-lysine) in dimethylsulfoxide in the presence of diisopropyllethylamine and reacted for 24 hours at room temperature. For fluorescent labeling, glycosylated poly-L-lysine (10 mg) was dissolved in dimethylsulfoxide containing diisopropylethylamine (5.0 μl). FITC isomer (1.0 mg in 400 μl DMSO) was added and the mixture was incubated in the dark at room temperature for one hour. The polymer was precipitated by the addition of 10 volumes of isopropanol. After centrifugation the isopropanol was removed. Subsequently the FITC-labeled glycosylated poly-L-lysine was dissolved in distilled water and lyophilized for storage at -10°C. For experiments a mixture of FITC-labeled glycosylated poly-L-lysine and unlabeled glycosylated poly-L-lysine in a 1:7 (wt:wt) ratio in distilled water was used.

Plasmid.

A rhodamine-labeled plasmid (pGeneGrip) with hCMV IE promotor/enhancer driving the β-galactosidase gene was obtained from Gene Therapy Systems (San Diego, CA).

Cell culture.

Airway epithelial cells in primary culture were prepared from tracheal and bronchial explants from CF patients obtained during lung transplantation at the time of surgery and from nasal polyps from CF patients at the time of polypectomy in accordance with the Institutional Review Board of The Children's Hospital of Philadelphia. Stripped and gently minced pieces were placed on 25 cm^2 flasks (Becton Dickson Labware, Franklin Lakes, NJ), coated with collagen and incubated in SAGM medium (Clonetics, Walkersville, MD) at 37˚C in a humidified atmosphere of 5% CO_2. When epithelial cells grew out from the tissue, the pieces were removed to a new flask until additional epithelial cells grew out and so forth. Cells were also obtained by protease treatment of the tissue and grown as described [1]. CF/T43, an immortalized nasal epithelial cell line from a CF patient homozygous for the ΔF508 mutation, was obtained from Dr. J. Yankaskas (University of North Carolina, Chapel Hill, NC) The immortalized cells were passaged once a week and cultured as described [37].

Conditions of transfection.

Airway epithelial cells in primary culture were seeded at 1.2 X 10^5 on 25-mm collagen coated glass coverslips and grown 48 hour in SAGM medium prior to transfection. CF/T43 cells were seeded at 1.2 X 10^5 on 25-mm glass cover slips and grown for 24 hours in KGM medium (Clonetics, Walkersville, MD) prior to transfection. When applicable cells were incubated with 12 μg FITC-labeled wheat germ agglutinin (WGA, EY Laboratories, San Mateo, CA) in 300 μl DMEM for one hour prior to transfection.

FITC-labeled lactosylated poly-L-lysine was added dropwise to the rhodamine–labeled plasmid in a 4:1 (wt:wt) ratio and held for 30 min at ambient temperature. The complex was suspended in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD). When applicable 100 μM or 50 μM chloroquine (Sigma, St Louis, MO) was added to CF/T43 cells and cells in primary culture respectively. In some

cases, 25 μg E5CA peptide (Core Laboratories of the Louisiana State University Medical Center, New Orleans, LA), 5% glycerol (Fisher, Pittsburgh, PA) or combination of agents was added. After the growth medium was removed, 300 μl of the final mixture, which contained 3.3 μg DNA per 1 ml was added to the cells grown on the coverslip. The cells were incubated at 37ºC with 5% CO_2 for the specified times up to 6 hours.

At the designated times transfection medium was removed and cells were washed with phosphate-buffered saline (PBS) pH 7.3 and fixed with ice-cold methanol at 4˚C for 8-10 minutes. Subsequently Hoechst 33342 stain (Molecular Probes, Eugene, OR) was applied to label the nucleus. The coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Immunocyto chemistry.

For immunofluorescence cytochemistry cells were fixed in 1% paraformaldehyde in PBS, followed by washes in PBS with 5 mM NH_4Cl. Cells on coverslips were incubated in freshly prepared 0.1% sodium borohydride to reduce autofluorescence, followed by 5% BSA, 10% normal goat serum in PBS for 30 minutes at room temperature to reduce nonspecific binding. Cells were permeabilized using 0.3% Triton X-100 in PBS. Specific antisera were diluted in PBS containing 0.3% Triton X-100 in PBS + 5% BSA and 10% normal goat serum. The unlabeled primary antibody was applied as followed: anti-lamin A&C JOL2 (Abcam, Cambridge, UK) 1:250 overnight at 4°C. After washing in PBS + 0.3% Triton-X in PBS, Alexa Fluor goat anti mouse IgG (Molecular Probes, Eugene, OR) was diluted at 2.5:1000 in the above described blocking solution and incubated for 2 hours at 4°C. Cells were washed in PBS+0.3% Triton-X. DAPI (4,6-diamidino-2-phenylidole, Molecular Probes, Eugene, OR) was applied to mark nuclei and coverslips were mounted using ProLong (Molecular Pobes, Eugene, OR) to reduce fading.

Confocal microscopy.

Confocal microscopic images were obtained using a computer-interfaced, laser-scanning microscope (Leica TCS4D), of the Confocal Core Facility, Children's Hospital of Philadelphia. Slides were sectioned optically at 1.0 -μm intervals through the cell monolayer to obtain the appropriate focal depth. The nucleus was typically scanned in 5 to 6 sections. The representative 1.0- μm image taken through the nucleus was chosen for photography. An argon/krypton mixed gas laser with excitation lines at 351, 488, 560 nm was used to induce fluorescence. The blue fluorescence (nuclear stain) was induced by the 351 nm wavelength. Excitation of the green fluorophore (FITC-signal) was achieved by using the 488 nm excitation wavelength, with resulting fluorescent wavelengths observed by using an optical filter. Red fluorescence (rhodamine-signal) was induced by the 560 nm excitation line. Images were digitalized with Leica Scanware. Obtained images were processed, including digital enlargement and embossed with a 10-μm bar using Adobe Photoshop.

Expression of the LacZ gene in CF/T43 cells.

In order to determine the expression of the rhodamine-labeled pCMV*LacZ*, CF/T43 cells were seeded 24 hours prior to transfection at 1.2 $X10^5$ cells per 25 mm well in a 12-well plate (Costar) and grown at 37°C. Subsequently cells were transfected for 4 hours using 2 μg rhodamine-labeled plasmid complexed to 8 μg FITC-labeled lactosylated poly-L-lysine or 2 μg plasmid alone in the presence of 100 μM chloroquine. Expression of the reporter gene was measured 48 hours after transfection with the Galacto-Star Chemiluminescent Reporter Gene Assay System (Tropix, Bedford, MA). Cells were processed according to manufacturer's instructions. 10 μl of the supernatant solution was added to Galacto-star substrate. After 90 minutes incubation luminescence was measured in a monolight 3010 luminometer (PharMingen, San Diego, CA).

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CHAPTER 4

Intracellular Transport of Lactosylated pDMAEMA-*co*-AEMA/DNA Complexes

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Abstract

We have developed a novel assay to determine the relationship between transduction efficiency and mitosis continuously monitoring cells with time-lapse microscopy. In this chapter we report that gene transfer in a model of human bladder carcinoma with a novel cationic polymer lactosylated pDMAEMA-*co*-AEMA requires a mitotic event. In addition, the intracellular transport of fluorescent-labelled lactosylated pDMAEMA-*co*-AEMA/cDNA complex was followed in live cells. Nuclear translocation into intact nuclei was not observed but the complex was detected in very near proximity of the nucleus. We conclude that nuclear transport through nucleopores in non-mitotic cells is not a likely explanation for the improved performance of lactosylated DNA carriers compared to non-lactosylated counterparts. Further, we propose that this assay provides a general method for the validation of karyotropic gene delivery systems.

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Introduction

At first sight, gene therapy, defined as the introduction of exogenous genes into human subjects with the aim of correcting phenotype or genotypic abnormalities, seems relatively simple to accomplish [1]. In reality this is far from true. Living organisms have evolved so as to ensure their own integrity and genetic stability and to protect themselves from intruding agents carrying foreign genomes. Almost two decades after the first reports of gene transfer experiments in animals with a non-viral vector [3], a non-viral GDS successfully applied in the clinic is yet to be reported. In Chapter 1 the problems of viral and non-viral gene delivery systems (GDS) and potential methods for gene therapy to overcome the problems are outlined [2]. A multitude of compounds is currently under investigation to develop a non-viral GDS for clinical applications. Although some are very effective in gene transfer *in vitro*, a major barrier has been that DNA uptake into the nucleus is an efficiency-limiting factor [4, 5].

Nuclear transfer of polyplexes.

There is still limited understanding of the cellular and molecular mechanisms involved in synthetic vector mediated gene transfer, especially in the trafficking of DNA to the site of transcription in the nucleus. A hydrophobic double membrane containing nucleopores bound the nucleus. In the nucleopores are NPCs that are composed of about 50 unique proteins, known collectively as nucleoporins (Nups) [6]. The Nups form a central cylinder, which is about 130 nm in diameter. Filamentous attachments that presumably interact with incoming and outgoing receptor complexes extend to 50–100 nm at both the cytoplasmic and nucleoplasmic faces [7]. Passive diffusion of metabolites and small macromolecules across the nucleopore is rapid but but larger cargo is transported in an active, signal- and energy mediated manner [8]. The diameter of the channel can expand from 10 to 25 nm to translocate cargo macromolecules that are as large as several Mega-Daltons (close to 40 nm in diameter) [9]. That nuclear transfer of very large protein/DNA complexes is actually possible, is shown by the fact that viral capsids are able to transduce nondividing cells. The capsid of adenovirus type 2 binds to the CAN/Nup214 nucleoporin and dissociates, followed by DNA entering the nucleus

through the nuclear pore accompanied by adenoviral protein VII [10]. The cigar-shaped capsid of baculovirus docks onto the nuclear pore of mammalian cells, followed by translocation of the 130 kb viral genome to the nuclear compartment [11]. The exact mechanism of these processes is still under investigation, but it is assumed that viral capsids make use of cellular translocation systems.

It was postulated that nuclear uptake of non-viral GDS, which do not contain intracellular transport features of viral capsids, occurs preferentially in cells entering mitosis, subsequent to disassembly of the nuclear membrane [12, 13]. However, there are reports that suggest a nuclear import mechanism through the NPC of transferrin/polylysine/DNA complexes. This was shown by the high transgene expression levels in both growing and growth-arrested fibroblasts [14]. Pollard *et al* [15] reported that some polycations facilitate the nuclear uptake of DNA complexes. The size-restrictions for NPC-mediated import were also challenged by reports that plasmid DNA up to 14.4 kbp were found localized in the nucleus in the absence of cell division. Such nuclear import required energy, cytoplasmic factors, and it was blocked by wheat germ agglutinin. Furthermore, plasmid DNA is thought to form complexes with proteins that carry nuclear localisation signals prior to transit to the NPC and translocation [16]. Likewise, it has been shown that single-stranded DNA/protein complexes were efficiently imported in mammalian nuclei following the classical importin-dependent nuclear import pathway [17].

Several synthetic GDS that display nuclear transport signals have been developed and studied [2, 18-20]. However, the results of these studies are inconclusive. Many of these studies were done with growth-arrested or synchronized cells, but several controls (i.e. mitotic activity and reporter gene expression throughout the experiment) are missing. It should be noted that non-viral GDS are generally cytotoxic. It is known that application of such agents can easily result in a transient burst of mitotic activity. Given the fact that *in vitro* all targeted cells internalise the complex, even a low mitotic activity of ten percent of the cells per 24 hours can result in considerable transfection efficiency. Mitosis gives rise to two daughter cells. In this case ten cell divisions per 100 cells yields 20 cells. Therefore 20 out of the now 110 cells can express marker gene resulting in a transfection efficiency of eighteen percent (20/(90+20)). Since mitosis takes only one hour, this level of mitotic activity

is difficult to detect: less than 1% of the cells will be in mitosis at any time. With regard to transport studies using fluorescence-labelled DNA, fragmentation of the DNA prior to nuclear transport cannot be excluded. It has been reported that nucleic acid fragments larger than 2 kbp are almost immobile in the cytosol, whereas fragments of up to 0.5 kbp diffuse freely into the nucleus after microinjection [21].

The role of lactosylation in nuclear translocation of polyplexes.

In Chapter 3 it was demonstrated that the nuclear presence of complexed DNA was enhanced by lactosylation of the lysine polymer carrier. This effect was sugar-specific and blocking of the nuclear pore complex (NPC) with wheat germ agglutinin prevented nuclear translocation. The findings suggest that lactose provides a nuclear targeting signal. However, the nuclear accumulation of complex was detected by optical slicing of cells that were fixed at various times post transfection. Consequently, it is impossible to determine the cell's history regarding cell cycle and mitosis. Therefore, although the nuclear presence of complex was detected, it was never established how the complex was translocated into the nucleus. Actual import could not be observed and previous nuclear membrane disassembly of nuclei that contained complex, could not be excluded. In summary, previous studies are suggestive for a role of lactose in the nuclear translocation but are inconclusive due to the methodology of the experiments.

We have sought to address this issue by developing an assay to study the transfection process in *real time* with confocal microscopy. Further, we chose to use a highly prevalent human epithelial carcinoma of the bladder as the target of gene transfer. This novel paradigm for establishing the relationship between mitotic activity and gene transfer was applied to a new lactosylated methyl-acrylate polymer. The data show that nuclear translocation of the complex was not observed, although the complex resided in very close proximity of the nucleus. Moreover, all cells that expressed eGFP had divided prior to reporter gene expression. We conclude that transfection with lactosylated pDMAEMA-coAEMA requires a mitotic event, apparently because the complex is unable to cross the nuclear membrane efficiently. In view of the limitations of alternative approaches, we propose to use real time fluorescence microscopy in all future studies of karyotropic (nucleus homing) GDSs.

Results and Discussion

Lactosylated pDMAEMA/cDNA complex does not enter intact nuclei.

A lactosylated polycationic methacrylate-based polymer, poly(2-dimethylamino) ethyl methacrylate (pDMAEMA), was prepared (see methods section). The characteristics of the parent polymer pDMAEMA (poly(2-dimethylamino) ethyl methacrylate) are described in Chapter 1 (1.4.3.3.). pDMAEMA is biocompatible and relatively easy to synthesize and modify. When complexed to DNA, the polymer protects the DNA from degradation and mechanical stress. Many cell types readily take up the complexes, due to the high positive surface charge of the particles. Furthermore, the complexes are able to escape from the endosomal compartment, allowing efficient transfection of target cells [22]

To observe the intracellular fate of the lactosylated pDMAEMA-*co*-AEMA/DNA complex, the fluorescence-labelled complex was followed in 1207 human bladder carcinoma cells for up to 6 hours after addition, with high power confocal microscopy. Plasmid DNA was labelled with the green fluorescent intercalating dye YOYO-1, the polymer was labelled covalently with the red fluorescent dye Alexa 546. To mark the nucleus of 1207 cells chromatin was stained with the blue fluorescent dye Hoechst 33342. Confocal scans were made every ten minutes for up to six hours, and rendered in 3D animations. The results of all combined experiments show that all cells (n=37) internalised a considerable number (32±8) of complex particles under these conditions, within 30 minutes. In the cell, the green and red fluorescence labels always co-localized, resulting in a yellow signal of lactosylated pDMAEMA-co-AEMA/cDNA complex, suggesting that significant dissociation of the complex does not occur in this time frame. As an example, the reader is referred to the animation, which shows the peripheral localisation of internalised polyplexes 60 minutes after application (t=0) (Fig 1A) and subsequently a more perinuclear localisation after three hours (Fig 1B) (Animation1). The complex could not be detected inside the nucleus of 37 cells analysed up to six hours after transfection. However, the complex was detected very close to the outer perimeter of the nucleus (Animation1). This suggests that the polyplex is transported from the periphery

towards the nucleus, presumably inside endosomes, but nuclear transfer of polyplexes does not occur efficiently.

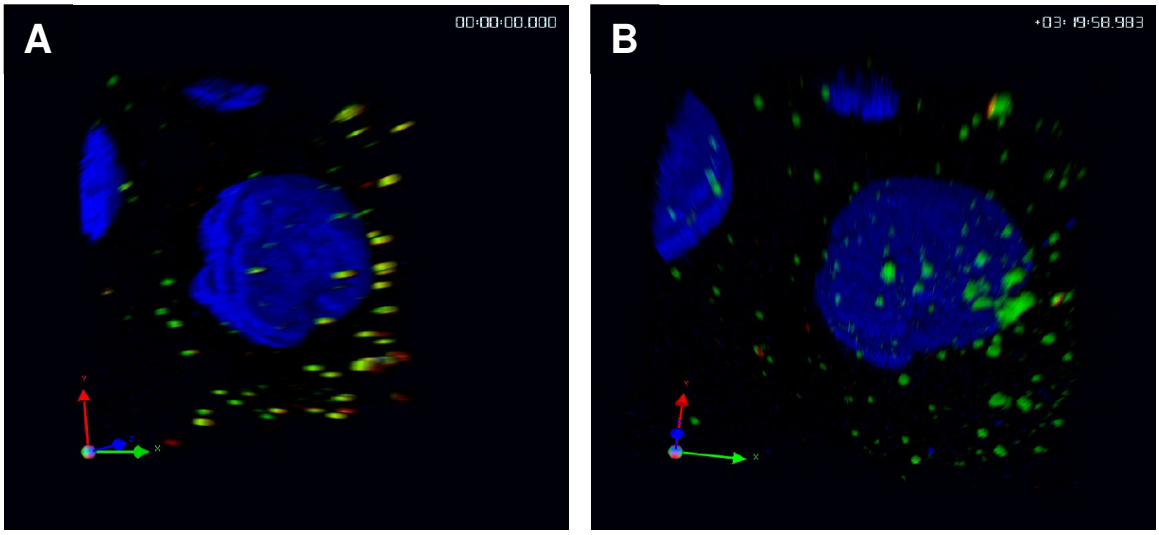

Figure 1 and Animation 1. At t=0 (A) the 3D reconstruction of the confocal scan is presented from different angles to demonstrate the location of the complex with respect to the nucleus. Subsequently, the time-lapse recording is followed from one angle to the time point t=3h 20' (B). At this time point the 3D reconstruction is shown from different angles to demonstrate the location of complex on the nucleus.

Reporter gene expression requires a mitotic event.

As explained above, a GDS can enter the nucleus either through the NPC, or during mitosis when the nuclear membrane is disassembled. This implies that, when translocated *via* the NPC, a GDS is able to transfer genetic material into a non-dividing cell. In our approach we have monitored non-dividing, as well as dividing cells. Living cells are monitored continuously from the moment of addition of the transfection agent until the detection of reporter gene expression. The results of one such experiment is shown in animation 2 (Animation2).

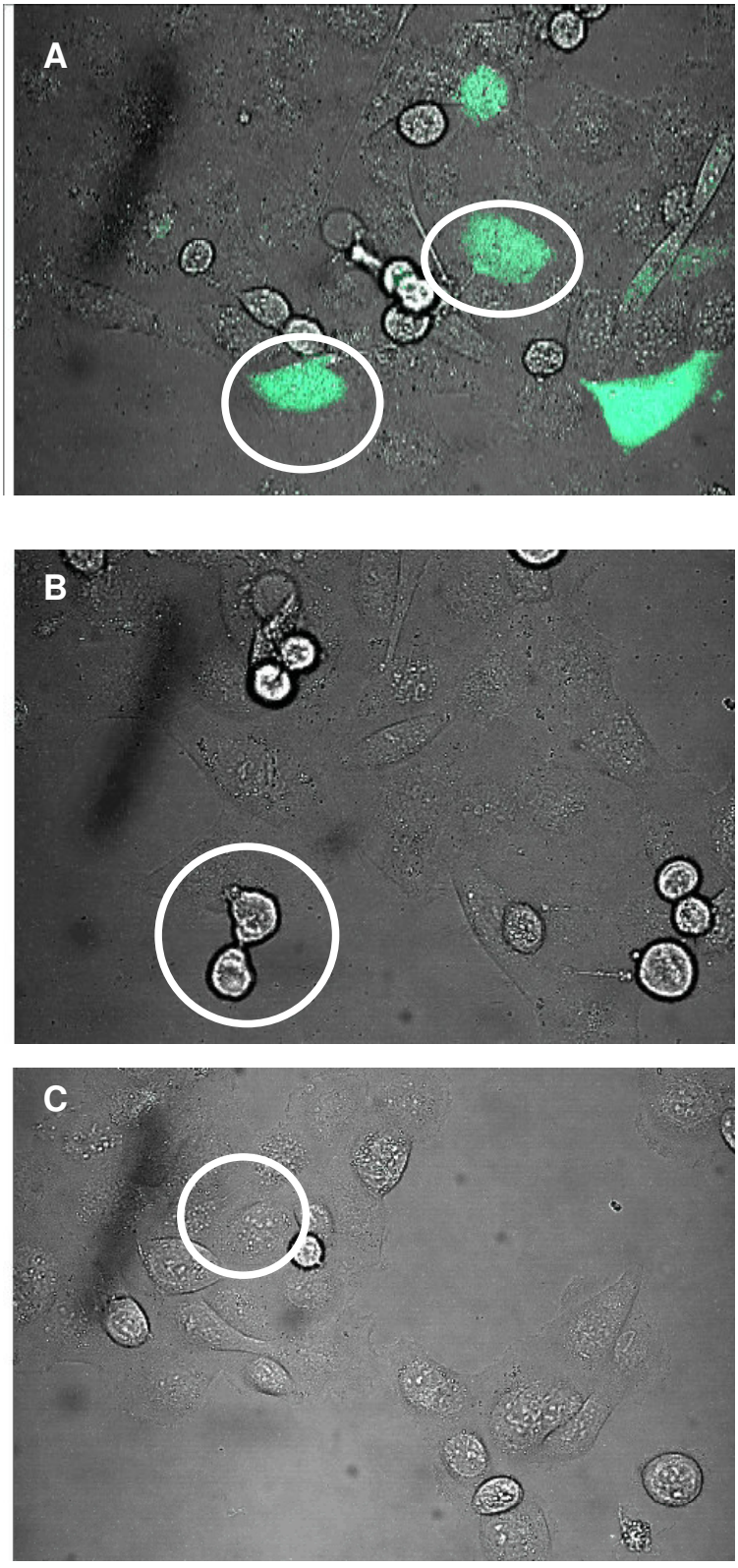

Figure 2 and Animation 2. Time-lapse microscopy of GFP gene transfer in 1207 cells using lactosylated pDMAEMA-co-AEMA.

At t=18 h (A) GFP expression is detected. When GFP expressing cells (circles) are backtracked, a mitosis is observed (B). At t=0 the parent cell is adhered to the glass cover slip. In Animation 2 the cells were followed from t=0 to t=18 hours and GFP expression can be observed. Halfway the animation, the scans are played backwards to demonstrate that all GFP-expressing cells had undergone mitosis previously. For details see *Methods* section.

The first half of the animation shows, in time-forward mode, GFP reporter gene expression is detected in mitotically active 1207 bladder carcinoma cells after transfection with lactosylated pDMAEMA-*co*-AEMA/pCMVeGFP. In the second half of the animation (animation2), the record is reversed, which allows us to follow cells that express GFP backwards in time. It can be observed that all GFP expressing cells have been in mitosis previously.

When transfected with lactosylated pDMAEMA-*co*-AEMA/pCMVeGFP complex around 15-20% of the original population expressed eGFP after 18 hours. All cells analysed that expressed eGFP (N=150, in 4 independent transfection experiments), had gone through mitosis prior to reporter gene expression. During the time course of this experiment approximately 50% of the cells divided, therefore nearly half of the mitotic cells were successfully transfected within 18 hrs. GFP expression was detectable as early as 4 hours after the addition of the transfection medium and was consistently correlated to the mitosis. Within approximately 2 hours following the mitotic event, GFP expression could be detected in the living cell. Our data is consistent with data obtained with nuclear micro-injection of plasmid DNA [13].

The results unambiguously demonstrate that for successful transfection with this carrier, a mitotic event is required. Despite the fact that all cells internalized the complex, GFP expression as only observed in post-mitotic cells. This is consistent with our intracellular tracking data, in which nuclear transfer of fluorescent polyplexes can not be observed. We therefore conclude that the nuclear import of lactosylated pDMAEMA-*co*-AEMA/DNA complex *via* the NPC does not occur at a detectable rate.

***Nuclear transport or retention of lactosylated carriers*.**

These findings appear to be in disagreement with the data of Chapter 3, in which it was shown that lactosylation of poly-L-lysine enhanced the nuclear presence of plasmid DNA compared to mannosylated poly-L-lysine and unsubstituted poly-L-lysine [23]. While a difference between the lactosylated poly-L-lysine and lactosylated pDMAEA-*co*-AEMA carriers cannot be excluded, translocation through the NPC does not appear to be a likely explanation for the observed effect. On the basis of the combined data, an alternative role of the lactosyl residue in the

intracellular trafficking can be postulated. Cytosolic and nuclear lectins have been described, and are shown to be involved in sugar-dependent nuclear transport and retention (reviewed in [24]. However, nuclear import of lactosylated BSA was not demonstrated in micro-injection experiments [25]. Instead, retention of a lactosylated complex through binding of a nuclear lectin seems possible. Galectin-3, is a nuclear galactose/lactose binding lectin that facilitates the transfer of spliceosomes out of the nucleus [26]. It's nuclear import and retention has been ascribed to the carbohydrate recognition domain (CRD) [27]. Thus, the lactosylated pDMAEMA-*co*-AEMA may target the polyplex towards the nucleus, subsequent to a mitosis, by association with a nuclear lectin during reassembly of the nuclear membrane.

A general method for testing the efficacy of karyotropic carrier systems.

These data demonstrate a general approach towards validation of karyotropic vector systems. As already pointed out in the introduction, all other methods presented suffer from temporal/dynamic limitations. Such static studies limit the extent of claims that can be made. An optical, i.e. non-intrusive, method to monitor cell movement and marker gene expression continuously in transfected viable cells determines unambiguously the relationship between mitosis and gene transfer with any delivery system. Transgene expression in the absence of a mitotic event in this system should be the golden standard of a vector that can be transported through the nucleopore.

Materials and Methods

Cell Culture.

1207, a bladder tumour cell line was obtained from Prof. van der Kwast *et al.* [28-30]. The cells were cultured at 37ºC with humidified 5% CO_2 on 75 cm^2 flasks (BD Labware Europe S.A., Meylan, France) in Dulbecco's modified Eagle medium (DMEM; BioWhittaker, Walkersville, MD), supplemented with penicillin/streptomycin (1.000 units per ml) and 10% fetal calf serum (FCS). The 1207 cells were passaged twice a week. For experiments the 1207 cells were seeded on fibronectin coated glass coverslips and grown overnight prior to the confocal microscopy experiment.

Preparation of lactosylated pDMAEMA-co-AEMA.

Poly(DMAEMA)-*co*-AEMA-lactosyl 20% was synthesized as follows. First, DMAEMA was copolymerized with amino ethyl methacrylate (AEMA) as described by van Dijk-Wolthuis *et al*. [31]. The primary amine content of the resulting copolymer was determined with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) as described by the same authors [31].

The lactosylation was performed according to a procedure described for PEI [32]. Because of the higher molecular weight of the monomers in pDMAEMA-*co*-AEMA, the (molar) concentration chosen was five times lower. pDMAEMA-*co*-AEMA (20% AEMA: 117 mg) was dissolved in borate buffer (5.0 mL, 200 mM, pH 8.5). For comparison, pDMAEMA (116 mg) was treated in a similar manner. For calculation of the amounts of reagent, the polymers were assumed to be fully hydrochlorinated. Lactose (1.1 equiv; 0% and 10% AEMA: 24 mg, 20% AEMA: 48 mg) was added, followed by $NaCNBH_3$ (5.5.equiv.; 0% 21 mg, 20% AEMA: 42 mg), and the reaction mixture was stirred for 3 days at 37 ℃. The clear solutions were dialyzed against water, and freeze-dried. The lactosylation is schematically illustrated in Figure 3.

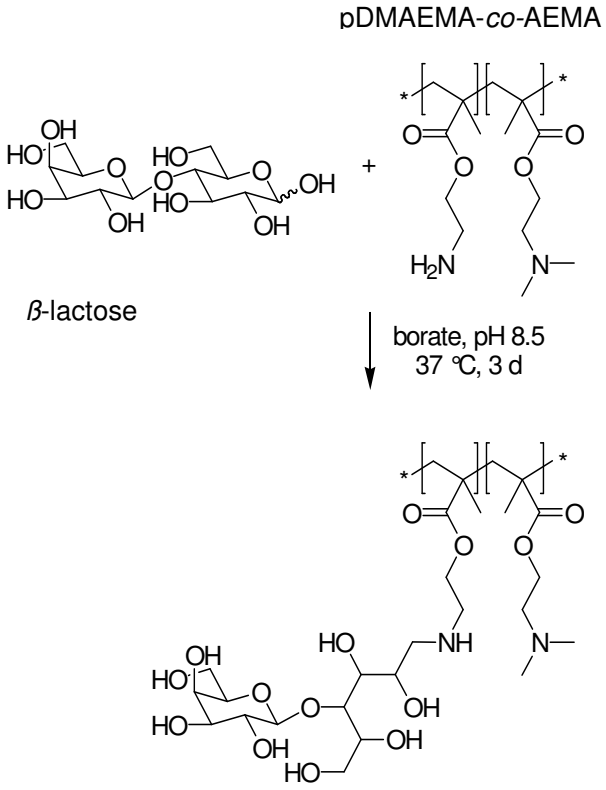

Figure 3. Scheme of lactosylation. After addition of the carbohydrate, the galactosyl residue is available for interaction

The incorporation of lactose (see Table 1) was analyzed by the resorcinol assay [33]. The polymer solution (200 µl 0.50 mg/mL in water) was put in a reaction tube. Resorcinol (200 µL, 6 mg/ml in water), and H_2SO_4 (75%) were added, and the solution was heated at 90 °C for 30 min and left in the dark at room temperature for another 30 min. The analysis was preformed at λ = 430 nm. The calibration curve was made with a 90.1 µg/ml (100 nmol/200 µl) galactose solution (eight data points, 10–175 µl diluted to 200 µl).

The incorporation was calculated using the following equation:

$$\text{MW (g/mol Gal)} = \frac{x \times \text{MW (DMAEMA)} + y \times \text{MW (AEMA)} + z \times \text{MW (AEMA-Lac)}}{z}$$

x = moles of DMAEMA in the polymer; y = moles of AEMA in the polymer; z = moles of AEMA-Lac in the polymer; $y + z = 1$; $x = 9$ (10% AEMA); $x = 4$ (20% AEMA)

Table 1. Incorporation of galactose into pDMAEMA-*co*-AEMA

AEMA (%)[a]	galactose (nmol/100 μg)	galactose (%)[a,b]
0	0	0
10	36.0 ± 1.1	6.7
20	75.9 ± 0.3	15.6

[a] mol (monomer)/mol (total monomers). – [b] weight of unprotonated monomer used to calculate content.

Fluorescent labelling of polymer.

Alexa Fluor 546 carboxylic acid succinimidyl ester (Molecular Probes, Leiden, The Netherlands) was dissolved in Dimethyl Sulfoxide (DMSO) resulting in a 1 mg/ml solution. Stock solution of pDMAEMA-*co*-AEMA was diluted in 0.1 M $NaHCO_3$ buffer (pH 8.4) to a 1 mg/ml solution and 100 μl of the Alexa Fluor 546 solution was added. The mixture was gently stirred for 1 hour at ambient temperature.

Plasmid.

The green fluorescent protein expression vector plasmid pCMVeGFP (GB #U55763) was obtained from Clontech (Palo Alto, Ca USA). Plasmid DNA was isolated from bacterial culture on milligram scale by alkaline lysis, purified with affinity chromatography and diluted to 1 mg/ml in TE buffer (Qiagen, Hilden, Germany). For fluorescent labelling, stock solution of plasmid was diluted in TE buffer (Qiagen, Hilden, Germany) to 1 mg/ml and or fluorescent labelling, YOYO-1 (1 mM in DMSOstock, Molecular Probes, Leiden, the Netherlands) was added to the DNA in a 20:1 w:w ratio. The mixture was continuously stirred for 1 hour at ambient temperature to allow for intercalation of the dye.

Preparation of polyplexes and transfection procedures.

Stock solutions of both polymer and plasmid were diluted in HEPES buffered saline (20 mM HEPES, 150 mM NaCl, pH 7..4; HBS) to give the appropriate concentrations. Volume ratio of polymer solution to DNA solution was 1:1. The polymer was added drop wise to the DNA under gentle vortexing in a 12:1 (w:w)

ratio. The solution was kept at ambient temperature for 20 minutes to allow for polyplex formation, resulting in a polyplex concentration of 1 µg DNA in 100 µl HBS.

Confocal microscopy.

For experiments, a Zeiss LSM510LNO confocal laser-scanning microscope with an axiovert microscope 100 driven by AIM software program (Carl Zeiss-Jena) was used. Transmitted-light differential interference contrast (DIC) images were taken together with images of GFP- or YOYO fluorescence, which were obtained after excitation with a 488-nm argon laser and using a 500-550 BP filter. Images of Alexa Fluor® 546 signal was acquired after excitation with a 543-nm Helium Neon laser and using a 560 nm longpass filter. The Hoechst 33342-labeled cell nuclei were visualised with a Coherent Verdi pump laser and Mira 900 multi-photon laser tuned at 800 nm. To maintain living cells, they were grown on coverslip and placed in a chamber warmed at 37ºC with 5% humidified CO_2.

Detection of intracellular location of lactosylated pDMAEMA-co-AEMA/cDNA in living cells.

To detect the lactosylated pDMAEMA-*co*-AEMA/cDNA complex both polymer and DNA were labelled. To visualize the polymer, Alexa Fluor 546-labeled pDMAEMA-co-AEMA (red fluorescence) was mixed with pDMAEMA-*co*-AEMA-lactosyl 20% in a 1 to 3 ratio (w:w). Subsequently, the polymer mixture was added to YOYO-labelled DNA (green fluorescence). When the polymer (red) and the DNA (green) would co-localise, the digital image overlay would result in a yellow signal, representing double fluorescent-labelled polyplex. Since main objective was to determine intracellular localisation of polyplex with respect to the nucleus, this organelle was counterstained by addition of Hoechst 33342 (Molecular Probes, Leiden, the Netherlands) to the growth medium with an end solution of 5 µg/ml.

Cells, cultured on coverslip and maintained as described above, were scanned using a heated 63X plan Apo 1.4 numeric aperture lens at ten-minute intervals for 4 to 6 hours after the addition of the fluorescent polyplex (end concentration 0.5 µg DNA/ml). Optical sections of 1 or 0.5-µm were made to obtain a detailed image of

the entire nucleus. After the time course, images were processed with LSM 5 software (AIM, Carl Zeiss Jena) to analyse the intracellular localization of the polyplex. Intranuclear localization of the polyplex was defined as follows: (1) in a given focal plane Z the polyplex can be detected in the nucleus in location XY; (2) the polyplex is absent in location XY of the neighbouring focal planes, i.e. the optical slices above Z (Z+1) and under Z (Z-1); (3) the nucleus is present in the neighbouring focal planes Z+1 and Z-1 in location XY. The 3-D reconstruction in time was done with Volocity 2.61 software.

Recording 1207 cells during transfection with lactosylated pDMAEMA-co-AEMA/cDNA.

To monitor 1207 cells during transfection, the cells were optically sectioned in 2-µm thick slices with a heated 40X plan apochromal lens numeric aperture 1.3. At ten-minute intervals, a GFP and DIC image was taken of the cells using a customized macro of the AIM software. To compensate for focal drift, the lens was auto-focussed prior to every scan using a 633-nm Helium Neon laser. Cells were followed from 10 minutes prior to addition of lactosylated pDMAEMA-*co*-AEMA/pCMVeGFP complex (final concentration 0.5 µg DNA /ml) until up to 18 hours after addition. At the end of the time course, GFP expressing cells were backtracked using the AIM software to analyse time of GFP expression and cell cycle motion, i.e. whether the cells had divided prior to reporter gene expression. The animation was constructed with Adobe Premiere software program.

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CHAPTER 5

Characterization of Lactosylated pDMAEMA-*co*-AEMA for Gene Therapy of Bladder Malignancies

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Submitted for publication

Abstract

The aim of this study was to investigate the effects of lactosylation in varying percentages of the gene delivery polymer pDMAEMA-*co*-AEMA on gene transfer into malignant bladder cells. Compared to the parent polymer lactosylation did not increase the efficiency of gene delivery to bladder tumor cells *in vitro,* but it did increase the level of reporter gene expression per cell. Since the binding and cellular uptake of complexed lactosylated pDMAEMA-*co*-AEMA did not differ from the unsubstituted polymer, the higher transgene expression may be due to a lactose-mediated modulating role in the intracellular trafficking of the polyplex. In a co-culture model of normal murine urothelium and human bladder tumor cells (1207 cells), specific uptake of lactosylated pDMAEMA-*co*-AEMA/DNA complex by the tumor cells and not the normal urothelial cells was demonstrated. In accordance with this, when reporter gene expression was examined 24 and 48 hours post transfection, the green fluorescence could be detected only in the tumor cells. Lactosylated pDMAEMA-*co*-AEMA is an efficient gene transfer agent and selectively transfects tumor cells when in co-culture with normal bladder cells. Therefore lactosylated pDMAEMA-*co*-AEMA may be further developed for clinical application in the treatment and diagnostics of bladder malignancies.

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Introduction

Bladder malignancies pose a significant public health problem with more than 130,000 deaths annually worldwide. The prevalence is also remarkable, with more than 500,000 currently diagnosed patients in the United States alone [1]. 95% of urothelial neoplasms are transitional carcinomas and they can range from papillary to flat, non-invasive to invasive, and well differentiated to highly anaplastic [2]. At clinical presentation, 75% of all bladder cancers are limited to the mucosa, submucosa and lamina priopria. The majority of these superficial tumors can be removed with surgery, but recurrence rates are high and can progress to muscle invasive disease with subsequent poorer prognosis [3]. Other therapeutic strategies include intravesical or systemic chemotherapy, immunotherapy with the intravesical administration of *Bacille Calmette-Guérin* (BCG), radiotherapy, photodynamic and laser treatment [4]. Each approach has its own strengths and limitations and currently it is thought that a combination of therapies will result in a more effective treatment [5].

Gene therapy, i.e. the transfer of genetic material, offers a novel approach towards the treatment of bladder malignancies. Strategies include correction of the physiological function of the mutated gene by supplying the correct genetic material. For example, the transfer of wild-type p53 gene into malignant cell lines restored physiological cell cycle control and reduced drug resistance. Moreover, a significant bystander effect was noted in animal experiments [6]. Another approach is aimed at stimulation of the patient's immune system to eliminate the bladder tumor. Immune modulatory strategies in cancer gene therapy include *in situ* transfection of tumor cells with immune response enhancing agents, or vaccination with *ex-vivo* transfected tumor cells [7]. A third approach: the cytotoxic strategy is aimed at selective killing of malignant cells, while sparing benign tissue. Commonly, tumor cells are transfected with genes encoding either a cytotoxic product or an enzyme that transforms a harmless prodrug to a toxic product. The latter strategy is also known as "suicide-gene therapy" [8].

For all these approaches a highly efficient and preferably selective transduction of the tumor cells is essential. For the delivery of genes to superficial bladder carcinomas, both viral and non-viral methods are currently under investigation [9]. Currently, clinical trials with viral-based gene delivery systems are being conducted. They include the use of adenoviral [10] and vaccinia-derived vectors [5, 11]. Under investigation in animal studies are adeno-associated, herpes-derived [12] and lentiviral vectors [13, 14]. However, environmental safety concerns, potential risks to the patient, and high costs of viral vector production and delivery may restrict their wide use in clinical applications.

Synthetic vectors may be an attractive alternative. They can be produced in large quantities in a cost-effective manner. Additional advantages are the absence of a size limit to the therapeutic gene and pose fewer safety and ethical concerns. The major drawback is that non-viral vectors may not have the high *in vivo* transfection efficiencies of viral vectors. A synthetic GDS consists of a polynucleotide, encoding the therapeutic gene and a carrier. The carrier, generally a positively charged lipid or polymer, has to condense the polynucleotide to protect it from mechanical stress and enzymatic attack. Subsequently, the carrier should facilitate transport of the therapeutic gene from the extracellular space into the nuclear compartment, where transcription can take place.

The realisation of an efficient synthetic vector suitable for clinical application has proven to be a daunting task. [15]. Modification of the carrier to mimic the viral capsid with respect to cell binding specificity and intracellular transport, is expected to improve synthetic GDS.

Glycosylation of synthetic GDS has been shown to improve gene transfer capacity. Glycosylated gene delivery vehicles can use membrane bound lectins, such as the asialoglycoprotein receptor on hepatocytes [16] and the mannose-receptor on macrophages [17], to enter a specific cell *via* receptor-mediated endocytosis. In addition, intracellular lectins are involved in trafficking, sorting and targeting of glycoproteins in the secretory pathways [18] and other pathways such as nuclear import [19, 20]. In gene therapy studies, both lactosylated poly-L-lysine [21] and galactosylated polyethyleneimine (PEI) [22] were reported to enhance nuclear

presence of the polycation/DNA complex *in vitro*. Considering the attractive features of a glycosylated GDS, we have sought to characterize a lactosylated metacrylate based polymer for its potential in gene transfer of bladder carcinoma cells.

Poly(2-dimethylamino) ethyl methacrylate (pDMAEMA) forms 100-200 nm complexes with plasmid DNA. Such complexes exhibit a positive zeta potential [23] and were efficient in transfection *in vitro* [24, 25]. pDMAEMA/plasmid complexes are thought to enter the cell *via* endocytosis [26] and have advantageous properties to allow escape from the endosome. According to the "proton sponge" hypothesis of PEI [27], the pH drop in the endosome causes protonation of the non-charged tertiary amines, which may lead to increased osmotic pressure and eventually disruption of the endosome [28, 29],.

To improve the intracellular trafficking of pDMAEMA-*co*-AEMA/DNA complexes, the effect of lactosylation was investigated. We observed that lactosylation of pDMAEMA-*co*-AEMA results in significantly higher marker gene expression. Further, in a co-culture model of superficial bladder carcinoma, delivery of the complexes and expression of the marker gene was observed exclusively in the malignant cells, and not in the normal urothelial cells.

Results

Lactosylated pDMAEMA-co-AEMA condenses plasmid DNA into small positively charged particles.

It was previously shown that when pDMAEMA was complexed with plasmid DNA, the initial negative zeta potential (-22 mV) of the plasmid levelled off at +30 mV with increasing polymer to DNA ratios. The size of the particles at different ratios pDMAEMA to DNA was 150 nm in diameter at polymer:DNA ratio >2 (w:w) [30].

Lactosylated pDMAEMA-*co*-AEMA was synthesized and added to plasmid DNA at different ratios as described in the methods section and the particle size and zeta-potential was measured at different ratio's polymer to DNA (w:w). At 10% substitution with lactose (Fig 1A) complexes with plasmid DNA were formed with an average size of 125 nm. The zeta potential remained virtually constant at around 30 mV. Complexes of the polymer with 20% substitution (Fig 1B) demonstrated the same trend with a slightly larger increase in particle size at higher ratios.

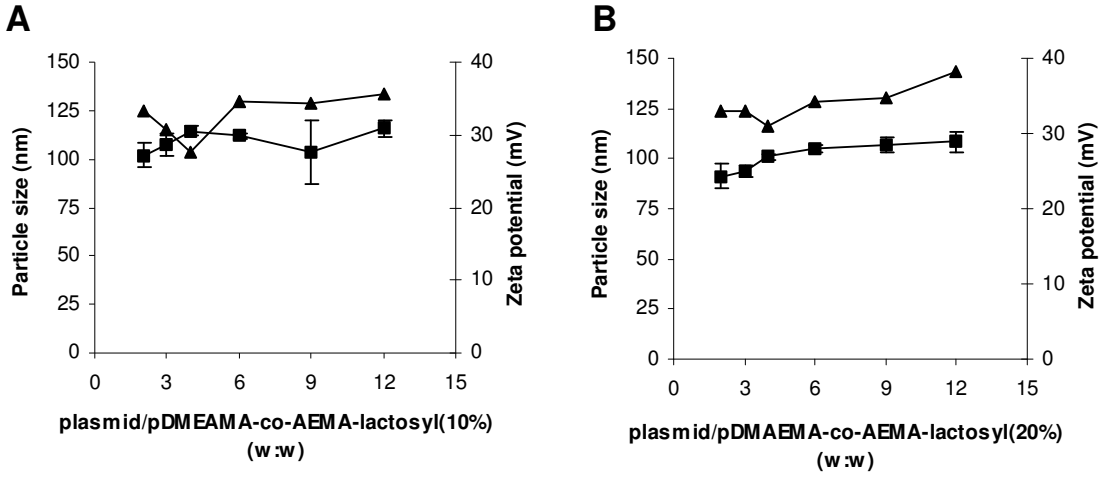

Figure 1. Particle size (▲) and zeta potential (■) of pDMAEMA-*co*-AEMA-lactosyl 10% (A) and 20% (B) complexed to plasmid. Measurements performed in 5 mM Hepes, pH 7.4. Data represent the mean of three indepently prepared formulations ± SEM.

The findings show that lactosylation of the pDMAEMA-*co*-AEMA does not impede the DNA-condensing properties of the polymer, and does not significantly affect the surface charge of the complexes. The ability to condense the extended structure of

plasmid is the first prerequisite of a non-viral vector. The extent of cellular uptake of a GDS depends on the positive surface charge that allows for electrostatic interaction with the negatively charged cell membrane [31].

Lactosylation of pDMAEMA-co-AEMA increases the mean reporter gene expression but not the percentage of transfected cells.

pDMAEMA and its derivatives are efficient in gene transfer to tumour cells in culture [32]. Glycosylation can enhance the gene transfer capacities of DNA-condensing agents [33]. To investigate the effect of lactosylation on pDMAEMA-*co*-AEMA, human bladder carcinoma (1207) cells were transfected with pDMAEMA-*co*-AEMA or lactosylated pDMAEMA-*co*-AEMA complexed at different ratios to a plasmid encoding green fluorescent protein (pCMVeGFP).

The lactosylated polymers were slightly more efficient than the parent polymer with respect to the percentage of cells successfully transfected (Fig 2). At high lactosylated polymer to plasmid ratios the transfection efficiency was 42 % (+/-15), compared to 33% (+/-7) for the parent polymer. While the transfection efficiency expressed as the percentage of positive cells did not differ substantially, lactosylation of the polymer significantly increased the reporter gene expression per cell. At high lactosylated polymer to plasmid ratios, a two to fourfold increase compared to pDMAEMA-*co*-AEMA was observed (Fig 3, average ratio : 2.6, $P<0.01$).

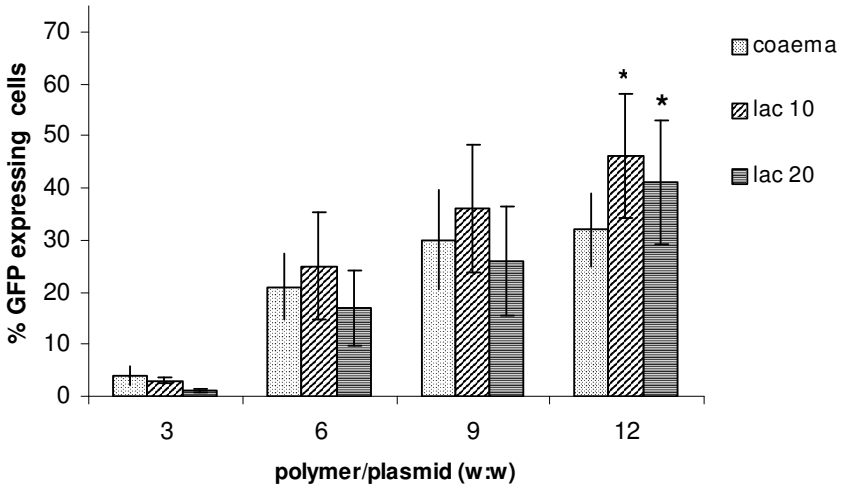

Figure 2. Transfection efficiency *in vitro* of lactosylated pDMAEMA-*co*-AEMA compared to pDMAEMA-*co*-AEMA. 1207 cells were transfected with an enhanced green fluorescent protein expression vector (pCMVeGFP) complexed to pDMAEMA-*co*-AEMA (coaema), pDMAEMA-*co*-AEMA-lactosyl 10% (lac 10) and pDMAEMA-*co*-AEMA-lactosyl 20% (lac 20), respectively. Preparation of transfection mixtures and conditions of transfection are described in the *Methods* section: 1 µg DNA is complexed to varying amounts of polymer. Eighteen hours after seeding, the cells were incubated for 3 h with the polyplexes diluted in serum-free medium. The percentage of GFP expressing cells was measured by FACS analysis 24 h post transfection as described. The bars represent the average of percentage cells with a fluorescence intensity above background (see *Methods* section). The difference between the data groups is statistically not significant, with the exception of lactosylated polymers at the highest ratio (*: $p<0.05$; two tailed Student's t test, n=3 separate experiments in duplicate).

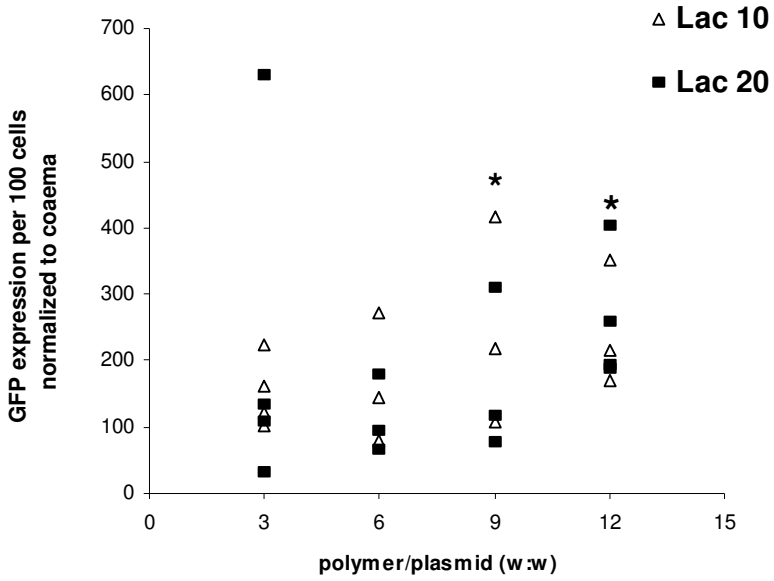

Figure 3. Mean total GFP expression per 100 cells transfected with lactosylated pDMAEMA-*co*-AEMA normalized to pDMAEMA-*co*-AEMA. 1207 cells were transfected with an enhanced green fluorescent protein expression vector (pCMVeGFP) complexed to pDMAEMA-*co*-AEMA (coaema), pDMAEMA-*co*-AEMA-lactosyl 10% (lac 10) and pDMAEMA-*co*-AEMA-lactosyl 20% (lac 20), respectively. Preparation of transfection mixtures and conditions of transfection are as described in the *Methods* section: 1 µg DNA is complexed to varying amounts of polymer. Eighteen hours after seeding, the cells were incubated for 3 h with the polyplexes diluted in serum-free medium. eGFP expression was measured 24 h post transfection as described. Data represent the total expression of eGFP per 100 cells, transfected with lactosylated pDMAEMA-*co*-AEMA, normalized to the expression using pDMAEMA-*co*-AEMA. (*: $p<0.05$, Two tailed Student's t test n=3 separate experiments in duplicate).

***Binding and uptake of lactosylated pDMAEMA-co-AEMA*/DNA complex.**

The higher reporter gene expression per cell demonstrated here could be due to an increased uptake of the lactosylated pDMAEMA-*co*-AEMA/DNA complex by the bladder tumour cells. The lactosyl residue of the polymer may target a membrane-bound lectin [34]. The binding and internalisation of lactosylated and unmodified pDMAEMA-*co*-AEMA/DNA complex was investigated by incubating the cells with fluorescence-labelled complex, followed by FACS analysis (Fig 4). Complexes were labelled with the DNA binding dye YOYO-1. At all ratios, the quenching of YOYO-1 fluorescence by all polymers was the same (data not shown). This rules out a possible confounding factor of lactosylation.

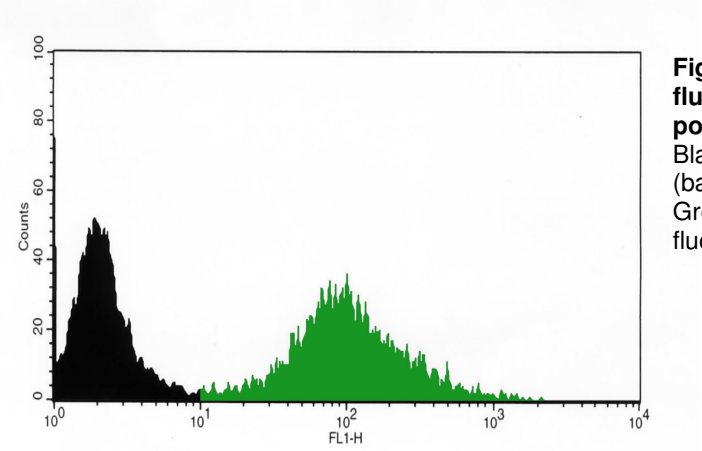

Figure 4. histogram illustrating the fluorescence intensity of cell populations.
Black represents untreated cells (background intensity).
Green represents cells incubated with fluorescent polyplex.

It was found that the binding and internalisation of the lactosylated pDMAEMA-*co*-AEMA/DNA complex was not substantially increased compared to pDMAEMA-*co*-AEMA (Fig 5). The binding and internalisation of pDMAEMA-*co*-AEMA-lactosyl 10% and 20% complexes was 132 (±16) % and 127 (± 10) % respectively, at the highest polymer to DNA ratios. These results suggest that the observed increase in reporter gene expression (Fig 3) was not due to increased targeting of a carbohydrate-binding moiety on the cell surface of 1207 cells.

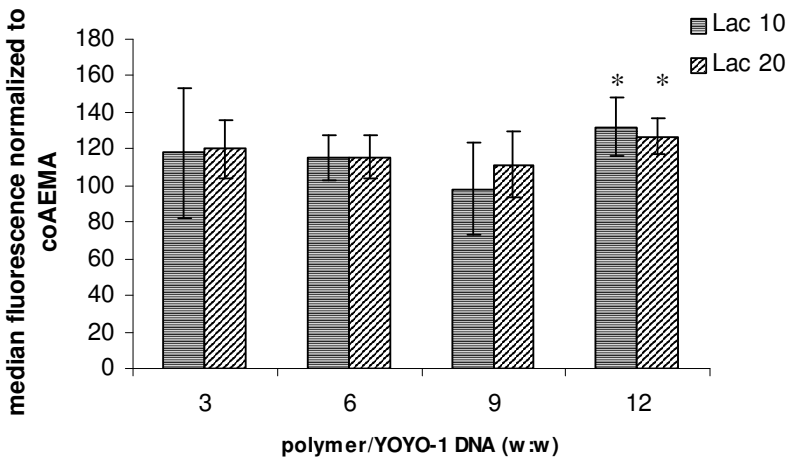

Figure 5. Relative binding and internalisation of the lactosylated polyplexes by 1207 human bladder carcinoma cells, compared to pDMAEMA-*co*-AEMA. Cells were incubated with YOYO-1 labelled pCMVeYFP, complexed to varying amounts of pDMAEMA-*co*-AEMA (coAEMA), pDMAEMA-*co*-AEMA-lactosyl 10% (Lac 10), pDMAEMA-*co*-AEMA, respectively. Preparation of transfection mixtures is described in *Methods* section. After 3 h transfection medium was removed and the cells were washed 3 times with PBS. Subsequently cells were harvested and analysed through FACS, as described. Bars represent the median fluorescence of the entire cell population incubated with lactosylated polymer/YOYO-1 labeled DNA complex normalised to non-glycosylated polymer/YOYO-1 labeled DNA complex. Error bars indicate ± standard deviation. The difference between lactosylated and unmodified polymers is statistically not significant, with the exception of lactosylated polymers at the highest ratio (*: $p<0.04$; Two tailed Student's t test, n=3 experiments in duplicates)

Selective gene transfer to bladder tumor cells in co-culture with normal urothelial cells.

Our data show that lactosylated pDMAEMA-*co*-AEMA is very efficient in the transduction of cultured 1207 cells. Transfection efficiency ranged from 30% to 60% and an increased reporter gene expression per cell was found when we compared non-glycosylated polymer to glycosylated polymers. All the preceding experiments were performed on a monoculture of bladder tumor cells. For potential clinical applications it would be of utmost importance to study how bladder carcinoma cells

compare to normal urothelial cells with respect to their interaction with gene delivery systems. To study this aspect *in vitro,* a co-culture model that more closely resembles the conditions *in vivo* was used. Murine urothelial cells grown in primary culture on membrane inserts in selective growth medium develop into a differentiated urothelium with the typical morphology of 'umbrella' cells (Fig 6A) [35-37]. In confluent and fully differentiated urothelial culture, circular areas (3 mm) of the membrane were exposed with a biopsy punch to allow the attachment of 1207 bladder carcinoma cells, creating an area of carcinoma *in situ.* The carcinoma cells form circular patches with morphology distinct from the normal urothelium (Fig 6B). Under these conditions, uptake of fluorescently labelled polymer/DNA complexes is completely restricted to the carcinoma cells (Fig 6 C to F). This probably reflects the different organisation of cell junctions and endocytotic activity of luminal membranes of carcinomas cells, compared to normal urothelium [38].

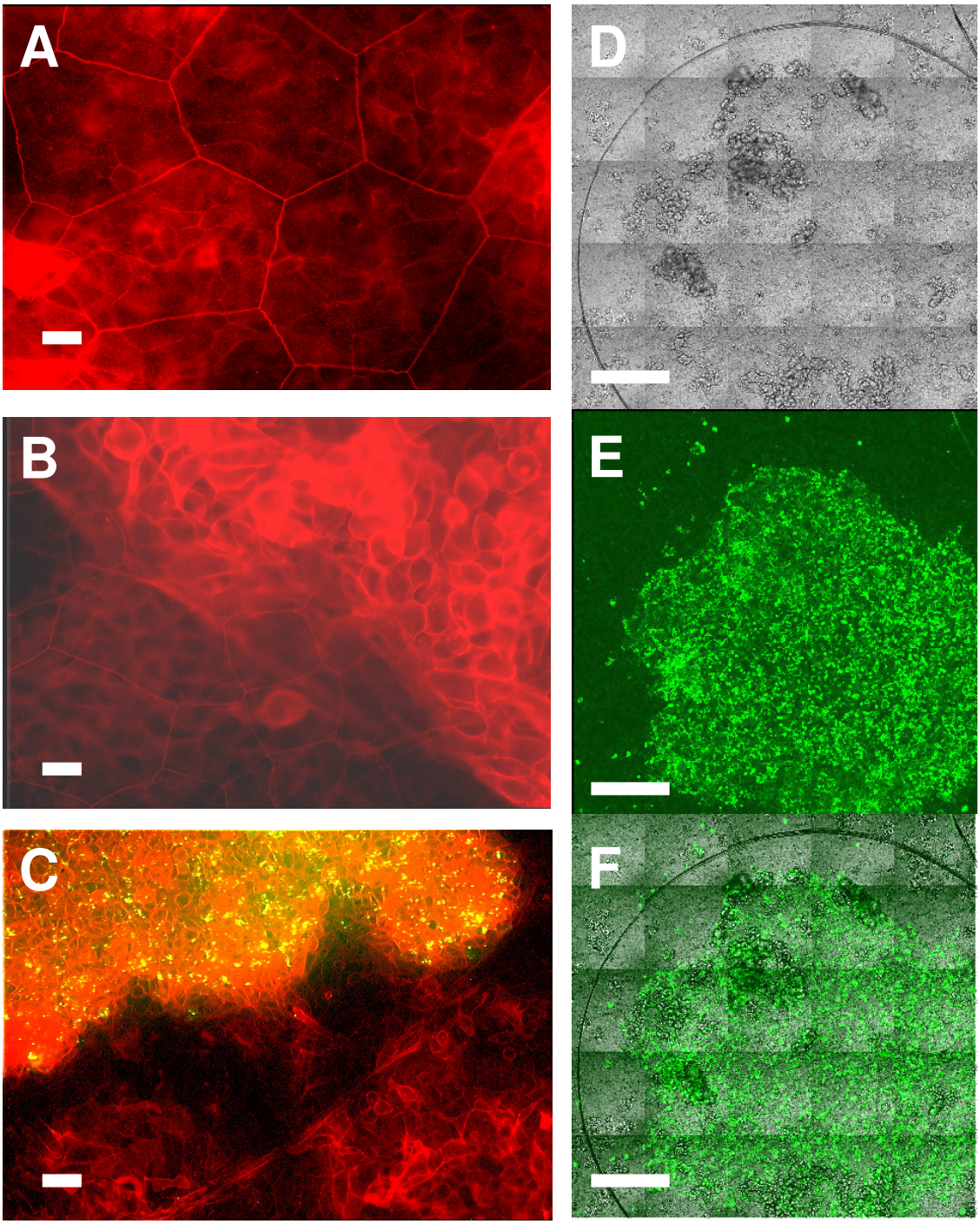

Figure 6. Lactosylated pDMAEMA-*co*-AEMA/YOYO-1 labeled pCMVeYFP is taken up by tumour cells and not by normal urothelial cells. 1207 cells were grown overnight on a partially denuded multi-cell layer derived from murine bladder mucosa and were subsequently transfected with pDMAEMA-*co*-AEMA-lactosyl 20%/YOYO-1 labeled pCMVeYFP complex. After 3 h incubation, transfection medium was removed and cells were washed three times with PBS. The living cells were examined for fluorescence with confocal microscopy. For details see *Methods* Section. In the three

left panels, cells were fixed and stained with Rhodamine labelled phalloidin, to visualise actin filaments (red). Images represent (A) normal urothelium; (B) a boundary between normal cells and tumour cells (untransfected); (C) tumour cells stained with fluorescent polyplex, in contrast to normal epithelium; (D) DIC image of co-culture at low magnification; (E) selective uptake by tumour cells of green fluorescent pDMAEMA-*co*-AEMA-lactosyl 20%/YOYO-1 labeled pCMVeYFP; (F) merged pictures of previous. Sizebar 20 µm (A,B); 60 µm (C); 1000 µm (D,E,F)

After transfection with the pDMAEMA-*co*-AEMA-lactosyl 20%/DNA or pDMAEMA-*co*-AEMA-/DNA complex, the co-cultures were examined for eGFP expression using fluorescence microscopy. Consistent with the data shown in figure 6, the overlay of the green fluorescent image and DIC image demonstrated that only tumor cells (10-15%) and not the urothelial cells expressed the reporter gene (Fig 7A). When the tumor areas were analysed for total reporter gene expression, it was found that lactosylated polyplex was substantially more efficient than the non-glycosylated polyplex (Fig 7B).

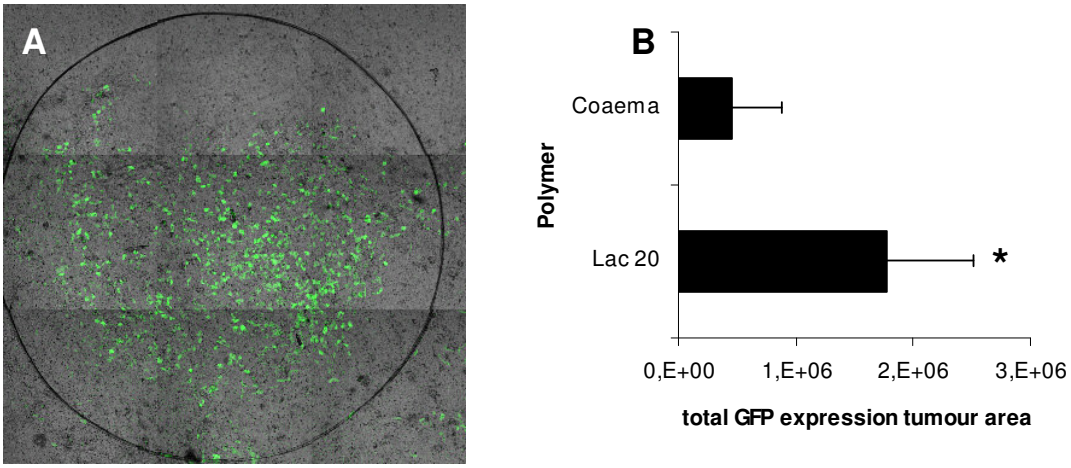

Figure 7. Selective transfection of 1207 human bladder carcinoma cells, growing in co-culture with primary murine urothelial cells by both lactosylated and standard pDMAEMA-*co*-AEMA polyplexes. 1207 human bladder carcinoma cells were seeded overnight on a partially denuded confluent multi-cell layer of normal urothelial cells derived from murine bladder mucosa, and were subsequently transfected with either pDMAEMA-*co*-AEMA-lactosyl 20%/pCMVeGFP complex (Lac 20) or pDMAEMA-*co*-AEMA/pCMVeGFP complex (Coaema). After 48 h the tumour areas were examined for fluorescence with confocal microscopy and data were analysed as described in the *methods section*. (A) composite image of bright field and fluorescence, a typical result with Lac 20 polyplexes. Only tumor cells (inside circle) express GFP. Quantitative analysis of GFP expression per area was performed as described in *methods section*, after transfection with Lac 20 (C) and Coaema (D) polyplexes. Combined results show a significantly higher expression level with Lac 20 polyplexes

(ratio of averages 2.5, P<0.01) (B). Bars represent the total GFP fluorescence detected in the tumour area. Error bars indicate ± standard deviation (*: P<0.01; Two tailed Student's t test N=5 in both series).

In conclusion, lactosylated (and parent) pDMAEMA-*co*-AEMA/DNA complexes selectively target malignant bladder cells when grown in co-culture with normal differentiated urothelial cells. Lactosylation of the polymer significantly enhanced reporter gene expression in the tumour areas. Therefore, lactosylated pDMAEMA-*co*-AEMA may provide for a novel powerful tool in the gene therapy of bladder malignancies.

Discussion

Gene therapy with the transfer of genetic material is a relatively novel approach to the treatment of bladder malignancies and could be useful as an *adjuvans* to established procedures, such as surgical tumor removal, irradiation and *BCG* immunotherapy [39-41]. For pre-clinical researchers, malignancy of the bladder is an advantageous model system to develop gene transfer methods. Intravesical instilment circumvents the toxicity and host defense mechanisms triggered by systemic administration. In addition, the repeated lavage of the bladder offers the possibility to exercise control over the conditions of transfection. The target cells are well-defined and can be readily distinguished from the normal cells that remain unaffected by the treatment. Once applied *in situ*, side effects are more likely to be limited to the site of administration, since the bladder is a confined compartment in the human body. Also, cystoscopy offers a non-invasive method for the evaluation of therapeutic effects.

We have characterised lactosylated pDMAEMA-*co*-AEMA for used gene transfer into bladder tumor cells. It was found that lactosylated and parent pDMAEMA-*co*-AEMA/DNA complexes are of similar size and surface charge (Figures 1, 2). Reporter gene expression in human bladder carcinoma cells was higher with lactosylated polymer compared to the parent compound (Fig 3). In a co-culture system of normal urothelial cells with bladder carcinoma cells, we could show that polymer/DNA complexes selectively target tumor cells (Fig 6). Also under these

conditions, we could show that lactosylation of the carrier polymer enhances the reporter gene expression.

Selective uptake of polymer/DNA complexes by tumor cells.

Specific cell targeting in cancer gene therapy is highly preferred to minimize side effects such as the loss of normal non-malignant tissue. The transitional epithelium of the normal bladder mucosa forms a barrier between urine and tissue fluid. The barrier consists of the apical membrane and tight junctions of the uppermost urothelial layer, the umbrella cells [42, 43]. Transitional cell carcinomas (TCC) comprise 90 to 95% of all bladder malignancies. 70% of all TCCs are papillary non-invasive and begin as areas of hyperplasia, followed by a process of dedifferentiation [2]. The thirty percent non-papillary invasive TCC are believed to arise from non-invasive carcinoma *in situ* (CIS), which is a flat lesion of cells that are very distinct from normal urothelial cells as it displays criteria of dedifferentiation [44]. The human bladder carcinoma cell line used in this study, 1207 was derived from TCC [45] and has a morphology distinct from normal urothelial cells (Fig 6). It exposes integrins at the luminal membrane (D Klink, data not shown) indicating absence of functional tight junctions, and is non-invasive in a mouse model of bladder carcinoma [35, 37]. Invasive growth and metastasis is characterised by a more motile and invasive phenotype, which characterized by a loss of intercellular adhesion and interaction with the lamina propria. Adhesion molecules are involved in control of cell proliferation and morphological differentiation. Decrease of adhesion molecule expression [3] and an altered glycosylation pattern [46, 47] reflects this phenotypic transformation. We propose that this phenotypic change is also associated with increased endocytic activity compared to normal urothelium. This would explain the selective uptake of polymer/DNA complexes and expression of the marker gene by tumor cells in co-culture (Fig 6 and 7). Considering the difference in uptake activity between normal urothelial and tumor cells, it can be postulated that lactosylated pDMAEMA-*co*-AEMA will be a suitable gene therapy vector for less differentiated tumors, such as sessile invasive TCC, arising from carcinoma *in situ*. More differentiated tumors, such as non-invasive papillary TCC, may be less likely candidates.

Enhanced marker gene expression by lactosylation of carrier polymer.

Lactosylation of pDMAEMA-*co*-AEMA enhanced the mean reporter gene expression per cell (Fig 3). While others have reported that particle size and surface charge of the complex are of importance for cellular uptake [48], our lactosylated pDMAEMA-*co*-AEMA/DNA complexes with a similar size, and a surface charge to the parent polymer (Fig. 1) were appropriate for cellular uptake We have further shown that lactosylation of pDMAEMA-*co*-AEMA/DNA complexes did not substantially increase the cellular uptake by tumor cells (Fig 4). Therefore, it seems likely that the cellular uptake of lactosylated pDMAEMA-*co*-AEMA/DNA complexes does not depend on the carbohydrate element of the polyplex. A more likely explanation for the enhanced marker gene expression would be a role of lactose in the intracellular trafficking of the polyplex. To avoid degradation, and allow transfer to the nuclear compartment, the polyplex has to escape from the endosome prior to lysosomal fusion. pDMAEMA has intrinsic properties to destabilise the endosomal membrane, but its endosomal escape could not be detected with confocal microscopy, indicating that the process was not very efficient [29]. Lactosylation may enhance endosomal escape. It was reported that mannosylated poly-L-lysine based polyplex remained longer in endosomes than their lactosylated counterparts. Also, twice as much of mannosylated poly-L-lysine based polyplex was detected inside lysosomes 24 h post transfection [49]. Thus, it has been postulated that intracellular trafficking of glycosylated polyplexes may be different according to the sugar signal recognition, since ligands are conveyed to different vesicular compartments after binding to different membrane lectins [50].

The discussion on how transport of genetic material across the nuclear membrane is accomplished by non-viral GDS has not yet reached a consensus. In principle there are two strategies. The first is to reside inside the cytosol until the cell enters mitosis, during which disassembly of the nuclear membrane occurs. This allows the vector to enter the nuclear compartment of the resultant daughter cells. Alternatively, the DNA can be delivered through the envelope of the interphase nucleus *via* the nuclear pore complex (NPC) [51]. The NPCs are very large protein structures [52] that form aqueous channels through the double membrane of the nucleus and allow for facilitated transport of cargo up to up to 25 nm in diameter, or

free diffusion of molecules up to ~1000 Da in weight [53]. Evidently, polyplexes containing large DNA molecules and lacking the sophisticated nuclear transfer machinery that viral capsids use to enter the nucleus, are not likely to be transported in this way [54]. The data in Chapter 4 demonstrated that reporter gene expression in transfection experiments using lactosylated pDMAEMA-*co*-AEMA/pCMVeGFP was only detected in post-mitotic cells. This virtually rules out active nuclear transport as the dominant mechanism of gene transfer. When intracellular transport of the polyplex was followed using time-lapse confocal microscopy, polyplexes were not observed inside interphase nuclei, but close to or on the nuclear membrane, as early as 30 min after transfection. Therefore, we currently favour a model of targeted delivery of the polyplex in close proximity of the nucleus, followed by nuclear entry during mitosis. The lactosyl residue may enhance this process since galectin-3, a galactose/lactose binding lectin, has been located in the nucleus [55, 56] and its nuclear import and retention has been ascribed to the carbohydrate recognition domain (CRD) [57]. In addition, it has been shown that lactosylation of PLL enhanced the nuclear presence of plasmid DNA compared to mannosylation, in cystic fibrosis airway epithelial cells transfected with glycosylated poly-L-lysine/DNA complex [58]. Thus, the lactosyl residue of lactosylated pDMAEMA-*co*-AEMA may modulate intracellular trafficking of the polyplex, resulting in an increased mean reporter gene expression.

***Towards clinical application of lactosylated pDMAEMA-co-AEMA*.**

To achieve a therapeutical effect in bladder cancer gene therapy, several strategies are under investigation: immune modulatory, cytotoxic and corrective strategies. Immune modulatory strategies aim to stimulate the patient's immune system to eliminate the tumour [8]. Transfection can be performed either *in situ* [59] or *ex vivo* after isolation of patient's tumor cells. Subsequently, transduced cells can be expanded and administered to the patient like a vaccine [60, 61]. Cytotoxic strategies are aimed at a selective killing of malignant cells while sparing benign tissue. In "suicide-gene therapy" target cells are transfected with genes coding for enzymes that convert harmless prodrugs into toxic substances that kill the cell [8]. Several enzyme/prodrug combinations are available for investigation, including Herpes simplex virus thymidine kinase/ganciclovir [62, 63] and nitroreductase

(NTR)/dinitroaziridinylbenzamide (CB 1954) [64]. In one instance pDMAEMA was used to transfect OVCAR-3 cells (an ovary epithelial cancer cell line) with *β*-glucuronidase to sensitize tumor cells to doxorubin-GA3. The cells were equally sensitive to the prodrug as to the toxic product, indicating an efficient conversion of the prodrug to drug [32]. Since it is unlikely that all cells within a tumour will be transfected, a strong bystander effect is crucial to achieve a therapeutic effect. The bystander effect is the ability of cells that are expressing the produg-activating enzyme to kill neighbouring non-expressing cells [65]. Both *β*-glucuronidase/doxorubin [32] and NTR/CB 1954 [66] are reported to have considerable bystander effect.

In corrective strategies the aim is to correct the causal mutations that lead to the oncogenic transformation of a cell. Molecular targets for corrective strategies have been reviewed [67] and include the retinoblastoma and p53 cell cycle regulator genes. Transfection of wild-type p53 into p53 mutated tumor cells can restore cell cycle and apoptosis control [68]. However, a phase 1 clinical trial of adenovirus-mediated gene transfer of p53 demonstrated that clinical benefit did not arise from a p53-mediated antitumour effect but rather from a non-specific inflammatory response to the vector [10]. Lactosylated pDMAEMA-*co*-AEMA can potentially be used in all 3 strategies, either by delivery of a therapeutic gene *in situ,* making use of the difference in uptake activity between normal and transformed cells, or in transfection *ex vivo* of tumor cells.

Materials and Methods

Co-culture of primary murine normal urothelium and 1207 cells.

Six to eight weeks old male C3H mice (Harlan, Austerlitz, The Netherlands) were sacrificed by CO_2 asphyxiation. After extraction of the bladder, the mucosa was resected and placed on a P.E.T. track-etched membrane (0.4 µm pore size; BD Labware Europe S.A., Meylan, France) with the urothelial layer upwards. The ex-plants were allowed to grow for 10-14 days until confluent, on HAM's F-12/DMEM (v:v 1:1) supplemented with 10% FCS, 0,1 µg/ml hydrocortisone, 10 µg/ml transferrin, 10 µg/ml insulin (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands) and penicillin/streptomycin (1.000 units per ml). With a biopsy punch (3 mm diameter) circular areas of the culture were denuded and subsequently, 1207 human bladder carcinoma cells (see Chapter 4) were seeded onto the culture. The co-cultures were used in experiments the following day, after removal of the non-attached cells.

Preparation of polyplexes and transfection procedures.

Lactosylated pDMAEMA-*co*-AEMA and plasmid DNA were prepared as described in chapter 4. Equal volumes of both polymer and plasmid in HEPES buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4; HBS) were mixed to give a final concentration of 10 µg DNA per ml, at different ratios (w:w)of polymer. The polymer solution was added drop wise to the DNA under gentle vortexing. The solution was kept at ambient temperature for 20 minutes to allow for polyplex formation,

For transfection experiments, 1207 cells were seeded on 6-well plates (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands) at a density of 10^5 cells per well and grown overnight in the medium described above prior to transfection. Subsequently the cells were washed with 2 ml PBS and 2 ml DMEM. Generally, 100 µl of polyplex solution was added per well under gentle swirling, resulting in an end polyplex concentration of 0.5 µg DNA per 1 ml. Cells were subsequently incubated for 3

hours at 37ºC with humidified 5% CO_2. To determine transfection efficiency, transfection medium was removed and fresh growth medium was added. Reporter gene expression was measured after 24 hours. To study the binding and internalisation of the polyplexes, cells were washed with phosphate buffered saline (pH 7.4; PBS) three times and harvested by trypsine/EDTA digestion directly after the 3-hour incubation period.

Zeta potential and DLS measurements.

Electrophoretic mobility measurements of polyplexes prepared at low ionic strength (i.e. 5 mM Hepes, pH 7.4) were performed on a Zetasizer 2C, equipped with an aqueous DTS5001 dip cell. Zeta potentials of the polyplexes were calculated using the Smoluchowski approximation of the Henry equation [69]. Dynamic lightscattering (DLS) measurements were performed on a Malvern 4700 system at 25ºC using an argon laser at 648 nm equipped with photon correlation spectrometry software (version 3.2 for Windows; Malvern Ltd., Malvern, UK) under the same conditions as for the electrophoretic mobility measurements.

FACS analysis.

At designated times after transfection, cells were harvested by trypsinization and centrifuged to remove the medium. Pellets were resuspended in FACSflow medium (BD, Erembodegem-Aalst, Belgium). Cells were fixed for 15 min in 2 % (wt/vol) paraformaldehyde in PBS (PFA-PBS), washed by centrifugation in 5% bovine serum albumin in PBS (PBS-BSA). Samples were analysed by a Becton Dickinson FACScan (BD, Erembodegem-Aalst, Belgium). Forward scatter and GFP- or YOYO fluorescence (detected in the fluorescein isothiocyanate [FITC] fluorescence channel) were analyzed using the CellquestPro software (BD, Erembodegem-Aalst, Belgium). For analysis of the reporter gene (GFP) expression, cells were transfected as described above. Percentage of GFP expressing cells was determined by counting cells with a fluorescence intensity > 10 units, the threshold for non-fluorescent cells. In addition, the total mean GFP expression of the viable cell population was expressed as the mean fluorescence value of positive cells multiplied by the percentage of positive cells. For determination of complex-to-cell-

binding, cells were incubated with polymer/YOYO-labelled DNA complex for 3 hours, followed by harvest. The total binding and internalisation of complex was expressed as the mean fluorescence of the total live cell population.

Confocal Microscopy.

Co-cultures of normal urothelium and bladder carcinoma cells on a supporting membrane were transferred to a coverslip placed in a chamber warmed at 37ºC with 5% humidified CO_2. The cells were examined on a Zeiss LSM510LNO confocal laser-scanning microscope with an axiovert microscope 100 driven by AIM software (Carl Zeiss-Jena) as described in Chapter 4. To comprise the entire tumor area 3X3 adjacent square fields (Area 1X1 mm) at 20X magnification were scanned. For analysis of GFP expression in the co-culture experiments, the total fluorescent signal of the tumour area defined as the product of the mean fluorescence and the fluorescent area was quantified using the AIM software.

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CHAPTER 6

Towards Targeted Gene Delivery

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Abstract

Polycations, when complexed to DNA, result in positively charged particles which have non-specific electrostatic interaction with negatively charged blood components and with the negatively-charged proteoglycans of the cell membrane and subsequent endocytosis, leading to the transfection of non-target cells. In this chapter we present an approach to develop a poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA) based gene delivery system, which would not display non-specific electrostatic interaction with the host and specifically targets the cell of interest. Addition of poly(ethylene glycol) (PEG) to the polymer would create a hydrophilic shell that prevents electrostatic interaction. To direct cell specific uptake of a PEGylated polyplex, a targeting ligand must be added. pDMEAMA-*co*-AEMA-*graft*-avidin was used to direct binding to biotinylated targeting ligands. We have characterized polyplexes consisting of pDMAEMA-*co*-AEMA-*graft*-PEG and pDMAEMA-*co*-AEMA-*graft*-avidin with plasmid DNA. With increasing percentage PEGylated polymer, the zeta potential of the polyplex decreased but cellular uptake was not similarly reduced. However, the reduction in transfection efficiency was more pronounced than the uptake data would predict, suggesting a reduced capacity of PEGylated polymers in endosomal escape. This was supported by the relatively strong rescue effect of chloroquine on the transfection efficiency of PEGylated complexes. We propose that PEGylation of pDMAEMA-*co*-AEMA causes reduction in interaction of the polymer with the plasma membrane and the endosomal membrane, resulting in reduced uptake and endosomal escape activity. Further studies are warranted to develop methods to effectively add biotinylated targeting ligand to the complex and to approach the problem of impaired endosomal escape.

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Introduction

Targeted delivery of therapeutical molecules to abnormal cells is one of the hallmark goals of cancer gene therapy. Generally, in conventional cytostatic drug therapy the drug distributes over the body according to its farmacokinetic characteristics and will affect both cancer and normal cells. Gene therapy offers the opportunity to target cells specifically and so minimalise the side effects on normal cells [1]. Viruses have a natural tropism for their host cells and viral-based gene therapy vectors take advantage of the same binding moieties on the cell surface [2]. Synthetic gene therapy vectors can be modified with targeting ligands. The first targeting ligand used was asialoorosomucoid for hepatocytes [3]. Numerous other ligands are under investigation and include transferrin, folate, monoclonal antibodies [4], [5], invasin [6] and several carbohydrates [7].

The addition of a targeting ligand to a synthetic vector enhances the transfection efficiency *in vitro*. When applied *in vivo* however, results have not been as expected. Polycations, when complexed to DNA, result in positively charged particles. The positive charge of the particle causes non-specific electrostatic interaction with negatively charged blood components and undesired cell types. Moreover, interaction with blood components such as serum proteins and physiological salt conditions cause polycation/DNA complexes to form aggregates [8]. The stability of complexes in the serum is compromised and they are rapidly cleared from the bloodstream [9].

Indeed, it is thought that pDMAEMA-based positively charged polyplexes interact with negatively charged blood constituents, including albumin and erythrocytes [10, 11]. Such interactions result in the formation of large polyplex aggregates which are trapped in the capillaries of the lungs and are then cleared. In addition, the positive charge of the complexes can activate the complement system leading to cell death [12]. Another major problem is the non-specific interaction with the negatively-charged proteoglycans of the

cell membrane and subsequent endocytosis [13] leading to the transfection of non-target cells.

In this chapter we present an approach to develop a poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA) based gene delivery system, which would not display non-specific electrostatic interaction with the host but would specifically target the cell of interest. The interaction of polycations with biomolecules can be reduced by addition of hydrophilic elements such as poly(ethylene glycol) (PEG) to the polymer. The PEGylated polymer attached to a complex creates a hydrophilic but non-ionogenic shell that prevents electrostatic interactions [14], resulting in less aggregation and reduced transfection of non-target cells.

The presence of such a protective layer on the polyplex also implies that the interaction, i.e. binding and uptake, with target cells is reduced. Specific binding and uptake of a PEGylated complex can be achieved by the subsequent addition of a targeting ligand, such as a synthetic receptor binding peptide, or an antibody to a cell surface marker. The conjugation of peptide to pDMEAMA based polymers has been described [15]. However this technique is inefficient and requires large amounts of peptide, which may not always be available. To approach this issue we have synthesized pDMEAMA-*co*-AEMA-*graft*-avidin. This would allow us to make use of the high binding affinity of avidin for biotin ($K_d=10^{-15}$ M) [16], for the efficient directed targeting of various biotinylated ligands.

In the present study we report the preparation of complexes consisting of pDMEAMA-*co*-AEMA-*graft*-avidin and poly(DMAEMA)-*graft*-PEG with plasmid DNA at different ratios. The aim of these studies is to arrive at a formulation that can be used in a cell targeting strategy. The structural properties, biological activity and uptake of the compound polyplexes are reported.

Results

PDMAEMA-co-AEMA-graft-avidin transfects 1207 cells efficiently.

pDMAEMA-*co*-AEMA-*graf*t-avidin had been newly synthesized and therefore needed characterisation. First, by SDS PAGE we established that the polymer contained 0.7 µg Avidin covalently attached per 1 µg polymer, i.e. 0.7 biotin binding-sites per 1 ug polymer. Using varying amounts of polymer with a eGFP reporter gene plasmid, the transfection efficiency in 1207 cells was investigated. eGFP expression was measured 24 hours post transfection, using fluorescence flow cytometry.

It was found that at low weight ratio (3:1 polymer:DNA) the polyplex was not active. With higher ratios the percentage of transfected cells increased. An optimum was reached at 12:1 with a percentage reporter gene expressing cells reaching 50 (±15)% (Fig 1.). Therefore, a 12:1 ratio is a good starting point for the partial substitution of pDMAEMA-*co*-AEMA-*graft*-avidin with pDMAEMA-*co*-AEMA-*graft*-PEG to reduce cell membrane interaction.

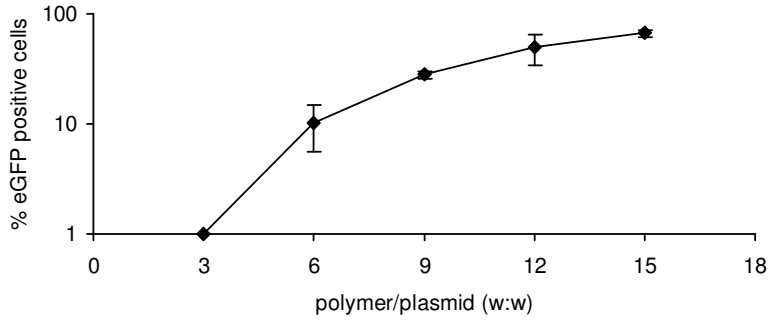

Figure 1. Transfection efficiency of pDMEAM-*co*-AEMA-*graft*-avidin in 1207 cells. The cells were transfected with an enhanced green fluorescent protein expression vector (pCMVeGFP) complexed to pDMEAM-*co*-AEMA-*graft*-avidin. Preparation of transfection mixtures and conditions of transfection are described in the *Methods* section: 1 µg DNA is complexed to varying amounts of polymer. Eighteen hours after seeding, the cells were incubated for 3 h with the polyplexes diluted in serum-free medium. eGFP expression was measured 24 h post transfection as described. The bars represent the average of percentage

cells with a green fluorescent intensity >10. Error bars indicate ± standard deviation. (n=2 experiments in duplicates).

Presence of pDMAEMA-co-AEMA-graft-PEG in the polyplex increases the particle size but reduces the zeta potential.

To study the effect of PEGylated polymer on complex formation, particle size and zeta potential were measured at various percentages of substitution. Two pDMAEMA-*co*-AEMA-*graft*-PEG polymers with different substitution ratios were investigated, pDMAEMA-*co*-AEMA-*graft*-PEG 8% (pDMAEMA-PEG8) and pDMAEMA-*co*-AEMA-*graft*-PEG 25% (pDMAEMA-PEG25).

The dynamic light scatter (DLS) measurements demonstrated that partial substitution of pDMAEMA-*co*-AEMA-*graft*-avidin with PEGylated polymer increased the particle size of the complex (Fig 2). This increase was higher for pDMAEMA-PEG25 (Fig 2B) than pDMAEMA-PEG8 (Fig 2A) with increases of 50% and 30% at equal polymer ratios, respectively. The polyplexes with only one of the polymers had a smaller particle size. The zeta potential of the polyplex on the other hand decreased from around 20 mV at 0% PEGylated polymer to nearly 0 mV at 100% (Fig 2). The zeta potential drop was more pronounced for pDMAEMA-PEG25 compared to pDMAEMA-PEG8 (Fig 2).

When PEGylated polymer is mixed with pDMAEMA-*co*-AEMA-*graft*-avidin the particle size is increased to around 140 nm. This size is still well under the norm for efficient transfection [17] and is sufficiently small (i.e. <200 nm [18, 19] to allow systemic circulation , and to be able to extravasate through the leaky tumor endothelium into tumor tissue via the so-called enhanced permeation and retention (EPR) effect [20]. The zeta potential was reduced significantly by the presence of PEG-chains in the polyplex. This reduction was more pronounced at the higher substitution of PEG-chains thus, as expected, the shielding of the polyplex depended on the amount of PEG-chains.

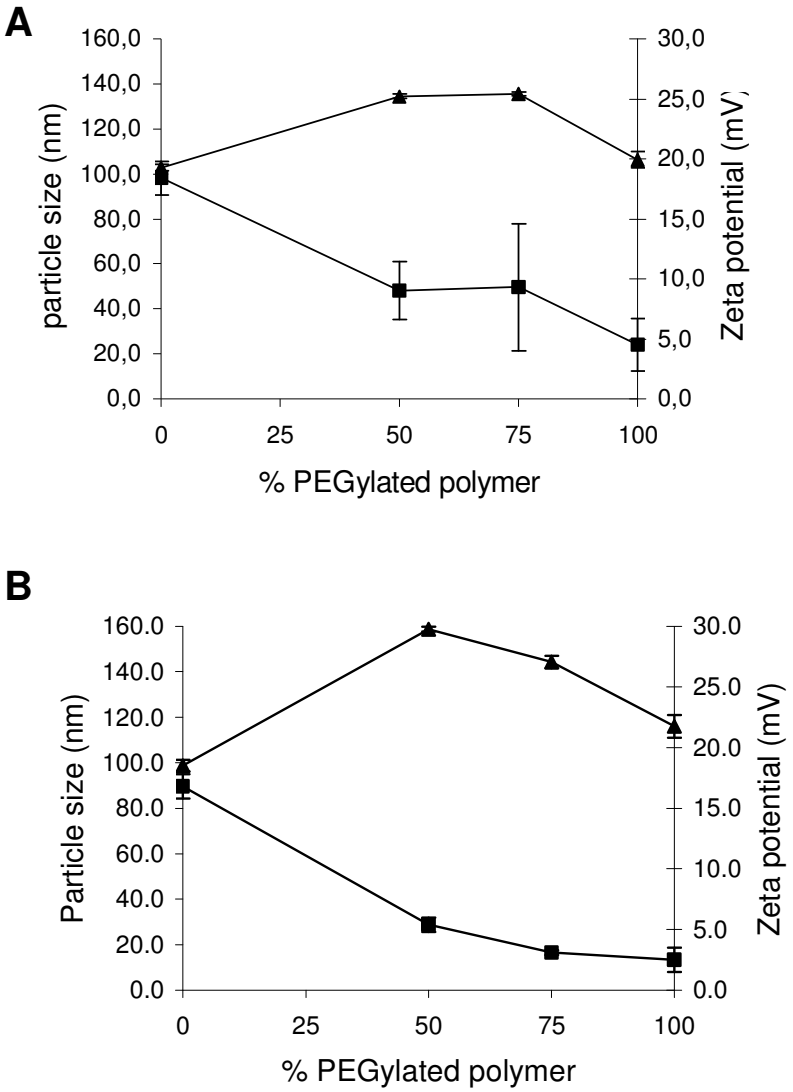

Figure 2. Particle size (▲) increases but zeta potential (■) decreases with increasing amount of pDMAEMA-*co*-AEMA-*graft*-PEG in the polyplex. pDMAEMA-*co*-AEMA-*graft*-avidin was mixed with designated amounts of pDMAEMA-*co*-AEMA-*graft*-PEG and subsequently added to pCMVluc in a 12:1 polymer:DNA ratio (w:w). Measurements were performed in 5 mM Hepes, pH 7.4. Details are described in *Methods* section. Data represent the mean of 2 indepently prepared formulations ± standard deviation. (A) pDMAEMA-PEG8; (B) pDMAEMA-PEG25. ■-■: Zeta potential (mV); ▲-▲ particle size (nm).

Presence of pDMAEMA-co-AEMA-graft-PEG in the polyplex reduces the binding and internalisation.

Presence of PEGylated polymer in the polyplex resulted in particles with a nearly neutral zeta potential. Therefore interaction with biomolecules, including negatively charged cell membrane components should be reduced. The effect of PEGylated polymer in the polyplex on the binding and internalisation was investigated by incubating the cells with complexes of fluorescent-labelled plasmid (YOYO-1) and polymers at different ratios, followed by fluorescence flow cytometry. First, the fluorescence quenching of YOYO-1 fluorescence by the polyplexes was measured to determine whether the readings needed to be compensated for the different ratios of pDMAEMA-*co*-AEMA-*graft*-avidin to PEGylated polymer (Fig 3). Indeed, the quenching by pDMAEMA-*co*-AEMA-*graft*-avidin containing polyplexes was significantly higher than by both pDMAEMA-*co*-AEMA-*graft*-PEG polymers.

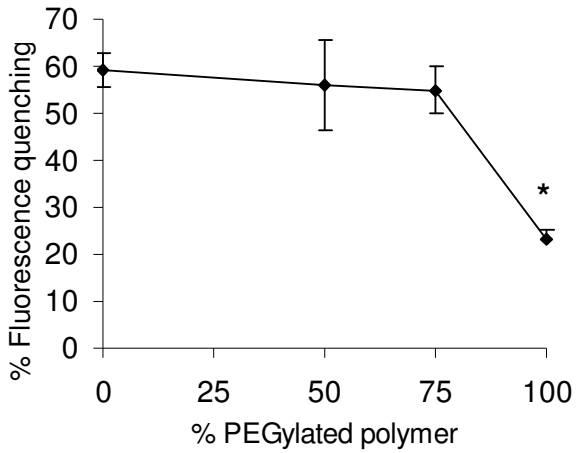

Figure 3. Quenching of fluorescent cDNA by pDMAEMA-*co*-AEMA-*graft*-avidin/pDMAEMA-*co*-AEMA-*graft*-PEG is not determined by the PEG-chain. YOYO-1 labeled pCMVeYFP was complexed to varying amounts of pDMAEMA-*co*-AEMA-*graft*-avidin with pDMAEMA-*co*-AEMA-graft-PEG (*See Methods* Section). The transfection mixtures were diluted in PBS and fluorescence intensity was measured with a standard Fluorimeter. Bars represent the relative quenching of YOYO-1 labeled DNA by the polymers ± standard deviation. (* P<0,05, Two-tailed Student's t test, n=3 experiments in duplicate.

As expected, the binding and internalisation of the polyplexes by 1207 human bladder carcinoma cells was reduced with increasing percentage PEGylated polymer (Fig 4). There was a difference for the two PEGylated polymers. At the 50% substitution, the pDMAEMA-PEG8 did not show a reduction, whereas pDMAEMA-*co*-AEMA-PEG25 did. At 75% substitution, the reduction was 35 (±8) % for pDMAEMA-PEG8 and 53 (±8) % for pDMAEMA-PEG25. At 100% substitution the reduction was 83 (±6) % and 82 (±6) %, respectively.

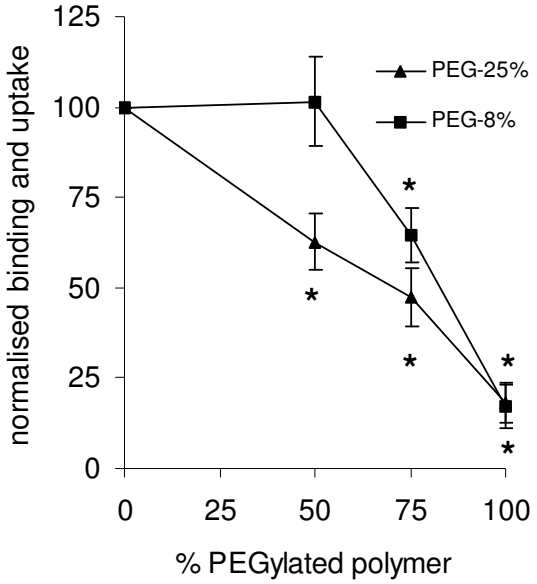

Figure 4. The binding and internalisation is reduced by the presence of pDMAEMA-*co*-AEMA-*graft*-PEG in the polyplex. Cells were incubated with YOYO-1 labeled pCMVeYFP, complexed to varying amounts of pDMAEMA-*co*-AEMA-*graft*-avidin with pDMAEMA-PEG8 (PEG 8%) and pDMAEMA-PEG25 (PEG 25%), respectively. Preparation of transfection mixtures is described in *Methods* section. After 3 h incubation, transfection medium was removed and the cells were washed 3 times with PBS. Subsequently cells were harvested and analysed through FACS, as described. Data point represents the median fluorescence of the entire cell population incubated with complex containing PEGylated polymer normalised to non-PEGylated polymer/YOYO-1 labeled cDNA complex. Fluorescence was corrected for quenching differences of polymers. Error bars indicate ± standard deviation. (* P<0,05, Two-tailed Student's t test, n=3 experiments in duplicates).

The results show that the presence of PEGylated polymer reduces binding and internalisation considerably but not completely. Although the zeta potential (Fig 2) is near 0 mV at 75 % and 50 % substitution, substantial amounts PEGylated polymer/cDNA complex can still be detected on, and inside the cell. This is illustrated by a confocal picture of 1207 cells incubated with pDMAEMA-*co*-AEMA-*graft*-PEG/ pDMAEMA-*co*-AEMA-*graft*-*avidin/YOYO-1 labeled DNA (wt:wt:wt ratio 3:1:0.3.)* (Fig 5 and Animation3). Clearly, perinuclear complexes can be observed in all cells scanned.

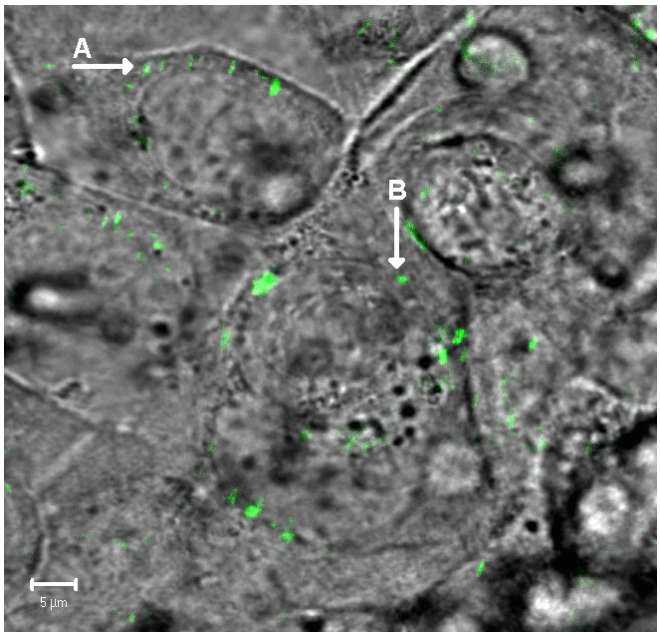

Figure 5 and Animation 3. Intracellular localization of fluorescent-labeled PEGylated polyplex. 1207 cells seeded on membrane were incubated with pDMAEMA-*co*-AEMA-*graft*-PEG/pDMAEMA-*co*-AEMA-*graft*-avidin/YOYO-1labeled DNA (wt:wt:wt ratio 3:1:0.3). After 3 h the cells were washed with PBS and subsequently examined with confocal microscopy (See *Methods* section). The polyplex could be detected in the cell (arrows A and B). The animation shows the optical sectioning starting from the membrane (basal side) to the top of the cells (apical side).

Presence of pDMAEMA-co-AEMA-graft-PEG in the polyplex reduces the transfection efficiency in vitro.

A reduction in cellular internalisation (Fig 4) should have implications for the transfection efficiency. 1207 cells were transfected with polyplexes containing various amounts of pDMAEMA-*co*-AEMA-*graft*-PEG, followed by reporter gene expression measurement using fluorescence flow cytometry.

When the polyplex contained PEGylated polymer, the transfection efficiency decreased significantly (Fig 6). It was noted that pDMAEMA-PEG8 and pDMAEMA-PEG25 displayed equal transfection reduction. The transfection

efficiency was halved when 50% of pDMAEMA-*co*-AEMA-*graft*-avidin was replaced with PEGylated polymer. At 75% substitution the transfection efficiency was reduced by approximately 90%. Polyplexes containing only PEGylated polymer did not transfect at all.

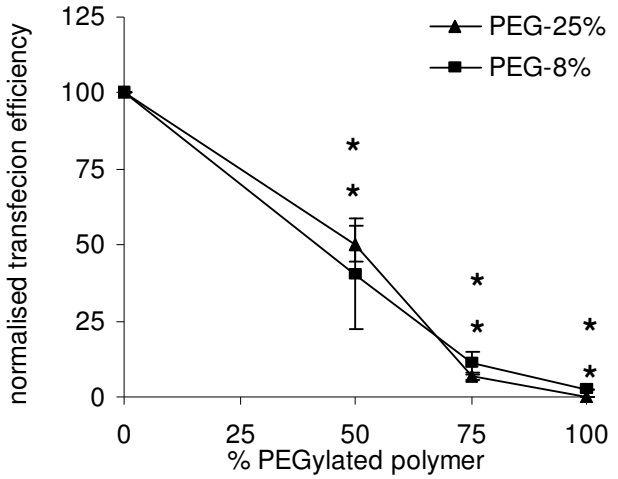

Figure 6. The transfection efficiency *in vitro* is reduced by the presence of pDMAEMA-*co*-AEMA-*graft*-PEG in the polyplex. 1207 cells were transfected with an enhanced green fluorescent protein expression vector (pCMVeGFP) complexed to a mixture of pDMAEMA-*co*-AEMA-*graft*-avidin with pDMAEMA-PEG8 (PEG-8%) and pDMAEMA-PEG25 (PEG-25%), respectively. Preparation of transfection mixtures and conditions of transfection are described in the *Methods* section: 1 µg DNA is complexed to varying ratios of PEGylated and non-PEGylated polymer. Eighteen hours after seeding, the cells were incubated for 3 h with the polyplexes diluted in serum-free medium. eGFP expression was measured 24 h post transfection as described. The data points represent the average of percentage cells with a green fluorescent intensity >background normalised to transfection efficiency of pDMAEMA-*co*-AEMA-*graft*-avidin. Error bars indicate ± standard deviation (* $P<0.05$ Two-tailed Student's t-test, n=2 experiments in duplicates for PEG-8%; n=2 experiments in triplicates for PEG-25%).

Endosomal entrapment is a rate-limiting factor for pDMAEMA-co-AEMA-graft-PEG containing polyplexes.

Comparing the data in Fig 4 and 6 we observe that reduced transfection efficiency does not in all cases correspond with reduced binding to and uptake by the cell. 50% pDMAEMA-PEG8 reduced transfection efficiency equally well as pDMAEMA-PEG25, whereas the binding and cellular

uptake was not reduced at 50% substitution with pDMAEMA-PEG8 (Fig 4). At 75% substitution both PEGylated polymers show reduced but still significant internalisation (Fig 4, Fig 5), whereas transfection efficiency is reduced to 10% of the control value (Fig 6). Apparently, there are other factors than uptake that impede gene transfer with PEGylated polymers. After internalisation, the polyplex has to escape from the endosome to avoid lysosomal degradation [4]. pDMAEMA based polyplexes are reported to have intrinsic properties to escape from the endosome without the aid of lysosomolytic agents [21]. However, it is not known how PEGylation of the polymer affects these advantageous characteristics. To investigate the role of endosomal escape, 1207 cells were transfected with PEGylated polymer containing polyplex, in the presence and absence of chloroquine, a widely used agent that enhances endosomal escape of synthetic vectors [22]. Subsequently, the difference in gene transfer efficiency was assessed by reporter gene expression measurement 24 hours post transfection.

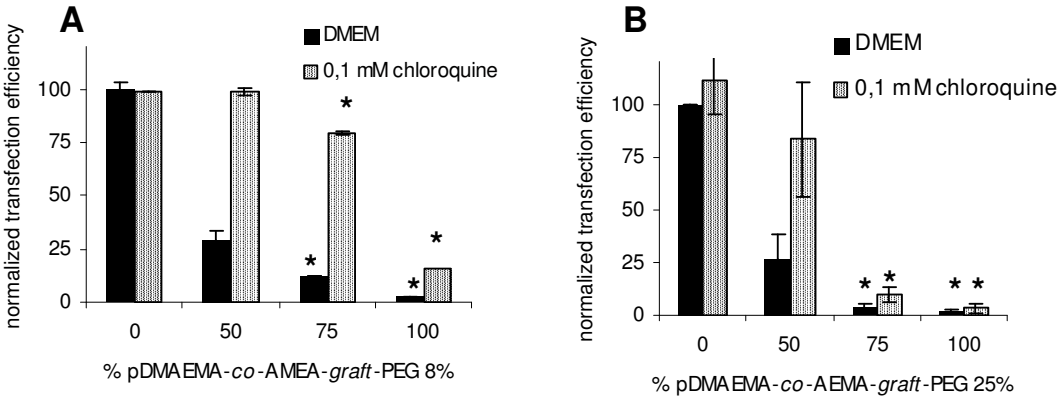

Figure 7. Transfection efficiency is restored by the presence of 0.1 mM chloroquine. 1207 cells were transfected with an enhanced green fluorescent protein expression vector (pCMVeGFP) complexed to a mixture of pDMAEMA-*co*-AEMA-*graft*-avidin with (A) pDMAEMA-PEG8 and (B) pDMAEMA-PEG25, respectively. Preparation of transfection mixtures and conditions of transfection are described in the *Methods* section: 1 µg DNA is complexed to varying ratios of PEGylated and non-PEGylated polymer. Eighteen hours after seeding, the cells were incubated for 3 h with the polyplexes diluted in serum-free medium in the presence or absence of 0.1 mM chloroquine. eGFP expression was measured 24 h post transfection as described. The bars represent the average of percentage cells with a green fluorescent intensity >10 normalised to transfection efficiency of pDMAEMA-*co*-AEMA-*graft*-avidin in the absence of chloroquine. Error bars indicate ± standard deviation (*P< 0,05, Two-tailed Student's t-test, n=2 experiments in duplicates for PEG-25%; n=1 experiment in triplicates for PEG-8%)

The presence of 0,1 mM chloroquine fully restores the transfection efficiency for polyplexes containing 50% pDMAEMA-PEG8 (Fig 7A) or pDMAEMA-*co*-AEMA-PEG25 to control values (Fig 7B). At 75% substitution a difference is detected for the two PEGylated polymers. The presence of chloroquine increases the transfection efficiency for pDMAEMA-PEG8, to 80% of control (Fig 6A). However, transfection efficiency of 75% pDMAEMA-PEG25 was not restored by chloroquine (Fig 7B). Polyplexes containing only PEGylated polymer did not transfect in the presence of chloroquine (Fig 7A,B)

Discussion

The specific targeting of a GDS to specific cells for therapeutic purposes would be of great advantage to medicine. Unfortunately, synthetic vectors that are highly efficient for gene transfer *in vitro* have not lived up to expectations when applied *in vivo.* As described in Chapter 1, gene transfer is a complex multi-step process. Briefly, a synthetic vector must a) condense DNA, b) bind to specific cells through interaction with surface proteins and promote uptake, c) escape from the endosomal compartment, and d) provide for nuclear translocation either by nuclear import or by entry during mitosis. Most current carrier systems are comprised of polycations. However, such carriers that suffer from non-specific electrostatic interaction with negatively charged blood components, resulting in aggregate formation [8] complement activation [12] and rapid clearance from the bloodstream [9].

We aimed to develop a novel pDMAEMA copolymer that addresses the problems associated with the positive surface charge of the vector and allows for specific targeting of the cell of interest. Covalently attaching hydrophilic PEG chains to the vector would mask the surface charge of the polyplex and thus reduce interaction with negatively charged biomolecules and cellular membranes. The addition of a targeting ligand would restore gene transfer of the only target cells. To establish a construct that has shielding PEG-chains and has the possibility for the attachment of targeting ligand, pDMAEMA-*co*-AEMA-*graft*-PEG and pDMAEMA-*co*-AEMA-*graft*-avidin were used.

pDMAEMA-*co*-AEMA-*graft*-avidin would allow for the linkage of a biotinylated targeting ligand, exploiting the marked avidity of avidin for biotin (K_d=10^{-15} M) [16].

We have characterized polyplexes consisting of pDMAEMA-*co*-AEMA-*graft*-PEG, pDMAEMA-*co*-AEMA-*graft*-avidin and plasmid DNA. Two types of PEGylated polymer namely pDMAEMA-PEG8 and pDMAEMA-PEG25 were investigated. For gene transfer the optimal ratio of pDMAEMA-*co*-AEMA-*graft*-avidin to DNA was determined to be 12:1 (w:w)(Fig 1). At this polymer:DNA ratio pDMAEMA-*co*-AEMA-*graft*-avidin was substituted with PEGylated polymer to obtain an inert polyplex that would not bind to negatively charged biomolecules and thus limit cellular binding and internalisation of the polyplex. PEGylated polymer may adversely affect complex formation but it was found that the presence of PEGylated polymer did not impede complex formation and particles around 140 nm in size were formed. With increasing percentages of PEGylated polymer, a decrease in the zeta potential was observed. The uptake data show that addition of PEGylated polymer does reduce internalisation of compound polyplexes, as predicted (Fig 4, Fig 5). However, this moderate reduction in uptake does not seem to match the larger reduction in surface charge (Fig 2). At present we cannot rule out a contribution of the avidin protein element in the uptake of the complexes to the cells. Further studies will have to clarify this.

Increasing amounts of PEGylated polymer dramatically reduced transfection efficiency, as expected (Fig 6, Fig 7). Surprisingly, the effect appeared stronger than the uptake data (Fig 4, Fig 5) would predict. This suggests that the capacity of PEGylated polymers to escape from the endosomal compartment is reduced, compared to the graft-avidin polymer. This is supported by the relatively strong effect of chloroquine on the transfection efficiency of PEGylated complexes (Fig 7A,B). In the case of 75% pDMAEMA-*co*-AEMA-*graft*-PEG25, restoration of transfection by chloroquine was not observed. Since uptake of this compound still was considerable (Fig 4, Fig 5) this is unexpected. Possibly, the lysolytic effect of chloroquine requires a synergistic contribution of the carrier complex, which may be much

reduced in this highly PEGylated polymer. Another explanation for the apparent discrepancy between uptake and transfection data follows from the observation that there is a non-linear relationship between transfection efficiency and the amount of complex per cell. Apparently, a minimum loading of the endosomal system is required to result in efficient escape. This could explain the 10% relative transfection efficiency at a 50% relative uptake, as observed for the 75% substituted PEGylated complexes (Fig 4, Fig 6). However, it does not explain the 50% transfection efficiency at 100% relative uptake observed with 50% pDMAEMA-PEG8 (Fig 4, Fig 6), nor does it explain the absence of a chloroquine effect at 75% substitution with pDMAEMA-PEG25 (Fig 7).

In conclusion, we propose that PEGylation of pDMAEMA-co-AEMA causes reduction in endosomal escape capacity, in addition to reduced cellular binding and internalisation. Considering that PEGylation reduces the interaction of the polymer with the plasma membrane, it is quite probable that is also reduces the interaction with the endosomal membrane, and thus its escape activity. This would impair the efficacy of PEGylated pDMAEMA polymers as delivery systems after addition of a targeting ligand. Therefore, after the establishment of effectively linking biotinylated targeting ligand to the avidin-residue of the complex, further studies are required to remedy this problem of reduced endosomal escape capacity. A potential solution is the attachment of PEG moieties via a biodegradable bond, to allow shedding of the PEG and exposure of the cationic polymer after internalisation.

Materials and Methods

Synthesis of polymers.

Poly(DMAEMA)-*graft*-PEG copolymers were synthesized as follows. First, DMAEMA (Sigma-Aldrich, Zwijndrecht, The Netherlands) was copolymerized with aminoethyl methacrylate (AEMA, Sigma-Aldrich, Zwijndrecht, The Netherlands) as described by van Dijk-Wolthuis *et al*. [22]. The primary amine content of the resulting copolymer was determined with *N*-succinimidyl3-(2-pyridyldithio)propionate (SPDP) as described by the same authors [22]. Next, methoxyPEG-succinimidyl propionate-*N*-hydroxy-succinimide-ester (Mw 5000; Shearwater Corporation Huntsville, AL, USA) dissolved in PBS was added to a solution of pDMAEMA-*co*-AEMA in the same buffer. The concentration of the polymer was 50 mg/ml and the molar ratio NH2/PEG was 1:2. The reaction time was 16 h at room temperature. Excess of PEG was removed by incubation of the polymer with an excess of negatively charged equivalents of Macroprep High S support, strong cation exchanger (Biorad, Life Science Research, Veenendaal, The Netherlands) and subsequent washing with PBS to remove unbound PEG. Bound polymer was eluted with PBS containing 1.5 M NaCl. The products were extensively dialyzed against water and subsequently collected after freeze-drying. The extent of grafting was established by determination of the remaining (unreacted) primary amine groups. The polymers were dissolved in 5 mM Hepes, pH 7.4, and filtered through a 0.45 µm filter before use.

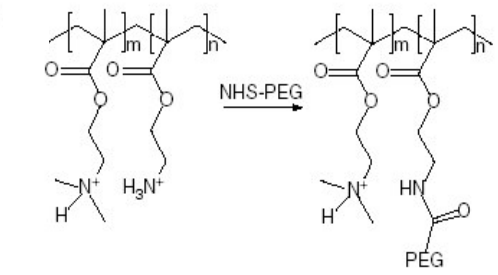

Figure 8. Derivatization of pDMAEMA-*co*-AEMA with NHS-PEG to yield pDMAEMA-*graft*-PEG

pDMAEMA-*co*-AEMA-*graft*-Avidine was synthesized by conjugation of pyridyldisulfide-substituted pDMAEMA-co-AEMA and thiolated avidin (see Figure 9). Pyridyldisulfide-substituted pDMAEMA-*co*-AEMA was prepared as described above. For the thiolation of avidin (ICN Biomedicals Inc), SPDP (0.56 mg in ethanol, 70 µl) was added to a solution of avidin (20 mg in PBS, 10 ml). After 40 min, the mixture was purified over a PD-10 column (PBS/EDTA). The 2-pyridyldisulfide content was determined by reduction of a sample (10 × diluted) with dithiothreitol, and measuring the absorbance at 343 nm (caused by 2-thiopyridinone, ε = 8080). Of a 5.9 mg sample (2.5 ml), the pyridyldisulfide groups were reduced by incubation with dithiothreitol (DTT, 11.4 mg) for 30 min, after which the modified protein was purified over a PD-10 column (PBS/EDTA). For the conjugation, an aliquot of the pyridyldisulfide-substituted polymer (8.82 mg in PBS/EDTA, 0.494 ml) was added to a solution of thiolated avidin (5.88 mg in PBS/EDTA, 3.5 ml). The mixture was stirred overnight, and purified over Sephadex G-25 (PBS/EDTA). The occurrence of covalent conjugation was verified by comparing the results of SDS-PAGE (7.5% polyacrylamide gel) under reducing and non-reducing conditions.

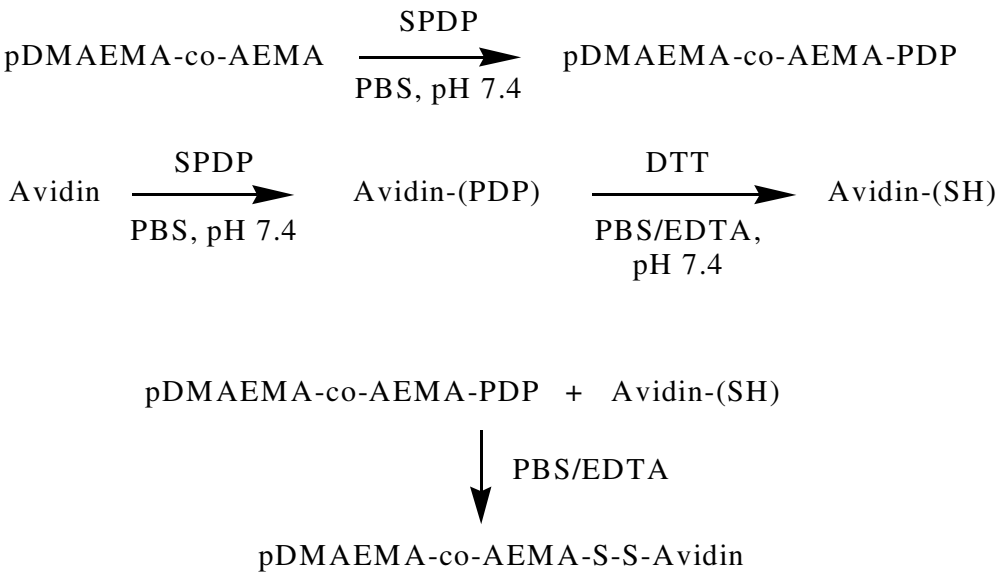

Figure 9. Conjugation of avidin to pDMAEMA-*co*-AEMA

Plasmid.

The fluorescent labelling of expression plasmids pCMVeGFP and pCMVeYFP were obtained and isolated as described in Chapter 4.

Preparation of polyplexes and transfection procedures.

Stock solutions of both polymer and plasmid were diluted in HEPES buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4; HBS) to give the appropriate concentrations. pDMAEMA-*co*-AEMA-*graft*-avidine was mixed with pDMAEMA-*graft*-PEG at designated ratios (w:w). Volume ratio of polymer mixture solution to DNA solution was 1:1. The polymer mixture was added drop wise to the DNA under gentle vortexing. The solution was kept at ambient temperature for 20 minutes to allow for polyplex formation, resulting in a polyplex concentration of 1 µg DNA in 100 µl HBS.

For experiments, cells on 6-well plates (for details see *Cell culture* in *Methods* section of Chapter 4) were washed with 2 ml PBS once and 2 ml DMEM was added. Generally, 100 µl of polyplex solution was added per well under gentle swirling, resulting in an end polyplex concentration of 0.5 µg DNA per 1 ml. Cells were incubated for 3 hours at 37ºC with humidified 5% CO_2. To determine transfection efficiency, transfection medium was removed and fresh growth medium was added. Reporter gene expression was measured after 24 hours. To study the binding and internalisation of the polyplexes, cells were washed with phosphate buffered saline (pH 7,4; PBS) three times and harvested by trypsine/EDTA digestion directly after the 3-hour incubation period.

Zeta potential and DLS measurements.

Electrophoretic mobility measurements, zeta potentials calculations and dynamic light scattering (DLS) measurements of polyplexes are described in Chapter 5.

FACS analysis

Analysis of the reporter gene (GFP) expression and determination of complex-to-cell-binding is described in Chapter 5.

Confocal Microscopy

The details of the confocal microscopy of living cells are outlined in the *Methods* section of Chapter 4 and Chapter 5.

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CHAPTER 7

General Discussion

Introduction.

As outlined in the introductory chapter of this thesis, the concept of gene therapy is based on the introduction of genetic material into living cells, in order to achieve a biological effect in the treatment of pathological processes (Chapter 1). Gene delivery systems (GDS) or vectors consist of a polynucleotide, encoding the therapeutic gene, and a carrier. The carrier has several important properties. First, it condenses the polynucleotide, protecting it from mechanical stress and enzymatic attack. Second, the carrier should facilitate transport of the therapeutic gene from the extracellular space into the nuclear compartment, where transcription can take place. For this task two classes of GDS are available: those based on viral vectors and those that are synthetic self-assembling systems. Viral vectors are generally highly efficient when applied *in vivo*, and the use of integrating vectors offers the possibility of stable transduction of a population of mitotically active target cells. However, the use of viral gene therapy poses serious safety concerns, since the host defense response to the viral vector or its components may damage the host and limit the efficacy of the vector. In addition the viral genome of the vector can recombine with or be activated by wild type viral genome, giving rise to potentially hazardous agents. When integrating vectors are used, there is a risk of insertional mutagenesis. Therefore, although viral vectors are potentially highly efficient for gene transfer *in vivo,* their clinical applications may be restricted due to their safety concerns. These potential restrictions have prompted the development of synthetic GDSs, such as cationic lipids and polymers, which may overcome the current problems in safety, immunogenicity and mutagenesis. In addition, the size of the therapeutic gene is unlimited for self-assembling synthetic vectors and cost-effective large-scale production is feasible. However, non-viral vectors lack the high transfection efficiency *in vivo* of viral vectors. This is because they lack the highly sophisticated mechanisms of viral capsids for binding to target cells, penetration of the plasma- or endosomal membrane, facilitation of intracellular transport, and subsequent import of the genome into the nucleus. Future non-viral vectors must be designed to mimic these properties. In this thesis the interaction of lactosylated polycation/DNA complexes with clinically relevant target cells is studied to further develop cationic polymer based gene delivery vehicles for targeted DNA delivery.

Lactosylated cationic polymers: binding and internalisation.

Lectins are proteins that bind to specific carbohydrate structures. Some membrane-bound lectins, for example the mannose receptor on macrophage subtypes and the asialoglycoprotein receptor on the hepatocyte membrane, are internalized upon binding to ligands. The covalent linkage of carbohydrates to non-viral GDS may enhance cell specificity by targeting membrane-bound lectins. Lactosylated poly-L-lysine (PLL), substituted with lactosyl residues on approximately 40% of the ε-amino groups of L-lysine proved most effective in gene transfer into CF airway epithelial cells in primary culture when compared to other glycosylated PLL [1]. The potential role of the lactosyl residue in the cellular internalization and trafficking of lactosylated PLL was investigated in a model of immortalised human airway cells (Chapters 2, 3). Using fluorescent and phase/contrast microscopy, it was found that lactose and lactosylated PLL inhibited the binding of lactosylated PLL/plasmid complex to CF and non-CF airway epithelial with approximately 80%. In contrast, the presence of Glc*NAc*, an irrelevant sugar, did not affect complex-to-cell binding. In addition, the binding of mannosylated PLL or unsubstituted PLL/DNA complex to airway epithelial cells was unaltered in the presence of lactose. To visualize the complex-to-cell membrane binding in detail, electron microscopy was applied. It was found that complex attached to the cell membrane was located in clathrin-coated pits. The vesicles that lactosylated PLL/DNA complex employed for internalization were further characterized. Immunocytochemistry showed that early endosome antigen 1 was present on the complex containing vesicles. Confocal microscopy demonstrated that during the internalization, transferrin, (marker for early endosomes) and lactosylated PLL/DNA complex co-localized. These results demonstrated that lactosylated PLL/DNA complexes enter airway epithelial cells *via* receptor-mediated endocytosis utilizing lactose-binding moieties, which employ the clathrin-coated pits for internalization. Therefore, we conclude that the addition of lactosyl-residues to non-viral GDSs improves uptake by of airway epithelial cells, and may provide a targeting ligand for this organ. The receptor involved in this uptake process has not been identified; further studies have to clarify this. In a model of human bladder carcinoma, uptake of lactosylated poly-methylacrylate was not

substantially enhanced compared to the parent polymer (Chapter 5). Whether this is due to the absence of a lectin that is expressed specifically on human airway epithelium, or reflects structural differences between lactosylated methyl-acrylate polymers and PLL remains to be established.

Nuclear transfer, an essential step in transfection.

After cell entry, a GDS must deliver the DNA into the nucleus in order to be transcribed. In chapter 3, the intracellular fate of the lactosylated PLL/DNA complex was studied using confocal microscopy. CF airway epithelial cells in primary culture were transfected using lactosylated PLL/DNA complex in the presence of lysolytic agents such as chloroquine, glycerol or fusogenic peptide. Optical sectioning of the cells demonstrated that the complex remained intact during internalization, intracellular transport and, most importantly, transport into the nucleus. When none of the lysolytic agents were present, nuclear localization of complex could not be detected. When compared, both mannosylated PLL and unsubstituted PLL complexed to plasmid DNA were much less efficient in the nuclear translocation of the DNA. Wheat germ agglutinin (WGA) blocks the nuclear pore complex (NPC) and inhibited the nuclear translocation of lactosylated PLL/DNA complex.

Although the results of chapter 3 demonstrated that the presence of lactosyl residues on PLL enhanced nuclear localization of DNA, the mode of nuclear entry was not established. There are two ways for a GDS to deliver its genetic material across the nuclear membrane into the nucleus. One is to reside in the cytosol until the nuclear membrane is disassembled during mitosis and then enter the nucleoplasm of the resulting daughter cells. Alternatively, the DNA can be delivered through the NPC *via* a nuclear import mechanism. The optical sectioning of the nuclei, as described in Chapter 3, was performed on cells fixed at different times after transfection. Actual nuclear import could not be observed, and previous nuclear membrane disassembly, could not be excluded. To address this problem a more sophisticated approach was developed: time-lapse confocal microscopy of living cells *in real time*. With this method, the intracellular path of a GDS could be tracked throughout the time in one particular cell and the cell cycle motion could be carefully monitored. This is the method of choice to establish nuclear transfer of GDS in non-mitotic cells. The

potential role of the lactosyl residue in intracellular trafficking of lactosylated polycation-based GDS was further explored using this approach (Chapter 4). Fluorescence-labelled lactosylated pDMAEMA-*co*-AEMA/pCMVeYFP was added to cultured cells, which were followed for 4 to 6 hours. In 3D rendering of the confocal images, the complex could be detected within near proximity of, and on the nucleus. However, neither nuclear translocation nor nuclear presence was observed in non-mitotic cells. Further, the relation between mitosis and reporter gene expression was established in cultured human bladder carcinoma cells, monitored for up to 18 hours after the addition of lactosylated pDMAEMA-*co*-AEMA/pCMVeGFP complex. It was demonstrated that all cells that expressed the reporter gene had gone through mitosis previously. The results from Chapter 4 indicate that lactosylated pDMAEMA-*co*-AEMA /DNA complex is located near and on the nuclear membrane but is not able to translocate across an intact nuclear membrane. On the basis of the available data it seems likely that this also applies to lactosylated PLL complexes. However, this does not explain the increased nuclear presence of this compound in transfected cells.

Lactosylated pDMAEMA-*co*-AEMA was further investigated for gene transfer efficiency in bladder malignancies (Chapter 5). Two different degrees of lactosylation (10% and 20%) were studied. When complexed to plasmid DNA, both polymers formed 125 nm particles with a zeta potential of +25 mV. Lactosylation did not increase the percentage of transfected bladder tumour cells *in vitro,* but it did prove to increase the reporter gene expression per cell. Since the binding and cellular uptake of complexed lactosylated pDMAEMA-*co*-AEMA did not differ substantially from the unsubstituted polymer, the higher transgene expression supports our hypothesis for a lactose-mediated modulating role in the intracellular trafficking or retention of the polyplex. In this context, intracellular lectins that are able to shuttle between cytoplasm and the nucleus are of interest, [2]. In particular, members of the galectin family, which have an affinity for galactose and lactose, are likely to interact with lactosylated polymer complexes. Galectins play a role in inflammation, tissue remodeling and organogenesis. These proteins have a remarkable lifestyle, with different binding partners for each individual galectin not only in the extracellular space, but also in the cytoplasm and the nucleus [3]. Surprisingly, Galectins have neither a classical secretion signal, nor a nuclear

location signal. Apparently these lectins move from one compartment to another by virtue of the capacity to change partners. In the nucleus, galectins 1 and 3, which are ubiquitously expressed, colocalize with snRNP, and may therefore be involved in mRNA splicing. A possible scenario that would explain the extraordinary efficacy of lactosylated GDS supposes an interaction of the complex with a galectin. This could occur either outside the cell, or in the cytoplasm, after endosomal escape of the complex. It is conceivable that this interaction promotes nuclear retention of the complex in mitotic cells, or even a better access of the vector DNA to the RNA production machinery. This would in part explain the observations reported in this thesis.

Application of lactosylated polymers to superficial bladder carcinoma.

To mimic the condition of bladder cancer *in vivo* more closely, a co-culture model of normal murine urothelium and human bladder tumour cells (1207 cells) [4] was used in the study of lactosylated GDS. Normal murine urothelial cells in primary culture were allowed to form a layer of 3-4 cells, resembling the luminal side of bladder mucosa. With a biopsy punch, standarized areas of the culture were denuded and subsequently 1207 human bladder carcinoma cells were seeded onto the culture. Incubation with fluorescent lactosylated pDMAEMA-*co*-AEMA/cDNA complex demonstrated selective uptake by the tumour cells and the absence of polyplexes in the normal urothelial cells. Correspondently, when reporter gene expression (GFP) was examined 24 and 48 hours post transfection, only in the tumour cells the green fluorescence could be detected. Also in this model, lactosylated pDMAEMA-*co*-AEMA was more effective than the parent compound. Therefore, lactosylated pDMAEMA-*co*-AEMA may be further developed for a clinical application in the treatment and diagnostics of superficial bladder malignancies.

Targeted delivery with cationic polymers.

Luminal application of a GDS to airway epithelium or to superficial bladder carcinoma partly avoids the problems associated with systemic application. When introduced into the bloodstream, non-specific interactions with blood proteins and irrelevant cells, e.g. macrophages and erythrocytes, are dominant and severely reduce the efficacy of a GDS. The construction of a GDS that selectively targets the cells of interest, even in such a challenging environment was pursued. pDMAEMA-*co*-AEMA was chosen to serve as the backbone for its DNA-binding capabilities, intrinsic endosomolytic properties and its relative ease to synthesize and derivatize. To shield the cationic aspect of the complexes, and thus reduce non-specific interactions, polyethylene glycol (PEG) moieties were linked to the polymer. Addition of a specific targeting ligand is another requirement, and avidin was chosen to link a targeting ligand to the polymer, using the high-affinity binding of avidin to biotin (Chapter 6). We have found that pDMAEMA-*graft*-avidin is highly efficient in gene transfer. As predicted transfection efficiency and cellular uptake decreased when the polymer was partially replaced with DMAEMA-*co*-AEMA-*graft*-PEG. However, the data reported in chapter 6 suggest that in addition to a reduction in internalisation, PEGylation of the polymer also results in a reduction of the capacity to escape from the endosomal compartment. This would require a different approach, such as a biodegradable attachment of the PEG moieties to the polymer.

Perspectives.

Gene therapy offers the potential of correcting the underlying cause of monogenetic diseases such as cystic fibrosis (CF) [5] and haemophilia B [6] for which the responsible gene is known. Therapeutic benefits of gene therapy can be expanded to a wide range of diseases that are not strictly hereditary, such as cancer [7] and cardiovascular disease [8]. In addition, applications of gene therapy can reach much further: introducing disease-modifying genes in already dysfunctional organs may alter the course of diseases [9]. When an infectious agent is involved, gene therapy can be directed towards elimination of the agent from the organism or towards prevention of infection in the form of vaccination [10]. Besides delivery of

dsDNA, other applications such as delivery of RNA sequences, RNAi, and RNA decoys that bind regulatory proteins [11] are currently under investigation. The potential safety concerns of the use of viral-based GDSs prompted the development of synthetic GDSs. Feasible applications *in vivo* of non-viral vectors are still hampered by low efficiency. A thorough understanding of the transfection process contributes to development of more efficient non-viral GDSs. In this thesis methods were developed to characterize the role of the lactosyl residue on the biological activity and intracellular transport of lactosylated polycation-based GDSs. We have found that lactosylated polymers constitute transfection systems with superior qualities, possibly due to an interaction with endogenous lectins, but that this probably does not reflect a capacity to transfer vector DNA through the NPC of non-mitotic cells. Therefore, application of such polymers to intact airways in Cystic Fibrosis is likely to be limited by the low mitotic activity of this tissue. The selective uptake of these complexes by superficial bladder carcinoma in culture warrant further studies aimed at clinical application.

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CHAPTER 8

Summary/Samenvatting

Summary

Chapter 1. Gene therapy offers a possibility to treat pathological processes by introduction of genetic material into living cells to achieve a therapeutic biological effect. A gene delivery system (GDS) or vector consists of a polynucleotide, encoding the therapeutic gene, and a carrier. The carrier has several important properties. A GDS should a) condense DNA, b) bind to a cell surface and promote uptake, c) escape from the endosomal compartment, and d) allow nuclear translocation. Two classes of GDS are available: those based on viral vectors and those that are synthetic self-assembling systems. Viral vectors take advantage of mechanisms that viruses have developed to allow viral genome to reach the cell nucleus, to be transcribed and to replicate. When applied *in vivo*, viral vectors are highly efficient in gene transduction. In addition, the use of certain viral vectors offers the possibility of stable integration of exogenous DNA in the genome of the cell. However, viral-based gene therapy poses serious safety concerns since the host defense response to the viral vector or its components may result in deliterious host reactions. Moreover, the viral genome of the vector can recombine with or be activated by wild type viral genome, giving rise to potential hazardous agents. When integrating vectors are used, there is a risk of insertion mutagenesis. Therefore, their clinical applications may be restricted due to their safety concerns. These potential restrictions have led to the development of synthetic GDSs such as cationic lipids and polymers, which may overcome the current problems in safety, immunogenicity and mutagenesis. In addition, the size of the therapeutic gene is unlimited for self-assembling synthetic vectors and cost-effective large-scale production is feasible. However, non-viral vectors lack the high *in vivo* transduction efficiency of viral vectors. Non-viral vectors must be designed to mimic the modulating properties of a viral capsid. An understanding of the pathways used by non-viral GDSs is needed for further development. In this thesis the interaction of polycation/DNA complexes with target cells is studied

Chapter 2. It was previously shown that lactosylated poly-L-lysine (PLL) was most effective in gene transfer into CF airway epithelial cells when compared to other glycosylated PLL. In this chapter, the potential role of the lactose-residue in the cellular internalisation of lactosylated PLL was investigated. Using fluorescent and phase/contrast microscopy, it was found that cell binding of lactosylated PLL/DNA was inhibited by lactose and lactosylated PLL but not by Glc*NAc*. Electron microscopy demonstrated that the lactosylated PLL/DNA complex attached to the cell membrane was located in clathrin-coated pits. The vesicles that lactosylated PLL/DNA complex employed for internalisation were further identified using early endosome markers. These results showed that lactosylated PLL/DNA complexes enter airway epithelial cells *via* receptor-mediated endocytosis utilizing lactose-binding moieties, which employ the clathrin-coated pit for internalisation. Therefore the addition of lactosyl-residues to non-viral GDSs may provide for the targeting of airway epithelial cells.

Chapter 3. After cell entry a GDS must deliver the DNA into the nucleus in order to be transcribed. Previous studies in the field have shown that this is an important barrier for synthetic GDS. The intracellular fate of the lactosylated PLL/DNA complex was studied using confocal microscopy. CF airway epithelial cells in primary culture were transfected using lactosylated PLL/DNA complex in the presence of lysolytic agents such as chloroquine, glycerol or a fusogenic peptide. Optical sectioning of the cells demonstrated that the complex remained intact during internalization, intracellular transport and, most importantly, transport into the nucleus. When none of the lysolytic agents were present, nuclear localization of complex could not be detected. When compared, both mannosylated PLL and unsubstituted PLL complexed to plasmid DNA were much less efficient in the nuclear translocation of the DNA. Wheat germ agglutinin blocks the nuclear pore complex (NPC) and inhibited the nuclear translocation of lactosylated PLL/DNA complex. These results provide support for the concept of a galactose/lactose binding protein, possibly a galectin, which may be involved in the nuclear translocation or retention of lactosylated PLL/DNA complex.

Chapter 4. It was shown that the presence of lactose on PLL enhanced nuclear localization of DNA but the mode of nuclear entry was not established. To address this, time-lapse confocal microscopy of living cells was applied. With this method the intracellular path of a GDS can be tracked throughout time in one particular cell and the cell cycle motion can be carefully monitored. The potential role of the lactose in intracellular trafficking of lactosylated polycation-based GDS was further explored. Fluorescence-labelled lactosylated pDMAEMA-*co*-AEMA (lac-pD)/DNA was added to cultured cells, which were followed for 6 hours. The complex could be detected near and on the nucleus but neither nuclear translocation nor nuclear presence was observed. To study the relation between mitotic activity and reporter gene expression, cultured cells were followed for up to 18 hours immediately after the addition of lac-pD/DNA complex. It was shown that all cells that expressed the reporter gene had divided prior to expression. The results indicate that lac-pD /DNA complex is located near and on the nucleus but is not able to translocate across an intact nuclear membrane

Chapter 5. Lactosylated pDMAEMA-*co*-AEMA (Lac-pD) was investigated for gene transfer in bladder malignancies. When complexed to plasmid DNA, 125 nm particles with a zeta potential of 25 mV were formed. Lactosylation did not increase the percentage of transfected bladder tumor cells *in vitro,* but it did increase the reporter gene expression per cell. Since the binding and cellular uptake of complexed Lac-pD did not differ from the unsubstituted polymer, the higher transgene expression supports our hypothesis for a lactose-mediated modulating role in the intracellular trafficking of the polyplex. Incubation of human bladder tumor cells, grown in co-culture with normal murine urothelium, with fluorescence-labeled Lac-pD/DNA complex showed specific uptake by tumor cells and the absence of polyplexes in normal urothelial cells. Correspondently, transgene expression (GFP) was only detected in the tumor cells. Lac-pD is a more efficient gene transfer agent, compared to the parent compound and transfects tumor cells in co-culture with normal bladder cells selectively. Therefore Lac-pD may be further developed for its clinical application in the treatment and diagnostics of bladder malignancies.

Chapter 6. It was pursued to construct a novel pDMAEMA co-polymer that allows specific cell targeting and addresses the problems associated with the positive surface charge of the vector. The addition of polyethylene glycol (PEG) chains to polymer reduces interaction with negatively charged biomolecules. To add a targeting ligand pDMAEMA-*co*-AEMA-*graft*-avidin (pD-av) was made to allow linkage of biotinylated targeting ligand. Polyplexes consisting of pDMAEMA-*co*-AEMA-*graft*-PEG (pD-PEG), pD-av and plasmid DNA were characterized. It was found that the high transfection efficiency of pD-av decreased when parts of the amount of polymer were replaced with pD-PEG. With increasing percentage pD-PEG, the polyplex zeta potential decreased but this reduction does not appear to correspond with the observed moderate reduction in internalisation of compound polyplexes into cells. In addition, the reduced transfection efficiency appeared stronger than the internalisation data would predict. The relatively strong effect of chloroquine on the transfection efficiency of PEGylated complexes suggests that the capacity of PEGylated polymers to escape from the endosome is reduced. We propose that PEGylation of pDMAEMA causes reduction in endosomal escape capacity, in addition to reduced cellular uptake. This would impair the efficacy of PEGylated pDMAEMA polymers as GDS after addition of a targeting ligand. A potential solution is the attachment of PEG via a biodegradable bond, to allow shedding of the PEG and exposure of the cationic polymer after internalisation.

Chapter 7. The results described in this thesis, and their possible implications for clinical use of lactosylated GDS, are discussed. These results identify a modulatory role for the lactosyl residue on polycation-based GDSs. In gene transfer of CF airway epithelial cells lactose enhances both uptake of the complexes and nuclear localization of plasmid DNA. The cell targeting capacity of lactose is not present in bladder tumor cell, but lactosylation of the carrier did enhance reporter gene expression. We propose that interaction with intercellular galectins may be advantageous in the route to the cell nucleus, but a nuclear import mechanism of lactosylated polyplex is not likely, since our studies also showed the prerequisite of mitosis prior to transgene expression. The methods used to obtain these data proved to be valid tools in

the development of new GDSs and may aid in the future design of polycation-based vectors which transfect cells in a living organism efficiently. Feasible applications *in vivo* of non-viral vectors are still hampered by low efficiency, but tremendous potential of gene therapy prompts the continuing development of synthetic gene delivery systems.

Samenvatting

Hoofdstuk 1. Gentherapie biedt de mogelijkheid pathologische processen te behandelen door het inbrengen van genetisch materiaal in de cel ten einde een therapeutisch biologisch effect te bereiken. Een gentransfer systeem (GTS), ook wel vector genoemd, bestaat uit een polynucleotide dat het therapeutisch gen codeert en een component dat de polynucleotide transporteert. Deze component heeft enkele belangrijke eigenschappen. Het moet a) DNA condenseren, b) binden aan het celoppervlak en opneming bevorderen, c) uit het endosomale compartement geraken en d) translocatie naar de celkern bewerkstelligen. Er zijn twee klassen van GTS: virale vectoren en non-virale vectoren. Virale vectoren maken gebruik van mechanismen die virussen ontwikkeld hebben om het trafficking probleem aan te pakken. Wanneer zij worden toegepast *in vivo*, zijn zij zeer efficiënt in transfectie. Bovendien biedt het gebruik van intergrerende vectoren de mogenlijkheid tot een stabiele transfectie. De veiligsheidaspecten van virale gentherapie zijn echter aanzienlijk. Het afweermechanisme gericht tot de virale vector of zijn componenten kan het gastheerlichaam schaden. Bovendien kan het genoom van virale vectoren recombineren met of geactiveerd worden door wild type viraal genoom met de mogelijkheid tot het onstaan van potentieel schadelijke agentia. Aan het gebruik van intergrerende vectoren zit het risico van insertie mutagenese. De klinische toepassing van virale vectoren kan dus beperkt worden door deze veiligheidsaspecten. Deze mogelijke beperkingen heeft geleid tot de ontwikkeling van synthetische GTS zoals cationische lipiden en polymeren. Deze zouden de huidige problemen met de veiligheid, imunnogeniciteit en mutageniciteit kunnen overkomen. Bovendien is voor non-virale vectoren de

grootte van het therapeutische gen niet beperkt en is een kost-effectieve productie op grote schaal uitvoerbaar. Non-virale vectoren zijn echter niet zo hoog efficiënt in transfectie als virale vectoren, wanneer toegepast *in vivo*. Bij het ontwerp van non-virale vectoren moeten zij de modulerende eigenschappen van het viruskapsel hebben. Voor de verdere ontwikkeling is een beter begrip van de werkingsmechanismen van non-virale vectoren nodig. In dit proefschrift wordt de interactie van polykation/DNA complex met "target" cellen bestudeerd en gekarakteriseerd ten einde te ontwikkelen GTS op basis van polycation polymeren voor celgerichte toediening van DNA.

Hoofdstuk 2. Het was eerder aangetoond dat gelactosyleerd poly-L-lysine (PLL) hoogst effectief was in de gentransfer van CF luchtweg-epitheelcellen in primaire kweek vergeleken met andere geglycolyseerde PLL. In dit hoofdstuk werd de mogelijke rol van de lactose-residu in de cellulaire internalisatie onderzocht. Met fluorescentie en fase/contrast microscopy werd aangetoond dat de binding van gelactosyleerd PLL/DNA complex aan CF en Non-CF luchtweg-epitheelcellen werd geïnhibeerd door lactose en gelactolyseerd PLL, maar niet door Glc*NAc*. Electron microscopie toonde aan dat gelactosyleerd PLL/DNA complex gebonden aan het celmebraan zich in de *clathrin-coated pit* bevond. De vesiculae die gelactosyleerd PLL/DNA complex gebruikte voor internalisatie werd nader gekarakteriseerd met endosomale markers. Deze resultaten tonen aan dat gelactosyleerd PLL/DNA complex luchtweg-epitheelcellen internaliseren via *receptor-mediated* endocytose, gebruikmakende van een lactose-bindende receptor gelocaliseerd in de *clathrin-coated pit.* Met de additie van lactose op non-virale GTS zou het dus mogelijk zijn luchtweg-epitheelcellen gericht te bereiken.

Hoofdstuk 3. Na internalisatie moet een GTS het DNA afgeven in de celkern voor transcriptie. Eerdere studies op dit gebied hebben aangetoond dat dit een belangrijke barrière is voor non-virale gentherapie. Met confocale microscopie werd de intracellulaire bestemming van gelactosyleerd PLL/DNA complex bestudeerd. CF luchtweg-epitheelcellen in primaire kweek werden getransfecteerd met gelactosyleerd PLL/DNA complex in de aanwezigheid van lysolytische agentia zoals chloroquine, glycerol en fusogeen peptide. De

optische secties van de cellen toonden aan dat het complex intact bleef gedurende opname, intracellulair transport en, meest belangrijk, translocatie naar de celkern. Bij afwezigheid van lysolytische agentia werd er geen nucleaire localisatie waargenomen. Gemannosyleerd en ongesubstitueerd PLL waren minder effectief in de nucleaire translocatie van plasmide DNA. *Wheat germ agglutinin* blokeert de celkern porie complex en belemmerde de nucleaire translocatie van gelactosyleerd PLL/DNA complex. Deze resultaten ondersteunen het concept van een galactose/lactose bindend eiwit, mogelijk een galectin, die betrokken kan zijn in de nucleaire translocatie of retentie van gelactosyleerd PLL/DNA complex.

Hoofdstuk 4. Hoewel de resultaten aantoonde dat de lactose-residu op PLL de nucleaire localisatie van plasmide DNA bevorderde, was het niet vastgesteld hoe het complex in de celkern kwam. Om dit probleem aan te pakken werd *time-lapse* confocale microscopie van levende cellen toegepast. Met deze methode kan in de tijd het intracellulaire pad van een GTS in een bepaalde cel worden vastgelegd en kan de celcylcus beweging worden gevolgd. De mogelijke rol van lactose-residu in intracellulair trafficking van gelactosyleerde polycation GTS werd nader onderzocht. Gelactosyleerd pDMAEMA-*co*-AEMA (lac-pD)/DNA complex, voorzien van een fluoriserend label, werd toegevoegd aan cellen in kweek, welke 6 uur werden vervold. Het complex werd gedetecteerd in de nabijheid van en op de celkernmembraan maar nucleair import of nucleaire localisatie werd niet waargenomen. Om de relatie tussen mitose en reportergen-expressie te onderzoeken werden celllen vervolgd voor maximaal 18 uur direct vanaf de toediening van lac-pD/DNA. Er werd aangetoond dat alle cellen die het reportergen tot expressie brachten, vooraf in mitose waren gegaan. De resultaten tonen aan dat lac-pD/DNA gelocaliseerd is nabij en op de celkernmembraan maar niet in staat is het DNA over een intacte nucleair membraan te transporteren.

Hoofdstuk 5. Gelactosyleerd pDMAEMA-*co*-AEMA (lac-pD) werd onderzocht voor de gentransfer van blaasmaligniteiten. Gecomplexeerd aan plasmide DNA werden 125 nm grote partikels met een zeta-potentiaal van 25 mV gevormd. Lactosylering verhoogde niet het percentage getransfecteerde

blaastumor-cellen *in vitro* maar wel de reportergen-expressie per cell. Daar de binding en opname lac-pD/DNA niet verschilde van de ongesubstitueerde polymer, ondersteunde de hogere transgen-expressie onze hypothese van een lactose gemedïeerde modulerende rol in het intracellulair trafficking van de polyplex. Incubatie van humane blaaskankercellen, in co-cultuur met normale muis-urotheelcellen, met fluorescentie-gelabeld lac-pD/DNA complex toonde de specifieke opname van complex door de tumor cellen en de afwezigheid van complex in de normale urotheel cellen aan. Overeenkomstig werd alleen transgen-expressie waargenomen in de blaaskankercellen. Gelactosyleerd pDMAEMA-*co*-AEMA is meer efficiënt voor gentransfer dan het moeder polymer en transfecteert selectief tumorcellen in co-cultuur met normale urotheelcellen. Derhalve kan gelactosyleerd pDMAEMA-*co*-AEMA verder ontwikkeld worden voor klinische toepassingen in de behandeling en diagnostiek van blaasmaligniteiten.

Hoofdstuk 6. De constructie van een nieuw pDMAEMA co-polymer werd nagestreefd dat de problemen, geassociëerd met de positief geladen oppervlakte lading, aanpakt en gericht de cellen van interesse bereikt. De binding van hydrofiele polyethyleenglycol (PEG) ketens aan het polymer zou de interactie met negatief geladen biomoleculen reduceren. De additie van een specifieke ligand kan de transfectie van de beoogde cellen herstellen. Hiervoor werd pDMAEMA-*co*-AEMA-graft-avidine (pD-av) gesynthetiseerd, waaraan gebiotinyleerde liganden kunnen binden. We hebben gekarakteriseerd: polyplexen bestaande uit pDMAEMA-*co*-AEMA-*graft*-PEG (pD-PEG), pD-av en plasmide DNA. De hoge efficiëntie van pD-av in gentransfer daalde wanneer er delen van de hoeveelheid polymer werden vervangen door pD-PEG. Bij oplopend percentage pD-PEG werd een afname van de zeta-potentiaal gemeten maar deze afname lijkt niet overeen te komen met de waargenomen afname in de celbinding en opname van de polyplex. Bovendien was de daling in transfectie-efficiëntie bij oplopende hoeveelheden pD-PEG markanter dan op grond van de binding- en opnameresultaten voorspeld zou worden. Het relatief sterke effect van chloroquine op transfectie-efficiëntie van gePEGyleerde complexen suggereert de capaciteit van gePEGyleerde polymeren om uit het endosoom te geraken is

gereduceerd. In conclusie, stellen wij dat PEGylering van pDMAEMA-*co*-AEMA het vermogen het endosomale compartement te verlaten reduceert, naast gereduceerde celbinding and internalisatie. Hierdoor kan de effectiviteit van gePEGyleerde pDMAEMA polymers als GTS na toevoeging van een specifieke ligand gehinderd worden. Een mogelijke oplossing is binding van PEG-ketens aan het polymer via een bioafbreekbare verbinding. Na internalisatie zouden PEG-ketens loslaten van het polymeer en zo het polykation polymer blootstellen.

Hoofdstuk 7. De resultaten van dit proefschrift en mogelijke implicaties daarvan voor het gebruik van gelactosyleerd GTS in een klinische setting worden besproken. Deze resultaten identificeren een modulerende rol voor de lactose-residue op polykationische GTS. De lactose-residu bevordert de opname en nucleaire translocatie van polyplex in gentransfer van CF luchtweg-epitheelcellen. Het vermogen de cel specifiek te bereiken is niet aanwezig in blaastumor-cellen, maar lactosylering van het polymeer verhoogde transgen-expressie. Wij stellen dat interactie met intercellulaire galectins de weg naar de celkern bevordert, maar dat een nucleair import mechanisme van gelactosyleerd polyplex onwaarschijnlijk is, daar onze studies tevens aantoonde de noodzakelijkheid van een celdeling voor reportergen-expressie. De methoden om deze data te verkrijgen bleken valide instrumenten in de ontwikkeling van nieuwe GTS en kunnen toegepast worden in de toekomstige constructie van polykation vectoren, welke efficiënt zijn in de gentransfer van cellen in een levend organisme. Haalbare toepassingen *in vivo* van non-virale vectoren zijn nog steeds belemmerd door de lage efficiëntie, maar het geweldige potentiëel van gentherapie in de behandeling van aandoeningen spoort aan tot verdere ontwikkeling van synthetische gentransfer systemen.

List of Abbreviations

AAV	adeno-associated virus
AEMA	amino ethyl methacrylate
BSA	bovine serum albumin
BCG	*bacille Calmette-Guérin*
CB 1954	dinitroaziridinylbenzamide
CF	cystic fibrosis
CRD	carbohydrate recognition domain
CIS	carcinoma *in situ*
DAPI	4,6-diamidino-2-phenylidole
DNA	deoxyribonucleid acid
EEA1	early endosome antigen
FACS	fluorescence activated cell-sorting
FITC	fluorescein-5-isothiocyanate
GDS(s)	gene delivery system(s)
GFP	green fluorence protein
HBS	Hepes buffered saline
Hepes	4-(2-hydroxy ethyl)-1-piperazine-ethanesulfonic acid
NPC	nuclear pore complex
NTR	nitroreductase
PEI	poly(ethyleneime)
PBS	phosphate-buffered saline pH 7.3
PCMVeGFP	plasmid containing the enhanced GFP gene under control of the CMV-promotor
pDMAEMA	poly(2-(dimethylamino)ethyl methacrylate
pDMAEMA-co-AEMA	poly(2-dimethylamino)ethyl methacrylate-*co*-2-aminoethyl methacrylate
pDMAEMA-PEG8	pDMAEMA-*co*-AEMA-*graft*-PEG 8%
pDMAEMA –PEG25	pDMAEMA-*co*-AEMA-*graft*-PEG 25%
PEG	poly(ethylene glycol)
PFA	paraformaldehyde
PLL	poly(L-lysine)
TCC	transitional cell carcinomas
TSA-poly-L-lysine	poly-L-lysine *p*-toluenesulfate salt
TEM	transmission electron micoscopy

Curriculum Vitae

Daniel Klink werd geboren op 10 maart 1973 te Schagen. In 1991 behaalde hij het V.W.O diploma aan het Canisius College-Mater Dei. In datzelfde jaar werd begonnen aan de studie geneeskunde aan de Katholieke Universiteit Nijmegen. In 1995 werd het doctoraal examen behaald. In het najaar van 1995 werd onder begeleiding van Prof. Dr. L.J. Takemoto (Manhattan) en Prof. Dr. J.J.H.M. de Pont (Nijmegen) bij de Biology Department van Kansas State University, Manhattan, KS USA een onderzoeksstage gelopen gericht op de karakterisatie van protease uit de lens. Na het afronden van de co-schappen behaalde hij het artsexamen in 1997.

Na een periode als arts-assistent werkzaam zijn geweest, begon hij in 1999 aan een research fellowship aan The Children's Hospital of Philadelphia te Philadelphia, PA USA gericht op non-virale gentherapie bij cystische fibrose onder leiding van Prof. Dr. M.C. Glick en Dr. T.F. Scanlin. Vanaf oktober 2002 was hij werkzaam als Assistent In Opleiding onder leiding van prof. Dr. F.G. Grosveld en Dr. B.J. Scholte aan het ErasmusMC te Rotterdam. Het onderzoek was gericht op de ontwikkeling non-virale vectoren en hun werkingsmechanismen *in vitro.* De resultaten van het onderzoek in Philadelphia en Rotterdam zijn beschreven in dit proefschrift.

Sinds april 2004 is Daniel Klink in opleiding tot kinderarts aan het VUmc te Amsterdam.

List of Publications

Peer-reviewed articles

Klink, D.T., Chao, S., Glick, M.C., and Scanlin, T.F.
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Intracellular transport of lactosylated pDMAEMA-*co*-AEMA/DNA complexes
Submitted

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Characterization of lactosylated pDMAEMA-*co*-AEMA for gene therapy of bladder malignancies
Submitted

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