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The role of the carrier and the endocytic pathway in non-viral gene delivery

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ACADEMIC DISSERTATION

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Abstract

Gene therapy offers promise for the treatment of both inherited and acquired diseases through the introduction of genetic material into target cells. The primary challenge for gene therapy is to develop a safe and efficient method for the delivery of therapeutic genetic material to the specific intracellular target. Non-viral carriers have received significant attention because of their potential to overcome the limitations of viral-based systems. However, their relatively low efficacy is a major obstacle to their clinical application. A thorough understanding of the key factors affecting the gene delivery process will provide clues on how to develop more effective carriers. This thesis focuses on the role of the carrier and the endocytic pathway in non-viral gene delivery, and also suggests improvements in the experimental methodology that would make it possible to obtain more reliable results in nanoparticle uptake studies.

More effective carriers for gene delivery are very much needed. We tested the novel pentaspheric lysine-based dendrimer and its analog, modified with fatty acid residues, for their gene delivery capacity. We demonstrated that despite their relatively low *in vitro* transfection efficacy, lysine dendrimers have good plasmid DNA (pDNA) binding and protective properties, and can therefore be used as the basis for the development of more effective carriers.

A detailed understanding of the cellular kinetics of gene delivery systems is critical to the further development of more effective carriers. We studied the impact of the carrier and of the endocytic pathway on cell uptake and the intracellular processing of genetic material (pDNA). The highly sensitive method of quantitative real-time PCR was applied to the study of the intracellular kinetics of pDNA introduced by the cationic polymer PEI, cationic lipid DOTAP, and CaP precipitates at multiple time points after transfection. The results obtained indicate that the carrier affects the cell uptake and the intracellular kinetics, and therefore predetermines the main transfection-limiting step. Furthermore, we demonstrated the important role of the post-nuclear processes in efficient non-viral gene delivery.

The impact of a specific endocytic pathway was studied by the inhibition of either the clathrin- or dynamin-mediated endocytosis. Selective blockage of endocytosis was achieved by two approaches, namely the genetically manipulated cell lines and the chemical inhibitors of endocytosis. Analysis of the intracellular kinetics of pDNA in the genetically blocked cells revealed that neither the amount of pDNA taken up by the cell, nor the intracellular pDNA elimination, but the amount of pDNA delivered to the nucleus was indicative of the significance of the particular pathway in the resulting efficacy of the carrier. A comparison of chemical and genetic means for blocking endocytosis revealed the limitations of both these methods. A careful optimization of the method and the use of several alternative approaches is recommended in order to obtain more reliable data.

We suggest that the characterization of *in vitro* cell models for the expression and activity of specific endocytic pathways (endocytic profiling) would facilitate the interpretation of the data obtained in nanoparticle uptake studies. Endothelial and epithelial cells are widely utilized in such studies because they form substantial barriers en route from the administration site to the target tissue. We performed endocytic profiling of

the epithelial CaCo-2 cells and the endothelial hCMEC/D3 cells at different stages of differentiation. The expression of genes involved in specific endocytic pathways was analysed at the mRNA level by quantitative real-time PCR, and at the protein level by Western blotting. The endocytic activities of the cells were analyzed by flow cytometry. We concluded that the mRNA expression of the endogenous proteins involved in particular endocytic pathways can be indicative of the expression and activity of these pathways. Furthermore, we showed that the differentiation status of the cells affects their endocytic activity, and must therefore be taken into account when designing nanoparticle uptake and transcellular permeability experiments. A comparison of the endocytic profiles of cell lines with primary cells revealed clear discrepancies, pointing to the importance of careful selection of *in vitro* models for endocytosis-related studies.

Overall, our study has improved the mechanistic understanding of the non-viral gene delivery process. We have described how the carrier and endocytic pathway both affect intracellular kinetics and the efficacy of gene transfer. Furthermore, we have demonstrated the importance of method optimization and the endocytic profiling of *in vitro* cell models in improving the quality of the obtained data.

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Contents

Abstract 3

Acknowledgements 5

List of original publications 9

Abbreviations 11

1 Introduction 12

2 Review of the literature 14

2.1 Non-viral gene delivery systems 14

2.1.1 Polymeric nanoparticles 14

2.1.2 Dendrimers 15

2.1.3 Liposomes 16

2.2 Barriers to non-viral gene delivery 16

2.2.1 Extracellular barriers 16

2.2.2 Cell entry and intracellular barriers 18

2.3 Endocytic pathways and their role in gene delivery 21

2.3.1 Phagocytosis 21

2.3.2 Macropinocytosis 22

2.3.3 Clathrin-mediated endocytosis 24

2.3.4 Caveolae-mediated endocytosis 24

2.3.5 Flotillin-mediated endocytosis 25

2.3.6 Other clathrin-independent pathways 25

2.4 Endocytosis in polarized cells and its role in gene delivery 26

2.5 Experimental approaches to the study of endocytic pathways of gene delivery systems 27

2.5.1 Chemical inhibition of endocytosis 28

2.5.2 Genetic inhibition of endocytosis 28

2.5.3 Fluorescent microscopy and other methods 32

3 Aims of the study 35

4 Overview of materials and methods 36

5 Lysine dendrimers as vectors for delivering genetic constructs to eukaryotic cells 39

6 Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems 49

7 Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models 63

8 Overview of the results 78

9 General discussion 81

9.1 The role of the carrier in non-viral gene delivery 81

9.2 The role of the endocytic pathway in non-viral gene delivery 83

9.3 Improving experimental methodology in cell uptake studies 85

9.3.1 Searching for an optimal approach to block the endocytic pathway 85

9.3.2 Endocytic profiling of cell models 85

10 Conclusions 88

11 Future prospects 89

References 91

List of original publications

This thesis is based on the following publications:

I. Kiselev A.V., **Il’ina P.L.**, Egorova A.A., Baranov A.N., Gur'janov I.A., Bayanova N.V., Tarasenko I.I., Lesina E.A., Vlasov G.P., Baranov V.S. Lysine dendrimers as vectors for delivering genetic constructs to eukaryotic cells. *Russian Journal of Genetics* 43(6):725-33, 2007.

II. **Ilina P.**, Hyvonen Z., Saura M., Sandvig K., Yliperttula M., Ruponen M. Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems. *Journal of Controlled Release* 163(3):385-395, 2012.
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III. **Ilina P.**, Partti S., Niklander J., Ruponen M., Lou Y.R., Yliperttula M. Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models. *Experimental Cell Research* 332(1):89–101, 2015.
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The publications are referred to in the text by their Roman numerals.

Author's contribution

Publication I: Lysine dendrimers as vectors for delivering genetic constructs to eukaryotic cells. The author participated in the design of the study together with supervisors and coauthors. The author performed most of the experiments and participated in the analysis of the data and the manuscript preparation together with supervisors and coauthors.

Publication II: Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems. The author designed this study together with Dr. Marika Ruponen. Experiments with the chemical inhibitor dynasore were performed by exchange student Maeva Saura under the author's supervision. Cell uptake experiments and some of nuclear uptake experiments were performed by Dr. Zanna Hyvönen and Dr. Marika Ruponen. All other experiments were performed by the author. The author analysed the data, wrote the first draft of the manuscript and prepared all figures and tables. She furthermore was responsible for the subsequent corrections and was a corresponding author of the manuscript.

Publication III: Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models. The author designed this study together with supervisors and performed most of the experiments. Some experiments were performed by undergraduate student Susanna Partti and research assistant Johanna Niklander under the author's supervision. The author analysed most of the data, wrote the first draft of the manuscript, prepared all figures and tables and actively participated in all the steps of the manuscript preparation.

Abbreviations

ALP	Alkaline phosphatase
BHK	Baby hamster kidney cell line
CaP	Calcium phosphate
CavME	Caveolin-mediated endocytosis
CHC	Clathrin heavy chain
CLIC/GEEC	Clathrin-independent carrier/glycosylphosphatidylinositol (GPI)- anchored protein-enriched endosomal compartments
CME	Clathrin-mediated endocytosis
CTB	Cholera toxin β-unit
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
FACS	Flow activated cell sorting
FME	Flotillin-mediated endocytosis
GPI	Glycophosphatidylinositol
GTP	Guanosine triphosphate
HeLa	Human cervix epithelial carcinoma cell line
LacCer	Lactosylceramide
miRNA	Micro ribonucleic acid
NPs	Nanoparticles
PAMAM	Polyamidoamine
pDNA	Plasmid deoxyribonucleic acid
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly-L-lysine
qRT-PCR	Quantitative real-time polymerase chain reaction
siRNA	Small interfering ribonucleic acid
SV40	Simian virus 40
Tf	Transferrin

1 Introduction

Conventional drugs often treat symptoms, but not the cause of the disease. Recent advances in molecular biology allow us to understand the molecular mechanisms of many diseases. The vast majority of these diseases are caused by a disturbance in normal protein production. The identification of the genes coding for various proteins and the genes involved to regulation of protein production has opened possibilities for more specific therapeutic approaches. The principle of gene therapy is to cure the disease by introducing genetic material into the target cells. The strategies include the introduction of DNA in order to produce therapeutic protein, or the introduction of siRNA, miRNA or antisense oligonucleotides to knock-down the expression of the gene involved in the diseased condition. Gene therapy offers promise for the treatment of both inherited and acquired diseases, including those which are currently regarded as incurable (Verma, Weitzman 2005). By June 2014, worldwide over 2000 gene therapy clinical trials had been approved, as either ongoing or completed (The Journal of Gene Medicine Clinical Trial site). The first gene therapy products have been approved as gene medicines, including Gendicine in China (2003) as an anti-cancer treatment and Glybera in Europe (2012) for the treatment of lipoprotein lipase deficiency - a rare inherited disease (Wirth, Parker & Yla-Herttuala 2013).

The biopharmaceutical properties of genetic material are poor. Its large size and negative charge renders it unable to cross biological barriers and prone to degradation and/or clearance from the body.Therefore, there is a need for an excipient termed a carrier or vector, which would be able to protect and specifically deliver the genetic material to the target site. Currently, more than 65% of all gene therapy clinical trials involve viral-based carriers (The Journal of Gene Medicine Clinical Trial site, updated June 2014); all currently approved gene therapy products are viral-based (Wirth, Parker & Yla-Herttuala 2013). However, several cases of cancer have developed in patients who received viral-based gene therapy treatment in clinical trials, raising important safety concerns (Ginn et al. 2013). Further limitations of the viral systems include their ability to raise immunological response, broad tropism to various cell types, and low loading capacity. Non-viral carriers are synthetic or natural substances which are able to bind and condense genetic material into nanoparticles and deliver it into cells. They provide easier, cheaper, and, most importantly, safer alternatives to viral systems (Niidome, Huang 2002 ; Pezzoli et al. 2012). Over the last decade, thousands of compounds have been tested for their gene delivery capacities. However, low delivery efficacy remains a serious problem, preventing the wider introduction of non-viral gene delivery systems into clinical trials.

To facilitate the development of more effective carriers, a thorough understanding of the delivery process is essential. Cell internalization is especially important, as target sites for gene-based therapeutics are intracellular. Gene delivery systems enter cells mainly by endocytosis, the process naturally used by cells for the uptake of solutes and particles (Iversen, Skotland & Sandvig 2011; Nguyen, Szoka 2012). Many endocytic pathways are known, and more are being discovered. The endocytic pathway is believed to define the intracellular trafficking and the final destination of the endocytosed entity, including the trafficking to the endolysosomal compartment for degradation, recycling to the cell

surface, and transport to the other side of the cell (transcytosis). Therefore, the identification of the endocytic pathways used by the gene delivery systems of different structures provides the basis for the development of more effective carriers. This type of study is methodologically very challenging, as the endocytic pathway of the gene delivery system is strongly affected by multiple factors, including the physico-chemical properties of the particle and the cell type (Vercauteren et al. 2012). To improve the reliability of the obtained data, comparative studies using different methods are required. Another important prerequisite for progress in the field is the use of suitable cell models which have been properly characterized in terms of their operating endocytic pathways.

The aim of this thesis is to improve the understanding of the role of the carrier and of the endocytic pathway in non-viral gene delivery. The problem is dealt with from several perspectives: testing of novel carriers for their gene delivery capacity; studying the relation between structure of the carrier, its endocytic pathway and its gene delivery efficacy; justification of methods and characterization of the *in vitro* cell models used to study the role of the endocytic pathway in gene delivery.

2 Review of the literature

2.1 Non-viral gene delivery systems

Thousands of molecules, both of natural origin and chemically synthesized, have been tested for their gene delivery properties. The vast majority of gene delivery systems have sizes in the submicron range and can therefore be classified as nanoparticles (NPs). The main classes of non-viral gene delivery systems include:

- polymer-based systems (polymeric NPs, polymerosomes, polymeric micelles)
- lipid-based systems (liposomes, nanoemulsions, solid lipid NPs)
- inorganic systems (calcium phosphate, gold and silica NPs, carbon nanotubes, etc.)
- hybrid particles (combinations of the aforementioned types)

Typically, molecules tested as potential non-viral gene delivery carriers are positively charged, in order to be able to spontaneously bind the negatively-charged genetic material. In general, an excess of the carrier is used to generate particles with a small size and positive surface charge. A size of about 100 nm seems to be the most favourable for cell uptake, although bigger particles (up to several μm) can be taken up, especially by cell types capable of phagocytosis (Hillaireau, Couvreur 2009). The positive surface charge facilitates the binding to the negatively charged cell membrane. Nevertheless, some examples of efficient gene delivery mediated by overall neutral or negatively charged NPs have also been reported (Patil, Rhodes & Burgess 2004; Green et al. 2007).

The performance of the carrier has often been improved by chemical modification with functional groups. For example, the attachment of polyethylene glycol (PEG) is widely used to increase water solubility and blood circulation time, to decrease renal clearance, and to reduce the immunogenicity of the gene delivery system (Ikeda, Nagasaki 2014). The active targeting of specific cell types can be achieved by the attachment of ligands that are recognized by particular cellular receptors. Examples of ligands successfully used for the targeting of gene delivery systems include transferrin (Dufes, Al Robaian & Somani 2013), folate (Zhao, Li & Lee 2008), epidermal growth factor (Ye, Gao & Cai 2010; Taylor, Furnari & Cavenee 2012), and many others.

In the following review, polymeric NPs and liposomes are discussed in more detail because these carriers have been included in the studies reported in this thesis.

2.1.1 Polymeric nanoparticles

Cationic polymers are able to condense genetic material into small complexes called polyplexes. Typical polyplexes are 10-100 nm in size, which is favourable for gene delivery to non-phagocytic cells. Polyplexes can be globular, toroid or rod-like in morphology depending on the type of polymer. Based on their molecular structure, cationic polymers can be divided into linear and branched (Figure 1, A,B). Polymer-based systems have the advantage of high carrying capacity and molecular diversity. Their physicochemical properties, such as molecular weight, polydispersity, composition, and

density of side chains, can be modulated in order to achieve better delivery efficacy, biocompatibility, and improved safety (Pezzoli et al. 2012).

Both synthetic polymers, such as polyethylenimine (PEI) and polyphosphoesters, and natural polymers, such as proteins, peptides, and polysaccharides, have been used for gene delivery. The most studied and promising polymers include the biodegradable polyesters (PLGA and PLA) (Avgoustakis 2004), PEI (Lungwitz et al. 2005), chitosan (biodegradable derivative of chitin) (Buschmann et al. 2013) and the dendrimers. The dendrimers are discussed in more detail in the next chapter.

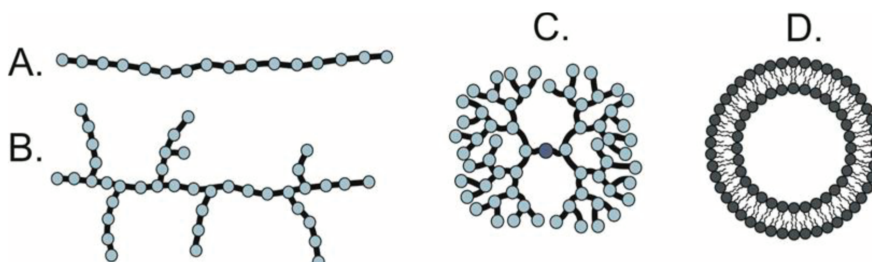


Figure 1. *Structures of carriers used for gene delivery: linear polymer (A), irregularly branched polymer (B), dendrimer (C), and liposome (D).*

2.1.2 Dendrimers

Dendrimers are globular, nano-sized macromolecules with regularly branched “arms” originating from a central core (Figure 1, C). The number of repeating branch units is referred as the “number of spheres”, or the “generation” of the dendrimer. The dendritic structure provides several advantages over conventional linear and irregularly branched structures. The controlled synthesis of dendrimers results in low polydispersity, and therefore more reproducible pharmacokinetic behavior. The high density of the surface groups provides various possibilities for surface functionalization in order to improve water solubility, reduce toxicity, target a specific tissue or cell type, etc. Due to the well-defined structure of the dendritic molecule, surface functionalization can be performed in a more controlled manner (Gillies, Frechet 2005; Nanjwade et al. 2009).

In recent years, many types of dendrimers have been synthesized and tested as potential gene delivery carriers. The gene delivery properties of the dendrimer are highly dependent on the generation number and the surface functionalization, as well as on the target cell type. Polyamidoamine (PAMAM) dendrimers, introduced by Tomalia et al. in 1984 (Tomalia et al. 1984), are probably the mostt studied ones, and some of them have shown efficient delivery of DNA, siRNA and oligonucleotides, both *in vitro* and *in vivo* (Xu, Wang & Pack 2010; Wu, Huang & He 2013). Polypropyleneimine dendrimers were demonstrated to induce intratumoral transgene expression after intravenous administration (Dufès et al. 2005) and to be efficient in siRNA delivery (Wu, Huang & He 2013).

Significant effort has been devoted to the development of biodegradable dendrimers. For example, some polyglycerol-based (Fischer et al. 2010) and peptide-based (Luo et al. 2012) dendrimers have been shown to have potential in gene delivery applications [reviewed in (Luo et al. 2014)].

2.1.3 Liposomes

Liposomes are spherical particles formed by one or more lipid bilayers with an aqueous central core (Figure 1, D). Liposomes have been shown to be efficient in the delivery of DNA, siRNA and oligonucleotides, both *in vitro* and *in vivo* (Fenske, Cullis 2008), and they are the most frequently used non-viral gene delivery systems in gene therapy clinical trials (Ginn et al. 2013). The main advantages of liposomes as gene delivery carriers are their low toxicity and high carrying capacity.

The liposomes used in gene delivery are typically composed of a cationic lipid responsible for the binding of the genetic material, and a neutral lipid, often called a "helper lipid", that improves stability and gene delivery efficacy. DOPE (dioleoylphosphatidylethanol-amine) and cholesterol are examples of the most frequently used helper lipids. Cationic lipids consist of a cationic headgroup and a hydrophobic domain connected by a linker. The properties of the head group, rather than those of the hydrophobic domain, are believed to be critical for gene transfer (Horobin, Weissig 2005). Examples of widely used cationic lipids include DOTAP (1,2-dioleoyloxy-3-(trimethylammonio)propane), DOTMA (N-[1-(2,3-dioleyl)propyl]-N,N,N-trimethylammonium chloride) and DOGS (dioctadecylamidoglycyl-spermine) (Balazs, Godbey 2011; Tros de Ilarduya, Sun & Duzgunes 2010).

Complexes consisting of cationic liposomes and nucleic acids are usually formed by self-assembly and are called lipoplexes. The lipoplex structure depends on the liposome lipid composition. Multilamellar (composed of DNA layers alternating with cationic lipid bilayers), inverted hexagonal, and other structures have been reported (Tros de Ilarduya, Sun & Duzgunes 2010).

2.2 Barriers to non-viral gene delivery

2.2.1 Extracellular barriers

In order to reach the target tissue/cell after *in vivo* administration, non-viral gene delivery systems need to pass multiple barriers created by extracellular biomolecules, non-target tissues, and cells (Wiethoff, Middaugh 2003; Uchegbu, Schatzlein 2010). The number of barriers to be overcome depends on the administration route (Figure 2).

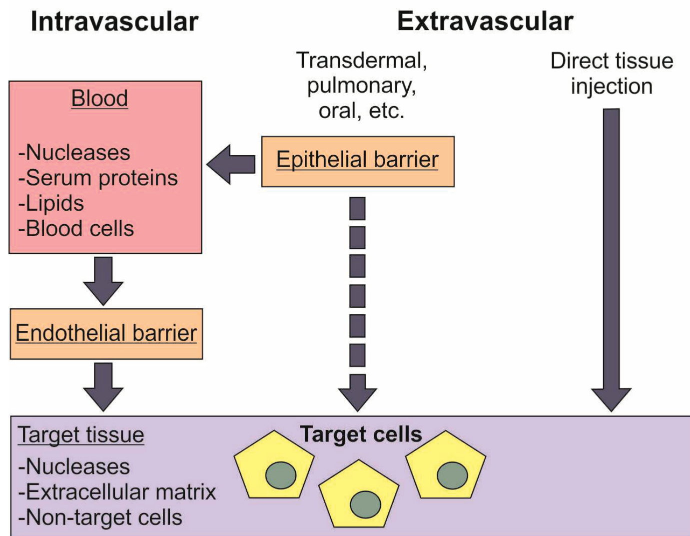


Figure 2. *Main extracellular barriers in the way of therapeutic nanoparticles administered by different routes. It must be noted that target cells can also be endothelial, epithelial or blood cells, depending on the pathological condition to be treated.*

Direct injection into target tissue, such as a tumor, is a widely used administration route for gene delivery systems as it brings them into immediate proximity to the target cells (Figure 2). However, the carrier needs to shield the genetic material efficiently from degradation by the extracellular nucleases present in the tissue. Moreover, the delivery system must avoid interaction with non-target cells and entrapment by the extracellular matrix including the proteoglycans. Negatively charged glycosaminoglycan chains of proteoglycans may bind positively charged gene delivery complexes and limit their mobility towards the target cells or cause premature release of the cargo (Ruponen et al. 2003).

Many *in vivo* tissues are not accessible for direct injection and can only be reached via intravascular administration. Once in the blood, delivery systems come into contact with nucleases, blood lipids, and negatively charged serum proteins such as albumin, fibronectin, complement, immunoglobulins, and apolipoproteins. Adsorption of proteins and lipids on the surface of the gene delivery system results in the formation of a so-called “biomolecular corona”. The composition of the biomolecular corona is dependent on both the NP properties and the composition of the biological environment and is subject to dynamic changes (Monopoli et al. 2012; Pearson, Juettner & Hong 2014). The biomolecular corona has been shown to critically affect the delivery properties of NPs, including their biodistribution, circulation time, targeting, and toxicity. For example, in the case of intravascular administration, adsorption of immunoglobulins and the complement on the NP surface can cause their recognition and rapid clearance from the circulation by the immune system (Pearson, Juettner & Hong 2014). Proteins on the

surface of targeted NPs were shown to perturb their binding to the target cells by shielding the receptor-specific ligands (Salvati et al. 2013). Biomolecular corona may also lead to premature cargo release (Azagarsamy, Yesilyurt & Thayumanavan 2010). Furthermore, delivery systems can unspecifically bind, enter, and be degraded within the blood and other non-target cells. The most common strategy to reduce unspecific interactions is to coat the gene delivery systems with stabilizing/shielding agents, such as PEG (Wiethoff, Middaugh 2003; Pezzoli et al. 2012) or albumin. For example, in the recent study of Peng and co-authors, a preformed albumin protein corona was shown to increase circulation time and reduce the toxicity of intravenously injected NPs (Peng et al. 2013).

The vascular endothelium creates an important extracellular barrier for systemically administrated delivery systems. Intracellular spaces between the endothelial cells are typically sealed with junctional complexes, including both tight junctions and adherens junctions. In the liver and spleen, the vascular endothelium has openings, or fenestrae, which allow enhanced paracellular permeation and the accumulation of nanoparticulate delivery systems in these organs (Garnett, Kallinteri 2006). Endothelia of other tissues, especially of the central nervous system, are continuous, tight, and therefore impermeable for the positively charged and relatively large in size gene delivery systems.

Junctional complexes are also very tight in epithelial tissues, forming a substantial extracellular barrier for delivery systems administered extravascularly, for example by transdermal, oral, or pulmonary administration routes.

To cross the epithelial/endothelial barriers by a paracellular pathway, the integrity of the adhesive junctions has to be perturbed. Intracellular junctions are known to be disrupted in some pathological conditions, for example in inflammation (Kumar et al. 2009; Ivanov, Parkos & Nusrat 2010). Disruption of intracellular junctions can also be achieved by applying permeation enhancers. For example, PAMAM dendrimers have been shown to have intrinsic permeation enhancer activity (Sadekar, Ghandehari 2012). In the absence of permeation enhancers, the only pathway by which gene delivery systems can overcome healthy epithelial/endothelial barriers is by transcytosis, which is discussed in greater detail in chapter 2.4.

2.2.2 Cell entry and intracellular barriers

After reaching the target cell, the gene delivery system needs to efficiently cross the plasma membrane, composed of a lipid bilayer, and then bypass multiple intracellular barriers (Figure 3). By now, it is well established that the main route for the uptake of NPs, including gene delivery systems, is endocytosis (Iversen, Skotland & Sandvig 2011; Treuel, Jiang & Nienhaus 2013). Typically, endocytosis occurs after cell surface binding, which can be either specific (if the surface of the gene delivery system is decorated by the ligand targeted to a certain receptor), or non-specific (hydrophobic and electrostatic interactions). NPs may also penetrate living cell membranes by generating ruptures, which often cause undesirable cytotoxic effects (Treuel, Jiang & Nienhaus 2013). However, membrane disruption can be reduced, or even avoided, by modulation of the surface structure and/or charge density (Verma et al. 2008; Lin et al. 2010; Wang et al. 2012).

Cytoplasm appears to be a considerable intracellular barrier, as it is crowded with macromolecules, is highly structured due to the dense cytoskeleton, and also contains active nucleases. Diffusion of genetic material through the cytoplasm is very limited due to its large size (Lukacs et al. 2000). It has been reported that both naked plasmid DNA (pDNA) and gene delivery systems (Vaughan, Dean 2006; Hasegawa, Hirashima & Nakanishi 2001; Suh, Wirtz & Hanes 2003) can be trafficked through the cytoplasm towards the nucleus along microtubules. With gene delivery systems, most of the microtubule-mediated transport occurs while still within the endosomes (Suh, Wirtz & Hanes 2003; Akita et al. 2010).

For small RNAs and oligonucleotides, cytoplasm is the final destination, whereas DNA has to penetrate into the nucleus in order to be transcribed (Figure 3). The nuclear envelope has been proposed as being one of the most substantial barriers. The nuclear envelope is fenestrated with nuclear pores that limit the passive diffusion of molecules with sizes larger than 50 kDa (Vaughan, DeGiulio & Dean 2006). Being relatively large in size, gene delivery systems, or released DNA, can enter the nucleus passively during cell division when the nuclear envelope is disassembled. This has been suggested as a major way for the entry of pDNA into the nuclei of rapidly dividing cells, such as immortalized cell lines (Brunner et al. 2000; Grosse et al. 2006). An alternative strategy is active transport through the nuclear pores utilizing the endogenous nuclear import machinery. Molecules modified with the nuclear targeting signal (NLS) can be recognized and transported into the nucleus by the nuclear pore complex (Vaughan, DeGiulio & Dean 2006).

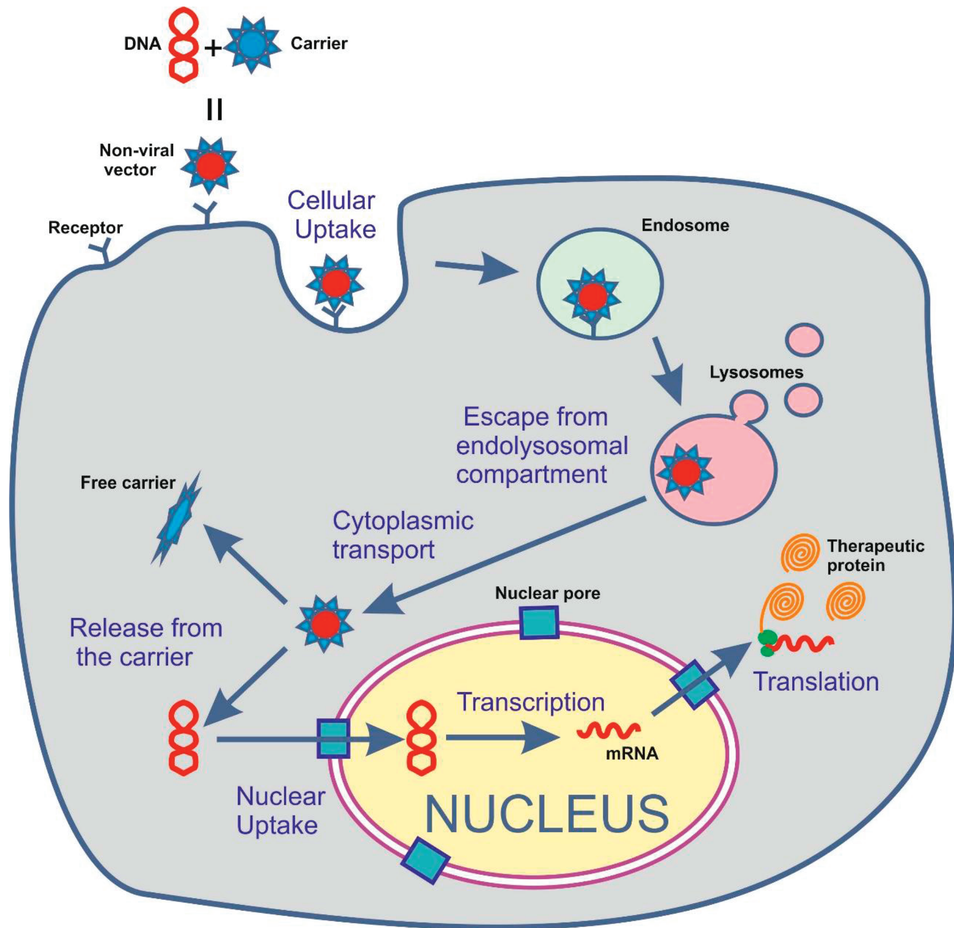


Figure 3. *Main intracellular steps and barriers to non-viral gene delivery.*

The release of genetic material from the carrier is another important intracellular barrier (Figure 3). The mechanism and intracellular location of this process is carrier-dependent. It is believed that lipoplexes release genetic material upon fusion with the endosomal membrane, whereas polyplexes escape from the endosomes intact and release DNA either in the cytoplasm or inside the nucleus. In general, binding by the carrier should be strong enough to prevent enzymatic degradation of the genetic material by intracellular nucleases, yet allow its further processing such as transcription. Indeed, insufficient release of DNA from a cationic carrier has been shown to be responsible for strikingly reduced transcription efficiency after gene delivery by the lipid-based carrier Lipofectamine in comparison to adenovirus (Hama et al. 2007).

Knowledge of the impact of the carrier on the functions of the cell is still very limited. It can be assumed that the positively charged carrier can interact with endogenous molecules causing toxicity and interfering with various intracellular processes including

the translation of the genetic material (Hama et al. 2007). One way to circumvent this problem is to use biodegradable carriers.

2.3 Endocytic pathways and their role in gene delivery

Endocytosis is a general term for the formation of membrane vesicles on the cell plasma membrane and the subsequent internalization of these vesicles. Endocytosis constantly occurs in living cells, serving their nutrient uptake, receptor signaling, regulation of cell shape, and volume. In certain cell types, endocytosis is involved in transcellular transport and pathogen removal (Doherty, McMahon 2009). The endocytic pathway is very important with respect to NP-mediated drug and gene delivery as it defines the intracellular routing, cytoplasmic release, final destination, and therefore the efficacy of the delivery system (Khalil et al. 2006a; Vercauteren et al. 2012).

Endocytosis is typically classified into phagocytosis, used for the uptake of large particles and mainly restricted to specialized mammalian cells, and pinocytosis, used for uptake of fluids and small particles and occurring in all cell types. Based on the proteins involved, pinocytosis can be further subdivided into macropinocytosis, clathrin-mediated endocytosis, and a diverse group of clathrin-independent pathways, the majority of which have been discovered only recently and are therefore relatively poorly characterized (Doherty, McMahon 2009; Sandvig et al. 2011). Alternatively, endocytic pathways can be classified based on their relation to lipid rafts. Lipid rafts are "small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes" (Pike 2006). Some types of endocytosis are believed to be restricted to certain types of lipid rafts. Therefore, lipid raft-based classification can be helpful in identifying the specific regulators of endocytic pathways as well as in selecting the specific inhibitors for particular pathways (El-Sayed, Harashima 2013). Other important characteristics of the endocytic pathways include the involvement of actin cytoskeleton and their dependence on dynamin - a large GTPase implicated in the fission of the endocytic vesicle from the plasma membrane. The main features of each endocytic pathway are represented in Figure 4. Their mechanisms and role in gene delivery will be discribed in greater detail in the following sections.

2.3.1 Phagocytosis

Phagocytosis refers to the uptake of large particles, of up to several micrometers in diameter. Being an important defence mechanism against foreign substances, phagocytosis is usually confined to specific cells of the immune system called professional phagocytes such as the polymorphonuclear granulocytes, the macrophages and monocytes. However, the non-professional phagocytes, for instance the fibroblasts and epithelial cells, are also able to uptake big particles under specific conditions (Rabinovitch 1995). Unlike macropinocytosis, phagocytosis involves the recognition and binding of the cargo to specific receptors. Recognition of particles lacking specific "eat me" signals on their

surface occurs after their opsonisation (binding of immunoglobulins and other components of the complement system). Actin-driven engulfment and internalization of the recognized cargo results in phagosome formation. Phagosomes fuse to early and late endosomes and form increasingly acidic phagolysosomes where the cargo is degraded by hydrolases and other antimicrobial enzymes (Flannagan, Jaumouille & Grinstein 2012).

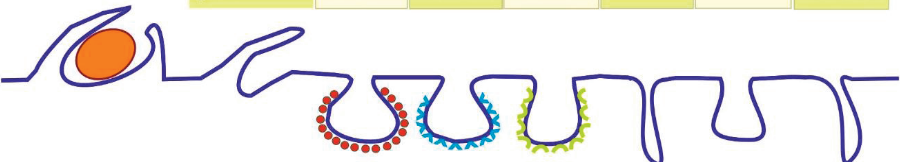
The fact that phagocytosis is mainly limited to specialized cell types has been successfully utilized for the passive targeting of gene delivery systems. Microparticles of the size of 1–10 μm, containing DNA vaccines, are too big for endocytosis by normal cells but small enough for phagocytosis by antigen presenting cells such as macrophages or dendritic cells (Little et al. 2005; Zhang et al. 2013). Phagocytosis, or a phagocytosis-like mechanism, has also been shown to be involved in the uptake of large gene delivery systems by non-professional phagocytes including airway epithelial cells (Matsui et al. 1997), fibroblasts (Santos et al. 2012), and retinal pigment epithelial cells (Vercauteren et al. 2011b).

2.3.2 Macropinocytosis

Macropinocytosis is a larger-scale type of pinocytosis in which the plasma membrane forms actin-driven protrusions, or ruffles, engulfing surrounding fluid. The resulting vesicles, termed macropinosomes, are usually 0.2-10 μm in size. This type of endocytosis is possible for any cell type, with only a few exceptions, and it is used for the massive uptake of solutes (Doherty, McMahon 2009).

Macropinocytosis is generally believed to be induced by growth factors, although it can also be induced by some viral and bacterial pathogens. In some cell types, for example in dendritic cells, macropinocytosis is a constitutive process not requiring activation (Jones 2007). Conventional macropinocytosis occurs at the cell periphery and does not require dynamin. However, a type of macropinocytosis called circular dorsal ruffles has been shown to be dynamin-dependent (Orth et al. 2006). The formation of circular dorsal ruffles has been observed in several cell types, including fibroblasts and differentiated epithelial cells, in response to growth factor activation (Buccione, Orth & McNiven 2004). The intracellular fate of macropinosome seems to vary between cell types. In most cases, macropinosomes mature, acquiring early and late endosome markers, and then fuse with lysosomes. However, in human epidermoid A-431 cells macropinosomes do not contact with the endolysosomal system and recycle their contents to the cell surface (Hewlett, Prescott & Watts 1994). In the human osteosarcoma cell line macropinosomes stay intracellularly, without fusion with lysosomes or recycling (Rintanen et al. 2012).

Macropinocytosis has been shown to be involved in the uptake and efficient transgene expression mediated by cell penetrating peptides (Wadia, Stan & Dowdy 2004; Khalil et al. 2006b; Al Soraj et al. 2012), lipoplexes (Pozzi et al. 2014), and PEI (Hufnagel et al. 2009). However, it must be noted that involvement of macropinocytosis in the gene delivery process is difficult to study due to the lack of specific inhibitors as well as characteristic membrane lipid or protein markers (El-Sayed, Harashima 2013).



	Phagocytosis	Macropinocytosis	Clathrin-mediated E.	Caveolae-mediated E.	Flotillin-mediated E.	GLIC/GEEC E.	RhoA-mediated E.	Arf6-mediated E.
Morphology and size	cargo-dependent, Up to 6-8 µm	ruffled, Up to 10 µm	vesicular, 150-200 nm	vesicular, ~100 nm	vesicular	tubular, diameter ~50-80 nm	vesicular	tubular
Cell type	mainly specialized cell types	all mammalian	all mammal.	endothelial, fibroblasts, muscular, some other	all mammal.	at least fibroblasts, epithelial	at least lymphocytes, epithelial	at least fibroblasts, epithelial
Marker/coating protein	-	-	clathrin	caveolin1 cavins	flotillin1 flotillin2	Graf 1	-	-
Membrane domains	mixed	mixed	non-lipid rafts	lipid rafts	lipid rafts	lipid rafts	lipid rafts	most likely lipid rafts
Dynamin involvement	+	+ and - reported	+	+	+ and - reported	-	+	+ and - reported
Actin involvement	+	+	-	+	-	+	?	+
Other regulators	Arf6 Cdc42 Rac1 RhoA	Arf6 Cdc42 Rac1 Pak1	Rab5 Arf6	?	?	Arf1 Cdc42	RhoA Rac1 Pak1	Arf6
Example cargos	pathogens, apoptotic remnants	fluid, some pathogens	Tf EGF LDL	CTB, SV40, GPI - anchored proteins	CTB, Proteoglycans	fluid, CTB, GPI - anchored proteins	IL2-R	MHC-I

Abbreviations:
E.=endocytosis
CLIC/GEEC= clathrin-independent carrier/GPI-anchored protein-enriched endosomal compartments
GPI= glycophosphatidylinositol
Tf=transferrin
EGF=epidermal growth factor
LDL=low density lipoprotein
CTB=cholera toxin β-unit
SV40= simian virus 40
IL2-R=receptor to interleukin 2
MHC=major histocompatibility complex

Figure 4. *Main endocytic pathways and their characteristics.*

2.3.3 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is one of the first discovered and most studied endocytic pathways (McMahon, Boucrot 2011). It occurs in all mammalian cell types and serves the continuous uptake of essential nutrients, antigens, growth factors, and receptors. CME is a rapid and highly dynamic process, which is usually triggered by the ligand binding to its specific receptor. With the help of adaptor and accessory proteins, of which AP2 seems to be the most important, clathrin is recruited from the cytosol to the plasma membrane where it forms hexagonal lattices. Polymerization of clathrin stabilizes the membrane curvature and results in the formation of clathrin-coated pits that are separated from the membrane by dynamin in an energy-dependent manner. The vesicles formed usually have a diameter of 100-150 nm and undergo rapid uncoating, resulting in early endosome formation. Upon endosome acidification, the receptors release ligands and are usually transported back to the cell surface membrane. Ligands are sorted according to their intracellular destinations, such as lysosomes, Golgi apparatus, or nucleus (Takei, Haucke 2001; Doherty, McMahon 2009; McMahon, Boucrot 2011).

Gene delivery systems can be targeted to CME by the attachment of ligands to specific receptors of which the transferrin and low-density lipoprotein (LDL) receptors are the most widely used. CME can also occur in a nonspecific manner. Nonspecific CME is involved in the internalization of carriers not coupled with any targeting moieties. As gene delivery systems taken up by CME generally follow the endolysosomal pathway, it is essential that they have an effective mechanism for endosomal escape. Therefore, a high level of uptake by CME does not necessarily lead to high gene delivery efficacy. For example, Rejman and co-workers demonstrated that although both PEI- and DOTAP-based complexes entered HeLa (cervix epithelial carcinoma) and A549 (pulmonary epithelial carcinoma) cells by CME, only in the case of DOTAP did this uptake pathway result in high transgene expression (Rejman, Bragonzi & Conese 2005).

2.3.4 Caveolae-mediated endocytosis

Caveolae-mediated endocytosis (CavME) originates from lipid rafts of 60-80 nm in size enriched with caveolin1 protein. The proteins termed cavins are essential for caveolae formation and may function as coat proteins, stabilizing caveolae. CavME is dynamin-dependent and is present in many cell types, including endothelial cells, adipocytes, and fibroblasts, but it is absent in some cell types such as kidney proximal tubule cells (Parton, Del Pozo 2013), lymphocytes, and neurons of the central nervous system (Razani, Woodman & Lisanti 2002). In contrast to the rapidly internalizing clathrin-coated pits, caveolae are very stable structures at the plasma membrane. In addition to their role in endocytosis, they participate in lipid regulation and signalling.

CavME has attracted considerable attention in NP-mediated gene and drug delivery, as it was long believed that cargo entering the cell by this pathway avoids lysosomal degradation. However, it is now clear that caveolae fuse with endosomes, thus following a conventional endocytic pathway (Sandvig et al. 2011; Parton, Del Pozo 2013). Caveolae have been shown to be involved in the uptake and efficient gene delivery mediated by

chitosan (Garaiova et al. 2012), PEI, DOTAP (Rejman, Bragonzi & Conese 2005; Yamano et al. 2011), anionic PAMAM dendrimers (Perumal et al. 2008), and some other carriers. However, the vast majority of these studies used chemical inhibitors (such as filipin and genistein) and caveolae markers (such as shiga toxin and CTB), all of which have lately been shown to be non-specific for the caveolae-mediated pathway (Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012). In addition, some of the ligands (such as lactosylceramide, a widely used specific marker of CavME) have been reported to induce internalization of otherwise immobile caveolae (Sharma et al. 2004). Therefore, the role of CavME in gene delivery might have been overestimated.

2.3.5 Flotillin-mediated endocytosis

Flotillin-mediated endocytosis (FME) was first reported in 2006. It occurs in lipid rafts enriched with the FME specific proteins flotillins -1 and -2, but devoid of caveolin1. Flotillins form microdomains in the plasma membrane of all studied mammalian cell types. The dynamin-dependency of this pathway is unclear, and the studies in this field are controversial (Otto, Nichols 2011). FME was reported to be responsible for the uptake of GPI-linked proteins, proteoglycans, and partly of CTB. As with caveolae, flotillin-enriched vesicles are internalized infrequently (Sandvig et al. 2011). They are believed to fuse with late endosomes/lysosomes thereby directing their content to a conventional endocytic pathway (Riento et al. 2009).

Several studies have demonstrated the involvement of FME in effective gene delivery. Vercauteren et al. have shown that amidoamine polymer-based gene delivery systems are internalized in retinal pigment epithelial cells by both FME and a phagocytosis-like mechanism, but only the FME mediated pathway was beneficial in terms of transgene expression (Vercauteren et al. 2011b). Other studies have demonstrated flotillin-mediated uptake of silica NPs (Kasper et al. 2013) and pegylated polylysine-based delivery systems (Chen et al. 2011).

2.3.6 Other clathrin-independent pathways

The CLIC/GEEC (clathrin-independent carrier/glycosylphosphatidylinositol (GPI)-anchored protein-enriched endosomal compartments) endocytic pathway has been shown to be involved in the uptake of GPI-anchored proteins, bacterial toxins, and extracellular fluid (Doherty, McMahon 2009; Sandvig et al. 2011). In fibroblasts, CLIC/GEEC endocytosis is responsible for at least half of the total fluid-phase uptake (Lundmark et al. 2008). It also plays an important role in cell spreading and migration (Doherty et al. 2011). This pathway requires the activity of small GTPases Cdc42 and Arf1, and is dependent on cholesterol and actin polymerization. GRAF1 protein has been proposed as a specific endogeneous marker (Lundmark et al. 2008), and cell-surface glycoprotein CD44 as a specific cargo for this pathway (Howes et al. 2010). Molecules endocytosed by CLIC/GEEC pathway are delivered to early endosomes. Their further destination appears

to be cell-type dependent, including both recycling endosomes and late endosomes/lysosomes (Fivaz et al. 2002).

Another clathrin-independent pathway is Arf6-dependent endocytosis, which has been found to be responsible for the uptake of major histocompatibility complex class I proteins and some integrins. The interleukin2 (IL2) receptor was shown to enter cells by a distinct endocytic pathway called RhoA-dependent endocytosis (Doherty, McMahon 2009; Sandvig et al. 2011).

All these pathways are now being extensively studied. Their role in non-viral gene delivery is not known due to their recent discovery and poor characterization.

2.4 Endocytosis in polarized cells and its role in gene delivery

So far, the vast majority of studies in the field of molecular biology involving endocytosis have been performed in non-polarized cells (Sandvig et al. 2011). A few studies in polarized epithelial and endothelial cells suggest differential localization and/or regulation of endocytic processes in apical and basolateral compartments. In particular, a predominant localization of caveolae on the basolateral membrane was found in endothelial cells of retinal and brain capillaries (Gardiner, Archer 1986), as well as in the MDCK (canine kidney epithelial) cell line (Vogel, Sandvig & van Deurs 1998). Although clathrin-coated pits are present at both the apical and basolateral sides of MDCK cells, they deliver their cargo to distinct intracellular compartments (Rodriguez-Boulan, Kreitzer & Musch 2005), suggesting differential regulation of CME. Soluble factors, such as proteinkinases A and C, were demonstrated to selectively stimulate clathrin-independent endocytosis on the apical side of MDCK cells without affecting the basolateral uptake (Eker et al. 1994). These experiments demonstrate that clathrin-independent endocytic processes can also be subject to differential regulation.

Endocytosis is a first and critical step in transcytosis: the transport of macromolecular cargo from one side of a cell to the other within membrane vesicles. This process is believed to be of major importance in epithelial and endothelial cells as it mediates the delivery of macromolecules through polarized cell barriers to the underlying tissue or blood circulation. *In vivo* transcytosis has been documented in the endothelial cells of heart, lung, skeletal muscle, and brain capillaries, as well as in the epithelial cells of intestine, trachea, lung, thyroid, and some others. In addition, some other cell types, such as bone-resorbing osteoclasts and neurons, are also capable of transcytosis (Tuma, Hubbard 2003).

Although the regulation of transcytosis remains largely unknown, the endocytic pathways involved seem to be highly dependent on the cell type. There is strong evidence that the transcytosis of macromolecules through endothelial cells occurs by CavME. The transendothelial transport of molecules, such as albumin, iron-transferrin, low density lipoproteins, insulin, and chemokines, has been shown to involve caveolae (Frank, Pavlides & Lisanti 2009; Wang et al. 2011). It has been proposed that in endothelial cells the endocytic pathway dictates the intracellular fate of the endocytosed cargo. Internalization by CME directs the cargo to lysosomal degradation, whereas caveolar

uptake ensures transcytosis (Tuma, Hubbard 2003). In epithelial cells transcytosis seems to be the branch of the conventional endocytic pathway mediated by CME. The best example studied is the transcytosis of Immunoglobulin A (IgA) and its receptor by epithelial cells in the intestine, kidney, and trachea (Tuma, Hubbard 2003).

Information about the involvement of endocytic pathways, other than CME and CavME, in transcytosis is very scarce. For example, FME has been shown to be involved in the transport of GPI-anchored proteins through polarized hepatocytes (Aït-Slimane et al. 2009).

Detailed understanding of the endocytic processes in polarized cells and their regulation can provide tools to improve the efficacy of gene delivery systems. The targeting of gene delivery systems to the transcytotic pathway would allow crossing of the endothelial and epithelial barriers on the way from the site of administration to the target tissue. For example, gene delivery systems decorated with ligands targeted to insulin and transferrin receptors, which are known to undergo transcytosis, have been shown to cross the blood-brain barrier endothelial cells and deliver genetic material to the brain (Zhang, Schlachetzki & Pardridge 2003; Shi et al. 2001). In contrast, the transcytotic pathway should be avoided in gene therapy approaches where the endothelial and epithelial cells are viewed not as a barrier, but as the target. For example, in gene therapy of hemophilia, intestinal epithelial cells can be transformed to become platforms for the expression of functional coagulation factors (Lozier et al. 1997); blood endothelial cells can be targeted for gene therapy for stroke, atherosclerosis, and tumors (Mukerjee et al. 2011).

2.5 Experimental approaches to the study of endocytic pathways of gene delivery systems

Studying the role of the endocytic pathway in NP-mediated delivery is a very challenging task, as the uptake pathway is affected by multiple factors, such as particle size, shape, surface charge, and ligand coupling, as well as the cell type. Besides, the same particles have been shown to utilize more than one uptake pathway (Sahay, Alakhova & Kabanov 2010; Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012).

Due to the lack of appropriate methodology for *in vivo* endocytosis studies, the vast majority of data in the field has been generated *in vitro*. Although cell culture and isolated tissue studies do not fully reconstitute a complex *in vivo* environment, especially the signals provided by the vascular, nervous, and lymphatic systems, they can provide fundamental information on the basic uptake and trafficking mechanisms of NPs in real-time and at high resolution.

Currently available *in vitro* tools for the study of endocytic pathways can be roughly divided into two groups:

- Inhibition studies, where one or several pathways are blocked by pharmacological agents, gene silencing, or genetic manipulation of cell lines.
- Colocalization studies of NPs with specific endocytic pathways under normal (not-blocked) conditions.

The advantages and potential drawbacks of these methods will be discussed briefly in the following chapters. (For a detailed review see: Sahay, Alakhova & Kabanov 2010; Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012.)

2.5.1 Chemical inhibition of endocytosis

Blockage of endocytosis with pharmacological agents is the oldest, but still widely used, approach for studying the role of the endocytic pathway in the delivery of NPs. This approach has several advantages. It is relatively easy, cheap, and all cells in the culture dish are equally affected by the treatment. As a short incubation period is usually sufficient to achieve the blocking effect, the cells do not have time to develop delayed side effects or compensatory mechanisms. However, this method has lately raised a lot of concerns due to the toxicity, low specificity, and side effects of the chemical inhibitors (Ivanov 2008; Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012). The undesirable effects of chemical inhibitors can largely be attributed to their unspecific interactions with multiple intracellular targets such as structural proteins, enzymes, and lipids. For example, many of the currently used chemical inhibitors were shown to interfere with the formation and distribution of F-actin fibers (Ivanov 2008). The actin cytoskeleton plays a critical role in many intracellular processes, including cell motility, division, signalling, and organelle and vesicular trafficking. Therefore, the preturbation of the normal functioning of this protein results in multiple side effects, as well as in unspecific blockage of all actin-dependent endocytic pathways. It must also be noted that the performance of chemical inhibitors has been shown to vary between cell types (Vercauteren et al. 2010). The inhibitors mostly used in NP studies, their mechanism of action, and their main drawbacks are presented in Table 1.

When using chemical inhibitors one should remember, that (1) concentrations and exposure time should be carefully optimized for each cell line in order to find the window where inhibitors are efficient but do not exhibit significant toxicity and side effects, and (2) inhibitor specificity to a particular endocytic pathway should be verified individually for each cell line. In general, a combination with more modern approaches is highly recommended in order to draw reliable conclusions (Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2010).

2.5.2 Genetic inhibition of endocytosis

Gene silencing. Blockage of the endocytic pathway can be achieved by silencing the expression of key endocytic proteins by siRNA technology. This method is believed to be more specific and less cytotoxic than chemical inhibitors. For example, siRNAs have been used against the clathrin heavy chain (CHC), flotillin1, dynamin2, caveolin1, p21-activated kinase 1 (Pak1) (Gabrielson, Pack 2009; Sahay et al. 2013; Vercauteren et al. 2011b; Al Soraj et al. 2012), Cdc42 and Rab1 (Sahay et al. 2013) to study the role of the endocytic pathway in non-viral gene delivery. However, the selection of the target protein requires detailed knowledge of the specific endocytic pathways at the molecular level.

Additionally, most of the endocytosis-related proteins are known to be involved in more than one pathway. Moreover, many proteins, including clathrin and caveolin, have other functions in the cell unrelated to endocytosis (Brodsky 2012; Liu, Rudick & Anderson 2002). It must also be noted that the siRNA-mediated blockage of the endocytic pathway usually takes 2-5 days. Therefore, the cells have time to adjust to the new condition, for example by activating compensatory endocytic pathways. It has also been reported that siRNA-mediated down-regulation does not occur equally in all cell populations. Another concern is the efficacy and duration of the silencing after siRNA treatment. In addition, the transfection agent used to deliver siRNA may cause side effects (Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012).

Other genetic manipulations. One more tool that selectively perturbs one or more endocytic pathways is the expression of mutant endocytosis-related protein that interferes with the function of endogenous protein. Benmerah and co-workers transiently blocked CME by transfecting cells with genetic construct coding for Epsin15 mutant lacking EH domains (Benmerah et al. 1999). Similarly, Roy et al. used amino-terminal truncation mutants of caveolin to perturb CavME (Roy et al. 1999). For a more controllable expression, genetically manipulated stable cell lines can be generated. For example, a DynK44A dominant-negative mutant was introduced to human epithelial carcinoma HeLa cells to block dynamin-dependent endocytic pathways (Damke et al. 1994). Another example is the hamster kidney fibroblast BHK cell line expressing CHC antisense RNA in order to inhibit CME (Iversen et al. 2003). In these cell lines the blockage of endocytosis can be induced by the removal of the antibiotic from the cell culture medium.

As with the gene silencing approach, this method is laborious, requires detailed knowledge of the molecular biology of endocytosis, and may result in the activation of compensatory endocytic mechanisms. In addition, abnormally high intracellular levels of mutant protein may lead to low-affinity interactions, not observed under normal conditions, and causing side effects and artefacts (Iversen, Skotland & Sandvig 2011).

Table1. *Chemical inhibitors of endocytosis, their main features/drawbacks and examples of their use in gene delivery studies.*

Pathway to be blocked	Inhibitor	Mechanism of action	Specificity	Drawbacks	Examples of use in non-viral gene delivery studies
CME	Hypertonic sucrose	Removes of clathrin lattices from the PM	Affects lipid raft-mediated E. and macropinocytosis	Multiple side effects including cell shrinkage, activation of ion pumps and redistribution of F-actin	Sahay, Batrakova & Kabanov 2008; Perumal et al. 2008; Hsu, Uludag 2012
	Potassium depletion	Removes of clathrin lattices from the PM	Affects fluid-phase uptake	Redistribution of F-actin	Zuhorn, Kalicharan & Hoekstra 2002; Rejman, Bragonzi & Conese 2005
	Chlorpromazine	Dissociates clathrin and adaptor proteins from the PM	Interferes with macropinocytosis and phagocytosis	Interacts with intracellular lipids and cytoskeletal regulators; toxic and inefficient in some cell lines	Zuhorn, Kalicharan & Hoekstra 2002; Vercauteren et al. 2011b; Al Soraj et al. 2012; Gabrielson, Pack 2009; von Gersdorff et al. 2006; Rejman, Bragonzi & Conese 2005; Perumal et al. 2008; Wong, Scales & Reilly 2007
Caveolae/lipid raft mediated endocytosis	Statins (lovastatin, simvastatin, pravastatin)	General inhibition of cholesterol synthesis	Interferes with CME, phagocytosis and fluid-phase uptake	Disturbs biosynthesis of various intracellular proteins	Zuhorn, Kalicharan & Hoekstra 2002; Vercauteren et al. 2010
	Methyl-β-cyclodextrin	Extracts cholesterol from the PM	Interferes with lipid raft-mediated E. and CME, inhibits fluid-phase uptake	Disturbs cytoskeleton, cell signalling	Zuhorn, Kalicharan & Hoekstra 2002; Rinne et al. 2007; Vercauteren et al. 2011b; Sahay, Batrakova & Kabanov 2008; Hsu, Uludag 2012; Wong, Scales & Reilly 2007
	Filipin	Binds cholesterol in PM	Affects other cholesterol-dependent mechanisms, such as macropinocytosis	Possible PM permeabilization, dissociates cortical F-actin from PM; unstable	Zuhorn, Kalicharan & Hoekstra 2002; von Gersdorff et al. 2006;

					Rejman, Bragonzi & Conese 2005; Perumal et al. 2008; Wong, Scales & Reilly 2007; Zuhorn, Kalicharan & Hoekstra 2002
	Nystatin	Binds cholesterol in PM	Affects other cholesterol-dependent mechanisms, such as macropinocytosis		Arukuusk et al. 2013
	Genistein	Tyrosine kinase inhibitor	Affects CME of receptors that need tyrosine phosphorylation for accumulation in clathrin-coated pits.	Efficiency depends on cell line	Vercauteren et al. 2011b; Gabrielson, Pack 2009; Rejman, Bragonzi & Conese 2005; Hsu, Uludag 2012; Wong, Scales & Reilly 2007; van der Aa et al. 2007
Macro-pinocytosis and phagocytosis	Amiloride and its derivatives (EIPA, DMA and HOE-694)	Inhibits sodium-proton exchange	Possibly affects CME and lipid raft-mediated E.	Reorganization of F-actin affecting vesicular trafficking and cytoskeleton	Rinne et al. 2007; Al Soraj et al. 2012; Hsu, Uludag 2012; Wong, Scales & Reilly 2007
	Rottlerin	Inhibits protein kinases C	Reported to be a selective inhibitor of constitutive macropinocytosis. Possibly affects also fluid phase endocytosis and phagocytosis		Hufnagel et al. 2009; Vercauteren et al. 2011b
	Wortmannin	Inhibits phosphatidylinositol 3-kinase	Specificity questionable		Hsu, Uludag 2012; van der Aa et al. 2007
Others	Dynasore	Inhibits GTPase activity of dynamin1 and -2	Affects CME, CavME and phagocytosis		Vercauteren et al. 2011b; Al Soraj et al. 2012
	Cytochalasin D	Inhibits filamental F-actin elongation	Affects mainly CME, fluid phase endocytosis and phagocytosis	Impairs other processes where actin filaments are involved.	Rinne et al. 2007; Vercauteren et al. 2011b; Al Soraj et al. 2012; Perumal et al. 2008; Zuhorn, Kalicharan & Hoekstra 2002

Abbreviations: *E.= endocytosis; CME=clathrin-mediated endocytosis; CavME=caveolae-mediated endocytosis; PM=plasma membrane.*

2.5.3 Fluorescent microscopy and other methods

Fluorescent microscopy is a widely used technique for the study of uptake and intracellular trafficking of NPs, including gene delivery systems. In one approach, cells are treated with fluorescently labelled NPs, fixed, and stained by immunocytochemistry for pathway-specific or intracellular compartment-specific marker proteins (see Table 2 for examples). After imaging by confocal microscopy, colocalization analysis is performed. Alternatively, colocalization studies can be performed in living cells. In this case, prior to exposure to NPs, cells are transfected with genetic constructs encoding for endocytosis-related proteins fused with fluorescent proteins like green fluorescent protein (GFP) (Table 2). Such an approach avoids artefacts related to cell fixation and permeabilization, and allows real-time imaging. However, possible artefacts related to protein overexpression, including toxicity, aggregation, and low affinity interactions, should be considered (Vercauteren et al. 2012).

Another way to study the endocytosis of NPs is through colocalization with marker molecules with known uptake and/or trafficking pathways (Table 2). However, many of these markers have been shown to utilize multiple endocytic pathways in the same cell, and/or different pathways in different cell types. Therefore, for most of the pathways, no specific marker is available at the moment. It must also be noted that electrostatic interactions between cell membrane and cationic NPs often result in a significant surface-bound fraction, which might be difficult to dissect from the intracellular fraction (Iversen, Skotland & Sandvig 2011).

In general, low resolution is the common disadvantage of all fluorescent microscopy methods. Parallel analysis of NP uptake by microscopic techniques with higher resolution, such as atomic force microscopy, or different types of electron microscopy, can provide more detailed information.

The activity of a specific endocytic pathway can be quantitatively estimated by measuring the uptake of fluorescently labelled pathway-specific markers by flow cytometry. As with fluorescent microscopy, special attention should be paid to verifying the specificity of the markers and to the removal of cell surface-bound markers and NPs (Iversen, Skotland & Sandvig 2011).

Table 2. *Endocytic pathway-specific or intracellular compartment-specific markers typically used in colocalization studies in non-viral gene delivery.*

Endocytic pathway-specific markers

Endocytic pathway	*Marker*	*Description*	*Examples of use in non-viral gene delivery studies*
CME	CHC	Component of coating protein of CME	Immunofluorescence: Gabrielson, Pack 2009; Kasper et al. 2013 Fusion protein: Vercauteren et al. 2011a
	Transferrin	Glycoprotein that binds to iron and transports it inside cells	Rinne et al. 2007; Vercauteren et al. 2011b; Al Soraj et al. 2012; von Gersdorff et al. 2006; Hufnagel et al. 2009; Sahay, Batrakova & Kabanov 2008; Perumal et al. 2008
CavME	Lactosyl-ceramide	Glycosphingolipid ubiquitously present in mammalian tissues	Vercauteren et al. 2011b; Al Soraj et al. 2012; Perumal et al. 2008
	CTB	Cholera toxin B subunit	von Gersdorff et al. 2006; Hufnagel et al. 2009; Sahay, Batrakova & Kabanov 2008
	Caveolin-1	Coating protein of caveolae	Immunofluorescence: Kasper et al. 2013; Rinne et al. 2007; Gabrielson, Pack 2009 Fusion protein: Vercauteren et al. 2011b; Billiet et al. 2012; Vercauteren et al. 2011a
FME	Flotillin-1	Membrane-associated marker protein of FME	Immunofluorescence: Kasper et al. 2013 Fusion protein: Vercauteren et al. 2011b
	Flotillin-2	Membrane-associated marker protein of FME	Immunofluorescence: Kasper et al. 2013; Vercauteren et al. 2011a
Fluid-phase uptake (group of constitutive pathways)	Dextran	Polysaccharide	Rinne et al. 2007; Vercauteren et al. 2011b; Al Soraj et al. 2012; Perumal et al. 2008
	HRP	Horseradish peroxidase, enzyme	Sahay, Batrakova & Kabanov 2008

Intracellular compartment-specific markers

Intracellular compartment	***Marker***	***Description***	***Examples of use in non-viral gene delivery studies***
Early endosome	Rab5	Small GTP-binding protein	Immunofluorescence: Rinne et al. 2007; Billiet et al. 2012 Fusion protein: Billiet et al. 2012; Wong, Scales & Reilly 2007; Vercauteren et al. 2011a
	EEA1	Endosomal membrane-bound protein	Immunofluorescence: Gabrielson, Pack 2009
Late endosome	Rab7	Small GTP-binding protein	Immunofluorescence: Billiet et al. 2012 Fusion protein: Billiet et al. 2012; Wong, Scales & Reilly 2007; Vercauteren et al. 2011a
	LysoTracker	Fluorescent dye selectively accumulating in cellular compartments with low internal pH	Perumal et al. 2008
Recycling endosome	Rab11	Small GTP-binding protein	Fusion protein: Vercauteren et al. 2011a
Lysosome	LAMP1	Transmembrane protein associated with lysosomes	Immunofluorescence: Gabrielson, Pack 2009 Fusion protein: Vercauteren et al. 2011a
	LysoTracker	See above	Perumal et al. 2008
	Dil-LDL	Fluorescently labelled low density lipoprotein, known to be transported to lysosomes	Karjalainen et al. 2011

Abbreviations: *CME=clathrin-mediated endocytosis; CavME= caveolae-mediated endocytosis; FME=flotillin-mediated endocytosis.*

3 Aims of the study

The general aim of this study was to evaluate the impact of the carrier and the endocytic pathway on the non-viral gene delivery process and to improve the methodology used in this type of study.

The more specific aims were:

1. To test the gene delivery properties of novel lysine-based dendrimers.

2. To clarify the impact of the carrier and endocytic pathway on cell uptake and the intracellular kinetics of the introduced plasmid DNA.

3. To compare the genetic and chemical means of blocking endocytosis as tools for studying the role of the endocytic pathway in nanoparticle-mediated gene delivery.

4. To suggest methods for endocytic characterization of endothelial and epithelial cell lines utilized in nanoparticle uptake and transcellular permeation studies.

4 Overview of materials and methods

Main materials used in the studies:

Material	Description	Source/Preparation method	Publication
Plasmids			
pCMV-nls*lac*Z	Plasmid DNA containing marker gene for bacterial β-galactosidase modified with the nuclear localization signal	Provided by B. Scholte (Erazmus University, Rotterdam, the Netherlands)	I
pCLuc4	Plasmid DNA containing marker gene for luciferase	Provided by Dr. F.C. Szoka (UCSF, San-Francisco, USA)	II
Gene delivery carriers			
D5	Pentaspheric lysine dendrimer	Synthesized at the Institute of Macromolecular Compounds, St-Petersburg, Russia	I
D5C10	Pentaspheric lysine dendrimer modified at the surface with capric acid	Synthesized at the Institute of Macromolecular Compounds, St-Petersburg, Russia	I
Polyfect	Polyamidoamine (PAMAM) cationic dendrimer	Commercially available (Qiagen)	I
PEI	Polyethylenimine, cationic polymer, branched, 25kDa	Commercially available (Sigma-Aldrich)	II
DOTAP liposomes	1,2-dioleoyloxy-3-(trimethylammonio)propane –based cationic liposomes	Prepared from commercially available lipid (Avanti Polar Lipids)	II
CaP precipitates		Prepared by calcium phosphate coprecipitation with pDNA	II
Fluospheres, 200 nm	Fluorescently-labelled polystyrene particles	Commercially available (Molecular Probes)	III

Cell lines

HeLa	Human cervix epithelial carcinoma cells	Cell culture collection of the Institute of Cytology, St. Petersburg, Russia	I
HeLaK44A	Human cervix epithelial carcinoma cells with inducible overexpression of dynamin 2	Provided by Prof. K. Sandvig (Institute for Cancer Research at the Norwegian Radium Hospital, Oslo, Norway)	II
BHK21tTA	Hamster kidney fibroblasts with inducible expression of clathrin antisense RNA		
hCMEC/D3	Human brain endothelial cells	Provided by Dr. P.O. Couraud (Institute of Cochin, Paris, France)	III
CaCo-2	Human colon epithelial carcinoma cells	ATCC (American Type Culture Collection)	III

Chemical inhibitors of endocytosis

Chlorpromazine	Inhibitor of CME	Commercially available (Sigma-Aldrich)	II
Monodansyl-cadaverine	Inhibitor of CME	Commercially available (Sigma-Aldrich)	II
Nystatin	Inhibitor of caveolae/lipid raft-mediated endocytosis	Commercially available (Sigma-Aldrich)	II
Dynasore	Inhibitor of dynamin-mediated endocytosis	Commercially available (Sigma-Aldrich)	II

Markers of endocytic pathways

Tf	Fluorescently labeled transferrin, marker of CME	Commercially available (Molecular Probes)	II, III
LacCer	Fluorescently labeled lactosylceramide, marker of caveolae/lipid raft mediated endocytosis	Commercially available (Molecular Probes)	III
Dextran	Fluorescently labeled polysaccaride, molecular weight 10kDa, marker of fluid phase uptake	Commercially available (Sigma-Aldrich)	III

Methods used in the studies:

Study objective	**Method**	**Publication**
pDNA binding efficiency of gene delivery carrier	Ethidium bromide displacement assay	I
pDNA protection capacity of gene delivery carrier	Resistance to DNase I digestion test	I
Gene delivery complex size	Dynamic light scattering	II
Cytotoxicity	MTT assay	II
pDNA delivery (transfection) efficacy	β-galactosidase activity assay	I
	Luciferase activity assay	II
Cellular uptake of gene delivery systems	qRT-PCR	II
Quantification of nuclear-associated pDNA	Nuclear isolation, qRT-PCR	II
Blockage of endocytic pathways	Chemical inhibitors	II
	Genetically manipulated cell lines	II
Activity of endocytic pathways	FACS-analysis	II, III
Gene expression at the protein level	Western blotting	III
Gene expression at mRNA level	qRT-PCR	III
Protein intracellular localization	Immunofluorescence	III

5 Lysine dendrimers as vectors for delivering genetic constructs to eukaryotic cells

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6 Genetic blockage of endocytic pathways reveals differences in the intracellular processing of non-viral gene delivery systems

II

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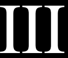
7 Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models



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8 Overview of the results

The main results reported in this thesis are summarized in the table below. More detailed information can be found in the original publications.

Publication I: Novel lysine dendrimers as gene delivery carriers.

pDNA binding efficacy	Lysine based dendrimers D5 and D5C10 completely bound pDNA at DNA/carrier charge ratio 1/1 and 1/2, respectively. The pDNA/dendrimer complex density increased with the increasing amount of the carrier
pDNA protection efficacy	The minimal DNA/carrier charge ratio ensuring complete protection of pDNA against nuclease degradation was 1/5 for D5 and 1/2 for D5C10
***In vitro* pDNA delivery efficacy**	The maximum pDNA delivery efficacy was 0.25% of positive cells for D5 and 2.5% for D5C10
Effect of endosomolytic peptide JTS-1 on the pDNA delivery efficacy	JTS-1 increased the pDNA delivery efficacy by 1.5 times for D5 and 4 times for D5C10
Summary: Modification of lysine dendrimers with capric acid residues was favourable for DNA binding and protective properties, and resulted in increased delivery efficacy. The endosomolytic agent further improved the delivery efficacy of the lysine dendrimers, however it still remained relatively low.	

Publication II: Intracellular processing of non-viral gene delivery systems.

	HeLaK44A cells	**BHK21tTA cells**
pDNA cell uptake	PEI>DOTAP>CaP	PEI>DOTAP>CaP
pDNA nuclear association	PEI>DOTAP>CaP	PEI>DOTAP>CaP
Rate of cell-associated (=intracellular+cell surface bound) pDNA elimination	CaP>DOTAP>PEI	CaP>DOTAP>PEI
Transgene expression	DOTAP >PEI~CaP	DOTAP>PEI>CaP
Summary: Neither cellular uptake nor nuclear association of pDNA correlates with transgene expression; transfection-limiting steps differ between carriers. Similar trends were observed in two cell lines.		

Publication II: Comparison of genetic and chemical approaches to block the endocytic pathway.

	Genetic block	**Chemical block**
Effect on transfection efficacy of non-viral gene delivery systems	PEI: reduced DOTAP: not changed, or reduced (depending on the cell line) CaP: increased	PEI: reduced DOTAP: reduced CaP: reduced

Summary: In many cases similar trends were observed between the two methods. However, chemical inhibitors resulted in a stronger inhibitory effect on transgene expression than the usage of genetically manipulated cell lines. The most serious discrepancies between the two approaches were observed for CaP precipitates.

Publication II: The role of the endocytic pathway in the cellular kinetics of non-viral gene delivery systems.

	HeLaK44A cells with blocked dynamin-mediated endocytosis	**BHK21tTA cells with blocked CME**
pDNA cell uptake	Reduced for all carriers	Reduced for all carriers
pDNA nuclear association	PEI: reduced DOTAP: not changed CaP: increased	PEI: reduced DOTAP: not changed CaP: increased
Rate of cell-associated (=intracellular+cell surface bound) pDNA elimination	PEI: reduced DOTAP: increased CaP: not changed	PEI: not changed DOTAP: not changed CaP: not changed
Transgene expression	PEI: reduced DOTAP: not changed CaP: increased	PEI: reduced DOTAP: reduced CaP: increased

Summary: The effect of a specific blockage of either clathrin- or dynamin-dependent endocytosis on transfection efficacy (=transgene expression) depends both on the carrier and the cell type. Changes in transfection efficacy after genetic blockage of a specific endocytic pathway correlated best with the level of nuclear-associated pDNA.

Publication III: Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models.

	CaCo-2 intestinal epithelial cells	**hCMEC/D3 brain endothelial cells**
Tightness and differentiation status	Differentiated, tight monolayer barrier with low proliferation	Low differentiation, multilayer, leaky barrier
Expression of the endocytosis-related genes at mRNA level during differentiation	Decreased	No change
Expression of the endocytosis-related genes at protein level during differentiation	Correlated to the mRNA levels in some, but not all cases	Correlated to the mRNA levels
Activity of endocytic pathways during differentiation	General endocytic activity, CME and lipid raft-mediated endocytosis was reduced	No change
Nanoparticle uptake during differentiation	Decreased	No change
Comparison of endocytic	No caveolin expression;	High PAK1 expression;

profiles of epithelial and endothelial cell lines	Generally lower mRNA expression of endocytosis related genes	Generally higher mRNA expression of endocytosis related genes
Comparison of endocytic profiles of cell lines to primary cells	Discrepancies in expression of endocytosis-related genes detected at both mRNA and protein level	
Summary: Cell differentiation is correlated with a clear reduction in endocytosis; mRNA levels of some endocytosis-related proteins are indicative of cell endocytic activity. Differences in endocytic profiles were revealed between endothelial and epithelial cells and also between cell lines and primary cells.		

9 General discussion

9.1 The role of the carrier in non-viral gene delivery

An “ideal” gene delivery carrier should fulfil many strict requirements. In particular, it should be able to bind and compact nucleic acid to small particles, protect it from undesirable interactions with biomolecules, deliver and release the cargo specifically to the intracellular target site, and possess low toxicity (Pezzoli et al. 2012). The molecular structure of the carrier is a key parameter affecting its gene delivery properties.

Developments in modern chemistry permit the synthesis of numerous compounds potentially applicable for gene delivery purposes. Lysine dendrimers combine the benefits of the dendritic structure, such as low polydispersity and ease of functionalization, with the biodegradability of lysine (Boyd et al. 2006). In the publication I, we studied the gene delivery properties of novel asymmetric lysine dendrimers: pentaspherical lysine dendrimer D5 and its analog D5C10, modified with capric acid residues at the outer sphere. Indeed, both molecules were able to bind and compact pDNA, protect it from nuclease degradation, and deliver to the cell nuclei as was indicated by the transgene expression. We demonstrated that modification of the dendrimer surface with capric acid residues increased its DNA-binding and nuclease protective properties and its gene delivery efficacy. Lipid modification has also been shown to improve the gene delivery properties of other cationic polymers (Incani, Lavasanifar & Uludag 2010). This positive effect may be attributed to the hydrophobic interactions between carrier molecules and lipid membranes. The addition of endosomolytic peptide JTS-1 resulted in a considerable increase of transfection efficacy, indicating that endosomal release is one of the important transfection-limiting steps for lysine dendrimers. Low endosomal release has been also demonstrated in experiments with other PLL-based carriers (Akinc, Langer 2002).

In our study, the efficacy of the lysine dendrimers did not exceed 15% of the treated cells (when the endosomolytic peptide was used), whereas the efficacy of some viral systems approaches 100%. Low transfection efficacy is a problem typical of non-viral systems, including those based on lysine (Pezzoli et al. 2012). Although significant efforts have been exerted to improve the efficacy of non-viral carriers, it must be noted that low efficacy is sufficient, and is even desirable, in some applications, for example with DNA vaccination (Chen, Huang 2005).

Overall, the data reported in the publication I demonstrate that lysine dendrimers can be used as a basis for the development of more effective carriers. The superior gene delivery properties of the modified over the unmodified dendrimer prove the validity of the concept of modular multifunctional carriers. This concept suggests improving the gene delivery properties of the basic carrier by the addition of certain blocks or complex components targeted to overcome specific barriers (Sanvicens, Marco 2008). Further illustrations indicating the validity of this concept for lysine dendrimers have been provided by later studies performed by other groups. For example, PEG has been used to improve the pharmacokinetics and biodistribution of lysine dendrimers upon *in vivo*

administration (Kaminskas et al. 2008). In another study, the addition of a porphyrine core resulted in an increased endosomal release of the lysine dendrimer-based carrier (Ma et al. 2013).

So far, the search for an optimal non-viral carrier has been based mainly on the trial-and-error principle. This approach has shown itself to be highly inefficient in terms of cost, time and outcomes. Although thousands of compounds have been tested during recent decades, the number of non-viral carriers that demonstrate an efficacy comparable to that of the viral vectors is negligible, and none of the non-viral agents is currently approved for clinical use. Building up quantitative structure-activity relationships is promising as a potent approach, enabling a more rational carrier design. However, a comparison of the results obtained in the different studies reported in the literature is limited by the great diversity of carrier structures and methods. From this perspective, parallel studies of structurally related compounds, similar to our comparison of the unmodified dendrimer with its modified analog, are of especially great value. Indeed, the transfection-favorable structural features of lipids (Ivanova et al. 2013), dendrimers (Kwok et al. 2013; Li et al. 2014), and some other groups of carriers has recently been revealed through this approach. Modern, high-throughput screening technologies are able to increase the speed and efficiency of analysis. It should be possible to perform simultaneous screenings of multiple compounds for such parameters as complex formation, pDNA release, cytotoxicity, and transfection efficacy (Rinkenauer et al. 2013). Moreover, comparative studies on cellular uptake and intracellular trafficking would allow the determination of the structural features of the carrier that are critical for improved penetration via distinct intracellular barriers. Based on these data, more specific structure-activity relationships could be established.

In the publication II, we studied in greater detail the relationship between the carrier and its delivery efficacy. In particular, we questioned how the carrier affects cell uptake and intracellular processing of genetic material (pDNA). For this study we selected three carriers: cationic polymer PEI, cationic lipid DOTAP, and CaP co-precipitated with pDNA, all of which are widely used in gene delivery studies. We applied a highly sensitive qRT-PCR based method to quantify the amount of cell-associated and nuclear-associated pDNA at multiple time points. The data obtained was correlated to the transfection efficacy of the carrier as indicated by the reporter protein expression. In contrast to our study, in most of the published studies, the analysis of the intracellular kinetics of pDNA has been performed by the semi-quantitative method of fluorescent microscopy, with transfection efficacy estimated only at a single time point.

Cell uptake was efficient for all the carriers, and seemed not to be a transfection-limiting step. The amount of internalized pDNA varied between carriers, but did not correlate with transgene expression. We suggest that positively charged gene delivery systems bind non-specifically to various negatively charged cell surface molecules, which results in internalization by multiple endocytic pathways. This is especially true for the high dose gene delivery systems, typically used for *in vitro* experiments, and should be taken into account when extrapolating *in vitro* data to the *in vivo* situation where the doses reaching target cells are generally low.

The elimination of cell-associated pDNA differed drastically between the carriers, being the fastest for the CaP- and the slowest for the PEI-mediated gene delivery. The same trend was observed in both the cell lines studied in this work, as well as in other cell lines (Ruponen et al. 2009; Billiet et al. 2012; Nomani et al. 2014), indicating that pDNA elimination rate is carrier rather than the cell line dependent. The rapid degradation of pDNA after the CaP-mediated transfection resembled the elimination of naked DNA and most likely resulted from the inability of CaP to protect pDNA from enzymatic degradation.

Furthermore, we compared the amount of pDNA delivered to the nucleus by different carriers. As with the cell uptake data, the nuclear delivery of pDNA varied between carriers, but showed no correlation with transfection efficacy. The data obtained was in line with the literature as several groups showed no correlation between either cellular or nuclear uptake of pDNA and the transfection efficacy of various polymer- and lipid-based carriers (Cohen et al. 2009; Ruponen et al. 2009; Hyvonen et al. 2012; Billiet et al. 2012). To the best of our knowledge, this was not studied earlier for CaP precipitates. The lack of correlation between nuclear delivery and transfection efficacy can be explained by the insufficient release of pDNA from the carrier, as well as by carrier interaction with various endogenous molecules. Cationic carriers were indeed shown to disturb transcription and/or translation of genetic material (Hama et al. 2007). Highly positively charged polymers, such as PEI, seem to affect the processing of genetic material to a greater extent than lipid-based cationic carriers such as DOTAP. Our studies indicate the importance of postnuclear processes as a barrier in non-viral gene delivery. Future studies should pay more attention to the biocompatibility of the carrier. In particular, the use of biodegradable carriers might help to avoid the problem. In general, the results obtained indicate that the carrier affects cell uptake and the intracellular kinetics of the introduced genetic material and, furthermore, predetermines the main transfection-limiting step, which seems to be the rapid elimination of cell-associated pDNA in CaP-, prenuclear transport in DOTAP-, and postnuclear events in PEI-mediated transfections.

9.2 The role of the endocytic pathway in non-viral gene delivery

The endocytic pathway is currently believed to play a critical role in the gene delivery process as it is linked to intracellular trafficking and the final destination of the endocytosed cargo. Although gene delivery systems can enter cells by many endocytic pathways, it has been shown that some pathways result in higher gene delivery efficacy than others (Rejman, Bragonzi & Conese 2005; Douglas, Piccirillo & Tabrizian 2008; Gabrielson, Pack 2009; Vercauteren et al. 2011b). By now, it is clear that for carriers of different structure different pathways can be beneficial. During the last decade, considerable effort has been devoted to obtaining a more detailed understanding of the underlying mechanisms; however, we are still far from the complete picture.

As reported in publication II, we performed a systematic study of the role of specific endocytic pathways in gene delivery mediated by PEI, DOTAP and CaP precipitates. The

impact of clathrin- and dynamin-dependent endocytosis on cell uptake, and cell-associated pDNA elimination and transport to the nucleus, were evaluated quantitatively in two genetically manipulated cell lines with inducible blockage of these pathways. Blocking of CME in the genetically manipulated BHK (hamster kidney fibroblast) cell line was induced by the expression of antisense RNA to the clathrin heavy chain protein. Blocking of the dynamin-dependent endocytosis in the genetically manipulated HeLa (human epithelial carcinoma) cell line resulted from the inducible expression of mutant dynamin1. It should be noted that dynamin-dependent endocytosis is a group of pathways including CME, CavME and some other, less characterized endocytic mechanisms.

Blocking of either the CME or dynamin-dependent endocytosis decreased the cellular uptake of all gene delivery systems studied. This result was not surprising, as positively charged gene delivery systems are known to utilize multiple endocytic pathways due to their ability to electrostatically and non-specifically bind to negatively charged plasma membrane (Vercauteren et al. 2012). Interestingly, in the case of the CaP precipitates, the lower pDNA uptake in the cells with blocked endocytic pathways resulted in greater reporter protein expression indicating possible activation of alternative endocytic pathways favourable for this carrier. Indirect evidence of alternative pathway activation upon genetic blockage of endocytosis has been reported previously in the literature (Damke et al. 1994).

The elimination of cell-associated pDNA was little changed after the blockage of endocytosis. This probably results from the fact that practically all known pathways are known to direct their cargo to the endolysosomal compartment with high nuclease activity (El-Sayed, Harashima 2013). Moreover, intracellular pDNA can also be degraded by cytoplasmic nucleases after complex release to the cytoplasm (Sasaki, Kinjo 2010). We conclude that intracellular pDNA elimination does not correlate to the changes observed in reporter protein expression upon blockage of endocytosis, and seems not to be an informative parameter when determining the endocytic pathway most beneficial for gene delivery. In contrast, the amount of pDNA transported to the nucleus in the blocked versus the not-blocked cells demonstrated good correlation with transfection efficacy in most cases.

Finally, we aimed to study the role of specific endocytic pathways in the overall transfection efficacy of gene delivery systems. In line with other studies, we confirmed that the uptake pathway most beneficial for transfection depends on both the carrier and the cell type. We demonstrated the important role of CME in DOTAP- but not in PEI-mediated gene delivery in the BHK cell line, and the more important role of dynamin-mediated endocytosis in PEI- than in DOTAP-mediated gene delivery in HeLa cells. The literature reports on the relative importance of different endocytic pathways in effective transfection are rather controversial. For example, CME (von Gersdorff et al. 2006), macropinocytosis (Hufnagel et al. 2009), and clathrin-independent pathways (Rejman, Bragonzi & Conese 2005; Gabrielson, Pack 2009) have all been suggested as being critical for PEI-mediated transfection. One of the main reasons for the controversy observed in the field is the diversity of the data in terms of the carriers used, the cell lines, and the experimental methods (Vercauteren et al. 2012), all of which make comparisons between different studies and the drawing of general conclusions challenging. In subsequent

studies we addressed some of these methodological problems, which will be discussed in greater detail in chapter 9.3.

9.3 Improving experimental methodology in cell uptake studies

9.3.1 Searching for an optimal approach to block the endocytic pathway

Selective blockage of endocytosis is a valuable tool when studying the role of the endocytic pathway in gene delivery. According to the literature, pathway-specific chemical inhibitors have been the most frequently used approach in blocking endocytosis. However, the specificity and the side effects of chemical inhibitors have raised serious concerns in the scientific community (Ivanov 2008; Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012). Genetically modified cell lines provide more specific and less cytotoxic alternatives. Although this approach has been used in virus uptake studies (Wang et al. 1998; Lakadamyali, Rust & Zhuang 2004), we were the first to use it in the non-viral gene delivery field.

Our studies showed that genetically manipulated cell lines with inducible blocking of endocytosis provide a useful tool for non-viral gene delivery studies. In most cases the chemical inhibitors and genetically modified cell lines demonstrated similar trends. However, some discrepancies were also revealed. In particular, the chemical inhibitors resulted in a stronger inhibition of transgene expression when compared with the genetically modified cell lines. Moreover, in the case of CaP precipitation, the two methods demonstrated opposite trends. The observed discrepancies may be attributed to the side effects of the inhibitors as well as to the possible activation of alternative endocytic pathways in the genetically blocked cells. Our study, together with studies done by others (Iversen, Skotland & Sandvig 2011; Vercauteren et al. 2012), provide strong evidence that, to date, none of the methods is ideal, all being a combination of benefits and drawbacks. More specific and targeted inhibitors of endocytosis are urgently needed. Computational tools, enabling the prediction of biological targets for chemical compounds, and high-throughput screening of chemical compound libraries are promising strategies for identification of new specific chemical inhibitors of endocytosis (Ivanov 2014). Moreover, increased knowledge about the regulators of endocytosis will make possible the generation of new mutant cell lines with disrupted functioning of endocytosis-related genes and improved gene silencing protocols.

9.3.2 Endocytic profiling of cell models

Studies on the role of the endocytic pathway in gene delivery have been performed in multiple cell culture models. In many studies it has been assumed that all pathways are present in all cell types. However, it is now becoming clear that different cell types are not

equal with respect to the expression and activity of endocytic pathways. For example, morphological data reveal the presence of particularly high numbers of caveolae in some cell types, including the adipocytes and endothelial cells (Razani, Woodman & Lisanti 2002). In contrast, no caveolae have been found in lymphocytes or the neurons of the central nervous system (Razani, Woodman & Lisanti 2002). Expression of flotillins -1 and -2 also varies between cell types (Volonte et al. 1999), although it is not yet clear how observed differences are related to the presence and activity of FME. Overall, the information available on the presence and activity of endocytic pathways in different cell types is still very fragmented. We suggest that detailed characterization of cell lines in terms of functional endocytic pathways (endocytic profiling) would significantly facilitate interpretation of the data on the uptake and cellular processing of NPs. In addition, the question of how faithfully *in vitro* models recapitulate *in vivo* endocytic processes should be more carefully addressed.

Endothelial and epithelial cells create a substantial barrier for gene delivery systems administered by all routes except by direct injection into target tissue. These cells are widely used in NP uptake studies and therefore represent an interesting subject for endocytic profiling. *In vivo* these cell types are highly differentiated. It has been reported that the differentiation is accompanied by a reduced endocytic activity of both fluid uptake markers and NPs (Matsui et al. 1997; Foerg et al. 2007; O'Neill et al. 2011). However, the effect of differentiation on specific endocytic pathways has not been addressed.

In the study III we performed endocytic profiling of CaCo-2 cells modelling intestine epithelium and hCMEC/D3 cells modelling brain endothelium, and compared them to primary cells. Moreover, we studied how cell endocytic profiles are dependent on cell differentiation.

Differentiation of CaCo-2 cells was accompanied by a very significant reduction of mRNA expression of most endocytosis-related genes, and a reduction in uptake of endocytic markers and NPs. In contrast, brain endothelial cells were poorly differentiated, demonstrated no major changes in the mRNA expression of endocytosis-related genes, and retained significant endocytic activity. Our studies emphasize that the differentiation status of *in vitro* cell models should be taken into account when designing NP permeation experiments.

The lack of correlation between the cell lines and the corresponding primary cells for some endocytosis-related proteins was demonstrated. In particular, the mRNA expression profile for endocytosis-related genes in CaCo-2 cells was lower than in the primary cells. In the hCMEC/D3 cells the opposite trend was observed. These differences may, at least partially, arise from the cancerous/immortalized nature of *in vitro* models. For example, an overexpression of both caveolin and clathrin heavy chain in hepatic cancer, in comparison to normal cells, has been reported (Tse et al. 2012; Seimiya et al. 2008). The physiological relevance of the data obtained in the immortalised cell lines is a question of debate in the literature (Bouis et al. 2001). Although primary cells are generally believed to be more representative of the *in vivo* situation, they are known to lose their initial properties soon after isolation (Lacorre et al. 2004), and their properties may vary between isolations (Bouis et al. 2001). Further development of a methodology for *in vivo* endocytosis studies would help to answer the question of the representativity of continuous cell lines and

primary cells. Moreover, this would shed light on the cellular processing of NPs in the living body. Conventional electron microscopy and immunofluorescence techniques of fixed tissue samples do not take into account the highly dynamic nature of the endocytic processes. Recent developments in intravital microscopy have allowed the visualization of cell uptake and the distribution of fluorescently labelled molecules in living animals (Sandoval, Molitoris 2008). Although insufficient tissue penetration, low resolution, and high costs remain serious limitations for this technique, it definitely has great potential for *in vivo* uptake studies of therapeutic NPs.

In our work, we emphasize the importance of the endocytic profiling of cell lines prior to studies on the cellular processing of therapeutic NPs. The most straightforward method for endocytic characterization of the cell lines is through a direct measurement of the endocytic uptake of pathway-specific markers. The characteristics of an ideal endocytic marker would include (1) specificity to one or several endocytic pathways, (2) lack of stimulation of the target pathway or other pathways, and (3) low toxicity. Although Tf is considered to be relatively specific for CME, well-established markers for other pathways are currently lacking. SV40, CTB and LacCer have been suggested as markers for caveolae-mediated endocytosis. However, SV40 was later shown to enter cells also by CME; CTB was demonstrated to utilize multiple pathways within the same cell type (Torgersen et al. 2001); and LacCer seems to be a marker of many clathrin-independent pathways (Hansen, Nichols 2009) as is also indicated by our data. Moreover, LacCer has been shown to stimulate caveolar uptake (Sharma et al. 2004). The discovery of new molecules that are taken up by specific endocytic pathways is an extremely important direction for future research.

Taking into account the current lack of pathway-specific markers, we suggest that the mRNA expression of endogenous proteins involved in certain endocytic pathways can be indicative of the expression and activity of those pathways. This novel approach is relatively cheap and fast, and could therefore be routinely utilized for the screening of cell lines for the presence of specific endocytic pathways prior to studies on the cellular processing of therapeutic NPs. A comparison of the endocytic profiles of cell lines with those of primary cells and human- and animal-derived tissues would allow the selection of the most representative *in vitro* models. Several important factors must be considered when discussing the feasibility of this approach. Firstly, mRNA levels do not necessarily correlate with the actual protein levels. Secondly, for proteins with multiple functions in the cell (such as the protein kinases included to our study), high protein levels do not necessarily suggest high activity of the corresponding endocytic pathway. The data obtained in our studies indicate that the mRNA expression of some proteins (caveolin1 and flotillin2) have the potential to be used as pathway activity markers. Further studies are needed to firmly establish this method.

10 Conclusions

This thesis provides insight into the role of the carrier and the endocytic pathway in non-viral gene delivery. Furthermore, the reported studies contribute to improvements in the methodology used in the field. The main conclusions are as follows:

1. Lysine dendrimers are able to bind and compact pDNA, protect it from nuclease degradation, and deliver it to cell nuclei. Although modification with capric acid and the utilisation of an endosomolytic agent improve the gene delivery properties, transfection efficacy is generally low. Lysine-based dendrimers can be used as a basis for the development of more effective carriers.

2. Neither the cellular, nor nuclear uptake of cationic delivery systems correlates with transgene expression. Post-nuclear processes are an important barrier in non-viral gene delivery.

3. The main transfection-limiting intracellular step of the non-viral gene delivery system is strongly carrier-dependent and seems to be the rapid elimination of cell-associated pDNA in CaP-, pre-nuclear transport in DOTAP-, and post-nuclear events in PEI-mediated gene delivery.

4. The endocytic pathway most beneficial for gene delivery is both carrier and cell type dependent. Neither the amount of pDNA taken up by the cell, nor the intracellular pDNA elimination, but only the amount of pDNA delivered to the nucleus is indicative of the significance of the particular pathway in the ultimate delivery efficacy of the particular carrier.

5. Genetically manipulated cell lines can be used as a tool to specifically block endocytosis in gene delivery studies. A comparison of chemical and genetic means to block endocytosis revealed the limitations of both those methods. Careful method optimization, and parallel studies using several alternative approaches, are recommended in order to obtain more reliable data.

6. The expression and activity of endocytic pathways are dependent on the cell type and the differentiation stage of the cell. Performing cell line characterization for the expression and activity of endocytic pathways (endocytic profiling) could facilitate the interpretation of data on the cellular processing of gene and drug delivery systems.

11 Future prospects

The clinical significance of biopharmaceuticals, including the peptides, recombinant therapeutic proteins, and nucleic acids is increasing. The number of peptide- and protein-based products launched onto the market is high and is constantly growing (Mitragotri, Burke & Langer 2014). Although nucleic acid-based products are generally at an early stage of development, the first gene therapy drugs have been approved for clinical use (Wirth, Parker & Yla-Herttuala 2013). The structural complexity of biopharmaceuticals assures their high specificity and potency as compared to conventional small drug molecules, but it also creates multiple challenges in their formulation and delivery.

As the successful delivery of macromolecular drugs is critically dependent on the carrier, the development of new carriers, including those for non-viral gene delivery, will remain an active area of research. The design of more effective carrier molecules should be more rational and not based on the trial-and-error principle. One of the prospective directions for future studies is the building of quantitative structure-activity relationships in order to relate the efficacy of every step of the delivery process to the molecular architecture of the carrier. Advances in computational modelling, combinatorial chemical synthesis, and the high throughput screening of libraries of chemical compounds will all help to identify potential highly effective carriers.

Due to their large size, the pharmacokinetics of macromolecular drugs is drastically different from that of small molecules. The main mechanism for small drug molecules to be absorbed and distributed inside the body is passive transcellular diffusion, whereas, for biological drugs, it is the endocytosis-based mechanisms. During recent decades the endocytic pathways and the subsequent intracellular trafficking of many macromolecular drugs, including gene delivery systems, have been described for various cell types. How the intracellular fate of the delivery systems can be controlled by their properties is another important question to be answered. Progress in this field will be further facilitated by new findings in fundamental cell biology, as well as by the further development of experimental tools. In particular, more specific markers and inhibitors of endocytosis are greatly needed.

Most of the currently available data on the mechanisms of drug and gene delivery have been obtained in cell and tissue cultures. The relevance of the models utilized to the physiological conditions is of utmost importance and should be addressed in a more careful and systematic manner. The characterization of existing cell models and the development of new models that represent the *in vivo* situation better should definitely be the focus of future research. Because of the importance of endocytosis in the pharmacokinetics of biological drugs, it is necessary to characterize not only the target cells but also the cells composing the biological barriers on the way to the target tissue. Additionally, *in vivo* studies on cell uptake and the intracellular trafficking of macromolecular drugs would provide extremely valuable information. The development of appropriate methods, including intravital microscopy, is currently in progress.

The huge amount of work performed in recent decades by researches all over the world has significantly improved our understanding of the critical parameters for the successful

delivery of macromolecular drugs. However, many pieces of the puzzle are still missing. We strongly believe that future studies will help to build a more comprehensive picture of the delivery process of macromolecules, and will allow the development and clinical approval of new potent macromolecular medicines, including non-viral gene therapy products.

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