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“Parameters influencing transfection efficiency of mRNA lipoplexes“

„mRNA-Lipoplexe: Transfektionseffizienz, Zytotoxizität und Dauer der Proteinexpression“

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1. INTRODUCTION

Gene therapy is a promising approach for treatment of both acquired and inherited diseases by correcting the underlying genetic disorder. To achieve this correction, so-called vectors transport therapeutic nucleic acids into the targeted tissue and further into the cells. Two major groups are used as vectors, namely viral and non-viral ones. Even though viral vectors reach the highest transfection efficiencies, numerous limitations concerning safety, immunogenicity, transgene size as well as costs involved in their production call for development of alternative systems. Cationic lipids and polymers are the most commonly used non-viral vectors and exhibit several advantages over viral vectors. They do not induce immune responses; their production is not very expensive and easy to be up-scaled. However, it is generally known that cationic vectors show poor transfection efficiencies as well as induce some cytotoxicity.

The main objective of this study was to optimize the conditions of the transfection process mediated by cationic lipids complexed to messenger RNA (mRNA). Since both transfection efficiency and cytotoxicity have been demonstrated to depend on the ratio, at which cationic lipids and nucleic acids are mixed together, it is imperative to find the optimal ratio. The second objective was to estimate the influence of serum on transfection efficiency. To that end influence of different serum concentrations on transfection efficiency was tested.

Since size is one of the parameters that might influence the mechanism of particle uptake and thus transfection efficiency, differently composed mRNA complexes were characterized in terms of their size. Furthermore, toxicity of the complexes was evaluated by analyzing the viability of cells following transfection. Finally, for a better understanding of the kinetics of the protein production upon transfection, duration of the protein production was followed over time.

2. BACKGROUND

2.1 Gene therapy

2.1.1 Definition

Gene therapy is a technique which uses nucleic acids as agents to prevent or treat genetic disorders as well as numerous acquired diseases.

Genes, as the units of heredity, encode proteins. As a consequence of mutations, proteins with altered functions are produced or the whole protein is missing. This results in genetic disorders. Gene therapy repairs those faulty genes by inserting nucleic acids (DNA or RNA) into cells or tissues in order to:

- “Replace a mutated gene” (U.S. National Library of Medicine®, 2014)
- Silence, ‘knock-out’ a gene
- Encode a protein which is therapeutic

(U.S. National Library of Medicine®, 2014)

In most cases the mutated gene is replaced by a functional one. To that end, a so-called vector is needed to transport a new gene sequence into a specific tissue and deliver it into the cell. This can be achieved by two types of vectors, viral and non-viral ones.

2.1.2 Viral vectors

This approach employs genetically modified viruses, which cannot cause diseases but are able to carry cargo in form of human DNA. Introduced into a specific cell, those viruses transfer genetic material as part of their replication cycle and sometimes integrate it into chromosomes of the host cell (Gene Therapy Net, 2013). Different virus-types are used as vectors in gene therapy:

2.1.2.1 Double-stranded DNA virus (group I of Baltimore classification)

- Adenovirus (*Adenoviridae*)

The adenovirus is the largest non-enveloped virus which contains double-stranded linear DNA. After the viral infection, the DNA molecule is inserted into the host cell, but will not be integrated into its genome. The DNA stays free in the nucleus, genes of the viral genome are transcribed as any other normal genes, but in contrast to other types of viruses, they will not be replicated during cell division. (Gene Therapy Net, 2013)

This is an advantage as well as an extra challenge for their use in gene therapy approaches. The viral DNA will not be integrated into the genome of the host cell and therefore will not occur in the descendants after cell division. Problems with uncontrolled cell division, as it is likely to happen with retroviruses, could be avoided. (Gene Therapy Net, 2013)

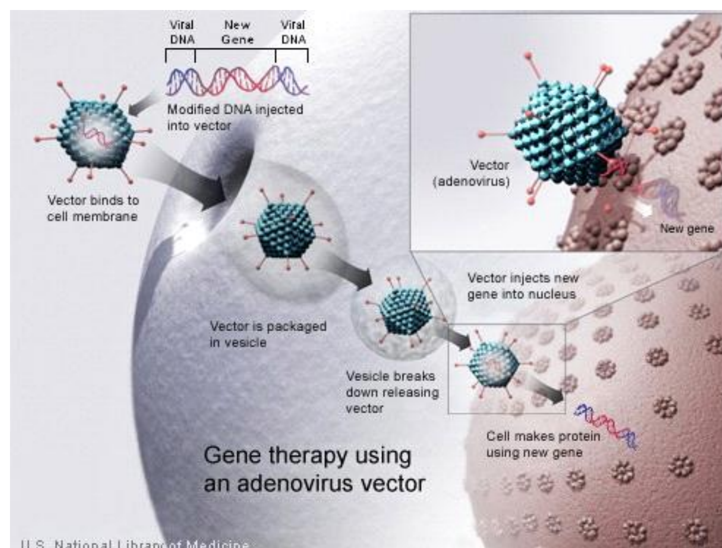


Figure 2.1: Introduction of nucleic acids into the nucleus by an adenoviral vector (U.S. National Library of Medicine®, 2014)**.** After binding to the plasma membrane, the viral vector is transported to the nucleus in vesicles.

- Herpes simplex virus (*Herpesviridae*)

This virus contains a large double-stranded, linear DNA genome, which encodes proteins that form the capsid, the tegument and the envelope of the virus and regulate its replication and infectivity. (Gene Therapy Net, 2013)

To enter the host cell, the glycoproteins of the viral envelope interact with receptors on the membrane of the host cell. As a consequence of the following fusion, a pore is formed, which facilitates the viral capsid to enter the host cell. The capsid releases the viral genome into the nucleus of the host cell as soon as it enters this compartment. (Gene Therapy Net, 2013)

Genetically modified herpes simplex viruses are used as vectors for gene therapy. Those vectors are not able to replicate anymore, but still keep the property to infect and establish latency. Herpes simplex viruses are especially used for gene delivery into the cells of the nervous system. (Gene Therapy Net, 2013)

2.1.2.2 Single-stranded DNA virus (group II of Baltimore classification)

- Adeno-associated virus (*Parvoviridae*) (AAV)

This non-enveloped virus contains a single-stranded DNA and is one of the smallest viruses. It belongs to the genus *Dependovirus*, meaning the virus itself needs a so-called helper virus to replicate in the host cell. This helper virus co-infects the host cell and produces proteins, which are necessary for the replication of the adeno-associated virus. Helper viruses are for example adenoviruses, herpes viruses or vaccinia viruses. (Gene Therapy Net, 2013)

Since this virus is not associated with any human disease, plus the fact that it infects both dividing and non dividing cells, it is often used as a vector for gene therapy. (Gene Therapy Net, 2013)

Recent discoveries suggest that AAV vectors may be associated with insertional mutagenesis and cancer, and therefore, may not be as safe as previously thought. Moreover, they can induce male infertility, and be able to replicate without a helper virus (Division of Research Safety, 2010). Further disadvantages are the low capacity due to the virus small size and a rather complicated production process. (Gene Therapy Net, 2013)

2.1.2.3 Double-stranded RNA viruses (group VI of Baltimore classification)

- Retrovirus (*Retroviridae*)

The virus contains a double-stranded RNA. Before the viral genome can be integrated into the host cell, the enzyme, reverse transcriptase, produces a DNA copy of the viral genome. As soon as this is completed, another enzyme called integrase incorporates the viral DNA into the genome of the host cell. (Gene Therapy Net, 2013)

To use the retrovirus as a vector, genes encoding the information for the group-specific antigen (gag), the polymerase (pol) and the envelope (env) genes are replaced by therapeutic genes. (Gene Therapy Net, 2013)

The major problem for using retroviruses as vectors is the unspecific integration catalyzed by integrase, which results in random virus integration in the host genome. In case that the viral genome is placed within a gene regulating cell division, uncontrolled cell division can happen, which can lead to cancer (Montini *et al.,* 2009).

- Lentivirus (Retrovirus – *Retroviridae*)

Lentiviruses are one of the most important vectors for efficient introduction of genetic material into host cells. A long stable expression of the introduced gene, an efficient infection of dividing as well as non-dividing cells, and its low immunogenicity make this viral vector a very promising candidate for gene therapy. Examples for lentiviruses are: human immunodeficiency virus (HIV), simian immunodeficiency virus or African Green Monkey virus (SIV), and feline immunodeficiency virus (FIV). (Gene Therapy Net, 2013)

2.1.2.4 Conclusions

In vitro as well as *in vivo* studies proof the dominance of viral vectors over non-viral systems. Presently, the adenovirus, the adeno-associated virus and the retrovirus are commonly used carrier systems in clinical trials.

Viral vectors reach high transfection efficiencies with the potential to infect different cell types, both dividing and non-dividing. In addition, these carrier systems appear to have wide tropism to the host cell, which is necessary for an efficient transfection. Nevertheless, it is important to take possible risks into consideration. First of all, administration of foreign material always bares the risk of acute toxicity in the human body as well as strong immune responses. Moreover, the introduction of foreign genetic material into the human DNA involves the risk of insertional mutagenesis, possibly leading to cancer. For example, in 2003 a clinical trial for patients suffering from severe combined immunodeficiency (SCID), the X-linked (X-SCID) or the adenosine deaminase (ADA) deficiency were treated by gene therapy using a gammaretroviral vector. While 9 of 10 patients were successfully treated, 4 of the 9 developed T-cell leukemia 31 – 68 months after gene therapy (Hacein-Bey-Abina *et al.*, 2008). Further investigations revealed that the gammaretroviral vector was inserted near a proto-oncogene, which led to the activation of its transcription. This caused high clonal proliferation resulting in uncontrolled T-cell growth (Hacein-Bey-Abina *et al.*, 2008).

The viral potency to infect more types of cells is an advantage at the same time it is a disadvantage. For gene therapy uses, the viral carrier does not only transfect the target cells but also healthy ones.

Summarizing, a lot of work needs to be done to reduce possible risks and to make the viral vector safe enough for human administration.

2.1.3 Non-viral vectors

This approach has been developed to avoid some of the problems associated with viral vectors. Non-viral gene delivery systems can be divided into two major groups (Niidome and Huang, 2002):

- Physical methods
- Chemical carriers

2.1.3.1 Naked DNA delivered by physical methods

The simplest way to introduce genetic material into a cell is by direct injection of the naked DNA. Direct injection of DNA seems to have potential for treating genetic diseases such as Duchenne/Becker muscular dystrophy (Romero *et al.,* 2004). After *intramuscular* injection of a full-length human dystrophin plasmid, dystrophin expression was detected in 6 out of 9 patients. Results showed a low level of protein expression, but no side effects or immune responses were observed. Furthermore, a clinical trial for patients suffering critical limb ischemia due to

atherosclerotic occlusive disease assessed the potential of hepatocyte growth factor (HGF) plasmids for improving the limb perfusion. Results showed a decent level of gene expression, and no toxicity was observed (Powell *et al.*, 2008; Kotzamanis *et al.*, 2011).

- Jet injection – Hydrodynamic pressure

A solution of naked DNA is injected with a narrow jet, which penetrates the skin and underlying tissue. Compressed air allows the *intramuscular*, *subcutaneous* as well as *intratumoral* delivery of the genetic material. Several *in vivo* studies have demonstrated this gene delivery method as safe and easy to perform. Focus of these jet injection-based studies was mainly put on antitumor treatment. Advantages such as feasibility, low costs and broad application areas make jet injection a considerable alternative for local application of naked DNA (Walther *et al.*, 2004; Walther *et al.*, 2008).

- Electroporation – Electric pulses

This physical method is based on the application of short electric pulses leading to an increase in permeability of the cell membrane. Furthermore, enhanced membrane permeability increases the uptake of substances, for example DNA (Neumann *et al.*, 1982).

Numerous studies have optimized parameters such as the size of an electroporation cell, field strength as well as duration, frequency and total number of applied pulses (Somiari *et al.*, 2000) to minimize possible toxic effects.

- Sonoporation – Ultrasound

The same effect, as caused by electric pulses, can be achieved by ultrasound waves. Even though the exact mechanism is not clear yet, the sonic forces possibly lead to acoustic cavitation, resulting in the formation and subsequent collapse of so-called microbubbles. Consequently, the cell membrane gets transiently permeable, facilitating the uptake of the genetic material. Microbubbles are small gas vesicles surrounded by a lipid monolayer. They were originally developed as a contrast agent for diagnostic purposes.

However, electroporation achieves higher transfection efficiency levels than sonoporation, the latter one has one major advantage: it is less invasive compared to electroporation. In order to balance

the disadvantages of each method, electric pulses and ultrasound can be combined. This so-called electro-sonoporation reaches higher expression levels and lower tissue damage compared to electroporation or sonoporation alone (Yamashita *et al.*, 2002).

2.1.3.2 Delivery mediated by chemical carriers

Chemical carriers represent a very promising alternative to viral vectors and their improvement is one of the major aims of nowadays research. Most of the synthetic vectors are positively charged. Electrostatic interaction between a carrier and a negatively charged genetic material leads to the complex formation. These complexes help protecting the DNA or RNA from degradation by nucleases and other components present in the blood. In addition, they facilitate their entry into the cell by endocytosis. Depending on the nucleic acid used, place of destination differs: DNA needs to be delivered into the nucleus of the cell, whereas RNA performs its biological function in the cytosol.

Chemical carriers are divided in two major groups:

2.1.3.2.1 Cationic lipids

- Definition

First introduced by Felgner *et al.* in 1987 lipoplexes are stable formulations of liposomes complexed with nucleic acids. Liposomes consist of a double layer of phospholipids with their hydrophilic heads turned outside and hydrophobic tails turned inside. The core of this vesicle contains an aqueous solution. Liposomes occur in two different formations: unilamellar and multilamellar. Whereas unilamellar liposomes (average diameter around 0.1 – 0.5 µm) are composed of one phospholipid double layer, multilamellar vesicles (average diameter around 0.2 - 5 µm) are formed by several concentric phospholipid bilayers (Figure 2.2).

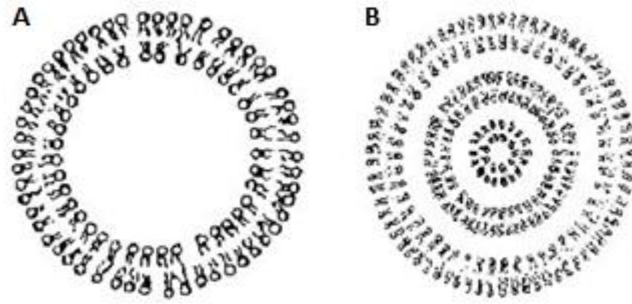


Figure 2.2: Structure of unilamellar (A) and multilamellar (B) liposomes (Ye *et al.*, 2000)**.** A double layer of phospholipids composes the unilamellar vesicle with an average diameter around 0.1 – 0.5 µm (A). Multilamellar vesicles contain more phospholipid bilayers and have an average diameter around 0.2 - 5 µm (B).

During lipoplex formation a topological change of both its components – the cationic lipid as well as the nucleic acid – leads to complexes with different morphologies. As presented in Figure 2.3, two structural models are generally accepted, namely the lamellar structure (L^{C}_{α}) and the inverted hexagonal structure (H^{C}_{II}).

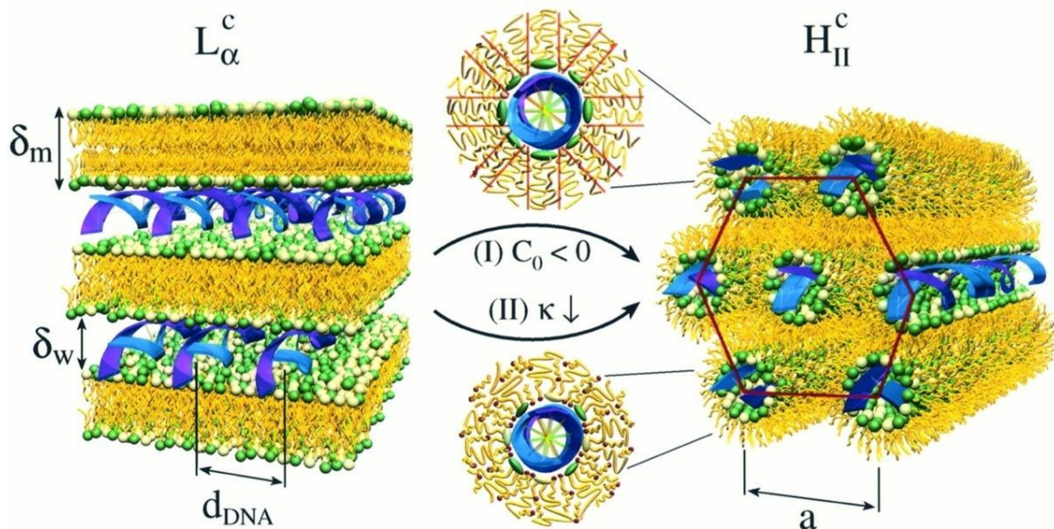


Figure 2.3: Structures of lipoplexes (Koltover *et al.*, 1998)**.** The lamellar structure (L^{C}_{α}) contains the DNA rods entrapped between lipid bilayers, whereas in the inverted hexagonal structure (H^{C}_{II}) a lipid monolayer envelopes the DNA rods on a hexagonal lattice.

Depending on the structure of the lipid that accompanies the cationic lipid, the complexes occur in one those different structures (Koltover *et al.*, 1998).

- Formation

So-called helper lipids (*e.g.* dioleyl phosphatidylethanolamine (DOPE), cholesterol or dioleoyl phosphatidyl choline (DOPC)) are utilized to enhance transfection efficiencies. In presence of DOPE, lipoplexes prefer the inverted hexagonal structure (H^{C}_{II}), whereas in complexes with DOPC the lamellar structure (L^{C}_{α}) dominates. Contrary to the lamellar structure, the hexagonal structure facilitates the fusion and release of the nucleic acid as they come in contact with negatively charged vesicles (*e.g.* anionic endosomes), which is necessary for a successful transfection (Koltover *et al.*, 1998).

Interaction between a positively charged vector and a negatively charged nucleic acid leads to the lipoplex formation. Finding the driving force behind the formation of lipoplexes is more complex than first expected. Electrostatic attraction between the positively charged liposome and the negatively charged nucleic acid was first stated as a reason (Kreiss *et al.*, 1999).

Several more recent studies demonstrated that surface interaction of the oppositely charged liposomes and nucleic acids is an endothermic process with a positive enthalpy. Like any thermodynamically favored process, entropy growth has to be the driving force behind this reaction (Pozharski *et al.*, 2003). Measurements of the zeta potential as well as fluorescence spectroscopy results confirm this so far hypothetical process, which furthermore leads to the essential condensation of the genetic material (Rodríguez-Pulido *et al.*, 2008).

Even though it seems to be a very simple process, this step is crucial for a successful transfection. Moreover, its optimization will be essential in the further development of gene transfer vehicles.

2.1.3.2.2 Cationic polymers

- Definition

Polymers are repeating units (so-called monomers), which together form so-called macromolecules. Those macromolecules can be divided into natural and synthetic polymers. Natural polymers include proteins, nucleic acids, fibers, carbohydrates and are easily biodegradable. Synthetic polymers can be naturally occurring polymers that undergo chemical modifications (*e.g.* synthetic rubbers or fibers) or fully industrially developed (polyethylene, polypropylene or polyvinylchloride (PVC)).

Polyplexes consist of cationic polymers complexed with nucleic acids. Poly(ethylenimine) (PEI) and poly(L-lysine) (PLL) are the most commonly used cationic polymers. In aqueous solutions,

protonation of the amino groups leads to a positive charge. Synthetic polymers can be chemically modified to change their molecular weight, degree of branching or charge of the surface (Tros de Ilarduya *et al.*, 2010).

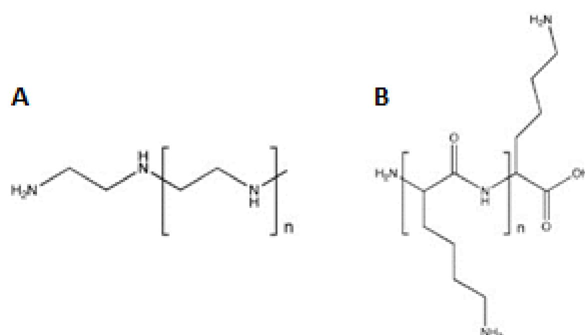


Figure 2.4: Chemical structure of linear poly(ethylenimine) (PEI) (A) and poly(L-lysine) (PLL) (B) (Storrie and Mooney, 2006)**.**

- Structure

Cationic polymers can be divided into linear (*e.g.* PLL, linear PEI) and branched (*e.g.* branched PEI). The degree of branching has a great influence on transfection efficiency as well as other parameters (Tros de Ilarduya *et al.*, 2010).

In addition, changes can be made in types and numbers of protonatable amines of the polymers. At physiological pH (pH 7,4) those amines (in particular the primary ones) are protonated and responsible for the positive charge of the polymer. As a consequence, an excess of primary amines in the cationic polymer/nucleic acid charge ratio results in highest transfection efficiencies (Tang and Szoka, 1997). This is the reason why linear PEI with a lower level of primary amines reaches not as high transfection efficiencies as branched PEI with more protonatable amines (Tros de Ilarduya *et al.*, 2010).

- Formation

Cationic polymers interact electrostatically with anionic nucleic acids and form stable complexes (*i.e.* polyplexes). As some cationic polymers (*e.g.* PLL) are not able to yield high transfection activities, due to a reduced endosomal escape, conjugation as well as incorporation of membrane destabilizing agents and target ligands is necessary (Tang and Szoka, 1997). Other polymers such as PEI function as proton sponges, which facilitates their endosomal escape. In those cases, transfection efficiency is sufficiently high and therefore additional conjugations are not required.

Another factor to take into consideration for a successful transfection seems to be the medium composition. PEI/DNA complexes prepared in 5 % glucose solution are much smaller (mean diameter 30 to 100 nm) than those made in a physiological salt solution (mean diameter > 1 μm). Strong repulsion between the cationic particles, which is present in the 5 % glucose solution, prevents particles from aggregation and smaller complexes are formed. In contrast, physiological conditions lower these repulsions and interactions between particles lead to aggregation. As a consequence, polyplexes are larger. This indicates that both size and stability of polyplexes is strongly influenced by the salt concentration (Goula *et al.*, 1998).

2.1.3.2.3 Conclusions

Despite the high transfection efficiencies and their broad range of cell targets, viral vectors have several disadvantages. This calls for development of alternatives, namely non-viral vectors. Lipid- as well as polymer-based carrier systems show many advantages compared to viral vectors. For example, they do not induce immune responses and their synthesis can be easily done at large-scale. However, lipoplexes and polyplexes are generally known to have low transfection efficiencies (in particular *in vivo*) and ensure only a short-termed transgene expression. Numerous extracellular and intracellular barriers limit their transfection efficiency. Optimization of those carrier systems is necessary in order to ensure a targeted and controlled delivery of nucleic acids (Niidome and Huang, 2002).

2.2 Cellular uptake pathways and intracellular trafficking

The next step after formation of complexes is their interaction with the plasma membrane. Cationic complexes interact with the negatively charged plasma membrane through non-specific electrostatic interactions. It is still uncertain how and to what extent certain cell surface components (*e.g.* glycoproteins or phospholipids) are involved in this process. Subsequently, endocytic mechanisms enable the uptake of lipo- and polyplexes into the cell.

2.2.1 Endocytosis and endocytic pathways

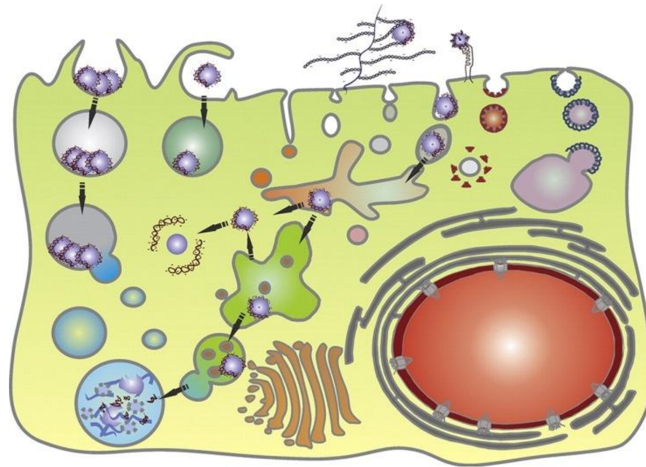


Figure 2.5: Intracellular uptake of cationic carriers complexed with nucleic acids via endocytosis (Vercauteren *et al.*, 2012)**.**

Vesicle-mediated internalization of the extracellular material can follow along different pathways. Once inside the cell, the vesicles with their cargo fuse with early endosomes, where sorting of the contents takes place. This step decides over the further fate of the cargo. Inside early endosomes a relatively mild pH (pH 6,2 – 6,5) allows the dissociation of receptor and ligand. Recycling vesicles transport the free receptors back to the plasma membrane, where they are again available for ligand-binding and internalization. Ligands destined for degradation in the lysosomes (*e.g.* infectious agents) are carried via endosomal carrier vesicles to late endosomes. Endosomal carrier vesicles function as intermediates between early and late endosomes (Gruenberg, 2001). Late endosomes interact with lysosomes characterized by the presence of hydrolytic enzymes.

Endocytosis can be divided in two major types:

- Pinocytosis
- Phagocytosis

2.2.1.1 Pinocytosis

Pinocytosis is a form of endocytosis, which in contrast to phagocytosis, is active in all cells. Pinocytosis can be further sub-divided into:

- Macropinocytosis
- Caveolae-mediated endocytosis

- Clathrin-mediated endocytosis
- Clathrin-independent endocytosis

- Macropinocytosis

Depending on the cell type particular signals, such as growth factors, induce invagination of the plasma membrane (*i.e.* ruffles), which subsequently leads to the formation of so-called macropinosomes.

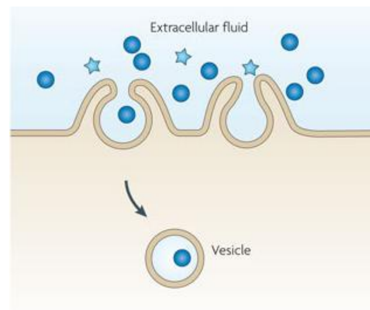


Figure 2.6: Macropinocytic uptake of extracellular fluid containing dissolved substances (Petros and DeSimone, 2010).

- Caveolae-mediated endocytosis

Caveolae are flask-shaped invaginations of the plasma membrane that were first observed on the surface of endothelial cells where they are very abundant (Conner and Schmid, 2003).

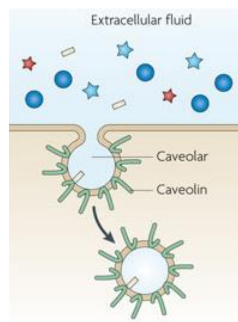


Figure 2.7: Caveolae-mediated uptake of extracellular fluid containing solute particles (Petros and DeSimone, 2010).

Caveolae occur as round formations with diameters between 50 – 80 nm, which are present on many, but not on all, cells. Depending on the cell type, caveolae differ in their morphology, function

as well as their composition. Caveolins, a family of integral membrane proteins, build the coat around those invaginations, which is responsible for their stability and formation. Caveolins are present in the plasma membrane areas reached in sphingolipids and cholesterol (Pelkmans *et al.*, 2002).

- Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the most common pathway for the selective internalization of nutrients, signaling molecules, antigens as well as pathogens in eukaryotic cells (Ungewickell and Hinrichsen, 2007). Clathrin is a protein, which builds a coat around the vesicle. The coating formation is triggered by the binding of adapter-protein complexes to clathrin, other proteins involved in the regulation of endocytosis, and the cargo (Le Roy and Wrana, 2005). After invagination, the vesicles pinch off into the cytosol (Conner and Schmid, 2003). In early endosomes sorting of the cargo decides whether it is recycled or degraded. Recycling brings it back to the plasma membrane, whereas for degradation it is transported to the late endosomal-lysosomal compartment (Le Roy and Wrana, 2005).

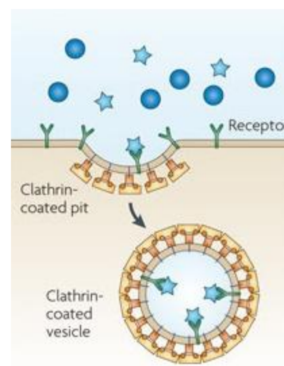


Figure 2.8: Invagination and formation of the clathrin-coated vesicle (Petros and DeSimone, 2010).

- Clathrin-independent endocytosis

Parts of the membrane as well as the fluid are internalized via clathrin-independent endocytosis. Clathrin-independent endocytosis terms different internalization pathways, including caveolin-mediated endocytosis, flotillin-mediated endocytosis, clathrin-independent carriers/GPI-enriched compartments endocytosis and Arf6-dependent endocytosis. To date, those endocytic routes seem to derive from so-called lipid rafts that are hydrophobic microdomains in the membrane. It is

believed that these lipid rafts – abundant in cholesterol and glycosphingolipids – aggregate receptors, which are important for cellular processes like endocytosis and signal transduction (Vercauteren *et al.*, 2012).

2.2.1.2 Phagocytosis

Phagocytosis is responsible for the uptake of solid particles with sizes over 500 nm. Some highly specialized cells (*e.g.* macrophages, dendritic cells and neutrophils) use this mechanism to degrade pathogens, bacteria and yeast as well as apoptotic cells. Thus, phagocytosis plays a crucial role in the immune response.

Various receptors on the plasma membrane specifically recognize ligands and initiate phagocytosis by facilitating their adhesion and internalization. Extensions of the plasma membrane, so-called pseudopodia, surround the particle to ingest and pinch off intracellularly by forming a vesicle (*i.e.* phagosome) (Aderem and Underhill, 1999).

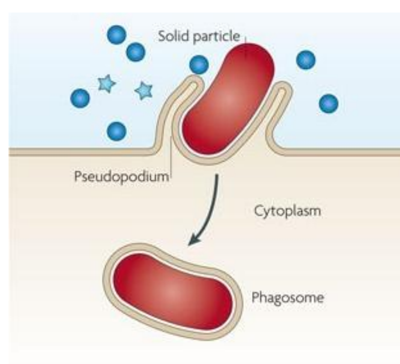


Figure 2.9: Phagocytic uptake of solid particulate material (Petros and DeSimone, 2010)**.**

2.2.2 Endosomal escape

To ensure transfection, the nucleic acid cargo must be released from the endosomal compartment before it reaches the lysosomal compartment. Whereas many viral vectors exhibit efficient escaping techniques and reach high transfection levels both *in vitro* and *in vivo*, endosomal escape is still a problem for some non-viral vectors (Varkouhi *et al.*, 2011). Different endosomal escape mechanisms have been suggested for different vectors.

2.2.2.1 Proton sponge effect (pH buffering effect)

A so-called proton sponge hypothesis has been proposed to explain the endosomal release mechanisms of polymer-based complexes. Polyplexes have high buffering capacity due to the presence of protonatable amines. After their endosomal uptake, the ATPase proton pumps, located in the endosomal membrane, actively transfer protons inside. Polymers compensate this acidification with their buffer capacity and get further protonated. This induces an inflow of chloride ions together with water. This leads to a swelling of the endosomes, which eventually rupture and this way the cargo is released (Behr, 1997).

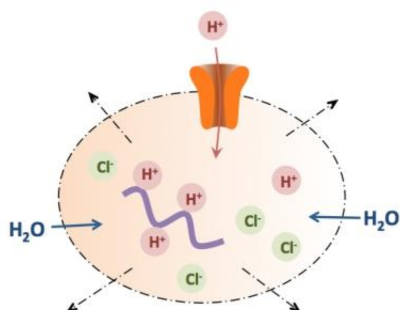


Figure 2.10: Proton sponge or pH buffering effect (Liang and Lam, 2012)**.**

2.2.2.2 Endosomal membrane destabilization

Fusogenic peptides, being part of polymer- as well as lipid-based complexes, destabilize the endosome membrane and this way facilitate the nucleic acid release into the cytosol. Those so-called cell-penetration peptides (CPP) are a sequence of neutral or positively charged amino acids. A lot of fusogenic agents derive from viruses (Liang and Lam, 2012).

2.2.2.3 Flip-flop mechanism

The so-called flip-flop mechanism occurs in lipid bilayers and terms the spontaneous diffusion of lipids between the leaflets resulting in their translocation. Three different enzymes, namely flippases, floppases and scramblases, mediate this normally slow event. The flip-flop mechanism was proposed to explain the endosomal release of nucleic acids from lipoplexes. Displacement of anionic lipids from the outer cytosolic to the inner endosomal layer via flip-flop mechanism leads to their interaction with the cationic lipids in lipoplexes. Consequently, neutrally charged ion pairs are

formed, which facilitate the nucleic acid release into the cytosol (Xu and Szoka, 1996; Harashima *et al.*, 2001).

2.2.3 Nuclear entry

In case of RNA the cytosol is the place of its destination, whereas DNA needs to enter the nucleus for performing its function. DNA can enter the nucleus via two different mechanisms:

- Active diffusion
- Passive diffusion

2.2.3.1 Active diffusion – Nuclear pore complex

The nuclear membrane of eukaryotic cells is permeable to solutes not larger than 9 nm (*e.g.* 40- 60-kDa proteins). Transport of larger molecules through nuclear pores is energy-dependent, signal-mediated and involves shuttle molecules (Zanta *et al.*, 1999).

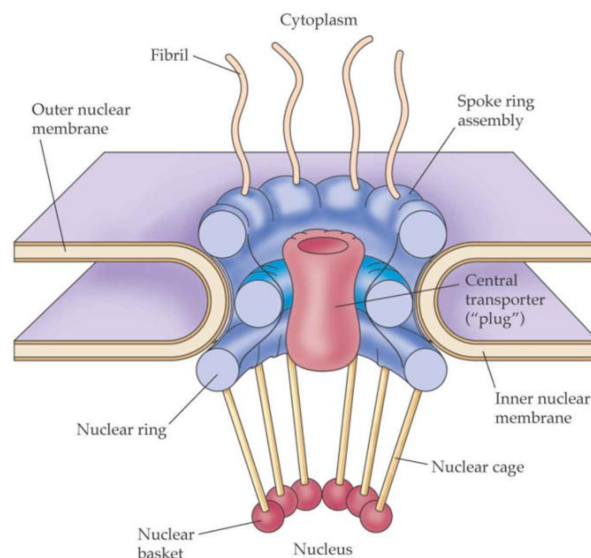


Figure 2.11: Nuclear pore complex – import and export of molecules is regulated by pores within the nuclear envelope (Deitzer, 2008)**.**

Translocation of DNA into the nucleus requires so-called nuclear localization signals (NLS). NLSs are sequences of amino acids triggering the transfer of the molecule into the nucleoplasm. For gene delivery, conjugation with a single peptide from the NLS was reported to improve transfection (Zanta *et al.*, 1999; Hu *et al.*, 2012).

2.2.3.2 Passive diffusion – during cell division

Another hypothesis suggests passive diffusion of DNA into the nucleus during mitosis. A so-called breakdown of the nuclear envelope due to cell division allows entry of the DNA into the nucleus. Transfection efficiency of cationic lipid-mediated delivery systems is enhanced, when transfection is performed during or right before mitosis. As a consequence, a relationship between cell cycle status and a successful DNA delivery into the nucleus has been proposed (Mortimer *et al.*, 1999).

Even though the mechanisms of formation and intracellular trafficking have been studied extensively, delivery of the genetic material into the nucleus still is a fundamental barrier for a successful transfection, with many factors involved.

3. MATERIALS AND METHODS

3.1 Cell culture

Cervical carcinoma (HeLa) cells were cultured in DMEM/F-12 medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12; GIBCO-Invitrogen®, Merelbeke, Belgium) supplemented with 10 % heat-inactivated FBS (fetal bovine serum; HyClone®, Pierce®, Rockford, IL, USA), 100 U/ml penicillin/streptomycin (GIBCO-Invitrogen®, Merelbeke, Belgium) and 5 ml L-glutamine (GIBCO-Invitrogen®, Merelbeke, Belgium). Cells were incubated at 37°C in a humidified 5 % CO_2-containing atmosphere.

Cells were seeded in 24-well plates (60.000 cells/well) 24 hours before transfection experiments.

3.2 Preparation of mRNA

mRNA encoding firefly luciferase was prepared according to the procedure described previously (Rejman *et al.*, 2010). mRNA was stored at -80°C, at the concentration 1 µg/µl.

3.3 Preparation of DOTAP/DOPE liposomes

100 µl of DOTAP (1,2-dioleoyl-3-trimethylammonium-propane)/DOPE (1,2 dioleoyl-sn-glycero-3-phosphoethanolamine) mixture (molar ratio 1:1 in chloroform, 10 mg/ml) (Avanti® Polar Lipids, Alabaster, Alabama, USA) was transferred to a sterile glass flask. The solvent was evaporated under nitrogen atmosphere. This resulted in formation of a lipid film. The addition of 1 ml of nuclease-free water in the presence of glass beads was followed by sonication, yielding DOTAP/DOPE liposomes. The total lipid concentration was 1 mg/ml.

3.4 Preparation of lipoplexes

1. Condition 15 µl DOTAP/DOPE + 35 µl Medium
4 µl mRNA + 46 µl Medium

2. Condition 20 µl DOTAP/DOPE + 30 µl Medium

4 µl mRNA + 46 µl Medium

3. Condition 25 µl DOTAP/DOPE + 25 µl Medium

4 µl mRNA + 46 µl Medium

Medium was added to DOTAP/DOPE liposomes, and then mixed with mRNA solution. After 10 minutes of incubation at room temperature, 900 µl of medium was added to this dispersion, which was then divided into 2 wells.

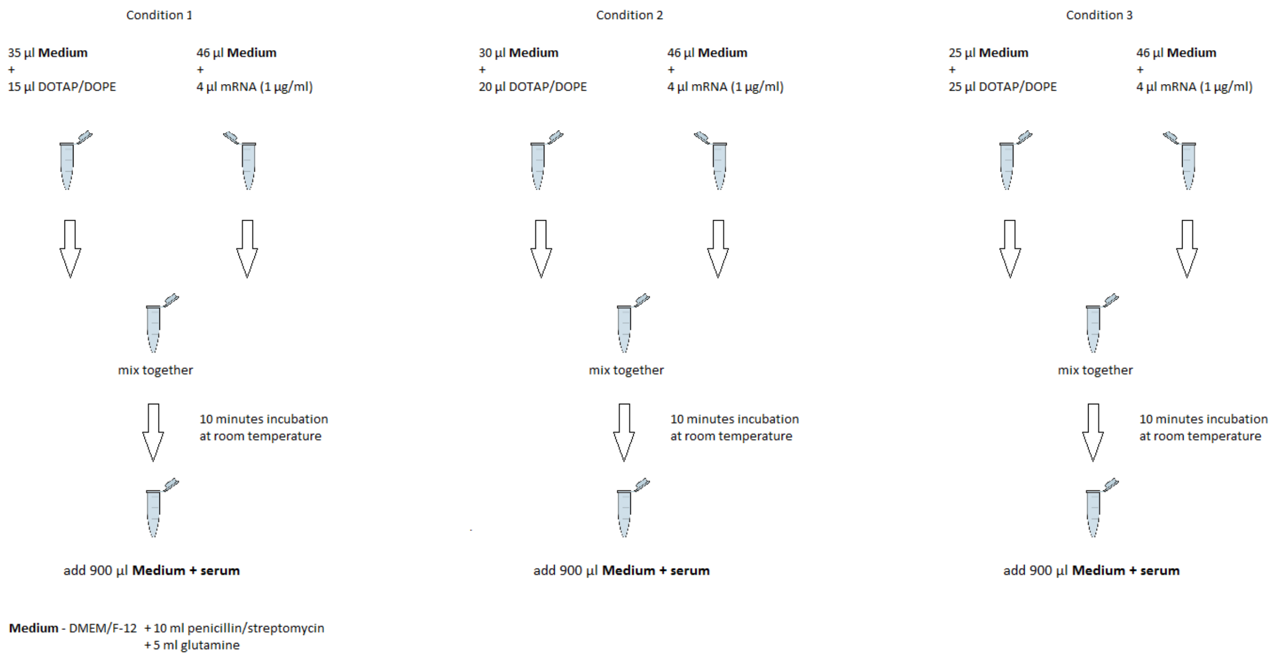


Figure 3.1: Preparation of DOTAP/DOPE/mRNA lipoplexes in the absence of serum.

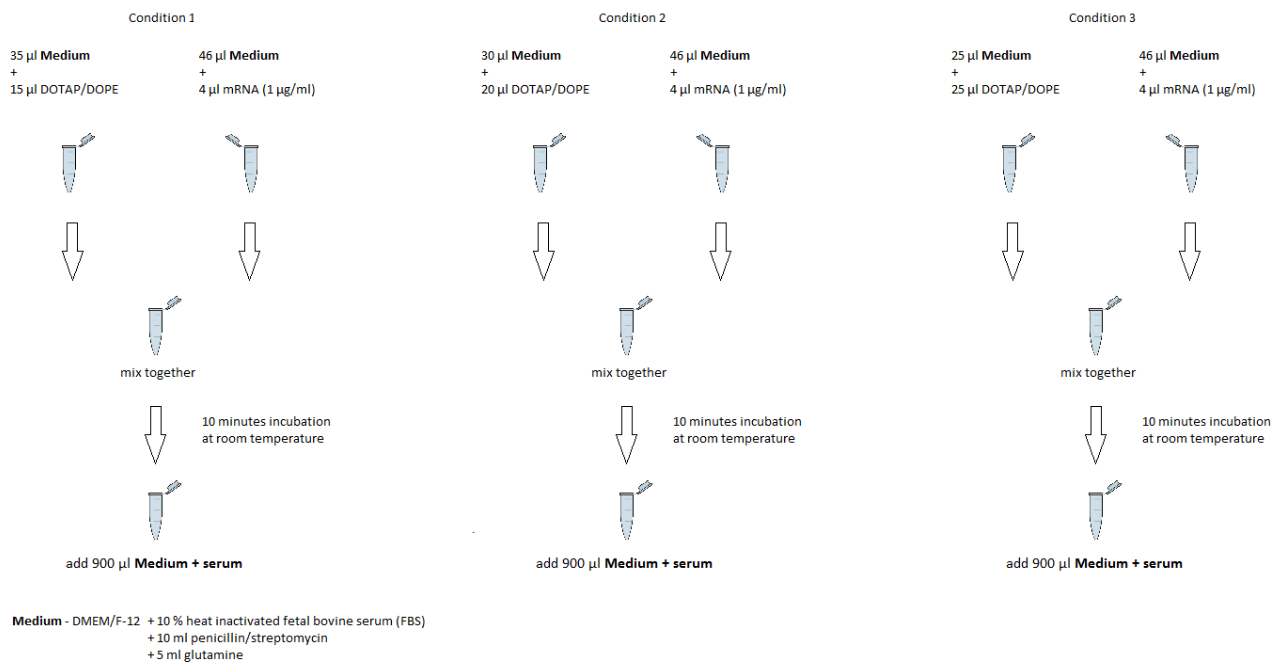


Figure 3.2: Preparation of DOTAP/DOPE/mRNA lipoplexes in the presence of serum.

3.5 Transfection

HeLa cells were seeded in 24-well plates 24 hours before transfection experiments. After removing growth medium, DOTAP/DOPE/mRNA complexes were added to the cells and incubated for 2 hours at 37°C in a humidified 5 % CO_2-containing atmosphere. Then the complexes were removed and 1 ml of fresh medium was added to the cells.

A luciferase assay was performed at different time points to evaluate the transfection efficiency.

3.6 Luciferase assay

3.6.1 Principle

3.6.1.1 Luciferase assay

The activity of luciferase was evaluated with a luciferase assay.

In the bioluminescence reaction, luciferin (substrate) is oxidized to oxyluciferin (product) through an electron transition being catalysed by luciferase. Firefly luciferase needs ATP and Mg^{2+} as co-

factors. During this chemical reaction an unstable reaction intermediate (oxyluciferin) is formed, which emits light while returning to the ground state.

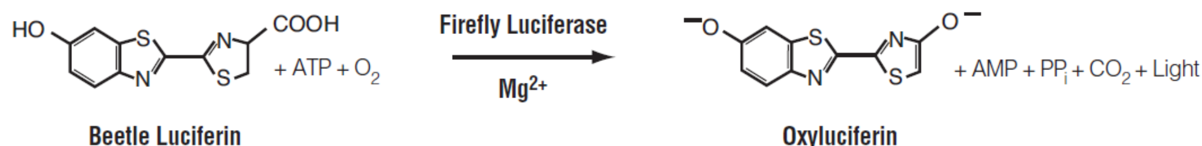


Figure 3.3: Bioluminescence reaction: firefly luciferase catalyses conversion of luciferin to oxyluciferin (Promega®, 2012)**.**

3.6.1.2 Protein assay

A Bio-Rad® (Bio-Rad®, Nazareth Eke, Belgium) protein assay was performed to determine the protein concentration. This assay is based on the Bradford method which uses Coomassie® Brilliant Blue G-250 as dye. When a protein binds in acidic conditions to the dye, the dye changes its form. Free dye is cationic and green or red, it converts into its anionic and blue form when bound to proteins. Consequently, the absorbance maximum shifts from 465 nm to 595 nm and its intensity relates to the amount of protein in the solution. The dye preferentially binds to basic and aromatic acid residues. The absorbance was measured with a NanoDrop® spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) at 595 nm.

3.6.2 Practical

3.6.2.1 Luciferase assay

After removing the medium, the cells were washed with DPBS (Dulbecco's Phosphate-Buffered Saline; GIBCO®-Invitrogen, Merelbeke, Belgium) and incubated with 100 µl Cell Culture Lysis Buffer (Promega®, Leiden, The Netherlands) for 15 minutes. The lysates were transferred to Eppendorff tubes and centrifuged for 5 minutes at 14.000 RPM. 40 µl of these supernatants were used for the luciferase assay. The luciferase activity was measured in a GloMax®-96 Microplate Luminometer (Promega®, Leiden, The Netherlands). 100 µl of luciferase substrate was injected into each well.

3.6.2.2 Protein assay

BSA (bovine serum albumin) (Bio-Rad®, Nazareth Eke, Belgium) was used as a protein standard. The concentration of BSA in the standard solution was 0.2 mg/ml. To prepare a series of protein dilutions, 10, 20, 30, 40 and 50 µg/ml of BSA solution were added to water (final volume 800 µl). Subsequently, 200 µl of Bradford solution were added. The absorbance was measured with a NanoDrop® spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) at 595 nm. The values were used to prepare a standard curve. The absorbance was plotted as a function of the known concentration.

3.7 MTT-based colorimetric assay

3.7.1 Principle

This colorimetric assay is based on the reduction of the yellow, water-soluble tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple, insoluble formazan crystals, which depends on the activity of reductases (see Figure 3.4). These mitochondrial enzymes catalyze the reduction to formazan only in metabolically active cells, using the pyridine nucleotides NADH and NADPH as co-factors. The purple formazan crystals are solubilised with a buffer (10 % SDS in 0.01 M HCl). The absorbance of resulting coloured solution can be measured at 600 nm.

The amount of formazan produced correlates with the amount of living cells present in the sample. Therefore, this assay can be used to determine the viability or proliferation of cells.

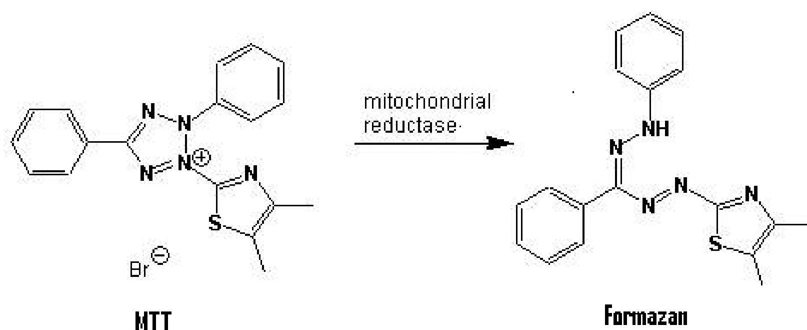


Figure 3.4: Reduction of MTT to formazan by mitochondrial reductase (Brescia and Banks, 2009).

3.7.2 Practical

An MTT assay was performed to estimate lipoplex cytotoxicity. HeLa cells were incubated with DOTAP/DOPE/mRNA lipoplexes for 2 hours. Subsequently, the medium with the lipoplexes was removed and 1 ml of fresh medium was added. Cell viability was measured with an MTT assay 4 hours after adding the complexes to the cells. To that end 500 µl of fresh medium was added. Subsequently, 50 µl of MTT reagent (Roche® Applied Science, Indianapolis, IN, USA) was added and incubated with the cells for 4 hours at 37°C in a humidified 5 % CO_2-containing atmosphere. Subsequently, 500 µl of solubilising solution was added and incubated at 37°C in a humidified 5 % CO_2-containing atmosphere overnight. The next day, the absorbance was measured with a NanoDrop® spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) at 600 nm.

3.8 Dynamic Light Scattering (DLS)

This physical technique determines the size of small particles in solution by measuring the fluctuations of the scattered light. Particles in solution follow the mathematical model of the Brownian motion. The smaller the particles, the quicker their movements. As particles become larger in size, the Brownian motions slow down. If monochromatic light shines on those particles, light scatters due to the Brownian motion. As size of the particles changes, fluctuations in the intensity of the scattered light appear. With these fluctuations in intensity of the scattered light it is possible to reveal the velocity of particles in solution. The velocity allows estimation of the particle size, in particular the hydrodynamic diameter, using the Stokes–Einstein equation. This equation relates the size of a particle with its velocity due to the Brownian motion.

$$d\,(H) = \frac{k\,T}{3\,\pi\,\eta\,D}$$

d (H) = hydrodynamic diameter

k = Boltzmann's constant

T = absolute temperature

η = viscosity

D = translational diffusion coefficient

A Zetasizer® Nano Range (Malvern®, Hoeilaart, Belgium) was used to determine the size of particles. Lipoplexes were prepared as described above and equilibrated at 37°C prior to measurements to mimic the conditions during transfection.

4. RESULTS

In order to develop safe and efficient gene therapy methods suitable for clinical applications, it is necessary to optimize both the complexes carrying the genetic material as well as the conditions of its application. In this study, a non-viral vector was used to deliver genetic information in form of messenger RNA (mRNA). For many years research has mainly focused on plasmid DNA (pDNA) instead of mRNA due to the presumed instability of the latter. However, in the last decade several studies have demonstrated the superiority of transfection based on mRNA over that of pDNA at least for some applications. mRNA is safer (no risk of insertional mutagenesis) and it does not need to enter the nucleus to perform its function. In contrast to pDNA, mRNA causes transient protein expression, which can be an advantage as well as a disadvantage depending on the clinical application required (Tavernier *et al.*, 2010).

Because of its negative charge, mRNA cannot enter the cell by itself.

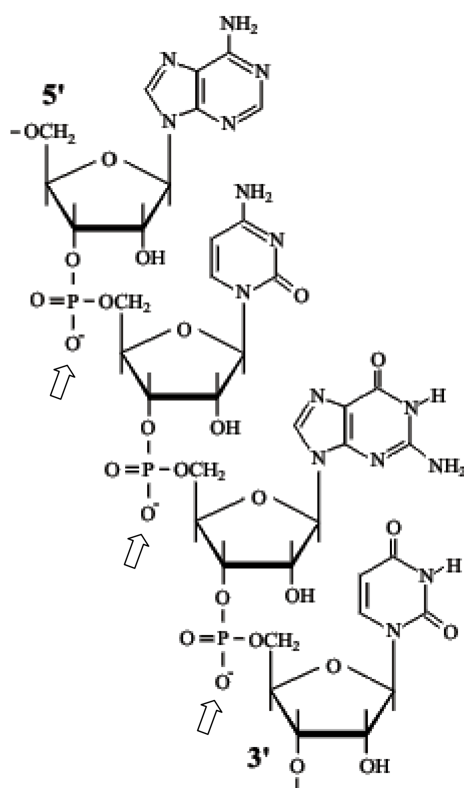


Figure 4.1: Chemical structure of ribonucleic acid (RNA) (Generalic, 2013)**.** The negative charge comes from the phosphate groups in the backbone of the mRNA (indicated with an arrow).

Introducing a positive charge compensates the negative charge, and enables the entry into the cell. This positive charge is provided by the cationic lipid DOTAP. To facilitate endosomal escape and improve transfection efficiency, a so-called helper lipid (DOPE) should be introduced.

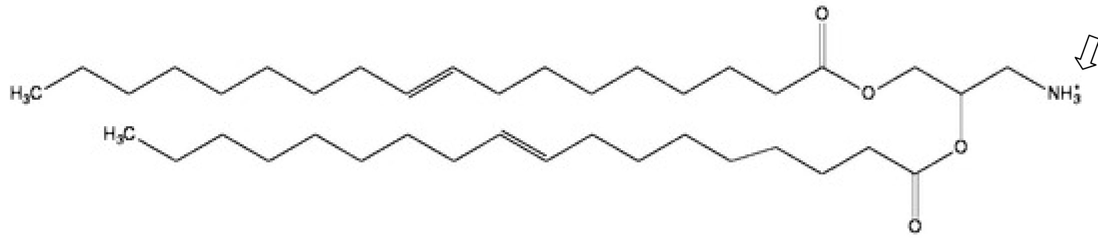


Figure 4.2: Chemical structure of cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Bergen *et al.*, 2008)**.** The positive charge comes from the amine group (indicated with an arrow).

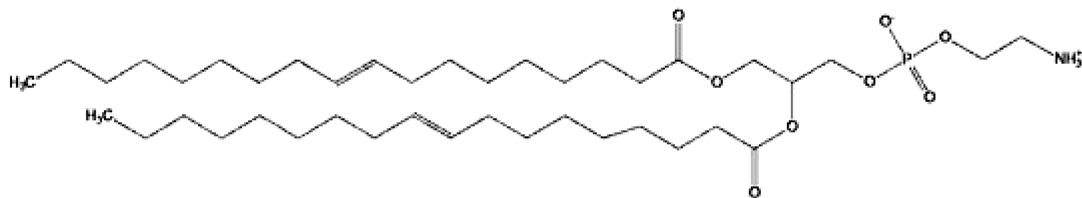


Figure 4.3: Chemical structure of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Bergen *et al.*, 2008)**.**

4.1 Finding optimal mRNA to cationic lipid ratio

4.1.1 Medium without serum

It has been demonstrated that both transfection efficiency and cytotoxicity of complexes made of plasmid DNA (pDNA) and cationic carriers depend on the ratio at which they are mixed together. Therefore, in the first set of experiments we aimed at finding the optimal condition to prepare DOTAP/DOPE/mRNA complexes. To that end 15, 20 or 25 µl of DOTAP/DOPE solution were mixed with 4 µg of mRNA encoding luciferase and transfection efficiency was evaluated 24 hours later. The complexes were prepared and incubated with the cells in the absence of serum. In order to evaluate transfection efficiency, luciferase activity was measured. Subsequently, the amount of total protein, evaluated by a Bradford protein assay, was correlated with the luciferase activity. Results are shown in Figure 4.4 as relative light units per mg of protein (RLU/mg protein).

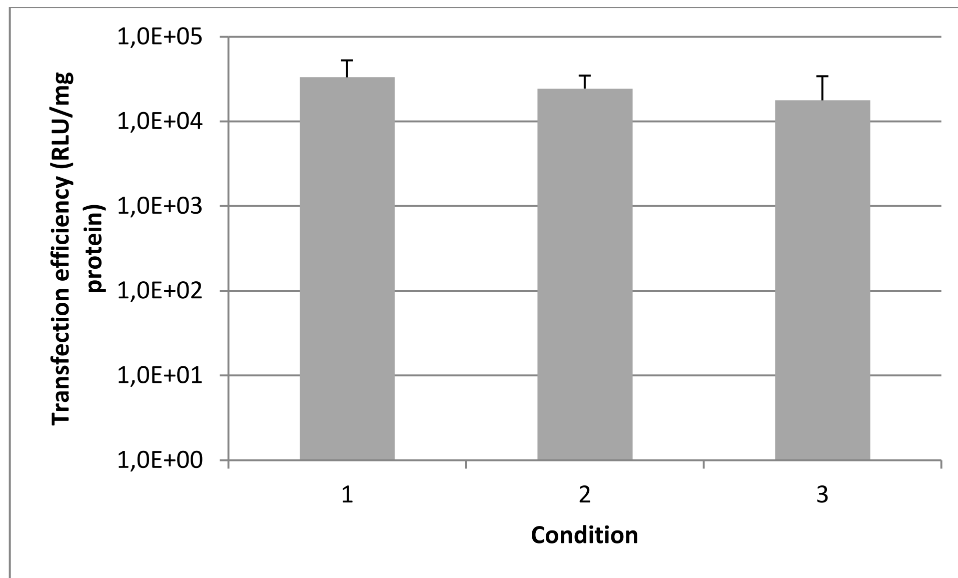


Figure 4.4: Transfection efficiency of complexes made of DOTAP/DOPE and mRNA mixed at different ratios – medium without serum. Complexes were prepared and incubated with HeLa cells in the absence of serum. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE solution were mixed with 4 µg of mRNA. The complexes were incubated with the cells for 2 hours. A luciferase assay was performed 24 hours later. Luciferase activity is expressed as relative light units per mg of protein.

When mRNA and DOTAP/DOPE were mixed at the first ratio, the activity of luciferase was 33.100 RLU/mg of protein. The values for the second and the third condition were lower: 24.400 and 17.700, respectively. However, the statistical analysis revealed that the differences are not significant.

4.1.2 Medium with 10 % serum

To estimate the influence of serum on the transfection efficiency, complexes were prepared in medium without serum but incubated with HeLa cells in medium supplemented with 10 % serum.

The luciferase activity was measured 24 hours after adding the complexes to the cells. The same conditions as those presented in Figure 4.4 were tested. The results are shown in Figure 4.5.

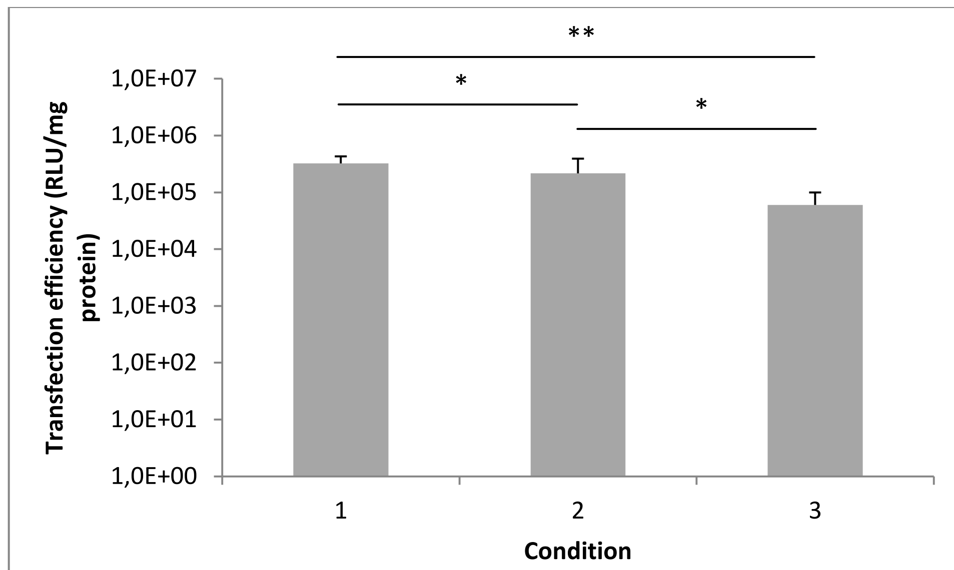


Figure 4.5: Transfection efficiency of complexes made of DOTAP/DOPE and mRNA mixed at different ratios – medium with serum. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE were mixed with 4 µg of mRNA. Complexes were prepared in medium without serum but incubated with HeLa cells in the presence of serum. The complexes were incubated with the cells for 2 hours. A luciferase assay was performed 24 hours later. Luciferase activity is expressed as relative light units per mg of protein. The results were analysed for the statistical significance with a Student's *t* test: * $p < 0.05$ and ** $p < 0.005$.

When DOTAP/DOPE and mRNA were mixed at the first ratio, the activity of luciferase was 323.600 RLU/mg of protein. The values for the second and the third condition were lower: 216.200 and 60.000, respectively.

The data obtained in the two sets of experiments are compared in Figure 4.6. As demonstrated there, for all three conditions tested, transfection efficiency was higher when DOTAP/DOPE/mRNA complexes were incubated with the cells in the presence of serum.

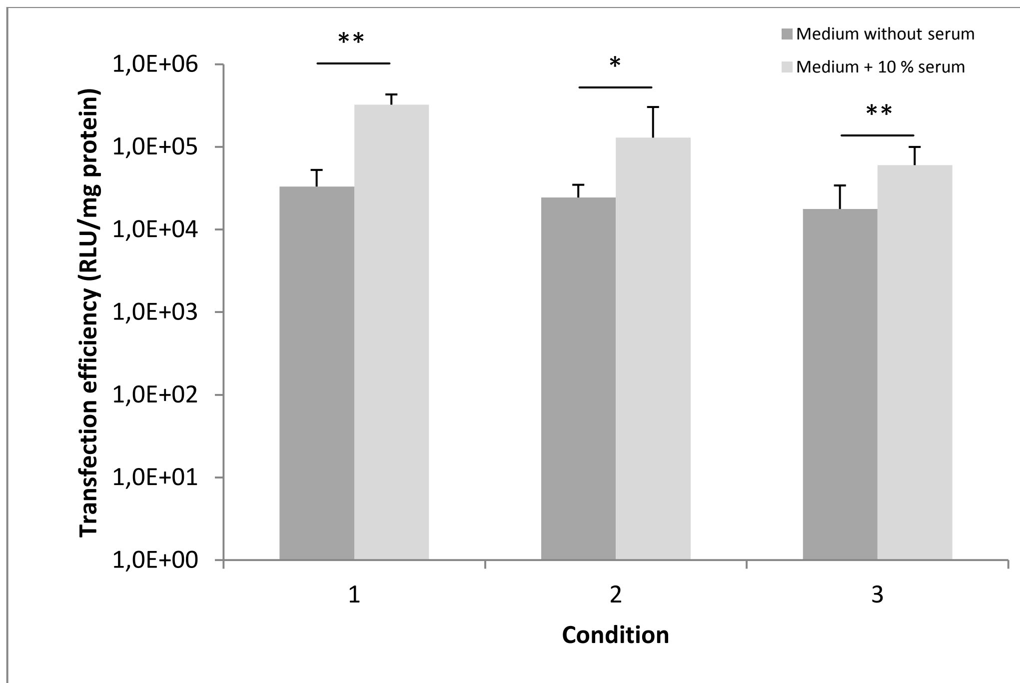


Figure 4.6: Transfection efficiency of mRNA complexed with DOTAP/DOPE – effect of serum. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE were mixed with 4 µg of mRNA. Complexes were prepared in medium without serum and incubated with HeLa cells in the absence (dark grey bars) or presence of serum (light grey bars). The complexes were incubated with the cells for 2 hours. A luciferase assay was performed 24 hours later. Luciferase activity is expressed as RLU/mg protein. The results were analysed for the statistical significance with a Student's *t* test: * $p < 0.05$ and ** $p < 0.005$.

To better evaluate the influence of serum on transfection efficiency, we tested three other serum concentrations, namely 2.5, 5 and 7.5 %.

Figure 4.7 demonstrates the impact of serum on transfection efficiency of complexes prepared by mixing 15 µl DOTAP/DOPE with 4 µg of mRNA (condition 1). The complexes were prepared in the absence of serum and incubated with the cells in the presence of different amount of serum. The data are compared to the condition in which complexes were prepared and incubated with the cells in the absence of serum.

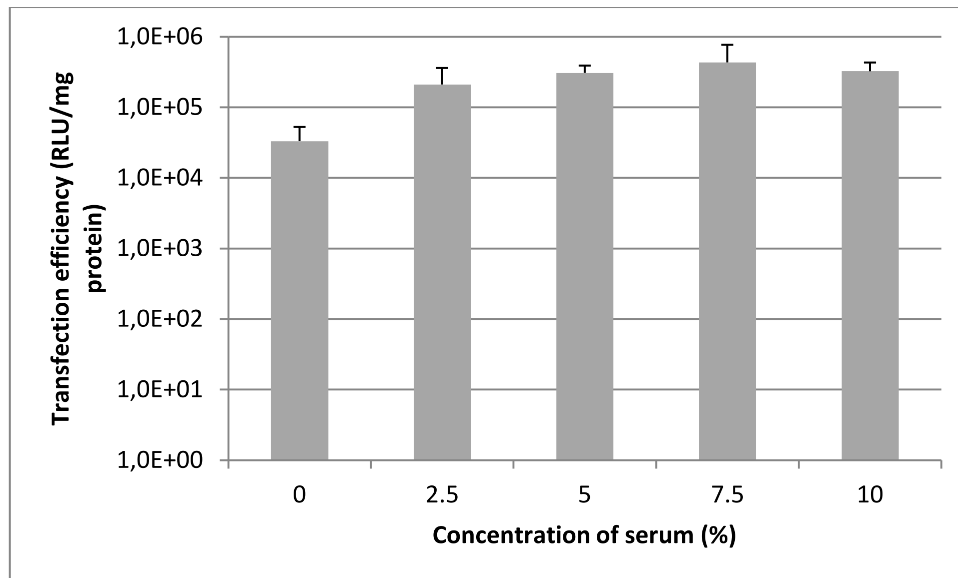


Figure 4.7: Transfection efficiency of complexes formed by mixing 15 µl DOTAP/DOPE and 4 µl mRNA (condition 1) – effect of different concentrations of serum. The complexes were prepared in the absence of serum but incubated with HeLa cells for 2 hours in medium supplemented with 2.5, 5, 7.5 and 10 % of serum. A luciferase assay was performed 24 hours later to determine transfection efficiency. Results are shown as percentage of luciferase activity (RLU/mg protein) of cells incubated with the complexes in the presence of 10 % serum (100 %).

The presence of as little as 2.5 % of serum significantly increased transfection efficiency. The activity of luciferase was the highest when the complexes were incubated with the cells in the presence of 7.5 % of serum.

Figure 4.8 demonstrates the impact of serum on transfection efficiency of complexes prepared by mixing 20 µl DOTAP/DOPE with 4 µg of mRNA (condition 2). The complexes were prepared in the absence of serum and incubated with the cells in the presence of different amount of serum. The data are compared to the condition in which complexes were prepared and incubated with the cells in the absence of serum. For this condition the highest transfection efficiency was observed for 5% of serum.

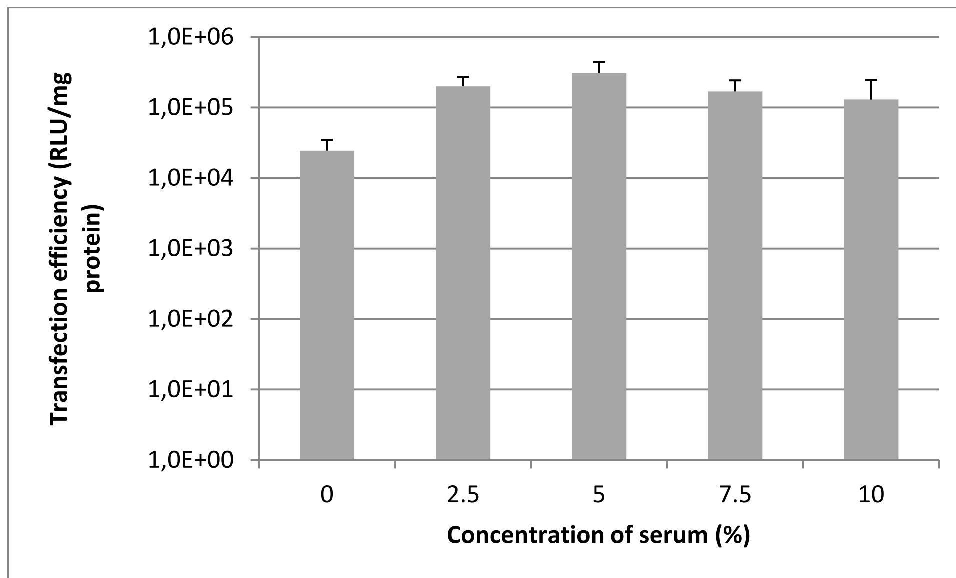


Figure 4.8: Transfection efficiency of complexes formed by mixing 20 µl DOTAP/DOPE and 4 µl mRNA (condition 2) – effect of different concentrations of serum. The complexes were prepared in the absence of serum but incubated with HeLa cells for 2 hours in medium supplemented with 2.5, 5, 7.5 and 10 % of serum. A luciferase assay was performed 24 hours later to determine transfection efficiency. Results are shown as percentage of luciferase activity (RLU/mg protein) of cells incubated with the complexes in the presence of 10 % serum (100 %).

Figure 4.9 demonstrates the impact of serum on transfection efficiency of complexes prepared by mixing 25 µl DOTAP/DOPE with 4 µg of mRNA (condition 3). The complexes were prepared in the absence of serum and incubated with the cells in the presence of different amount of serum. The data are compared to the condition in which complexes were prepared and incubated with the cells in the absence of serum. The highest transfection efficiency was measured for 5 % of serum.

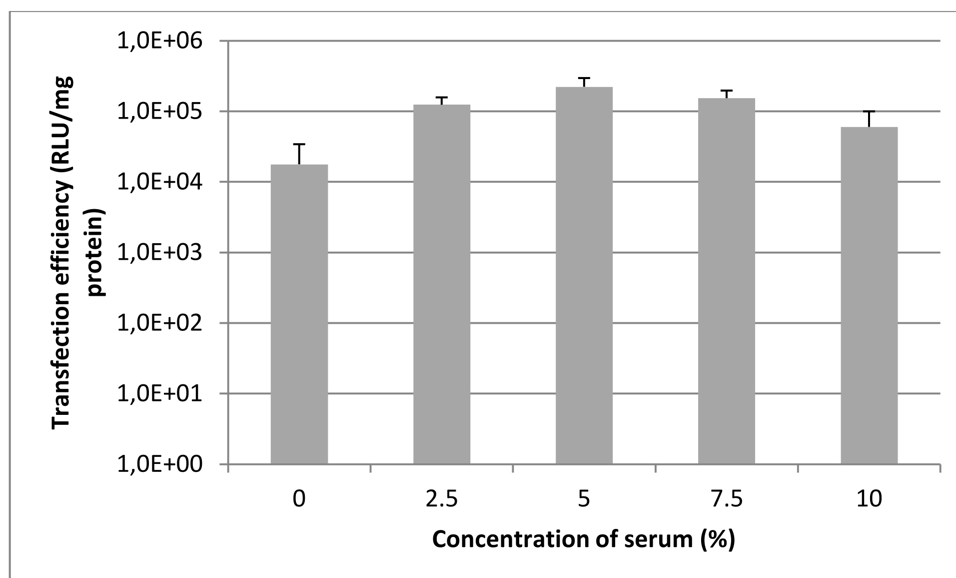


Figure 4.9: Transfection efficiency of complexes formed by mixing 25 µl DOTAP/DOPE and 4 µl mRNA (condition 3) – effect of different concentrations of serum. The complexes were prepared in the absence of serum but incubated with the HeLa cells for 2 hours in medium supplemented with 2.5, 5, 7.5 and 10 % of serum. A luciferase assay was performed 24 hours later to determine transfection efficiency. Results are shown as percentage of luciferase activity (RLU/mg protein) of cells incubated with the complexes in the presence of 10 % serum (100 %).

4.1.3 Conclusions

- When the complexes with the lowest positive charge were incubated with the cells in the presence of 7.5 % of serum, the transfection efficiencies obtained were the highest.
- For two other conditions, the highest transfection efficiencies were obtained for 5 % of serum.

4.2 Size

Size is one of the parameters that might determine the mechanism of particle uptake and this way influence their transfection efficiency. To further characterize the complexes, it was therefore necessary to evaluate their diameter. The average diameter was measured in medium with or without serum for the three conditions. The results for medium without serum are shown in Figure 4.10.

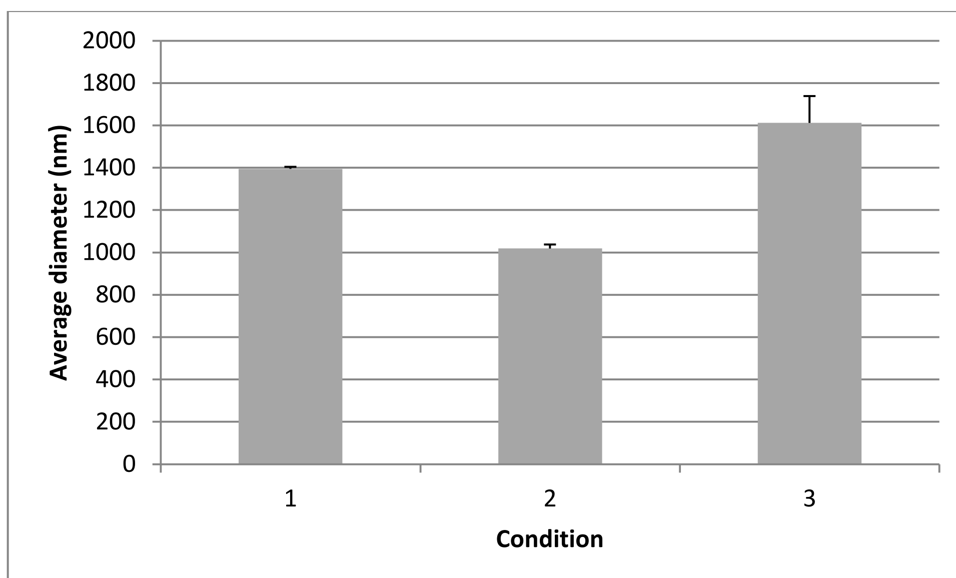


Figure 4.10: Average diameter of DOTAP/DOPE/mRNA complexes – medium without serum. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE were complexed with 4 µl of mRNA in medium without serum. The complexes were further diluted in the same medium.

The average diameter of particles prepared by mixing 15 µl of DOTAP/DOPE with 4 µl mRNA, was 1.395 nm. Increasing amount of DOTAP/DOPE to 20 µl resulted in formation of smaller particles (1.018 nm). The complexes prepared by mixing 25 µl of DOTAP/DOPE with 4 µl mRNA were the largest (1.612 nm).

The results for medium with serum are presented in Figure 4.11.

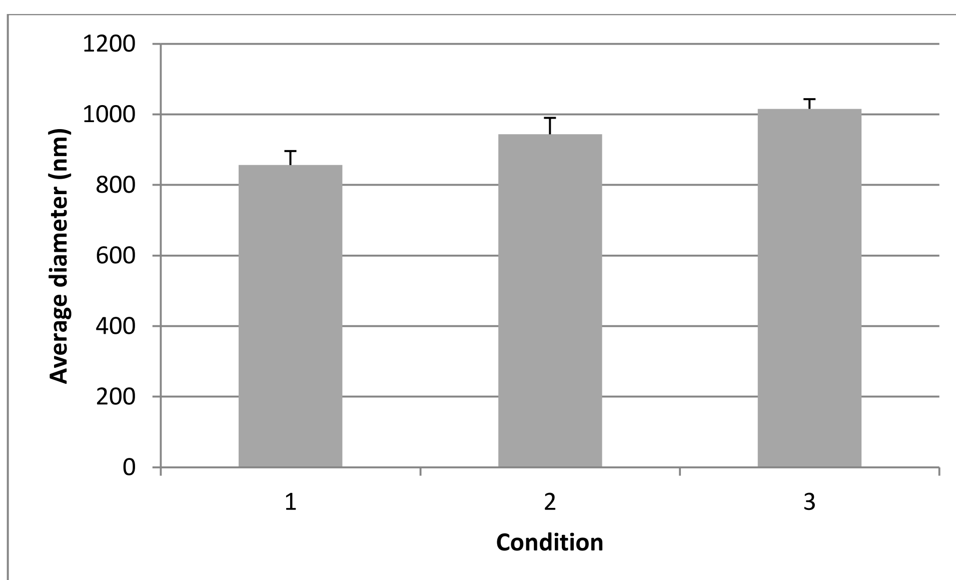


Figure 4.11: Average diameter of DOTAP/DOPE/mRNA complexes – medium with serum. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE were complexed with 4 µl of

mRNA in medium without serum. The complexes were further diluted in medium supplemented with 10 % serum.

The average diameter of particles prepared by mixing 15 µl of DOTAP/DOPE with 4 µl mRNA, was 856 nm. Increasing amount of DOTAP/DOPE to 20 µl resulted in formation of larger particles (944 nm). The complexes with the highest positive charge were the largest (1.016 nm).

4.2.1 Conclusions

- The complexes diluted in medium supplemented with serum were smaller than those diluted in medium without serum for all conditions tested.
- The average diameter of complexes prepared by mixing 25 µl of DOTAP/DOPE with 4 µl mRNA was always the largest.

4.3 Toxicity

To further characterize the mRNA-carrying complexes, it is imperative to determine their toxicity. Therefore an MTT assay was performed. The lipoplexes were prepared the same way as for transfection experiments (see 3. Materials and Methods).

4.3.1 Medium without serum

The complexes were prepared by mixing 15, 20 or 25 µl DOTAP/DOPE with 4 µg mRNA. The complexes were made and incubated with HeLa cells in medium without serum. Toxicity was evaluated 4 hours after adding the complexes to the cells.

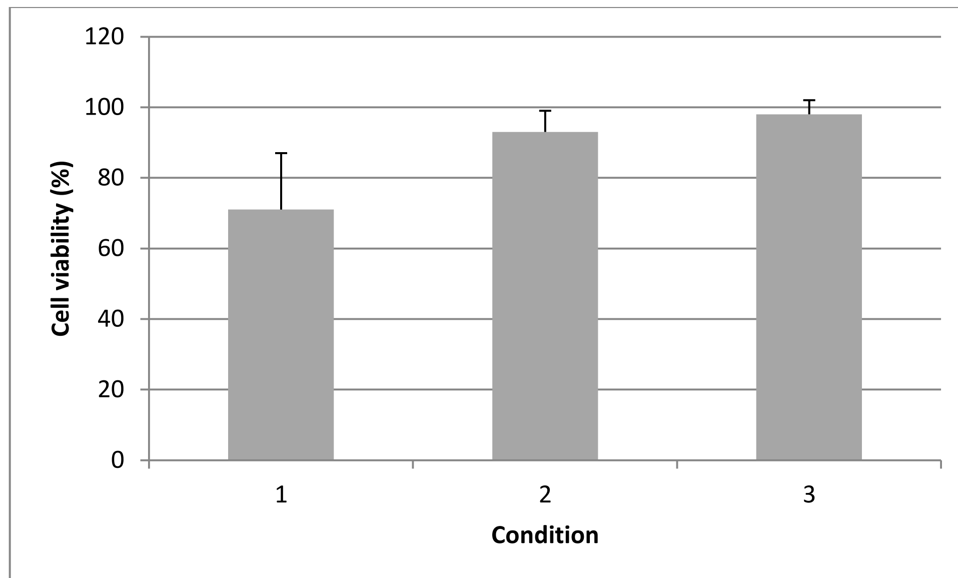


Figure 4.12: Toxicity of DOTAP/DOPE/mRNA lipoplexes – medium without serum. An MTT assay was performed to determine the viability of HeLa cells. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE were complexed with 4 µl of mRNA in medium without serum. The complexes were incubated with HeLa cells in medium without serum. Results are shown as percentage of untreated cells (100 %).

As shown in Figure 4.12 only complexes prepared by mixing 15 µl DOTAP/DOPE and 4 µl of mRNA considerably reduced viability of HeLa cells.

4.3.2 Medium with 10 % serum

The complexes were prepared by mixing 15, 20 or 25 µl DOTAP/DOPE with 4 µg mRNA. The complexes were made in the absence of serum but incubated with HeLa cells in medium supplemented with 10 % serum. Toxicity was evaluated 4 hours after adding the complexes to the cells.

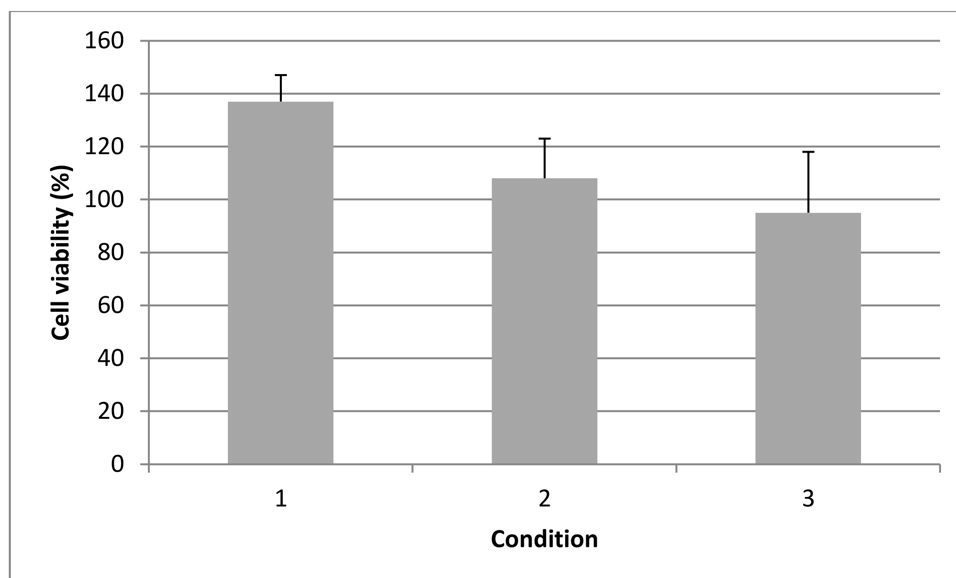


Figure 4.13: Toxicity of DOTAP/DOPE/mRNA lipoplexes – medium with serum. An MTT assay was performed to determine the viability of HeLa cells. 15 (condition 1), 20 (condition 2) or 25 µl (condition 3) of DOTAP/DOPE were complexed with 4 µl of mRNA in medium without serum. The complexes were incubated with HeLa cells in medium supplemented with 10 % serum. Results are shown as percentage of untreated cells (100 %).

As shown in Figure 4.13 none of the formulations induced any toxicity in HeLa cells.

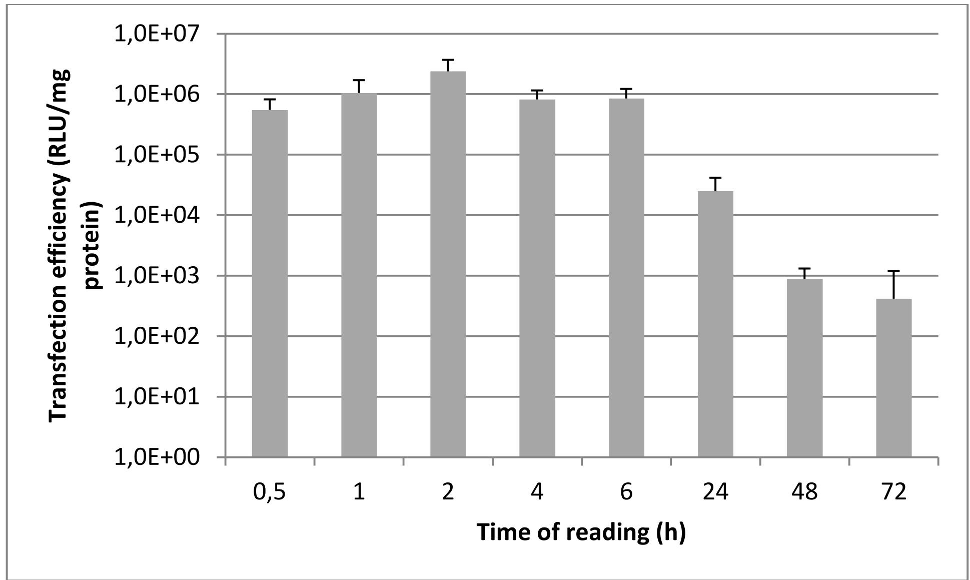
4.3.3 Conclusions

Hardly any of the formulations tested significantly compromised viability of HeLa cells.

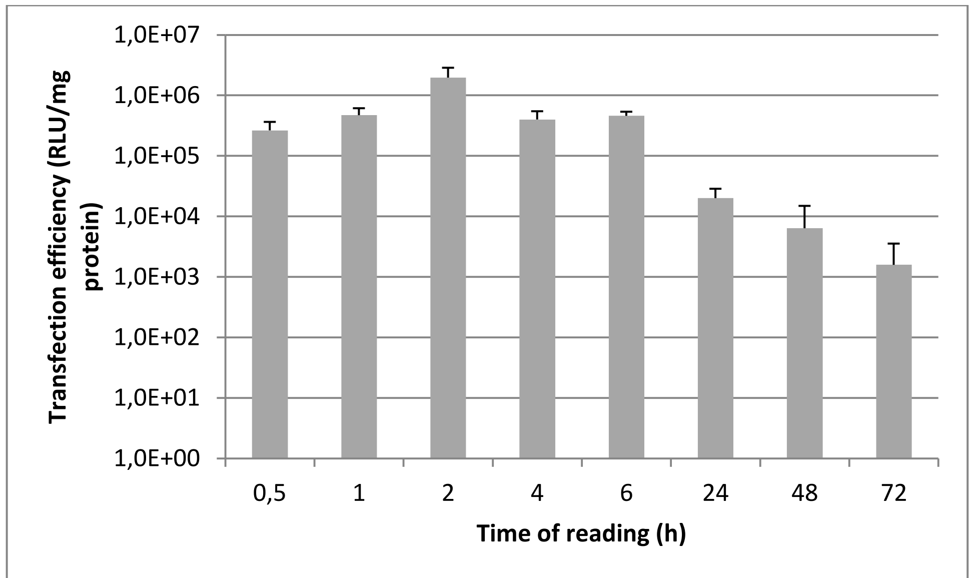
4.4 Duration of protein production following transfection with DOTAP/DOPE/mRNA complexes

4.4.1 Medium without serum

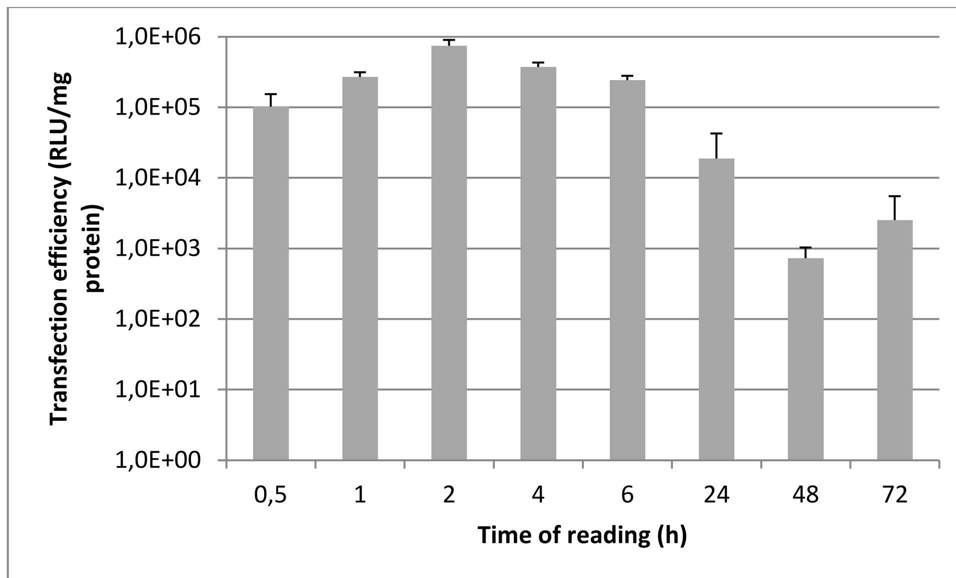
To better understand kinetics of protein production upon transfection with DOTAP/DOPE/mRNA complexes luciferase activity was measured 0.5, 1, 2, 4, 6, 24, 48 and 72 hours after adding the complexes to HeLa cells. The results for complexes made by mixing 15 µl of DOTAP/DOPE with 4 µl of mRNA in medium without serum are shown in Figure 4.14 (A), for complexes made by mixing 20 µl of DOTAP/DOPE with 4 µl of mRNA in Figure 4.14 (B) and for complexes made by mixing 25 µl of DOTAP/DOPE with 4 µl of mRNA in Figure 4.14 (C).



(A)



(B)



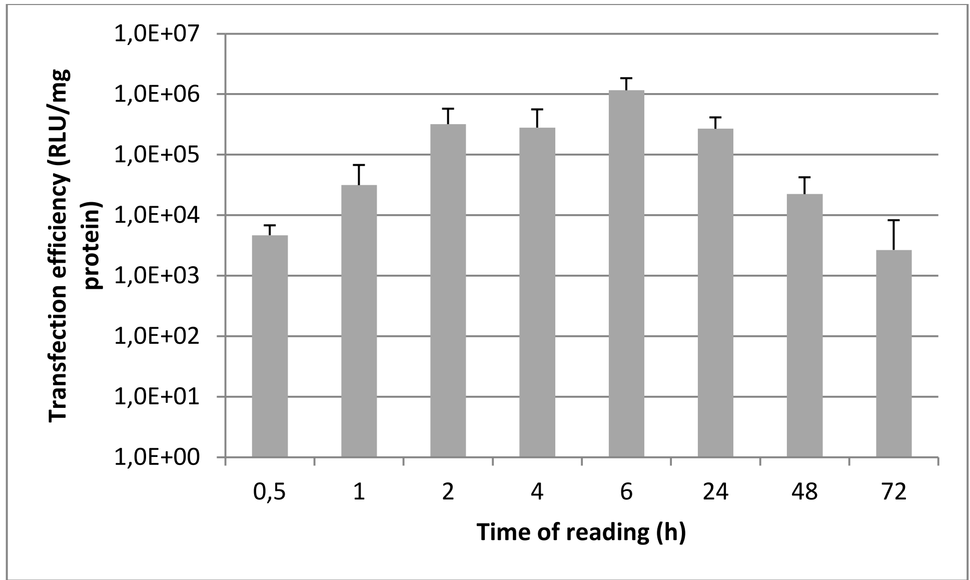
(C)

Figure 4.14 (A-C): Time course of luciferase expression upon transfection with DOTAP/DOPE/mRNA complexes – medium without serum. 15 (A), 20 (B), or 25 µl (C) of DOTAP/DOPE were mixed with 4 µl of mRNA in medium without serum. Complexes were incubated with HeLa cells in the absence of serum. The complexes were incubated with the cells maximally for 2 hours. Luciferase assay was performed at different time points. Luciferase activity is shown as RLU/mg protein.

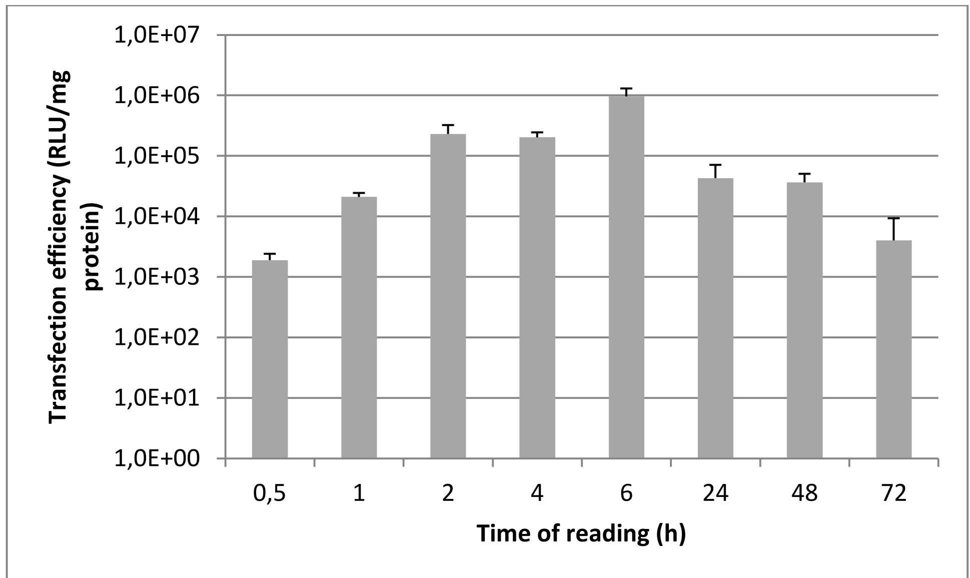
As shown in Figure 4.14 significant levels of luciferase activity were detectable as early as 30 minutes after adding the complexes to the cells. The protein expression reached its maximum 2 hours post transfection. Transfection mediated by DOTAP/DOPE/mRNA complexes lasted up to 3 days. This was observed for all the conditions tested.

4.4.2 Medium with 10 % serum

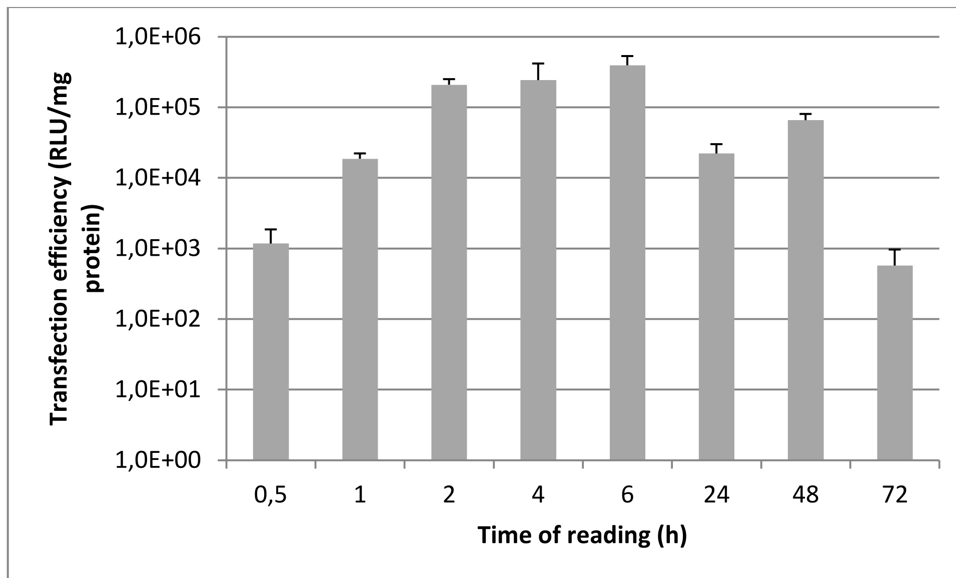
In the next set of experiments we evaluated kinetics of protein production upon transfection with DOTAP/DOPE/mRNA complexes which were incubated with HeLa cells in the presence of serum. To that end luciferase activity was measured 0.5, 1, 2, 4, 6, 24, 48 and 72 hours after adding the complexes to the cells. The results for complexes made by mixing 15 µl of DOTAP/DOPE with 4 µl of mRNA are shown in Figure 4.15 (A), for complexes made of 20 µl of DOTAP/DOPE with 4 µl of mRNA in Figure 4.15 (B) and for complexes made of 25 µl of DOTAP/DOPE with 4 µl of mRNA in Figure 4.15 (C).



(A)



(B)



(C)

Figure 4.15 (A-C): Time course of luciferase expression upon transfection with DOTAP/DOPE/mRNA complexes – medium with serum. 15 (A), 20 (B) or 25 µl (C) of DOTAP/DOPE were mixed with 4 µl of mRNA in medium without serum. Complexes were incubated with HeLa cells in the presence of serum (10 %). The complexes were incubated with the cells maximally for 2 hours. Luciferase assay was performed at different time points. Luciferase activity is shown as RLU/mg protein.

The data presented in Figure 4.15 demonstrate that significant levels of luciferase activity were detectable as early as 30 minutes after adding the complexes to the cells. The protein expression reached its maximum 6 hours post transfection. Transfection mediated by DOTAP/DOPE/mRNA complexes lasted up to 3 days. This was observed for all the conditions tested.

5. DISCUSSION

Finding a safe and efficient vector for gene therapy is essential for clinical applications. Despite the immense amount of research done in this field, a lot of work is still required to reach that point.

The main goal of this study was to evaluate whether DOTAP/DOPE/mRNA complexes are good candidates for their use in gene therapy. Therefore the lipoplexes were assessed in regard to their transfection efficiency as well as cytotoxicity, both important parameters for a successful delivery of the genetic material into the cell. Transfection efficiency and cytotoxicity depend on the ratio at which nucleic acids and cationic carriers are mixed together. Thus, the first aim was to find the optimal conditions for the preparation of the complexes. The smallest amount of DOTAP/DOPE mixture ensured the highest transfection efficiencies. To determine how and to what extent serum influences transfection, 10 % of serum was added to the transfection medium. As before, the highest transfection efficiency was observed with the lowest cationic carrier/mRNA ratio. Comparing those complexes to the complexes, which were incubated with cells in the absence of serum, the presence of serum revealed higher transfection efficiencies. Since these data suggested that serum enhances transfection, three other serum concentrations were tested, namely 2.5, 5 and 7.5 %. Transfection efficiencies of all three conditions tested were higher than those obtained in the absence of serum. Complexes characterized by the lowest cationic charge reached the highest transfection efficiency with 7.5 % serum. This transfection efficiency was even higher than that achieved in medium supplemented with 10 % serum. For the other two conditions, the highest transfection efficiency was reached in the presence of 5 % serum.

It has been shown, that structure and stability of the lipoplexes are affected by a number of parameters, including the ratio of cationic lipids to nucleic acids (Ma *et al.*, 2007). This is supported by the results of this study.

Our results show the highest transfection efficiencies when cells were incubated in the presence of serum – depending on the condition – either with 7.5 (condition one) or 5 % (condition two and three) serum. The interaction between serum and lipoplexes is another important parameter affecting transfection efficiency, particularly for *in vivo* applications. It has been suggested that as the lipoplexes come in contact with the serum, it has not only an impact on the structure of the lipoplexes, but also on the transport and ultimately on the transfection efficiency (Simberg *et al.*, 2003).

Our results indicate the negatively charged proteins present in the serum interact with the positively charged lipoplexes, which forms a stable structure in a way that allows the endosomal transport and escape and finally a successful transfection.

The size of lipoplexes is another important parameter that should be taken into account especially for *in vivo* applications. It has been suggested that particle size might influence the endocytic pathway by which the complexes are taken up by the cells (Rejman *et al.*, 2004). This in turn might determine their transfection efficiency. The exact correlation between the size of lipoplexes and the transfection efficiency is not clear to date. Some reports suggest that larger particles reach higher transfection efficiencies, while others state something opposite. Size itself increases along with an increasing lipid to nucleic acid ratio (Eastman *et al.*, 1997). This matches with most of our results. In the absence of serum in condition two the measured particles were smaller than condition one and therefore are not supporting this report. Both in the presence and in the absence of serum, the largest diameters were observed using the highest DOTAP/DOPE to mRNA ratio. Supplementing medium with serum caused formation of smaller particles than using medium without serum. Again, this was to be expected, since serum inhibits the lipoplexes aggregation (Konopka *et al.*, 2006).

To further characterize the lipoplexes it was essential to evaluate their cytotoxicity. As mentioned before, toxicity induced by cationic carriers was and still is, together with inefficient transfection, one of the major barriers for their therapeutic application (Zhang *et al.*, 2004).

In medium without serum, complexes with the lowest amount of cationic lipid reduced cell viability, whereas they had no toxic effect at the other ratios. In the presence of serum, none of the formulations significantly reduced cell viability.

Final objective of our study was to evaluate the duration of protein expression following the transfection with the mRNA-carrying complexes. For a long time, mRNA was believed to be too unstable and therefore not suitable for gene therapy. Only recently more interest was put on the employment of mRNA instead of pDNA (Tavernier *et al.*, 2011).

In the absence as well as in the presence of serum, protein production was detected as early as 30 minutes after adding the complexes to the cells and lasted up to 3 days. In the absence of serum, maximal expression was observed 2 hours after adding the complexes to the cells. In the presence of serum the highest levels of protein was detected 6 hours after transfection.

Concluding, the results presented in this thesis support the notion that DOTAP/DOPE/mRNA complexes are a promising alternative to viral vectors. One should be aware, however, that further research needs to be done in the field before one day they can be used in the clinics.

6. ABSTRACT

Gene therapy is a promising approach to treat both acquired and inherited diseases by correcting the underlying genetic disorder. To achieve that, so-called vectors transport therapeutic nucleic acids into the targeted tissue and further into the cells. Viral and non-viral ones are two major groups of vectors used. Even though viral vectors reach the highest transfection efficiencies, numerous limitations concerning safety, immunogenicity, transgene size as well as high production costs call for the development of alternative systems. Cationic lipids and polymers are the most commonly used non-viral vectors and exhibit several advantages over virus-based systems. They do not induce immune responses, their production is inexpensive and easy to be up-scaled. However, it is generally known that cationic vectors show poor transfection efficiencies and might induce some cytotoxicity.

In this study we evaluated cationic lipoplexes as vectors to be used in gene therapy. The cationic lipoplexes tested consisted of the positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) forming liposomes, which complexed messenger RNA (mRNA).

DOTAP/DOPE/mRNA complexes were evaluated in terms of their transfection efficiency as well as cytotoxicity, both important parameters for a successful delivery of the genetic material into the cell. First aim was to find the optimal conditions to prepare the complexes. Therefore the cationic vector DOTAP/DOPE was mixed at three different ratios with mRNA encoding luciferase. 24 hours later transfection efficiency was determined by using a luciferase assay. Our results showed that transfection efficiency depends on the amount of cationic lipid present. The highest transfection efficiency was achieved with the lowest amount of the cationic lipid used. To study the influence of serum on the transfection efficiency, the cells were incubated in the presence of 10 % serum. Our results revealed that serum enhances transfection efficiency. To better evaluate the influence of serum, we tested three other serum concentrations, namely 2.5, 5 and 7.5 %. With the lowest amount of cationic lipid, 7.5 % serum reached the highest transfection efficiency. Increasing the amount of the cationic part, the maximum transfection efficiency was observed in the presence of 5 % serum.

Since size is one of the parameters that might influence the mechanism of particle uptake and thus transfection efficiency, differently composed mRNA complexes were characterized in terms of their size by dynamic light scattering (DLS). Complexes with the highest positive charge were the largest. In the presence of serum, the particle size decreased.

Toxicity of the complexes was evaluated with an MTT assay by analyzing the viability of cells following transfection. Except one, none of the formulations tested significantly reduced viability of the cells and therefore did not show any cytotoxicity.

Finally, for a better understanding of the kinetics of the protein production upon transfection, duration of the protein production was followed over time. Luciferase activity was detected as early as 30 minutes after adding complexes to the cells and lasted up to 3 days. This was observed in the absence as well as in the presence of serum.

Our results suggest that mRNA complexed with DOTAP/DOPE present a promising alternative for viral vectors. The cationic mRNA lipoplexes can reach high transfection efficiencies and do not induce any cytotoxicity, the two major concerns when it comes to non-viral vectors. However, one should keep in mind that more research needs to be done for further approaching the final aim, which is their clinical use.

7. ZUSAMMENFASSUNG

Gentherapie eröffnet vielversprechende Möglichkeiten zur Behandlung von erworbenen Erkrankungen und auch Erbkrankheiten. Dabei soll der zugrunde liegende genetische Defekt durch therapeutische DNA oder RNA gebunden an Transportvehikel, sogenannte Vektoren, korrigiert werden. Derzeit werden zwei Gruppen von Vektoren eingesetzt, nämlich virale und nicht-virale.

Obwohl mit viralen Vektoren deutlich höhere Transfektionseffizienzen erzielt werden, ist diese Art des Gentransfers mit Problemen, wie ungenügender Sicherheit, möglicher Immunogenität, begrenzter Transgengröße aufgrund der geringen Transportkapazität des Virus und hoher Kosten verbunden. Aufgrund dieser Nachteile ist eine Entwicklung von alternativen Transportsystemen notwendig.

Kationische Lipide und Polymere gehören zu den am häufigsten verwendeten nicht-viralen Vektoren und besitzen einige Vorteile gegenüber dem viralen System. Sie induzieren keine Immunantwort, ihre Produktion ist kostengünstiger und auch in größerem Maßstab möglich. Allerdings sind kationische Vektoren häufig mit geringer Transfektionseffizienz und Zytotoxizität verbunden.

Im Rahmen dieser Studie wurden kationische Lipoplexe auf ihren Einsatz als Vektoren in der Gentherapie untersucht und bewertet. Die Komponenten der Lipoplexe in dieser Arbeit waren das positiv geladene Lipid 1,2-Dioleoyl-3-trimethylammonium-propan (DOTAP) und das Helferlipid 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamin (DOPE) sowie messenger RNA (mRNA) als genetisches Material.

Sowohl Transfektionseffizienz als auch Zytotoxizität der DOTAP/DOPE/mRNA Komplexe wurden beurteilt, da diese beiden Faktoren den Erfolg des Transportes von genetischem Material in die Zelle bestimmen.

Erstes Ziel war die Optimierung der Herstellungsparameter. Zu diesem Zweck wurde der kationische Vektor DOTAP/DOPE in drei unterschiedlichen Verhältnissen mit einer Luciferase-kodierenden mRNA gemischt. 24 Stunden später wurde die Transfektionseffizienz in HeLa-Zellen mit Hilfe eines Luciferase Assays bestimmt. Die höchste Transfektionseffizienz wurde bei der Verwendung der geringsten Menge an kationischem Lipid erzielt. Um den Einfluss von Serum auf die Transfektionseffizienz zu untersuchen, wurden die Zellen in Gegenwart von 10 % Serum inkubiert. Die Ergebnisse zeigten, dass Serum die Transfektionseffizienz steigert. Zur genaueren Beurteilung des Einflusses von Serum auf die Transfektionseffizienz wurden drei weitere Serum-Konzentrationen getestet, und zwar 2.5, 5 und 7.5 %. Mit der geringsten Menge an positiv geladenem Lipid wurde die höchste Transfektionseffizienz bei 7.5 % Serumgehalt erreicht. Bei

Erhöhung des kationischen Anteiles wurde die maximale Transfektionseffizienz bei 5 % Serumzusatz beobachtet.

Größe ist ein weiterer wichtiger Parameter, der sich auf den Aufnahmemechanismus von Partikeln und damit auf die Transfektionseffizienz auswirkt. Die Größenbestimmung unterschiedlich zusammengesetzter mRNA Komplexe mit Hilfe von dynamischer Lichtstreuung (DLS) ergab die größten Komplexe bei der höchsten positiven Ladung. Durch Zugabe von Serum nahm die Partikelgröße ab.

Die Toxizität der Komplexe wurde durch Bestimmung der Zellviabilität nach der Transfektion ermittelt. Mit Ausnahme einer Formulierung reduzierte keines der untersuchten Transfektionssysteme die Zellviabilität signifikant und zeigte daher keine Zytotoxizität.

Um auch die Kinetik der Proteinexpression nach der Transfektion zu erfassen, wurde die Dauer der Proteinproduktion nach der Transfektion zeitlich verfolgt. Bereits 30 Minuten nach Zugabe der Komplexe konnte Luciferase-Aktivität in den Zellen detektiert werden, und hielt bis zu 3 Tagen an. Dies wurde sowohl in der Anwesenheit als auch in der Abwesenheit von Serum beobachtet.

Unsere Ergebnisse bestätigen, dass DOTAP/DOPE/mRNA Komplexe eine vielversprechende Alternative zu viralen Vektoren darstellen. Die kationischen mRNA-Lipoplexe erzielen hohe Transfektionseffizienzen und induzieren keine Zytotoxizität, womit die zwei Hauptbedenken gegenüber nicht-viralen Vektoren ausgeräumt werden konnten. Allerdings ist noch intensive Forschung notwendig, um das Ziel einer klinischen Anwendung zu erreichen.

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