CHITOSAN AND QUATERNISED CHITOSAN POLYMERS AS GENE TRANSFECTION AGENTS

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Thesis submitted for the degree

PHILOSOPHIAE DOCTOR (PHARMACEUTICS)

in the

School of Pharmacy

at the

NORTH-WEST UNIVERSITY
(POTCHEFSTROOM CAMPUS)

Promotor: Prof. A.F. Kotzé

Potchefstroom

2005

"Logic will get you from A to B.
Imagination will take you everywhere."

Albert Einstein

To my parents
Chris and Bessie Jonker

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ABSTRACT

Several approaches have been employed for directing the intracellular trafficking of DNA to the nucleus. Cationic polymers have been used to condense and deliver DNA and a few specific examples using chitosan as cationic polymer have been described. The concerted efforts in gene therapy to date have provided fruitful achievements toward a new era of curing human diseases. A number of obstacles, however, still must be surmounted for successful clinical applications.

Therefore, chitosan-plasmid and quaternised chitosan-plasmid complexes (polyplexes) were investigated for their ability to transfect COS-1 cells and the results were compared with Transfectam®/DNA lipoplexes for transfection efficiency. All of the chitoplexes utilised in this study proved to transfect COS-1 cells, however to a lesser extent than the Transfectam®/DNA lipoplexes, which served as a positive control. Complexes formed with quaternised trimethyl and triethyl chitosan oligomers, specifically TMO L and TEO L, proved to be superior transfecting agents compared to other chitosans. The molecular mass of chitosan is considered to influence the stability of the chitosan/DNA polyplex, the efficiency of cell uptake and the dissociation of DNA from the complex after endocytosis.

In literature it was shown that the toxicity of the chitosan/DNA polyplexes is relatively low compared to viral gene and lipid non-viral delivery vectors. This study showed that the percentage viable COS-1 cells when transfected with the chitosan polymers, oligomers, quaternised chitosan polymers and quaternised chitosan oligomers (chitoplexes) was higher than the percentage viable cells when transfected with lipoplexes prepared with Transfectam® with the MTT assay. The Transfectam®/DNA lipoplexes induced cell damage and a decreased viability of COS-1 cells were found. Chitosan/DNA and quaternised

chitosan/DNA complexes did not affect the viability of the cell line. The degree of quaternisation of the polymers and oligomers and molecular size proved to be two important factors when considering effective non-viral gene delivery.

It can be concluded that chitosan, especially quaternised oligomeric derivatives are polysaccharides that demonstrate much potential as a gene delivery system. The high solubility and low toxicity of chitosan allow its use in a wide variety of applications in the pharmaceutical industry and, as shown in this study, in gene delivery.

Keywords: Gene delivery; Quaternised chitosan; Oligomeric chitosan, Polyplexes; Transfectam®; Lipoplexes, COS-1.

UITTREKSEL

Verskeie benaderings kan gebruik word vir die intrasellulêre vervoer van DNA na die selkern. Kationiese polimere is reeds gebruik vir die aflewering van DNA en 'n paar spesifieke voorbeelde waar kitosaan gebruik is, is reeds beskryf. Die huidige pogings vir geenaflewering het alreeds tot suksesvolle resultate gelei en kan lei tot 'n nuwe era vir die uitwissing van siektetoestande in mense. Daar is egter nog 'n aantal struikelblokke wat oorkom moet word vir die suksesvolle kliniese toepassings daarvan.

Kitosaan-plasmied en kitosaan derivaat-plasmied komplekse (poliplekse) is ondersoek vir hul vermoë om COS-1 selle te transfekteer en die resultate is vergelyk met die transfeksie-effektiwiteit van Transfectam®/DNA lipoplekse. Al die kitoplekse wat in hierdie studie gebruik is het die COS-1 selle getransfekteer, maar in 'n baie mindere mate as die positiewe kontrole, Transfectam®/DNA lipoplekse. Daar is bewys dat komplekse wat gevorm is met die gekwaterniseerde trimetiel kitosaan oligomere en gekwaterniseerde tri-etiel kitosaan oligomere, spesifiek TMO L en TEO L, beter transfeksie lewer as ander kitosane. Daar is aangetoon dat die molekulêre massa van kitosaan en die stabiliteit van die kitosaan/DNA polipleks, die effektiwiteit van selopname en die dissosiasie van die DNA vanuit die kompleks na endositose beïnvloed.

In die literatuur is reeds aangetoon dat die toksisitiet van kitosaan/DNA poliplekse relatief laag is wanneer dit vergelyk word met virale and nie-virale lipied vektors. In teenstelling met transfeksie deur bereide Transfectam® lipoplekse het die MTT gehaltebepaling in hierdie studie getoon dat die persentasie lewensvatbare COS-1 selle hoër was wanneer dit met kitosaan polimere, kitosaan oligomere, gekwaterniseerde kitosaan polimere en gekwaterniseerde kitosaan oligomere (kitoplekse) getransfekteer is. Die Transfectam®/DNA lipoplekse induseer selskade en 'n verlaagde

lewensvatbaarheid van COS-1 selle is aangetoon. Kitosaan/DNA en gekwaterniseerde kitosaan/DNA komplekse het nie die lewensvatbaarheid van die sellyn beïnvloed nie. Die graad van kwaternisering van die polimere en oligomere en die molekulêre grootte is twee belangrike faktore wanneer daar na effektiewe nie-virale geen aflewering gekyk word.

Opsommend kan gesê word dat kitosaan, veral gekwaterniseerde oligomeriese derivate van kitosaan, polisakkariede is wat baie potensiaal toon as 'n geen afleweringsisteem. Die hoë oplosbaarbaarheid en lae toksisiteit van kitosaan bevorder die gebruik daarvan vir 'n verskeidenheid toepassings in die farmaseutiese industrie asook, soos aangetoon in hierdie studie, in geenaflewering.

Sleutelwoorde: Geenaflewering, gekwaterniseerde kitosaan, oligomeriese kitosaan, poliplekse, Transfectam®, lipoplekse, COS-1.

INTRODUCTION AND AIM OF THE STUDY

Future treatment of disease on a genetic level may well be made possible due to encouraging progress with gene delivery systems in recent years. However, gene transfer still faces major obstacles in many applications. Laboratory applications promise efficient and safe gene delivery systems, although the clinical applications remain limited due to low *in vivo* expression. Barriers/obstacles include poor delivery efficiency, high cost, time-consuming vector preparation, toxicity, immunogenicity & oncogenicity, transient transgene expression and poor expression levels. The central focus for scientists therefore remains to explore ways to improve *in vivo* gene delivery in order to enhance the expression of the gene of interest. Major challenges remain for designing gene delivery systems that are biodegradable, non-toxic and able to access only the desired target tissues.

The growing interest in using viruses for gene therapy is due to their high efficiency in gene delivery. Viral vectors allow a rapid transfection rate and a high transcription of the inserted material in the viral genome (Mansouri *et al.*, 2004:2). However, the limitations of viral vectors that include toxicity, immune and inflammatory responses, make synthetic vectors an attractive alternative for gene delivery due to better safety and stability profiles (Tomlinson and Rolland, 1996:357). Engineering, evaluation and development of alternative non-viral vector systems prove to be a great challenge for scientists. Some of the potential advantages over viral vectors are the fact that non-viral vectors are usually able to carry more DNA than viruses, allowing the delivery of larger genes, non-immunogenicity, low acute toxicity, simplicity and feasibility to be produced on a large scale.

In previous studies it was reported that chitosan is a good candidate for gene delivery because cationic charged chitosan easily forms complexes with negatively charged DNA (Mao *et al.*, 1996:402). Promising results were reported in the formation of complexes between chitosan and DNA and low cytotoxic activity was demonstrated (Roy *et al.*, 1997:674). These results suggest that chitosan has comparable efficacy without the associated toxicity of viral and other synthetic vectors and can, therefore, be an effective gene-delivery vehicle *in vivo*. The aim of this study was to evaluate the gene transfection efficacy of a range of chitosan and quaternised chitosan polymers.

The specific objectives of this study were to:

1. Conduct a literature study to select a suitable delivery vector for studying luciferase gene transfer.
2. Conduct a literature study on chitosan and quaternised chitosan polymers as non-viral gene transfection agents.
3. Describe the synthesis and characterisation of a range of quaternised chitosan polymers.
4. Isolate and purify the pGL3 plasmid and to characterise DNA/chitosan complexes for their stability with gel electrophoresis.
5. Determine the cytotoxicity of different chitosan and quaternised chitosan polymers alone and as complexes in different ratios using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on COS-1 (monkey kidney fibroblasts).
6. Evaluate the transfection efficiency of the different polymers using the pGL3 luciferase transporter gene in COS-1 (monkey kidney fibroblasts) cells.
7. Determine the best possible formulation from the ratios and different chitosan polymers tested.

Chapter 1 will provide more information on gene delivery and explain the different types of vectors available for gene delivery while chapter 2 will focus specifically

on chitosan in gene delivery. In chapter 3 the synthesis and characterisation of several quaternised chitosan polymers are documented. Chapter 4 will focus on the propagation, isolation and purification of the selected pGL3 luciferase plasmid. This chapter also contains the procedures for culturing and maintenance of the COS-1 cell culture line, the *in vitro* gene transfection experiments and toxicity experiments. All the results obtained with these studies are presented and discussed in chapter 5.

CHAPTER 1

GENE DELIVERY VECTORS

Gene therapy has been progressively developed with the hope that it will be an integral part of medical modalities in the future. The ultimate goal of gene therapy is to cure disorders in a straightforward manner by removing their causes, that is, by adding, correcting, or replacing genes and thereby offering a means of treating currently incurable genetic and acquired diseases such as cystic fibrosis and certain cancers, respectively (Huang and Viroonchatapan, 1999:4). For example, at present the most notable disease target for pulmonary gene therapy include genetic disorders arising from a single genetic defect, e.g. cystic fibrosis and neoplastic disease. However, getting DNA into the cell and nucleus, remains a crucially limiting factor that must be overcome for successful clinical applications. The main problem with the gene therapeutic approach is a lack of effective gene delivery systems.

1.1 INTRODUCTION

By definition, gene therapy requires effective gene transfer followed by adequate gene expression. Gene delivery for therapeutic benefit is a concept that holds enormous promise for treating a wide variety of conditions. Prominent examples are Parkinson's disease, haemophilia, chronic metabolic disorders, cardiovascular disease and various forms of cancer. The principle of gene therapy is very simple and involves the supplement of the body cells with the corrected copies of the malfunctioning genes. However, efficient delivery and expression of genes in the body cells (transfection) is easier said than done. Developing an efficient gene therapeutic approach and designing safe and efficient gene delivery reagents are inseparable. Shortcomings in one will adversely affect the success of the other. In other words, realization of the full

potential of gene therapy will depend, in a major way, on the future development of safe and efficient gene delivery reagents (Robbins *et al.*, 1998:35).

Currently, transport of exogenous DNA to cells can be achieved using vectors separated mainly into two categories: viral and non-viral vectors. In early clinical trials, both these biological and non-biological vectors have been used with some success particularly in cancer gene therapy (El-Aneed, 2004:1). Efficiencies of viral transfection vectors are unquestionably superior to their non-viral counterparts (McTaggart and Al-Rubeai, 2002:3). However, serious immunogenic concerns associated with the use of viral vectors were highlighted by the death of an 18-year-old gene-therapy patient (Marshall, 1999:2244). These drawbacks are increasingly making non-viral gene delivery reagents the vectors of choice for gene delivery. The non-viral systems are in general plagued by a lack of efficiency but offer flexibility and, most important, comparable safety as documented in table 1.1.

The ideal gene delivery system should be biodegradable, non-toxic and nonimmunogenic (safe), able to produce a high level of gene expression, be capable of being administered orally and suffer no degradation on storage under ambient conditions, able to access the target desired tissues or cells only and must be produced economically to pharmaceutical standards. Depending on the application, it should integrate into the genome. Some existing vectors can fulfil some of these criteria mentioned, but none can provide all of the necessary functions (Zauner *et al.*, 1998:98).

Table 1.1 Viral and non-viral gene delivery systems (Schätzlein and Uchegbu, 2001:17)

Gene delivery system	Delivers genes of a limited size	High level of sustained gene expression	Scale up problems	Safety problems
Cationic liposomes	No	No	No	No
Cationic polymers	No	No	No	No
Naked DNA	No	No	No	No
Retroviruses	Yes	Yes	Yes	Possible mutagenesis
Lentiviruses	Yes	Yes	Unknown	Possible mutagenesis
Adenoviruses	Yes	No	No	Yes
Adeno-associated viruses	Yes	Yes	Yes	Yes

1.2 DELIVERY BARRIERS FOR GENE VECTORS

Several steps are required for effective gene expression. DNA is a large polyanionic molecule which, per se, is not internalised by eukaryotic cells. Therefore, it has to be condensed to a size that allows it to be taken up into cells and the negative charges of the DNA have to be masked. To allow specific uptake and gene expression, the condensed DNA particle has to bind to a specific receptor on the surface of the target cell and be internalised. Then the particle has to enter the cytoplasm and travel into the nucleus. The DNA has to stay in the nucleus for a period of time long enough to lead to gene expression

(Wiethoff and Middaugh, 2003:208). For *in vivo* applications, DNA particles have to be able to resist the harsh environment (bloodstream, other body fluids, enzymes, etc.) presented and be able to penetrate to the tissue of interest. Furthermore, DNA particles should not lead to inflammation or other immunological responses.

The barriers that a gene delivery vector must overcome can be broadly divided into two classes, namely systemic and cellular barriers.

1.2.1 Systemic barriers

Systemic or biological barriers are the factors that hamper the specific transport of the transfection vectors to the specific organ sites following systemic administration. In the designing of efficient non-viral systems, it will sometimes be required to deliver the therapeutic genes to remote target cells via the systemic route of administration. Because the non-viral complexes will be confronted by a set of biological barriers, starting from the injection site to the target cell, including the blood compartment (serum proteins and cellular elements e.g. erythrocytes and platelets), the design of target-specific non-viral transfection vectors presents a large scientific challenge.

1.2.1.1 Stability in extracellular compartments

The stability of non-viral delivery systems in the extracellular milieu, such as intercellular or intravascular spaces, is related to the chemical stability of the DNA as well as the physical stability of the delivery system (Wiethoff and Middaugh, 2003:204).

1.2.1.2 Cellular association of DNA

The association of naked DNA with the cell surface is typically very low in the absence of any delivery agent as an immediate result of the relatively high

negative charge density of both the DNA and the cell surface. Polycations have been shown to substantially increase the cellular association of DNA by neutralisation of the DNA negative charge, with the charge ratio of the complex modulating the extent of this contact (Wiethoff and Middaugh, 2003:205; Pouton and Seymour, 2001:194).

The association of non-viral gene delivery systems containing either cationic lipids or polymers is thought to be mediated by interactions with cell surface heparin sulphate proteoglucans (HSPGs). These proteoglycans are present in the cell membranes of all cells and are involved in a variety of cellular processes, including differentiation, adhesion and migration (Yanagishita and Hascall, 1992:9452).

1.2.2 Cellular barriers

In addition to the systemic challenges described above that a complex have to confront, the design of efficient vectors also include strategies to deal with a multitude of cellular barriers presented once a complex reach a specific cell. The intracellular trafficking of the complex consists of a series of steps, including the specific binding of the vector/DNA complex to the target cell, the uptake of the complex into intracellular endosomes, trafficking in the endosome/lysosome compartment, escape or release into the cytoplasm, transport to the nucleus and decomplexation as depicted in figure 1.1.

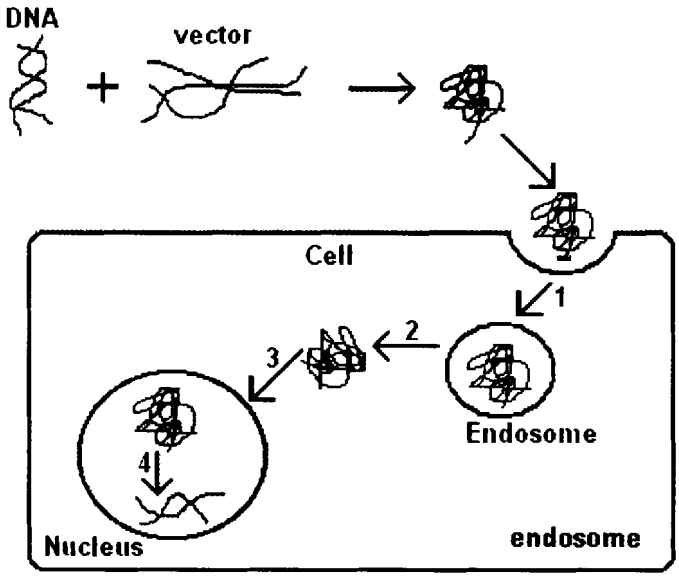


Figure 1.1 Schematic representation of the transfection process in target cells with plasmid-DNA. (1) uptake into intracellular endosomes, (2) successful release from endosomes into cytoplasm, (3) intracellular transport and nucleus localisation of DNA and (4) decomplexation (Borchard, 2001:146).

1.2.2.1 Intracellular trafficking of non-viral gene delivery systems

One of the major cellular barriers to overcome in non-viral gene delivery is the process of endosomal release of DNA into the cell cytoplasm. If the endosomes containing DNA fuse with lysosomes before endosomal release of DNA, lysosomal degradation is likely to be the fate of the DNA. It is proposed that the mechanism of endosomal escape probably relies, in one form or another, on the disruption of endosomal membranes (Felgner *et al.*, 1987:7413).

Once internalized, the intracellular vesicles carrying the vectors fuse with organelles collectively referred to as the endocytic compartment. It has been well documented that escape of the DNA from these structures is one of the major barriers to efficient gene delivery (Pouton and Seymour, 2001:194). Initially the vectors appear in vesicles known as early endosomes. An inability of non-viral vectors to escape the endosomal compartment will presumably result in

the degradation of DNA by lysosomes. Therefore, efficient endosomal escape is necessary for effective delivery and gene transfection.

1.2.2.2 Cytosolic transport of DNA

After endosomal escape, the vector must cross the cytosol to access the nucleus. It has been suggested that DNA is released from complexes with cationic lipids or cationic polymers during endosomal escape leaving DNA free in the cytosol (Xu and Szoka, 1996:5620). The metabolic instability of plasmid DNA following its endosomal release, possibly due to degradation by cytosolic nucleases, forms another important potential cellular barrier to gene transfer.

1.2.2.3 Nuclear localization of plasmid DNA

Ultimately the last major significant cellular barrier that the DNA has to confront is the barrier to nuclear trafficking. Delivery of DNA to the nucleus must occur for transcription of the transgene to take place. The mechanism of DNA nuclear translocation and whether the DNA is still associated with the delivery system are not fully understood but appear to depend on the type of delivery vehicle employed. At least three possible routes exist for DNA transport to the nucleus. Firstly, the DNA can pass into the nucleus through nuclear pores, or secondly, it can become physically associated with chromatin during mitosis when the nuclear envelope breaks down, or thirdly, it could cross the nuclear envelope. The efficiency of nuclear trafficking is very low, a probability documented is of 1 in 104 - 105 plasmids taken up by the cell that eventually gets expressed. Facilitation of DNA transport through nuclear pores is possible by modification of the DNA sequence to include binding sites for karyophilic proteins that aid nuclear pore transport. This phenomenon was first demonstrated for plasmids that contain the SV40 enhancer region, which is known to bind a variety of transcription factors (Wiethoff and Middaugh, 2003:209).

1.3 VECTORS FOR GENE DELIVERY

Several different delivery systems can be used to transfer foreign genetic material into the human body. Vectors proposed for DNA delivery systems fall into two major categories, recombinant viral and non-viral vectors.

1.3.1 Viral vectors (Biological gene delivery systems)

1.3.1.1 Introduction

Viral vectors are replication-defective viruses with part of or all of the viral coding sequence replaced by that of therapeutic genes (Ledley, 1996:1596). Viral vectors are used to exploit their natural ability to enter the cells and express their own proteins. The interest in using viruses for gene therapy is their high efficiency in gene delivery. This type of vector allows a high transfection rate and a rapid transcription of the foreign material inserted in the viral genome (Mansouri *et al.*, 2004:2). However, depending on the type of virus some of the problems arising include insufficient pharmaceutical quantities, toxicity and the potential replication of competent viruses (Oligino *et al.*, 1997:17). A few of the more important viral vectors that will be discussed below are: (1) Retrovirus, (2) Adenovirus, (3) Adeno-associated virus, (4) Herpes simplex virus and (5) other viral vectors.

1.3.1.2 Retrovirus

Retroviral vectors consist of two identical copies of single-stranded, positive-sense RNA, plus integrase and reverse transcriptase enzymes, contained in a protein shell surrounded by a lipid membrane (McTaggart and Al-Rubeai, 2002:4). Retroviruses can stably infect dividing cells by integrating into the host DNA without expressing any immunogenic viral proteins to produce long-term gene expression (Robbins *et al.*, 1998:35). In theory, the integrated retroviral

vector will be maintained for the life of the host cell, continuing to express the gene of interest. Three subgroups of the retrovirus can be identified: (1) oncoretrovirus (e.g. Moloney Murine Leukaemia Virus), (b) lentivirus (e.g. HIV) and (3) spumaviruses. The lentivirus vectors will almost certainly confront big regulatory hurdles because this therapeutic delivery system is derived from the same virus that causes AIDS (Buchschacher and Wong-Staal, 2000:2499).

1.3.1.3 Adenovirus

Adenoviruses are double-stranded DNA viruses that can infect both dividing and non-dividing cells (Li *et al.*, 1993:405). Transfection with adenoviruses is short-lived since the DNA genome does not integrate permanently into the host cell's genetic material. Therefore, repetitive administration of the adenoviral vectors is needed to obtain the desired therapeutic outcomes.

Adenovirus, one of the causes of the common cold, has been engineered to be defective of its replicating genes. Each adenovirus can quickly deliver a big genetic payload, but because of the virus' complexity, it is rapidly detected by the human immune system and eliminated. Also, at high doses it can cause a toxic immune response. Adenoviral vectors accounted for the first reported death in clinical gene therapy trails (Raper *et al.*, 2002:165).

1.3.1.4 Adeno-associated virus

Adeno-associated viruses are single stranded DNA viruses. Similar to adenoviruses, these viruses can infect both dividing and non-dividing cells. Their DNA, however, integrate into the host cell genome similar to retroviruses. Adeno-associated viral vectors pose little toxicity since their wild type version does not cause any pathologic effect in humans. The main drawback in this system, however, is the need for helper viruses (adenovirus or Herpes simplex virus) for adeno-associated virus production (Janik *et al.*, 1989:323). This may

result in contaminated adeno-associated viral vectors during preparation. Despite the fact that adeno-associated viral vectors have been used in cancer gene therapy, it has been shown that other viral systems such as adenoviruses posses better transfection ability (Ponnazhagan *et al.*, 2001:6318; Su *et al.*, 1997:13893).

1.3.1.5 Herpes simplex virus (HSV)

The HSV family naturally infects the human eye, the oral and vaginal mucosa causing lytic curable effects (El-Aneed, 2004:3). During their life cycle, they infect the sensory nerve ending and migrate to the neuronal cells resulting in a latent infection. This feature was utilized to deliver genes effectively to brain tumours (Parker *et al.*, 2000:2208). The large linear double strand genome of HSV virus (about 150kb), which is almost 15 and 4 times bigger than that of lentiviruses and adenoviruses respectively, can be replaced by almost 40kb of foreign genes, ranking at the top of viral vectors capacity (Latchman *et al.*, 2001:4). Both the original pathologic and latent infectious nature of these viruses can limit their therapeutic applications.

1.3.1.6 Poxvirus (Vaccina virus)

These viruses were used as vaccines, which eradicated smallpox worldwide. They are double-stranded DNA viruses that can infect both dividing and non-dividing cells. Similar to HSV, they have a large genome (about 186 kb) such that they can accommodate up to 25 kb transgenic sequence (Smith and Moss, 1983:24). Because of the success in recombinant vaccination via poxviruses, which can induce T cell-mediated immune reaction against infectious and malignant diseases, they were successfully tested for *in vivo* cytokine gene delivery against cancer in pre-clinical studies (Qin *et al.*, 2001:555).

Table 1.2 Summary of the main advantages and disadvantages of using various virus types for the development of vectors for gene transfer in humans (McTaggart and Al-Rubeai, 2002:4).

Gene delivery	Advantages	Disadvantages
Naked DNA	• Non-pathogenic • Low immunogenicity of DNA	• Efficiency of DNA transfer can be low • Transfection of therapeutic gene only transient
Non-viral vectors	• Non-pathogenic	• Very low efficiency of DNA transfer
Retrovirus vector (1) Oncovirus	• Integration of therapeutic gene • Highly efficient gene transfer • Wide host range • Low immunogenicity • Vectors proteins not expressed in host • Well-studied system	• Risk of insertional mutagenesis / activation of oncogenes • Difficult to target • Difficult to obtain high titre from packaging cells • Only infects dividing cells
(2) Lentivirus	• Integration of therapeutic gene • Highly efficient gene transfer • Wide host range • Low immunogenicity • Vectors proteins not expressed in host • Well-studied system • Infect nondividing cells	• Risk of insertional mutagenesis / activation of oncogenes • Difficult to target • Difficult to obtain high titre from packaging cells
(3) Spumavirus	• Integration of therapeutic gene • Highly efficient gene transfer • Wide host range • Low immunogenicity • Vectors proteins not expressed in host • Well-studied system • Large insert size	• Risk of insertional mutagenesis / activation of oncogenes • Difficult to target • Difficult to obtain high titre from packaging cells

Gene delivery	Advantages	Disadvantages
Adenovirus vector	• Infects both dividing and quiescent cells • Insertational mutagenesis unlikely • High transduction efficiency • High titre easily obtained • Relatively stable	• Expression of therapeutic gene is short-lived • Viral proteins expressed • Potentially immunogenic • Common human virus (cause disease)
Adeno-associated virus vector	• Can induce gene expression for years • Infects nondividing cells • Relatively easy regulation of inserted genes • No immunity detected	• Mechanism for transduction unclear • Unknown effects of subsequent infection of patient with adeno- or herpes virus • Small insert size (4 kb)
Herpes simplex virus (HSV) vector	• Large insert size (20-30 kb) • High titres • Episomal, but latent infection can be lifelong • Neuron specificity	• Difficult to avoid cytotoxicity • Wide tissue tropism • Lower transduction efficiency • Potential to generate infectious HSV in humans

1.3.1.7 Concluding remarks

The use of viruses in gene therapy is mostly very effective, but could be limited by various factors, such as toxicity, immune and inflammatory responses. Several safety questions have been asked about viral vectors after the death of a clinical trial patient. Gene therapy using viral vectors is also limited by the fact that only small sequences of DNA can be delivered by the viral vector and large-scale production on the manufacturing side may also be difficult to achieve. These limitations of viral vectors have led to the engineering, evaluation and development of alternative non-viral vector systems.

1.3.2 Non-viral vectors (Non-biological gene delivery systems)

1.3.2.1 Introduction

Non-viral delivery systems can be broadly divided into two categories, namely synthetic transfection particles and physical transfection vectors. Synthetic or chemical vectors resemble a classical pharmaceutical formulation in that the compounds used (e.g. plasmid DNA, polymers and lipids) are well-defined. These synthetic transfection particles involve the use of plasmid DNA complexed to synthetic carrier molecules, such as cationic lipids or polymers to deliver DNA. (Wiethoff and Middaugh, 2003:204). Physical transfection vectors involve taking plasmids and forcing them into cells through such means as electroporation, sonoporation, DNA injection and the gene gun (Zauner *et al.*, 1998:98).

The limitations of viral vectors make synthetic vectors an attractive alternative for gene delivery. Non-viral gene delivery vectors have several potential advantages over viral vectors. Non-viral vectors can usually carry more DNA than viruses, allowing the delivery of larger genes. Advantages of non-viral vectors include their nonimmunogenicity, low acute toxicity, simplicity and feasibility to be produced on a large scale. However, some drawbacks with these non-viral vectors include their lower efficiency than viral vectors in gene transfer and their transient or short-lived gene expressions.

1.3.2.2 Synthetic or chemical non-viral vectors

For non-viral vectors there is no need for replication, a characteristic of the biological viral system, therefore chemical design can explore and exploit a larger spectrum of candidate molecules. With some imaginative leads and a great deal of 'evolutionary' trial and error, two classes of synthetic vectors have been developed over the last decade. Since these are synthetic compounds, many modifications such as molecular weight alterations and ligand attachments can

be easily achieved (El-Aneed, 2004:4). These non-viral synthetic compounds, whether lipids or polymers, are all cationic, like their classical predecessors (calcium phosphate, DEAE-dextran) used for gene transfer *in vitro* (Remy *et al.*, 1998:86).

1.3.2.2.1 Lipoplexes

Liposomes or microscopic bubbles of fatty molecules (lipids) surrounding a watery interior, have long been viewed as promising biocompatible drug delivery systems because of their similarity to cell membranes. Cationic lipids have been used for the delivery of encapsulated drugs, as well as vectors for gene therapy. They are able to interact spontaneously with negatively charged DNA to form clusters of aggregated vesicles along the nucleic acid (Wheeler *et al.*, 1999:273). A distinct advantage of liposomal drug delivery systems is their ability to entrap both water-soluble hydrophilic drugs inside their watery core and water-insoluble hydrophobic drugs within their membrane bilayers.

Because of their opposite surface charge, cationic liposomes can form an overall positively charged complex with a negatively charged DNA molecule. The resulting positively charged biocompatible lipid-DNA complex formed, known as lipoplexes, do not face the electrostatic barrier faced by the naked DNA and easily get endocytosed by the cell plasma membrane. In addition, cationic liposomes also protect DNA from attack by DNases. Thus, broadly speaking, cationic transfection lipids are designed to compact DNA so that favourable interactions with the plasma membrane can occur, leading to efficient endocytosis and subsequent destabilization of endosomes.

The track record of liposomal transfection vectors is indeed encouraging. Many investigations have demonstrated powerful DNA condensing and high transgene expression properties (both *in vitro* and *in vivo*) of cationic liposomes (Porteous *et al.*, 1997:210; Perez *et al.*, 2001:211). Lipoplexes have been shown to

transfect the endothelial cells of lungs reasonably well following injection into the tail vein of mice and the airway epithelial cells following direct administration into the lungs (Brigham *et al.*, 1989:278; Alton *et al.*, 1993:135). Gene expression and appropriate physiological effects have been observed following lipoplex-mediated administration of the cystic fibrosis transmembrane receptor gene into the nose and lungs of cystic fibrosis patients. Cancer gene therapeutic strategies involving the use of cationic liposomes have recently progressed to Phase II clinical studies and such studies have shown that only brief expression of transgene is required for killing of tumour cells. The visible fruits of such intense global efforts toward developing safe and efficient cationic liposomes for use in gene therapy are a number of commercially available cationic lipid-based transfection kits (Pedroso de Lima *et al.*, 2001:278).

Despite early excitement, there are serious limitations to these cationic lipid systems, some of which are similar to those exhibited by viral vectors. Unfortunately, these cationic lipids can be highly cytotoxic *in vitro* (Brown *et al.*, 2001:18). Prolonged incubation of macrophages with cationic liposomes results in high levels of toxicity. Furthermore, they induce potent anti-inflammatory activity *in vivo* (Filion and Phillips, 1998:165).

A few of the commercially available cationic lipid vectors presented in figure 1.2 are: (1) *N*[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), (2) 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), (3) dioctadecylamido-glycyl spermine (DOGS) or Transfectam® and (4) 2,3 dioleyloxy-*N*-[2(spermine carboxaminino)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA). Besides DOTMA and DOTAP, two of the most popular cationic lipids, numerous new lipids have become commercially available for transfection purposes. DOTMA and DOTAP are both two-chained amphiphiles, whose acyl chains are linked to the propyl ammonium group (through ether and ester bonds, respectively). A direct correlation between the nature of the cationic lipids, their

ability to mediate transfection and cause cytotoxicity has been established, but the categorisation of this correlation has not yet been completely established.

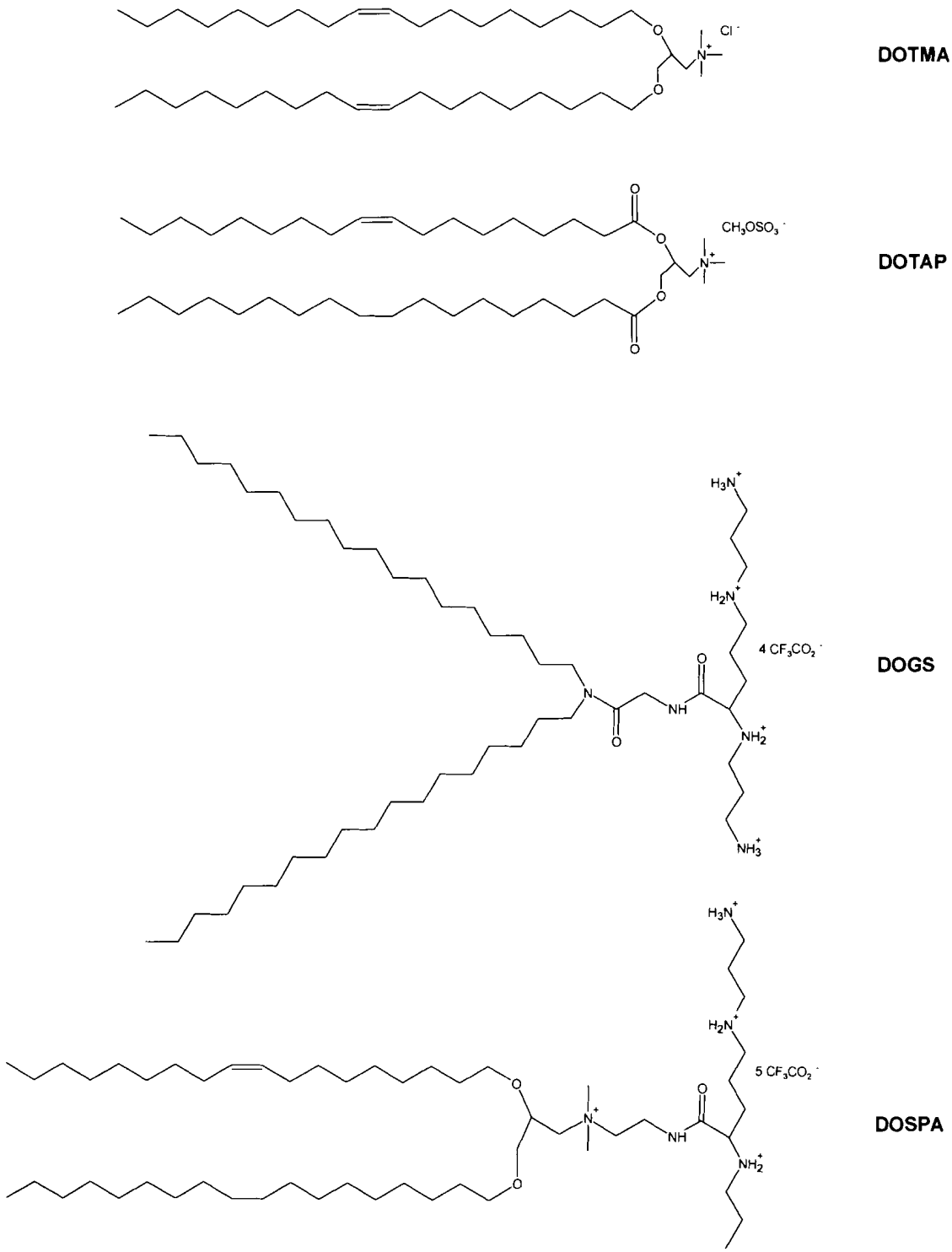


Figure 1.2 Structures of some cationic lipids used in gene therapy (Pedroso de Lima *et al.*, 2001:280).

DOSPA and DOGS, which are multivalent cationic lipids, exhibit a higher efficacy in condensing DNA than monovalent lipids (e.g. DOTMA, DOTAP). However, this property does not necessarily lead to higher transfection efficiency, since the intracellular dissociation of DNA from the complexes is expected to be more difficult (Ferrari *et al.*, 1998:341). Lipids with stable ether linkages, e.g. DOTMA proved to be more toxic than those containing labile ester linkages, e.g. DOTAP (Pedroso de Lima *et al.*, 2001:279).

1.3.2.2.2 Polyplexes

Although non-viral cationic lipid-based gene carriers are currently being clinically evaluated more than polyplexes, several arguments remain considering polyplexes as valuable candidates for gene carriers. Firstly, depending on specific therapeutic application and location, it is very likely that several types of gene carriers may be ultimately applied to humans. Secondly, while for some therapeutic applications lipoplexes are sufficiently active *in vivo*, they may fail in other applications. To illustrate this, Duncan *et al.* (1997:431) warns that pulmonary surfactants may inhibit cationic liposome-mediated gene delivery to respiratory epithelial cells. Thirdly, using viral carriers there remains the risk of an immune response to the viral particle, not allowing repeated *in vivo* transfection administration while using the same carrier (De Smedt *et al.*, 2000:113).

With regard to polymers, major attention is given to cationic polymers, which are able both to condense large genes into smaller structures and to mask the negative DNA charges. Polymers can be natural or synthetic cationic substances that form complexes with anionic DNA by electrostatic interaction. These cationic polymer/DNA complexes are known as polyplexes. The complexes normally have an overall positive charge. These polyplexes formed are taken up by cells through the electrostatic interaction with the negatively charged cell surface (Sato *et al.*, 2001:2075). The polyplexes associated with

the cell membrane are then internalized into the cytoplasm by cell surface receptors via receptor-mediated endocytosis. The properties of polymers can be easily controlled through chemical manipulations and the polymers can be designed to be biocompatible and biodegradable. Cationic polymers offer structural variability and versatility including the possibility of surface modification with targeting ligands for gene expression mediated through specific receptors. If these properties are controlled accurately the polymers would be metabolised efficiently and therefore toxicity in the body would be lowered.

Several cationic polymers e.g. poly(*L*-lysine), poly(ethyleneimine) and chitosan have been shown to from complexes with DNA and thus facilitated gene transfer because the DNA becomes better protected (Han *et al.*, 2000:303). Even neutral polymers like poly(vinylalcohol) which do not condense DNA are evaluated to protect 'naked' genes from extracellular nuclease degradation and to retain them better at the site of administration after intracellular administration (Mumper and Rolland, 1998:151).

A few of the cationic polymer vectors that will be further discussed are: (1) Poly(*L*-lysine) (PLL), (2) Poly(ethyleneimine) (PEI), (3) Chitosan, (4) Poly(2-dimethylamino)ethylmethacrylate (pDMEAMA), (5) Poly(*D,L*-lactic acid-*co*-glycolic acid) (PLGA) and (6) Polyvinylpyrrolidone (PVP).

1.3.2.2.2.1 Poly(*L*-lysine) (PLL)

Poly(*L*-lysine) (PLL) polymers are one of the first cationic polymers employed for gene transfer. They are linear polypeptides with the amino acid, lysine, as the repetitive unit (figure 1.3), therefore, they possess a biodegradable nature (Wu *et al.*, 1987:4430). PLL has a sufficient number of primary amines with positive charges to interact with the negatively charged phosphate groups of DNA. PLL/DNA complexes are prone to aggregation under physiological conditions.

Many PLL polymers with different MW were tested and evaluated for gene transfer. It has been shown that DNA condensation and transfection efficiency increased with high molecular weight PLL, which was also associated with undesirable high toxicity (Mannisto *et al.*, 2002:179). To increase the solubility and decrease the toxicity modifications to the PLL molecule has been made (Choi *et al.*, 1998:40).

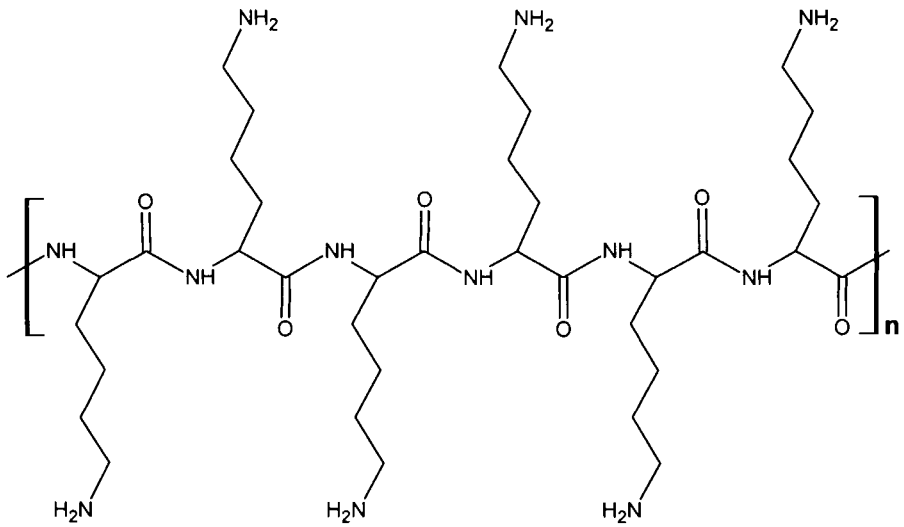


Figure 1.3 Chemical structure of PLL (Han *et al.*, 2000:304).

1.3.2.2.2.2 Poly(ethyleneimine) (PEI)

Poly(ethyleneimine) (PEI) is a cationic polymer composed of 25 % primary amines, 50 % secondary amines and 25 % tertiary amines (figure 1.4). It has been shown to effectively transfect plasmid DNA into a variety of cells both *in vitro* and *in vivo* (Boussif *et al.*, 1995:7297). PEI has a high charge density, due to every third atom on the PEI backbone being a nitrogen atom. In linear PEI, all of these nitrogen atoms are protonable, whereas in branched PEI, only two-thirds of them can be charged (Garnett, 1999:152). The homogeneous nature and small size of the PEI/DNA complex has been shown to produce high levels of gene expression in mature mouse brain.

Many factors affect the efficiency/cytotoxicity profile of PEI polyplexes such as molecular weight, degree of branching, ionic strength of the solution, zeta potential and particle size (Kunath *et al.*, 2003:114). Florea *et al.* (2002:1) evaluated PEI for its transfection efficiency in non-differentiated COS-1 (green monkey fibroblasts) and well-differentiated human submucosal airway epithelial cells (Calu-3). PEI of high MW (600-1000 kDa), medium MW (60 kDa) and low MW (25 kDa) was used and particle size, zeta potential, presence of serum proteins or chloroquine was studied. In COS-1 cells the transfection efficiency was 3 orders of magnitude more effective than in Calu-3 cells. However, some evidence of apoptosis in both cell lines was found.

It was noted that more studies is needed to produce optimum PEI carriers with respect to efficiency/toxicity behaviour (El-Aneed, 2004:5). However, PEI is not the ideal transfection agent. Delivering undamaged DNA into cells is important, but of little importance if the carrier also acts to kill the host cells. A great deal of toxicity on cellular level is observed with PEI-mediated gene delivery (Fischer *et al.*, 1999:1275; Godbey *et al.*, 1999:476).

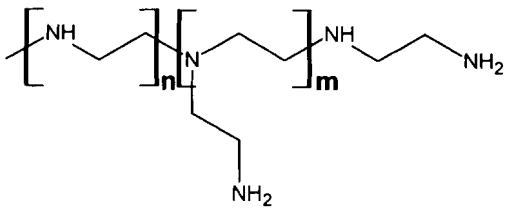


Figure 1.4 Chemical structure of PEI (Han *et al.*, 2000:304).

1.3.2.2.2.3 Chitosan

Chitosans (figure 1.5) are natural polysaccharides obtained in various molecular weights (MW) and represent a novel class of cationic carriers for gene delivery that are potentially safe, efficient and cost-effective. General lysosomes in the body degrade chitosan into a common amino sugar, *N*-acetyl glucosamine, which

is incorporated into the synthetic pathway of glycoproteins, and is subsequently excreted as carbon dioxide.

Chitosan and chitosan derivatives effectively condense plasmid-DNA, protecting it from DNase degradation. These chitosan-based gene delivery systems can also be equipped with ligands for specific cell interaction. A number of *in vitro* and *in vivo* studies showed that chitosan is one of the most suitable materials for efficient non-viral gene and DNA vaccine delivery (Mao *et al.*, 2001:418; Sato *et al.*, 2001:2079; Kiang *et al.*, 2004:5300). The potential application of this polymer in gene transfection will be described in more detail in section 1.5 and in chapter 2.

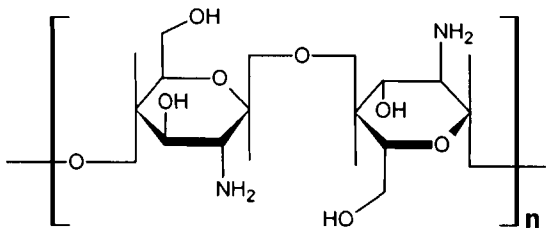


Figure 1.5 Chemical structure of chitosan (Han *et al.*, 2000:304).

1.3.2.2.2.4 Poly(2-dimethylamino)ethylmethacrylate (pDMEAMA)

Poly(2-dimethylamino)ethylmethacrylate (pDMEAMA) (figure 1.6) is a water-soluble cationic polymer that can efficiently bind and condense DNA and can mediate transfection. The optimal transfection efficiency was found at a pDMAEMA/DNA ratio of 3:1 (w/w), a ratio at which homogenous complexes of ~ 150 nm in diameter could be formed (Cherng *et al.*, 1996:1040). The transfection efficiency of the complexes was not affected by the presence of serum proteins.

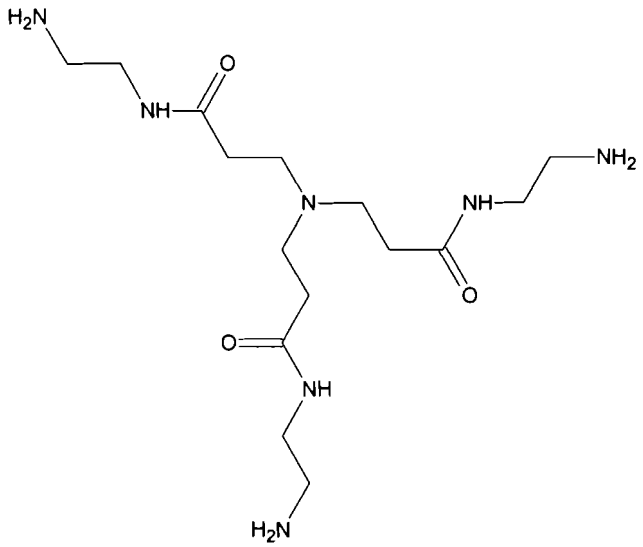


Figure 1.6 Chemical structure of pDMEAMA (Han *et al.*, 2000:304).

1.3.2.2.2.5 Poly(*D*,*L*-lactic acid-*co*-glycolic acid) (PLGA)

Poly(*D*,*L*-lactic acid-*co*-glycolic acid) (PLGA) (figure 1.7) is a commonly used biodegradable and biocompatible polymer. Nanospheres were prepared containing plasmid DNA using a water-in-oil-in-water (W/O/W) double-emulsion and solvent evaporation method. PGLA microspheres have been shown to protect DNA from degradation by nuclease (Wang *et al.*, 1999:11).

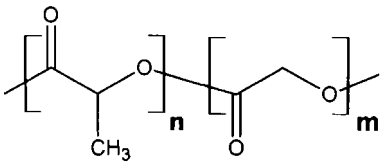


Figure 1.7 Chemical structure of PLGA (Han *et al.*, 2000:304).

1.3.2.2.2.6 Polyvinylpyrrolidone (PVP)

Noncondensing polymers, such as polyvinylpyrrolidone (PVP) (figure 1.8) are amphiphilic molecules, having both a hydrophilic and a hydrophobic portion. The hydrophilic portion of these polymers may interact with plasmid DNA by hydrogen bonds, van der Waals interactions, and/or by ionic interactions (Han *et al.*, 2000:306). These interactions between the polymer and the DNA protects the DNA from nuclease degradation and facilitate its cellular uptake via hydrophobic interactions with cell membranes.

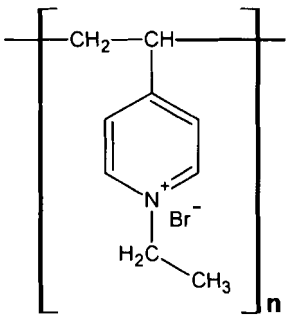


Figure 1.8 Chemical structure of PVP (Han *et al.*, 2000:304).

1.3.2.3 Physical non-viral vectors

Physical transfection techniques, such as electroporation suffer from poor viability of transfected cells, whereas direct injection of plasmid DNA appears to be limited only to muscle tissue (Zauner *et al.*, 1998:98). These physical methods, categorised as part of non-viral gene delivery vectors involve taking plasmids and forcing them into cells through such means as electroporation, sonoporation, or using a gene gun.

1.3.2.3.1 Electroporation (EP)

Electroporation (EP) involves the application of short, controlled electric pulses to target tissue, and has been shown to significantly improve transfection of skin cells (Zhang *et al.*, 2002:4). EP permeabilises cell membranes by forming transient aqueous pathways across these membranes. These pathways, along with other membrane changes allow DNA molecules that cannot normally penetrate the cell membrane to enter the cell for short periods of time without losing integrity. Transfected cells after EP are primarily located in the dermis and some transfected cells are also observed in the draining lymph nodes (Peachman *et al.*, 2003:238). EP has been used as a research tool in molecular biology since the early 1980's, but only recently has it been developed for potential medical applications.

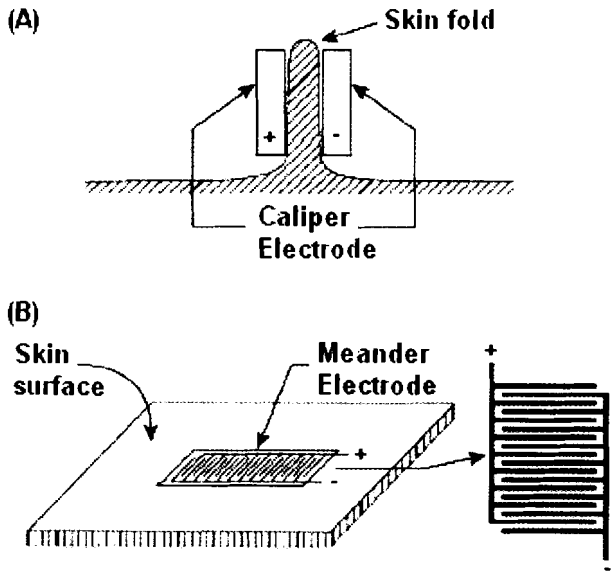


Figure 1.9 Caliper and meander electrodes that are both applied topically (Zhang *et al.*, 2002:3).

EP can be achieved by using calliper electrodes or meander electrodes (figure 1.9). The calliper electrode squeezes the skin into a skin fold between two metal plates, and the uniform electrical field is applied to this skin fold. The meander

electrode is a gentler, more patient friendly device that is placed on the surface of the skin. The electrical field is generated between each positive and negative electrode, with a component of the field penetrating into the skin. This flat, flexible electrode can be made to adhere and conform to the specific shape and area of skin to be treated.

An advantage of EP is the broad transfection efficiency of a variety of cells, including keratinocytes. One disadvantage of ER is DNA stability. The use of DNase inhibitors caused increased transfection of the gene of interest, suggesting that DNA introduced by this technique in the absence of DNase inhibitors may not be stable (Peachman *et al.*, 2003:238).

1.3.2.3.2 Sonoporation

DNA transfer *in vivo* can be accomplished with high-level therapeutic ultrasound. One effect of shockwave ultrasound, is sonoporation (transient permeabilised with resealing) of cells by acoustic cavitation. This allows large molecules, which are normally excluded by the cell membrane, to become trapped inside surviving cells. The ability of ultrasound to load large molecules into cells with subsequent survival opens the possibility of DNA transfer and expression of foreign gene products.

Although the primary effect of ultrasound exposure *in vitro* is cell lysis induced by ultrasonic cavitation (Miller *et al.*, 1996:1131), sublethal damage may also occur with passage of large molecules. Ultrasound treatment in a 20 kHz cell disruption apparatus has been reported to induce a transient permeabilisation of cell membranes *in vitro*, which leads to the uptake of external molecules into cells (Bao *et al.*, 1997:953). Fluorescent-labeled dextrans, which are normally not taken up by cultured cells, were loaded into the cells by shock wave exposure in the presence of the molecules (Gambihler *et al.*, 1994:267).

1.3.2.3.3 DNA injection

The simplest physical non-viral gene delivery system simply utilises naked DNA. Naked (free) DNA has been injected directly into certain tissues, particularly muscle, and shown to produce gene expression. The simplicity of this approach has led to its inclusion into a number of experimental protocols.

Although direct injection of DNA has been shown to lead to gene expression, the overall level of expression is much lower than with either viral or liposomal vectors. Furthermore, naked DNA is also unsuitable for systemic administration due to the presence of serum nucleases. Therefore, direct injection of DNA seems to be limited to tissues that are easily accessible to direct injection such as skin and muscle (Mansouri *et al.*, 2004:2).

1.3.2.3.4 Gene gun

Particle-mediated gene transfer - biolistics - has been used for transferring genes into plants for many years, but only recently has this technique been modified for use in mammalian cells. Today, there are two types of gene gun models in use: the in-chamber type, which needs a vacuum for transfection, and the hand-held gene gun, which requires no vacuum. With the in-chamber gene gun, the target sample is placed in a small bombardment chamber, the overlying air is removed with a vacuum pump, and the microcarriers coated with the gene of interest are fired into the target. This type of device has been reasonable successful, although it has a number of serious drawbacks including frequent cell damage (many biological materials are sensitive to vacuum) and the use of a small bombardment chamber, which restricts the application to small, *in vitro* samples. The hand-held gene gun can be used for a much wider range of applications including cultured cells, tissues, and whole animals (Barry and Johnston, 1997:788). These hand-held devices have been shown to be effective in

transfecting tissues, but again there is the problem of cell damage as high gas pressures are required for effective transfection (O'Brien and Lummis, 2002:14).

The particle-mediated gene gun technology uses compressed helium to propel micrometer-sized colloidal gold particles coated with plasmid through the stratum corneum, where the particles lodge in the epidermal and dermal layers of the skin (Peachman *et al.*, 2003:237).

Advantages include that small quantities of DNA are required to transfect a broad variety of cells. Gene gun immunizations have a 10 to 100-fold higher expression level of the DNA-encoded protein than regular intra-muscular vaccinations (Barry *et al.*, 1997:788). A disadvantage of the technique is the frequent and multiple sites required per immunization for eliciting immune responses.

1.3.2.4 Concluding remarks

Non-viral vectors can be though of as an attempt to create synthetic viruses that are engineered to exploit the advantages of viral carriers but without their drawbacks. Several recent developments in non-viral gene delivery have been examined and it is clear that much work still need to done for a viable option for treating conditions with non-viral gene vectors. Toxicity, transfection efficiency and host response remain some of the problems for gene delivery applications. However, non-viral gene vectors may become a valuable method for safe and effective gene therapy in future.

1.4 CHARACTERISTICS OF NON-VIRAL VECTORS

1.4.1 Self-assembly process

Through a process of condensation, DNA molecules organise into highly ordered structures at higher concentrations or upon the addition of chemical agents, such as multivalent cations, alcohols, basic proteins, cationic liposomes, to DNA. The complex of cationic liposomes and/or cationic polymers with DNA can be employed to deliver and transfer genes into target cells. The process of complex formation is governed by multiple types of weak molecular forces including ionic, H-bonding and hydrophobic forces (Huang and Viroonchatapan, 1999:9).

1.4.2 Interactions with DNA

Since DNA is negatively charged, both cationic polymers and liposomes will form a complex with DNA through charge interaction. The interaction between DNA and cationic polymers and liposomes is inadequately understood and it is therefore difficult to control the size and distribution of the complex produced. Electron microscopy further illustrates that at a 1:1 lipid/DNA ratio, approximately half of the DNA molecules are bound to liposomes. When the liposome concentration is increased, all of the DNA is covered by the lipids. At low liposome/DNA ratios, the liposome/DNA complex is nearly spherical; but at high ratios, the liposome/DNA complex turns into smooth rod-like structures. These structures contradict the popular belief that the liposomes bound the DNA on their surface while maintaining their original size and shape (Huang and Viroonchatapan, 1999:9).

1.4.3 Target specificity

Mammalian cell surfaces are coated with negatively charged sialic acid and these anionic biological surfaces can be targeted through charge interaction by a cationic delivery system. Among the different methods of targeting, attachment of a ligand on the surface of a vector is the most efficient method of delivery to a selective site. Both antibodies and low molecular weight ligands have been used to target vectors to cell surface receptors (Huang and Viroonchatapan 1999:10).

1.4.4 Stability

Li *et al.* (1999:589) reported that cationic lipid vectors become negatively charged, significantly increase in size and aggregate to each other after exposure to mouse serum. The rate of vector disintegration depends on the lipid composition of the vector. Incorporating dioleoylphosphatidylethanolamine (DOPE) into the composition accelerates the rate of vector disintegration. To prevent aggregation, the surface of the complex can be modified with polyethylene glycol which also decreases the interaction of the complex with the blood components (Huang and Viroonchatapan 1999:11).

1.5 STRATEGIES TO IMPROVE GENE TRANSFECTION EFFICACY WITH CATIONIC POLYMERS

1.5.1 Chitosan/DNA complexes

Non-viral chitosan-DNA complexes with a mean size smaller than 100 nm and a homogenous distribution of DNA were prepared by Mansouri *et al.* (2004:4). Larger chitosan-DNA nanoparticles ranging from 20 to 500 nm have been prepared by Illum *et al.* (2001:83). The smaller size complexes have the advantage of entering the cells through endocytosis and/or pinocytosis, and thereby increasing the transfection rate. The lack of toxicity of chitosan when

compared to cationic lipids such as lipofectamine was also confirmed (Sato *et al.*, 2001:2075; Thanou *et al.*, 2001:153). However, one major pitfall of these *in vitro* studies is the lack of reproductibility *in vivo*.

1.5.2 Modified chitosans for gene therapy

1.5.2.1 Lactosylated chitosan

Chitosan and lactosylated chitosan vectors were investigated by Erbacher *et al.* (1998:1332) for their transfection efficiencies *in vitro*. The transfection efficiency of chitosan was compared to PEI in HeLa cells in the presence of 10 % fetal calf serum. This study proved that the transfection efficiencies obtained from the two vectors were comparable. However, in another study it was found that these vectors were not efficient in transfecting galactose-specific membrane lectin (HepG2 cells). The addition of chloroquine, (a weak base preventing lysosome acidification) did not improve transfection efficiency (Pouton *et al.*, 1998:289).

1.5.2.2 Galactosylated chitosan-graft-polyethylenglycol (GCP)

Galactosylated chitosan-graft-polyethylenglycol (GCP) have been used as a DNA vector and the complexes formed between GCP and DNA proved to be small with a minimum size of about 27 nm (Park *et al.*, 2001:349). The GCP/DNA complexes were stable and were protected against enzyme degradation by DNase. The transfection efficiency of these complexes, however, was very low, mainly because of plasma interactions which led to dissociation of the GCP/DNA complexes. Gao *et al.* (2003:57) proved that galactosylated-low molecular weight chitosan (Gal-C_{LMW}) complexed with DNA were more effective in transfecting HepG2 cells than naked DNA, high molecular weight chitosan (C_{HMW})/DNA and C_{LMW}/DNA complexes, but that the highest transfection efficiency was achieved with lipofectin/DNA complexes. The C_{HMW} and C_{LMW} were found to be less toxic than lipofectamine in HepG2 cells.

1.5.2.3 Quaternisation of oligomeric chitosan

Thanou *et al.* (2001:153) prepared *N*-trimethylated chitosan oligomers (TMO) in another approach to increase the transfection rate using chitosan as vector. Two different TMO polymers were synthesised with different degrees of trimethylation. These polycations were tested for their efficacy in transfection COS-1 (kidney) and Caco-2 epithelial cells. At both the 2:100 and 2:10 DNA/oligomer ratios, the TMO polymers formed smaller complexes compared to unmodified chitosan oligomers. The transfection efficiency proved to be substantially lower in Caco-2 cells than in COS-1 cells. The authors noted that these results proved that differentiated Caco-2 cell cultures were resistant to transfection, compared with early stage dividing cultures. A similar observation was made by Uduehi *et al.* (1999:1805).

1.5.2.4 Chitosan/DNA/ligand complexes

The transferrin receptor was one of the first receptors to be targeted in an approach to increase transfection rate by using ligands coupled to polymer/DNA. The number of transferring molecules attached to each nanoparticle surface varies according to the molecular weight of the polymer (Sato *et al.*, 2001:2075). Compared to non-modified chitosan, the methods results in a fourfold enhanced transfection efficiency, depending on the cell line. Transfection was also increased when chloroquine was added.

1.5.2.5 Deoxycholic acid modified-chitosan vector

Lee *et al.* (1998:215) proposed to hydrophobically modify chitosan with deoxycholic acid. Deoxycholic acid is the main component of bile acid and since bile acid can assemble in water, the deoxycholic acid-modified chitosan also self-associates to form complexes. Hydrophobically modified chitosan containing 5.1 deoxycholic acid groups per 100 anhydroglucose units was synthesized. The

formation and characteristics of self-aggregates formed with hydrophobically modified chitosan were studied by fluorescence spectroscopy and a dynamic light scattering method. The transfection efficiency of the complexes in COS-1 cells obtained was enhanced when compared to naked DNA, but lower to that achieved by Lipofectamine.

1.6 CONCLUSION

Gene therapy is a concept that can change the world of medicine as it is known today. Parkinson's disease, cardiovascular disease and cancer are just a few examples of disease that may someday be erased by gene therapy. Developing safe and effective gene delivery agents is one of the most important factors that impair this therapeutic treatment.

None of the current vectors used in gene delivery fulfil all the requirements for gene delivery vectors. Viral or non-viral vectors are the two delivery systems currently employed for gene delivery. The viral vectors are very efficient vectors but their use is limited due to factors such as toxicity and immune responses. Non-viral vectors express lower toxicity and non-immunogenicity, but transient gene expressions hamper their effectivity. Lipoplexes were one of the first non-viral vectors to be explored and as a success a few cationic lipid-based transfection kits are currently commercially available. Polyplexes from cationic polymers also proved to be valuable candidates for gene therapy. Several cationic polymers have shown to facilitate gene transfer due to better protected DNA.

Different strategies have been used to improve transfection efficacy with cationic polymers. A few specific examples using chitosan as cationic polymer have been described. Furthermore, gene vectors also need to overcome different barriers to delivery. For DNA to be expressed by a cell, the cell must endocytose the DNA, passes it along the cytoplasm and allows passage into the nucleus for

a period of time. Taken together, the complex machinery possessed by viruses to overcome the cellular and systemic barriers, more and more inclusion of viral-mimicking structural components are likely to be found in the future design of gene vectors to ensure the clinical success of non-viral gene therapy.

The concerted efforts in gene therapy to date have provided fruitful achievements toward a new era of curing human diseases. A number of obstacles, however, still must be surmounted for successful clinical applications. For that purpose, investigators from various areas of expertise are dealing with the problems, and the dream of exploiting gene therapy for medical modalities should become reality in the near future.

CHAPTER 2

CHITOSAN IN GENE DELIVERY

2.1 INTRODUCTION

Chitosan as a delivery system for gene therapy was first described by Mumper *et al.* (1995:178). Chitosan is a biodegradable mucopolysaccharide of two units (D-glucosamine and *N*-acetyl-D-glucosamine) linked together by β(1,4) glycosidic bonds (MacLaughlin *et al.*, 1998:259). The amino group has an intrinsic pK_a value of 6.5 and therefore chitosan behave as a polycation at acidic and neutral pH values (Schipper *et al.*, 1996:1686).

Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is almost never complete (Ravi Kumar, 2000:1). Chitin is a material found in abundance in shells of crustacea such as lobsters, prawns and crabs. Chitin is also found in some microorganisms and in yeast and fungi (Illum *et al.*, 1994:1186) and chitosan is available in a range of molecular weights.

Chitosan possesses certain properties that make this polymer a potentially useful system for DNA delivery. Chitosan is generally regarded as non-toxic (Illum *et al.*, 1994:1186) and biocompatible. General lysozymes in the body degrade chitosan into *N*-acetyl glucosamine, a common amino sugar, which is ultimately excreted as carbon dioxide. Chitosan salts can bind strongly to negatively charged materials such as cell surfaces and mucus (Illum *et al.*, 2001:83). Mucus contains mucins that have different chemical constitutions but contain significant proportions of sialic acid. At physiological pH, sialic acid carriers a net negative charge and, as a consequence, mucin and chitosan can demonstrate strong electrostatic interaction when in solution. Ligands may be conjugated to the chitosan/DNA microspheres to stimulate receptor mediated endocytosis and to target specific cells or tissues (Mao *et al.*,1996:401).

2.2 PREPARATION OF GENE DELIVERY SYSTEMS BASED ON CHITOSAN

The development of new carrier systems for gene delivery represents an enabling technology for treating many genetic disorders. However, as explained in chapter 1 a critical barrier for successful gene therapy remains the formulation of an efficient and safe delivery vehicle. Non-viral delivery systems have been increasingly proposed as alternatives to viral vectors owing to their safety, stability and ability to be produced in large quantities (Tomlinson and Rolland, 1996:357). Several approaches have been employed for directing the intracellular trafficking of DNA to the nucleus. As such, promising results were reported in the formation of complexes between chitosan and DNA (Mao *et al.*, 1996:402). Although chitosan increases transfection efficiency, the addition of appropriate ligands to the DNA/chitosan complex seems to achieve a more efficient gene delivery via receptor-mediated endocytosis (Murata *et al.*, 1997:105). Furthermore, incubation of cells with chitosan demonstrated low cytotoxic activity (Roy *et al.*, 1997:674). These results suggest that chitosan has comparable efficacy without the associated toxicity of other synthetic vectors and can, therefore, be an effective gene-delivery vehicle *in vivo*.

2.2.1 Preparation of chitosan/plasmid complexes

Complex coaservation is a process of spontaneous phase separation that occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution. The electrostatic interaction between the two species of macromolecules result in the separation of a coacervate (polymer-rich phase) from the supernatant (polymer-poor phase). This phenomenon can be used to form microspheres and encapsulate a variety of compounds (Leong *et al.*, 1998:184). In order to increase the phase separation, a coaservation agent (Na_2SO_4) is sometimes used. In a study by Romøren *et al.* (2003:125) the same method was used, without a coaservation agent and larger compounds were formed. Mao *et al.*

(2001:401) have performed similar experiments, however, they did not observe significant influence when they increased the content of Na_2SO_4.

Thanou *et al.* (2001:155) diluted the respective chitosan to the specific concentration in cell culture medium, either with or without serum. The RSV (Rous Sarcoma Virus promotor)-α3 luciferase plasmid used was in an aqueous solution. For preparation of the different ratios of chitosan to plasmid chitoplexes specific quantities of polymer solution were added to the aqueous solution containing the plasmid. The mixed solutions were vortexed and shaken at ambient temperature.

MacLaughlin *et al.* (1998:261) dissolved each chitosan oligomer or polymer in an acetic acid solution by sonicating it for 30 minutes. Once in solution water was added to produce a specific concentration, which was then sterile filtered through a 0.2 µm filter. The required volume of chitosan solution was added to an aqueous suspension containing the plasmid. The solution was vortex and left at ambient temperature for the complexes to completely form.

2.2.2 Complex size and charge

Mumper *et al.* (1995:178) described self-assembling of polymeric and oligomeric chitosan/DNA complexes by mixing a solution of the respective chitosan with plasmid DNA. The DNA/chitosan complex sizes varied from 150 to 500 nm and were found to depend on the molecular weight of the chitosan (108, 230 or 540 kDa) used, and not on saline, different buffers added or the presence of sugars. As the molecular weight of chitosan decreased, smaller, more monodispersed particles were formed. Toroid formation of the DNA/chitosan was shown with transmission electron microscopy (TEM) and it was proposed that this structure may promote endocytosis of the DNA/chitosan complex.

MacLaughlin *et al.* (1998:265) used chitosan with MW of 102, 230, 540 kDa respectively and supported Mumper's discovery. They concluded that a general trend showed that a decrease in the size of the complexes occurred as the molecular weight of the chitosan decreased. It was also noted that although it may be expected that a higher MW chitosan can interact with, and therefore condense the plasmid more efficiently than a lower MW chitosan, this is outweighed by the fact that higher MW chitosan is less soluble. As a result an increase in the particle diameter or even aggregation may occur. Complexes of 100 to 500 nm in size were reported. Plasmid concentration and charge ratio also influenced the complex diameter.

Other authors also reported that the complex size showed dependence on the chitosan/DNA ratio. The size was inversely proportional to the amount of chitosan added (Thanou *et al.*, 2001:156).

The chitosan backbone of glucosamine units shows a high density of amino groups, and requires pH values below 6 to be soluble. At physiological pH, not all of the amino groups are protonated. Chitosan/DNA ratios in complexes are therefore better expressed as N/P ratios (number of polymer nitrogen (N) per DNA phosphate (P)).

Erbacher *et al.* (1998:1334) found an increase in the complex size ranging from 1 to 5 μm at a nitrogen/phosphate (N/P) ratio of 2. This coincided with a zeta potential of the complexes close to 0 mV, indicating full retardation of plasmid DNA and aggregation of complexes as neutral complex charges. It was also noted that donut or rod shapes of chitosan/DNA complexes were visible with TEM.

Chitosan, hydrophobically modified by binding of deoxycholic acid, yielded spherical self-aggregates with a mean diameter of 160 nm (Lee *et al.*, 1998:213).

Mao *et al.* (1996:401) prepared nanospheres of chitosan/DNA by using a modified complex coaservation method yielding particle sizes ranging from 200 to 500 nm. Measurements of zeta potential of the nanoparticles were performed by laser Doppler anemometry and the zeta potential of the nanoparticles at various pH-values were recorded. This study examined the influence of several parameters on the size of DNA-chitosan nanoparticles. A range of sodium sulphate concentrations (2.5-25 mM) did not have an effect on particle size. A pH-value of 5.0 to 5.8 for the buffer, and temperature of the solution above 50 °C resulted in chitosan-DNA nanoparticles with the least aggregation (Mao *et al.*, 2001:402). Particle sizes between 150 and 250 nm were obtained at N/P ratios between 3 and 8 when particles were prepared at a temperature of 55 °C and pH of 5.5. It was observed that the size of the nanoparticles depended on the N/P ratios while the incorporation of plasmids of various sizes (5.1, 5.2, 6.6 and 11.9 kb), proved to have no influence on the particle sizes of the nanoparticles. The zeta potential at the complexation pH (~5.7) was approximately +12 to +18 mV. The zeta potential of the nanoparticles only increased slightly with an increase in the N/P ratio (Mao *et al.*, 2001:408).

Kiang *et al.* (2004:5294) used 3 chitosans with different MW (138, 209, 390 kDa) and degrees of deacetylation (61.7 – 93.2 %) and no significant differences in particle size were obtained. In testing a selection of formulation variables (e.g. MW, degree of acetylation of chitosan, pH and ionic strength) other authors also concluded that MW was not found to have significant influence on the particle size (Romøren *et al.*, 2003:125).

2.2.3 DNA loading

The electrostatic nature of interaction between chitosan and plasmid DNA can be examined by competitive binding studies using ethidium bromide (EtBr) as DNA stain (Mao *et al.*, 2001:401). When chitosan is added to a solution of EtBr-stained DNA, fluorescence decrease as a result of the competitive binding of

cationic chitosan to DNA. Leong *et al.* (1998:190) used confocal laser scanning microscopy in combination with EtBr staining to determine the distribution of DNA in chitosan nanoparticles. It was observed that the DNA was uniformly distributed in the nanospheres.

The extent of DNA complexation into chitosan nanoparticles can be determined by various methods. Gel electrophoresis was used by Mumper *et al.* (1995:178) to determined the loading efficiency indirectly by demonstrating the absence of free DNA after formation of complexes. Mao *et al.* (2001:402) determined non-entrapped DNA in the supernatant after sucrose gradient centrifugation by staining with Hoechst 33258 dye and a fluorescence measurement. The concentration of free chitosan in the supernatant was detected quantitatively by a ninhydrin assay. The amount of chitosan entrapped in the nanoparticles was also calculated from this measurement.

Another method for determining DNA loading is based on the PicoGreen® assay after the nanospheres were digested with chitosanase and lysozyme (Leong *et al.*, 1998:186). Usually, loading efficiencies of more than 95 % are reported. A result of encapsulation is the shift of DNA conformation from the supercoiled to the relaxed state, as detected by gel electrophoresis.

The release of plasmid DNA from chitosan microparticles in phosphate buffered saline (PBS, pH 7.4) at 37 °C was measured by Özbas-Turan *et al.* (1999:645). The DNA released was detected photometrically at 260 nm. A significant initial burst effect was observed followed by a sustained release of DNA from the microparticles over a period of 50 days. The authors concluded that their *in vivo* transfection studies showed that the chitosan microparticles used were capable of mediating efficient *in vivo* gene transfer.

Romøren *et al.* (2003:118) quantatively determined the association between chitosan and plasmid with PicoGreen® and reported that only the charge ratio

could be distinguished as an important formulation variable for the association efficiency. They concluded that the methods used may probably not be specific enough for formulation studies.

2.2.4 Stability of chitosan/DNA complexes

As described in chapter 1, DNA delivered by non-viral carriers is vulnerable to degradation by DNase. It is therefore critical for the carrier material to have the ability to protect the incorporated DNA against degradation by DNase. As physiological concentrations of the enzyme can merely be estimated, protection against DNase is routinely checked by incubation of the chitosan/DNA complexes or nanoparticles with DNase I or II as a model enzyme at different concentrations, followed by gel electrophoresis (Borchard, 2001:147).

The effect of protection to plasmid DNA was examined using 25 U/ml of a model enzyme (DNase I) at 37 °C. After an incubation period of 15 minutes naked plasmid DNA showed significant degradation whereas the plasmid DNA recovered from nanoparticles after the same treatment remained intact (Mao *et al.*, 2001:409). At higher DNase I concentrations (250 U/ml) plasmid DNA was completely degraded during the 15 minute incubation period, while the degradation of plasmid from nanoparticles was also evident, but a significant portion of non-degraded plasmid remained. It was concluded that at physiological conditions, where the nuclease concentration is markedly lower than the tested concentration, such a formulation should render a significant protection of the plasmid.

Studies have also shown that crosslinked chitosan/DNA nanoparticles stored in water remained stable for more than 3 months, whereas non-crosslinked nanoparticles stored in PBS or serum culture media remained stable for only a few hours. However, non-crosslinked gelatin-DNA nanospheres would spontaneously dissociate under the same conditions (Leong *et al.*, 1998:187).

Lyophilised chitosan/DNA nanoparticles with ligands prepared by Mao *et al.* (1997:671), could be easily resuspended and maintained its transfection potency for at least one month in storage.

Complexation of DNA with chitosan fractions (MW of < 5000 Da, 5000-10 000 Da and > 10 000 Da) at all charge ratios formed (1:1, 1:0.1 and 1:0.05) resulted in a significant decrease in degradation by DNase II. At a charge ratio of 1:1 (chitosan:DNA) almost complete inhibition of degradation by DNase II was observed, while at a charge ratio of 1:0.05 80 % less degradation was observed (Richardson *et al.*, 1999:236). In summary, chitosans are able to protect DNA against degradation more efficiently, probably by a change in the tertiary DNA structure (complexation-induced) that is causing a steric hindrance.

2.2.5 Evaluation of cytotoxicity

A 3-[4,5-Methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used by Richardson *et al.* (1999:234) to determine cell viability. The cells were incubated with polymer before the addition of the MTT in PBS. After this incubation period with MTT, the cells in suspension were centrifuged and the media was removed. Dimethylsulphoxide (DMSO) was added and the cells were left for a period. Absorbance was then read and the results were expressed as percentage viability with respect to control cell cultures with no polymer added. It was found that the purified, low molecular mass fractions of chitosan were non-toxic to L132 human embryonic lung cells or CCRF-CEM human lymphoblastic leukaemia cells up to concentrations of 500 μg/ml. In contrast, poly(L-lysine) was toxic in all concentrations above 50 μg/ml. The authors concluded that their chitosan fractions were suitable for intravenous administration.

Thanou *et al.* (2001:155) also evaluated cytotoxicity with the MTT assay. The DOTAP cationic lipid used induced serious cell damage and decreased viability of COS-1 and Caco-2 cell lines to 50 to 57 %, respectively. Chitosan and

trimethylated chitosan oligomers did not affect the viability of both cell lines; instead an increase of mitochondrial activity compared to the controls was noted.

Viability of A549 human lung carcinoma cells, incubated with the DNA/chitosan complexes at the experimental conditions employed in a study by Sato *et al.* (2001:2079), was more than 90 % as determined by MTT assay. Cell morphologies observed by optical microscopy also showed that the cytotoxicity of chitosan was quite low. These results suggest that chitosan is a low toxic and biocompatible gene transfer reagent. Because of low cytotoxicity of chitosan, it was possible to increase the dose of the DNA/chitosan complex and this resulted in high transfection efficiency. While investigating these reports about cytotoxicity and the biological effect of chitosan, we concluded that chitosan is a non-toxic, useful system for drug-delivery.

2.3 TRANSFECTION STUDIES WITH CHITOSAN AND CHITOSAN DERIVATIVES

Several transfection studies involving chitosan derivatives have been reported in the last few years. Chitosans polymers have been investigated for their ability to condense and deliver plasmid DNA in COS-1, which are routinely used for screening transfection studies (Borchard, 2001:148).

In one specific study 1:2 (-/+) complexes were chosen for *in vitro* evaluation. It was hypothesised that if the affinity of the chitosan for plasmid was critical in the transfection procedure, the transfection efficiency would be influenced by complexing plasmid with different MW chitosan oligomers and polymer. In the absence of serum, the highest transfection level produces by the complex were produced by the complex made with 102 kDa chitosan. Lipofectamine® which was used as positive control expressed a 250-fold higher transfection. Very low expression was observed with the 540 kDa chitosan (MacLaughlin *et al.*, 1998:269).

Another study prepared trimethyl chitosan oligomers (TMO) of 40 and 50 % degrees of quaternisation respectively (Thanou *et al.*, 2001:156). These oligomers were examined for their potency as DNA carrier systems in 2 cell lines, COS-1 and Caco-2. In this study it was proved that TMO is superior to oligomeric chitosan in transfecting COS-1 cells. It was also shown that none of the complexes used (chitosan/DNA, TMO/DNA or DOTAP/DNA) were able to increase transfection efficiency in differentiated Caco-2 cells.

Chitosan and lactosylated chitosan vectors were prepared and investigated for their transfection efficiencies *in vitro* (Erbacher *et al.*, 1998:1336). In this study, the transfection efficiency of chitosan in HeLa (human cervix epitheloid carcinoma) cells in the presence of serum was found to be comparable to that of another cationic polymer, polyethyleneimine (PEI).

2.4 CHITOSAN AS DELIVERY SYSTEM FOR VACCINES

Chitosan salts can bind strongly to negatively charged materials such as mucus and cell surfaces. At physiological pH, sialic acid carries a net negative charge and, as a consequence, mucin and chitosan can demonstrate strong electrostatic interaction in solution. When compared to a labeled saline solution in the nasal route it was evident that chitosan behaves as a bioadhesive material increasing the half-time of clearance significantly (Illum *et al.*, 2001:83). This mucoadhesive properties of chitosan and its ability to modulate tight junction and increase paracellular transport (Kotzé *et al.*, 1997:1200), make it an ideal candidate for the delivery of DNA to mucosal tissues. It has been shown that chitosan has a great potential for complexation with negatively charged DNA plasmids, due to its cationic characteristics, thereby forming nanoparticulate system for the improved delivery of DNA (Mao *et al.*, 2001:401; MacLaughlin *et al.*, 1998:261).

2.4.1 Nasal influenza vaccine

Influenza is a specific acute viral respiratory disease characterised by fever, weakness, myalgia, cough, headache, malaise, pharyngitis, chills and nasal discharge. It usually occurs as an epidemic in winter (Berkow, 1992:192). Recovery is usually rapid but some symptoms may persist for weeks and in some patients, especially in the elderly and in children, serious pulmonary complications may develop. All influenza vaccines in routine use are 'inactivated' or 'attenuated' viral vaccines.

In a study done by Illum *et al.* (2001:84) various chitosan-based influenza vaccine systems for nasal delivery have been evaluated in animal models as well as a phase I clinical investigation. An increase in haemagglutinin inhibition (HI) titre values in more than 40 % of the volunteers tested for all three influenza strains was reported and this is indicative of an effective immune response to the vaccine. A candidate vaccine was selected for further clinical evaluation.

2.4.2 Pertussis vaccine

Pertussis or whooping cough is an infectious disease caused by the bacterium *Bordetella pertussis*. A definition is an acute, highly communicable bacterial disease, characterised by a paroxysmal or spasmodic cough that usually ends in a prolonged, high-pitched, crowing inspiration (the whoop) (Berkow, 1992:2151). The disease is spread by airborne droplets. The bacteria attached to the respiratory cilia, produce toxins that paralyse the cilia, cause inflammation and suppress the clearance of pulmonary secretions. This can potentially cause pneumonia.

In non-immunised populations in the world, pertussis remains a major health problem among children, with an estimated 300 000 deaths per year due to the disease. The most commonly used vaccines against pertussis are three to four

component acellular vaccines combined with vaccines against diphtheria and tetanus toxoids. These vaccines are all given by injection (Illum *et al.*, 2001:86).

A number of different chitosan-based nasal vaccines for pertussis have been evaluated (Illum *et al.*, 2001:86). A subcutaneous (s.c.) dose was used as the control. Nasal formulations were given with or without chitosan. The s.c. injection provided a slightly higher antibody titre than for the intranasal chitosan formulations. The chitosan-based systems elicited higher levels of both IgG and secretory IgA than nasal systems formulated without chitosan.

2.4.3 Diphtheria vaccine

Diphtheria is an acute contagious disease caused by *Corynebacterium diphtheriae*, characterised by the formation of a fibrinous pseudomembrane, usually on the respiratory mucosa, and by myocardial and neural tissue damage secondary to an exotoxin (Berkow, 1992:2148). It is a highly contagious disease that spreads by aerosolisation (droplet infection). When diphtheria develops in an immunised person, the mortality rate is 10 times lower than in non-immunised patients.

The most commonly used vaccines against diphtheria is combined vaccines against pertussis and tetanus toxoids (DtaP). These vaccines are all given by injection. Illum *et al.* (2001:88) tested both powder and solution formulations of chitosan for the diphtheria vaccine. Amoung others significant levels of serum IgG titres were found for both the powder and solution chitosan formulations after nasal adminstration.

2.4.4 Toxoplasmosis vaccine

Toxoplasmosis is a generalised or central nervous system (CNS) disease caused by *Toxoplasma gondii*. *T. gondii* is a small intracellular protozoan parasite that

can infect any warm-blooded animal. The infection occurs worldwide; those at risk for severe disease are the developing fetus and the immunocompromised. The infection may reactivate under conditions of immunosuppression resulting in toxoplasma encephalitis and other complications (Berkow, 1992:235). Control of the infection in the host occurs through the induction of strong and persistant cell mediated immunity, characterised by production of gamma-interferon (IFN-γ), immunoglobulin (Ig) IgG2a and cytotoxic T cells.

In a preliminary *in vivo* study conducted by Bivas-Benita *et al.* (2003:18) in mice it was concluded that oral vaccination by using chitosan-based formulations as particulate delivery systems can prime the immune response.

2.4.5 Tuberculosis vaccine

Tuberculosis is a chronic, recurrent infection most common in the lungs, caused by *Mycobacterium tuberculosis* (Berkow, 1992:131).

The currently available means for vaccination against tuberculosis are inadequate, the highly variable level of protection induced by the commonly used vaccine *Mycobacterium tuberculosis* bacillus-Calmette-Guérin (BCG) and the increasing multidrug-resistance of *M. tuberculosis* strains are stressing the need for new and more effective vaccines (Bivas-Benita *et al.*, 2004:1609).

The effect of the use of chitosan nanoparticles as vaccine carriers in endotracheal immunisation was analysed. Systemic T-cell activation was evaluated by IFN-γ secretion (ELISA). It was shown that pulmonary delivery of chitosan-DNA nanoparticles resulted in higher IFN-γ secretion in comparison to both pulmonary DNA solution and intramuscular injection. The chitosan nanoparticles described here, were shown to protect plasmid DNA from DNase I degradation, which may explain the higher immunogenicity achieved in this immunisation group (Bivas-Benita *et al.*, 2004:1614).

2.5 CONCLUSION

Chitosan is a biodegradable polysaccharide that proved to be non-toxic. Because of an electrostatic interaction chitosan and chitosan derivatives effectively condense plasmid DNA, protecting it from DNase degradation. Chitosan/DNA nanoparticles of specific size ranges can be formulated by optimised preparation processes. Some studies proved that a decrease in the size of the DNA/chitosan complex occurred with an increase in the MW of the chitosan. Others concluded that the complex size was dependent on the DNA/chitosan ratio. EtBr can be used as a DNA stain to examine the electrostatic interaction between DNA and chitosan. EtBr and confocal microscopy can be used to locate the distribution of DNA in the complexes. Gel electrophoresis was also used to prove the absence of free DNA after formation of the complexes, while others used the PicoGreen® assay. The non-viral DNA carrier has to protect the DNA against degradation by DNase. DNase I and II are routinely used to check the release of DNA from the complexes. The cytotoxicity caused by the non-viral DNA carrier is also a very important factor for gene delivery. MTT assays proved to be useful in this regard. Various vaccines encorporating chitosan were also described as an application. A number of *in vitro* and *in vivo* studies showed that chitosan is a suitable material for efficient non-viral gene and DNA vaccine delivery.

CHAPTER 3

CHITOSAN POLYMERS FOR GENE TRANSFECTION: SELECTION AND CHARACTERISATION

3.1 INTRODUCTION

Recently, a number of techniques have been developed to introduce foreign DNA into cells. It is well documented that cationic polymers, like chitosan, have been employed as non-viral gene transfecting agents (Lee *et al.*, 1998:213; Sato *et al.*, 2001:2075). The cationic polymers form complexes with anionic DNA by electrostatic interaction. These cationic DNA complexes formed were then taken up in cells through an electrostatic interaction, because the cell surface is negatively charged.

Chitosan is a linear cationic polysaccharide composed of β1 → 4 linked glucosamine partly containing *N*-acetyl glucosamine. Recently, chitosan has been considered for pharmaceutical formulation and drug delivery applications in which attention has been focused on its absorption enhancing, controlled release and bioadhesive properties. Because chitosan is obtained from a natural occurring source, the polymer has been shown to be both biocompatible and biodegradable. When chitosan was administered orally in mice, the LD_{50} was found to be in excess of 16 g/kg, which is higher than that of sucrose (Dodane and Vilivalam, 1998:246).

N-trimethyl chitosan chloride (TMC) is a partly quaternised derivative of chitosan with improved solubility and ease of preparation (Sieval *et al.*, 1998:157). This chitosan derivative has excellent absorption enhancing effects, especially in neutral environments were chitosan is ineffective as an absorption enhancer

(Kotzé *et al.*, 1998:38). In previous studies it was shown that the degree of quaternisation has an effect on the absorption enhancing properties (Kotzé *et al.*, 1999:255), as well as on the mucoadhesive properties of TMC (Snyman, 2003:65).

Both chitosan, and to a lesser extend, quaternised chitosan polymers has been shown to be able to transfect cells *in vitro* (Thanou *et al.*, 2001:153; Mao *et al.*, 2001:399). To increase the understanding of what effect the degree of quaternisation (DQ) of different chitosan derivatives will have when employed as a non-viral gene delivery system, a range of quaternised chitosan polymers with different DQs and MWs were evaluated. All of the quaternised chitosan polymers used in this study were generously donated by the Department of Pharmaceutics in the School of Pharmacy at the North-West University (Potchefstroom campus). The polymers were used as received. Only a brief description of the synthesis procedures and characterisation will therefore be given below (3.2 – 3.4).

The aim of this study was therefore to evaluate the different chitosan polymers and quaternised chitosan polymers to identify the most effective polymer for transfection of the luciferase gene in COS-1 cells in a neutral environment. The charge density, as determined by the degree of quaternisation is an important factor that influences the absorption enhancement effect and probably the toxicity of these polymers (Hamman *et al.*, 2000:38).

3.2 SYNTHESIS OF QUATERNISED CHITOSAN POLYMERS

N-trimethyl chitosan chloride (TMC) and *N*-triethyl chitosan chloride (TEC) polymers were synthesised from chitosan polymers with different molecular weights (MW) by varying the number and duration of the reaction steps and were characterised by ^{1}H-NMR spectroscopy to determine the degree of quaternisation. *N*-trimethyl chitosan oligomer (TMO) and *N*-triethyl chitosan

oligomer (TEO) polymers were synthesised from a chitosan polymer with low MW (chitosan oligomer) and were characterised by ^{1}H-NMR spectroscopy to determine their degree of quaternisation (DQ) as been described elsewhere (Domard *et al.*, 1986:105; Sieval *et al.*, 1998:157; Hamman *et al.*, 2000:36). The properties of the different polymers is summarised in table 3.1.

Table 3.1 Summary of the polymers used in this study.

Polymer	Parent chitosan	MW	DQ
TMC HH	Chitoclear® High MW	High	High
TMC HL	Chitoclear® High MW	High	Low
TMC MH	Chitoclear® Medium MW	Medium	High
TMC ML	Chitoclear® Medium MW	Medium	Low
TMC H	Seacure® 244	High	High
TMC M	Seacure® 244	High	Medium
TMC L	Seacure® 244	High	Low
TMO H	Primex oligosaccharide	Low	High
TMO L	Primex oligosaccharide	Low	Low
TEC MH	Chitoclear® Medium MW	Medium	High
TEC ML	Chitoclear® Medium MW	Medium	Low
TEO H	Primex oligosaccharide	Low	High
TEO L	Primex oligosaccharide	Low	Low

3.2.1 Materials

Seacure® 244 chitosan were a gift from Pronova Biopolymers (Drammen, Norway). Chitoclear® High MW, Chitoclear® Medium MW and chitosan oligosaccharide were all gifts from Primex Ingredients ASA (Avaldsnes, Norway). Certificates of analysis for the parent chitosans are provided in annexure 1. All parent chitosan polymers (table 3.2) were used without further purification. Sodium hydroxide pellets, sodium chloride, acetic acid, ammonium acetate, absolute ethanol and diethylether were obtained from Merck (Kempton Park, South Africa). *N*-methyl-2-pyrrolidone was obtained from Riedel-de Haën (Seelze, Germany) and sodium iodide and iodoethane were obtained from Fluka (Basel, Switzerland). All chemicals used were of analytical grade and used as received.

Table 3.2 MW and degree of deacetylation (DDA) of parent chitosan polymers.

Manufacturer	Retail description	MW (Da)	DDA (%)
Pronova	Seacure®244	148 000	93
Primex	Chitoclear® High MW	263 700	97
Primex	Chitoclear® Medium MW	77 670	93
Primex	Oligosaccaride	13 500	79

In future text Primex H will refer to Chitoclear® High MW, Primex M will refer to Chitoclear® Medium MW and Primex O will refer to the oligosaccharide. Seacure will refer to the chitosan obtained from Pronova Biopolymer.

3.2.2 Method

TMC polymers were synthesised by reductive methylation of chitosan accomplished by a chemical reaction between chitosan and iodomethane in the presence of sodium hydroxide at 60 °C according to the method described by Sieval *et al.* (1998:157). It was shown that the degree of quaternisation of TMC could be controlled by the number and duration of the reaction steps involved in the synthesis process (Hamman *et al.* 2000:36; Snyman, 2003:30). The TEC derivatives were synthesised by substituting the iodomethane with iodoethane at a temperature at 80 °C. The reaction steps were repeated several times with the product obtained after precipitation from each step to increase the degree of quaternisation of the different polymers. The reaction steps for each of the polymers are shown in table 3.3. The reaction time was decreased for the low MW polymers as the same conditions for the high and medium MW polymers would have resulted in significant polymer degradation (Snyman, 2003:32). The synthesis procedure for the polymers can be summarised as follows:

Chemical reaction: A mixture of 2 g chitosan, 4.8 g of sodium iodide, 11 ml of a 15 % w/v aqueous sodium hydroxide solution and 11.5 ml of iodomethane/iodoethane in 80 ml of *N*-methylpyrrolidone was magnetically stirred on a water bath at a fixed temperature for a predetermined period of time. The product was precipitated from solution with 99 % ethanol and isolated by centrifugation.

Sequential chemical reactions: The product obtained from the previous step was dissolved in 80 ml *N*-methylpyrrolidone and 4.8 g of sodium iodide, 11 ml of a 15 m/v % aqueous NaOH solution and 7 ml of iodomethane/iodoethane were added. This mixture was stirred on a water bath at a fixed temperature for a predetermined period of time. The product was precipitated from solution with ethanol and isolated by centrifugation.

Table 3.3 Conditions of the synthesis for each of the polymers (Adapted from Snyman, 2003:33; Hamman *et al.*, 2002:238).

Polymer	Temperature (°C)	Chemical reaction	Time (min)	Sequential chemical reactions	Time (min)	Adding step	Time (min)
TMC HH	60	1	45	2	45	1	30
TMC HL	60	1	45	-	-	-	-
TMC MH	60	1	45	2	45	1	30
TMC ML	60	1	45	-	-	-	-
TMC H	60	1	45	2	45	1	30
TMC M	60	1	45	1	45	-	-
TMC L	60	1	45	-	-	-	-
TMO H	60	1	30	2	30	-	-
TMO L	60	1	30	-	-	-	-
TEC MH	80	1	45	2	45	1	30
TEC ML	80	1	45				
TEO H	80	1	30	2	30	-	-
TEO L	80	1	30	-	-	-	-

Adding step: An additional 2 ml iodomethane/iodoethane and 0.6 g NaOH pellets were added to the mixture at the end of the chemical reaction while stirring was continued for another 30 min at the specific temperature. The product was precipitated from solution with ethanol and isolated by centrifugation.

Ion exchange step: After washing the final product with 99 % ethanol and diethylether the product was dissolved in 40 ml of a 5 % w/v aqueous NaCl solution to exchange the iodide-ion with a chloride-ion. The polymer was precipitated from solution using ethanol and isolated by centrifugation.

The final product was dissolved in 40 ml distilled water to remove the remaining NaCl from the product and precipitated from solution with ethanol. The product was dried in a vacuum oven at 40 °C.

3.3 CHARACTERISATION OF POLYMERS

The characterisation of the synthesised polymers is important for further studies relying on the differences between the polymers. The quaternised chitosan polymers obtained from the Department of Pharmaceutics were characterised with nuclear magnetic resonance (^{1}H-NMR) spectroscopy. From these spectra the degree of quaternisation (DQ) was determined. Molecular weights of the polymers were determined with SEC/MALLS and IR spectroscopy (IR) was used for further characterisation.

3.3.1 ^{1}H-NMR analysis

The synthesised quaternised chitosan polymers were characterised with nuclear magnetic resonance (^{1}H-NMR) spectroscopy to determine the degree of quaternisation. The ^{1}H-NMR spectra were recorded in D_2O with a 600 MHz DMX

Bruker spectrometer (Bruker, Karlsruhe, Germany) at 80 °C with suppression of the water peak.

3.3.1.1 Calculation of the degree of quaternisation (DQ)

The DQ was calculated form the ^{1}H-NMR spectra of the quaternised trimethylated chitosan (TMC and TMO) polymers by substituting the integrals of the identified peaks into equation 3.1. Sieval *et al.* (1998:157) documented assignments of the different peaks in a previous study.

$$DQ\,(\%) = [(\frac{\int TM}{\int H}) \times \frac{1}{9}] \times 100 \qquad (3.1)$$

where:

DQ(%) = degree of quaternisation of TMC/TMO expressed as a percentage

$\int TM$ = integral of the trimethyl amino group (quaternary amino) peak

$\int H$ = integral of the ^{1}H peaks

The DQ was calculated form the ^{1}H-NMR spectra of the quaternised triethylated chitosan (TEC and TEO) polymers by substituting the integrals of the identified peaks into equation 3.2.

$$DQ\,(\%) = [(\frac{\int TE}{\int H}) \times \frac{1}{9}] \times 100 \qquad (3.2)$$

where:

DQ(%) = degree of quaternisation of TEC/TEO expressed as a percentage

$\int TM$ = integral of the triethyl amino group (quaternary amino) peak

$\int H$ = integral of the ^{1}H peaks

All the ^{1}H-NMR spectra obtained for the polymers were published by Snyman (2003:38) and Hamman (2001:59). Clear differences in the size of the quaternary amino peaks were recorded in the ^{1}H-NMR spectra of the different TMC and TMO polymers. It was documented that the increase in the size of the quaternary amino group peak may be attributed to the increase in the number of trimethyl amino groups found as a result of the increase in the number and duration of the reaction steps. Differences in the size of the quaternary amino peaks were also seen in the ^{1}H-NMR spectra of the different TEC and TEO polymers. The increase in the size of the quaternary amino group peak was also attributed to the increase in the number of triethyl amino groups found as a result of the increase in the number and duration of the reaction steps.

The different DQ of the various polymers synthesised by Snyman (2003:45) and Hamman (2001:68) were calculated with equation 3.1 and 3.2. The results obtained are shown in table 3.4.

Table 3.4 DQ of the polymers used in this study (Adapted from Snyman *et al.*, 2003:706; Hamman, 2001:54).

Polymer	DQ (%)	Polymer	DQ (%)
TMC HH	65.50	TMO H	54.60
TMC HL	21.90	TMO L	31.20
TMC MH	62.73	TEC MH	26.71
TMC ML	23.90	TEC ML	10.21
TMC H	59.20	TEO H	27.90
TMC M	42.75	TEO L	15.10
TMC L	22.15	-	-

The DQ is increased with an increase in the number of reaction steps during the synthesis procedure. It was noted that although reaction procedures were identical, lower DQ values were obtained for TEC polymers than for TMC polymers. Similarly, lower DQ values were also obtained for TEO polymers than for TMO polymers in identical reaction procedures. This was attributed to the reactivity and molecular size of iodomethane compared to iodoethane. The reactivity of iodomethane is higher than the reactivity of iodoethane, it was therefore expected that the degree of substitution with iodoethane would be lower. Furthermore, the molecular size of iodoethane is also larger than iodomethane and this result in lower mobility and subsequently lower substitution on the chitosan backbone (Snyman, 2003:45).

3.3.2 Infrared (IR) analysis

IR spectra were recorded on a Nicolet Nexus 470 FT IR ESP spectrometer (Madison, USA) over a range of 4000 - 400 cm^{-1} using the KBr reference technique. Samples of the different polymers, each weighing approximately 2 mg were dried for 1 hour at 40 °C and then mixed with 200 mg of KBr (Merck, Germany) before analysis. With the use of infrared spectrometry the stretching and bending vibrations of different groups on a molecule can be identified and analysed.

IR spectra of all the polymers were compared to determine possible significant differences with regard to structural changes during synthesis. IR spectra were published by Snyman (2003:50) and Hamman (2001:73). Clear differences were observed between each parent chitosan and the polymers synthesised thereof. These differences can be attributed to the increase in the degree of quaternisation. No differences were found between the IR spectra of methylated and ethylated quaternised chitosan polymers (e.g. TMC and TEC), as the important peaks was only indicative of changes in the amount of C-N bonds, and

these bonds increased to the same extent for both TMC and TEC (Snyman, 2003:51).

3.3.3 Determination of molecular weight (MW)

The average molecular weight of the synthesised polymers was determined in a previous study by Snyman (2003:36) with size exclusion chromatography (SEC) coupled to a multi angle light scattering device (MALLS) that consisted of a laser photometer (Dawn DSP, Wyatt Technology Corporation, USA) connected to a refracting index detector (ERC 7515A, Japan).

Table 3.5 MW of the polymers used in this study (Adapted from Snyman *et al.*, 2003:706; Hamman, 2001:54).

Polymer	MW (g/mole)	Polymer	MW (g/mole)
TMC HH	167 700	TEC MH	82 810
TMC HL	244 000	TEC ML	91 120
TMC MH	62 250	TEO H	9 000
TMC ML	74 180	TEO L	11 000
TMC H	143 000	Primex H	263 700
TMC M	211 000	Primex M	77 670
TMC L	247 000	Primex O	13 490
TMO H	6 600	Seacure	148 000
TMO L	8 500	-	-

A summary of the results obtained from the SEC/MALLS characterisation experiments for the different quaternised chitosan polymers used in this study is shown in table 3.5 (Snyman, 2003:48; Hamman, 2001:70).

3.4 CONCLUSION

In this chapter the synthesis and characterisation of quaternised chitosan polymers with different degrees of quaternisation and molecular weights were briefly described. These polymers were obtained from the Department of Pharmaceutics at the North-West University (Potchefstoom Campus). These quaternised chitosan polymers were evaluated for their efficacy as gene transfectors (chapter 4 and 5) to increase our understanding of the effect of the degree of quaternisation and molecular weight of quaternised chitosan polymers on the gene transfection process.

CHAPTER 4

EVALUATION OF THE TRANSFECTION PROPERTIES OF CHITOSAN AND QUATERNISED CHITOSAN POLYMERS: EXPERIMENTAL DESIGN AND METHODOLOGY

4.1 INTRODUCTION

In chapter 1 it was concluded that non-viral vectors offered some advantages for gene delivery when compared to viral vectors. In chapter 2 a closer look was taken at chitosan as non-viral vector for gene delivery. Chitosan and chitosan derivatives effectively condense plasmid DNA and thereby protecting it from DNase degradation. It was concluded that chitosan and chitosan derivatives may be one of the most promising classes of non-viral vectors. Therefore, quaternised chitosan polymers (TMC, TMO, TEC and TEO) with different degrees of quaternisation were described in chapter 3. In this chapter these chitosan polymers will be evaluated for their efficiency of delivering a selected plasmid to a selected cell culture line. The chitosan polymers were complexed with a plasmid in different P/N ratios and compared to a positive and negative control to determine the optimum ratio for gene transfection of each polymer. The toxicity of these polymers was studied by an *in vitro* MTT assay.

4.2 SELECTION AND PROPAGATION OF A SUITABLE PLASMID

4.2.1 Introduction

Plasmids are extrachromosomal genetic elements found in a variety of bacterial species that can replicate independently. A plasmid is a small circular piece of

DNA that range in size from 1 kb to greater than 200 kb that contains important genetic information for the growth of bacteria (Maniatis *et al.*, 1983:3). In nature, this information is often a gene that encodes a protein that will make the bacteria resistant to an antibiotic.

Plasmids were discovered in the late sixties, and it was quickly realized that they could be used to amplify a gene of interest. A plasmid containing resistance to an antibiotic (usually ampicillin) is used as a vector. The gene of interest is inserted into the vector plasmid and this newly constructed plasmid is then put into *E. coli* that are sensitive to ampicillin. The bacteria are then spread over a plate that contains ampicillin. The ampicillin provides a selective pressure because only bacteria that have acquired the plasmid can grow on the plate. Therefore, as long as you grow the bacteria in ampicillin, it will need the plasmid to survive and it will continually replicate it, along with your gene of interest that has been inserted into the plasmid. Large quantities of the gene product can be obtained with this technique of genetic engineering (Tamarin, 1993:304).

There are many different kinds of plasmids commercially available. All of them contain a selectable marker (i.e., a gene that encodes for antibiotic resistance), an origin of replication (the starting point to make a copy of the plasmid) and a multiple cloning site. The main differences among commercially available plasmids are the number of restriction enzyme sites, their order in the multiple cloning site, the type of antibiotic resistance that the plasmid confers, and some other genetic information that makes the plasmid useful for a specific purpose.

4.2.2 Selection of a suitable plasmid

Genetic reporters are used commonly in cell biology to study gene expression and other cellular events coupled to gene expression. The pGL3 luciferase reporter vectors provide a basis for the quantitative analysis of factors that potentially regulate the mammalian gene expression. The backbone of the

previously used pGL2 luciferase reporter vectors was redesigned for the pGL3 vectors for increased expression and contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimised for monitoring transcriptional activity in transfected eukaryotic cells. Firefly luciferase is widely used as a reporter for the following reasons: (1) the reporter activity is available immediately upon translation since the protein does not require post-translational processing, (2) the assay is very sensitive, because its light production has the highest quantum efficiency known for any chemiluminescent reaction and no background luminescence is found in the host cells or the assay chemistry, and (3) the assay is rapid, requiring only a few seconds per sample (Promega, 2002a:1). The assay of this genetic reporter is rapid, sensitive and quantitative. A pGL3 control vector (figure 4.1) was purchased from Promega (Madison, USA).

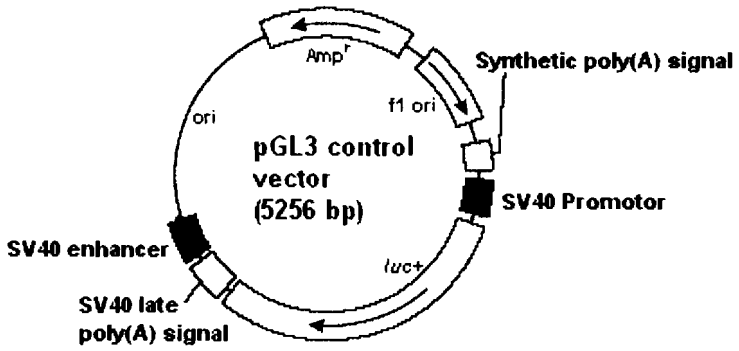


Figure 4.1 pGL3 control vector circle map. Additional description: *luc*+, cDNA encoding the modified firefly luciferase; Amp^r, gene conferring ampicillin resistance in *E. coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli*. Arrows within *luc*+ and the Amp^r gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis (Promega, 2002b:5).

4.2.3 Isolation of single colonies

Luria Bertani (LB) medium (table 4.1) was prepared (Promega, 2002b:16). The three ingredients were mixed together in 800 ml double distilled water (dd.H_2O).

The pH was adjusted to 7.50 with 0.3 % w/v NaOH (Saarchem (Pty) Ltd., Krugersdorp, RSA) and the final volume was kept to 1000 ml.

Table 4.1 Composition of LB medium per liter (Promega, 2002b:16).

Product	Quantity (gram)	Supplier
Bacto®-tryptone	10	Biolab, Victoria, Australia
Bacto®-yeast	5	Biolab, Victoria, Australia
NaCl	10	Saarchem (Pty) Ltd., Krugersdorp, RSA

The medium was divided into smaller aliquots of 50 ml, 100 ml and 200 ml in Schott glass bottles (Merck, Kempton Park, RSA). In two of the 100 ml LB media bottles, 1.5 g agar (Merck, Kempton Park, RSA) was added and marked accordingly. All the media bottles were autoclaved for 15 min at 121 °C. The media was allowed to cool down to ± 50 °C. In one of the 100 ml medium bottles containing agar, 1 ml of a sterilised 0.5 % w/v ampicillin in absolute EtOH was added and marked. Four agarplates were filled ⅓rd with the first bottle containing agar (1.5 % w/v) and ampicillin. Another four agarplates were filled ⅓rd with the second bottle containing only agar (1.5 % w/v) without ampicillin. All the plates were labelled on the bottom of the plate according to the presence of ampicillin and left to cool and set.

The JM109 bacterial cells (*E. coli* strain) (Promega Corporation, Madison, USA) was stored at -70 °C and removed just before use. A platinum transfer loop was sterilised by flaming the loop until it glowed light red. Using an aseptic technique, cells was scraped from the freezed culture and transferred with the platinum transfer loop to a 17 x 100 mm sterile tube (Elkay® Products, Massachusetts, USA) with 5 ml of LB medium. The culture was grown for 3 hours in a waterbath at 37 °C. Before transfer the tube was briefly vortexed to ensure that clumps of

bacteria are dispersed. The platinum loop was again sterilized by flaming, cooled, and dipped into the bacterial suspension. The bacteria adhering to the loop was streaked onto a segment of the plate containing agar medium (without antibiotic). The loop was again sterilised by flaming and cooled. The loop was passed once across one end of the primary streak and the bacteria that adhere to the loop were streaked into a fresh region of agar media (Secondary streak). This step was repeated twice more, serially (figure 4.2). The lid was replaced onto the plate. The plate was then incubated in a hybridisation oven (Hybaid™, Middlesex, UK) in an inverted position at 37 °C for 16 - 24 hours. Well separated single colonies were visible in the area of the final streak.

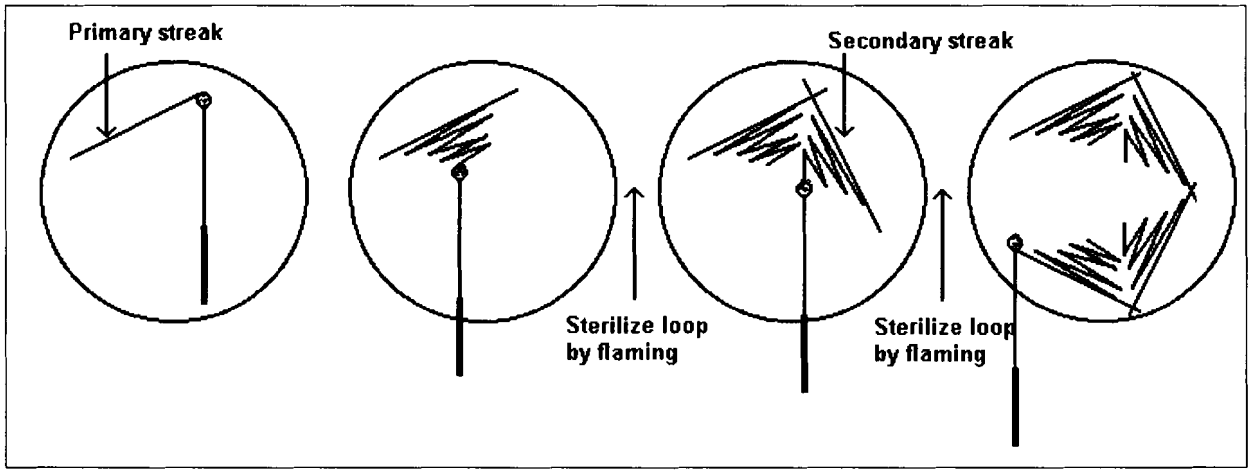


Figure 4.2 Streaking of bacteria onto agar medium (Maniatis *et al.*, 1983:58).

4.2.4 Bacterial transformation of plasmid DNA in *E. Coli*

The plasmids were next inserted into bacteria by a process called *transformation*. *Transformation* is the genetic alteration of a cell resulting from the introduction, uptake and expression of foreign DNA (Tamarin, 1993:144). The purpose of this technique is to introduce a foreign plasmid into bacteria and to use that bacteria to amplify the plasmid in order to obtain large quantities of plasmid. This is

based on the natural function of a plasmid, to transfer genetic information vital to the survival of the bacteria.

Most methods for bacterial transformation are based on an observation of Mandel and Higa (1970:160), who demonstrated that uptake of bacteriophage DNA is enhanced by treatment of bacterial cells with calcium chloride. Many variations in this basic technique have been developed, all directed toward optimising the efficiency of transformation for different bacterial strains (Cohen *et al.*, 1972:2110).

4.2.4.1 Preparing competent bacterial cells

This procedure must be and was performed under sterile conditions. Since DNA is a very hydrophilic molecule it won't pass the bacterial cell membrane. To force bacteria to take in the plasmid, the bacterial cells must first be made *competent* to take up DNA (Tamarin, 1993:144). This was done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium. Briefly, the plasmid DNA was then forced into the *competent* cells by incubating the cells and the plasmid together on ice, placing them briefly at 42 °C (heat shock), and then putting them back on ice. This shock treatment caused the bacteria to take in the DNA. The *competent* cells with the plasmid DNA inside, were then plated on the agar media plates containing the antibiotic ampicillin. Because bacteria are very labile in high calcium media/surroundings, the bacteria were kept on ice at all times to keep them viable.

The calcium chloride procedure (Maniatis *et al.*, 1983:250) was used to make the JM109 cells competent. A 100 ml TB media in a 500 ml flask was inoculated with 1 ml of an overnight bacterial culture. The cells were grown with vigorous shaking in a Gallenkamp orbital incubator (Optolabor (Pty) Ltd., Randburg, RSA) at 37 °C for 4 hours. The culture was then placed on ice for 10 minutes. The cell suspension was centrifuged at 4000 x g for 4 minutes at 4 °C. The supernatant

was discarded and the cells were resuspended in half of the original culture volume (50 ml) of an ice-cold, sterile solution of 50 mM $CaCl_2$ (SIGMA, Steinheim, Germany) and 10 mM Tris.Cl (pH 8.0) (SIGMA, Steinheim, Germany). The cell suspension was again placed on ice for 15 minutes and then centrifuged at 4000 x g for 5 minutes at 4 °C. The supernatant was discarded. The cells were now resuspended in $1/15^{th}$ of the original volume (6.7 ml) of an ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris.Cl (pH 8.0). Aliquots of 0.2 ml were dispensed into pre-chilled Eppendorf tubes (Eppendorf UK Ltd., Cambridge, UK). The cells were stored at 4 °C for 12 – 24 hours.

4.2.4.2 Transformation of the competent bacterial cells

The agar plates containing ampicillin were taken out of storage at 4 °C and left to equilibrate at room temperature (RT). The ligation buffer (Promega Corporation, Madison, USA), sterile 2 ml Eppendorf tubes and the pGL3-control DNA vector (Promega Corporation, Madison, USA) were all stored on ice. The DNA was diluted by adding 99 µl ligation buffer to 1 µl of DNA in a sterile Eppendorf tube and labelled accordingly. Four sterile Eppendorf tubes were used and the quantities reflected in table 4.2 of diluted DNA mixture, ligation buffer and competent cells were added.

Table 4.2 Quantities of diluted DNA mixture, ligation buffer and competent cells.

	Tube 1	Tube 2	Tube 3	Tube 4
Diluted DNA (pGL3-control vector)	0 µl	1 µl	2 µl	4 µl
	0 ng	10 ng	20 ng	40 ng
Ligation buffer	10 µl	9 µl	8 µl	6 µl
Competent cells	50 µl	50 µl	50µl	50 µl
Total volume in tube	**60 µl**	**60 µl**	**60 µl**	**60 µl**

The four filled Eppendorf tubes were kept on ice for 30 minutes. The tubes were then transferred to the waterbath at 42 °C for 2 minutes and returned to the ice for another 10 minutes. To each of the Eppendorf tubes, 1000 µl TB media was added and the tubes were then placed in an incubator at 37 °C for 20 - 30 minutes. The equilibrated agar plates were then labelled according to the diluted DNA quantity added (0, 1, 2, 4). A bent stainless steel spreader was dipped in EtOH, flamed sterile and left to cool. Each tube was emptied onto its corresponding agar plate (with ampicillin) and a stainless steel spreader was used to spread the liquid until dry. The agar plates were then incubated at 37 °C overnight. A few cultures formed on the agar plates where DNA was added and no cultures were visible on the agar plate where no DNA was added. This observation was very positive, since the ampicillin in the agar eliminated all the cells on the plate where no plasmid DNA was present to exhibit antibiotic resistance. A few cells were sterile transferred from each agar plate with a platinum transfer loop to 5 ml LB medium with ampicillin in a sterile tube. The LB medium contained ampicillin in a concentration of 50 µg/ml. This tube was placed in a shake-incubator at 37 °C and left to grow for 8 hours. The cell suspension was then transferred to a sterile 1 L flask containing 500 ml LB medium with ampicillin (50 µg/ml). This flask was returned to the shake-incubator and grown shaking overnight at 37 °C.

It is important to realize that only a very small proportion of the cells are competent to incorporate plasmid DNA in a stable fashion, and that only 1 DNA molecule in approximately 10 000 is successful at transformation. Once inside the bacterium, the plasmid DNA replicates and expresses the drug-resistance markers that allow the transformed cell to survive in the presence of an antibiotic.

The ability of bacteria to take up DNA is short-lived. After exposure to agents that enhance uptake, most strains of bacteria remain in a competent state for only 1-2 days. However, competent cells can be prepared in large quantities, tested and stored in frozen aliquots.

4.2.5 Growth, maintenance and preservation of bacterial strains

4.2.5.1 Growth of bacteria

Under ideal conditions, *Escherichia coli* (*E. coli*) will grow exponentially. The rate of growth is dependent on the medium, the genotype of the strain, the temperature and the degree of aeration. As the density of the culture increases, the rate of division decreases until the bacteria reach a concentration (saturation density) at which they no longer divide but are viable.

The rate of growth of a bacterial culture is most conveniently monitored by withdrawing aliquots at various times and reading the optical density (OD) at a wavelength of 600 nm. As a rough guide, 1 OD_{600} = 8 x 10^8 cells/ml. Aeration of cultures are best achieved by attaching a flask or tube to a rotary shaking platform or a rotating wheel. The volume of the vessel should be at least four times larger than the volume of medium it contains so as to allow vigorous shaking (Maniatis *et al.*, 1993:61).

4.2.5.2 Short-term storage

Colonies of most strains of bacteria can be maintained for periods of a few weeks on the surface of agar media if the plates are tightly wrapped in parafilm and stored inverted at 4 °C (Maniatis *et al.*,1983:61).

4.2.5.3 Long-term storage

Bacteria can be stored for many years in media containing 15 % glycerol at low temperature without significant loss of viability (Maniatis *et al.*, 1983:62).

A quantity of 10 ml TB medium in a culture flask was inoculated with a single bacterial colony of the JM109 cell culture and grown overnight. 0.85 ml of the

overnight culture was transferred to a sterile Eppendorf tube containing 0.15 ml of sterile glycerol (SIGMA, Steinheim, Germany). The cap on the vial was closed and the contents were mixed thoroughly by vortexing. The glycerinated cultures was then stored at - 20 °C. The cultures can be stored in this way for 1 to 3 years without loss of viability. Some of the tubes with glycerinated suspension were also stored at - 70 °C. In this case, viable bacteria can be recovered for many years after the initial freezing. The viable bacteria can be recovered by simply scratching the surface of the frozen stock with a sterile platinum loop and the frozen suspension can then be returned to the freezer (Maniatis *et al.*, 1983:62). Several vials were frozen for future use.

4.2.6 Isolation of the plasmid DNA from the *E. Coli* strain

The isolation of the plasmid DNA was achieved with the Wizard® *Plus* Maxipreps DNA purification system (Promega Corporation, Madison, USA). The kit consisted of 150 ml Cell Resuspension Solution (CRS), 150 ml Cell Lysis Solution (CLS), 300 ml Neutralization Solution (NS), 100 ml Wizard® Maxipreps DNA Purification Resin (PR), 125 ml Column Wash Solution (CWS), 10 Maxi/Megacolumns with Reservoirs, 10 Syringes (5 ml), 10 Syringe Filters (0.2 µm) and 1 protocol (Promega, 2001:1). TE buffer consisting of 10 mM Tris.Cl (pH 7.4) and 1 mM EDTA (pH 8.0) was prepared according to Maniatis *et al.* (1983:448). A stock solution of ethidium bromide (EtBr) (SIGMA, Steinheim, Germany) in dd.H_2O of 10 mg/ml was prepared and stored at 4 °C in a light-proof bottle.

The 500 ml overnight culture prepared (§ 4.2.4.2) were centrifuged in two 250 ml centrifuge bottles at 5 000 × g for 10 minutes in a RT rotor to form a pellet. The supernatant was poured off and the pellet was resuspended in 15 ml of CRS. (To aid with resuspension the pellet was manually pipetted until no clumps were visible. Complete resuspension is critical for optimal yields). 15 ml of CLS were added and mixed gently by inverting. Cell lysis was completed when the solution

became clear and viscous. Then 15 ml of NS was added and immediately mixed by gently inverting the centrifuge bottle several times. The solution was then centrifuged at 14 000 × g for 15 minutes in a RT rotor. The cleared supernatant was transferred by filtering it through filter paper (Whatman® #1, Whatman International Ltd., Maidstone, England) into a clean 100 ml graduated cylinder. The supernatant volume was measured and then transferred to a centrifuge bottle (44 ml). 0.5 volume of RT isopropanol (22 ml) was added and mixed by inversion. The solution was centrifuged at 14 000 × g for 15 minutes in a RT rotor. The supernatant was discarded and the DNA pellet resuspended in 2 ml of TE buffer. The walls of the bottle were washed thoroughly with TE buffer to recover the DNA. The pellet was not always visible at this stage.

4.2.7 Purification of the plasmid

10 ml of PR was added to the DNA solution from § 4.2.6 and swirled to mix. For each Maxiprep, one Maxicolumn was used. The Maxicolumn tip was inserted into the vacuum manifold port (Vacuum pump and compressor, Edwards High Limited, Sussex, UK). The resin/DNA mix was transferred into the Maxicolumn. A vacuum of at least 15 inches of Hg was applied to pull the resin/DNA mix into the Maxicolumn and the vacuum was maintained up to 30 seconds after all of the solution had flowed through the column. 25 ml of CWS was added to the Maxicolumn and again a vacuum was applied to draw the solution through the Maxicolumn. The resin was then washed by adding 5 ml of 80 % ethanol to the Maxicolumn and applying a vacuum to draw the ethanol through the Maxicolumn. The vacuum was allowed to draw for an additional 1 minute after all liquid has passed through. The Maxicolumn was placed in a 50 ml screw cap tube (Bibby Sterilin Ltd., Staffs, UK). A swinging bucket rotor (Roto-Uni II, BHG, Germany) was then used to centrifuge the Maxicolumn at 1 300 × g for 5 minutes. The Maxicolumn was removed and both the tube and the liquid were discarded. The Maxicolumn was placed back on the vacuum manifold and the resin dried by applying a vacuum for 5 minutes. The Maxicolumn was then removed from the

vacuum manifold and placed in the provided 50 ml screw cap reservoir tube. A volume of 1.5 ml of preheated (65 - 70 °C) nuclease-free water was added to the Maxicolumn and left for 1 minute. The DNA was eluted by centrifuging the Maxicolumn/Reservoir at 1 300 × g for 5 minutes in a centrifuge with a fixed-angle rotor (Beckman® J2-21 Centrifuge, Beckman Instruments, Inc., California, USA). A white pellet of resin fines was present in this final eluate. The plunger was removed from one of the 5 ml syringes and set aside. The syringe barrel was attached to the Luer-Lok® extension of a 0.2 µm syringe filter and the eluate was pipeted into the syringe barrel. The filter was then centered over a 15 ml plastic tube. The plunger was carefully inserted into the syringe barrel and gently pushed to transfer the liquid into the tube. The eluate was transferred to a 1.5 ml centrifuge tube and centrifuged at 14 000 × g for 1 minute. This additional step removed all resin fines that was present in the final eluate. The supernatant was immediately transferred to a new microcentrifuge tube. The eluted DNA was stored at 4 °C in TE buffer.

4.2.8 Agarose gel electrophoresis

The standard method used to separate, identify and purify DNA fragments is electrophoresis through agarose gels (Maniatis *et al.*, 1983:150).

The method used is an adaption of the method described by Maniatis *et al.* (1983:159) and the prepraration of TAE buffer and loading buffer were also published by the same authors. An amount of 0.35 g powdered agarose was added to the measured amount (35 ml) of electrophoresis buffer (1x TAE buffer). The slurry was heated in a microwave until the agarose has dissolved. The solution was then cooled down to 50 °C and EtBr was added to a final concentration of 0.5 µg/ml. EtBr is a fluorescent dye that contains a planar group that intercalates between the stacked bases of DNA and thereby offer the most convenient method to visualise DNA in agarose gels. The uncovered edges of the plastic electrophoresis plate (A) were covered with masking tape to form a

rectangular mold (figure 4.3). The comb (B) of which the teeth will form the sample wells were placed into position and the rest of the warmed agarose solution were poured into the mold. It was checked that there is al least 0.5 mm of agarose between the bottom of the plate (base of the gel) and the bottom of the teeth, in order for the wells to be completely sealed.

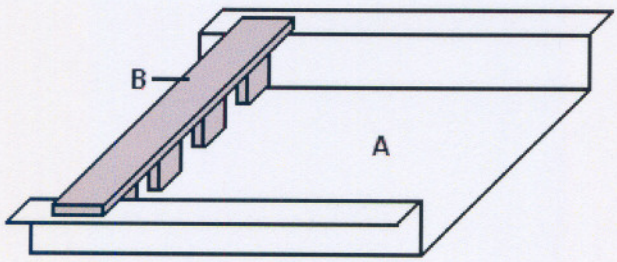


Figure 4.3 The electrophoresis plate as mold for the agarose gel where (A) represents the elctrophoresis plate and (B) the comb for the forming of wells.

After 30 - 45 minutes at room temperature the gel was completely set and the tape was then removed from the sides of the plate. The gel in the electrophoresis plate was then mounted in the electrophoresis tank (figure 4.4).

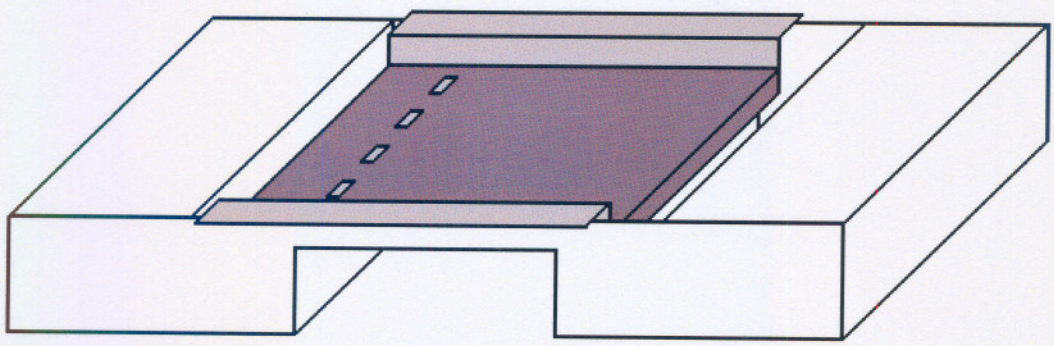


Figure 4.4 The electrophoresis tank with the agaose gel in the electrophoresis plate.

Enough electrophoresis buffer (1x TAE buffer) was added to cover the gel in the plate to a depth of about 1 mm. Samples (10 μl) were mixed with loading buffer (2 μl) and 10 μl of this mixed sample was loaded into the wells. A diluted sample of the pGL3-control plasmid mixed with loading buffer was loaded into the first well and samples from the plasmid propagated in *E. coli* mixed with loading buffer was loaded into the next wells. The resistance to the passage of electrical current of the gel is almost the same as that of the buffer, so a considerable fraction of the applied current passes along the length of the gel (Maniatis *et al.*, 1983:153). Micrographs of the gels were made with transmitted or incident UV light. This method was used to confirm the presence of DNA and to characterise the different polyplexes prepared (§ 5.2.1).

4.2.9 Conclusion

The JM109 bacterial cells obtained was made competent for the uptake of plasmid DNA through a calcium chloride procedure. The pGL3-control plasmid was then propagated in these competent *E. coli* strains. A quantity of these bacterial cells was also stored for future use. Isolation and purification of the plasmid was achieved by using Wizard® *Plus* Maxipreps and purity of the plasmid was confirmed by agarose elctrophoresis.

4.3 CULTURING AND MAINTENANCE OF THE COS-1 EXPERIMENTAL CELL CULTURE LINE

4.3.1 Introduction

COS-1 cells were derived from African green monkey kidney cells with SV40 transformation. This cell line is therefore able to handle vectors possessing SV40 elements and suitable for transfection by vectors requiring expression of the SV40 T antigen.

Because this study evaluated different chitosan and chitosan derivatives, a cell line was selected where differences in transfection efficiencies would be easily noticed. Secondly, two previous studies using chitoplexes also employed the COS-1 cell line (Thanou *et al.*, 2001:153; MacLaughlin *et al.*, 1998:259). Thanou *et al.* (2001:157) noted that the transfection efficiencies observed in the Caco-2 cell line were substantially lower compared to the levels observed in COS-1 cells. A possible reason might be that Caco-2 cells are a differentiated cell line that proved to be more resistant to transfection than COS-1 cells, an early stage dividing culture.

4.3.2 Culturing of COS-1 cells in culture flasks

The African green monkey (*Cercopithecus aethiops*) kidney fibroblast-like cell line, COS-1 (ATCC®, Virginia, USA), starting at passage 11, was grown in 150 cm^2 cell culture, canted neck, tissue culture treated, nonpyrogenic, polystyrene, sterile flasks (Costar®, Corning Incorporated, New York, USA) to produce cells for seeding on 24-well culture plates (Costar®, Corning Incorporated, New York, USA). The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose and L-glutamine (BioWhittaker™, Cambrex, Walkersville, USA) supplemented with 10 % fetal bovine serum (Delta Bioproducts, Kempton Park, RSA) and 1 % Pen/Strep Fungizone Mix [100X] (10 000 U penicillin/ml, 10 000 μg streptomycin/ml and 25 μg fungizone/ml, (BioWhittaker™, Cambrex, Walkersville, USA). The cell cultures were kept in an incubator (Forma scientific, Inc., Ohio, USA) at a temperature of 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2. The medium was changed three times per week in a laminar air-flow cabinet (Labotec, Midrand, RSA) and trypsinisation was performed as described in the following section.

4.3.3 Trypsination

The 150 cm^2 culture flasks were checked microscopically and only flasks with cells that have reached confluency were used for the trypsinisation procedure. The cells reached confluency within 6-7 days. Trypsin-Versene Mixture (BioWhittaker™, Cambrex, Walkersville, USA) and the culture medium were warmed in a waterbath (Wirsam Scientific and Precision Equipment (Pty) Ltd., Richmond, RSA) at 37 °C. The cell culture flask and the warmed solutions were wiped with 70 % ethanol and transferred to the laminar airflow cabinet. The medium in the 150 cm^2 culture flask was aspirated and 6 ml Trypsin-Versene Mixture was poured onto the bottom of the flask in order for all cells to be covered with the solution. The 150 cm^2 culture flask was then incubated for 15 minutes at a temperature of 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2. After 15 minutes the 150 cm^2 culture flask was removed from the incubator, microscopically cheched for detachment, wiped with ethanol and transferred to the laminar airflow cabinet. The cells were trypsinated 1:6 according to the product information sheet (ATCC®, Virginia, USA) and therefore 1 ml of detached cells were added to 29 ml of warmed culture medium in a new 150 cm^2 culture flask. The fresh cell suspension was agitated with a pipet to ensure that cell aggregates were dispersed. The new 150 cm^2 culture flask was labelled with the cell type, new passage number and the date. The new, labelled 150 cm^2 culture flask was placed in the incubator at a temperature of 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2. This trypsination procedure was repeated every 6-7 days when confluency was reached, until the cells were seeded on 24-well plates for the transfection experiments.

4.3.4 Seeding and culturing on 24-well plates

COS-1 cells (from passage 16) were seeded on 24-well tissue culture treated, polystyrene, sterile, plates. A cell suspension was obtained as described in

§ 4.3.3. The cells were counted on a Neubauer improved bright-line hemacytometer (Precicolor, HBG, Germany) and diluted with culture medium (DMEM supplemented with 10 % fetal bovine serum and 1 % Pen/Strep Fungizone Mix) until a concentration of 5.0 x 10^5 cells/ml was reached. A volume of 1 ml of this cell suspension was transferred to each of the wells in the 24-well plates. The 24-well plate was then placed in the incubator at a temperature of 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2. Seeding was done 24 hours before the start of transfection experiments.

4.3.5 Cryopreservation

Cryopreservation media consisting of modified culture medium (DMEM supplemented with 20 % fetal bovine serum and 1 % Pen/Strep Fungizone Mix) supplemented with 5 % (v/v) DMSO. Cryopreservation media was prepared and kept on ice. Cell suspensions were obtained as described in § 4.3.3. The cell suspensions were diluted 50:50 with culture medium and centrifuged at 2 000 x g for 5 minutes at 4 °C. The desired amount of cryopreservation media was added to the pellet obtained from centrifugation and this suspension was mixed well. Small aliquots (1 ml) of this mixed suspension were transferred to 2.0 ml cryovials (Costar®, Corning Incoporated, New York, USA) that was labelled with the cell type, passage number and the date. The filled cryovials were placed in a slow-rate freezer (Labotec, Midrand, RSA) and froze to - 90 °C within 1 hour. The frozen cryovials were then rapidly transferred to a Cryoplus 2 cryofreezer (Forma scientific, Inc., Ohio, USA) filled with nitrogen in the vapour phase.

4.3.6 Conclusion

The COS-1 cell line was selected as a suitable cell line to investigate the transfection efficiency of a pGL3-control DNA by experimental non-viral vectors. The culturing and trypsination procedures used for the cell line were explained, as well as the cryopreservation method used to preserve cells for future use.

The seeding and culturing procedure for the transfection experiments, 24 hour before the start of an experiment, was also explained.

4.4 *IN VITRO* TRANSFECTION EXPERIMENTS

4.4.1 Determination of DNA concentration

The DNA concentration of the experimental pGL3-control plasmid was determined by measuring UV absorbance at 260 nm and 320 nm and substituting the values into equation 4.1. The absorbance value obtained at 320 nm is a representation of the background and is therefore subtracted from the absorbance value obtained at 260 nm. Because of the following: 50 $A_{260} \approx 1\mu g$ DNA/ml, the corrected absorbance value is multiplied by 50. If a dilution for the DNA is used, the dilution factor will also be included into the equation to determine the DNA concentration (Strauss, 1987:2.2.1). The UV absorbance spectra obtained is shown in figure 4.5.

$$[DNA] = (A_{260} - A_{320}) \times 50 \times dilution\ factor \qquad (4.1)$$

where:

A_{260} = Absorbance value at 260 nm = 0.6328
A_{320} = Absorbance value at 320 nm = 0.0060
dilution factor = 5

$$[DNA] = (0.6328 - 0.0060) \times 50 \times 5 \quad \therefore [DNA] = 156.7\ \mu g/ml$$

The yield of plasmid, evident from the DNA concentration, will vary depending on a number of factors that includes the type of culture medium, the volume of bacterial culture and the bacterial strain (Promega 2001:4). The concentration obtained after purification was used for the experimental design without further additions or purifications.

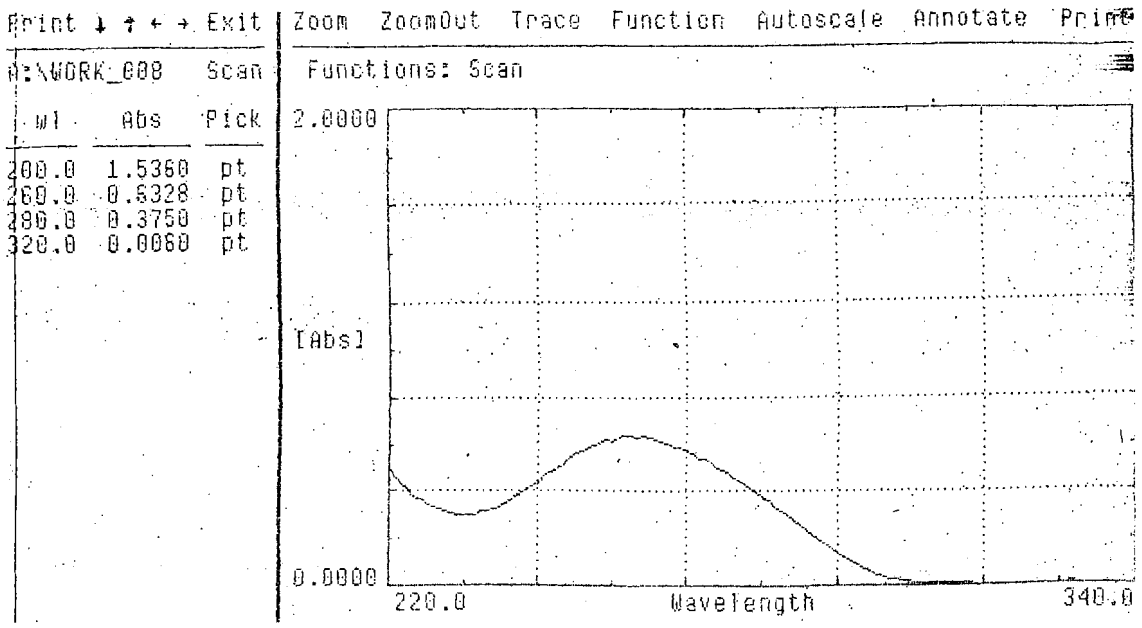


Figure 4.5 UV absorbance spectra for determination of plasmid DNA concentration.

4.4.2 Preparation of polymer solutions

Chitosan and quaternised chitosan polymer solutions were prepared using the coaservation method described by Mao *et al.* (2001:401). Sodium acetate buffer (5 mM) was prepared using sodium acetate trihydrate (Saarchem (Pty) Ltd., Krugersdorp, RSA) and acetic acid (Saarchem (Pty) Ltd., Krugersdorp, RSA). The pH was measured and adjusted to pH 5.5 with 0.1 M solutions of NaOH (Saarchem (Pty) Ltd., Krugersdorp, RSA) or HCl (Saarchem (Pty) Ltd., Krugersdorp, RSA). All solutions were freshly prepared each day when the polymer-DNA complexes were prepared and the experimental procedure performed. Chitosan polymers and quaternised chitosan polymers in different quantities were dissolved overnight in 100 ml of sodium acetate buffer (table 4.3). These solutions represented the 10x solution of each chitosan polymer. Dilutions from this solution were made in order to have 1x, 3x and 5x solutions. These solutions were then sterile filtered through 0.2 μm filters (Whatman®, Whatman International Ltd., Maidstone, England).

Table 4.3 Mass (g) of each chitosan polymer dissolved in 100 ml sodium acetate buffer (5 mM) to produce 10x polymer solution.

Polymer	Mass (g)	Polymer	Mass (g)
TMC HH	0.02872	TEC MH	0.02902
TMC HL	0.02754	TEC ML	0.02898
TMC MH	0.02858	TEO H	0.02904
TMC ML	0.02774	TEO L	0.02898
TMC H	0.02852	Primex H	0.02324
TMC M	0.02814	Primex M	0.03287
TMC L	0.02770	Primex O	0.02430
TMO H	0.02898	Seacure	0.02348
TMO L	0.02770	-	-

4.4.3 Preparation of polymer-DNA complexes (polyplexes)

DNA-polymer complexes for each of the chitosan polymers were made in different ratios of phosphor/nitrogen (P/N) of 1:1, 1:3, 1:5 and 1:10 (§ 2.2.2). P refers to the amount of phosphor (+) present on each DNA vector in relation to N, the amount of nitrogen (-) present on each polymer chain. Firstly, it was calculated what the average weight of each monomer of the chitosan polymers would be. The answer from this equation was divided by the average MW (Da) of the polymer as determined with SEC/MALLS analysis. This answer would give an indication of the amount of monomers in a polymer chain. Because of the fact that only one nitrogen molecule is present in each chitosan monomer (figure 1.5, § 1.3.2.2.2.3) the amount of nitrogen was indirectly determined. The same calculation was made for the phosphor in the DNA molecule. Because

DNA is a double helix, for each of the base pairs (bp) of the pGL3-control DNA vector there will be 2 phosphor atoms present. The pGL3-control vector consists of 5256 bp and therefore 10512 (5256 x 2) phosphor atoms. The average MW of the pGL3-control vector is 3 204 437 Da (determined by the Field Applications Specialist for Promega). From these results the correct amount of pGL3-control vector (in sodium sulphate solution) could be detemined to be added to each of the chitosan solutions to be able to produce complexes of P/N ratios of 1:1, 1:3, 1:5 and 1:10.

For each of the chitosan polymers its corresponding polymer solution (1x, 3x, 5x en 10x) prepared in 5 mM sodium acetate buffer and the DNA solution prepared in 5 mM sodium sulphate solution were preheated to 55 °C separately. The DNA solution contained the pGL3-control vector for each experimental well to receive 0.5 μg/well. An equal volume of each polymer and DNA solution were quickly mixed together in a sterile 1.5 ml Eppendorf microcentrifuge tube and vortexed for 30 seconds. As described by Mao *et al.* (2001:402) the final volume of the mixture in each preparation was limited to below 500 μl to yield uniform nanoparticles. The nanoparticles prepared by this procedure were used for the transfection experiments without further purification.

4.4.3.1 Characterisation of polyplexes with TEM

The complexes were examined by transmission electron microscopy (TEM) (Phillips CM10 TEM, Carl Zeiss (Pty) Ltd., New York, USA). The freshly made nanoparticles of each chitosan polymer in the different P/N ratios were placed on a glow discharged carbon grid and allowed to air-dry for a few minutes. The sample was negatively stained with 1 % (w/v) uranyl acetate (BioRad, Massachusetts, USA) for viewing on the microscope. After viewing of the complexes and measurement of the size of the complexes a TEM micrograph was taken.

4.4.3.2 Electrophoresis (gel agarose) of complexes

The complexes were electrophoresed on a 1 % (w/v) agarose gel in 1x TAE buffer with 0.01 % EtBr for 60 minutes at 80 V. After 60 minutes the gel was illuminated on an UV illuminator to show the location of the DNA. The volume of the sample loaded into the well was 10 µl of the DNA/chitosan complex suspension.

4.4.4 Preparation of Transfectam® complexes

Transfectam® reagent (annexure 2) for the transfection of eukaryotic cells is a synthetic, cationic lipopolyamine molecule named dioctadecylamidoglycyl spermine (DOGS) (figure 1.2, § 1.3.2.2.1). Transfectam® allows efficient transfection of a wide range of eukaryotic cells (Promega, 2002c:1). For this reason Transfectam® was used as the positive control in the transfection experiments conducted in the COS-1 cell line. For each 24-well plate used, two sterile microcentrifuge tubes were used to prepare the Transfectam® complexes. In the first tube 12.6 µl of pGL3-control vector DNA was mixed with 487.4 µl of cell culture medium (without serum). The second tube contained 6.0 µl of Transfectam® mixed with 494.0 µl of cell culture medium (without serum). These two microcentrifuge tubes were then mixed together by vortexing for 30 seconds. For each of the experimental wells of the positive control 250 µl of the Transfectam® complexes were used.

4.4.5 Cell culture seeding for transfection experiments

24 hours before the start of the transfection experiment COS-1 cells were seeded onto 24-well tissue culture plates. A cell suspension of 5.0 x 10^5 cells/ml was obtained as described in § 4.3.4. A volume of 1 ml of this cell suspension was transferred to each of the wells in the 24-well plates. Two 24-well plates were

used for each polymer tested. The 24-well plates were then placed in the incubator at a temperature of 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2.

4.4.6 Administration of experimental treatments to COS-1 cells

The confluency of the COS-1 cell culture was microscopically checked 24 hours after the seeding of the cells in the 24-well plates. If the confluency proved to be 80 % or higher the cell culture media was aspirated from the wells. Fenol red free Phosphate buffered saline (PBS) (BioWhittaker™, Cambrex, Walkersville, USA) was warmed to 37 °C and 500 µl was added twice to each well for washing and then discarded. Each chitosan or quaternised chitosan polymer solution with different P/N ratio (§ 4.4.3) was diluted with culture medium (without serum) in order for each well to receive 250 µl cell culture media (without serum) containing 3.2 µl DNA/well. The naked DNA served as the negative control and consisted of only 0.5 µg/well pGL3-control vector DNA and cell culture medium (without serum) to 250 µl/well. Transfectam® served as the positive control and again 250 µl of the complexes (§ 4.4.4) containing 3.2 µl DNA was added per well. The polymer complexes, Transfectam® complexes or naked DNA were added in triplicate to the respective wells (figure 4.6).

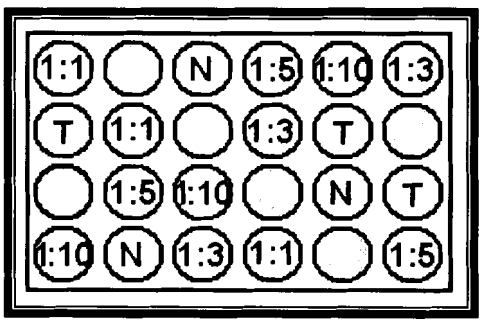


Figure 4.6 The 24-well plate layout of each transfection experiment. Polymer complexes (1:1, 1:3, 1:5, 1:10), Transfectam® complexes (T) or naked DNA (N) were randomly arranged on the plate. Coloured wells received no treatment.

The 24-well plate was then incubated at a temperature of 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2 for 4 hours. After this incubation period the experimental medium on the cells were aspirated and the wells were washed twice with 500 µl warmed PBS. Fresh, warmed (37 °C) culture medium (with serum) of 1 ml was added to each well of one 24-well plate and was again incubated at 37 °C in a humidified atmosphere of 92.5 % air and 7.5 % CO_2 for another 48 hours. The second 24-well plate was used after the 4 hour incubation period for the toxicity assay.

4.4.7 Toxicity determination

The MTT assay was conducted to determine the number of viable cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), a pale yellow substate is oxidised to a purple formazan crystal by the active mitochondria of living cells (Mosmann, 1983:57). Because the oxidation only takes place when mitochondrial reductase enzymes are active, the conversion can be directly related to the number of viable cells. These coloured formazan crystals can be spectrophotometrically analysed after dissolving the crystals and this then gives an indication of the viability of the cells. Mossman (1983:55) investigated several organic solvents for dissolving the formazan crystals and isopropanol was found to be the most suitable. Proof of linearity between different amount of cells and the optical densities should be established to ensure that the results can be expressed as a percentage of the control.

For the proof of linearity of MTT in the COS-1 cell line, the cells in the culture flasks were treated with trypsin-versene and incubated at 37 °C for 15 minutes (§ 4.3.3). Cells were seeded onto a third 24-well plate in the concentrations as depicted in figure 4.7.

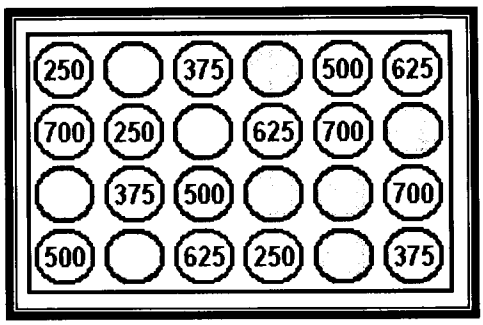


Figure 4.7 Cell quantities (x 10^3) per well used for the linearity graph in the MTT assay.

This 24-well plate was also incubated for 24 hours for the cells to adhere to the bottom of the plate. MTT solution, at a concentration of 0.25 mg/ml in PBS was prepared, filtered sterile through a 0.2 μm filter and stored at - 20 °C until use. After the initial 24 hour incubation period, the cell culture medium was aspirated. The cells were washed twice with preheated PBS to remove phenol red and FBS from the cell culture medium. After each wash the PBS was discarded. The prepared MTT solution was warmed to 37 °C and 200 μl was added to each well and incubated for another 4 hours for the formazan crystals to form. After the 4 hour incubation period the MTT solution was removed from the cells and 250 μl isopropanol (SIGMA, Steinheim, Germany) was added to each well to dissolve the crystals. The 24-well plate was placed back in the incubator for 5 minutes. The optical density of each well was measured with a Multiscan RC microtitre plate reader (Labsystems, Vantaa, Finland) at 560 nm. A graph was constructed with the *average of the optical densities* vs *cells/well.* A correlation coefficient was determined for the graph to prove linearity.

The cytotoxicity evaluation of the treatments was conducted similarly and simultaneously to the evaluation for the proof of linearity. The second 24-well plate that received treatments (§ 4.4.6) was removed after the 4 hour incubation period and the experimental media was removed from the cells. The cells were washed twice with preheated PBS and after each wash the PBS was removed. 200 μl of the prepared MTT solution (preheated) was added to each well and

incubated for another 4 hours. After the 4 hour incubation period the MTT solution was removed from the cells and 250 µl isopropanol was added to each well to dissolve the crystals. The 24-well plate was placed back in the incubator for 5 minutes. The optical density of each well was measured with the Multiscan RC microtitre plate reader at 560 nm. A bar graph was constructed and each treatment was expressed as a percentage of the control. The optical density control value for 5.0 x 10^5 cells/well was taken as 100 %.

4.4.8 Analysis of protein content

The protein content in the cells after transfection was determined with the Bradford protein assay for 96-well plates (Bradford, 1976:248). This assay determines protein concentrations between 0.1 - 0.4 mg/ml by measuring absorbance in the 595 nm region. The protein concentrations proved to be linear in this range (0.1 - 0.4 mg/ml) when using bovine serum albumin (BSA) (SIGMA, Steinheim, Germany) as standard.

Table 4.4 Dilutions of BSA used as protein standards.

Protein concentration	Dilution in test tubes	
	Volume of 2 mg/ml BSA	Volume of Reporter Lysis Buffer (RLB)
0 mg/ml (blank)	0 µl	100 µl
0.1 mg/ml	5 µl	95 µl
0.4 mg/ml	20 µl	80 µl
0.7 mg/ml	35 µl	65 µl
1.0 mg/ml	50 µl	50 µl
1.4 mg/ml	70 µl	30 µl

A blank sample (no protein), standard samples (range of known protein concentrations) and unknown samples (testing samples) were used in every assay. A sufficient amount of Bradford Reagent (SIGMA, Steinheim, Germany) was withdrawn and equilibrated to RT. A 2 mg/ml solution of BSA was prepared in dd.H_2O and dilutions as described in table 4.4 were accurately measured. The unknown samples were used as they were, or diluted for the concentration to fall between the specific ranges.

A 96-well cell culture cluster (Costar®, Corning Incorporated, New York, USA) was used for the assay procedure. All the samples were analysed in duplicate, therefore 2 x 5 µl of each tube (from the blank, standards and unknown samples) were added to separate wells of the 96-well plate. A volume of 250 µl of the equilibrated Bradford Reagent was added to each well using an Eppendorf micropipette. The plate was immediately mixed in the shaking facility of the Multiscan RC microtitre plate reader for 30 seconds. The plate was then incubated at RT for 15 minutes and the absorbance of the plate was read at 560 nm. The netto absorbance values were determined by subtracting the average blank value from the average of all other values. The netto absorbance was plotted against the known protein concentrations of the standards. The unknown protein concentrations were then determined from the graph constructed for the standard protein concentrations.

4.4.9 Luciferase reporter gene expression assay

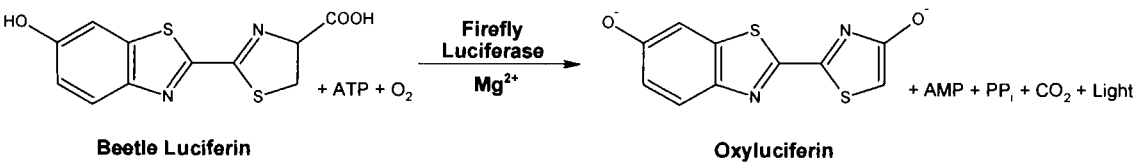


Figure 4.8 Bioluminescent reaction catalysed by firefly luciferase (Promega, 2002a:1).

Cell culture media from the first 24-well plate (§ 4.4.6) were aspirated 48 hours post-transfection. The cells were rinsed twice with PBS and care was taken not to dislodge the attached cells. As much as possible of the PBS were removed. Enough 1x Reporter Lysis Buffer (RLB) (Promega Corporation, Madison, USA) was added to cover the cells (200 µl/well). According to the manufacturer's instructions a single freeze-thaw cycle was performed to ensure complete lysis. The attached cells were scraped from the dish and all the liquid was transferred to a Eppendorf microcentrifuge tube and placed on ice. The microcentrifuge tube was vortexed for 15 seconds and then centrifuged at 12 000 x g for 2 minutes at 4 °C. The supernatant was transferred to a new microcentrifuge tube and luciferase reporter gene expression assay was then started. The luminometer was programmed for the appropriate delay and measurement times. The 96-well opaque, solid black plate (Costar®, Corning Incorporated, New York, USA) containing 20 µl of cell lysate per well was placed into the luminometer. An anount of 100 µl of Luciferase Assay Reagent (LAR) was added per well and then the well was read immediately. The light intensity of the reaction (figure 4.8) proved to be nearly constant for about 1 minute and then decayed slowly with the half-life being approximately 10 minutes (Promega, 2002a:6).

4.5 DATA ANALYSIS AND STATISTICAL EVALUATION

For each treatment of the different chitosan polymers tested, a luminescence value was obtained expressed in relative light units (RLU). This value was normalised against the protein value obtained from the protein assay. Toxicity values with the MTT assay were determined and expressed as a % of the control.

Statistical significant differences between the means of the transfection efficiency values of the different treatments of a specific polymer were evaluated with repeated variance analysis (Repeated ANOVA) with $p < 0.05$. To determine if statistical significant differences exists between the means of treatments

between the different polymers, Tukey intervals at a 5 % level of significance were calculated (STATISTICA (data analysis software system), version 6. 2003. StatSoft, Inc. Tulsa, USA).

4.6 CONCLUSION

In this chapter the selection of a suitable plasmid was described and some general information was given about pGL3 luciferase reporter vectors. The propagation of the pGL3-control plasmid in competent *E. coli* bacteria was described. The process *transformation* where plasmids were inserted into bacteria was explained. Competent bacterial cells were prepared to facilitate the transformation process. Growth of E. Coli bacteria was described as well as the storage of competent bacterial cells. After the propagation procedure the plasmids were isolated and purified by using the Wizard® *Plus* Maxipreps DNA purification system. The plasmid DNA obtained was identified with electrophoresis through an agarose gel.

The culturing and maintenance procedures used for the COS-1 cell culture was described. The *in vitro* transfection experiments conducted in this study was explained in detail and how the DNA concentration has been determined. The preparation of polymer solutions, polymer/DNA complexes (polyplexes), Transfectam® complexes and the characterisation of polyplexes with Transmission Electron Microscopy (TEM) was also described. The gel electrophoresis experiment for the synthesised complexes were also explained.

All the experimental treatments was administered to the COS-1 cell culture. The cytotoxicity assay (MTT) performed on the COS-1 cell culture proved to be a quick and easy way to determine cytotoxicity of the complexes. The results obtained with this assay will be very valuable to give an overall estimate of the true potential of the polymers tested. Analysis of protein content, necessary to

compare each set of data to another was conducted by the Bradford protein assay and the luciferase reporter gene expression assay was also conducted.

CHAPTER 5

EVALUATION OF THE TRANSFECTION PROPERTIES OF CHITOSAN AND QUATERNISED CHITOSAN POLYMERS: RESULTS AND DISCUSSION

5.1 INTRODUCTION

Research in gene therapy has been focused on the development of suitable vectors that, while exhibiting adequate features for *in vivo* use, would also mediate efficient intracellular delivery of genetic material. And although the majority of clinical trials have been based on the use of viral vectors, cationic polymers are emerging as promising non-viral vectors for genetic medicines due to their safety and versatility. The use of cationic polymers in the delivery of genes has been described by different authors (Briones *et al.*, 2001:709; Illum *et al.*, 2001:82; Thanou *et al.*, 2001:153; Sato *et al.*, 2001:2075).

The problems associated with vectors for transfection studies such as insufficient transfection efficiency, strong cytotoxicity and inhibition by serum have to be overcome before vectors can be applied for practical use.

Commercially available chitosan and chitosan oligomer as well as the synthesised quaternised chitosan polymers all display much potential as a gene delivery system. The ability of the chitosan, chitosan oligomer and the synthesised quaternised polymers to condense plasmid DNA was determined using TEM and agarose gel electrophoresis. Reporter gene expression was determined in COS-1 cells and toxicity was also determined.

This chapter investigates the effect of different quaternised chitosan polymers and quaternised chitosan oligomers as well as the parent chitosan on the

transfection efficiency of the pGL3 luciferase plasmid in COS-1 cells, as well as the toxicity of these complexes.

5.2 EVALUATION OF THE TRANSFECTION PROPERTIES OF CHITOSAN AND QUATERNISED CHITOSAN POLYMERS: RESULTS AND DISCUSSION

5.2.1 Gel electrophoresis of polyplexes

In figures 5.1 – 5.3 the results of the gel electrophoresis are depicted.

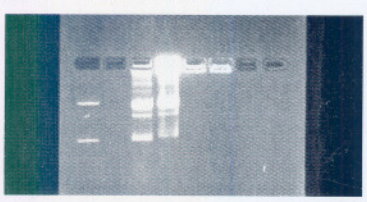
Gel electrophoresis	Polymers
Lane 1 2 3 4 5 6 7 8 	• TMC HH • TEC ML

Figure 5.1 Representation of the gel electrophoresis of the polymers listed above. Lane 1 represents naked DNA as control, lane 2 - empty, lane 3 – ratio 1:1, lane 4 – ratio 1:3, lane 5 – ratio 1:5 and lane 6 – ratio 1:10.

As observed by the gel electrophoresis depicted in figure 5.1, naked DNA and polyplexes with a substantial amount of free DNA (ratio 1:1 and 1:3) moved along the gel, whereas polyplexes where most of the DNA had been complexed (ratio 1:5 and 1:10) were retained inside the loading well.

Gel electrophoresis (figure 5.2) confirmed that the naked DNA and polyplexes or oligoplexes with a substantial amount of free DNA (ratio 1:1) moved along the gel, whereas polyplexes where most of the DNA had been complexed (ratio 1:3, 1:5 and 1:10) were retained inside the loading well.

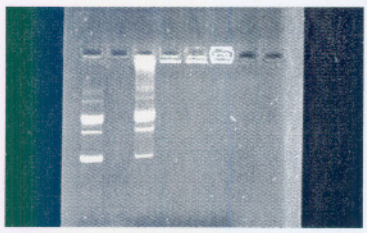
Gel electrophoresis	Polymers
Lane 1 2 3 4 5 6 7 8 	• TMC HL • TMC MH • TMC H • TEC MH • TEO H • Primex H • Primex O

Figure 5.2 Representation of the gel electrophophoresis of the polymers listed above. Lane 1 represents naked DNA as control, lane 2 - empty, lane 3 – ratio 1:1, lane 4 – ratio 1:3, lane 5 – ratio 1:5 and lane 6 – ratio 1:10.

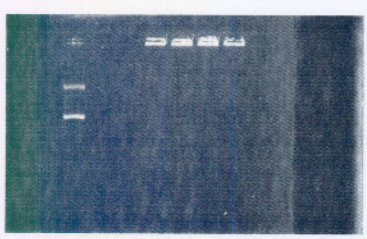
Gel electrophoresis	Polymers
Lane 1 2 3 4 5 6 7 8 	• TMC ML • TMC H • TMC L • TMO H • TMO L • TEO L • Primex M • Seacure

Figure 5.3 Representation of the gel electrophoresis of the polymers listed above. Lane 1 represents naked DNA as control, lane 2 - empty, lane 3 – empty, lane 4 – ratio 1:1, lane 5 – ratio 1:3, lane 6 – ratio 1:5 and lane 7 – ratio 1:10.

Gel electrophoresis (figure 5.3) confirmed that the naked DNA moved along the gel, whereas polyplexes or oligoplexes where most of the DNA had been complexed (ratio 1:1, 1:3, 1:5 and 1:10) were retained inside the loading well.

5.2.2 Transfection efficacy of chitosan polymers

5.2.2.1 TMC HH

5.2.2.1.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.4. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

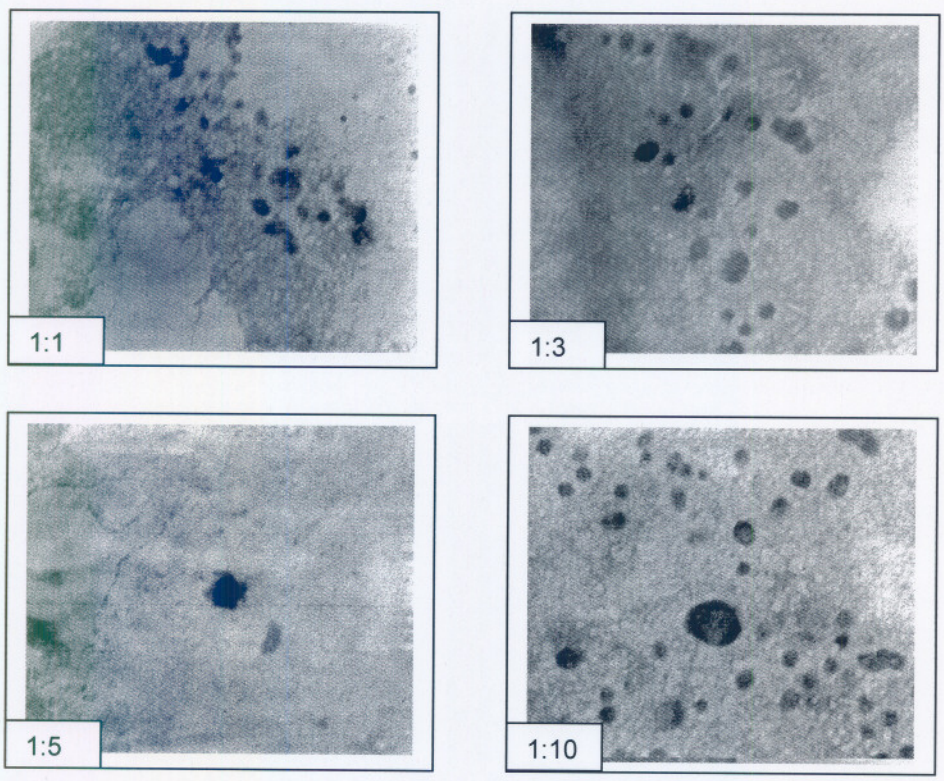


Figure 5.4 TEM micrographs of TMC HH/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TMC HH polymer condensed the plasmid into oval and round shaped particles with mean sizes of 35.3 nm (1:1), 36.4 nm (1:3), 47.1 nm (1:5) and 35.0 nm (1:10), respectively.

5.2.2.1.2 *Toxicity*

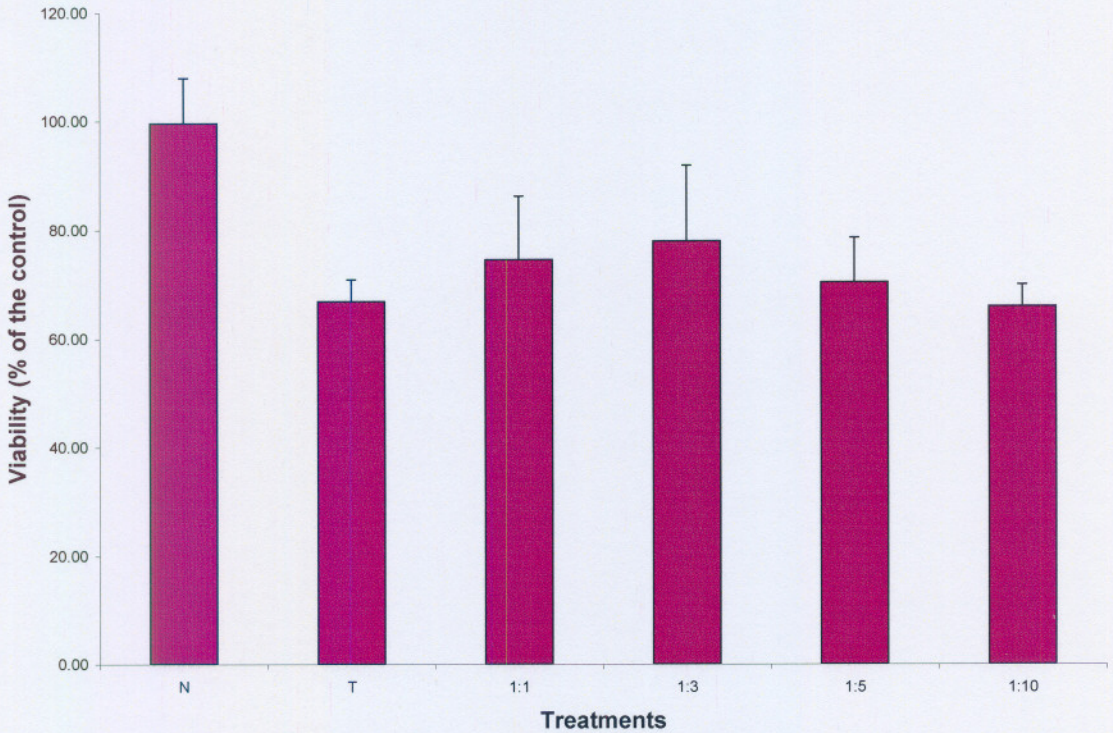


Figure 5.5 Cell viability after treatment of COS-1 cells with TMC HH/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC HH/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay (§ 4.4.7) after a 4 hour incubation period were found to be 99.53 ± 8.40 % for naked DNA (N), 66.86 ± 4.00 % for Transfectam®/DNA complexes (T), 74.55 ± 11.59 % for TMC HH/DNA polyplexes (1:1), 77.87 ± 13.82 % for TMC HH/DNA polyplexes (1:3), 70.28 ± 8.15 % for TMC HH/DNA polyplexes (1:5) and 65.81 ± 3.99 % for TMC HH/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.5, it can be conluced that the TMC HH/DNA polyplexes exhibited higher mitochondrial activity and are therefore considered less toxic compared to the Transfectam®/DNA complexes.

5.2.2.1.3 *Gene transfection*

TMC HH/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment. The Transfectam®/DNA complexes showed a 15-fold increase in transfection efficiency compared to the naked DNA (figure 5.6).

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.4-, 1.6-, 1.5- and 1.7 times respectively. Although an increased transfection in COS-1 cells with polyplexes was observed, no statistical significant difference between any of the tested polyplex ratios compared to naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

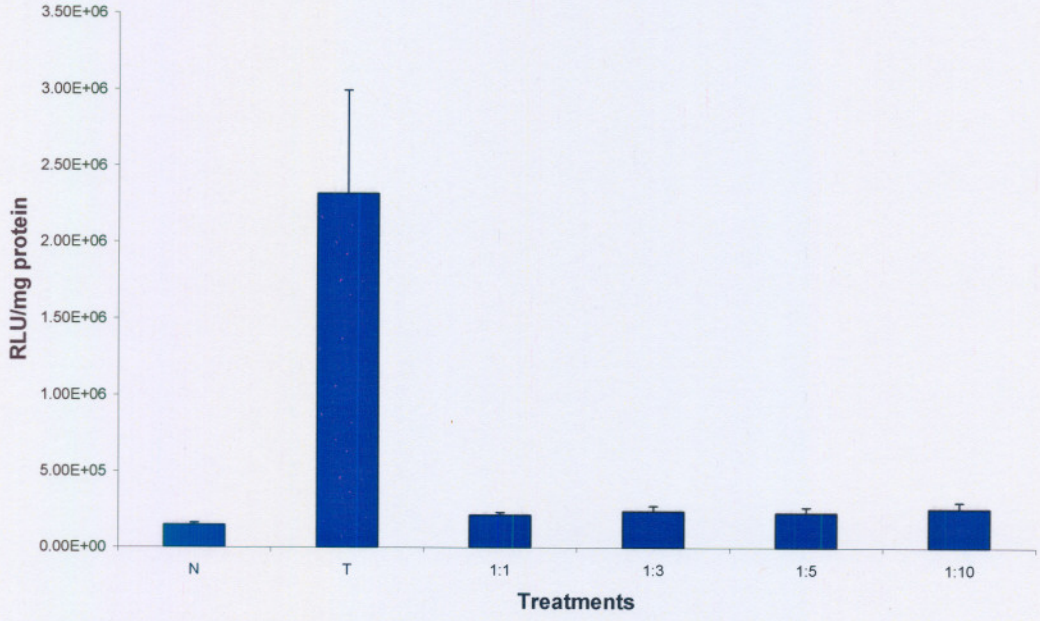


Figure 5.6 Transfection efficiencies in COS-1 cells obtained with TMC HH/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC HH/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.2 TMC HL

5.2.2.2.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.7 (magnification of the microscope set at 105 000 and voltage at 100 V). The formed polyplexes are indicated by darker regions on each micrograph.

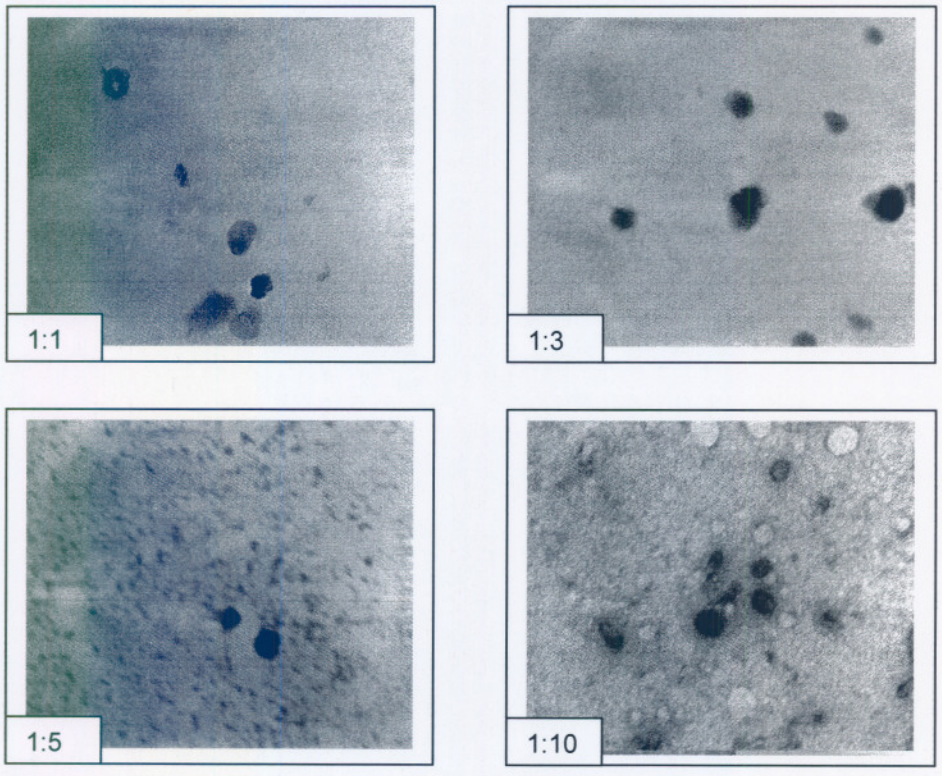


Figure 5.7 TEM micrographs of TMC HL/DNA polyplexes. Different ratios indicated in each micrograph.

The mean sizes measured with the microscope was 56.2 nm (1:1), 66.5 nm (1:3), 35.7 nm (1:5) and 51.2 nm (1:10), respectively.

5.2.2.2.2 *Toxicity*

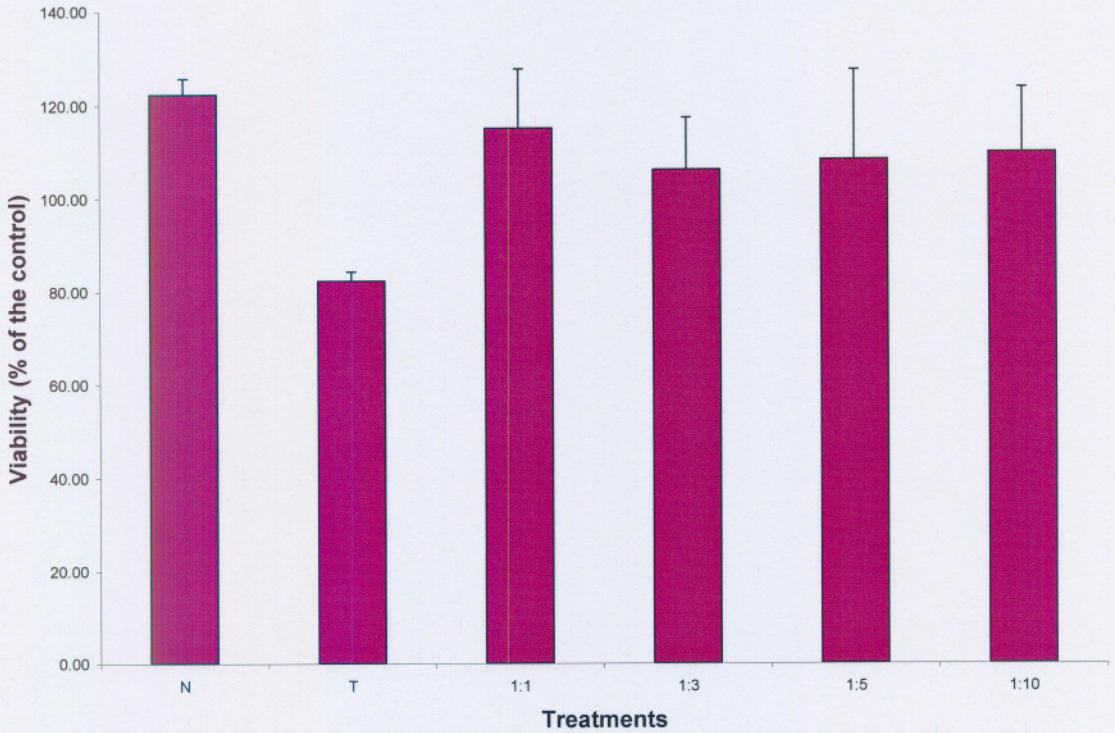


Figure 5.8 Cell viability after treatment of COS-1 cells with TMC HL/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC HL/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 122.29 ± 3.31 % for naked DNA (N), 82.34 ± 1.99 % for Transfectam®/DNA complexes (T), 115.01 ± 12.44 % for TMC HL/DNA polyplexes (1:1), 105.95 ± 11.24 % for TMC HL/DNA polyplexes (1:3), 108.12 ± 18.98 % for TMC HL/DNA polyplexes (1:5) and 109.54 ± 13.79 % for TMC HL/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.8, the TMC HL/DNA polyplexes exhibited higher mitochondrial activity when compared the Transfectam®/DNA complexes and therefore indicated less toxicity.

5.2.2.2.3 *Gene transfection*

TMC HL/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment. Transfectam®/DNA complexes showed a 20-fold increase in transfection efficiency compared to the naked DNA. Increased transfection efficiency with TMC HL/DNA polyplexes proved to be 1.6-fold for the 1:1 ratio, 1.66-fold for the 1:3 ratio, 1.82-fold for the 1:5 ratio and 1.85-fold for the 1:10 ratio when compared to the negative control (figure 5.9). Although a general increased transfection in COS-1 cells with polyplexes was observed, no statistical significant difference between any of the tested polyplex ratios and naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

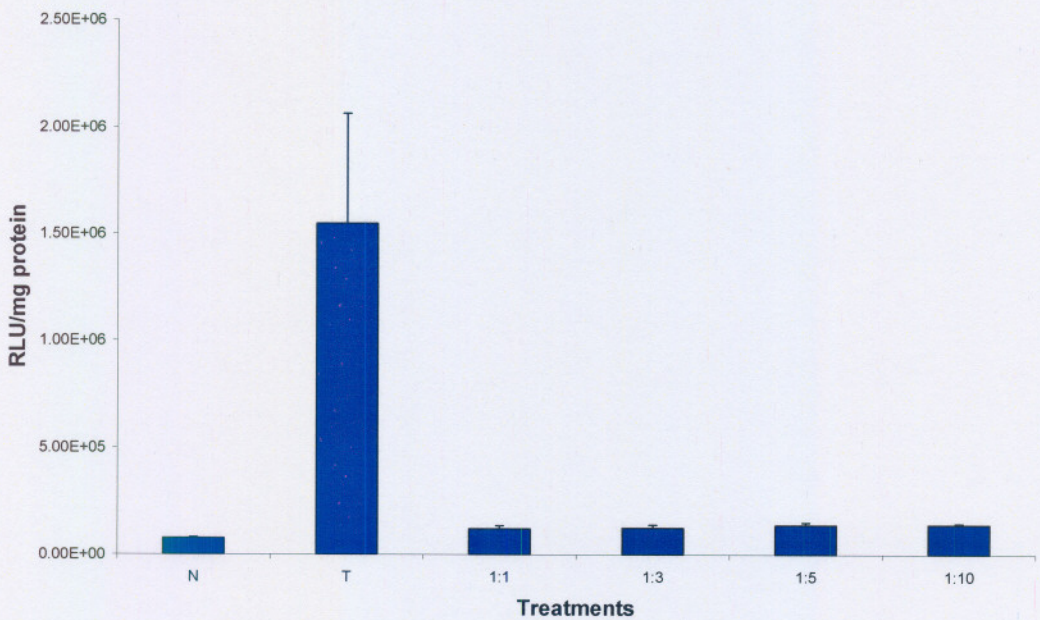


Figure 5.9 Transfection efficiencies in COS-1 cells obtained with TMC HL/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC HL/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.3 TMC MH

5.2.2.3.1 Confirmation of complex formation

TEM micrographs of TMC MH/DNA polyplexes are shown in figure 5.10. The darker regions on each micrograph indicate the polymer/DNA polyplexes.

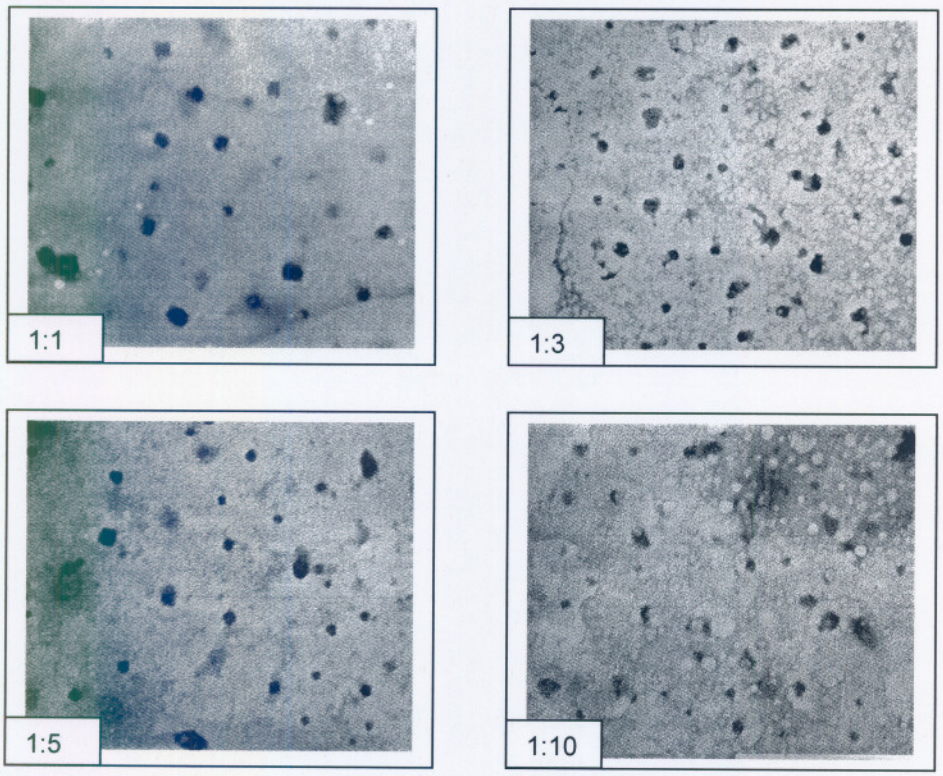


Figure 5.10 TEM micrographs of TMC MH/DNA polyplexes. Different ratios indicated in each micrograph.

The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. From the TEM micrographs it was observed that the TMC MH polymer condensed plasmid into particles with mean sizes of 37.2 nm for ratio 1:1, 40.1 nm for ratio 1:3, 41.7 nm for ratio 1:5 and 38.8 nm for ratio 1:10, respectively.

5.2.2.3.2 *Toxicity*

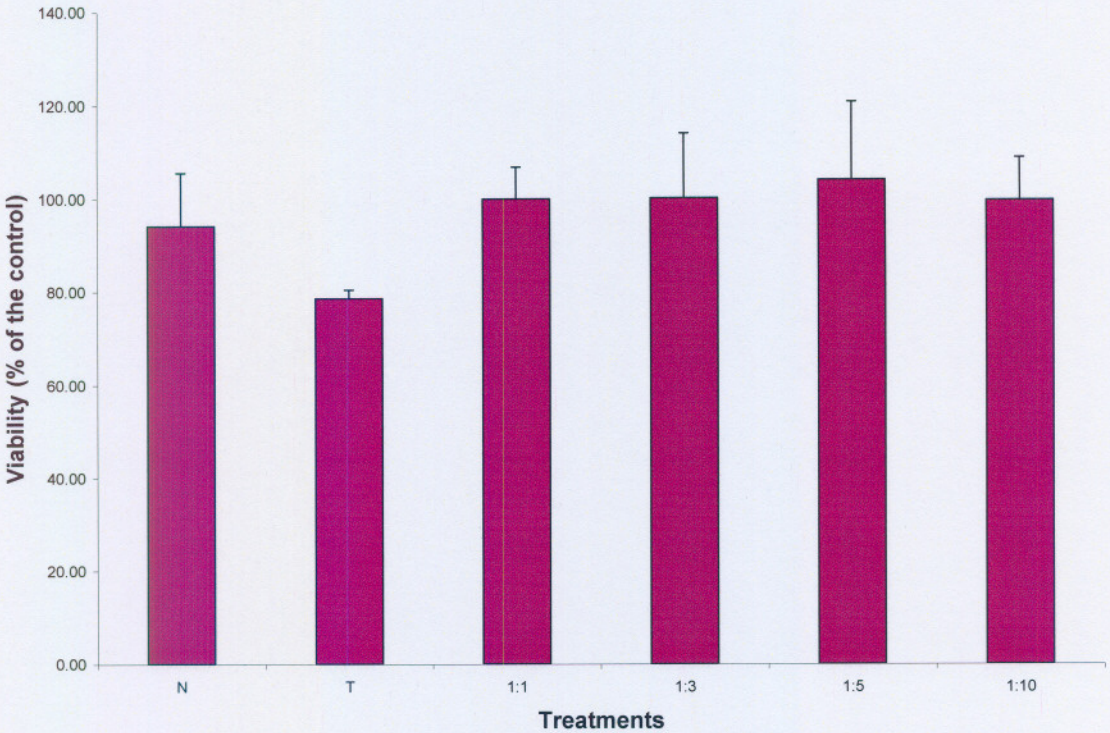


Figure 5.11 Cell viability after treatment of COS-1 cells with TMC MH/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC MH/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The MTT assay used to establish the viability of COS-1 cells after a 4 hour incubation period were found to be 94.18 ± 11.38 % for naked DNA (N), 78.61 ± 1.82 % for Transfectam®/DNA complexes (T), 99.87 ± 6.78 % for TMC MH/DNA polyplexes (1:1), 99.96 ± 13.93 % for TMC MH/DNA polyplexes (1:3), 103.81 ± 16.72 % for TMC MH/DNA polyplexes (1:5) and 99.36 ± 9.08 % for TMC MH/DNA polyplexes (1:10) compared to the control. All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.11, the TMC MH/DNA polyplexes exhibited higher mitochondrial activity when compared the Transfectam®/DNA complexes and therefore indicated less toxicity.

5.2.2.3.3 *Gene transfection*

TMC MH/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment. Transfectam®/DNA complexes, the positive control, showed a 10-fold increase in transfection efficiency compared to the naked DNA.

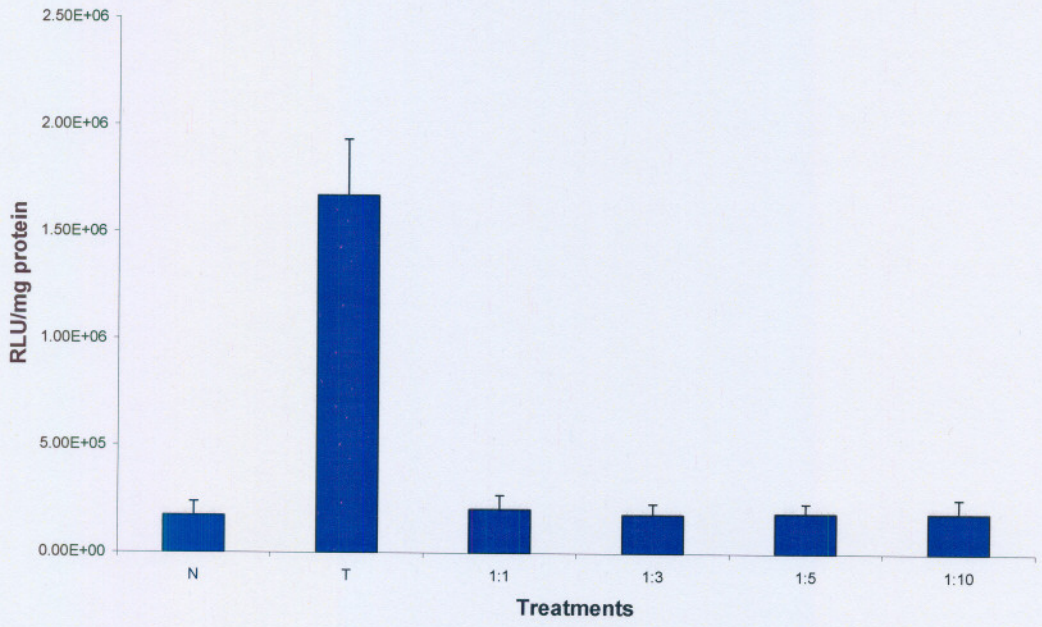


Figure 5.12 Transfection efficiencies in COS-1 cells obtained with TMC MH/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC MH/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.18-, 1.04-, 1.09- and 1.08 times respectively (figure 5.12). Although a general increased transfection in COS-1 cells with polyplexes was observed, no statistical significant difference between any of the tested polyplex ratios and naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.4 TMC ML

5.2.2.4.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM). The magnification and voltage of the microscope was set at 105 000 and 100 V respectively, as for all other polyplex micrographs. The polymer/DNA polyplexes are indicated by darker regions on each micrograph as shown in figure 5.13.

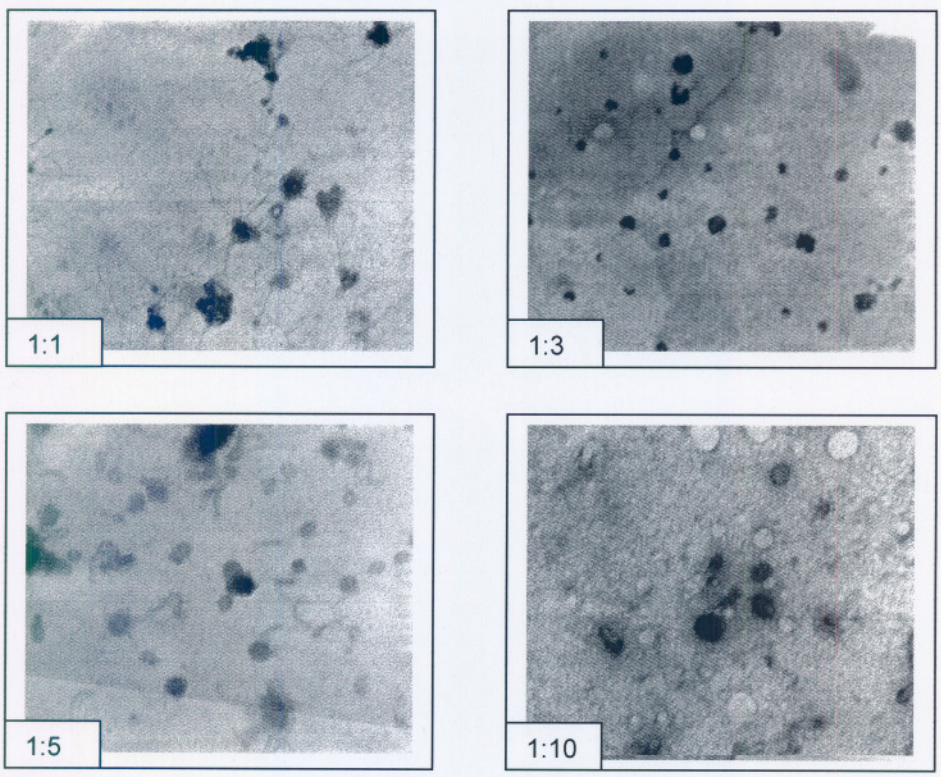


Figure 5.13 TEM micrographs of TMC ML/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TMC ML polymer condensed the plasmid into oval and round shaped particles with mean sizes of 45.8 nm (1:1), 36.4 nm (1:3), 42.3 nm (1:5) and 48.9 nm (1:10), respectively.

5.2.2.4.2 *Toxicity*

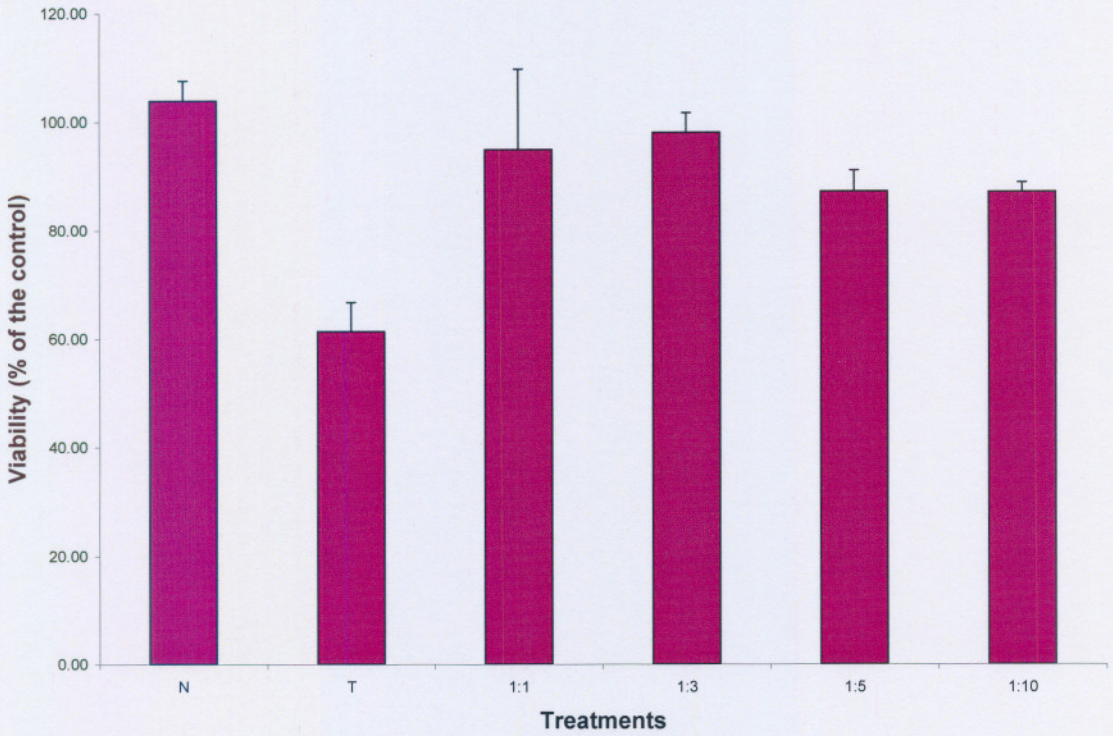


Figure 5.14 Cell viability after treatment of COS-1 cells with TMC ML/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC ML/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with MTT assay after a 4 hour incubation period were found to be 103.83 ± 3.75 % for naked DNA (N), 61.39 ± 5.27 % for Transfectam®/DNA complexes (T), 94.82 ± 14.77 % for TMC MH/DNA polyplexes (1:1), 97.87 ± 3.55 % for TMC MH/DNA polyplexes (1:3), 86.97 ± 3.88 % for TMC MH/DNA polyplexes (1:5) and 86.82 ± 1.75 % for TMC MH/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.14, all the TMC MH/DNA polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and therefore indicated less toxicity.

5.2.2.4.3 Gene transfection

TMC ML/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment. As expected Transfectam®/DNA complexes showed a 23-fold increase in transfection efficiency compared to the naked DNA.

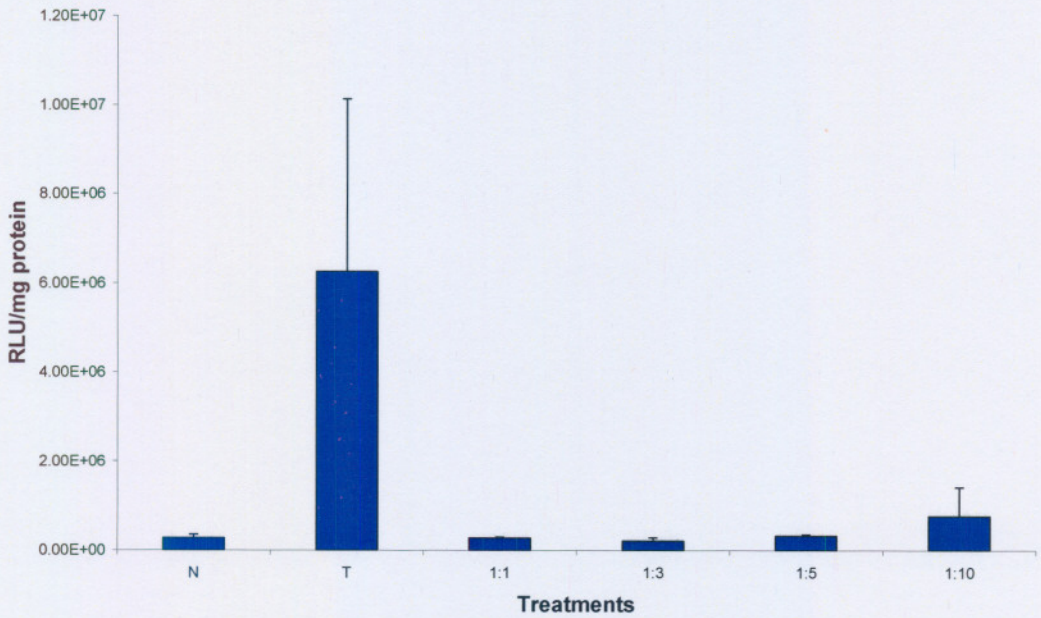


Figure 5.15 Transfection efficiencies in COS-1 cells obtained with TMC ML/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC ML/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed transfection of 1.0-, 0.8-, 1.2- and 2.8 times respectively (figure 5.15). Although an increased transfection in COS-1 cells with 1:5 and 1:10 polyplexes was observed, no statistical significant difference between any of the tested polyplex ratios and naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.5 TMC H

5.2.2.5.1 Confirmation of complex formation

The magnification and voltage of the TEM microscope was set at 105 000 and 100 V respectively and micrographs of the polymer/DNA polyplexes were recorded. The darker regions on each micrograph, as illustrated in figure 5.16 indicate the formed complexes.

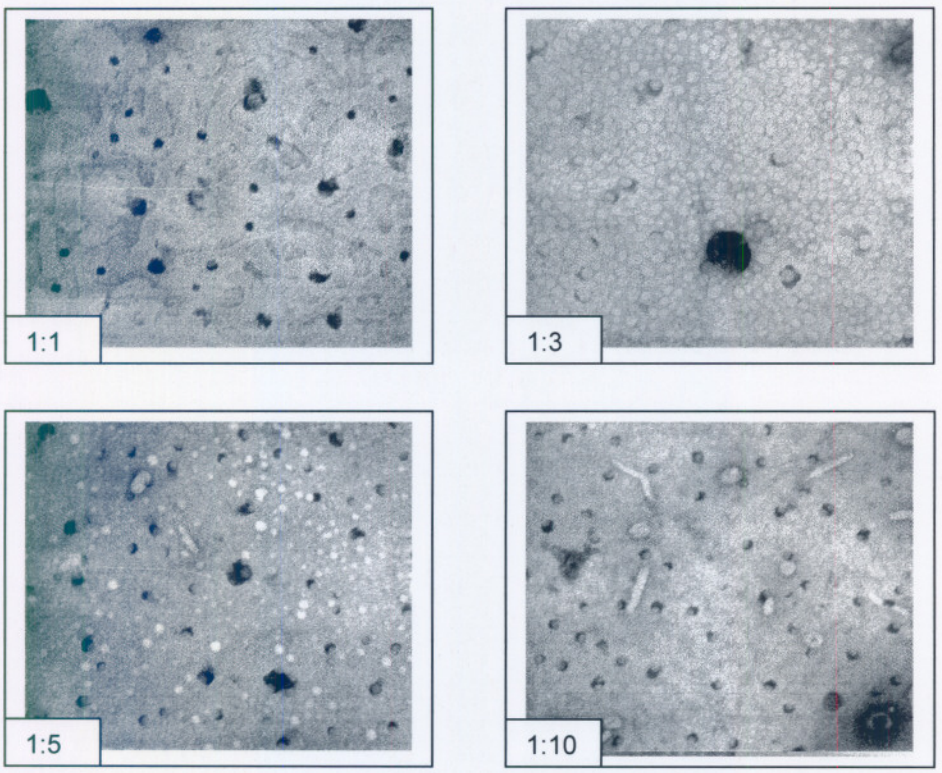


Figure 5.16 TEM micrographs of TMC H/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TMC H polymer condensed the plasmid into particles with mean sizes of 33.9 nm (1:1), 68.9 nm (1:3), 36.3 nm (1:5) and 45.8 nm (1:10), respectively.

5.2.2.5.2 *Toxicity*

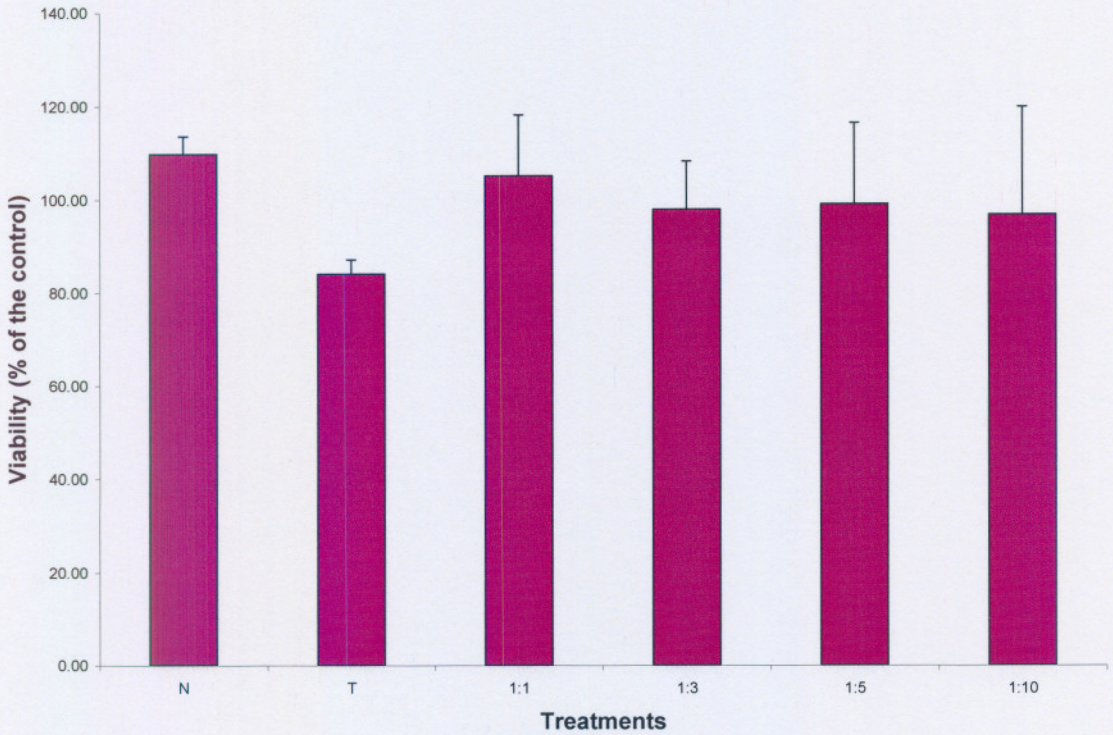


Figure 5.17 Cell viability after treatment of COS-1 cells with TMC H/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC H/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 109.80 ± 3.81 % for naked DNA (N), 84.08 ± 3.06 % for Transfectam®/DNA complexes (T), 105.07 ± 13.06 % for TMC H/DNA polyplexes (1:1), 97.81 ± 10.26 % for TMC H/DNA polyplexes (1:3), 98.95 ± 17.31 % for TMC MH/DNA polyplexes (1:5) and 96.59 ± 23.07 % for TMC H/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.17, all the TMC H/DNA polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.5.3 *Gene transfection*

TMC H/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.18). Transfectam®/DNA complexes showed a 16-fold increase in transfection efficiency compared to the naked DNA.

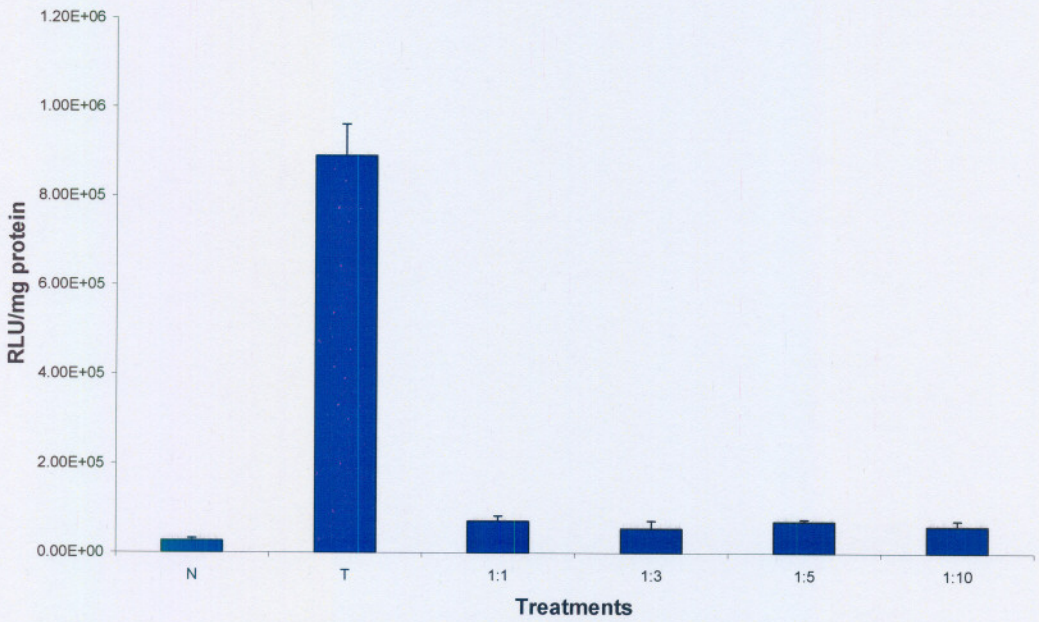


Figure 5.18 Transfection efficiencies in COS-1 cells obtained with TMC H/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC H/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 2.6-, 2.1-, 2.6- and 2.2 times respectively. An increased transfection in COS-1 cells with polyplexes was observed and the tested polyplex ratios differed statistically significant compared to naked DNA. However, the different polyplex ratios did not differ statistically significant from each other. The trend of increased transfection is however clear, compared to naked DNA.

The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.6 TMC M

5.2.2.6.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.19. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

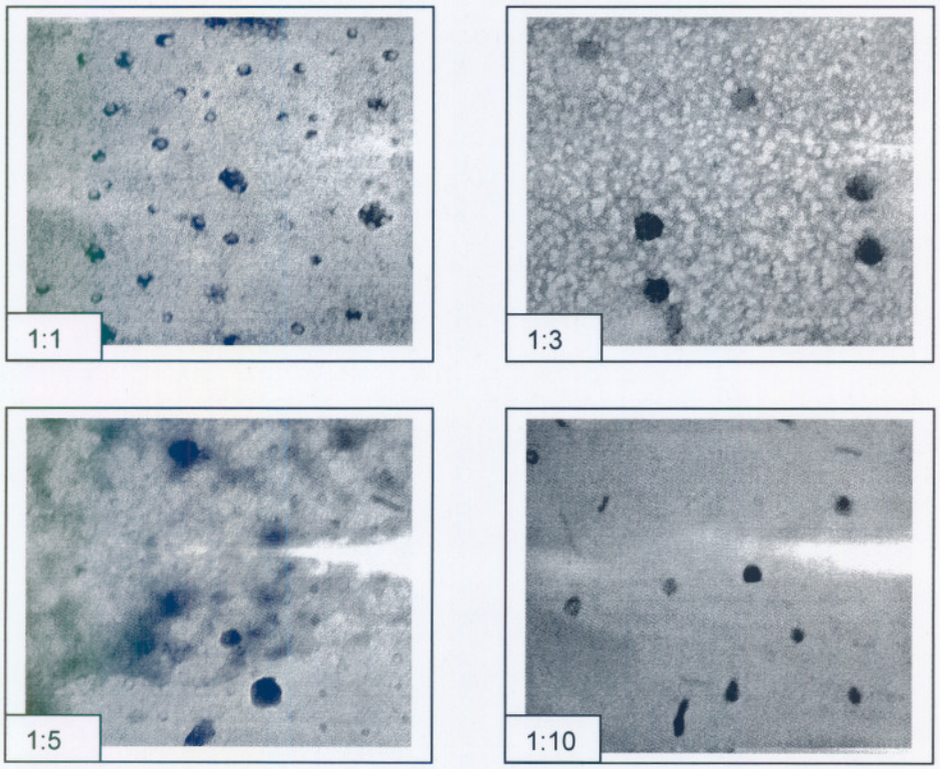


Figure 5.19 TEM micrographs of TMC M/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TMC M polymer condensed the plasmid into oval and round shaped particles with mean sizes of 52.3 nm (1:1), 55.7 nm (1:3), 57.6 nm (1:5) and 36.1 nm (1:10), respectively.

5.2.2.6.2 Toxicity

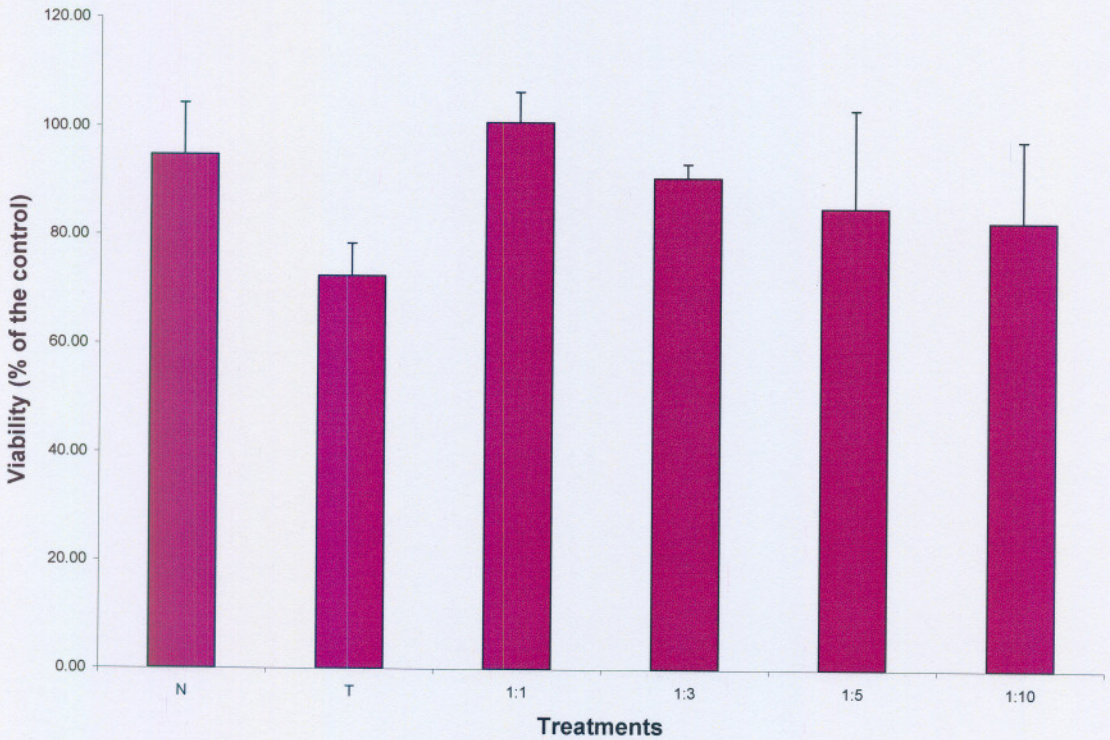


Figure 5.20 Cell viability after treatment of COS-1 cells with TMC M/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC M/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The MTT assay was used to determine the viability of COS-1 cells after a 4 hour incubation period. Values were found to be 94.67 ± 9.58 % for naked DNA (N), 72.35 ± 5.96 % for Transfectam®/DNA complexes (T), 100.75 ± 5.54 % for TMC M/DNA polyplexes (1:1), 90.48 ± 2.58 % for TMC M/DNA polyplexes (1:3), 84.90 ± 18.10 % for TMC M/DNA polyplexes (1:5) and 82.31 ± 14.97 % for TMC M/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.20, all the

TMC M/DNA polyplexes exhibited higher mitochondrial activity compared to the Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.6.3 Gene transfection

TMC M/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.21). As expected Transfectam®/DNA complexes showed a 12.5-fold increase in transfection efficiency compared to the naked DNA.

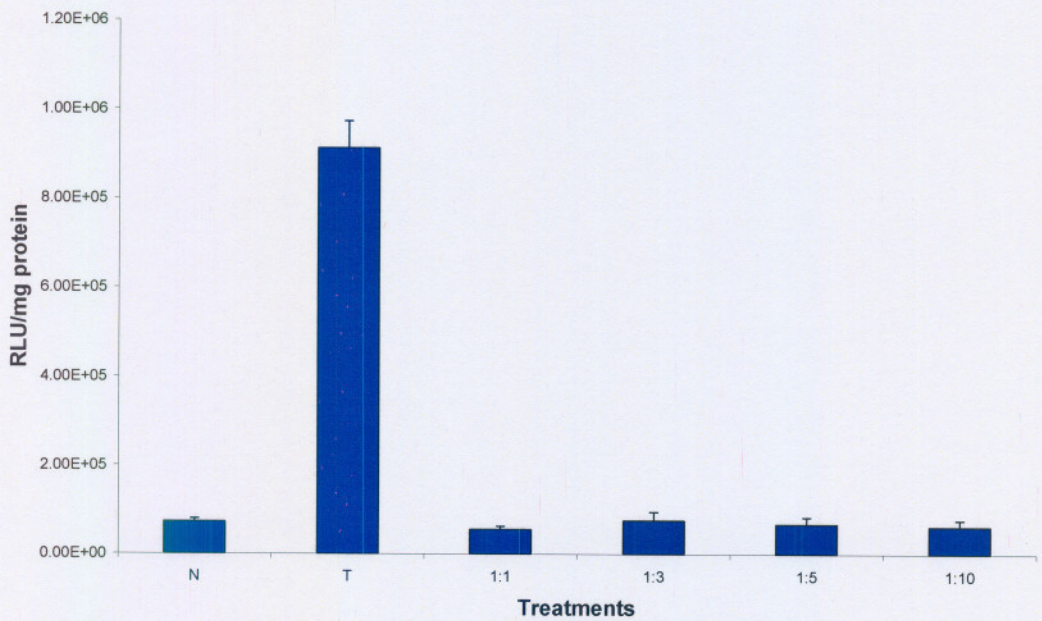


Figure 5.21 Transfection efficiencies in COS-1 cells obtained with TMC M/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC M/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed transfection of 0.77-, 1.05-, 0.93- and 0.85 times, respectively. These values did not differ statistically significant from the value obtained with naked

DNA. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.7 TMC L

5.2.2.7.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.22. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

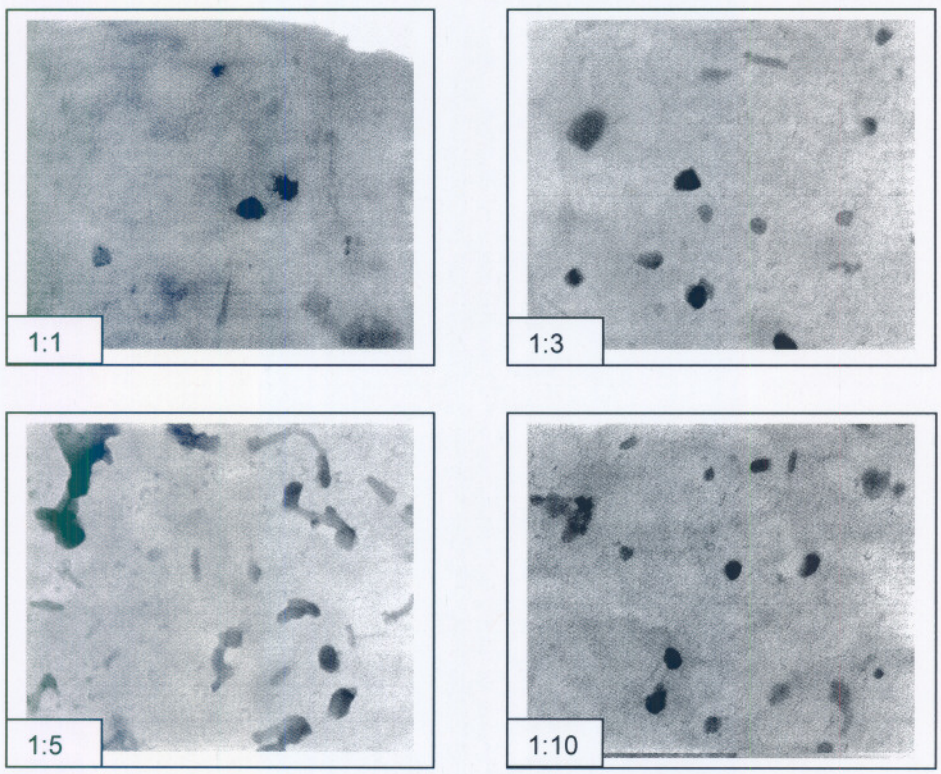


Figure 5.22 TEM micrographs of TMC L/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TMC L polymer condensed the plasmid into oval and round shaped particles with mean sizes of 21.5 nm (1:1), 26.4 nm (1:3), 25.8 nm (1:5) and 31.8 nm (1:10), respectively.

5.2.2.7.2 Toxicity

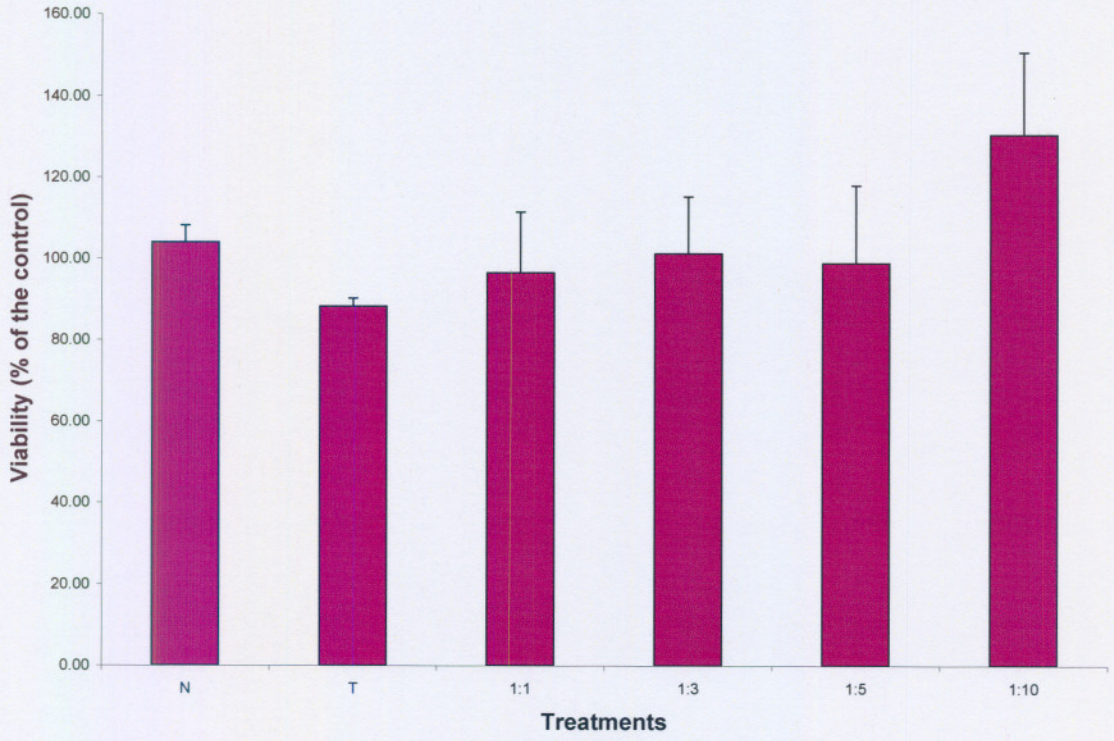


Figure 5.23 Cell viability after treatment of COS-1 cells with TMC L/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMC L/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells determined with the MTT assay after a 4 hour incubation period were found to be 103.95 ± 4.14 % for naked DNA (N), 88.26 ± 1.90 % for Transfectam®/DNA complexes (T), 96.42 ± 14.84 % for TMC L/DNA polyplexes (1:1), 101.07 ± 13.94 % for TMC L/DNA polyplexes (1:3), 98.68 ± 19.02 % for TMC L/DNA polyplexes (1:5) and 130.19 ± 20.14 % for TMC L/DNA polyplexes (1:10). All these values represent the percentage viable cells

compared to the control. Based on the results depicted in figure 5.23, all the TMC L/DNA polyplexes exhibited higher mitochondrial activity than the Transfectam®/DNA complexes, with TMC L/DNA (1:10) polyplex that indicated the least toxicity.

5.2.2.7.3 Gene transfection

TMC L/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.24). Transfectam®/DNA complexes showed a 41-fold increase in transfection efficiency compared to the naked DNA.

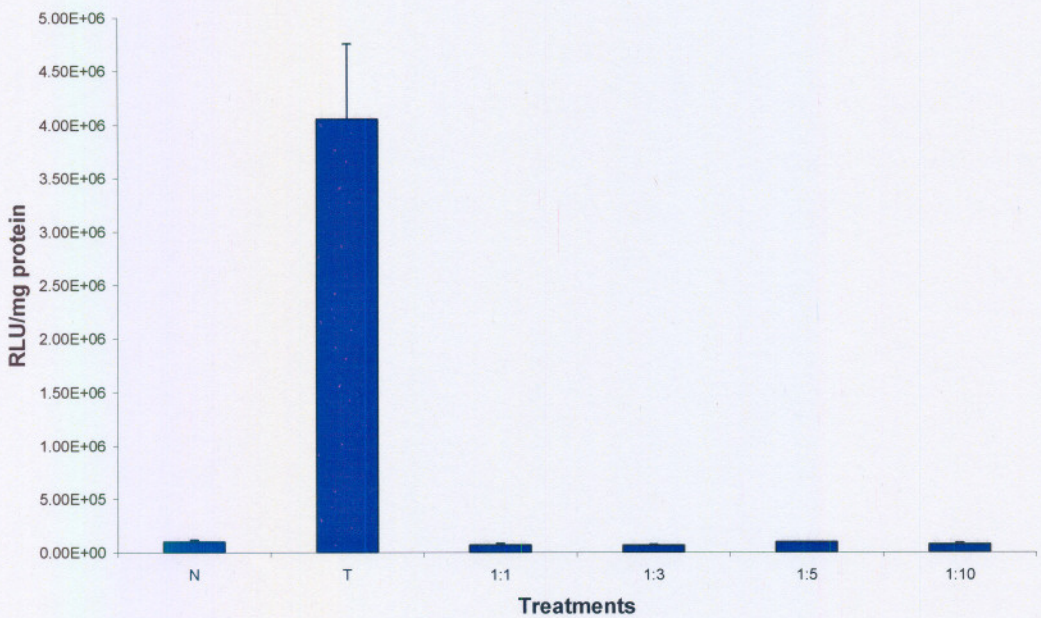


Figure 5.24 Transfection efficiencies in COS-1 cells obtained with TMC L/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMC L/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed transfection of 0.71-, 0.69-, 0.96- and 0.76 times respectively. These values did not differ statistically significant from the value obtained with naked DNA. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.8 TMO H

5.2.2.8.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.25. The magnification of the microscope was set at 105 000 and voltage was set at 100 V. The polymer/DNA oligoplexes are indicated by darker regions on each micrograph.

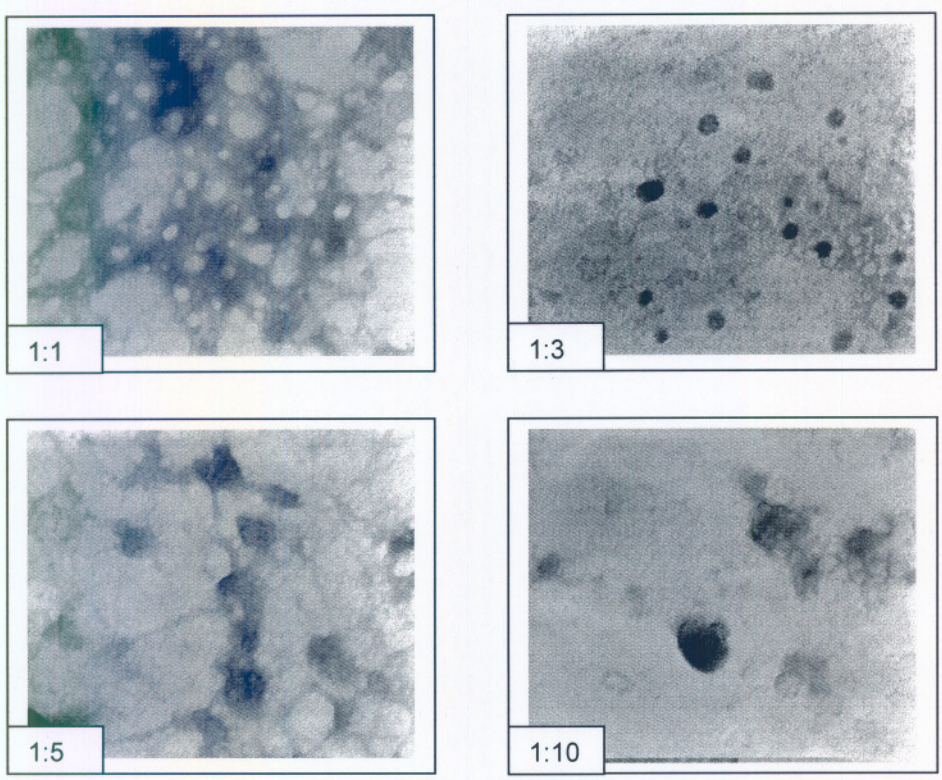


Figure 5.25 TEM micrographs of TMO H/DNA oligoplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TMO H oligomer condensed the plasmid into oval and round shaped particles with mean sizes of 55.7 nm (1:1), 31.8 nm (1:3), 48.2 nm (1:5) and 70.4 nm (1:10), respectively.

5.2.2.8.2 Toxicity

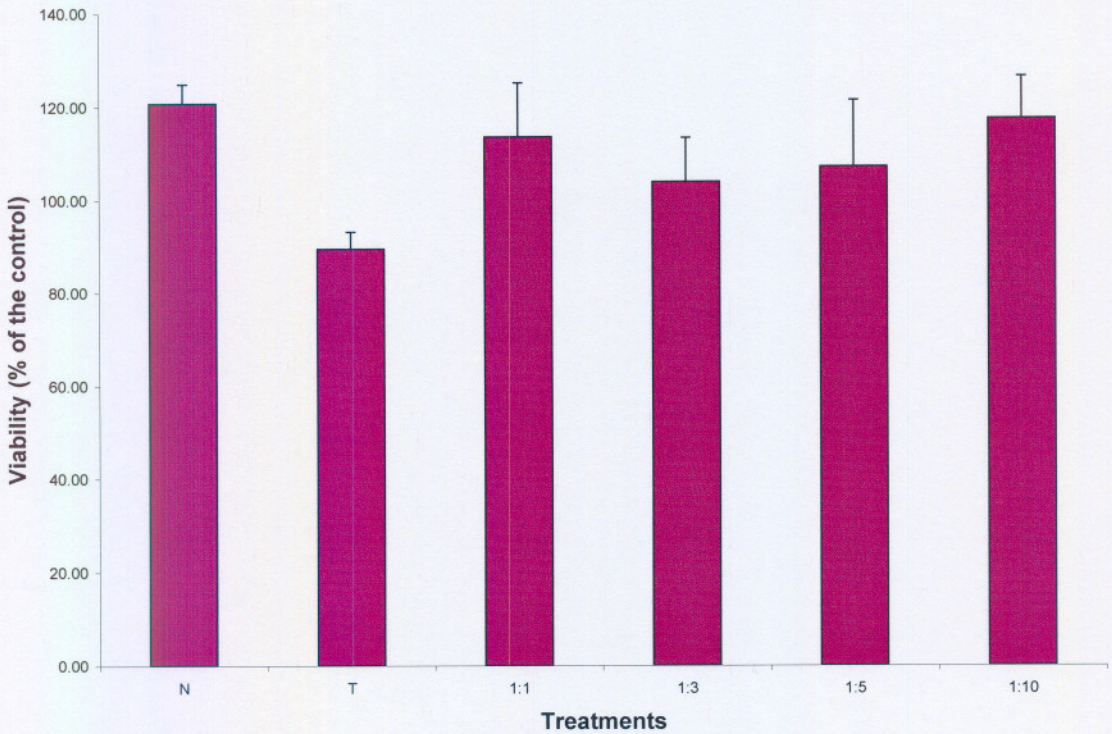


Figure 5.26 Cell viability after treatment of COS-1 cells with TMO H/DNA oligoplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMO H/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 120.76 ± 4.02 % for naked DNA (N), 89.46 ± 3.70 % for Transfectam®/DNA complexes (T), 113.38 ± 11.54 % for TMO H/DNA oligoplexes (1:1), 103.74 ± 9.36 % for TMO H/DNA oligoplexes (1:3), 106.86 ± 14.33 % for TMO H/DNA oligoplexes (1:5) and 117.16 ± 8.92 % for TMO H/DNA oligoplexes (1:10). All these values represent the percentage viable cells

compared to the control. Based on the results depicted in figure 5.26, all the TMO H/DNA oligoplexes exhibited higher mitochondrial activity when compared to Transfectam®/DNA complexes and were therefore considered to be less toxic.

5.2.2.8.3 Gene transfection

TMO H/DNA oligoplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.27). Transfectam®/DNA complexes showed a 16-fold increase in transfection efficiency compared to the naked DNA.

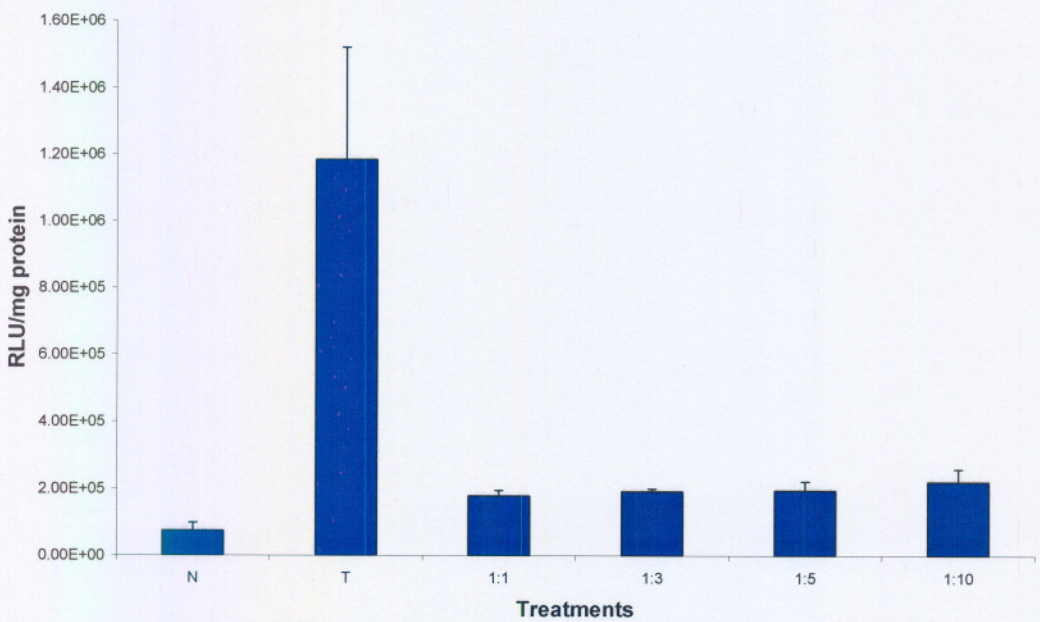


Figure 5.27 Transfection efficiencies in COS-1 cells obtained with TMO H/DNA oligoplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMO H/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 oligoplexes showed an increased transfection of 2.41-, 2.59-, 2.62- and 2.97 times respectively. A statistical significant difference between the tested oligoplex ratios and naked DNA was found, but the different oligoplex ratios did not differ statistically significant from each other. The trend of increased transfection is however clear, compared to naked DNA. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested oligoplex ratios.

5.2.2.9 TMO L

5.2.2.9.1 Confirmation of complex formation

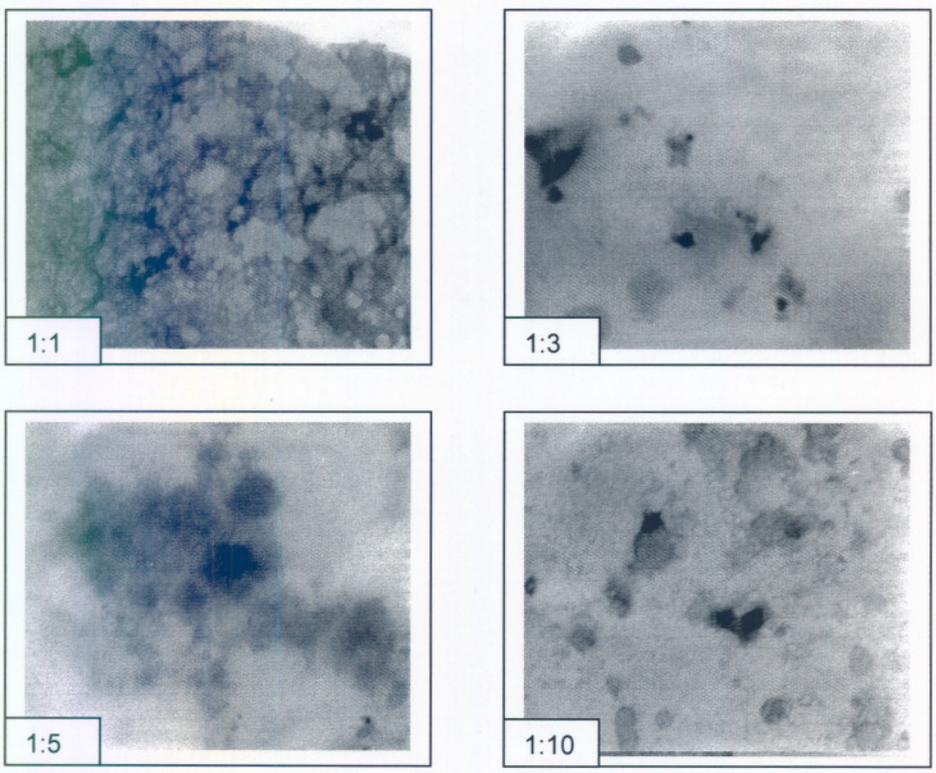


Figure 5.28 TEM micrographs of TMO L/DNA oligoplexes. Different ratios indicated in each micrograph.

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.28. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA oligoplexes are indicated by darker regions on each micrograph.

From the TEM micrographs it was observed that the TMO L oligomer condensed the plasmid into oval and round shaped particles with mean sizes of 65.2 nm (1:1), 56.8 nm (1:3), 78.3 nm (1:5) and 55.1 nm (1:10), respectively.

5.2.2.9.2 Toxicity

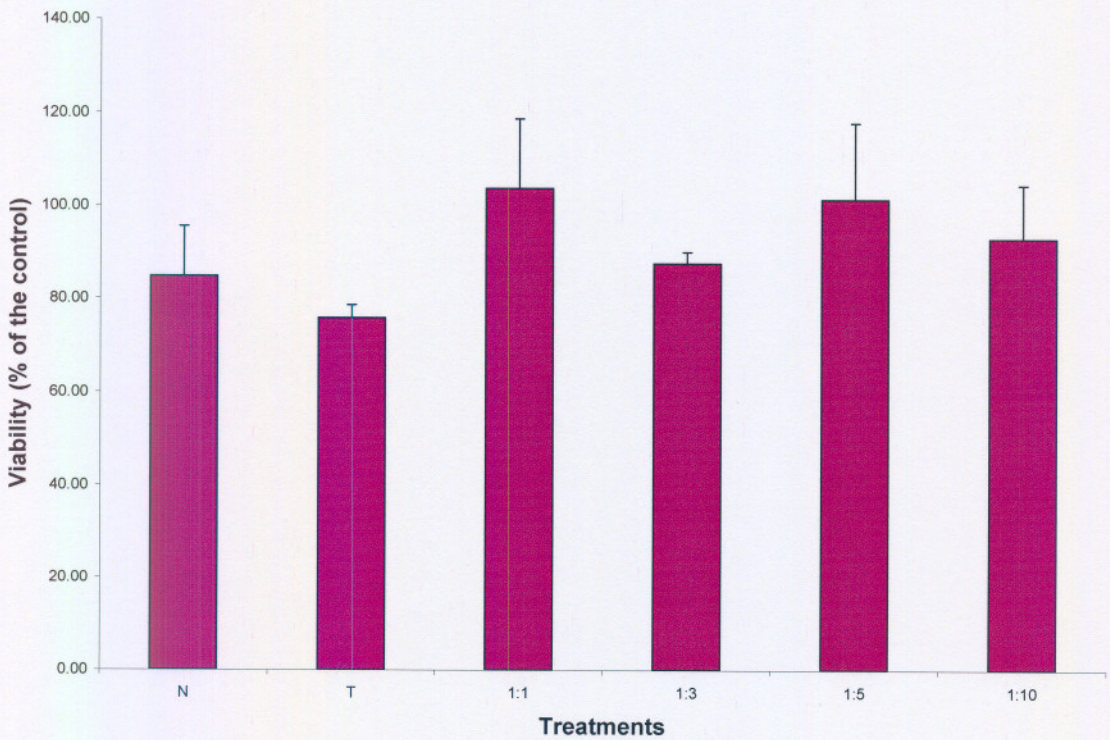


Figure 5.29 Cell viability after treatment of COS-1 cells with TMO L/DNA oligoplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TMO L/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

After a 4 hour incubation period the viability of the COS-1 cells, obtained with the MTT assay were found to be 84.75 ± 10.80 % for naked DNA (N), 75.76 ± 2.80 % for Transfectam®/DNA complexes (T), 103.59 ± 14.95 % for TMO L/DNA oligoplexes (1:1), 87.43 ± 2.41 % for TMO L/DNA oligoplexes (1:3), 101.12 ± 16.38 % for TMC TMO L/DNA oligoplexes (1:5) and 92.67 ± 11.46 % for TMO L/DNA oligoplexes (1:10). These values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.29, all the TMO L/DNA oligoplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.9.3 Gene transfection

TMO L/DNA oligoplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.30). Transfectam®/DNA complexes showed a 31-fold increase in transfection efficiency compared to the naked DNA.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 oligoplexes showed an increased transfection of 3.23-, 4.49-, 6.09- and 7.41 times respectively. A statistical significant difference between the tested oligoplex ratios and naked DNA was found, oligoplexes in ratios 1:1, 1:3 and 1:5 did not differ statistically from each other, but ratio 1:10 proved to be significantly different from ratio 1:1, 1:3 and 1:5. The trend of increased transfection is however clear, compared to naked DNA. Also, the Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

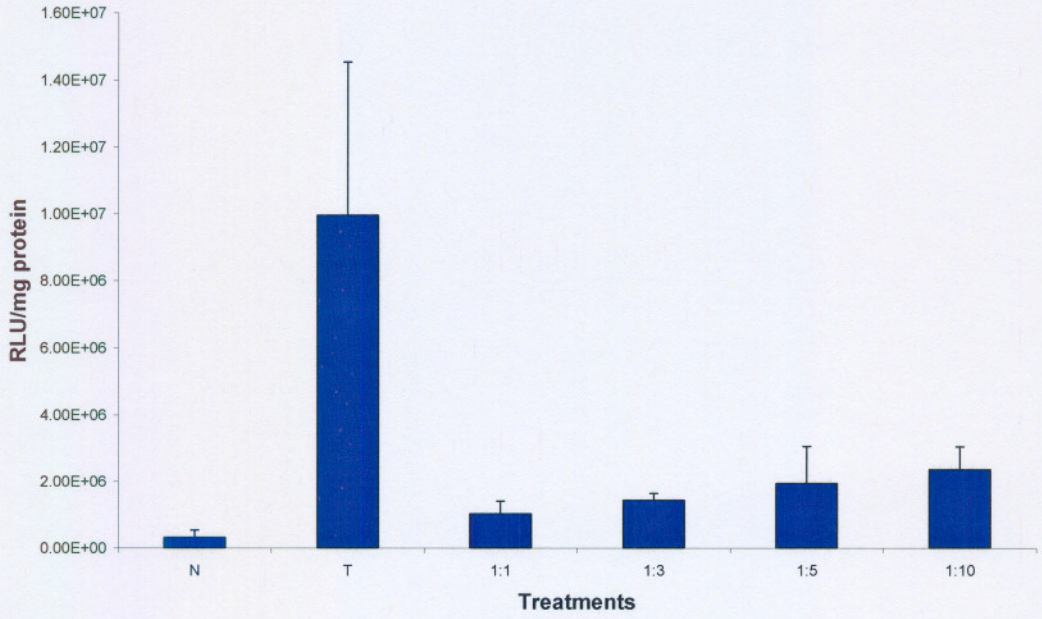


Figure 5.30 Transfection efficiencies in COS-1 cells obtained with TMO L/DNA oligoplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TMO L/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.10 TEC MH

5.2.2.10.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) with the magnification and voltage of the microscope set at 105 000 and 100 V respectively. The results are shown in figure 5.31 and darker regions on each micrograph indicated the polymer/DNA polyplexes.

From the TEM micrographs it was observed that the TEC MH polymer condensed plasmid into oval and round shaped particles with mean sizes of 45.7 nm (1:1), 31.4 nm (1:3), 45.2 nm (1:5) and 30.2 nm (1:10), respectively.

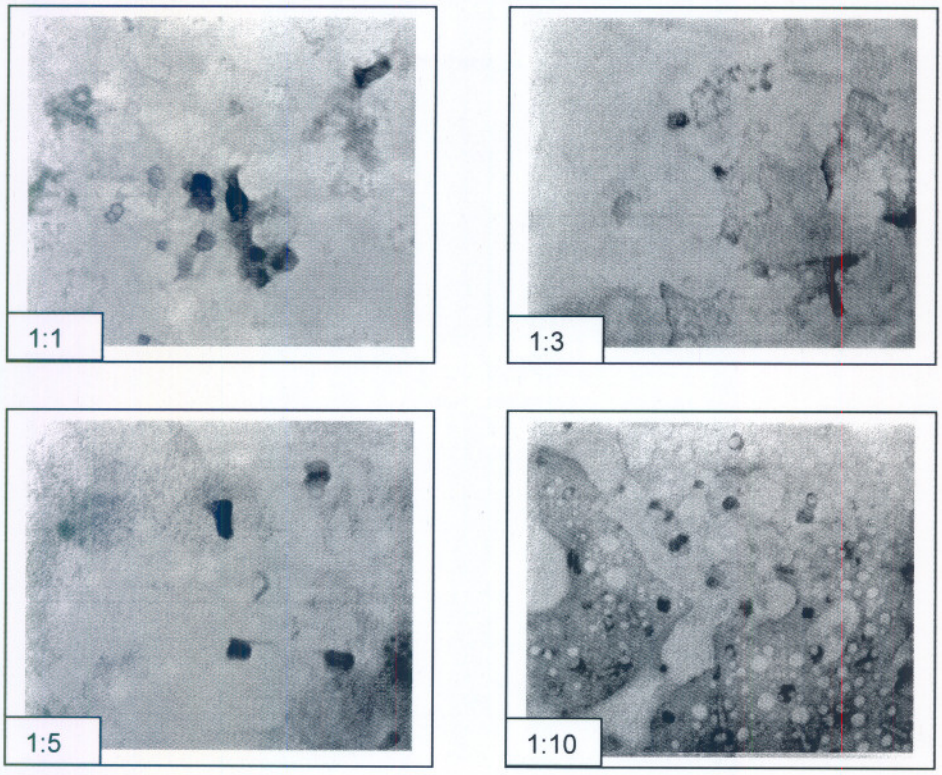


Figure 5.31 TEM micrographs of TEC MH/DNA polyplexes. Different ratios indicated in each micrograph.

5.2.2.10.2 Toxicity

The viability of COS-1 cells obtained with the MTT assay, after a 4 hour incubation period were found to be 100.80 ± 5.48 % for naked DNA (N), 66.87 ± 4.90 % for Transfectam®/DNA complexes (T), 101.58 ± 16.48 % TEC MH/DNA polyplexes (1:1), 104.33 ± 4.35 % for TEC MH/DNA polyplexes (1:3), 90.81 ± 1.63 % for TEC MH/DNA polyplexes (1:5) and 92.15 ± 2.18 % for TEC MH/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.32, all the TEC MH/polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

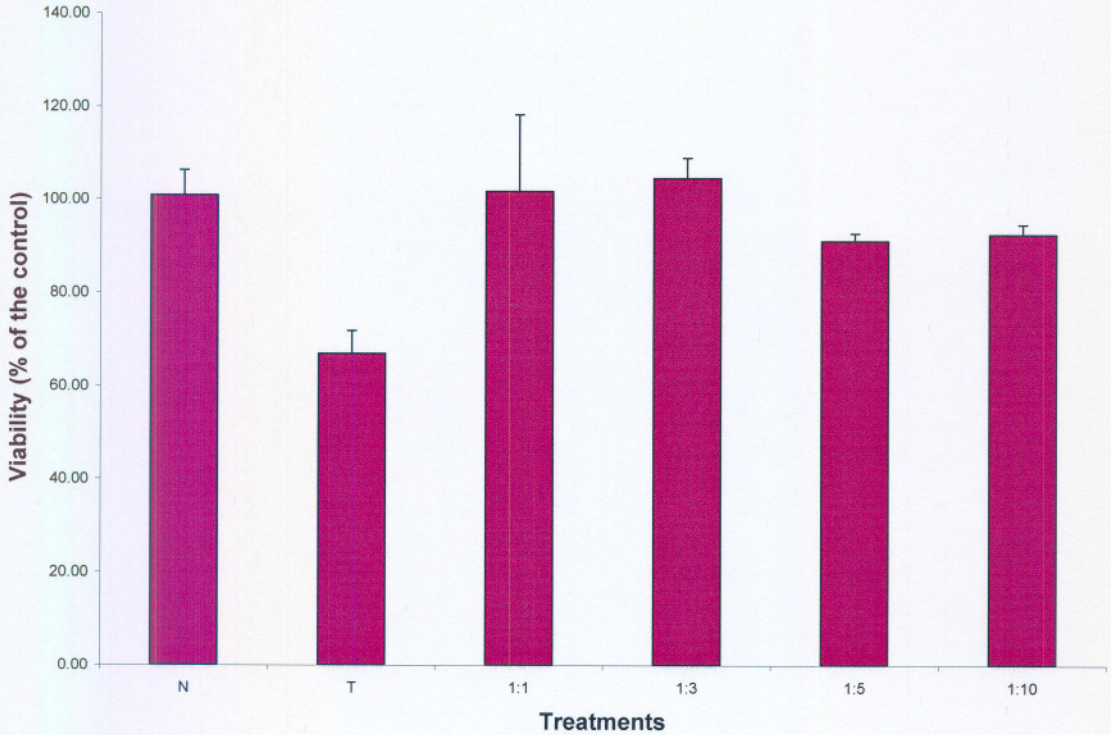


Figure 5.32 Cell viability after treatment of COS-1 cells with TEC MH/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TEC MH/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.10.3 Gene transfection

TEC MH/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.33). As expected Transfectam®/DNA complexes showed an 11-fold increase in transfection efficiency compared to the naked DNA.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.46-, 1.23-, 1.37- and 1.4 times respectively. Although an increased transfection in COS-1 cells with polyplexes was obtained, no statistical significant difference compared to naked DNA was

found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

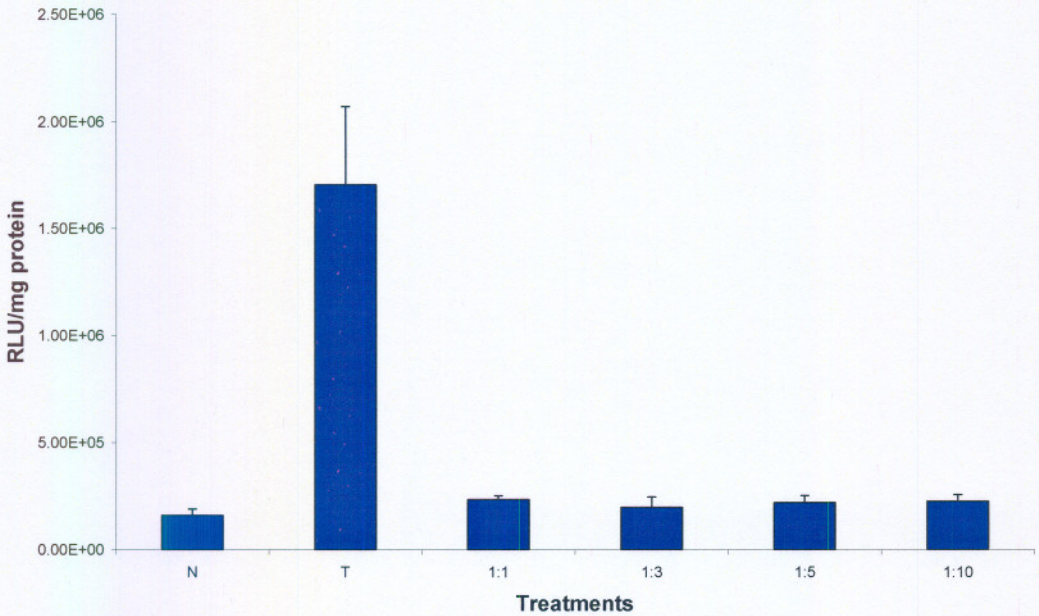


Figure 5.33 Transfection efficiencies in COS-1 cells obtained with TEC MH/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TEC MH/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.11 TEC ML

5.2.2.11.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.34. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

From the TEM micrographs it was observed that the TEC ML polymer condensed the plasmid into oval and round shaped particles with mean sizes of 43.3 nm (1:1), 46.5 nm (1:3), 37.5 nm (1:5) and 46.7 nm (1:10), respectively.

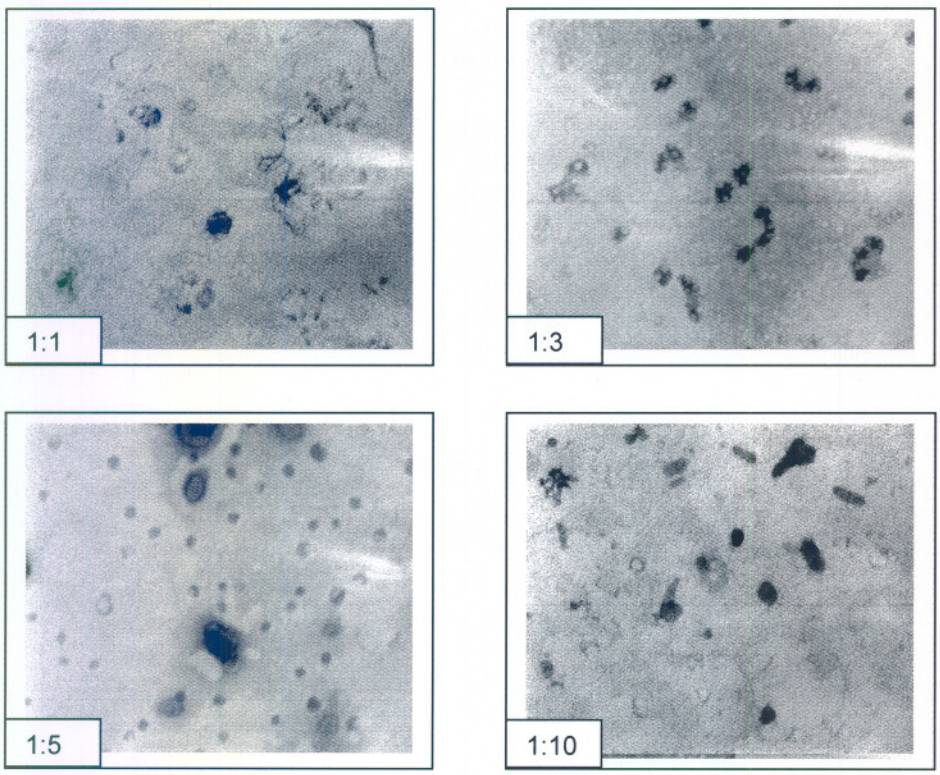


Figure 5.34 TEM micrographs of TEC ML/DNA polyplexes. Different ratios indicated in each micrograph.

5.2.2.11.2 Toxicity

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 95.12 ± 1.60 % for naked DNA (N), 80.77 ± 10.83 % for Transfectam®/DNA complexes (T), 118.08 ± 8.44 % for TEC ML/DNA polyplexes (1:1), 116.12 ± 16.17 % for TEC ML/DNA polyplexes (1:3), 121.70 ± 19.67 % for TEC ML/DNA polyplexes (1:5) and 117.57 ± 2.66 % for TEC ML/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.35, all the TEC ML/polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

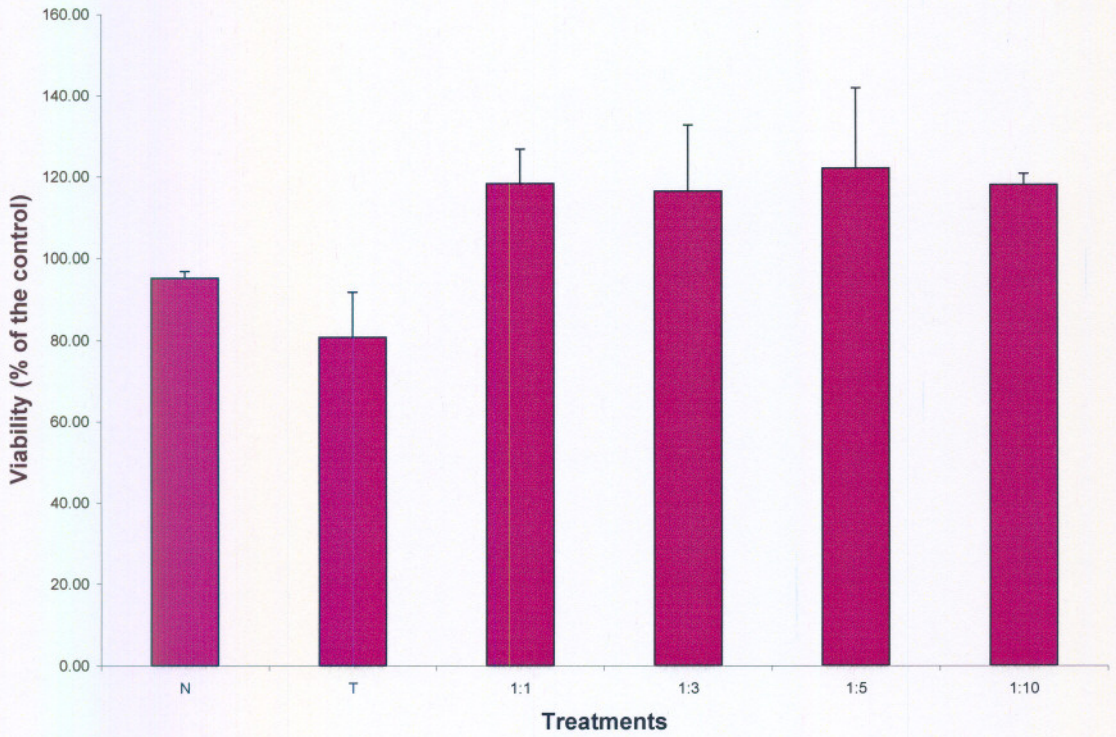


Figure 5.35 Cell viability after treatment of COS-1 cells with TEC ML/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TEC ML/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.11.3 Gene transfection

TEC ML/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.36). The Transfectam®/DNA complexes showed a 14-fold increase in transfection efficiency compared to the naked DNA.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.20-, 1.35-, 1.56- and 1.41 times respectively. Although an increased transfection in COS-1 cells with polyplexes was obtained, no statistical significant difference between any of the tested

polyplex ratios compared to naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

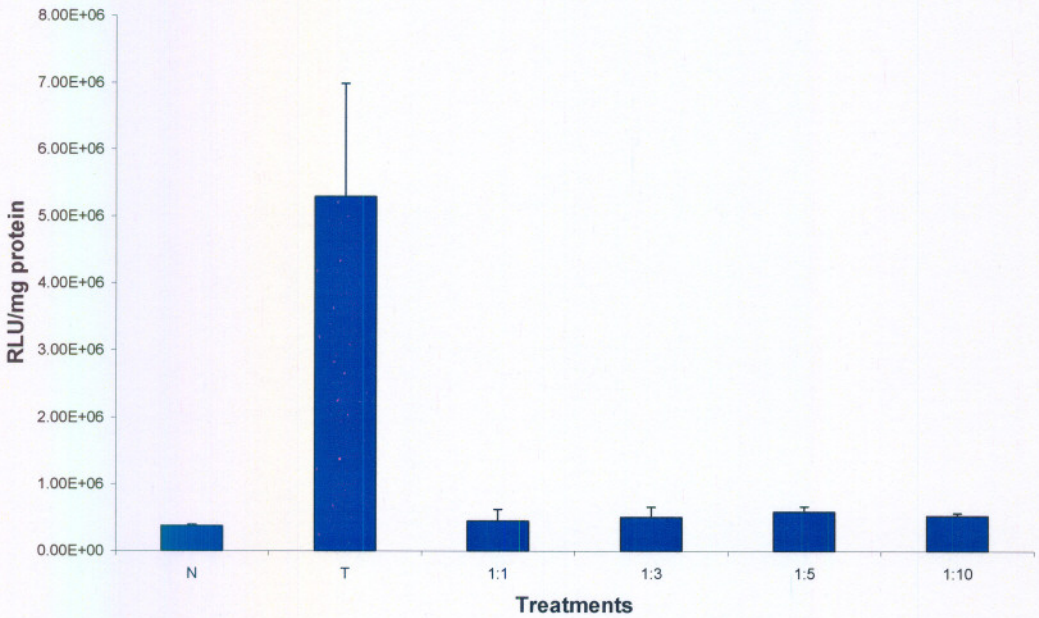


Figure 5.36 Transfection efficiencies in COS-1 cells obtained with TEC ML/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TEC ML/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.12 TEO H

5.2.2.12.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) with the magnification and voltage of the microscope set at 105 000 and 100 V respectively. The results are shown in figure 5.37 and darker regions on each micrograph indicated the oligomer/DNA oligoplexes.

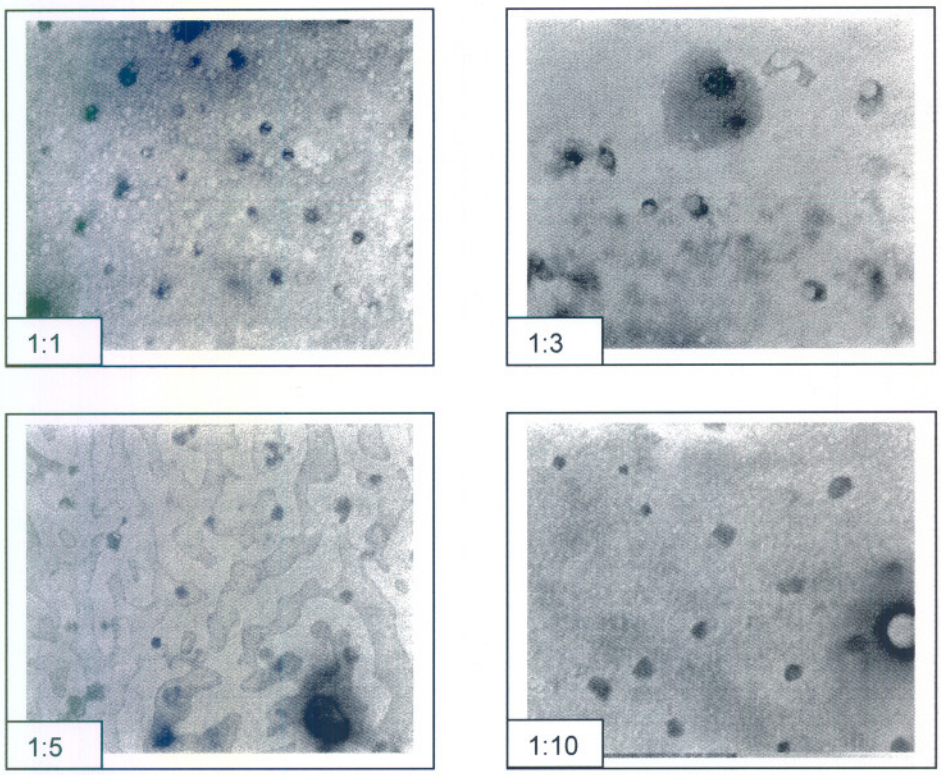


Figure 5.37 TEM micrographs of TEO H/DNA oligoplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TEO H polymer condensed the plasmid into oval and round shaped particles with mean sizes of 30.7 nm (1:1), 39.4 nm (1:3), 31.6 nm (1:5) and 38.8 nm (1:10), respectively.

5.2.2.12.2 Toxicity

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 120.56 ± 3.98 % for naked DNA (N), 66.19 ± 2.57 % for Transfectam®/DNA complexes (T), 112.41 ± 10.59 % for TEO H/DNA oligoplexes (1:1), 101.78 ± 13.57 % for TEO H/DNA oligoplexes (1:3), 107.28 ± 12.31 % for TEO H/DNA oligoplexes (1:5) and 119.24 ± 5.34 % for TEO H/DNA oligoplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.38, all the

TEO H/oligoplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

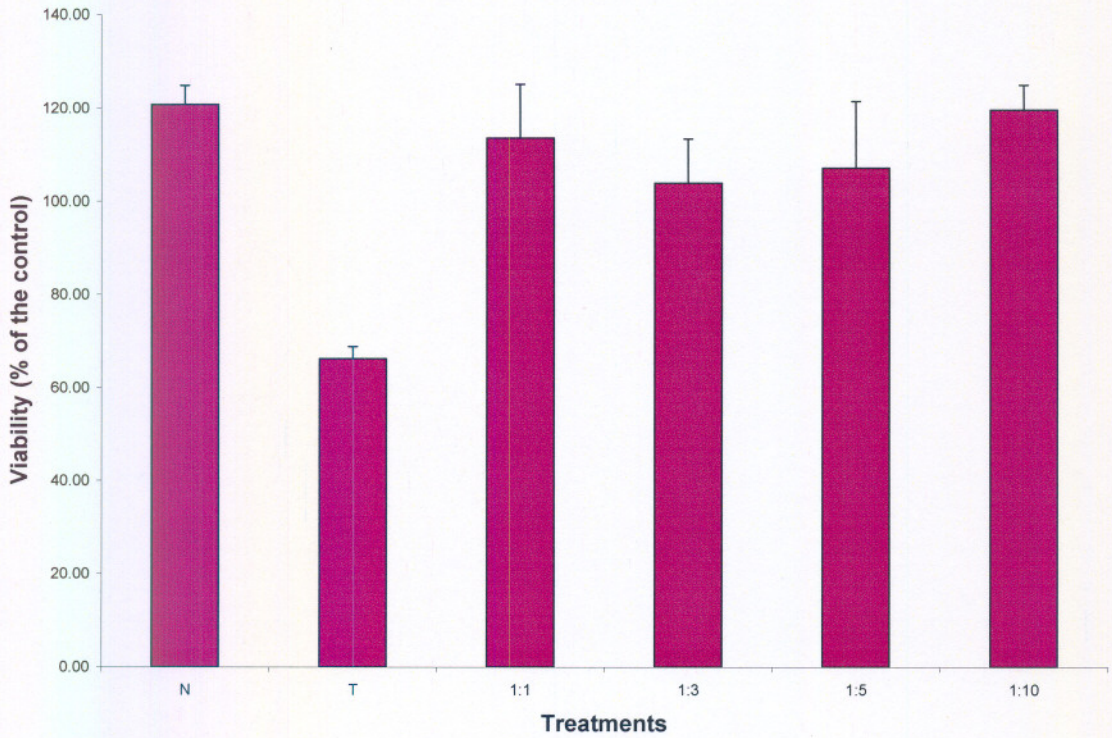


Figure 5.38 Cell viability after treatment of COS-1 cells with TEO H/DNA oligoplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TEO H/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.12.3 Gene transfection

TEO H/DNA oligoplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.39). Transfectam®/DNA complexes showed a 22-fold increase in transfection efficiency compared to the naked DNA.

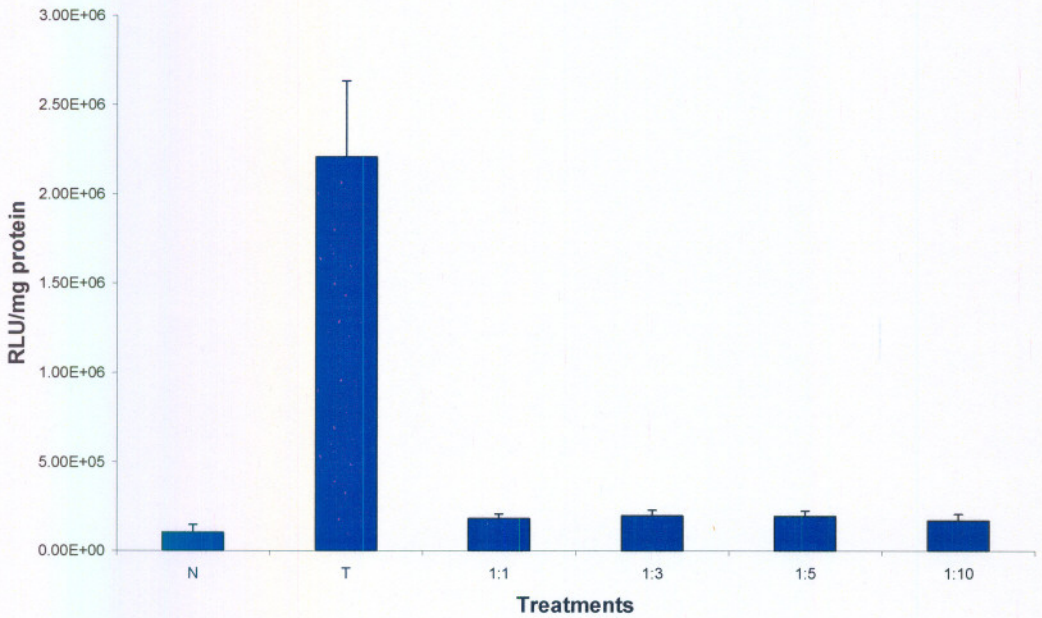


Figure 5.39 Transfection efficiencies in COS-1 cells obtained with TEO H/DNA oligoplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TEO H/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 oligoplexes showed an increased transfection of 1.77-, 1.92, 1.89- and 1.66 times respectively. Although an increased transfection in COS-1 cells with oligoplexes was obtained, no statistical significant difference between any of the tested oligoplex ratios compared to naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested oligoplex ratios.

5.2.2.13 TEO L

5.2.2.13.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.40. The magnification and

voltage of the microscope was set at 105 000 and 100 V respectively. The oligomer/DNA oligoplexes are indicated by darker regions on each micrograph.

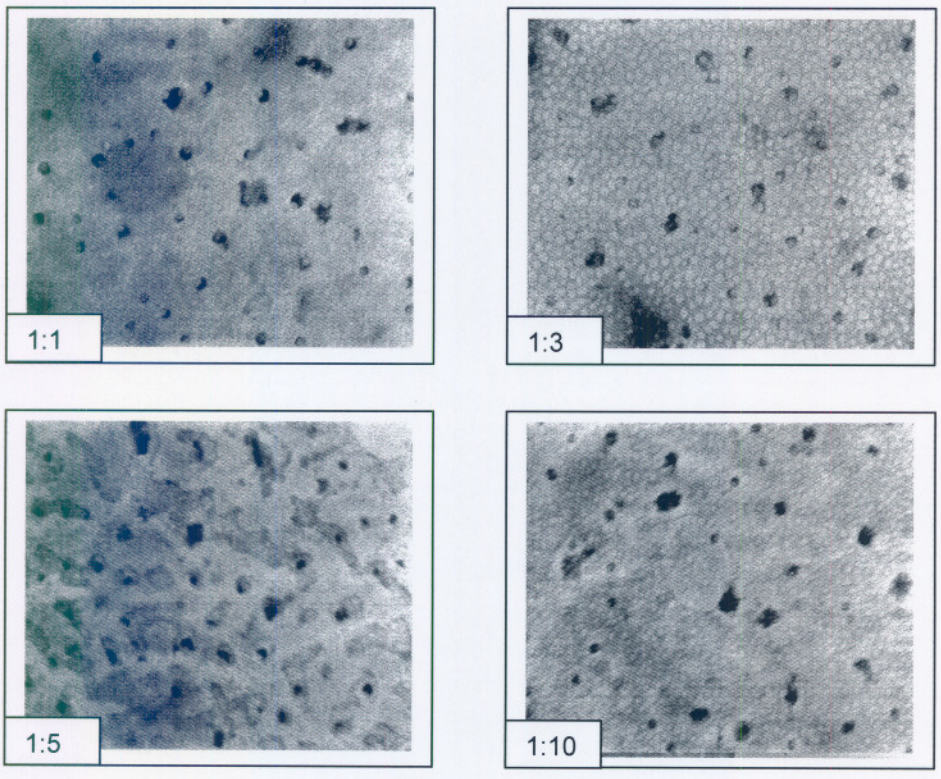


Figure 5.40 TEM micrographs of TEO L/DNA oligoplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the TEO L oligomer condensed the plasmid into oval and round shaped particles with mean sizes of 29.1 nm (1:1), 32.2 nm (1:3), 24.8 nm (1:5) and 45.2 nm (1:10), respectively.

5.2.2.13.2 Toxicity

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 105.69 ± 10.04 % for naked DNA (N), 83.18 ± 4.67 % for Transfectam®/DNA complexes (T), 119.69 ± 15.04 % for TEO L/DNA oligoplexes (1:1), 105.09 ± 10.68 % for TEO L/DNA oligoplexes (1:3), 120.77 ± 9.64 % for TEO L/DNA oligoplexes (1:5) and 103.29 ± 12.38 % for TEO L/DNA

oligoplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.41, all the TEO L/oligoplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

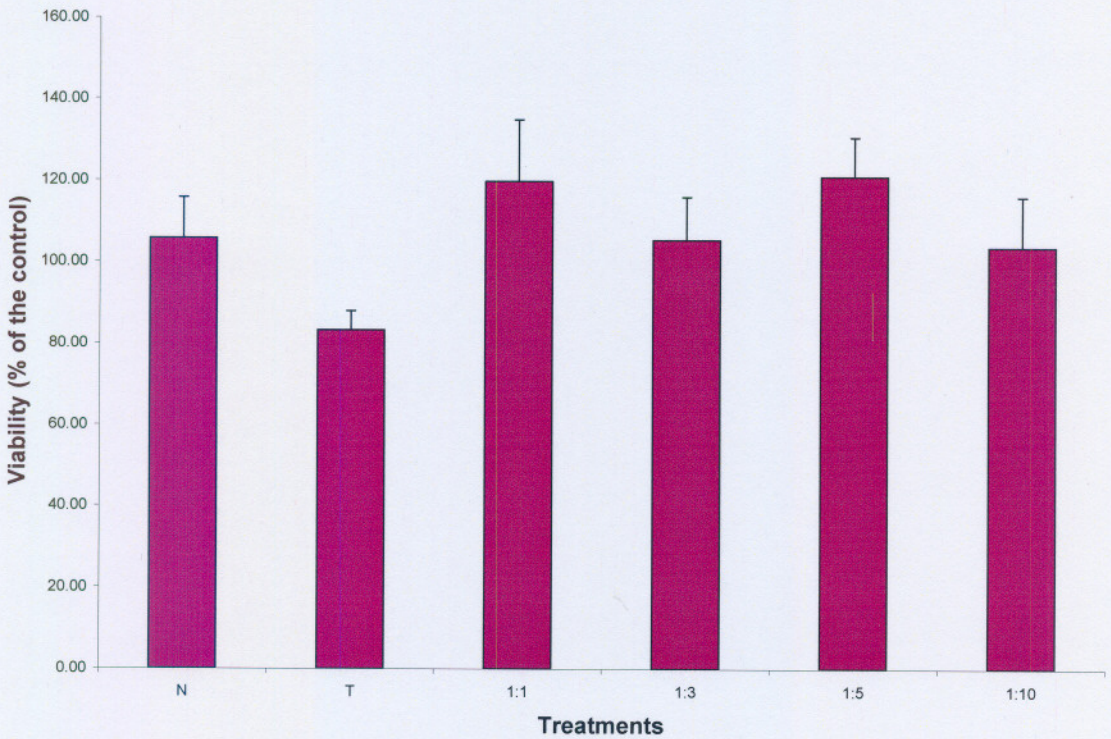


Figure 5.41 Cell viability after treatment of COS-1 cells with TEO L/DNA oligoplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of TEO L/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.13.3 Gene transfection

TEO L/DNA oligoplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.42). Transfectam®/DNA complexes showed a 47-fold increase in transfection efficiency compared to the naked DNA.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 oligoplexes showed an increased transfection of 2.82-, 6.16-, 5.08- and 7.21 times respectively. A statistical significant difference between the tested oligoplex ratios compared to naked DNA was found, oligoplexes in ratios 1:1, 1:3 and 1:5 did not differ statistically from each other, but ratio 1:10 proved to be significantly different from ratio 1:1, 1:3 and 1:5. The trend of increased transfection is however clear, compared to naked DNA. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested oligoplex ratios.

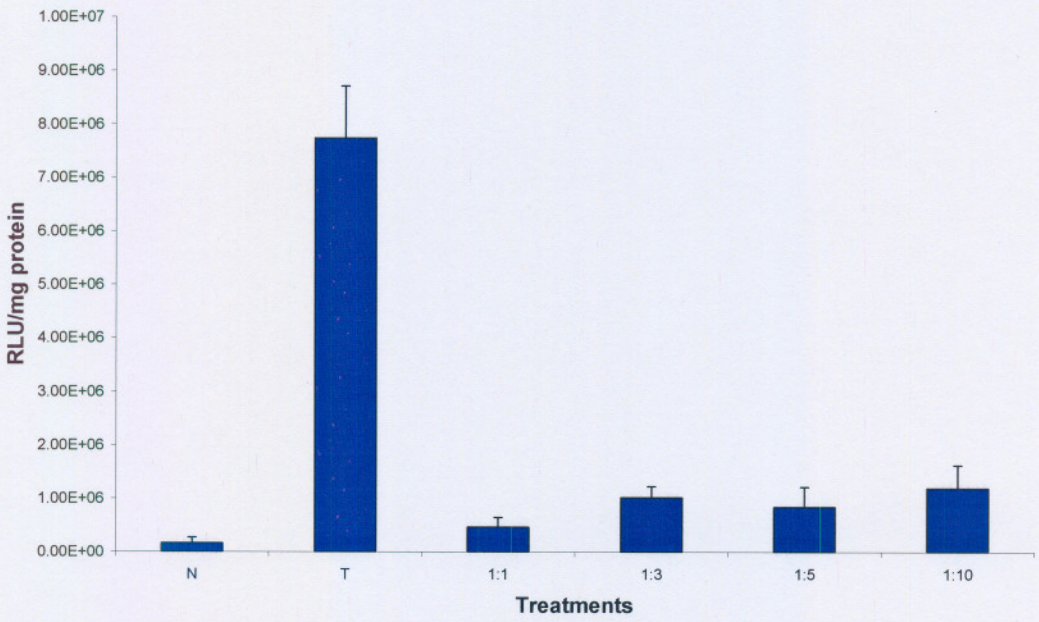


Figure 5.42 Transfection efficiencies in COS-1 cells obtained with TEO L/DNA oligoplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of TEO L/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.14 Seacure

5.2.2.14.1 *Confirmation of complex formation*

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.43. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

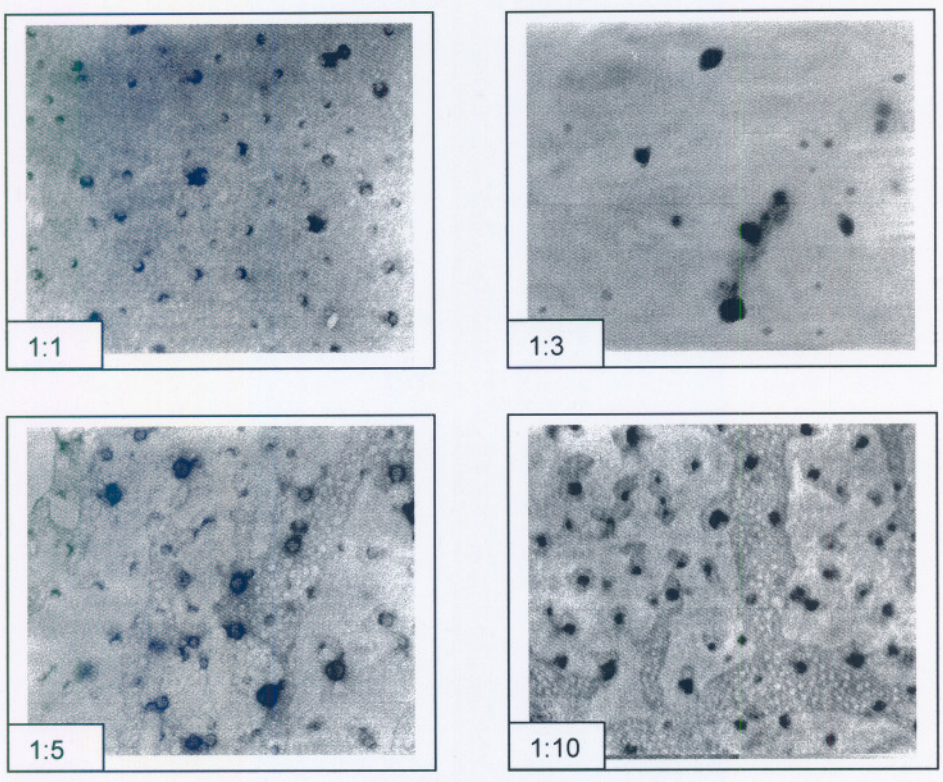


Figure 5.43 TEM micrographs of Seacure/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the Seacure polymer condensed the plasmid into oval and round shaped particles with mean sizes of 20.5 nm (1:1), 27.5 nm (1:3), 35.6 nm (1:5) and 25.6 nm (1:10), respectively.

5.2.2.14.2 *Toxicity*

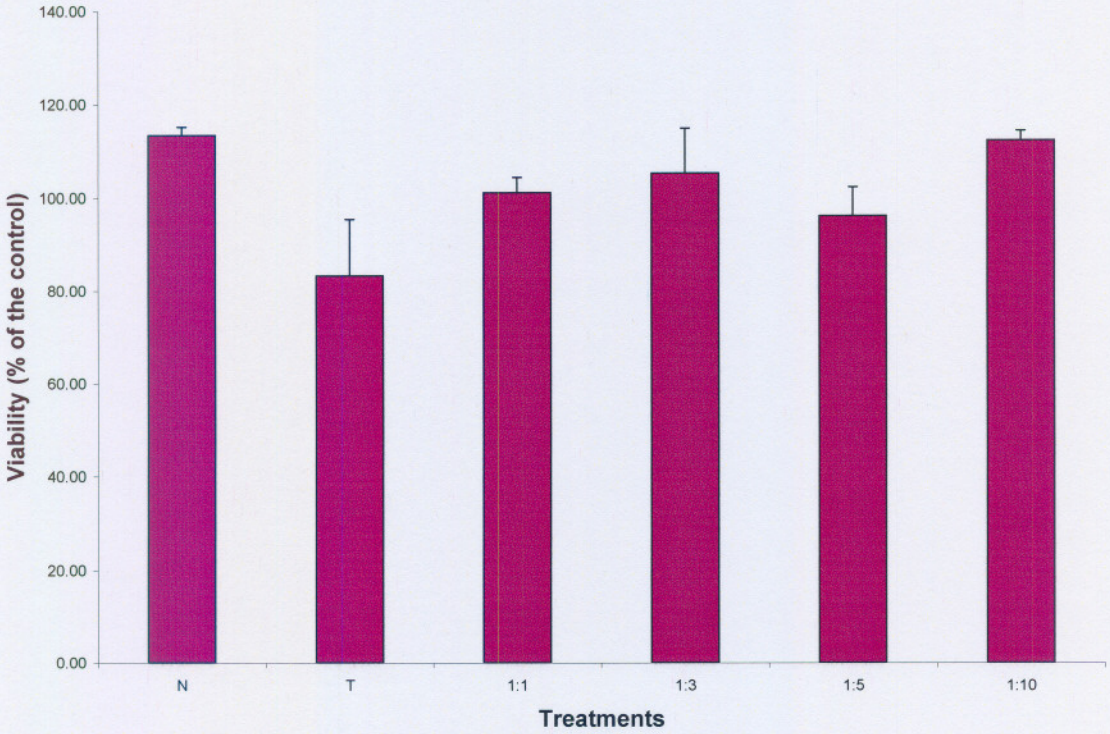


Figure 5.44 Cell viability after treatment of COS-1 cells with Seacure/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of Seacure/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 113.40 ± 1.73 % for naked DNA (N), 83.26 ± 12.16 % for Transfectam®/DNA complexes (T), 101.06 ± 3.20 % for Seacure/DNA polyplexes (1:1), 105.11 ± 9.60 % for Seacure/DNA polyplexes (1:3), 96.00 ± 6.13 % for Seacure/DNA polyplexes (1:5) and 111.99 ± 2.13 % for Seacure/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.44, the Seacure/DNA polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.14.3 Gene transfection

Seacure/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.45). Transfectam®/DNA complexes showed a 23-fold increase in transfection efficiency compared to the naked DNA.

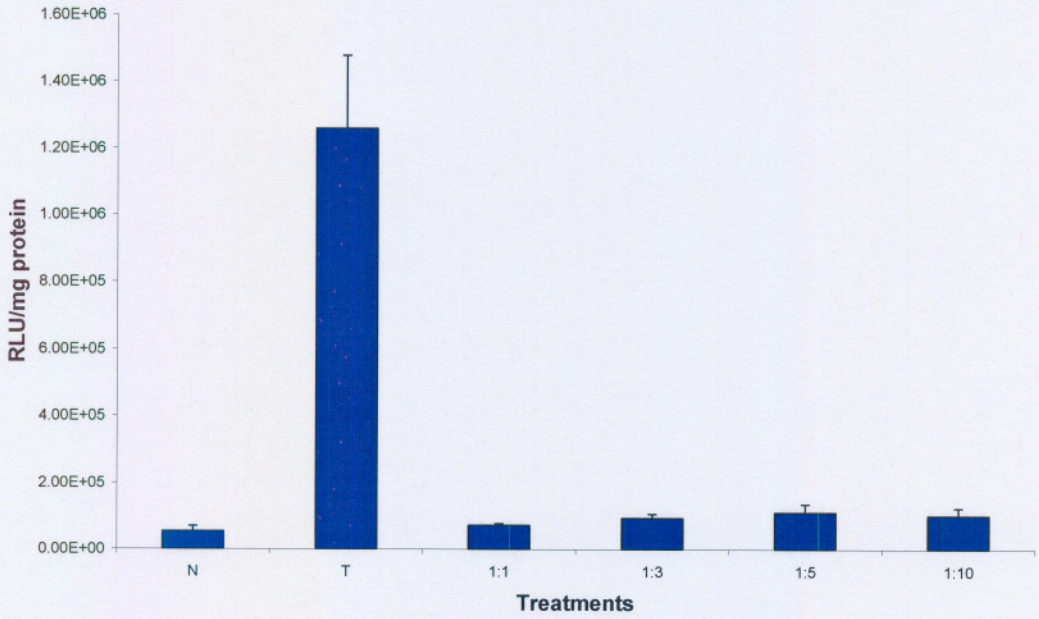


Figure 5.45 Transfection efficiencies in COS-1 cells obtained with Seacure/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of Seacure/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.34-, 1.75-, 2.06- and 1.88 times respectively. Although an increased transfection in COS-1 cells with polyplexes was observed, no statistical significant difference from naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.15 Primex H (Chitoclear High MW)

5.2.2.15.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.46. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

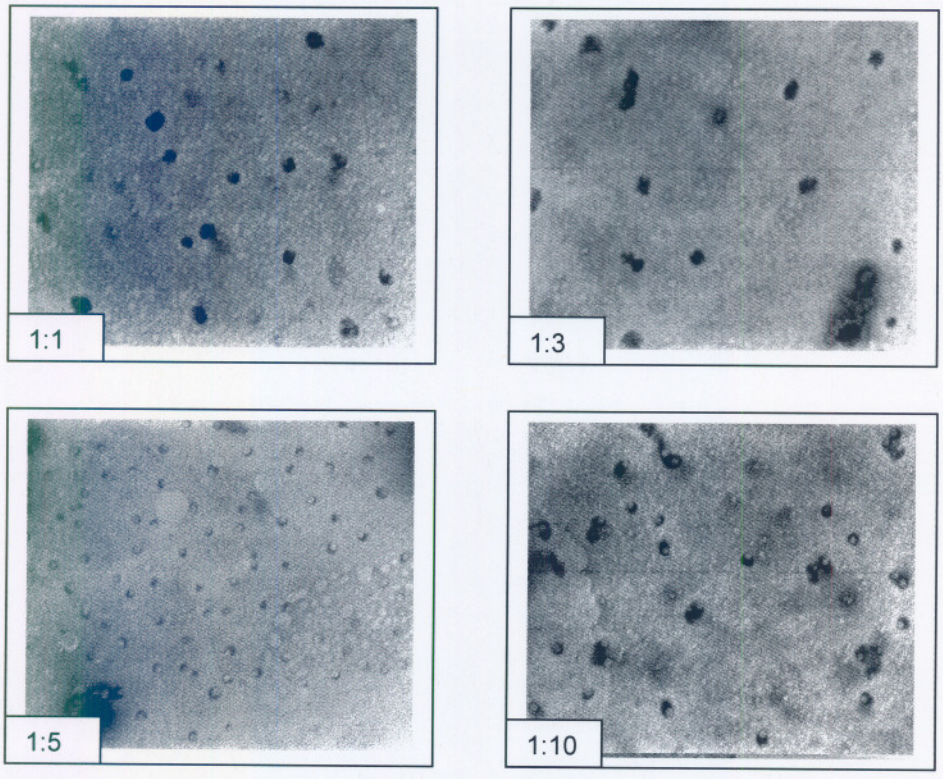


Figure 5.46 TEM micrographs of Primex H/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the Primex H polymer condensed the plasmid into oval and round shaped particles with mean sizes of 29.2 nm (1:1), 34.5 nm (1:3), 22.7 nm (1:5) and 32.3 nm (1:10), respectively.

5.2.2.15.2 Toxicity

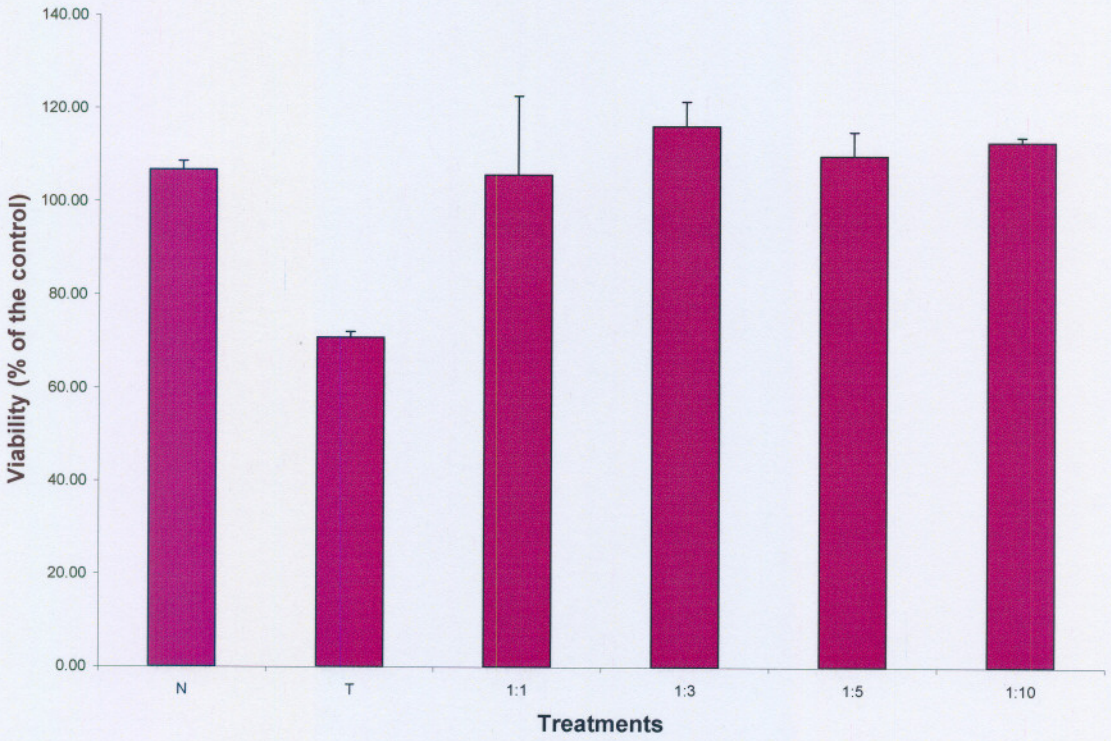


Figure 5.47 Cell viability after treatment of COS-1 cells with Primex H/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of Primex H/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 106.77 ± 1.85 % for naked DNA (N), 70.83 ± 1.21 % for Transfectam®/DNA complexes (T), 105.67 ± 16.90 % for Primex H/DNA polyplexes (1:1), 116.10 ± 5.26 % for Primex H/DNA polyplexes (1:3), 109.67 ± 5.18 % for Primex H/DNA polyplexes (1:5) and 112.61 ± 1.06 % for Primex H/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.47, the Primex H/DNA polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.15.3 *Gene transfection*

Primex H/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.48). Transfectam®/DNA complexes showed a 21-fold increase in transfection efficiency compared to the naked DNA.

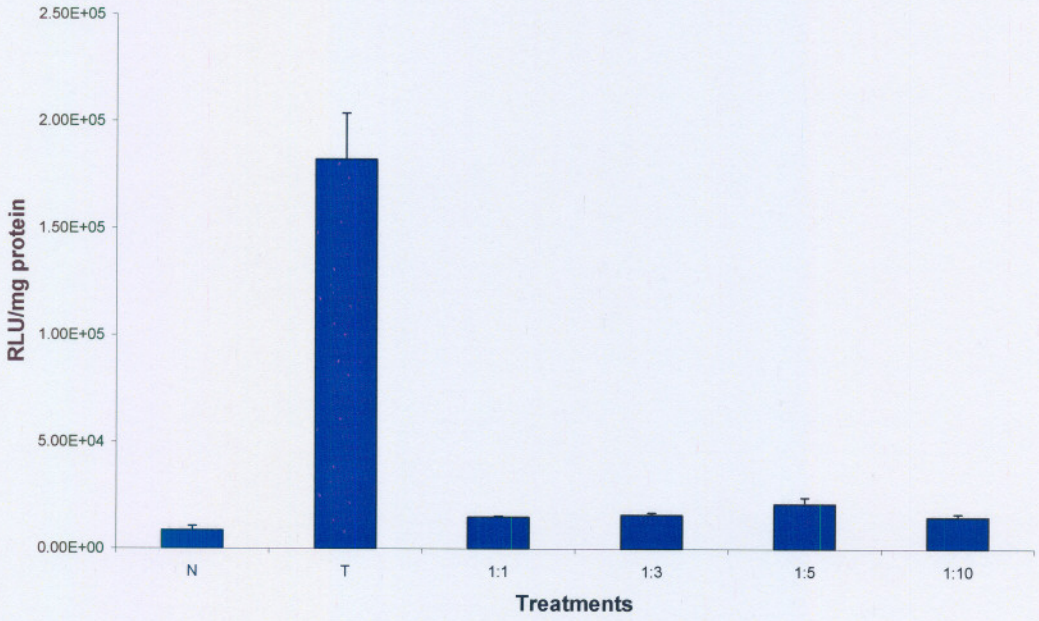


Figure 5.48 Transfection efficiencies in COS-1 cells obtained with Primex H/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of Primex H/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.72-, 1.85-, 2.45- and 1.74 times respectively. Although an increased transfection in COS-1 cells with Primex H polyplexes was observed, no statistical significant difference from naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.2.16 Primex M (Chitoclear Medium MW)

5.2.2.16.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.49. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The polymer/DNA polyplexes are indicated by darker regions on each micrograph.

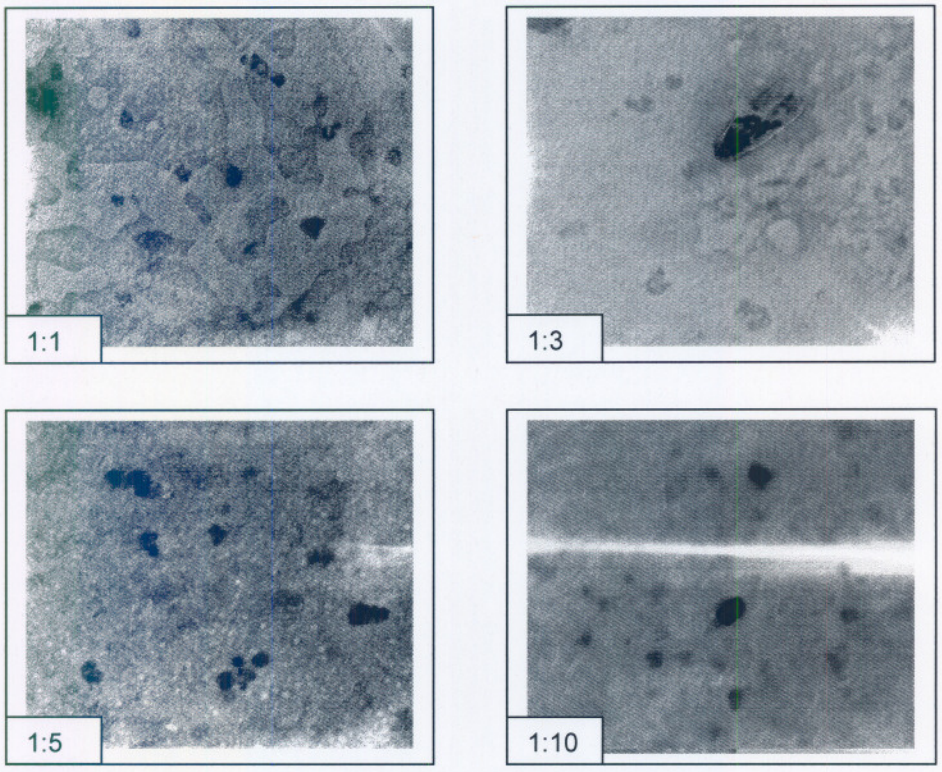


Figure 5.49 TEM micrographs of Primex M/DNA polyplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the Primex M polymer condensed the plasmid into oval and round shaped particles with mean sizes of 65.3 nm (1:1), 74.5 nm (1:3), 68.8 nm (1:5) and 65.1 nm (1:10), respectively.

5.2.2.16.2 *Toxicity*

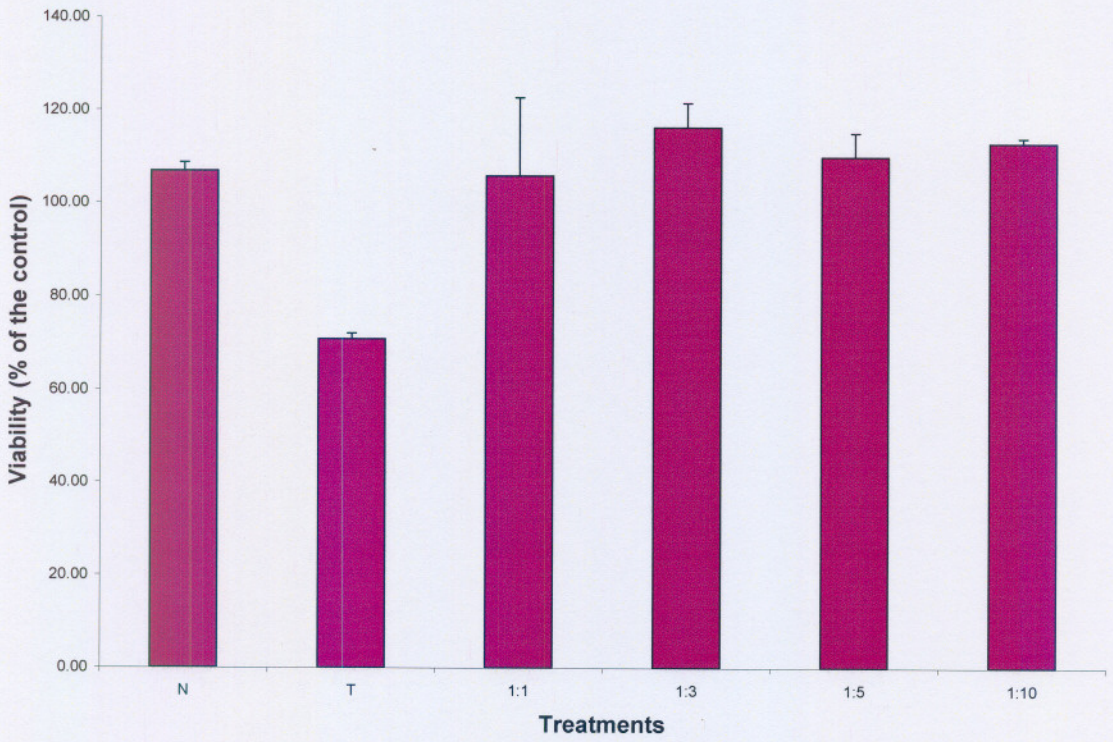


Figure 5.50 Cell viability after treatment of COS-1 cells with Primex M/DNA polyplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of Primex M/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 106.77 ± 1.85 % for naked DNA (N), 70.83 ± 1.21 % for Transfectam®/DNA complexes (T), 105.67 ± 16.90 % for Primex M/DNA polyplexes (1:1), 116.10 ± 5.26 % for Primex M/DNA polyplexes (1:3), 109.67 ± 5.18 % for Primex M/DNA polyplexes (1:5) and 112.61 ± 1.06 % for Primex M/DNA polyplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.50, the Primex M/DNA polyplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.16.3 *Gene transfection*

Primex M/DNA polyplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.51). Transfectam®/DNA complexes showed a 22-fold increase in transfection efficiency compared to the naked DNA.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 polyplexes showed an increased transfection of 1.50-, 1.56-, 2.20- and 2.24 times respectively. Although an increased transfection in COS-1 cells with Primex M polyplexes was observed, no statistical significant difference from naked DNA was found. The Transfectam®/DNA complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

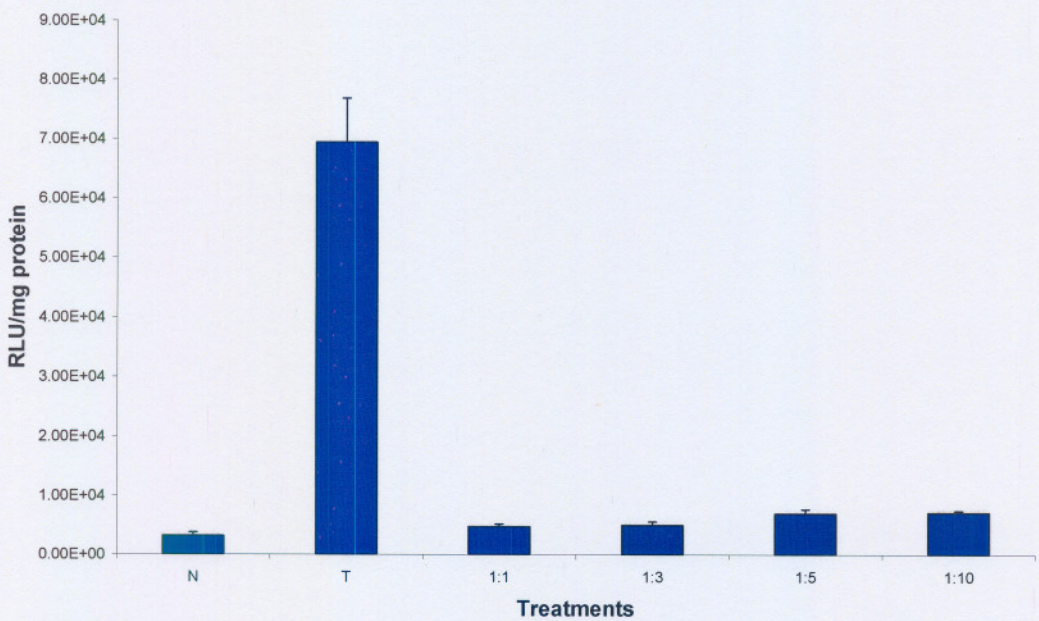


Figure 5.51 Transfection efficiencies in COS-1 cells obtained with Primex M/DNA polyplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of Primex M/DNA polyplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

5.2.2.17 Primex O (Oligosaccharide)

5.2.2.17.1 Confirmation of complex formation

Average size measurements of complexes were determined with transmission electron microscopy (TEM) as shown in figure 5.52. The magnification and voltage of the microscope was set at 105 000 and 100 V respectively. The oligomer/DNA oligoplexes are indicated by darker regions on each micrograph.

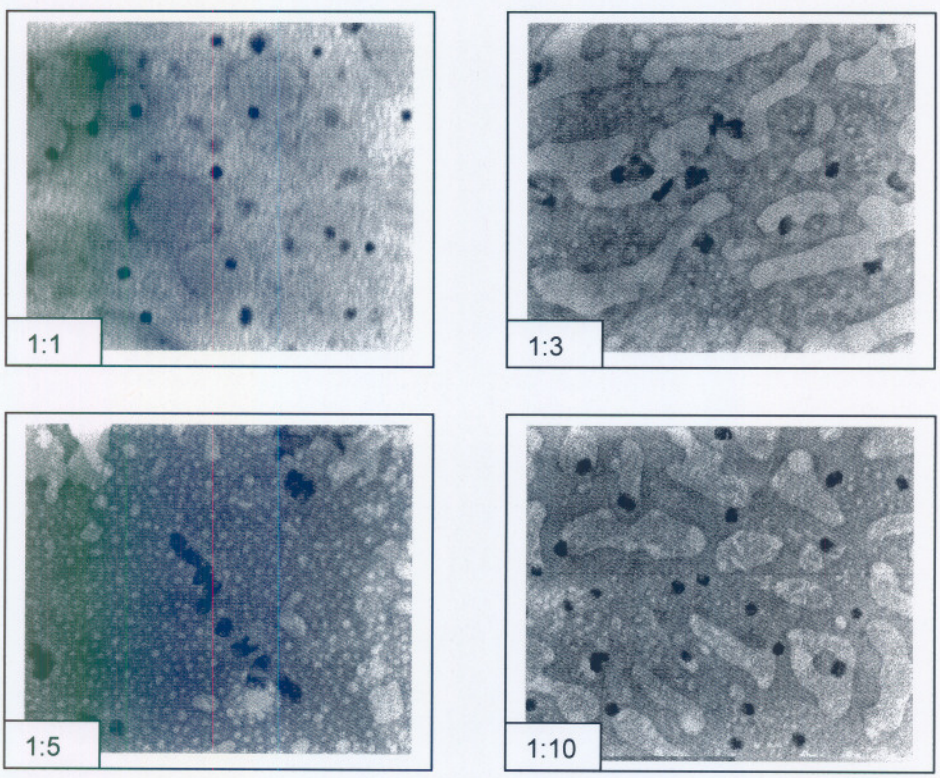


Figure 5.52 TEM micrographs of Primex O/DNA oligoplexes. Different ratios indicated in each micrograph.

From the TEM micrographs it was observed that the Primex O oligomer condensed the plasmid into oval and round shaped particles with mean sizes of 31.5 nm (1:1), 45.3 nm (1:3), 38.3 nm (1:5) and 33.4 nm (1:10), respectively.

5.2.2.17.2 *Toxicity*

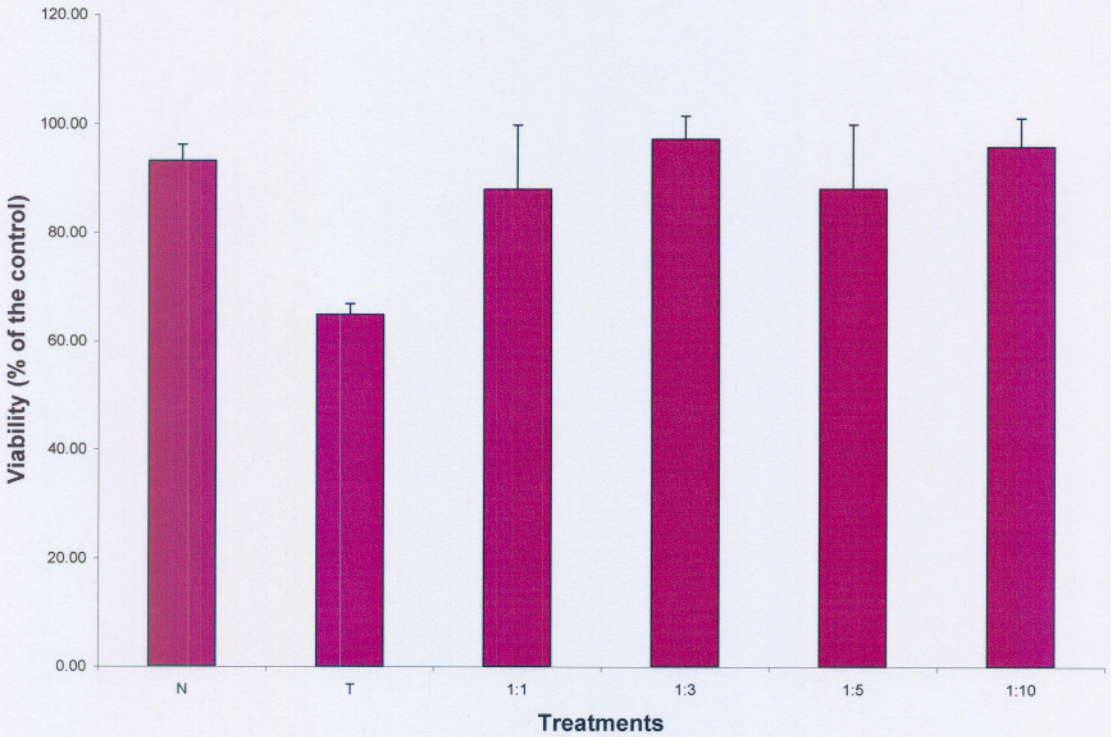


Figure 5.53 Cell viability after treatment of COS-1 cells with Primex O/DNA oligoplexes compared to naked DNA (N) and Transfectam®/DNA complexes (T) as controls. Ratios of Primex O/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

The viability of COS-1 cells obtained with the MTT assay after a 4 hour incubation period were found to be 93.23 ± 2.93 % for naked DNA (N), 64.93 ± 1.96 % for Transfectam®/DNA complexes (T), 85.86 ± 10.46 % for Primex O/DNA oligoplexes (1:1), 97.14 ± 4.21 % for Primex O/DNA oligoplexes (1:3), 87.91 ± 11.82 % for Primex O/DNA oligoplexes (1:5) and 95.62 ± 5.20 % for Primex O/DNA oligoplexes (1:10). All these values represent the percentage viable cells compared to the control. Based on the results depicted in figure 5.53, the Primex O/DNA oligoplexes exhibited higher mitochondrial activity compared to Transfectam®/DNA complexes and are therefore considered less toxic.

5.2.2.17.3 *Gene transfection*

Primex O/DNA oligoplexes were tested for their ability to transfect COS-1 cells in the presence of serum proteins. Transfectam®/DNA complexes (T) were used as positive control and naked DNA (N) as negative control during the experiment (figure 5.54). Transfectam®/DNA complexes showed a 21-fold increase in transfection efficiency compared to the naked DNA.

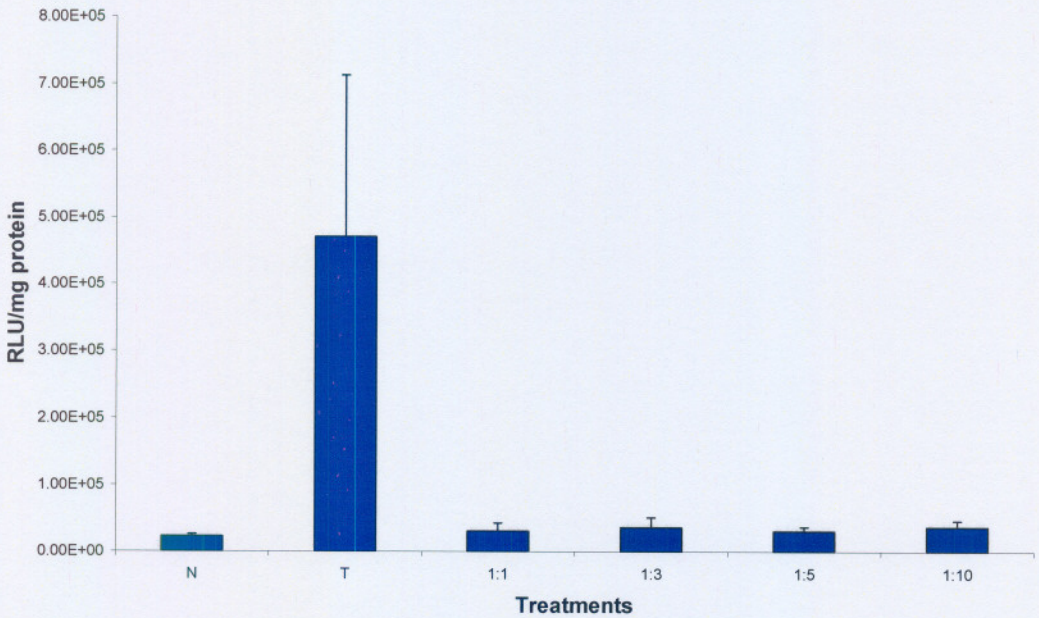


Figure 5.54 Transfection efficiencies in COS-1 cells obtained with Primex O/DNA oligoplexes compared to Transfectam®/DNA complexes (T) and naked DNA (N). Ratios of Primex O/DNA oligoplexes compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In comparison with the negative control, the 1:1, 1:3, 1:5 and 1:10 oligoplexes showed a general increased transfection of 1.36-, 1.61-, 1.36- and 1.63 times respectively. Although an increased transfection in COS-1 cells with oligoplexes was observed, no statistical significant difference between any of the tested oligoplex ratios compared to naked DNA was found. The Transfectam®/DNA

complexes proved to differ statistically significant from naked DNA and all tested polyplex ratios.

5.2.3 Comparison of transfection efficiencies and toxicity

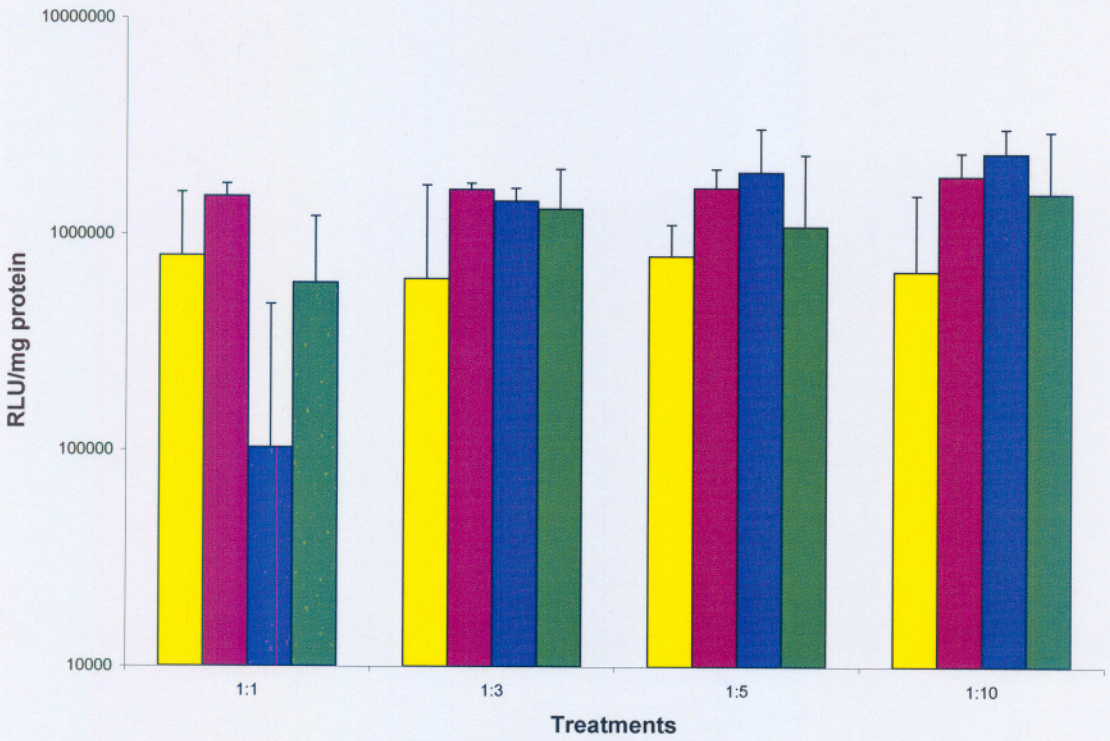


Figure 5.55 Transfection efficiencies in COS-1 cells obtained with TMC H/DNA polyplexes (●), TMO H/DNA oligoplexes (●), TMO L/DNA oligoplexes (●) and TEO L/DNA oligoplexes (●). Ratios compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In figure 5.55 all the polyplexes and oligoplexes were depicted that differed statistically significant when compared to naked DNA. These transfection efficiencies were normalised by adjusting the positive control to be similar for the tested polymer/DNA and oligomer/DNA complexes for the purpose of this graph. From the gel electrophoresis (figure 5.3) it was shown that all these complexes formed with TMC H, TMO L, TMO H, & TEO L were able to complex the plasmid in the ratios tested. The degree of quaternisation that seems to be optimum in

this study was between 15 % (TMO L) and 59 % (TMC H). A dependence on the size of the molecule seems to be present with the polymers and oligomers tested [TMC H (143 kDa), TMO H (6.6 kDa), TMO L (8.5 kDa) and TEO L (11 kDa)]. Only the 1:10 ratios of the TMO L and TEO L differed statistically significant from the other ratios tested, and both of these oligomers have a relatively small size, 8.5 kDa vs 11 kDa.

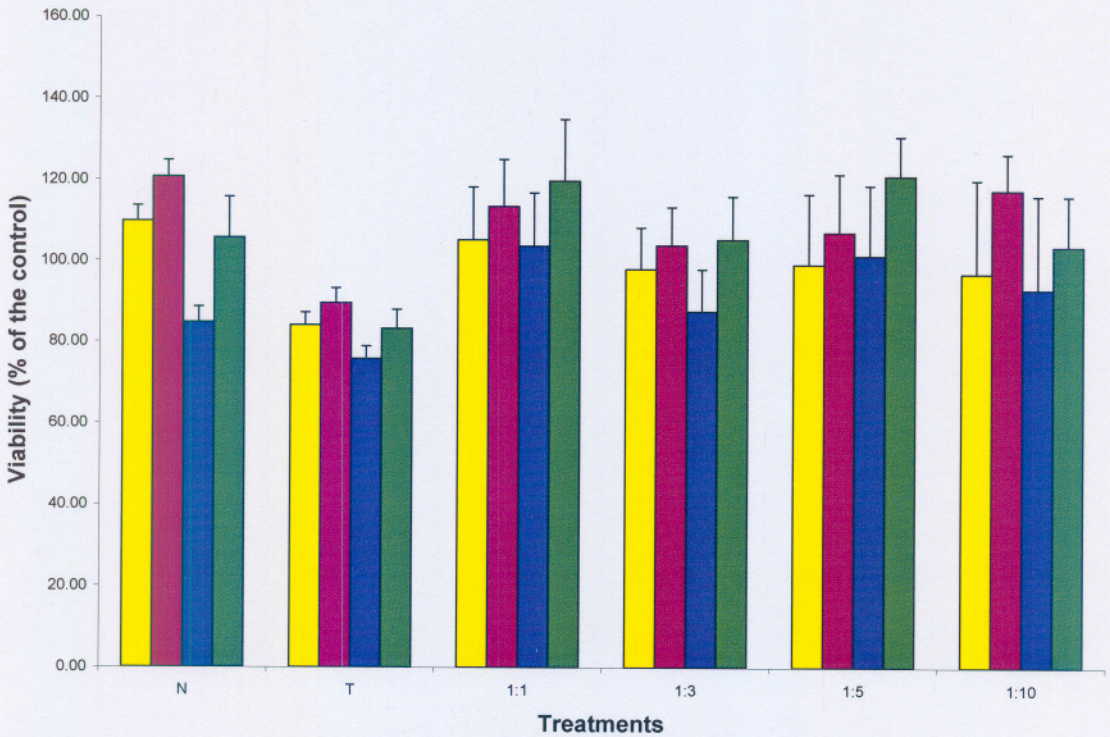


Figure 5.56 Cell viability after treatment of COS-1 cells with TMC H/DNA polyplexes (●), TMO H/DNA oligoplexes (●), TMO L/DNA oligoplexes (●) and TEO L/DNA oligoplexes (●) compared to naked DNA (N) and Transfectam®/DNA complexes (T). Ratios compared were 1:1, 1:3, 1:5 and 1:10. Each bar represents the mean value ± SD of 3 experiments.

In figure 5.56 the viability of the COS-1 cells is given for the 1:1, 1:3, 1:5 and 1:10 ratios of the polyplexes and oligoplexes that differed statistically in their transfection efficiencies, from the naked DNA. It was clear that the percentage viable COS-1 cells when transfected with the chitoplexes prepared with TMC H,

TMO L, TMO H, & TEO L was higher than the percentage viable cells when transfected with lipoplexes prepared with Transfectam®. The Transfectam®/DNA complexes induced cell damage and a decreased viability of COS-1 cells were found. Complexes (chitoplexes) from quaternised chitosan polymers and quaternised chitosan oligomers did not affect the viability of the cell line.

5.3 CONCLUSION

The relatively high solubility in acidic environments and low toxicity of chitosan allow its use in a wide variety of applications in the pharmaceutical industry and, as shown in this study, in gene delivery. Chitosan, especially quaternised oligomeric chitosan are polysaccharides that demonstrate much potential as a gene delivery system. In literature it was shown that the toxicity of the chitosan/DNA polyplexes is relatively low compared to viral gene and lipid non-viral delivery vectors.

This study showed that different chitosan polymers or oligomers and their quaternised chitosan polymers formed complexes with the plasmid DNA. The complexes formed with chitosan polymers, oligomers, quaternised chitosan polymers and quaternised chitosan oligomers was shown to be less toxic to COS-1 cells compared to the Transfectam®/DNA complexes.

The quaternised chitosan polymer TMC H, and the quaternised chitosan oligomers TMO L, TMO H, & TEO L proved to be statistically significant different compared to naked DNA in the ratios tested (1:1, 1:3, 1:5 and 1:10), with regard to their transfection efficiencies. Only the 1:10 ratios of the TMO L and TEO L differed statistically significant from the other ratios tested. The degree of quaternisation of the polymers and oligomers and their molecular size is therefore very important factors to consider for in gene transfection.

SUMMARY AND FUTURE PROSPECTS

The goal of gene therapy is to offer a means to treat currently incurable genetic and acquired diseases (Huang and Viroonchatapan, 1999:4). Gene delivery is a concept for treating a wide variety of conditions by supplementing the body cells with corrected copies of required genes. The ultimate therapeutic approach is to design safe and efficient gene delivery systems. Transport of the foreign genetic material to body cells can currently be achieved using viral or non-viral vectors. The efficiencies of viral vectors have proved to be superior to their non-viral counterparts, but toxicity, immune and inflammatory responses limit their use. Although non-viral vectors have inferior efficiency when compared to viral vectors, they offer flexibility and comparable safety. Non-viral vectors were created in an attempt to create synthetic viruses, engineered to exploit the advantages of viral carriers but without their drawbacks.

Different strategies have been used to improve transfection efficacy with cationic polymers. Cationic polymers have been used to condense and deliver DNA and promising results were reported (Mao *et al.*, 2001:399). A number of the properties of chitosan render it a potentially useful system for the delivery of plasmids (MacLaughlin *et al.*, 1998:259). Chitosan is non-toxic and biocompatible (Roy *et al.*, 1997:674). To increase the understanding of the effect of the degree of quaternisation (DQ) of different chitosan polymers when employed as a non-viral gene delivery system, a range of quaternised chitosan polymers, *N*-trimethyl chitosan chloride (TMC), *N*-triethyl chitosan chloride (TEC), *N*-trimethyl chitosan oligomer (TMO) and *N*-triethyl chitosan oligomer (TEO) were obtained from the Department of Pharmaceutics and evaluated for their gene transfection efficiencies. Polyplexes were formed and investigated for their ability to transfect COS-1 cells and the results were compared with Transfectam® lipoplexes.

A pGL3-control vector (Promega) was propagated in *Escherichia coli* (JM109) and isolated and purified using Wizard Maxipreps (Promega). The DNA concentration was determined by measuring UV absorbance at 260 nm and purity was confirmed by agarose gel electrophoresis. All complexes for each experiment were formed according to a calculated nitrogen/phosphate (N/P) ratio of 1:1, 1:3, 1:5 and 1:10. The chitosan and quaternised chitosan polymers were dissolved in 5 mM sodium acetate buffer and the plasmid was diluted in 5 mM sodium sulphate solution. Each solution was heated to 55 °C separately and then equal volumes were mixed together to keep the sizes of the nanoparticles uniform (Mao *et al.*, 2001:399). The complexes were characterised with transmission electron microscopy (TEM) and complex formation was confirmed. COS-1 cells were maintained in DMEM of pH value 7.4, supplemented with 10 % (w/v) fetal bovine serum (FBS) and 1 % (w/v) Pen-Strep-Fungizone®. The cells were seeded on 24-well plates at a seeding density of 10^5 cells/cm^2. Prior to transfection, culture medium was removed and the cells were rinsed twice with PBS (pH 7.4). The COS-1 cells were incubated with (a) naked DNA (N), (b) complexes of chitosan/quaternised chitosan and DNA (polyplexes) and (c) complexes of Transfectam® (T) and DNA (lipoplexes) at 37 °C in serum-free medium. After 4 h the formulations were removed, the cells were rinsed with PBS and grown in culture medium (with serum) for 48 h to allow for luciferase expression. The cells were harvested by adding Reporter Lysis Buffer and the luciferase activity was determined using a Luciferase Assay System and Luminometer. The protein content was determined using the Bradford protein assay and the results were expressed in Relative Light Units (RLU) per mg protein as an average of 3 experiments. The viability of the cell culture was investigated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tretrazolium bromide) assay (Thanou *et al.*, 2001:153).

The transfection results obtained indicated that TMC H polyplexes, TMO L, TMO H, and TEO L oligoplexes differed statistically significant when compared to naked DNA in the ratios tested. Only the 1:10 ratios of the TMO L and TEO L

differed statistically significant from the other ratios tested. The optimum degree of quaternisation for transfection in this study was between 15 % (TMO L) and 59 % (TMC H). The size of the molecule also seemed to play an integral role with the polymers and oligomers tested for transfection efficiency. It was clear that the percentage viable COS-1 cells when transfected with TMC H, TMO L, TMO H and TEO L was higher than the percentage viable cells when transfected with lipoplexes prepared with Transfectam® and that these chitoplexes did not affect the viability of the cell line. The Transfectam®/DNA complexes induced cell damage and a decreased viability of COS-1 cells was found.

It can be concluded that chitosan, especially oligomeric chitosan and its quaternised derivatives are polysaccharides that demonstrate much potential as a gene delivery system. In literature it was shown that the toxicity of the chitosan/DNA polyplexes is relatively low compared to viral gene and lipid non-viral delivery vectors. This study showed that oligomeric chitosan and trimethylated chitosan oligomers proved to be non-toxic in COS-1 cell cultures in contrast to Transfectam® that decreased viability. The high solubility and low toxicity of chitosan allow its use in a wide variety of applications in the pharmaceutical industry and, as shown in this study, in gene delivery.
Recommendations for future studies in this field are:

- New polymeric materials and derivatives need to be tested individually to assess their toxicity and transfection efficiency.
- The ratios for oligomer/DNA complexes should be optimised for TMO L and TEO L oligomers.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to all the following people.

My heavenly Father, the Creator and Guardian to whom I owe my very existence. I know that without your loving and caring hand upon me, none of this would be possible.

Johan, my dear husband, for always encouraging and motivating me. Thank you for always being there for me and for helping me with my 'baobab' trees in life. I love you.

Prof. Awie F. Kotzé, my supervisor and mentor, thank you very much for all your suggestions, encouragement, novel ideas and advice. Thank you for always believing in me, I greatly appreciate everything you have done for me. I could not have imagined having a better advisor and mentor.

Prof. Pieter J. Pretorius, Department of Biochemistry, for all the help and expert advice on the synthesis & purification of the plasmid and the use of your gel electrophoresis apparatus and capture system. I truly acknowledge everything that you have done for me.

Anne Grobler, Department of Pharmaceutics, thank you for all your positive input and helpful suggestions in the study.

Mev. Wilma Pretorius, Department Electron Microscopy, for all your help with my TEM micrographs and your friendship. I appreciate your efforts, time and advice.

Prof. Fanus Steyn, Statistical Consultation Services, North-West University, for his assistance with the statistical analysis of the data.

Sharlene Niewoudt, for all your time invested in the central laboratory. Thank you for always being the backbone of the system. I did not go unnoticed.

Mrs. Anriëtte Pretorius, thank you for all your help and advice with the bibliography.

My family, for your unconditional love, friendship and support. You're simply the best!

My friends, Sharlene, Jan, Karin, Ruaan, Jacques, Ian & Thersia, for your enjoyable friendship and support during this study.

All my colleagues, at the School of Pharmacy, thank you very much for valuable advice and support during this study.

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ANNEXURE 1

Certificate of analysis

ChitoClear™

PRODUCT:	Fine grade chitosan made from fresh shrimp shells. Batch no: TD 012
	CHEMICAL PARAMETERS:
DRY MATTER:	92.1%
ASH:	0.1 %
DEGREE OF DEACETYLATION	97.6% *(Titration method)*
SOLUBILITY:	> 99.9 % *(in 1 % acetic acid)*
TURBIDITY:	6 NTUs
VISCOSITY:	552 (mPa.s(cP)) *1 % solutions in 1 % acetic acid measured on a Brookfield RV DV-II viscometer, 25C, spindle no: RV2 at 60 rpm.*
	FEATURE:
FLAKES:	0.1 - 6 mm
	MICROBIOLOGICAL PARAMETERS:
TOTAL PLATE COUNT:	< 1 000 / g
ESCHERICHIA COLI:	absent
COLIFORME BACTERIA:	absent
SALMONELLA PR. 25 GRAM:	absent
MOULD AND YEAST:	< 100 / g

Primex Ingredients ASA

Christian Horst
Laboratory Manager

ANNEXURE 1 (cont.)

Certificate of analysis

ChitoClear™

PRODUCT:	Fine grade chitosan made from fresh shrimp shells. Batch no: TM 612

CHEMICAL PARAMETERS:

DRY MATTER:	95.7 %
ASH:	0.7 %
DEGREE OF DEACETYLATION	93.2 % *(Titration method)*
SOLUBILITY:	> 99.9 % *(in 1 % acetic acid)*
TURBIDITY:	9 NTUs
VISCOSITY:	12 (mPa.S(cp)) *1 % solutions in 1 % acetic acid measured on a Brookfield LVT viscometer, 25C, appropriate spindle At 30 rpm.*

FEATURE:

MICROMILLED POWDER:	100% finer than 60 mesh, 50% finer than 100 mesh
DENSITY:	0.46 g/cc *(USP method)*

MICROBIOLOGICAL PARAMETERS:

TOTAL PLATE COUNT:	< 1 000 / g
ESCHERICHIA COLI:	absent
COLIFORME BACTERIA:	absent
SALMONELLA PR. 25 GRAM:	absent
MOULD AND YEAST:	< 100 / g

Primex Ingredients ASA

Mette Aalstad Jansen
Laboratory Manager

ANNEXURE 1 (cont.)

PRIMEX CHITOSAN OLIGOSACCHARIDE

Certificate of Analysis

PRODUCT: Fine grade chitosan oligosaccharide made from fresh shrimp shells.
Batch no.: 1118372

CHEMICAL PARAMETERS:

DRY MATTER:	88,8 %
ASH:	0,4 %
DEGREE OF DEACETYLATION:	79,1 % *(UV-method)*
VISCOSITY:	1,4 (mPa.s(cP)) *1% solutions in 1% acetic acid measured on a Brookfield LV DV-II+ viscometer, 25C, spindle no ula at 60 rpm*
TURBIDITY:	8 NTUs
DENSITY (TAPPED):	0,56 g/cc *(USP method)*

MICROBIOLOGICAL PARAMETERS:

TOTAL PLATE COUNT:	< 1 000/g
ESCHERICHIA COLI:	absent
COLIFORME BACTERIA:	absent
SALMONELLA PR. 25 GRAM:	absent
MOULD AND YEAST:	< 100/g

Primex Ingredients ASA

Christian Horst
Laboratory Manager

ANNEXURE 1 (cont.)

CERTIFICATE OF ANALYSIS FOR CHITOSAN

Product name : SEACURE 244

Generic name : Chitosan

Product code : 27030244

Batch no. : 010-370-02R

				SME No.:
Viscosity	:	40	mPas	4004
Loss on drying	:	8.3	%	4018
Insolubles	:	0.3	%	4013
Deacetylation	:	93	%	4001
Ash content	:	0.1	%	4020
Particle size	:	Ground		

SME: Pronova Standard Method

Methods available upon request

POMOSIN-PECTACON B.V.
Röntgenweg 6
3752 LJ BUNSCHOTEN
HOLLAND

Drammen, September 12. 1995
Pronova Biopolymer
Bent Bogesen

Pronova Biopolymer a.s P.O. Box 494, N-3002 Drammen, Norway. Phone: + 47 32 83 73 00
Fax: + 47 32 83 34 88, Telex: 76594 pronb n

ANNEXURE 2

Promega

Cat. No. E1231

Lot No. 165217

Product: Transfectam® Reagent for the Transfection of Eukaryotic Cells

Storage Recommendations:

Store lipid film at room temperature. Once reconstituted in ethanol, store at 4°C.

PRODUCT USE LIMITATIONS, WARRANTY, DISCLAIMER
Promega manufactures products for a number of intended uses. Please refer to the product label for the intended use statements for specific products. Promega products contain chemicals which may be harmful if misused. Due care should be exercised with all Promega products to prevent direct human contact.

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Description: **1mg (Part # E123A)** or **0.5mg (Part # E123B)** of lipid film which has been dried down from ethanol. 1mg is sufficient Transfectam® to transfect 80-260µg of DNA depending on the Transfectam®:DNA ratio used. See Technical Bulletin accompanying this product for details on optimization.

Chemical Formula: $C_{49}H_{103}O_2N_6$ (Dioctadecylamidoglycyl spermine).

Molecular Weight: The molecular weight of dioctadecylamidoglycyl spermine is 807g. Transfectam® is supplied as a trifluoroacetate salt of this compound with a molecular weight of 1263g.

Reconstitution: Reconstitute 1mg of Transfectam in 400µl of 100% EtOH (0.5mg pack size should be reconstituted in 200µl) to yield a 2mM stock.

Stability: Transfectam® is stable for at least 12 months as supplied. Once reconstituted, it is stable for at least 6 months if stored at 4°C.

QUALITY CONTROL - this batch meets the following release criteria:

Purity: Chemical purity of Transfectam® is assessed by NMR.

Functional Testing: This product is tested for its ability to transfect NIH3T3 with pGL3-Control Vector (Cat# E1611) in the absence of serum. Cells are transfected with a Transfectam:DNA ratio of 2:1 in 60mm dishes following the protocol given in Technical Bulletin 116. Serum is added back to the medium 4 hours post-transfection and luciferase activity assayed 48 hours later using Promega's Luciferase Assay System (Cat# E1500).

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Unless otherwise stated on the label, the performance of this product is guaranteed for six months from the date of purchase if stored and handled properly.

Signed by: _____ Date: October 18, 2005
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