NANOPARTICULATE SYSTEMS FOR NUCLEIC ACID DELIVERY

Amir Khashayar Varkouhi
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NANOPARTICULATE SYSTEMS FOR NUCLEIC ACID DELIVERY

Nanodeeltjes als dragersystemen voor nucleïnezuren
(met een samenvatting in het Nederlands)

Proefschrift

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door

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geboren op 13 februari 1980, te Khorramabad, Iran

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Co-promotoren:	Dr. R.M. Schiffelers Dr. T.G.G.M. Lammers

"Nature is an unlimited broadcasting station, through which God speaks to us every hour, if we only will tune in."

George Washington Carver (1864-1943)

To my dear parents

Chapter 1

General Introduction

RNA interference (RNAi)-based therapeutics

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing based on translational repression or direct enzymatic cleavage of the target mRNA which results in inhibition of expression of the corresponding protein. Within cells, RNAi is mediated by double-stranded RNAs (dsRNAs) called microRNA (miRNA) and small interfering RNA (siRNA).

In the endogenous pathway, RNAs containing stem loops or short hairpin structures, encoded in the host genome are processed in the nucleus and exported to the cytoplasm as precursor molecules called pre-microRNAs. In the cytoplasm, these pre-microRNAs are further shortened and processed by an RNAse III enzyme called Dicer to produce double-stranded microRNA (miRNA). Dicer similarly processes long dsRNA into siRNA molecules in cytoplasm. Both miRNA and siRNA duplexes are incorporated into a protein complex called the RNA-induced silencing complex (RISC) which is responsible for cleavage of target messenger RNA (mRNA). Gene silencing is induced by siRNA through sequence-specific cleavage of complementary mRNA, whereas in case of the miRNAs, the mature miRNAs are incorporated into RISC, but rather than cleaving their target sequences, they generally mediate translational repression owing to incomplete complement formation with their target mRNA sequences [1-5].

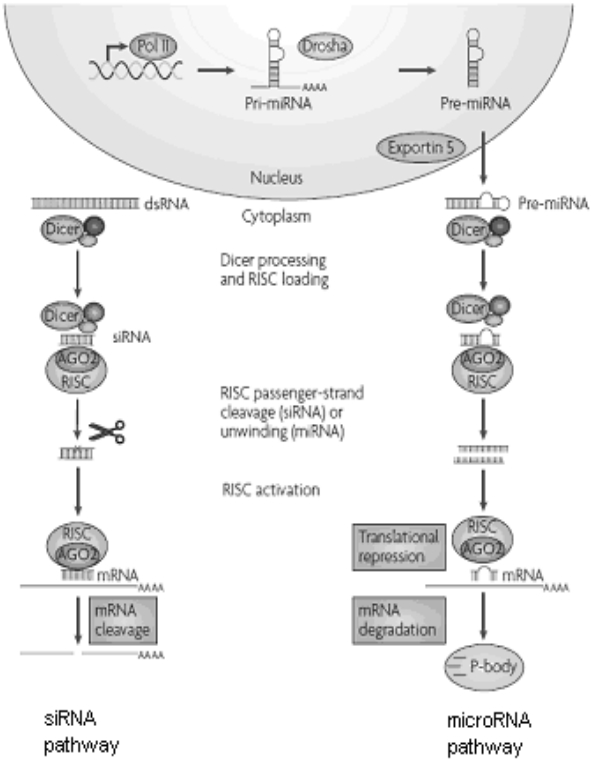

Figure 1. Mechanism of RNA interference (RNAi) in mammalian cells. RNAi pathways are guided by the siRNA pathway (left) and the microRNA pathway (right) (taken from De Fougerolles et al 2009 [4]).

The reduction in expression of target proteins through RNAi is applicable to all classes of molecular targets, including those that are difficult to modulate selectively with traditional pharmaceutical approaches involving small molecules or peptides/proteins [6]. Therefore, recently, considerable research efforts have been dedicated to the use of synthetic siRNAs as novel biotherapeutics for the treatment of several diseases with a genetic origin (Table 1).

Table 1. Small RNA-based therapeutics in clinical trials (taken from Lares et al 2010 [7]).

Diseases	Disease category	Drug name	Drug type	Target	Phase	Company/ Affiliation	Notes[a]
AMD	Ophthamology	Bevasiranib	siRNA	VEGF	III	Opko Health	Phase III was interrupted by Opko in 2009 (Box 1).
		PF-4523655 (RTP801^{n}i-14)	siRNA	RTP801	II	Quark	RTP801 is a Quarks proprietary target.
		AGN211745	siRNA	VEGF-R1	I/II	Allergan	AGN211745 is known as Sirna-027.
Macular edema	Ophthamology	Bevasiranib, Cand 5	siRNA	VEGF	II	Opko Health	
		PF-4523655 (RTP801^{n}i-14)	siRNA	RTP801	II	Quark	RTP801 is a Quark proprietary target.
Chronic optic nerve atrophy	Ophthamology	QPI-1007	siRNA	proNGF	I	Quark	
Pachyonychia congenita	Genetic disorder	TD1010	siRNA	Keratin K6a	I	Pachyonychia Congenita Project	
Chronic lymphocytic leukemia	Oncology	SPC2996	LNA oligo	Bcl-2	I/II	Santaris Pharma	
Metastatic lymphoma	Oncology	Proteasome siRNA	siRNA	Immuno-proteasome β-subunits LMP2, LMP7 and MECL1	I	Duke University	
		^{g}PLK SNALP	siRNA	PLK1	Pre-clinical	Tekmira	siRNA embedded in SNALP.
Solid tumors	Oncology	CALAA-01	siRNA	M2 subunit of ribonucleotide reductase	I	Pharmaceutical Inc	siRNA embedded in lipid nanoparticles.
		Atu027	siRNA	PKN3	I	Silence Therapeutics AG	
		EZN3042	LNA oligo	Survivin	I/II	Enzon	
		EZN2968	LNA oligo	HIF-1α	I/II	Santaris Pharma	
		FANG vaccine	shRNA	Furin	I	Gradalis	shRNA furin+ GMCSF expression.
		ALN-VSP	siRNA	KSP and VEGF	I	Alnylam	siRNA embedded in SNALP.
		eIF-4E ASO	LNA oligo	eiF-4E	I	Lilly	
		Survivin ASO	LNA oligo	Survivin	II	Lilly	For prostate cancer.
Delayed graft function	Inflammation	I5NP	siRNA	p53	I/II	Quark	Also known as QPI-1002.
Acute kidney injury	Inflammation	I5NP	siRNA	p53	I	Quark	Also known as QPI-1002.
Familial adenomatous polyposis	Inflammation	CEQ508	shRNA	β-Catenin	I	Cequent	Oral administration MDRNA acquired Cequent in 2010.
Hypercholesterolemia	Metabolic disease	PRO-040201	siRNA	ApoB	I	Pharmaceuticals Corp.	
		ApoB SNALP	siRNA	ApoB	I	Tekmira	siRNA embedded in SNALP.
		SPC4955	siRNA	ApoB	Pre-clinical	Santaris Pharma	
		ALN-PCS	siRNA	PCSK9	Pre-clinical	Alnylam	siRNA embedded in SNALP.
HCV	Viral infection	SPC3649	LNA oligo	miR-122	I	Santaris Pharma	
HIV	Viral infection	Lentivirus expressing shRNASI/ SII-TAR decoy and anti CCR5 ribozyme	shRNA+TAR decoy +CCR5 ribozyme	HIV Tat and Rev proteins, HIV TAR RNA, and human CCR5	I	City of Hope Medical Center/Benitec	
		pHIV7-shI-TAR-CCR5RZ	shRNA+TAR decoy +CCR5 ribozyme	HIV Tat protein, HIV TAR RNA, and human CCR5	Pre-clinical	City of Hope Medical Center/Benitec	Lentiviral expression in autologous CD4^{+}.
RSV	Viral infection	ALN-RSV01	siRNA	RSV nucleocapsids	II	Alnylam/Cubist	

Abbreviations: AMD, Age-related Macular Degeneration; Bcl-2, B-cell CLL/lymphoma 2; eiF-4E, eukaryotic translation initiation factor 4E; GMCSF, Granulocyte Macrophage Colony Stimulating Factor; HCV, Hepatitis C Virus; HIF-1a, Hypoxiainducible Factor 1 alpha; HIV, Human Immunodeficiency Virus; KSP, Kinesin Spindle Protein; LMP2, Large Multifunctional Peptidase 2; LMP7, Large Multifunctional Peptidase 7; LNA, Locked Nucleic Acid; MECL1, proteasome subunit beta type-10; PLK1, Polo-Like Kinase 1; PNK3, Protein Kinase N3; PSCK9, Proprotein convertase Subtilisin/kexin; proNGF, proform of Nerve Growth Factor; RSV, Respiratory Syncytial Virus; RTP801, DNA-damage-inducible transcript 4; shRNA, short Hairpin RNA; SNALP, Stable Nucleic Acid-Lipid Particles; TAR, Transactivating Region; VEGF, Vascular Endothelial Growth Factor.

However, the successful application of synthetic siRNA molecules designed to silence the expression of specific genes, is limited by their unfavorable biopharmaceutical properties. They are rapidly degraded by nucleases present in biological fluids, have low cellular membrane penetration capabilities, and show rapid renal elimination after i.v. administration. Therefore, they do not reach in sufficient amounts the cellular cytoplasm of the target cells, where the RNA interference (RNAi) machinery is situated. Consequently, a key challenge to the effective and widespread use of this new class of biotherapeutics is cytosolic delivery [8,9].

Gene therapy

Gene therapy is a therapeutic approach based on introducing therapeutic genes encoding missing, defective or new proteins into target cells of patients. A wide range of therapeutic DNA sequences and several therapeutic approaches have been investigated to achieve effective gene therapy [10]. The initial aim in the field of gene therapy was the correction of inherited genetic diseases by providing to the targeted cells a functional copy of the deficient gene responsible for the disease. Gene therapy can also be applied to the treatment of acquired diseases, in particular cancers. The situation is more complex in cancer gene therapy because cancer most often results from sequential genetic and epigenetic alterations, affecting oncogenes, tumour-suppressor genes and microRNAs [11-13] So far, several gene therapy clinical trials have been performed (Figure 2). During the last decade, a series of minor successes but particularly failures in treatment of diseases such as cancer have left the field somewhat frustrated [14].

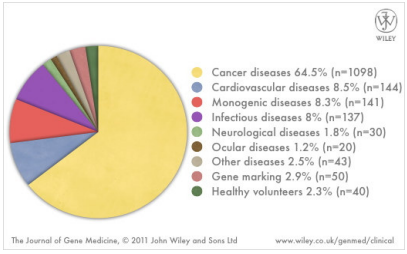

Figure 2. Gene therapy clinical trials approved worldwide 1989-2010 (taken from [15]).

Nucleic acid delivery

In both approaches of RNAi and gene therapy, an efficient delivery of the nucleic acids to their intracellular sites of action is an essential step [16]. Nucleic acids including siRNA and DNA are rapidly degraded by nucleases present in biological fluids and are unable to enter the cells by passage over the plasma membrane and to overcome the intracellular barriers such as the endosomal membrane (Figure 3).

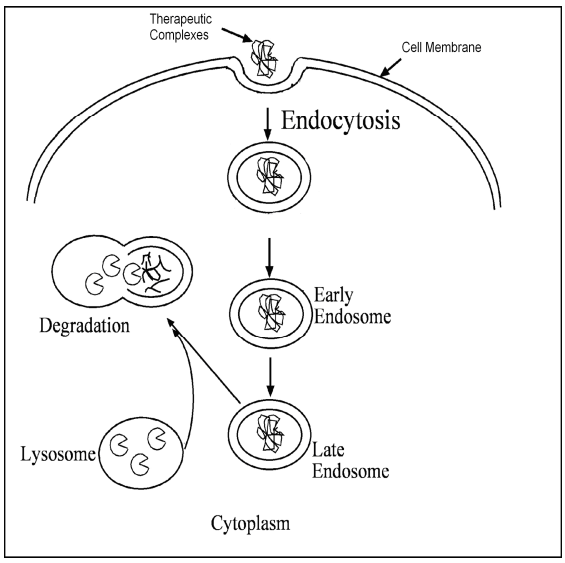

Figure 3. An artistic representation depicting the internalization of nanoparticulates into the cell by endocytosis. Early endosomes are vesicles containing therapeutic complexes coming from the cell surface. Late endosomes, which are thought to mediate a final set of sorting events prior to interaction with lysosomes, receive the internalized materials from early endosomes. Lysosomes, as last parts of the endocytic pathway, contain hydrolytic enzymes which digest the contents of the late endosomes. Therefore, nucleic acids need to escape from the endosomes before lysosomal degradation occurs.

Endocytosis is one of the cellular uptake mechanisms which a part of that involves vesicles known as endosomes with an internal pH around 5 that mature from early endosomes to late endosomes and fuse with intracellular organelles called lysosomes which contain certain enzymes.

Thus, endocytosed therapeutic cargos become entrapped in endosomes and eventually end up in the lysosomes, where active enzymatic degradation processes take place [17,18]. Therefore, in order to facilitate nucleic acid delivery to other intracellular compartments, like the cytosol for siRNA and the nucleus for plasmid DNA, several types of carrier systems have been studied. They can be subdivided in viral and nonviral carrier systems.

Viral delivery systems

Viruses are nature's own highly efficient gene delivery systems. By loading viruses with therapeutic genes, they can be applied as efficient vectors for gene therapy. The most commonly used viral vectors are derived from adenoviruses, adeno-associated viruses and retroviruses. Adenoviruses are highly efficient in DNA delivery and transduction of the cells but can not integrate the DNA into the host cell genome but also as a drawback induce high immunogenicity. Retroviruses and adeno-associated viruses are able to integrate their DNA into the host cell genome, resulting in persistent transgene expression. However, retroviruses are only able to efficiently infect dividing cells which limits their clinical application. Adeno-associated viruses have a low loading capacity for genetic material. So far, viral vectors have been applied in the large majority of clinical gene therapy trials. However, because of limited loading capacity and safety issues such as immunostimulatory effects and oncogenesis due to DNA integration, research efforts have also been dedicated to design efficient nonviral vectors [19,20].

Nonviral delivery systems

In order to overcome the disadvantages of viral vectors, synthetic nonviral vectors based on polymers, lipids and carbon nanotubes (CNTs) have been developed. As compared to viral vectors, nonviral vectors are superior in terms of safety and scale-up potential, but they show lower transfection activity. Positively charged polymers and liposomes can electrostatically interact with negatively charged nucleic acids. They form nano-sized complexes so called polyplexes and lipoplexes, respectively, which have been studied for their ability to enter cells and deliver nucleic acids to their intracellular targets. However, their delivery efficiency and toxicity

are still under intensive investigation [21,22]. In this thesis, several polymeric vectors and CNTs were studied for nucleic acid delivery.

Polymeric vectors. Cationic polymers with either permamently groups or protonable amines have been studied for nucleic acid delivery both *in vitro* and *in vivo*. The relative number and pKa of the protonable amines differs between cationic polymers. Some of them, such as poly (L-Lysine) (pLL), are linear polymers, while other ones, like poly (ethylenimine) (PEI) and dendrimers, consist of highly branched chains. Furthermore, some of them have the positive charges in the backbone (as in PEI) while others have these charges in side groups (as in e.g. pLL) [23]. Due to their positive charge, such polymers are able to complex via electrostatic interactions with anionic nucleic acids to form so called polyplexes. However, the delivery efficiency and stability of the complexes in biological fluids is still a matter of concern. Many cationic polymers have shown considerable toxicity *in vitro* and *in vivo*. The toxicity of these vectors is attributed to their cationic character, and additionally, to the fact that many of the studied polymeric vectors are not biodegradable. Therefore, the search for effective but non-toxic vectors is currently of prime interest. The recent literature shows that biodegradable polymers are attractive candidates for the design of such nucleic acid loaded polyplexes [24,25].

Carbon Nanotubes (CNTs). Carbon nanotubes (CNTs) (Figure 4) are nano-sized tubes of carbon with unique properties which have raised great enthusiasm in the nanomedicine field for their promise as potential drug, antigen and nucleic acid delivery vehicles. Because of their nano-needle structure (Figure 4), experimental observations suggest that they can cross cell membranes and translocate directly into the cytosol, utilizing an endocytosis-independent mechanism without inducing cell death. Obviously, if this mechanism is applicable to a great variety of target cell types, such an endocytosis-independent cell entry mechanism of CNTs is a valuable advantage. Moreover, CNTs offer a structural advantage due to their large surface area which can be modified with functional groups and be complexed with therapeutics such as nucleic acids, drugs and proteins. These properties account for the considerable scientific and industrial interest, as evidenced by many publications on nanotubes reported annually [26,27].

CNTs are categorized as single-wall CNTs (SWCNTs) and multi-wall CNTs (MWCNTs). SWCNTs consist of a single graphite lattice rolled into a perfect cylinder and MWCNTs contain several concentric cylindrical graphite shells. Several studies suggest that SWCNTs and MWCNTs can be used as efficient drug delivery systems *in vitro* and *in vivo*. Functionalized CNTs have been designed which are able to electrostatically bind nucleic acids and to deliver these macromolecules to their intracellular targets. Nevertheless, in recent years, conflicting data have been reported concerning delivery properties, and particularly regarding their safety and biocompatibility [28].

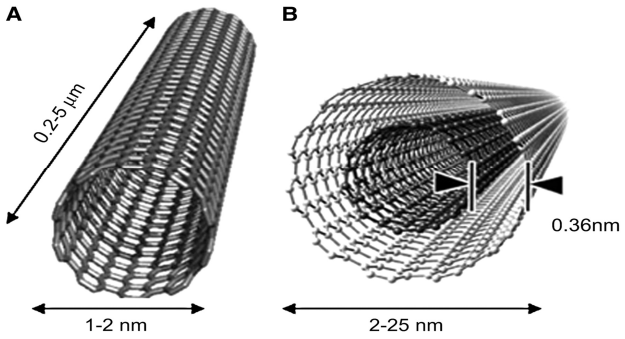

Figure 4. Schematic structures of a single-wall carbon nanotube (SWCNT) (A) and a multi-wall carbon nanotube (MWCNT) (B) (taken from Reilly et al 2007 [29]).

Aim and outline of this thesis

The aim of this study is to investigate the applicability of nanoparticulate systems based on polymers and carbon nanotubes (CNTs) for nucleic acid delivery. Several new biodegradable and non-degradable polymers as well as functionalized CNTs have been characterized and studied for their *in vitro* and *in vivo* nucleic acid delivery properties.

Chapter 2 is based on a literature study reviewing several mechanisms of endosomal escape to be utilized for overcoming the endosomal membrane as a barrier for the delivery of nucleic acids to intracellular sites other than endo/lysosomes.

In **Chapter 3**, two differently functionalized carbon nanotubes (CNTs) are studied for their siRNA delivery properties. CNTs have been proposed to

be able to penetrate the plasma membrane as nano-needles and to enter directly into the cytoplasm by an endocytosis-independent passage mechanism.
Most cationic polymers designed for nucleic acid delivery possess one cationic charge per monomer unit. In **Chapter 4**, a cationic polymer model with two cationic sites per monomer unit (quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxy styrene] (QNPHOS) as well as its block copolymer with PEG are used to investigate the hypothesis that the presence of two charges per monomer unit can enhance the nucleic acid binding and delivery properties of polyplexes.
Biodegradability of carrier systems is essential to minimize toxicity and to allow intracellular release of the bound pDNA/siRNA. In **Chapter 5**, two biodegradable polymers, pHPMA-MPPM (poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol)) and TMC (O-methyl free N,N,N-trimethylated chitosan), designed by our group are studied for siRNA delivery. In this study, photochemical internalization (PCI) is applied to enhance endosomal escape.
Introduction of thiol groups in several carrier systems has been shown to enhance their delivery properties due to formation of reducible disulfide bonds between thiol groups which leads to increased extracellular stability and improved intracellular release properties of the complexes. In **Chapter 6,** thiolated TMC (TMC-SH) is studied for siRNA delivery and compared to non-thiolated TMC.
In **chapter 7**, initial results are being presented on the application of selected polyplex systems for the treatment of rheumatoid arthritis. In this chapter, the *in vivo* anti-inflammatory properties of polyplexes containing anti-TNF-α siRNA and several biodegradable polymeric delivery systems are studied in a Rheumatoid Arthritis (RA) mouse model.
Chapter 8 provides a summary of the obtained results along with some future perspectives.

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Chapter 2

Endosomal Escape Pathways for Delivery of Biologicals

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Abstract

Despite continuous improvements in delivery systems, the development of methods for efficient and specificdelivery of targeted therapeutic agents still remains an issue in biological treatments such as protein and gene therapy. The endocytic pathway is the major uptake mechanism of cells and any biological agents, such as DNA, siRNA and proteins. These agents become entrapped in endosomes and are degraded by specific enzymes in the lysosome. Thus, a limiting step in achieving an effective biological based therapy is to facilitate the endosomal escape and ensure cytosolic delivery of the therapeutics. Bacteria and viruses are pathogenswhich use different mechanisms to penetrate themembranes of their target cells and escape the endosomal pathway. Different mechanisms such as pore formation in the endosomal membrane, pH-buffering effect of protonable groups and fusion into the lipid bilayer of endosomes have been proposed to facilitate the endosomal escape. Several viral and bacterial proteins have been identified that are involved in this process. In addition, chemical agents and photochemical methods to rupture the endosomal membrane have been described. New synthetic biomimetic peptides and polymers with high efficacy in facilitating the endosomal escape, low pathogenicity and toxicity have been developed. Each strategy has different characteristics and challenges for designing the best agents and techniques to facilitate the endosomal escape are ongoing. In this review, several mechanisms and agents which are involved in endosomal escape are introduced.

Keywords: endosomal escape, cytosolic delivery, protein, gene, siRNA

Introduction

The success in the application of nanomedicines and gene therapy is largely dependent on the development of the vectors that can selectively and efficiently deliver the gene or therapeutic agents to the target cells with minimal toxicity [1,2]. Despite the efforts given in vector technology, development of methods for efficient and protected delivery of therapeutic agents to
the target cells still remains a main issue [3,4]. On the other hand, principal considerations to design safe and reliable delivery systems led to the development of physically targeted delivery vehicles [5]. Despite the potent immunogenicity of viral vectors, their developed cell entry mechanism and high transfection efficiency in both dividing and non-dividing cells is desirable [6,7].
Nowadays, nonviral vectors with minimal toxicity and immuno-genicity have been developed to mimic the receptor-mediated cell entry mechanism of viruses. Although the early attempts to deliver biologicals, by application of nonviral vectors that follow the receptor-mediated endocytosis have fallen short of the goal of efficient delivery, mainly because of inability to escape the endosomal pathway [8,9]. Several approaches have been tested to facilitate the early release of therapeutic cargos from the endosomal pathway into the cytosol. These approaches were based on identified mechanisms for endosomal escape, like pore formation in the endosomal membrane, the pH-buffering effect and conformational changes in endosomal escape enhancers. These include the use of viral proteins, bacterial proteins and especially synthetic biomimetics as endosomal-releasing agents in nucleic acid and protein delivery systems. New synthetic biomimetic peptides are used as endosomal escape reagents; however, their usage is limited because of several potential problems and disadvantages such as immunogenicity and low stability. Considering these problems and also inspired by the principle behind these biological strategies, synthetic polymers that contain pH-sensitive chemical functionalities that mimic those of biological delivery systems have been designed and tested as new endosomal- releasing components [10]. However, an optimal agent for endosomal escape should have high efficiency with no toxicity.
The endocytic pathway (Figure 1) is one of the uptake mechanisms of cells. This pathway is composed of vesicles known as endosomes with an internal pH around 5 that mature in a unidirectional manner from early

endosomes to late endosomes before fusing with intracellular organelles called lysosomes which contain certain digestive enzymes [11]. Thus, particles entering the cells via the endocytic pathway become entrapped in endosomes and eventually end up in the lysosome, where active enzymatic degradation processes take place. This results in a limited delivery of therapeutic agents to the intracellular targets. Therefore, many compounds with a promising potential *in vitro*, can not be applied *in vivo* because of bioavailability problems. So far, several attempts have been made to deliver various macromolecular components directly into the cytosol, escaping the endocytic pathway to protect them from degradation [12-14].

While many viruses have evolved quite efficient systems for endosomal release [15,16], the situation is different for nonviral vectors, where in many cases the lack of endosomal escape is a major obstacle for efficient biological delivery, implying that more efficient methods for endosomal release would lead to improvements in designing synthetic transfection systems.

In contrast to synthetic vectors, viral vectors are known to be efficient both for *in vitro* and *in vivo* applications [17,18]. However, in the case of the adeno-associated viruses, intracellular barriers such as the endosomal membrane have been described [19,20] which highlights the potential beneficial effects of the enhanced endosomal escape for viruses [21-24].

The importance of preventing the degradation of therapeutics in the endosomes/lysosomes has been exemplified by the use of lysosomotrophic agents such as chloroquine which prevents the activity of lysosomal enzymes [25,26]. In this review, severalmechanisms which have been proposed for endosomal escape aswell as the agentswhich are known to have the endosomal release properties are introduced.

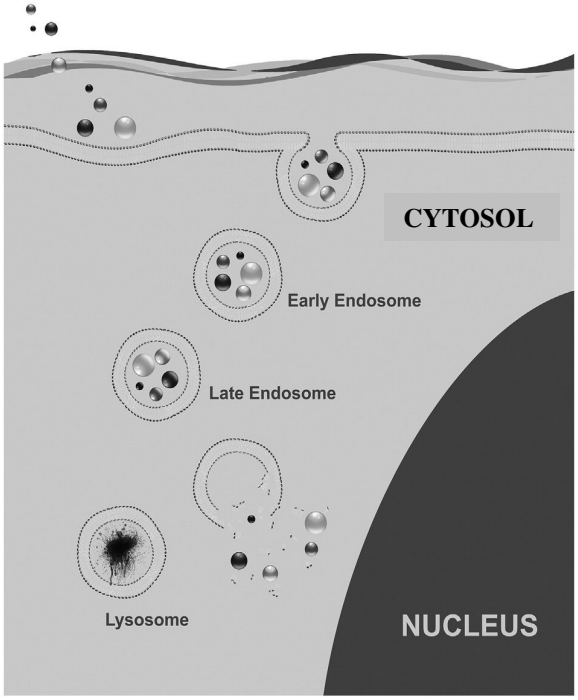

Figure 1. An artistic representation depicting the internalization of therapeutics into the cell through endocytosis and subsequent endosomal escape. Early endosomes consist of the vesicles containing the therapeutics coming from the cell surface. Late endosomes which are thought to mediate a final set of sorting events prior to interaction with lysosomes, receive the internalized materials from early endosomes. Lysosomes as the last parts of the endocytic pathway contain the hydrolytic enzymes which digest the contents of the late endosomes. Therefore, the endosomal release of the therapeutics is necessary before lysosome mediated digestion of the therapeutics.

The mechanisms of Endosomal escape

Understanding the mechanisms of viral and bacterial escape from endosomes is important for improving cellular delivery of therapeutic agents. The mechanisms of these processes have been intensely studied. Enveloped and non-enveloped viruses have evolved mech-anisms for membrane penetration, which are essential for endosomal escape. In enveloped viruses, the fusion of the viral envelope with the lipid bilayer may occur and non-enveloped viruses either lyse the vesicular membrane

or generate a pore through it to allow escape of the viral genome into the cytosol [27,28].
In the case of bacteria, pore formation is one of the basic methods for endosomal escape which is mediated by bacterial exotoxins [29]. The acidic pH of endosomes triggers the endosomal escape by affecting the peptides and leading to interactions between the peptides and the lipid bilayer of the endosomes. In some cases these peptides forma randomcoil structure at pH 7 and as the pH decreases, some domains of amino acids are protonated leading to the transition into an amphipathic α-helical conformation. Consequently, the peptides can interact with phospholipid membranes to form pores or induce membrane fusion and/or lysis [30].
In the following paragraphs a number ofmechanisms proposed for endosomal escape are described.

Pore formation in the endosomal membrane

In general, pore formation is based on the interplay between a membrane tension that enlarges the pore and a line tension that closes the pore. Some components like peptides have a high affinity for the rim of the pore. Binding of the peptides to the rim leads to reduction of the line tension which decreases the number of peptides causing the internal membrane tension and keeps the pore radius stable [31] (Figure 2).
It has been shown that binding of agents such as cationic amphiphilic peptides (AMPs) to the lipid bilayer leads to internal stress or internal membrane tension that can be sufficiently strong to create pores in the lipid membrane. Some models such as barrel-stave pores or toroidal channels have described these events which lead to formation of pores in the lipid bilayer. In the barrel-stave pore model, peptides reorient to become the staves which together form a barrel-shaped cluster. This cluster of peptides orients perpendicular to the plane of the lipid bilayer and forms the pore. Formation of toroidal pore model is mediated by aggregates of peptides which enter into
the membrane in a perpendicular orientation followed by membrane curving inward to form a hole which the peptides line it [32].

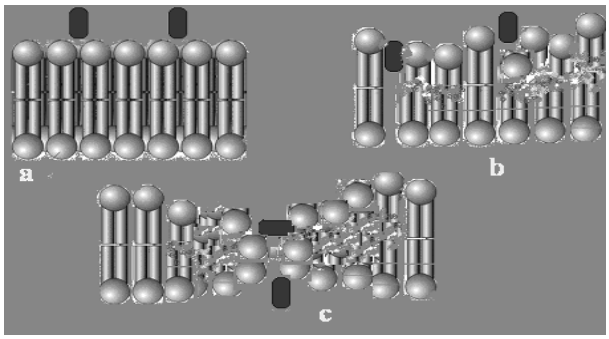

Figure 2. Proposed interactions between peptides and lipid bilayer. (a) Peptides are soluble in water but have a high affinity for binding to the lipid bilayer. (b) Peptides inserting into the membrane cause thinning of the chain region and thus create an internal membrane tension. (c) Peptides preferentially bind to the edges of the pores, which has the consequence of loosening the internal membrane tension.

pH buffering effect (the proton sponge effect)

The proton sponge effect is mediated by agents with a high buffering capacity and the flexibility to swell when protonated. Protonation, induces an extensive inflow of ions and water into the endosomal environment which subsequently leads to rupture of the endosomal membrane and release of the entrapped components (Figure 3). Tertiary amine groups that contain a hydrophobic chain, have been shown to accumulate in endosomes which have an acidic pH and become detergents upon protonation resulting in disruption of the membrane [33]. As an example, histidine-rich molecules show a buffering effect upon protonation of the imidazole ring of histidine, resulting in disruption of the endosomal membrane [34,35] and poly (amido amine) polymers have a high buffering effect due to the presence of protonatedamine groups in their structurewhich leads to an increase in osmotic pressure in the endosome that results in disruption of the endosomal membrane [36].

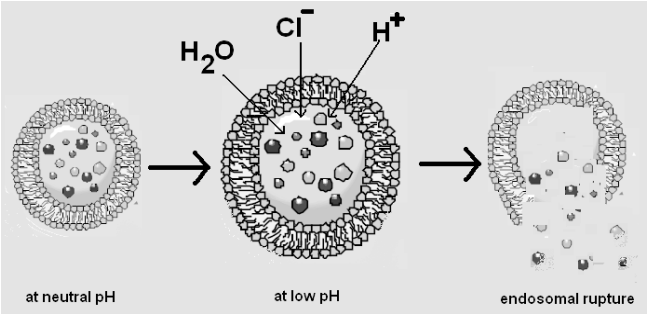

Figure 3. An artistic representation depicting the proton sponge hypothesis. The low pH in endosomal environment leads to protonation of the entrapped agents with a high buffering capacity. Protonation leads to inflow of H+ and Cl– and water into the endosomes, resulting in osmotic swelling and endosome rupture.

Fusion in the endosomal membrane

Another mechanism for endosomal escape is the destabilization of the endosomal membrane by fusogenic peptides which are used in a number of fusion systems. Membrane fusion plays an important role in cellular trafficking and endocytosis. The majority of viruses have single integral membrane peptides which undergo conformational changes upon a trigger such as a change in pH. These conformational changes allow the protein to induce the fusion in the lipid bilayer [37,38]. Haemagglutinin,which is a peptide of the influenza virus coat, acts as a fusogenic agent that is converted from an anionic, hydrophilic coil at pH 7.4 to a hydrophobic helical conformation at the acidic endosomal pH. This new α-helical structure leads to fusion of the viral membrane into the cellular membrane [39].

Photochemical disruption of the endosomal membrane

The possibility of photochemically releasing biologicals from endosomal pathway into the cytosol has been described as a technique called photochemical internalization (PCI). A number of photosensitizers, including TPPS4, TPPS2a, AlPcS2a and dendrimer-based photosensitizer (DP) (dendrimer phthalocyanine (DPc)) localize primarily in the membrane of the endosomes and lysosomes [40,41]. After exposure to light, these photosensitizers induce the formation of reactive singlet

oxygenwhich has a short lifetime and destroys the endosomal/lysosomal membrane, whereas the contents of the organelles remain intact and are delivered to the cytosol [42,43].
This technique has been applied for delivery of several formulations, such as lipid carriers [44,45] and polymers [46,47]. Moreover, in some studies photo stimulation has been applied to enhance the endosomal release of the therapeutic complexes containing cell-penetrating peptides [48,49].

Endosomal escape agents

Researchers have started to use the methods employed by viruses and bacteria for endosomal escape. So far several endosomal escape agents from different sources have been purified or synthesized (Table 1). Here several agents that are possibly useful to enhance endosomal escape are described.

Proteins and peptides

Protein and peptide-based agents are the principal groups of endosomal escape agents which are derived from several viral, bacterial, vegetal and human/animal sources. Furthermore, recombinant DNA technology has enabled the production of small and large molecular weight polypeptides with repeating blocks of amino acids with precise compositions, sequences and lengths.

Virus derived agents. In many cases, small peptide domains of viral proteins have been identified which have a crucial function responsible for endosomal escape. The HA2 subunit of haemagglutinin (HA) protein of the influenza virus with a short chain of an N-terminal amphiphilic anionic peptide has shown fusogenic activity. At low pH, the protonation of the glutamic acid and the aspartic acid causes a conformational change to a helical structure in this peptide which leads to activate its fusogenicity resulting in destabilization of the endosomal membrane. Therefore, in several studies this peptide has been used as a fusogenic agent for endosomal escape [50-52]. It was shown that conjugation of poly (L-lysine) (PLL) to HA-2 peptide can enhance the endosomal release properties of the HA-2 [53].
The influenza-derived fusogenic peptide diINF-7 has been used to facilitate the endosomal escape. The presence of this peptide, efficiently

enhanced the endosomal escape when used in complex with lipid carriers for siRNA [54] and protein [55] delivery and also in complex with polymeric vectors for gene delivery purposes [56-58].
The penton base protein is the most hydrophobic protein of adenovirus in acidic environments. This protein interacts with the lipid bilayer of the endosomes which disrupts it through an unclear mechanism. However, previous studies have shown that viral fusogenic peptides are able to formpores in the endosomal membrane in acidic environment which leads to release of biologicals into the cytosol [59].
Also gp41 transmembrane protein as a part of Env glycoprotein in HIV acts as a fusogenic agent that leads to formation of helical conformation. This protein has been used as a hydrophobic domain in combination with a hydrophilic domain derived from the nuclear localization sequence of SV40 large TY antigen. In the case of gp41, the fusion step is not well understood and the hypothesis of stalk-pore formation has been proposed which is based on making the hemifusion diaphragm by transmonolayers which is ruptured to form the complete fusion pore [60,61].
In another study, a gp41-derived peptide from HIV was covalently linked to polyethylenimine (PEI) and the peptide-modified polymer was complexed with DNA. The gp41-derived peptide demonstrated significant lytic activity both as free peptide and when conjugated to PEI [62].
TAT (HIV-1 Trans-activator gene product) is known as a powerful transcription factor of the integrated human immunodeficiency virus-1 (HIV-1) genome. This peptide was first identified to activate HIV-1 transcription when added extracellularly. Several studies have shown that TAT protein can efficiently facilitate gene transfer via membrane destabilization. This protein contains arginine and lysine amino acid residues which are involved in lipid membrane penetration and are
able to facilitate and enhance the internalization of large liposomes and largemolecules, such as dextran particles encapsulatingmagnetic beads [63]. Several studies have revealed that in the case of TAT-based gene delivery systems, the size of the peptide complexes plays an essential role in the uptake mechanism by endosomes. Particles smaller than 300 nm do not enter the cell through the endosomal pathway, in contrast to particles of 500-700 nm which are taken up by endocytosis [64-67]. In a study by Lung et al., sequences of histidine and cysteine residues incorporated into the TAT structure which led to promote the gene transfection efficiency by up to 7000-fold over the original TAT peptide. It is claimed that

presence of histidine and cysteine residues, promote the endosomal escape properties and stability of the TAT/DNA complexes respectively [68].
L2 peptide which is located at the C terminus of the minor capsid protein of Papillomavirus has shown a strong membrane-disrupting activity at low pH. This peptide leads to release of the viral genomes from endosomes and induces cytolysis of bacteria and eukaryotic cells [69].
The major envelope protein (E) of the West Nile virus is another example of a fusogenic agent which exerts its endosomal disruptive activities at an upper threshold of pH 7.0 and has a maximum activity at pH 6.4 and below which leads to maximum conformational change in the protein within seconds [70].
In addition, several groups have already shown that lysine-rich peptides and cationic peptides, derived from viral proteins which mimic the endosomal disruptive properties of viral particles, penetrate cells and facilitate the delivery of nucleic acids [71].

Bacteria derived agents. Bacteria are as opportunistic as viruses and toxins, but they are also manipulators. They introduce bacterial proteins, known as effectors, into the host cells tomodify their behavior. Some bacteria simply avoid their degradation by destabilizing the phagosome membrane and facilitate their own release into the cytosol of the host cells.
Listeria monocytogenes uses this strategy. This bacterium produces listeriolysin O (LLO) which is a cholesterol-dependent toxin that at acidic pH induces pore formation in the cholesterol containing lipid bilayers [72]. This protein is a known as a pore-forming hemolysin [73] that undergoes a rapid cytosolic degradation which in combination with its pH-dependent activity, reduces its potential cytotoxic effects [74]. In several studies, LLO has been used as an endosomal
escape agent, in combination with lipid carriers [75,76] and cationic polymers [77,78].

Table 1. Endosomal escape agents

Category	Agent	Mechanism of endosomal escape
Proteins and peptides	**Virus derived agents**	
	haemagglutinin (HA2) [50-52]	Fusion
	(HA2)/poly (L-lysine) (PLL) [53]	Fusion
	diINF-7 [54-58]	Fusion
	penton base [59]	Pore
	gp41 [60,61]	Pore/fusion
	gp41/polyethylenimine (PEI) [62]	Pore/fusion/Proton sponge
	TAT [63-68]	Unclear
	L2 from Papillomavirus [69]	Fusion
	envelope protein (E) of West Nile virus [70]	Fusion
	Bacteria derived agents	
	listeriolysin O (LLO) [72-78]	Pore
	Pneumococcal pneumolysin (PLO) [79]	Pore
	Streptococcal streptolysin O (SLO) [79]	Pore
	Diphtheria toxin (DT) [80-83]	Fusion
	Pseudomonas aeruginosa exotoxin A (ETA) [84-88]	Pore
	Shiga toxin [89]	Pore
	cholera toxin [90]	Pore
	Plant derived agents	
	Ricin [90,91]	Unclear
	Saporin [92-94]	Unclear
	Gelonin [92-94]	Unclear
	Human / animal derived agents	
	human calcitonin derived peptide, hCT [95-98]	Unclear
	fibroblast growth factors receptor(FGFR3) [99,100]	Unclear
	Melittin [101-106]	Pore
	Synthetic peptides	
	(R-Ahx-R)(4) AhxB [107-110]	Unclear
	glycoprotein H (gpH) from herpes simplex [111]	Fusion
	KALA [112-115]	Fusion
	GALA [116-120]	Fusion
	Synthetic surfactants [121,122]	Fusion
	Penetratin (pAntp) [123,124]	Unclear
	R6-Penetratin with arginine-residues [125]	Unclear
	EB1 [126]	Unclear
	bovine prion protein (bPrPp) [127-129]	Pore
	Poly (L-histidine) [130-134]	Proton sponge
	Sweet Arrow Peptide (SAP), proline-rich [135,136]	Fusion
Chemicals	polyethylenimine (PEI) [137]	Proton sponge
	Poly(amidoamine)s (PAAs) [141-143]	Proton sponge
	poly(propylacrylic acid) (PPAA) [144]	Proton sponge
	ammonium chloride [145]	Proton sponge
	chloroquine [145]	Proton sponge
	methylamine [145]	Proton sponge

Pneumococcal pneumolysin (PLO) and Streptococcal streptolysin O (SLO) are other members of the family of pore-forming toxins. The pore-forming ability of these bacterial membrane-disruptive agents is due to the presence of the amino acids sequence at their C. termini, containing Tryptophan residues which are crucial for pore formation and a Cysteine residue that makes them intolerant for oxidizing conditions [79].
Diphtheria toxin (DT) is a protein secreted by Corynebacterium diphtheria [80]. At endosomal pH, the T domain of this toxin undergoes conformational changes and acts as a fusogenic peptide which inserts into the endosomal membrane [81]. Diphtheria toxin T domain fuses with the endosome membrane by pH decrease and then enhances the endosomal escape of the diphtheria toxin C fragment. It was shown that conjugation of diphtheria toxin T domain to poly (ethylenimine) (PEI) polyplexes enhanced the endosomal escape and transfection efficiency of the system [82]. The endosomal release properties of DT were shown in other studies [83]. Pseudomonas aeruginosa produces a single-chain exotoxin with three major domains so called exotoxin A (ETA). Domain II can facilitate the endosomal escape resulting in translocation of the bacterium to the Cytosol [84-86]. In several studies, domain II of Pseudomonas exotoxin has been used as an endosome disruptive agent in fusion proteins and immunotoxins [86-88]. There are other bacterial toxins the same as ETA, with endosomal release properties which contain an enzymatically active part that is able to modify a cytosolic target. These toxins including Shiga toxin and cholera toxin exert their effect on cells by binding to the cell surface and internalization through endocytosis. Subsequently they are transported retrogradely all the way to the ER before translocation of the enzymatically active part to the cytosol [89].

Plant derived agents. Several protein toxins in plants have cytosolic targets and enter mammalian cells via endocytosis. For instance, Ricin is a ribosome-inactivating protein (RIP) originated from Ricinus communsis which is known as an anti-cancer agent with membrane-disruptive properties. It was shown that Ricin is able to facilitate the release of large and small molecules from lipid vesicles [90,91].
Saporin and Gelonin are other members of plant RIP family which enter the cells via receptor-mediated endocytosis. It was shown that Saporin does not require a low pH for membrane destabilization. The endosomal release properties of these vegetal toxins have been shown previously [92-94].

Human/animal derived agents. Several agents with human or animal sources are known which are effective in endosomal escape.The human calcitonin derived peptide, hCT (9-32) belongs to the cell-penetrating peptide family. This peptide and its derivatives enter the cells through lipid raft-mediated endocytosis. However, the endosome disruptive mechanism of these peptides is unclear [95]. Several studies have proven the high protease resistance and high efficiency of N-terminally truncated hCT (9-32) and its analogues in penetrating the membranes of various cell lines [96-98].
The family of fibroblast growth factors (FGFs) consists of 22 multifunctional, heparin-binding polypeptides which have key roles in several cellular processes. The FGFs perform their functions through binding to their cellular receptors called FGFRs. It was shown that activated FGFR3 which is normally targeted for lysosomal degradation, is able to escape lysosomal targeting. Furthermore, it was reported that FGFRs interact with Heparan Sulfate which is available in cellular membranes and cross the lipid bilayer. These evidences might show the possibility of using EGFR which are natural human proteins without toxicity and pathogenicity as endosomal/lysosomal escape components [99,100].
Melittin, a cationic peptide which is the major component of bee venom is able to disrupt membranes and exhibit a high cytotoxic activity. It was shown that melittin can form amphipathic α-helical structures in aqueous solution which in combination with its positive charge makes it able to insert into the lipid membrane and induce destabilization. Furthermore, it was shown that unmodified melittin can be used as a transfection agent which has a slight transfection activity with high toxicity [101,102]. This cationic peptide has been used as an efficient endosomolytic agent in several formulations [103-106].

Synthetic peptides. Nowadays several endosomolytic peptide/protein polymers with specific sequences and length are designed. Arginine-rich cell-penetrating peptides are known as efficient delivery systems for therapeutic agents such as peptides, proteins, and nucleic acids. These synthetic peptides are efficiently bound to proteoglycans on cell surface which leads to a dramatic uptake of conjugated molecules. The newly designed arginine-rich peptide called (R-Ahx-R)(4) AhxB showed promising results in nuclear delivery of splice correcting peptide nucleic

acids (PNA) and phosphorodiamidate morpholino oligomers (PMO) [107-110].

A synthetic analogue of glycoprotein H (gpH) which is a known fusogenic peptide from herpes simplex virus has been designed. This glycoprotein has a pH-sensitive fusogenicity and was used in combination with cationic liposomes to improve cellular internalization and endosomal release of the complexes. The results indicate on up to 30-fold increasing in the transgene expression in human cell lines [111].

The peptides which are in the N-terminal sequence of the influenza virus haemagglutinin subunit HA-2 have been used as models to construct two new peptides for endosomal escape. One of these peptides, KALA which is a cationic amphipathic peptide, was designed based on the wild type sequence of haemagglutinin subunit (HA-2) from influenza virus. This fusogenic peptide undergoes a conformational change from pH 5.0 to 7.5, resulting in effectively destabilizing the endosomal membranes which is reported in several studies [112-115].

GALA is another synthetic amphipathic peptide with a substitution of glycine for glutamic acid at position 4 from a mutant sequence of influenza virus. Also this peptide undergoes a pH-dependent conformational change resulting in formation of a helical structure that induces the leakage of the contents of large uni-lammellar phosphatidylcholine vesicles. This peptide is soluble at pH 7.5 and destabilizes the lipid bilayers at a pH less than 6.0 [116]. The membrane-disruptive properties of this peptide were shown after application in different formulations for drug and nucleic acid delivery purposes [117-120].

Synthetic surfactants with pH-sensitive amphiphilicity have been introduced as membrane-disruptive components. They contain a protonatable amino head group, two cysteine residues and two lipophilic tails. The pH-sensitive membrane-disruptive properties of these components were shown in rat red blood cells [121]. It was shown that at low pH, formation of mixed micelles between gemini surfactant and membrane phospholipids leads to endosomal disruption [122].

Penetratin (pAntp) is the third helix of the homeodomain of Antennapedia protein. This peptide is able to cross the lipidmembrane through an energy-independent mechanism. It was shown that peptides and oligonucleotides covalently attached to this peptide were efficiently delivered to the cytosol and nucleus in different cell lines [123,124]. Also, R6-Penetratin with arginine-residues in the N-terminus has been designed

which showed endosome disruptive activities [125]. EB1 which is the synthetic analog of penetratin, destabilizes the endosomal membrane after forming an amphipathic alpha helix upon protonation at endosomal pH. In case of the forming complexes and siRNA delivery, EB1 was more far more effective than penetratin [126].
The signal peptide located in the N-terminus of the unprocessed bovine prion protein (bPrPp) belongs to the family of cell-penetrating peptides. This peptide forms β-structure in interaction with negatively charged membranes and is able to transport large hydrophilic nanoparticles through the cell membrane [127-129].
Poly (L-histidine) posses a strong pH-buffering effect, because of presence of several aminoethyl groups in the structure. Therefore, this poly-peptide has efficient endosome-disrupting properties which make it a promising delivery system [130]. Histidine-rich peptides have been used in several studies for endosomal escape purposes [131-134].
The amphipathic Sweet Arrow Peptide (SAP), which is a proline-rich, gamma-zein-related sequence, has been designed for endosomal escape and delivery purposes. This peptide which is water soluble has shown good translocation properties with no cytotoxicity [135,136].

Chemical agents

Several chemical agents with endosome disruptive properties have been introduced. It has been hypothesized that polymers such as polyethylenimine (PEI) [137] and imidazole-containing polymers [138,139] which have buffering capacities in pH range 7.2-5.0, could rupture the endosome by the proton sponge effect. On the other hand, the proton sponge hypothesis seems not to be applicable for all polymers with buffering capacity at low pH [140].
Poly (amidoamine)s (PAAs) are a group of water-soluble synthetic polymers designed to be biodegradable and biocompatible. Most of the poly(amido amine)s possess buffer capacities in the pH range 7.4-5.1 that are even higher than polyethylenimine (PEI), a property that may favorably contribute to the endosomal escape [141-143]. It has been shown that the synthetic pH-sensitive anionic polymer, poly (propylacrylic acid) (PPAA) has been efficient in endosomal escape of nonviral vectors and improved their transfection efficiency [144].
Agents such as ammonium chloride, chloroquine, and methyl-amine are relatively lipophilic in their unprotonated form and they penetrate the

membranes of cells and vacuoles. Upon entering an acidic environment they become protonated and too polar to escape rapidly through the membrane [145]. Nevertheless, nowadays using of such chemicals for gene/siRNA delivery purposes is less than the other types of endosomolytic agents.

Concluding remarks

Facilitating the endosomal escape is an important strategy in therapeutic delivery of biologicals. Different strategies for endosomal escape have different characteristics; a safe endosomal escape agent, should have low immunogenicity and toxicity, high efficiency, ease of use and production, modular attachment of targeting ligands and the potential for cost-effective large-scale manufacture.

It seems that the strategy of synthetic biomimetic polymers that mimic the viral peptides and undergo a pH-dependent conformational change is an efficient motif for facilitating the endosomal escape. They have some advantages over those isolated from biological sources like controlling the amino acid sequence, hydrophobicity and peptide length.

Considering different proposed strategies for endosomal escape and characteristics of several agents, the best strategy would be to use a protein or peptide which is similar to natural human components and should preferably be a human component which interacts with endosomal membrane in a natural manner and facilitates the safe release of therapeutic agents. Such agent would be without immunogenicity and toxicity and should result in a safe delivery of therapeutic agents. Also fusion is a natural process in human and should be considered in endosomal escape too. In other words, fusogenic peptides lead to endosomal escape in a natural manner that subsequently leads to a safe release of therapeutic gene/protein/drug. FGFR3 (harbouring mutations) as a member of the family of fibroblast growth factors, has been efficient in endosomal escape and follows a natural manner. This natural human protein may form the basis for the development of proteins for endosomal escape. It would be preferable to design a peptide that is active under the milder conditions of the early endosome, to reduce the risk of degradation of the co-delivered therapeutic molecule.

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Chapter 3

SiRNA Delivery with Functionalized Carbon Nanotubes

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Abstract

Carbon nanotubes (CNTs) have been studied for drug, antigen and nucleic acid delivery both *in vitro* and *in vivo*. Due to their nano-needle structure, they are supposed to cross the plasma membrane and enter directly into the cytoplasm likely upon an endocytosis-independent mechanism without inducing cell death. In this study, two cationically functionalized CNTs (CNT-PEI and CNT-pyridinium) were investigated for siRNA delivery. Both functionalized CNTs complexed siRNA and showed 10-30% silencing activity and a cytotoxicity of 10% to 60%. However, in terms of reduced toxicity or increased silencing activity, CNT-PEI and CNT-pyridinium did not show an added value over PEI and other standard transfection systems. Probably, the type of functionalization of carbon nanotubes might be a key parameter to obtain an efficient and non-cytotoxic CNT-based delivery system. Nevertheless, in view of the present results and importantly also of the non-degradability of CNTs, preference should currently be given to designing biodegradable carriers which mimic the needle structure of CNTs.

Keywords: carbon nanotube, siRNA, gene silencing, cytotoxicity

Introduction

Carbon nanotubes (CNTs) have raised great enthusiasm in the nanomedicine field as potential drug, antigen and nucleic acid delivery vehicle [1-3]. Because of their nano-needle structure (Figure 1), they have been proposed to easily cross the plasma membrane and to translocate directly into cytoplasm of target cells, utilizing an endocytosis-independent mechanism without inducing cell death [4-6]. Obviously, if this interesting new mechanism is applicable to a great variety of target cell types, such an endocytosis-independent cell entry mechanism of CNTs is a valuable advantage. However, there are indications for an endocytosis-dependent cell entry of CNTs [7-10].

CNTs offer a structural advantage due to a very large surface which can be modified with functional groups and be loaded with therapeutics such as nucleic acids, drugs and proteins [1,11]. Furthermore, it is expected that CNTs can be scaled-up for industrial production relatively easily [12]. These properties set a foundation for further exploration of CNTs for biological and therapeutic applications.

Poor solubility of CNTs in water is one of the limitations for most of their applications. Several approaches such as introducing polar or charged groups on their surface, have been investigated to facilitate their solubilisation. In addition to increasing solubility, introduction of positively charged groups at their surface also serves another purpose: such functionalized CNTs can bind molecules such as siRNA or DNA [13,14]. In comparison with chemical conjugation of DNA/siRNA to CNTs [15], nanoparticle preparation through electrostatic interactions between the negatively charged nucleic acids and chemically functionalized cationic CNTs is much faster and convenient [16].

Considerable research efforts have been dedicated recently to the use of nucleic acids, especially siRNAs as novel biotherapeutics [17,18]. Both DNA and siRNA molecules are rapidly degraded by nucleases present in biological fluids and not able to enter the cellular interior, with the nucleus and the cytoplasm as intracellular targets, respectively [19-21]. Therefore, a key challenge to the effective and widespread use of nucleic acids is their intracellular delivery [22-25]. For this purpose, functionalized CNTs could be valuable candidates in particular in view of the reported needle-mechanism enabling direct translocation over the plasma membrane.

Several functionalized CNTs have been designed and tested for the purpose of nucleic acid delivery [1,26]. Phospholipid-coated CNTs

functionalized with amine-terminated polyethylene glycol (PL-PEG2000-NH_2) were shown to be efficient in siRNA and DNA delivery in human T cells and primary cells [27]. Ammonium-functionalized CNTs [28] and more recently dendron-CNT [26] were reported to be efficient in siRNA delivery with low cytotoxicity. After i.v. injection of PEGylated CNTs in mice, no toxicity over several months was observed [29,30]. Nevertheless, in several studies the potential toxicitiy of CNTs has been discussed and attributed to various factors such as, amongst others, length of the tubes, type of functionalization, dosage, duration of exposure, cell type, route of administration and tissue distribution. Still, most aspects regarding CNT toxicity remain uncertain [11,29,31]

In this study, two newly synthesized functionalized CNTs were investigated for siRNA delivery (Figure 1). One CNT type was functionalized covalently with the cationic polymer PEI (polyethylenimine) (CNT-PEI), which is a well-known transfection agent [32]. The silencing activity of the siRNA complexes based on CNT-PEI was compared to those based on PEI alone. The other studied CNT type was functionalized non-covalently with cationic pyridinium. The silencing activity and the cellular cytotoxicity of siRNA complexes based on these functionalized CNTs were compared with those based on the regularly used lipidic transfection agent Lipofectamine and the well-known polymeric transfectant poly(2-dimethylaminoethyl methacrylate), pDMAEMA [33].

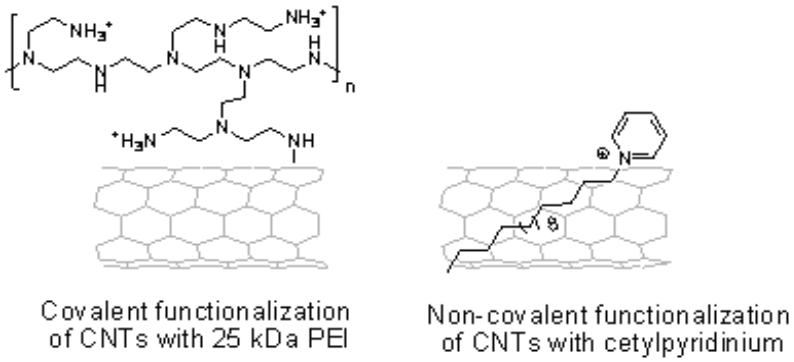

Figure 1. Schematic representation of the two functionalized CNTs under study.

Materials and Methods

Materials

Multi-wall Carbon nanotubes (MWCNT) (Nanocyl 3150) with a metallic catalyst content< 5% and average diameter and length of 9.5 nm and 1 μm, respectively, were purchased from Nanocyl (Sambreville, Belgium). PEI 25 kDa and other chemicals were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). The Fluc double stranded siRNA which specifically targets firefly luciferase (used against mRNA from pGL3; structure below) and anti-EGFP siRNA as non specific siRNA were obtained from Integrated DNA Technologies BVBA (Leuven, Belgium).
Sense strand: 5´- pGGUUCCUGGAACAAUUGCUUUUAca
Antisense strand: 3´- GACCAAGGACCUUGUUAACGAAAAUGU
Poly(2-(dimethylamino) ethylmethacrylate) (pDMAEMA) was synthesized and purified as described previously [33]. Lipofectamine 2000 was obtained from Invitrogen (Breda, The Netherlands). Luciferase assay reagent and reporter lysis buffer were obtained from Promega (Leiden, The Netherlands).

Shortening of carbon nanotubes

80 mg of CNTs were ultrasonicated in 30 ml of toluene for 4 days with an ultrasonicating Branson Sonifier 450 probe (25 W output power). The suspension was centrifuged for 30 min at 10000 rpm, and the supernatant was discarded. The precipitate was taken back in 30 ml of H_2O and centrifuged again. This operation was repeated twice using 30 ml of acetone. The sample was finally dried under vacuum to yield 75 mg of shortened CNTs [34].

Preparation of CNT-PEI

CNTs (50 mg) were dispersed in 10 ml of DMF using an ultrasonic probe (15 min, 10 W output power) before 250 mg of 25 kDa branched-PEI were added. The mixture was further sonicated for 10 min and stirred for 3 days at 50°C. The PEI-functionalized nanotubes were collected by filtration on 0.2 μm polypropylene membrane, washed with CH_2Cl_2 (15 ml), DMF (15 ml), and MeOH (3 x 15 ml). Nanotubes were taken back in 10 ml HCl (pH 1), sonicated for 10 min, filtered, washed with H_2O (3 x 15

ml) and MeOH (15 ml). CNT-PEI was redispersed in H_2O under sonication (10 min) and centrifuged (5 min, 2000 rpm) to remove aggregates. The black supernatant was collected and lyophilized to afford 28 mg of CNT-PEI. A control experiment was indeed carried out by mixing PEI with CNTs without heating. After the usual workup, XPS analysis showed only traces amounts of N on the nanotubes. This experiment clearly shows that there is no adsorbed PEI on the nanotube.

Preparation of CNT-Pyridinium

CNTs (50 mg) were dispersed in 10 ml of H_2O for 15 min using an ultrasonic probe (25 W output power). Cetylpyridinium chloride (10 mg) was then added and the mixture was further sonicated for 10 min and centrifuged for 5 min at 2000 rpm. The supernatant was collected and centrifuged for 90 min at 11000 rpm to remove cetylpyridinium. The precipitate was taken back in 10 ml of H_2O under sonication and centrifuged again. This operation was repeated twice. The precipitate was collected and taken back in 10 ml of H_2O under sonication (10 min, 25 W) and centrifuged for 5 min at 2000 rpm to remove aggregates. The black supernatant was collected and lyophilized to afford 20 mg of CNT-Pyridinium.

Physicochemical characterization

The functionalized CNTs were dispersed in water (1mg/ml) by bath sonication for 1 hour. Electron microscopy analysis was carried out on a Philips CM12 microscope at 100 kV. XPS spectra were recorded on a VG ESCALAB 210 spectrometer. The zeta potential of the CNT dispersions in 5 mM HEPES buffer (pH 7.4) was determined at 25°C in a DTS5001 cell using a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential. The zeta potential of the calibration standard was measured on different days and was -64 mV ± 5. This accuracy (around 95%) is reflected in the standard deviations of the zeta potentials of the CNTs.
Complexes of CNTs or PEI with siRNA were prepared by mixing 100 µl anti-luciferase siRNA (20µg/ml: in 5 mM HEPES buffer (pH 7.4)) with different concentrations of functionalized CNTs, PEI or pDMAEMA in the same buffer. The resulting mixtures were vortexed for 10 seconds and then used after 30 minutes storage at room temperature. Complexes were

prepared at different CNT, PEI or pDMAEMA to siRNA ratios (expressed as N/P ratios, where N is the number of moles of nitrogen in CNT, PEI or pDMAEMA and P is number of moles of P in siRNA). Complexes of either specific or non-specific siRNA with Lipofectamine 2000 were prepared by gently mixing 50 µl siRNA (20 µg/ml) in HEPES (5 mM, pH 7.4) with 3.7 µl Lipofectamine 2000 in 50 µl HEPES (5 mM, pH 7.4) followed by 30 min incubation at room temperature.

Agarose gel electrophoresis

The complexation of siRNA with the cationic vectors was investigated using agarose gel electrophoresis. The agarose gels (NuSieve® GTG® Agarose, Lonza, Rockland, ME, USA) were made at a concentration of 4% (w/v) in Tris-acetate-EDTA (TAE) running buffer and contained 0.5 µg/ml ethidium bromide. The complexes made at different N/P ratios were prepared as described above and were applied in the starting slots of the gel followed by electrophoresis at 60 V for 50 minutes. Naked siRNA was used as control. The siRNA bands, stained with ethidium bromide, were detected on a UV transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

Gene silencing and cytotoxicity

The human lung cancer cell line H1299 which expresses firefly luciferase was used to study the gene silencing activity of the different complexes. The cell line was maintained in RPMI 1640 medium with HEPES and L-glutamine (PAA laboratories GmbH, Pasching, Austria, catalog No. E15-842) completed with fetal bovine serum (FBS) (final concentration 10% v/v) and cultured at 37 °C at 5% CO_2 humidified atmosphere. The cells (8×10^3 cells/well) were seeded into 96-well plates and cultured overnight. The anti-Luciferase siRNA complexes were added to the cells and incubated at 37 °C for 2 h. Then, the medium was removed, and fresh medium was added. Subsequently, the cells were incubated at 37 °C for 48 hours, after which the luciferase protein expression was analyzed using a Luciferase reporter gene assay (Promega). The cytotoxicity of the complexes and cetylpyridinium (concentrations ranging from 1.3 to 8.6 µg/ml which correspond with its concentrations in siRNA complexes incubated with cells) was measured using the XTT colorimetric viability assay [35].

Determination of Luciferase activity

Luciferase activity was measured after removal of the growth medium and lysis of the cells by the addition of 100 µl reporter gene lysis buffer. After a freeze/thaw cycle at -80 °C/room temperature, 50 µl of luciferase assay reagent was added to 50 µl of the cell lysate and relative light units (RLU) were measured for 10 s at room temperature using a FLUOstar OPTIMA microplate based multi-detection reader with a microinjector.

Results and Discussion

Efficient gene silencing mediated by siRNA depends on an efficient and protected delivery of siRNA to the intra-cellular target site, the cytoplasm. CNTs offer several advantages which make them attractive carriers for nucleic acid delivery. They are thin and long, offering a large surface area to which siRNA can be bound. Remarkably, it has been observed that their nanoneedle structure facilitates their translocation over the plasma membrane into the cytoplasm via an endocytosis-independent pathway. In a study by Kostarelos et al [36], it was shown that CNTs were taken up by cells despite the presence of endocytosis inhibitors in the culture medium. Cell types studied included fibroblasts which are phagocytosis-deficient and prokaryotic cells (fungi, yeast and bacteria) which have no endocytosis machinery. Therefore, it was concluded that the uptake mechanism of the CNTs is passive and endocytosis-independent [4,6]. Considering the endosomal localisation as one of the most problematic barriers in siRNA cytosolic delivery, this endocytosis-independent cell entry mechanism makes the CNT delivery capacity independent from endosomal escape. However, the needle mechanism is still a controversial issue and several opposite observations have been reported in the literature pointing to an endocytosis-dependent cell entry of CNTs [7-10].
There have been several reports on the use of functionalized CNTs for siRNA delivery. Wang et al [37] reported that ammonium-functionalized CNTs could electrostatically bind siRNA against cyclin A2, inducing growth inhibition and apoptosis of a human erythroleukemic cell line (K526) *in vitro*. Liu et al [27] used CNTs functionalized with amine-terminated PEG (PEG; PL-PEG2000) for siRNA delivery into human T cells and observed 60-90% knockdown of CXCR4 receptors. Laderia et al

[38] used siRNA coiled into carboxyl-functionalized CNT and observed >95% silencing activity in different cell lines with no cytotoxicity. Bartholomeusz et al [39] used siRNA complexed to pristine CNT for silencing the hypoxia-inducible factor 1 alpha (HIF-1alpha) and observed a specific inhibition of cellular HIF-1alpha activity. Moreover, intra-tumoral administration of these complexes in mice bearing MiaPaCa-2/HRE tumors significantly inhibited the activity of tumor HIF-1alpha.
In this study, two types of functionalized CNTs (Figure 1) were investigated for their siRNA delivery properties *in vitro* and compared with the polymeric transfectant pDMAEMA developed in our group [33] and the well-known lipidic cationic transfectant Lipofectamine [40] and PEI.

Physicochemical characterization

Full-length carbon nanotubes were first mechanically shortened by ultrasonication. Transmission electron microscopy (TEM) pictures showed that ultrasonication reduced the length of the nanotubes to a range between a few tens of nm to ~500 nm (Figure 2a). The mean length of the nanotubes was approximately 200 nm (Figure 2b). This value has to be compared to the initial CNTs before ultrasonication which were typically above 1 µm in length.

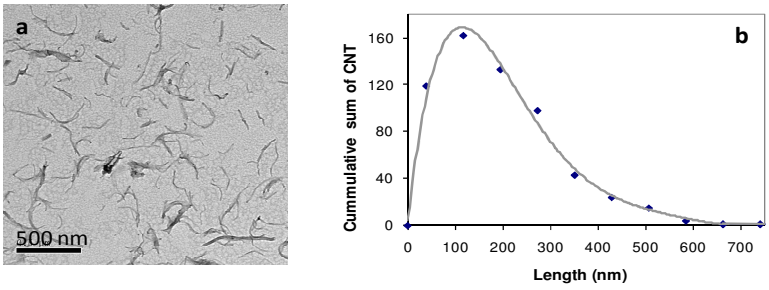

Figure 2. TEM picture (a) and size distribution of shortened CNTs (b).

Cationic functionalization of the surface of CNTs was envisaged according to two different functionalization pathways, one resulting in covalently bound and the other one in non-covalently bound surface

moieties. Covalent modification of the CNTs was achieved by direct amination of the graphene lattice with 25 kDa polyethyleneimine (PEI), as described by Basiuk et al [41]. Non-covalent functionalization was done by adsorption of cetylpyridinium chloride (Py^+) onto the surface of CNTs, as reported by Mackiewicz et al [42] (Figure 1). In the latter process, the hydrophobic portion of the amphiphile is adsorbed on CNT walls by van der Waals interactions while its hydrophilic head-group is oriented toward the aqueous phase. The CNT-PEI and CNT-Pyridinium samples were found to be dispersible in aqueous solutions and stable against aggregation for at least 72 hours. TEM pictures (Figure 3) show the grafted CNTs in dry state. Quantification of the degree of functionalization was achieved by X-ray photoelectron spectroscopy (XPS) which indicated the appearance of a N 1s peak at 404.7 eV which was attributed to the nitrogen atoms. From the nitrogen to carbon ratio we determined the level of functionalization of the CNTs being 28 wt% in case of 25 kDa PEI and 11 wt% in case of Pyridinium. These values indicate that the CNTs are densely covered with cationic amino groups to be used for siRNA complexation. Indeed, if we consider that all the amino groups are protonated, the cationic amino group per mg of complex is ca. 6 µmol for CNT-PEI and 0.3 µmol for CNT-Pyridinium.

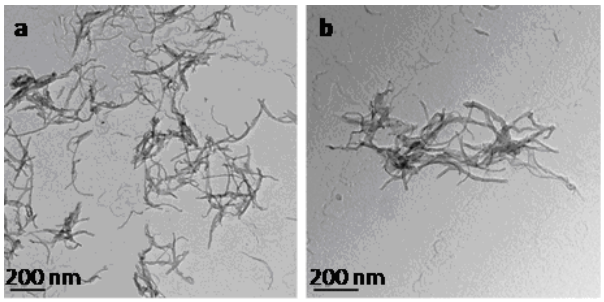

Figure 3. TEM pictures of CNT-PEI (a) and CNT-Pyridinium (b) in dry state.

Both types of functionalized CNT dispersions showed a positive zeta potential (10-15 mV ± 5) pointing to their ability to make complexes with negatively charged siRNA. The complex formation ability of CNT-PEI with siRNA in comparison to PEI at different N/P ratios was investigated by agarose gel electrophoresis. Figure 4 shows that the intensity of the free siRNA bands on the gel in case of CNT-PEI reduced gradually with

an increase of the N/P ratio (0.5-12) indicating complete complexation at N/P ≥ 14. Complete binding of siRNA to PEI alone was already observed at N/P ≥ 2.

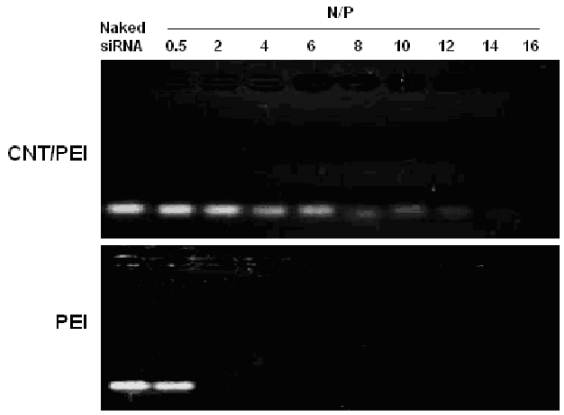

Figure 4. Complex formation of CNT-PEI 25kDa and PEI with siRNA as function of N/P ratio as studied by agarose gel electrophoresis.

In case of the CNT-Pyridinium, complete binding of siRNA to CNT-Pyridinium was observed at N/P≥3.5 (Figure 5).

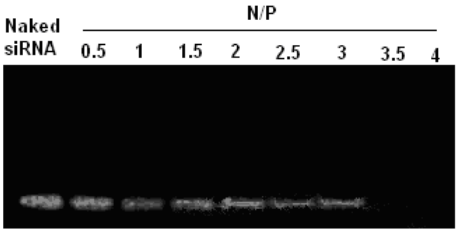

Figure 5. Complex formation of CNT-Pyridinium with siRNA as function of N/P ratio as studied by agarose gel electrophoresis.

These results clearly show that both types of functionalized CNTs are able to complex siRNA.

Silencing activity and cell viability

Human lung cancer cells (H1299) expressing luciferase were used for the gene silencing and cell viability studies. Figure 6a shows the gene silencing efficiency (corrected for cytotoxicity) of CNT-PEI/siRNA complexes compared to those based on PEI, pDMAEMA and Lipofectamine as reference transfectants. The silencing activity of the different formulations was corrected for cytotoxicity, so the percentage silencing reported is only related to viable cells. In the absence of serum, incubation of cells with the siRNA complexes based on CNT-PEI showed up to 20% silencing activity whereas incubation with PEI polyplexes showed 20-30% gene silencing. The results of the XTT cell viability assay showed higher cytotoxicity of CNT-PEI-based complexes than those based on PEI (Figure 6b) which suggests that CNT enhances the cytotoxicity of PEI, while, Liu et al showed that CNTs modified with PEI were less cytotoxic than PEI alone [43]. By increasing the dose of the siRNA complexes (at N/P 12) from 10 pmol to 30 pmol per well, no significant enhancement in gene silencing activity of CNT-PEI and PEI-based complexes was observed (Figure 6a) whereas the cytotoxicity of the CNT-PEI based complexes increased slightly up to 40% (Figure 6b). It was observed that the gene silencing activity of the CNT-PEI based complexes was much lower than that of complexes based on Lipofectamine 2000 (60%) and pDMAEMA (50%) (Figure 6a). In the presence of serum in the growth medium, the same results of gene silencing and cytotoxicity were observed for all siRNA formulations (data not shown). These results show that there is no added value of CNT-PEI over PEI and the reference transfectants Lipofectamine 2000 and pDMAEMA.

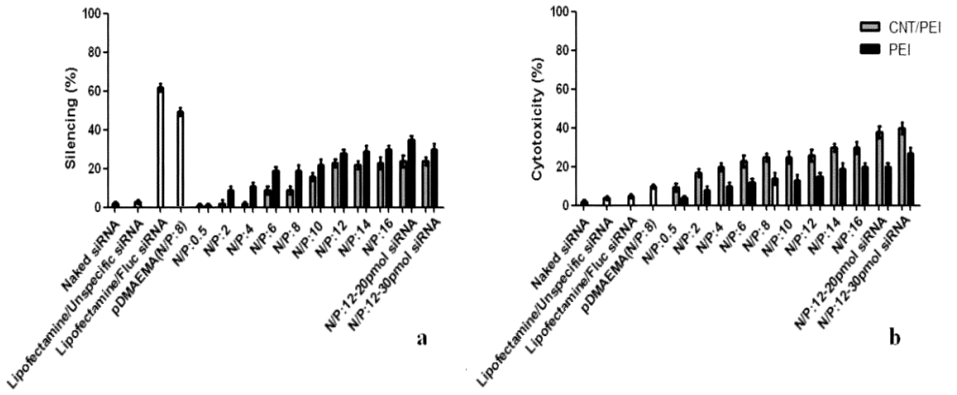

Figure 6. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes based on CNT-PEI and PEI alone made at different N/P ratios, in serum free medium. b. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum free medium (mean ± standard deviation (n=3)).

Figure 7 shows the findings obtained with CNT-pyridinium/siRNA complexes. In the absence of serum, incubation of cells with the CNT-pyridinium/siRNA complexes (N/P 3.5) showed about 30% silencing activity (corrected for cytotoxicity) (Figure 7a) and a relatively high cytotoxiciy of about 60% (Figure 7b). By increasing the dose of the siRNA complexes (N/P 3.5) from 10 pmol/well to 30 pmol/well, no significant enhancement in the gene silencing activity of CNT-Pyridinium based complexes was observed (Figure 7a) whereas the cytotoxicity of the complexes increased slightly up to 70% (Figure 7b) which is probably due to the increased amount of the CNT-pyridinium in the growth medium. Cetylpyridinium in different concentrations (corresponding with its concentrations in siRNA complexes incubated with cells) showed ≤ 5% cytotoxicity. It is clear from the results that the cytotoxicity of the CNT-Pyridinium/siRNA complexes at the same cetylpyridinium concentrations is substantially higher related to adverse effects of the CNTs (Figure 7b). These results reveal high cytotoxicity of the CNT-Pyridinium/siRNA complexes and limited silencing activity, with no added value of CNT-Pyridinium over Lipofectamine 2000, pDMAEMA and PEI (Figure 7a). In several studies the potential *in vitro* and *in vivo* toxicities of CNTs have been discussed and attributed to various factors such as, amongst others,

length of the tubes, type of functionalization, dosage, duration of exposure, cell type, route of administration and tissue distribution. Still, most aspects regarding CNT toxicity remain uncertain [44-50].

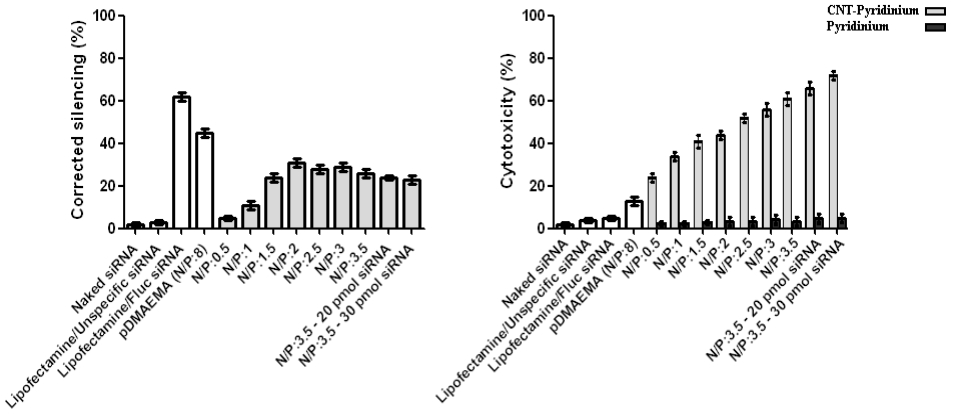

Figure 7. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes based on CNT-Pyridinium made at different N/P ratios, in serum free medium. b. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes and Pyridinium alone in serum free medium (mean ± standard deviation (n=3)).

Conclusion

In this study, CNT-PEI and CNT-pyridinium did not show any added value over PEI, pDMAEMA, and Lipofectamine used as reference transfection agents regarding siRNA silencing activity and cytotoxicity. Despite these disappointing results obtained with the two functionalized CNT types, other literature reports encourage further nucleic acid delivery studies with other types of functionalized CNTs. Probably, the type of functionalization of carbon nanotubes might be a key parameter to obtain an efficient and non-cytotoxic CNT-based delivery system. Nevertheless, in view of the present results and importantly also of the non-degradability of CNTs, preference should currently be given to designing biodegradable carriers which mimic the needle structure of CNTs.

Acknowledgment

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Chapter 4

Polyplexes Based on Cationic Polymers with Strong Nucleic Acid Binding Properties

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Abstract

Cationic polymers have been studied for nucleic acid delivery both *in vitro* and *in vivo*. However, many polymer-based formulations suffer from lack of stability in biological fluids due to interactions with anionic biomacromolecules such as proteins and polysaccharides. Likely, the stronger the electrostatic interactions between a cationic polymer and nucleic acids, the higher the stability of the polyplexes in biological fluids will be. To get evidence for this hypothesis, quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxyl styrene] (QNPHOS) with two permanently charged cationic sites per monomer unit as well as its block copolymer with PEG were synthesized and compared with the standard transfectant pDMAEMA, in terms of nucleic acid binding strength, gene silencing and transfection activities of the complexes which these polymers form with siRNA and plasmid DNA, respectively. It was shown that siRNA complexes based on QNPHOS and QNPHOS-PEG dissociate in the presence of a 4-fold higher heparin concentration than necessary to destabilize pDMAEMA complexes. Under the same conditions, complexes of DNA and QNPHOS or QNPHOS-PEG did not show any dissociation, in contrast to pDMAEMA polyplexes. The DNA polyplexes based on QNPHOS or QNPHOS-PEG did not show transfection activity, which might be ascribed to their high physicochemical stability. On the other hand, siRNA complexes based on QNPHOS and QNPHOS-PEG showed a low cytotoxicity and an improved siRNA delivery and high gene silencing activity, even higher than those based on pDMAEMA. This might be due to the excellent binding characteristics of QNPHOS and QNPHOS-PEG to siRNA which in turn is ascribed to the presence of two permanently charged cationic groups per monomer unit. Based on the results of this study, it is concluded that formation of strong siRNA complexes with polymers containing double charges per monomer is advantageous.

Keywords: siRNA, plasmid DNA, delivery, polymeric vectors, gene silencing, transfection

Introduction

Gene therapy has gained significant attention over the past two decades as a potential modality for treatment of life threatening diseases such as cancer [1-3]. A crucial item in gene therapy research is the delivery of sufficient quantities of therapeutic nucleic acid molecules such as plasmid DNA and siRNA into target cells [4-6]. However, nucleic acids have unfavorable biopharmaceutical properties, because they are rapidly degraded by nucleases present in biological fluids and have low membrane penetration capabilities. Therefore, research efforts are currently focused on designing effective vectors that stably complex the nucleic acids and facilitate their cellular recognition and internalization. Furthermore, the internalized nucleic acid carrier complexes have to escape from the endosome and deliver siRNA in the cytosol and plasmid DNA into the nucleus [7,8].

Cationic polymers have been studied for nucleic acid delivery both *in vitro* and *in vivo*. Due to their positive charge, these polymers are able to complex the anionic nucleic acids to form polyplexes which are internalized by cells [9-16]. However, it has been shown that polyplexes can have a limited stability in blood and other biological fluids, due to interactions with anionic biomacromolecules such as proteins and polysaccharides which may result in polyplex dissociation [17-19]. It can therefore be hypothesized that the stronger the electrostatic interactions between a cationic polymer and nucleic acids, the higher the stability of the polyplexes in biological fluids will be.

To substantiate this hypothesis, in the present study we synthesized a polymer with two permanently charged cationic sites per monomer unit (quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxy styrene] (QNPHOS) as well as its block copolymer with PEG (Figure 1). It is expected that the presence of the two quaternized nitrogen groups will confer strong nucleic acid binding properties of QNPHOS polymers. In this paper, we studied the nucleic acid binding capability of QNPHOS and QNPHOS-PEG, the gene silencing (with siRNA) and transfection (with plasmid DNA) activities of the formed polyplexes in comparison to the well-known transfectant pDMAEMA (poly(2-(dimethylamino)ethyl methacrylate)). In previous studies, a higher transfection activity of polyplexes based on pDMAEMA in comparision to quaternized pDMAEMA (pTMAEMA, poly (2- (trimethylamino)ethyl methacrylate chloride)) was reported [20,21]. The lower transfection activity of

pTMAEMA polyplexes is probably due to the strong binding of this permanently charged polymer to pDNA and its low intracellular dissociation. Therefore, in current study pDMAEMA was used as reference transfection system.

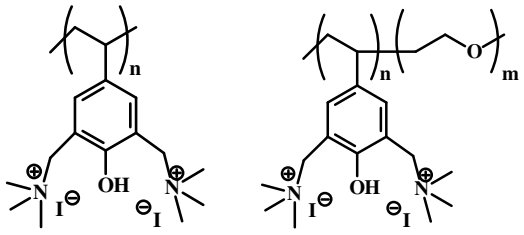

Figure 1. Structure of QNPHOS (left, quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxy styrene]) and QNPHOS-PEG block copolymer (right).

Materials and methods

Materials

The double-stranded siRNA which specifically targets firefly luciferase (used against mRNA from pGL3; structure below), was obtained from Dharmacon bioscience (Lafayette,USA) and anti-EGFP siRNA as nonspecific siRNA and Fluc-TYE563- labeled siRNA were obtained from Integrated DNA Technologies BVBA (Leuven, Belgium).
Sense strand: 5'- GAU UAU GUC CGG UUA UGU A UU
Antisense strand: UU CUA AUA CAG GCC AAU ACA U P-5'
Plasmid encoding for firefly Luciferase, pcDNA3Luc, which is under the transcriptional control of the cytomegalovirus immediate promoter was produced by Plasmid Factory (Bielefeld, Germany). QNPHOS homopolymer (M_w = 46000; M_w/M_n= 1.08) and QNPHOS-PEG block copolymer (M_w = 62700, 14% PEG; M_w/M_n= 1.13) were synthesized as described previously [22,23]. PDMAEMA (M_w= 130 kDa) was synthesized and purified as described previously [24]. The photosensitizer (PS) TPPS2a, meso-tetraphenylporphyrin was obtained from PCI Biotech AS (Oslo, Norway) [25]. LumiSource®, a bank of four light tubes

emitting light with a wavelength range of 375 - 450 nm and 13 mW/cm^2 irradiation intensity was provided by PCI Biotech AS, Oslo, Norway.
Polyplexes were prepared at different polymer-to-siRNA/DNA ratios (expressed as N/P ratios, where N is the moles of cationic nitrogens in the polymer and P is moles of phosphate in siRNA/DNA) varying from 0.5-16 by adding a siRNA/DNA solution (200 µl, 40 µg/ml) in HEPES (5 mM, pH 7.4) to polymer solutions (200 µl, various concentrations) in the same buffer. The resulting polyplex dispersions were vortexed for 10 seconds. After 30 minutes incubation at room temperature, Z- average diameters were measured with dynamic light scattering at 25 °C with a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectroscopy) software for Windows version 1.34 (Malvern, UK). The viscosity and refractive index of water at 25 °C were used. Calibration was done with an aqueous dispersion of polystyrene particles with a diameter of 100 nm. Particle size distribution is characterized by the polydispersity index (PDI), ranging from 0 for a monodisperse to 1 for a fully heterodisperse preparation. The zeta potential of the polyplexes prepared in HEPES (5 mM, pH 7.4) was determined at 25 °C in a DTS5001 cell using a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential.

Agarose gel electrophoresis

The complexation of nucleic acids with the different polymers and the heparin-induced destabilization of the polyplexes were investigated using agarose gel electrophoresis. The agarose gels (NuSieve® GTG® Agarose, Lonza, Rockland, ME, USA) were made in a concentration of 4% (w/v) (for siRNA complexes) and 0.8% (for DNA complexes) in Tris-Acetate-EDTA (TAE) running buffer which also contained 0.5 µg/ml ethidium bromide. Polyplexes (N/P ratios varying from 0.5 to 16) of different polymers with Fluc siRNA and Luciferase DNA were prepared as described above. Naked siRNA/DNA was also used. Polyplex dispersions (N/P 8) with or without heparin (0.5-4.5 USP/µg siRNA/DNA; molar ratio of amine groups of the polymer and sulfate groups of heparin varied from 2.8 to 0.23) were incubated for 5 minutes at room temperature. Polyplexes (10 µl, corresponding to 0.2 µg siRNA/DNA) were applied in the starting slot of the gel and electrophoresis was performed at 80 V for 45 min. The siRNA/DNA bands stained with ethidium bromide were detected on a UV

transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

Gene Silencing, transfection and cytotoxicity experiments

The human lung cancer cell line H1299 which expresses firefly luciferase provided by Marbourg University, Germany, was used to study the gene silencing activity of the siRNA complexes. The cell line was maintained in RPMI 1640 medium with HEPES and L-glutamine (PAA laboratories GmbH, Pasching, Austria) completed with fetal bovine serum (FBS) (final concentration 10% v/v). For transfection studies, COS7 African Green monkey kidney cells obtained from the American Type Culture Collection (ATCC, Maryland, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (PAA laboratories GmbH, Pasching, Austria) supplemented with 5% FBS. Both cell lines were cultured at 37 °C at 5% CO_2 humidified atmosphere. H1299 cells (8×10^3 cells/well) were seeded into 96-well plates and cultured overnight. The anti-luciferase siRNA polyplexes were added to the cells (10-20 pmol siRNA/well) in the presence or absence of serum in the medium and incubated at 37 °C for 4 hours. Then, the medium was removed, and fresh medium was added. Subsequently, the cells were incubated at 37 °C for 48 hours, after which the luciferase protein expression was analyzed using luciferase reporter gene assay (Promega). In all cases the gene silencing results were corrected for cytotoxicity. COS7 cells (4×10^4cells/well) were seeded into 24-well plates and cultured overnight. The luciferase DNA polyplexes were added to the cells (0.5-1 µg DNA/well) in the presence or absence of serum in the medium and incubated at 37 °C for 4 hours. Then, the medium was removed, and fresh medium was added. Subsequently, the cells were incubated at 37 °C for 48 hours, after which the luciferase protein expression was analyzed.
For photochemical internalization (PCI) treatment, different concentrations of photosensitizer (ranging from 0.05 to1.5 µg/ml) and different illumination times (15 to 90 seconds) were applied for COS7 cells. We selected a dose of photosensitizer of 0.5 µg/ml and illumination time of 75 seconds. Under these conditions the cytotoxicity was minimal (≤ 10%). One day after seeding, the cells were incubated at 37 °C with complete culture medium also containing the photosensitizer TPPS2a for 18 h. Next, the medium was removed and fresh medium was added to the wells and the luciferase DNA complexes were added to the cells and

incubated at 37 °C for 4 h. Then, the medium was removed, fresh medium was added, and the cells were exposed to the light source for 75 s. Subsequently, the cells were incubated at 37 °C for 2 days, after which the luciferase protein expression was analyzed. The cytotoxicity of the polyplexes was measured using the XTT colorimetric viability assay as previously described [26].

Determination of Luciferase activity

Luciferase activity was measured after removal of the growth medium and lysis of the cells by the addition of 100 µL reporter gene lysis buffer. After a freeze/thaw cycle at -80 °C/room temperature, 50 µl of luciferase assay reagent was added to 50 µl of the cell lysate and relative light units (RLU) were measured for 10 s at room temperature using a FLUOstar OPTIMA microplate based multi-detection reader with a microinjector.

Confocal laser scanning microscopy (CLSM)

The cellular uptake of polyplexes was investigated with confocal laser scanning microscopy (CLSM). In short, 8×10^3 cells were seeded in the wells of 12-well plates. The cells were cultured for 24 hours at 37° C and subsequently incubated with Fluc TYE563- labeled siRNA polyplexes. Then, the cells were fixed with 4% solution of formaldehyde in PBS for 30 minutes at room temperature. Next, the cells were washed twice with PBS and incubated with DAPI nucleic acid stain (300 nM) in PBS for 3 minutes. Subsequently, the cells were washed twice with PBS and mounted onto glass cover slides, using FluorSave. The cells were analyzed using CLSM with a SPE-2/DMI 4000 Leica confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with 405, 488, 561 and 635 nm diode lasers. Images were acquired sequentially using standard settings for DAPI and TYE563.

Results and Discussion

Physicochemical characterization of polyplexes

The average diameter of the siRNA/DNA polyplexes prepared at different N/P ratios was measured by dynamic light scattering (Figure 2 and Figure 3). The polymers were capable of forming complexes with both siRNA

and DNA over the whole N/P range of 0.5-16. The average size of both siRNA and DNA particles decreased with increasing N/P ratio, with small-sized nanoparticles of ≤ 200 nm being formed at N/P ratio ≥ 4 for siRNA complexes for all polymer types. In case of the DNA complexes, all polymer types except pDMAEMA formed nanoparticles of ≤ 200 nm in size at N/P ratio ≥ 2 [27]. This decrease in size of the complexes is due to the increased polymer amount in the formulations which leads to formation of tighter condensed complexes as also shown for other cationic polymers [15,16]. There was no significant difference in size of the nanoparticles based on QNPHOS and QNPHOS-PEG over the whole N/P range of 0.5-16. Furthermore, a polydispersity index (PDI) of ≤ 0.3 was observed for the different siRNA and DNA polyplex formulations prepared at N/P ratio ≥ 4 and N/P ratio ≥ 2 respectively, which indicates a rather narrow size distribution.

At N/P of 0.5, the siRNA/DNA polyplexes showed a negative zeta potential (-10 to -30 mV), which can be explained by the excess of siRNA/DNA present in the complexes. At N/P ≥ 2, positively charged particles for the different polyplexes (zeta potential +5 to +30 mV) were formed (Figure 2 and Figure 3). The zeta potential of polyplexes based on QNPHOS-PEG was lower than those based on QNPHOS which is explained by the charge shielding by PEG as also observed for other PEGylated polyplexes [28,29].

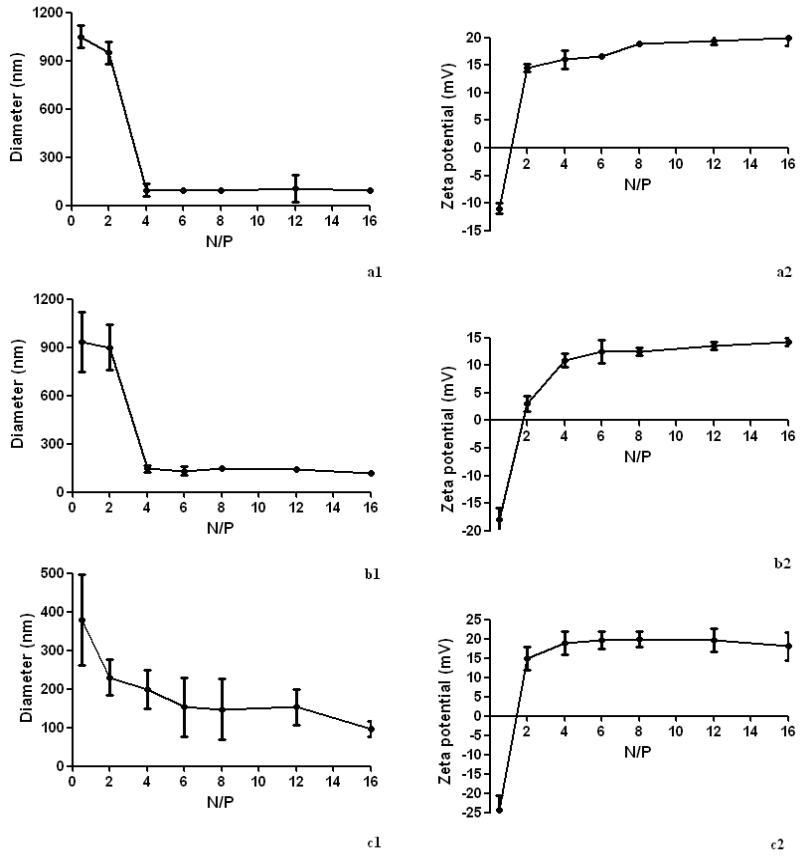

Figure 2. Average diameter and zeta potential of siRNA polyplexes prepared at different N/P ratios, measured after 30 min incubation at room temperature. **a1, a2**. QNPHOS; **b1, b2**. QNPHOS-PEG; **c1, c2**. pDMAEMA (mean ± standard deviation (n=3)).

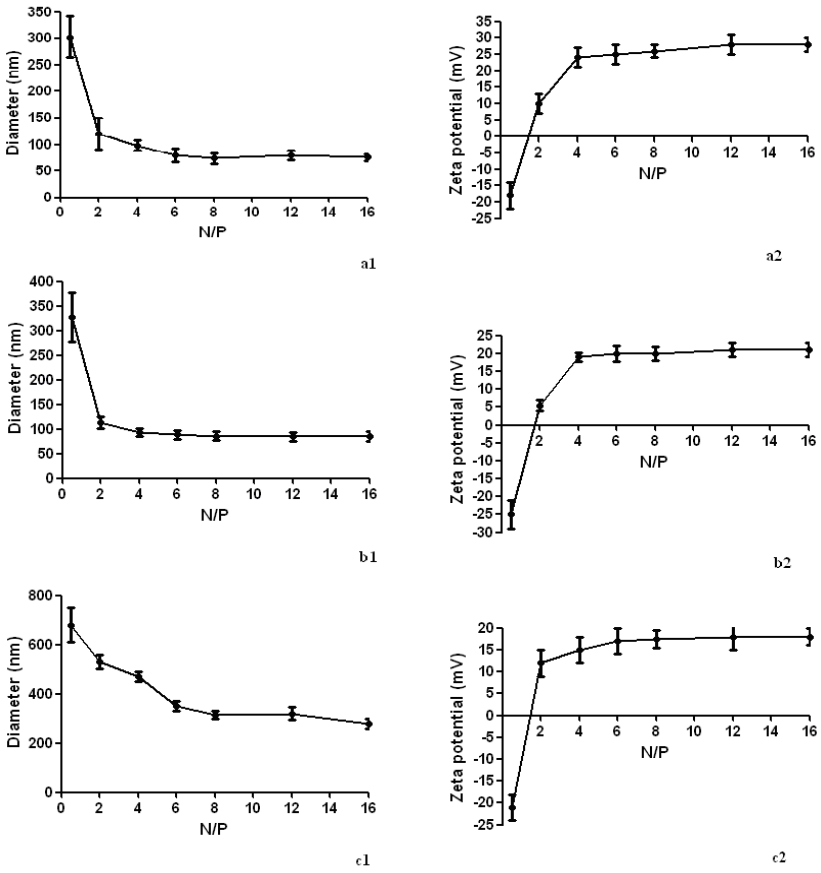

Figure 3. Average diameter and zeta potential of DNA polyplexes prepared at different N/P ratios, measured after 30 min incubation at room temperature. **a1, a2**. QNPHOS; **b1, b2**. QNPHOS-PEG; **c1, c2**. pDMAEMA (mean ± standard deviation (n=3)).

No change in size of the polyplexes was observed upon incubation for 24 hours at room temperature, indicating that the particles have a good colloidal stability.

Agarose gel eelectrophoresis

Binding of the cationic polymers to siRNA/DNA molecules and subsequent complex formation are essential for protection of the nucleic

acids against degradation by nucleases. On the other hand, polyplex destabilization and subsequent release of the nucleic acids has to occur inside the cell to achieve gene silencing or gene transfection for siRNA and DNA, respectively.

Figure 4 shows that siRNA/DNA remain in the starting slots of the gel for siRNA/DNA polyplexes prepared at N/P $\geq$ 2. For QNPHOS and QNPHOS-PEG complexes, siRNA and DNA are hardly detected in the starting slots, while, in case of the pDMAEMA nucleic acids are clearly detected, indicating a stronger binding of QNPHOS and QNPHOS-PEG polymers to nucleic acids in comparison to pDMAEMA. A stronger binding of QNPHOS and QNPHOS-PEG to nucleic acids was anticipated because of the two permanently charged amine groups per monomer unit. After incubation of the siRNA polyplexes with the highly negatively charged polysaccharide heparin, dissociation of polyplexes and subsequent release of the siRNA was observed, showing that complexation of QNPHOS and QNPHOS-PEG is reversible. Importantly, Figure 4 shows that siRNA complexes based on QNPHOS and QNPHOS-PEG are dissociated in presence of a four fold higher heparin concentration than used for pDMAEMA complexes, again indicating that QNPHOS and QNPHOS-PEG form stronger siRNA polyplexes than pDMAEMA.

The DNA polyplexes based on QNPHOS and QNPHOS-PEG did not show release of the nucleic acid after incubation with heparin, while pDMAEMA polyplexes showed already release of the DNA at N/S $\leq$ 0.7, indicating a strong binding of QNPHOS and QNPHOS-PEG to DNA. A strong binding of nucleic acids to polymers with quaternary amine groups has been observed previously [20,30].

Based on these observations, it is concluded that siRNA/DNA complexes based on QNPHOS and QNPHOS-PEG are much more stable than complexes based on pDMAEMA which is likely due to the presence of two permanently charged nitrogen groups per monomer unit.

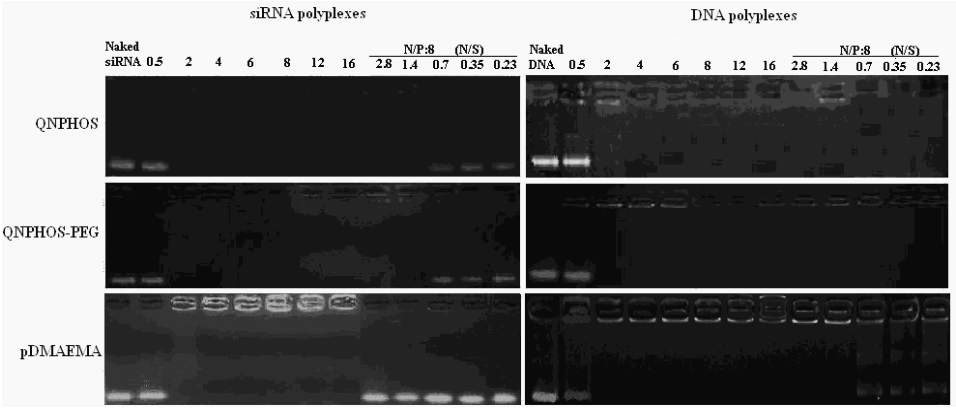

Figure 4. Agarose gel electrophoresis. SiRNA and DNA polyplexes made in 5 mM HEPES buffer (pH: 7.4) at N/P ratios 0.5 - 16 were applied on 4% and 0.8% agarose gels, respectively. To study polyplex destabilization and siRNA/DNA release, polyplexes made at an N/P ratio of 8 were incubated for 5 minutes with heparin (different polymer to siRNA ratios expressed as N/s ratios, where N is the moles of cationic nitrogens in the polymer and S is moles of sulphate in heparin) and subsequently applied on the gels.

The binding strength of the siRNA/DNA polyplexes based on QNPHOS and QNPHOS-PEG polymers after exposure to 150 mM NaCl, in the presence and absence of heparin was also studied. Figure 5 shows that exposure of the siRNA complexes to a solution of 150 mM NaCl does not lead to complex dissociation. However, after incubation of the polyplexes with a solution of heparin in 150 mM NaCl, complex dissociation and release of siRNA was observed. The DNA polyplexes based on QNPHOS and QNPHOS-PEG did not show release of the nucleic acid after incubation with solution of 150 mM NaCl with and without heparin, while incubation of the DNA complexes based on pDMAEMA with 150 mM NaCl and heparin resulted in release of the DNA, again indicating a stronger binding of QNPHOS and QNPHOS-PEG polymers to DNA as compared to pDMAEMA.

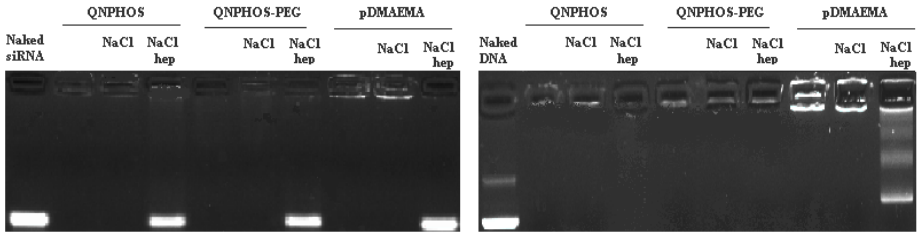

Figure 5. Agarose gel electrophoresis. SiRNA and DNA polyplexes (N/P: 8) made in 5 mM HEPES buffer (pH: 7.4) were incubated for 5 minutes in 5 mM HEPES buffer (pH: 7.4) containing150mM NaCl) with or without heparin (N/S: 0.23) and subsequently applied on 4% and 0.8% agarose gels, respectively.

Silencing activity and cell viability

Human lung cancer cells (H1299) expressing luciferase were used for gene silencing studies. SiRNA polyplexes based on QNPHOS and QNPHOS-PEG prepared at N/P ratios of 8 and 16 were selected for silencing studies, because these particles are able to bind to siRNA (Figure 4), have a positive mean zeta-potential and a small average size (Figure 2). Figure 6a shows that in the absence of serum, incubation of cells with polyplexes based on QNPHOS and QNPHOS-PEG prepared at N/P ratio of 8 and 10 pmol siRNA resulted in 30% and 50% silencing activity, respectively. Increasing the dose of the polyplexes to 20 pmol siRNA resulted in 40% and 60% silencing, respectively. Incubation of cells with both polyplex formulations made at an N/P ratio of 16 resulted in about 40-50% gene silencing, and increasing the dose of the polyplexes from 10 to 20 pmol siRNA, resulted in 60% and 70% silencing for QNPHOS- and QNPHOS-PEG-based polyplexes, respectively. These results illustrate the N/P- and dose- dependent gene silencing activity of the polyplexes as previously also observed for other polyplex formulations [15,16,31,32]. Incubation of cells with pDMAEMA-based polyplexes prepared at an N/P ratio of 8 and 10 pmol siRNA resulted in > 50% silencing. Incubation with polyplexes made at N/P ratio of 16 and increasing the dose of the polyplexes to 20 pmol siRNA, led to a drop in silencing activity which is explained by the enhanced cytotoxicity of the polyplexes due the higher concentrations of polymer to which the cells were exposed (Figure 6b). Figure 6b also shows that the QNPHOS- and QNPHOS-PEG-based polyplexes had a substantially better cytocompatibility (toxicity ≤20%)

than those based on pDMAEMA (up to 50%). This demonstrates that a high charge density of polymers is not necessarily accompanied by a higher cytotoxicity.

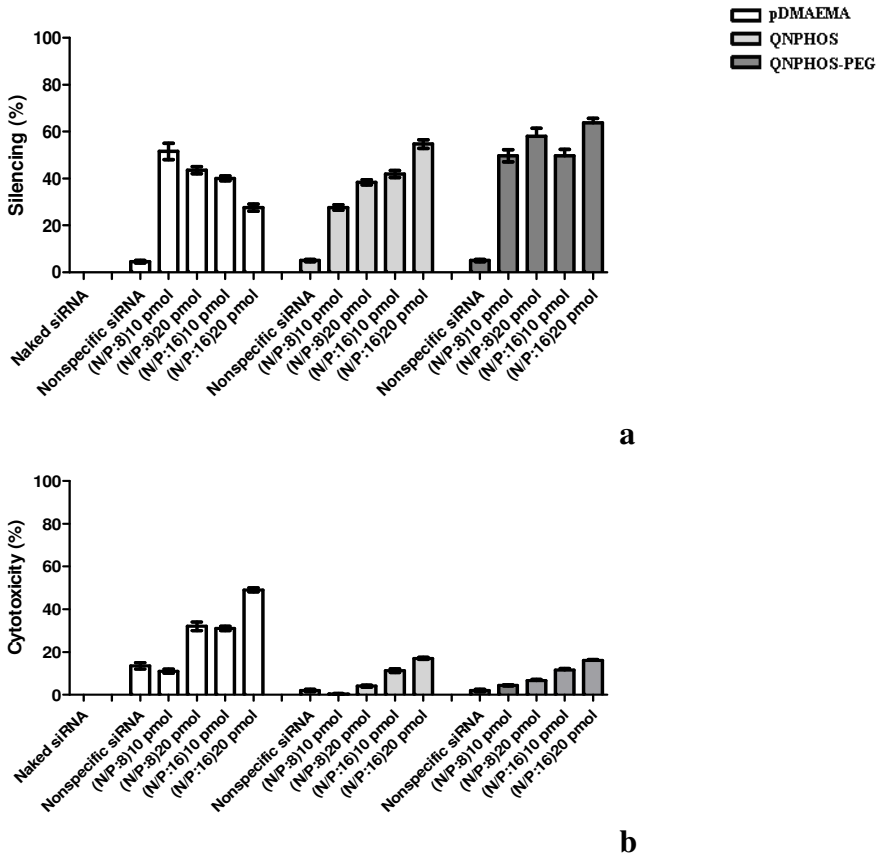

Figure 6. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes (10 to 20 pmol siRNA content per well) in the absence of serum. **b**. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum free medium (mean ± standard deviation (n=3)).

Figure 7a shows that the gene silencing activity of QNPHOS and QNPHOS-PEG polyplexes was affected by serum proteins and decreased to about 20% and 50%, respectively. This reduction might be partly

explained by the adsorption of negatively charged serum proteins on the polyplex surface which can result in polyplex destabilization and/or reduced cellular uptake due to charge reversal of the polyplex [33,34]. The cytotoxicity of QNPHOS and QNPHOS-PEG polyplexes remained low in the presence of serum (≤20%) whereas the cytotoxicity of pDMAEMA polyplexes was reduced (Figure 7b) as compared to the serum free condition. As suggested in literature, serum proteins might mask the cytotoxicity of cationic polyplexes and polymers [35-37].

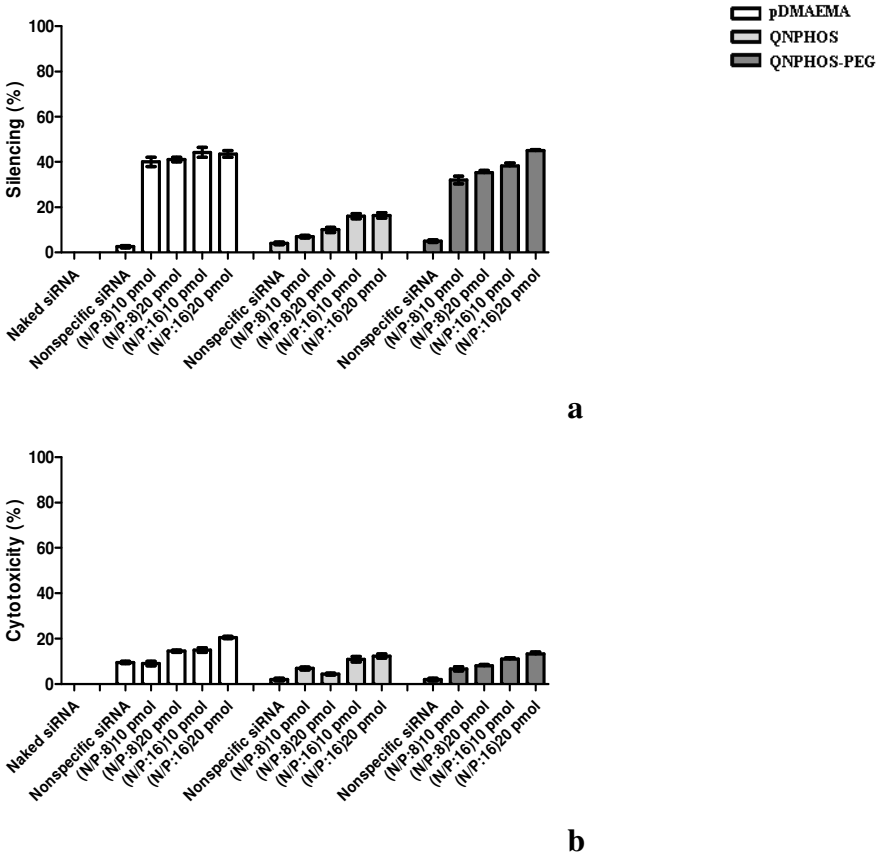

Figure 7. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes (10 to 20 pmol siRNA content per well) in presence of serum. **b**. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in the presence of serum (mean ± standard deviation (n=3)).

The transfection activity of the DNA polyplexes based on QNPHOS and QNPHOS-PEG as well as on pDMAEMA was investigated using COS-7 cells. In a previous study [38] it was shown that low molecular weight pDMAEMA (< 50 kDa) is unable to condense the structure of pDNA and polyplexes of > 500-1000 nm were formed. These polyplexes, likely due to their poor cellular uptake, showed low transfection activity. In the present study, we therefore used pDMAEMA with a molecular weight of 130 kDa that formed polyplexes of 100-300 nm, a size that allows uptake by endocytosis. Polyplexes prepared at N/P ratios of 8 and 16 were selected for transfection studies, because these particles had a positive mean zeta-potential and a small average size (Figure 3). Figure 8a shows that incubation of cells with luciferase DNA polyplexes based on QNPHOS and QNPHOS-PEG did not show detectable luciferase gene expression, whereas incubation with pDMAEMA polyplexes resulted in considerable transgene expression. In line with Figures 4 and 5, the lack of luciferase gene expression of QNPHOS and QNPHOS-PEG polyplexes is likely ascribed to the strong binding of these polymers to DNA due to the presence of two permanently charged nitrogen groups per cationic monomer block, leading to a limited (or no) DNA release from the polyplexes. Therefore, the presence of two charged groups per monomer unit limits the DNA release and therefore expression of the transgene is not obtained. In line with the cytotoxicity data of the siRNA complexes, DNA polyplexes based on QNPHOS and QNPHOS-PEG showed a substantially better cytocompatibility (cytotoxicity ≤20%) than those based on pDMAEMA (cytotoxicity 10-50%) (Figure 8b).

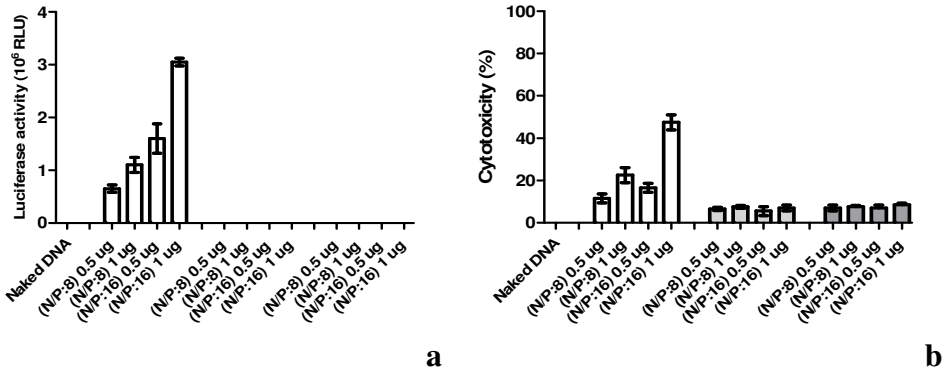

Figure 8. a. Luciferase expression after transfection of COS-7 cells with DNA complexes (0.5 to 1 µg DNA content per well) in serum free medium. **b**. Cell viability as measured by XTT assay after incubation of COS-7 cells with DNA complexes in serum free medium (mean ± standard deviation (n=3)).

It has been demonstrated in many papers that cationic polyplexes are essentially taken up by cells through endocytosis [39-41]. When no endosomal escape occurs, the particles remain entrapped in these vesicles and eventually end up in lysosomes where active degradation processes under the action of lysosomal enzymes take place [42]. Therefore, to study whether the lack of gene expression of QNPHOS and QNPHOS-PEG polyplexes is due to lack of endosomal escape, transfection in combination with photochemical internalization (PCI) was applied. PCI makes use of photosensitizers that localize in endosomal membranes upon addition to cells and photochemically destabilize these membranes after illumination, resulting in cytosolic release of the complexes [43]. However, application of PCI did not result in luciferase gene expression in COS7 cells treated with DNA polyplexes based on QNPHOS and QNPHOS-PEG (data not shown). It is therefore concluded that lack of transfection activity of QNPHOS and QNPHOS-PEG polyplexes is likely due to strong binding of these polymers to the DNA leading to a limited release of the DNA from the polyplexes [44].

Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to study the cellular uptake and internalization of siRNA complexes using

fluorescently labeled siRNA. Figure 9 shows that red spots originating from the labeled siRNA (images b) were intracellularly observed after incubation of the cells with the various siRNA formulations investigated in this study, demonstrating that the complexes were indeed internalized. For the cells treated with QNPHOS and QNPHOS-PEG based complexes, the fluorescence intensity of the internalized siRNA is considerably higher than cells treated with pDMAEMA-based complexes, which might indicate their higher cellular uptake/release, therefore higher gene silencing activity (Figure 6a).

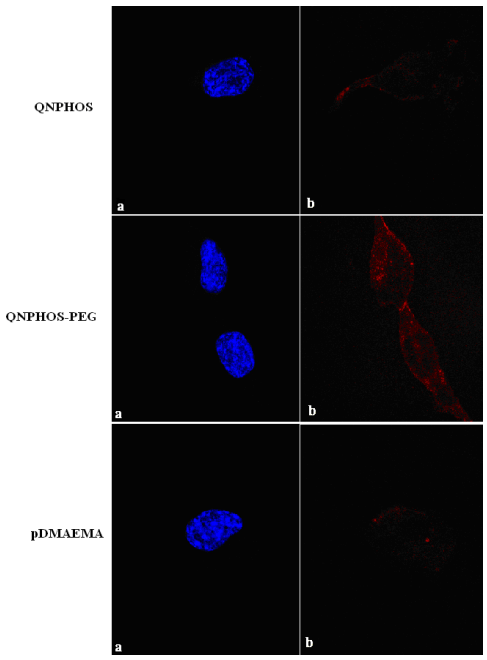

Figure 9. Confocal laser scanning microscopy images of H1299 cells incubated with complexes prepared with TYE 563 labeled siRNA (red). Images **a**: Nuclei of cells stained with Dapi, Images **b**: Complexes with TYE 563 labeled siRNA.

Conclusion

This study introduces QNPHOS and QNPHOS-PEG as polymeric model structures with two quaternized nitrogen groups per monomer unit which enables the polymers to form stronger complexes with nucleic acids in comparison to the well-known transfectant pDMAEMA. Based on the

data presented in this paper it is concluded that formation of strong siRNA complexes is indeed advantageous, since it enhances the stability of the polyplexes in biological fluids, contributing to an improved siRNA delivery resulting in a high gene silencing activity than pDMAEMA based systems. On the other hand, QNPHOS and QNPHOS-PEG form too stable polyplexes with DNA with a limited release, resulting in lack of gene expression. Furthermore, complexes based on QNPHOS and QNPHOS-PEG show lower cytotoxicity that those based on pDMAEMA.

Acknowledgment

This project was funded by MEDITRANS, an Integrated Project funded by the European Commission under the "Nanotechnologies and Nano-sciences, Knowledge-based Multifunctional Materials and New Production Processes and Devices" (NMP), thematic priority of the Sixth Framework Program. We would like to thank Dr. R.Wubbolts and ing. E.M. van ‘t Veld from the Center for Cellular Imaging (CCI) (Faculty of Veterinary Medicine, Utrecht University) for technical assistance with the CLSM and Prof. Thomas Kissel and Dr Olivia Merkel from Marbourg University, Germany, for providing the H1299 cell line.

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Chapter 5

Gene Silencing Activity of siRNA Polyplexes Based on Biodegradable Polymers

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Abstract

Cationic polymers are used as nonviral vectors for nucleic acid delivery. In this study, two biodegradable cationic polymers were evaluated for the purpose of siRNA delivery: pHPMA-MPPM (poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol)) and TMC (O-methyl free N,N,N-trimethylated chitosan). The silencing activity and the cellular cytotoxicity of polyplexes based on these biodegradable polymers were compared with those based on non-biodegradable pDMAEMA (poly(2-dimethylamino)ethyl methacrylate) and PEI (polyethylenimine), and with the regularly used lipidic transfection agent Lipofectamine. To promote endosomal escape, either the endosomolytic peptide diINF-7 was added to the formulations, or photochemical internalization (PCI) was applied. Incubation of H1299 human lung cancer cells expressing firefly luciferase with polyplexes based on pHPMA-MPPM and TMC showed 30-40% silencing efficiency. This silencing activity was equal to or better than that obtained with the standard transfectants. Under all experimental conditions tested, the cytotoxicity of the biodegradable polymers was low. The application of PCI, as well as the addition of the diINF-7 peptide to the formulations increased their silencing activity up to 70-80%. This demonstrates that pHPMA-MPPM- and TMC-based polyplexes benefit substantially from endosomal escape enhancement. Importantly, the polyplexes retained their silencing activity in the presence of serum and they showed low cytotoxicity. These biodegradable vectors are therefore attractive systems for further *in vivo* evaluations.

Keywords: siRNA, delivery, polymeric vectors, endosomal escape, photochemical internalization, fusogenic peptides, gene silencing, cytotoxicity

Introduction

Since the first description of RNA interference (RNAi), considerable research efforts have been dedicated to use small interfering RNAs (siRNA) as novel biotherapeutics [1]. These double stranded RNA molecules are able to silence genes effectively in a sequence specific manner [2,3]. However, siRNA has unfavorable biopharmaceutical properties. They are rapidly degraded by nucleases present in biological fluids and have low membrane penetration capabilities and therefore do not reach in sufficient amounts the cellular cytoplasm, where the RNA interference (RNAi) machinery is located [4,5]. Consequently, a key challenge to the effective and widespread use of this new class of biotherapeutics is their cytosolic delivery [6].

Cationic polymers have been studied for nucleic acid delivery in the last decade. Due to their positive charge, these polymers are able to complex with anionic nucleic acids to form polyplexes [7]. However, many of these cationic polymers show considerable toxicity both towards *in vitro* cultured cells and in animal models. The toxicity is attributed to their cationic character, and, additionally, to the fact that, many of the studied polymeric gene/siRNA vectors are non-degradable. Therefore, the search for effective but nontoxic vectors is currently of prime interest [8]. It has been shown that biodegradable polymers are attractive candidates for the design of siRNA-loaded polyplexes [9,10]. Our group has reported on several biodegradable polymers which have been evaluated for DNA delivery, both *in vitro* and *in vivo*. One example is pHPMA-MPPM (Figure 1). This polymer contains a biodegradable linker which is stable at pH 5 (pH in endo/lysosome) and is degraded at pH 7. The colloidal stablilty of polyplexes based on this polymer was investigated by Luten et al (2006). It was shown that the particles were stable for around 10 hours at 37°C and pH 7.4. DNA polyplexes based on this polymer are stable in serum and show a high *in vitro* transfection activity with minimal cytotoxicity [11].

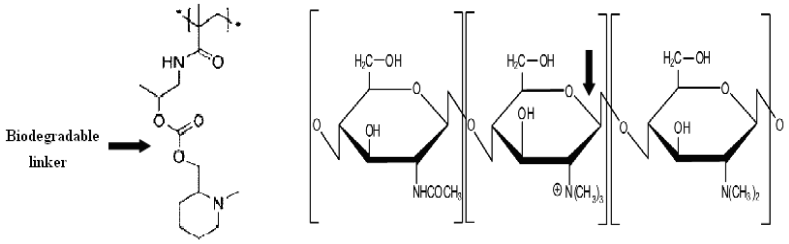

Figure 1. Structure of pHPMA-MPPM (left, poly(hydroxy propyl methacrylamide-1-methyl-2-piperidine methanol) and TMC (right, O-methyl free N,N,N-trimethylated chitosan). In TMC, the various substituents are randomly distributed over the polymer chain (acetyl groups (see block 1), quaternized amines (see block 2))

Trimethylated chitosan (TMC) has been studied for DNA and siRNA delivery [12]. The degradation of TMC is due to hydrolysis of the glycosidic bond (indicated with an arrow in Figure 1) [13].
Our group recently reported on the synthesis of a well defined and enzymatically degradable TMC (O-methyl free N,N,N- trimethylated chitosan) (Figure 1) [14].
Polyplexes can be taken up by cells through endocytosis [15,16]. Particles entering the cell through this route become entrapped in endosomes and eventually end up in lysosomes where active degradation processes under the action of special enzymes take place [17]. Thus, one of the crucial steps in successful nucleic acid delivery with cationic polymers is the escape of the polyplexes from endosomes [18]. Several approaches have been used to promote the endosomal escape of endocytosed polyplexes. One of these approaches is Photochemical Internalization (PCI), a technique based on the use of photosensitizers that localize in endosomal membranes upon addition to cells and photochemically destabilize endosomal membranes after illumination, with subsequent release of endocytosed material into the cytosol [19]. In addition, several endosomal membrane-disrupting peptides such as diINF-7, a peptide based on the fusion domain of the influenza virus, have been used to facilitate the endosomal escape of polyplexes. At endosomal pH, at which such peptides become fusogenic, the C- terminal side of the V-shaped peptide undergoes a conformational change, resulting in the formation of a helical

structure that allows insertion of the peptide into the lipid bilayer, leading to endosomal membrane destabilization [20].
In this study, pHPMA-MPPM and TMC were investigated for siRNA delivery. We compared these polymers with the non-degradable polymers poly(2-dimethylamino)ethyl methacrylate) (pDMAEMA) [21] and polyethylenimine (PEI) and the routinely used commercially available lipidic transfection agents Lipofectamine. In addition, the application of PCI and the use of the membrane-disrupting peptide diINF-7 on the silencing activity of the polyplexes were investigated.

Materials and Methods

Materials

The Fluc double stranded siRNA which specifically targets firefly luciferase (used against mRNA from pGL3; structure below), anti-EGFP siRNA as non specific siRNA and Fluc TYE563- labeled siRNA were obtained from Integrated DNA Technologies BVBA, Leuven, Belgium.
Sense strand: 5´- pGGUUCCUGGAACAAUUGCUUUUAca
Antisense strand: 3´- GACCAAGGACCUUGUUAACGAAAAUGU
Branched polyethylenimine (Mw 50-100 kDa) was obtained from Polysciences, Inc. (Warrington, Pennsylvania USA). PHPMA-MPPM (240 kDa) [11], TMC 33% (60 kDa) [14] and Poly(2-(dimethylamino) ethylmethacrylate) (pDMAEMA) [21] were synthesized and purified as described previously. Sodium heparin and gelatin solution were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) and Lipofectamine 2000 was obtained from Invitrogen (Breda, The Netherlands). Luciferase assay reagent and reporter lysis buffer were obtained from Promega (Leiden, The Netherlands). Formaldehyde was obtained from Fluka (Zwijndrecht, The Netherlands) and FluorSave for mounting the cells onto glass cover slides was obtained from Calbiochem (San Diego, CA, USA). INF-7 was synthesized as previously described [20]. Its fusogenic activity was assessed using a liposome leakage assay [22]. The photosensitizer (PS) TPPS2a, meso-tetraphenylporphyrin was obtained from PCI Biotech AS (Oslo, Norway) [23]. LumiSource®, a bank of four light tubes emitting light with a wavelength range of 375 - 450 nm and 13 mW/cm^2 irradiation intensity was provided by PCI Biotech AS, Oslo, Norway.

Physicochemical characterization of polyplexes

Polyplexes with different polymer to siRNA ratios (expressed as N/P ratios, where N is the moles of cationic nitrogens in the polymer and P is moles of phosphate in siRNA) between 0.5 - 20 were prepared by adding a Fluc siRNA solution (175 µl, 20 µg/ml) in HEPES (5 mM, pH 7.4) to polymer solutions (700 µl, various concentrations) in the same buffer. The resulting solutions were vortexed for 10 seconds. After 30 minutes incubation at room temperature, Z- average diameters were measured with dynamic light scattering at 25 °C with a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern, UK). Viscosity and refractive index of water at 25 °C were used. The system was calibrated with an aqueous dispersion of polystyrene particles with a diameter of 100 nm. Particle size distribution is described using the polydispersity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of the polyplexes prepared in HEPES (5 mM, pH 7.4) was determined at 25 °C in a DTS5001 cell with a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential. In some experiments, after 30 minutes incubation of polyplexes at room temperature, 50 µl aqueous solutions of INF-7 peptide (different concentrations) in HEPES (5 mM, pH 8) was added to the polyplex dispersion (final peptide concentration ranged from 10 to180 µg/ml). After vortexing for 5 seconds, the dispersions were incubated at room temperature for 15 minutes and characterized for size and charge. The colloidal stability of the polyplexes in HEPES (5 mM, pH 7.4) was studied by measuring their Z-average diameters during storage at room temperature.

Agarose gel electrophoresis

The complexation of siRNA with the various transfectants under study and the heparin-induced destabilization of the polyplexes were investigated using agarose gel electrophoresis. The agarose gels (NuSieve® GTG® Agarose, Lonza, Rockland, ME, USA) were made in a concentration of 4% (w/v) in Tris-Acetate-EDTA (TAE) running buffer and contained 0.5 µg/ml ethidium bromide. Polyplexes (N/P ratios varying from 0.5 to 20) of different polymers with Fluc siRNA were prepared as

described above. Naked siRNA was also used. Polyplex dispersions (N/P 8) with or without heparin (0.4 - 2.7 USP/µg siRNA; molar ratio of amine groups of the polymer and sulfate groups of heparin (N/S) varied from 0.4 to 3) were incubated for 5 minutes at room temperature. Polyplexes (15 µl, corresponding with 15 pmol siRNA) were applied in the starting slot of the gel and electrophoresis was performed at 60 V for 50 min. The siRNA bands stained with ethidium bromide were detected on a UV transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

Gene silencing and cytotoxicity experiments

The human lung cancer cell line H1299 which expresses firefly luciferase was used to study the gene silencing activity of the different polyplex formulations. The cell line was maintained in RPMI 1640 medium with HEPES and L-glutamine (PAA laboratories GmbH, Pasching, Austria, catalog No. E15-842) completed with fetal bovine serum (FBS) (final concentration 10% v/v) and cultured at 37 °C at 5% CO_2 humidified atmosphere. The cells (8×10^3 cells/well) were seeded into 96-well plates and cultured overnight. Different concentrations of photosensitizer (ranging from 0.05 to1.5 µg/ml) and different illumination times were applied (15 to 90 seconds). To achieve the strongest silencing with minimal cytotoxicity (≤ 10%), we selected a dose of photosensitizer of 0.5 µg/ml and illumination time of 75 seconds. One day after seeding, the cells were incubated at 37° C with complete culture medium containing photosensitizer for 18 h. Next, the medium was removed and fresh medium was added to the wells and the anti-Luciferase siRNA polyplexes (10 pmol siRNA/well) were added to the cells and incubated at 37° C for 4 h. Then, the medium was removed, fresh medium was added, and the cells were exposed to the light source for 75 s. Subsequently, the cells were incubated at 37 °C for 2 days, after which the luciferase protein expression was analyzed using Luciferase reporter gene assay (Promega). The cytotoxicity of the polyplexes was measured using the XTT colorimetric viability assay as previously described [24]. When the PCI treatment was not applied, the day after seeding, the cells were incubated with polyplexes with or without INF-7 peptide (final concentration of 1.5 to 9.5 µg/ml in growth medium) for 4 hours, followed by refreshing the medium and 2 days incubation at 37° C. Polyplexes of specific and non-specific siRNA with Lipofectamine 2000 were prepared by gently mixing

50 µl siRNA (20 µg/ml) in HEPES (5 mM, pH 7.4) with 3.7 µl Lipofectamine 2000 in 50 µl HEPES (5 mM, pH 7.4) followed by 30 min incubation at room temperature.

Determination of Luciferase activity

Luciferase activity was measured after removal of the growth medium and lysis of the cells by the addition of 100 µl reporter gene lysis buffer. After a freeze/thaw cycle at -80 °C/room temperature, 50 µl of luciferase assay reagent was added to 50 µl of the cell lysate and relative light units (RLU) were measured for 10 s at room temperature using a FLUOstar OPTIMA microplate based multi-detection reader with a microinjector.

Multi-Photon laser scanning microscopy

The cellular uptake of polyplexes was investigated with multi-photon laser scanning microscopy (MPLSM). In short, 8×10^3 cells were seeded in the wells of 12-well plates, pre-coated with 100 µl gelatin. The cells were cultured for 24 hours at 37° C and subsequently incubated with Fluc TYE563- labeled siRNA lipo/polyplexes (with or without photosensitizer (PS)).Then the cells were fixed with 4% solution of formaldehyde in PBS for 30 minutes at room temperature. After fixation, the cells were washed twice with PBS and incubated with DAPI nucleic acid stain (300 nM) in PBS for 3 minutes. Subsequently, the cells were washed twice with PBS and mounted onto glass cover slides, using FluorSave. The cells were analyzed using MPLSM (Bio-Rad, Hemel Hempstead, United Kingdom). Excitation of DAPI was achieved by multiphoton excitation at 780 nm using mode-locked Titanium: Sapphire laser (Tsunami; Spectra Physics, San Jose, CA) pumped by a 10-W solid state laser (Millennia Xs; Spectra Physics), whereas TYE563 was excited by confocal laser light (550 nm). Samples were viewed with a TE200 inverted microscope using a 60/1.4 oil objective (Nikon, Tokyo, Japan). Images were analyzed using LaserSharp 2000 software (Bio-Rad).

Results and Discussion

In this study, pHPMA-MPPM and TMC were investigated for their siRNA delivery properties and compared with those of well-known non-

degradable polymeric transfectants (pDMAEMA) [21], branched PEI [25,26] and the cationic lipid Lipofectamine [27].

Physicochemical characterization of polyplexes

The average diameter of the polyplexes prepared at different N/P ratios were measured by dynamic light scattering (Figure 2). All polymers were capable of forming complexes with siRNA. In case of the PEI- and pHPMA-MPPM-based polyplexes, small particles with an average size around 100-150 nm were formed over the whole N/P range of 0.5-20. The average size of pDMAEMA-based and TMC-based polyplexes decreased with increasing N/P ratio, with small-sized nanoparticles of 100-150 nm being formed at N/P ratio $\geq$ 8. This can probably be ascribed to an increased polymer amount in the formulations which leads to tighter complexes. Furthermore, a polydispersity index (PDI) of $\leq$ 0.3 was observed for all polyplex formulations made at N/P ratio $\geq$ 8, which indicates a rather narrow size distribution. At lower N/P ratios the pDMAEMA and TMC-based polyplexes were significantly larger than pHPMA-MPPM and PEI-based polyplexes. In case of TMC, this is most likely due to the lower charge density of TMC as compared to pHPMA-MPPM and PEI.

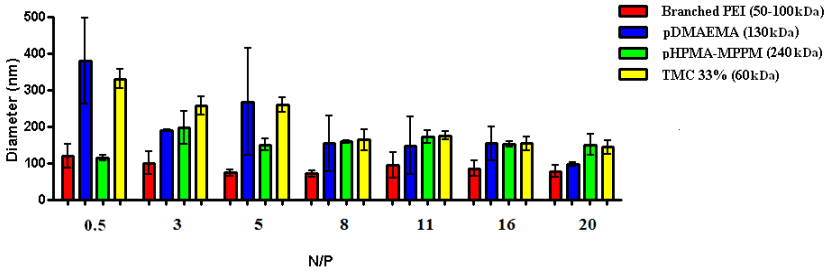

Figure 2. Average diameter of polyplexes prepared at different N/P ratios (mean ± standard deviation (n=3).

At N/P of 0.5, the different polyplexes had a negative zeta potential (about -25 mV), which can be explained by the excess of siRNA present in the formulations. At N/P $\geq$ 3, positively charged particles (zeta-potential

around 20 mV, Supporting information S1) were formed as a result of the increased polymer amount in the formulations.

Agarose gel electrophoresis

Binding of cationic polymers to siRNA molecules and subsequent complex formation are essential for protection of siRNA against degradation by endonucleases. On the other hand, polyplex destabilization also needs to occur as siRNA should be released in the cytosol in order to achieve gene silencing activity. Heparin is commonly used as a model anionic polyelectrolyte to monitor the sensitivity of polyplexes for dissociation and release of siRNA [28,29].
Figure 3 shows that siRNA remains in the starting slots of the gel for siRNA/polymer formulations prepared at N/P $\geq$ 3, indicating, in agreement with DLS analysis (Figure 2), that polyplexes were formed. In line with previous studies [30,31], no siRNA was detected in case of the PEI formulations (Figure 3d) pointing to the formation of strong complexes. After incubation of the polyplexes with heparin, dissociation of polyplexes and subsequent release of the siRNA was observed, except for the PEI/siRNA polyplexes, again indicating that PEI and siRNA form strong complexes. Figure 3a shows that polyplexes based on pHPMA-MPPM are dissociated at the lowest heparin concentration investigated (N/S=3) pointing to a low binding strength between this polymer and siRNA. In case of the TMC-based polyplexes, release of the siRNA was observed at N/S $\leq$ 1 (Figure 3b) which indicates a higher binding strength of TMC to siRNA than pHPMA-MPPM. Figure 3c shows that siRNA is released from pDMAEMA polyplexes at N/S $\leq$ 1.8 which indicates an intermediate binding strength between this polymer and siRNA.

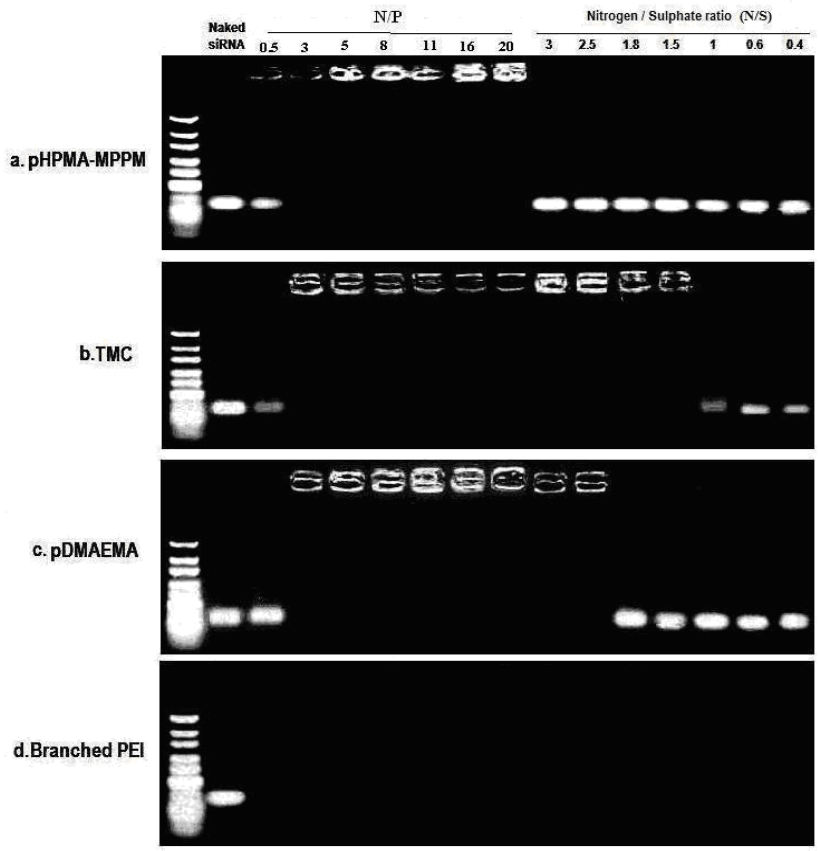

Figure 3. Agarose gel electrophoresis. Polyplexes made in 5 mM HEPES buffer (pH: 7.4) at N/P ratios 0.5 - 20 were applied on a 4% agarose gel. To study polyplex destabilization and siRNA release, polyplexes made at an N/P ratio of 8 were incubated for 5 minutes with heparin solution (different concentrations) and subsequently applied on the gel.

Silencing activity and cell viability

Human lung cancer cells (H1299) expressing luciferase were used for gene silencing studies. Polyplexes prepared at N/P ratios of 8 and 16 were selected for silencing studies, because these particles showed binding to the siRNA (Figure 3), had a positive mean zeta-potential (Supporting information S1), and small average size (Figure 2). Figure 4a shows that in the absence of serum, incubation of cells with polyplexes based on

pDMAEMA resulted in up to 50% silencing activity whereas incubation with pHPMA-MPPM and TMC polyplexes made at N/P ratios of 8 and 16 showed 30-40% gene silencing. The activity of these polyplexes is comparable with the lipofectamine formulation (60% silencing efficiency). Incubation of cells with polyplexes based on branched PEI led to 25-30% silencing of luciferase. In all cases the effect of the N/P ratio (8 vs 16) was small. The relatively low silencing activity of branched PEI/siRNA polyplexes can likely be explained by the strong binding of PEI to the siRNA which inhibits the liberation of the siRNA from the complexes (Figure 3d). Figure 4b shows low cytotoxicity of the lipofectamine and polymer formulations made at an N/P ratio of 8 (<15%) (except PEI formulation, ~25%). When compared to a N/P of 8, the cytotoxicity of polyplexes prepared at an N/P ratio of 16 was about two fold higher (in case of PEI less than 2-fold) which can be ascribed to the higher concentration of the cationic polymers in the formulations. In this study, all the gene silencing results were corrected for cytotoxicity.
Figure 4c shows that, importantly, the gene silencing activity of pHPMA-MPPM, TMC and pDMAEMA polyplexes was not affected by the presence of serum proteins. Moreover, in the presence of serum, the cytotoxicity of the polyplexes was slightly lower (Figure 4d) than that of the complexes incubated with the cells in medium without serum (Figure 4b). As suggested in literature, serum proteins might mask the cytotoxicity of cationic polyplexes and polymers [32,33].

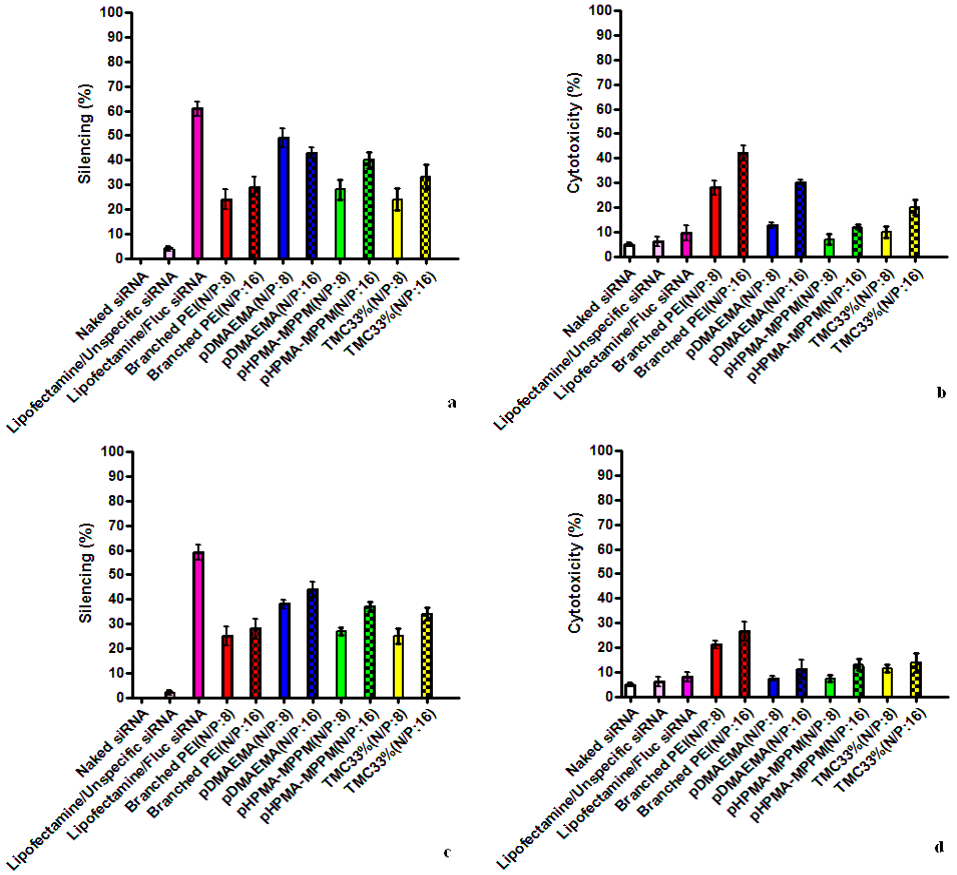

Figure 4. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes in serum free medium. b. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum free medium. c. Luciferase gene silencing of siRNA complexes after incubation with H1299 cells in the presence of 10% FBS. d. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in the presence of 10% FBS (mean ± standard deviation (n=3)).

Enhancement of endosomal escape

PCI is a technique based on the use of photosensitizers that photochemically destabilize endosomal membranes after illumination [34,35]. It appears that by application of PCI, the gene silencing activity of the pHPMA-MPPM and TMC polyplexes increased from 30-40%

(Figure 4a) to ~70-80% (Figure 5a). This means that the endosomal escape properties of these polymers are limited, as previously reported for pHPMA-MPPM/DNA complexes [11]. By application of PCI, the silencing efficiency of the pDMAEMA polyplexes only slightly increased which confirms previous findings that pDMAEMA polyplexes have intrinsic endosomal escape properties [36]. In a recent study by Boe et al, it was reported that gene silencing activity of PEI/siRNA complexes was enhanced after application of PCI [37]. In our study, the gene silencing activity of polyplexes based on PEI did not benefit from PCI treatment. In comparison to the study of Boe et al, we used PEI with a higher molecular weight (50-100kDa) which likely forms stronger complexes, with less release of the siRNA as confirmed by the heparin competition assay in Figure 3. Also, the cell line which we used was different than the one used in the study of Boe et al. For Lipofectamine-based complexes, silencing activity increased up to 70% after application of PCI (Figure 5a). Importantly, the application of PCI was not associated with an increase in cytotoxicity (compare Figure 4b and Figure 5b).

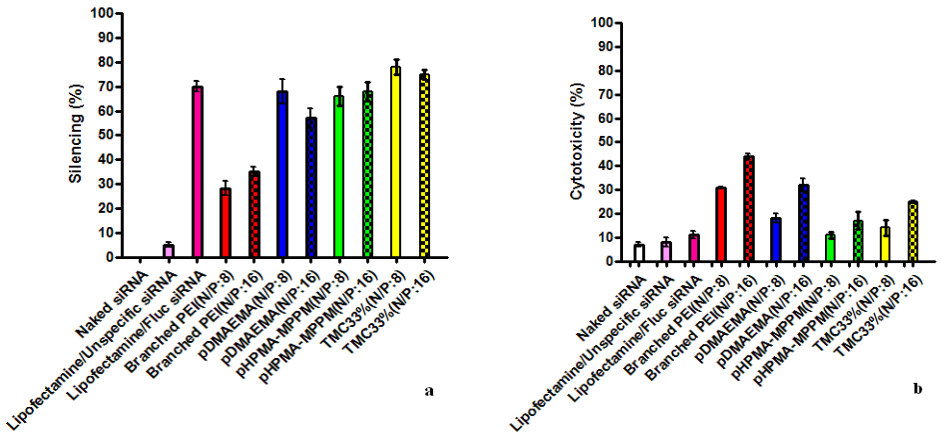

Figure 5. a. Effect of PCI on luciferase gene silencing activity of siRNA complexes after incubation with H1299 cells in serum free medium. b. Cell viability as measured by XTT assay after application of PCI for cells incubated with siRNA complexes in serum free medium (mean ± standard deviation (n=3)).

The effect of PCI on gene silencing activity of the siRNA complexes was also studied in the presence of serum in the medium. In the presence of serum, also an increased gene silencing activity was observed after application of PCI for the TMC, pHPMA-MPPM and pDMAEMA-based polyplexes (Supporting information S2a, compare with Figure 4c). In line with the results of Figure 5a, PCI has no effect on the activity of the PEI-based polyplexes. The cytotoxicity of PEI-based and pDMAEMA-based polyplexes after application of PCI was lower than in the absence of serum (Supporting information S2b).

Besides by PCI, endosomes can be destabilized by endosome-disruptive peptides. Therefore, we studied the effect of the endosome disruptive diINF-7 peptide on gene silencing efficiency of pHPMA-MPPM polyplexes. It was shown that this peptide is able to destabilize liposomes at pH 5 (Supporting information S3) [18,20,38,39]. DLS measurements showed that incubation of the polyplexes with the diINF-7 peptide did not affect their size distribution (data not shown). On the other hand, a gradual decrease in zeta potential of the polyplexes with increasing concentrations of diINF-7 was observed. At high concentrations of the peptide, the polyplexes became even negatively charged (Supporting information S4). At pH 7, diINF-7 is negatively charged and therefore binds via electrostatic interactions to the positively charged surface of the polyplexes. At high concentrations of the peptide, the surface is likely fully covered with the peptide by which the zeta potential of the polyplexes is reversed.

The gene silencing activity of diINF-7 coated pHPMA-MPPM polyplexes is shown in Figure 6a. The silencing activity of the Lipofectamine formulation slightly increased with increasing diINF-7 concentration (from about 55 to 70%). Incubation of cells with pHPMA-MPPM formulations containing 25-100 µg/ml diINF-7 peptide resulted in a gradual increase of the silencing activity from 25 to 50%, again indicating that the pHPMA-MPPM polyplexes have limited endosomal escape tendency. At the highest concentration of peptide (145 µg/ml) used, a drop in silencing activity to about 30% was observed, which may be explained by a too strong reduction of the zeta potential with less cellular interaction and uptake as a consequence [40,41] (Figure 6a). The XTT cell viability assay shows low cytotoxicity of the diINF-7-coated siRNA complexes (Figure 6b).

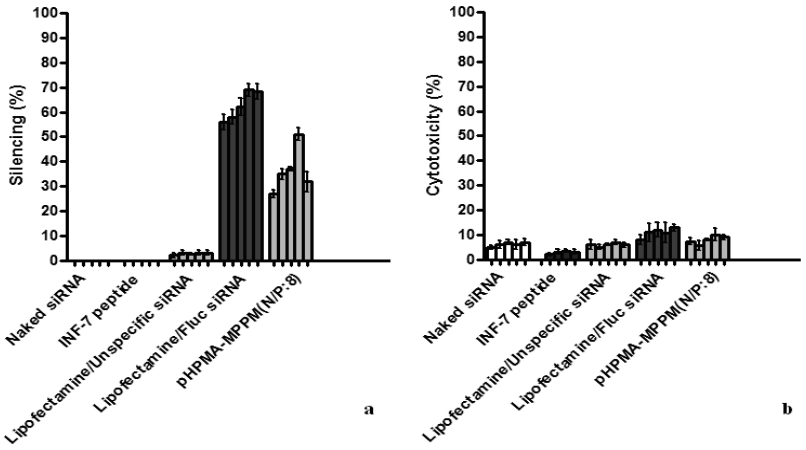

Figure 6. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes, with or without diINF-7 peptide in the medium containing 10% FBS. b. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes, with or without diINF-7 peptide, in the medium containing 10% FBS. In all formulations, the first bar shows the polyplexes without diINF-7 and second, third, forth and fifth bars show the polyplexes containing 25, 50.100 and 145 µg/ml diINF-7 (mean ± standard deviation (n=3)).

Multi-photon laser scanning microscopy

Multi-photon laser scanning microscopy (MPLSM) was used to study the cellular uptake and internalization of siRNA complexes using fluorescently labeled siRNA. Naked siRNA was not taken up by cells (not shown), in agreement with previous studies. Figure 7 shows that red spots originating from the labeled siRNA (images d and h) were intracellularly observed after incubation of the cells with the various siRNA formulations investigated in this study, demonstrating that the complexes were internalized. The fluorescence patterns did not change after application of PCI, even for polyplexes for which a PCI-mediated increase in gene silencing efficiency was noted (Figure 5a and supporting information S2a). These results indicate that the PCI-effect is below the detection limit of the MPLSM technique. The PCI effect is difficult to visualize as the punctuate fluorescence signal from endocytic vesicles is lost upon PCI when the siRNA appears through the cytosol, as it was shown previously by Oliveira et all (2008) [42]. The images of cells treated with

Lipofectamine/siRNA complexes do visualize release of siRNA into the both cytosol and nucleus, in agreement with previous observations [30,43].

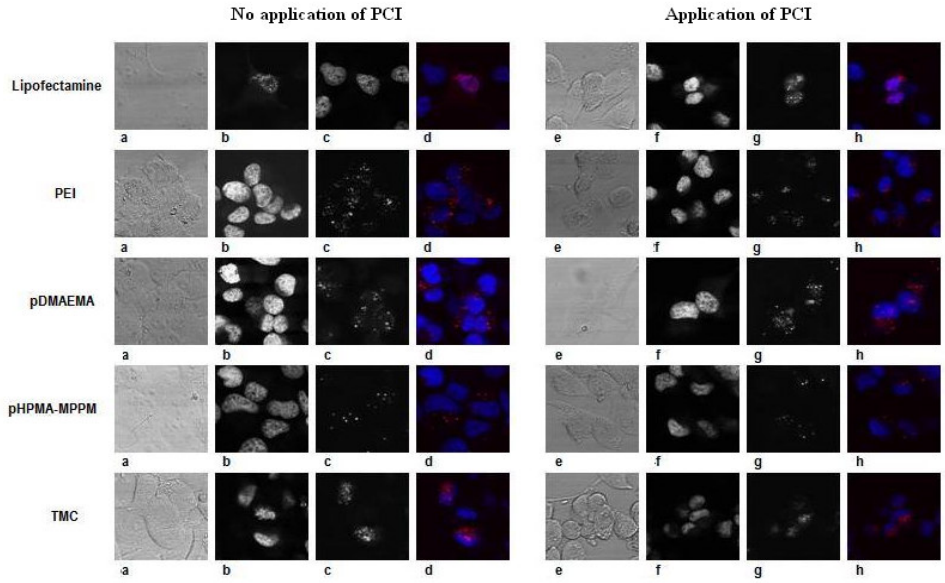

Figure 7. Multi-photon laser scanning microscopy images of H1299 cells incubated with complexes prepared with TYE 563 labeled siRNA (red). Images a and e: Pattern of cells with light microscopy, Images b and f: Nuclei of cells stained with Dapi, Images c and g: Complexes with TYE 563 labeled siRNA, Images d and h: Red, Complexes with TYE 563 labeled siRNA; Blue, DAPI stained nuclei.

Conclusion

This study introduces biodegradable pHPMA-MPPM and TMC as promising vectors for siRNA delivery. As compared to the nondegradable PEI- and pDMAEMA-bases polyplexes, their silencing activity was similar but their cytotoxicity profile more favorable. By promoting the endosomal escape of these polyplexes using either PCI or an endosome disruptive peptide, enhancement of their silencing activity was observed. Importantly, the polyplexes preserve their gene silencing activity in the

presence of serum proteins while their cytotoxicity remained low. These features make these polymers attractive candidates for further development and warrant application in animal studies.

Acknowledgment

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Supporting information (Chapter 5)

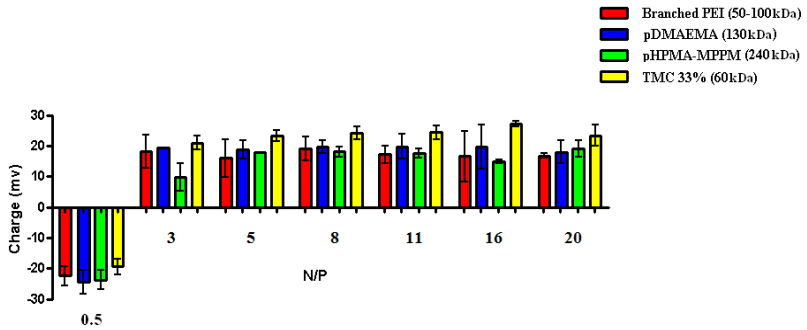

Figure S1. Zeta potential of polyplexes prepared at different N/P ratios (mean ± standard deviation (n=3))

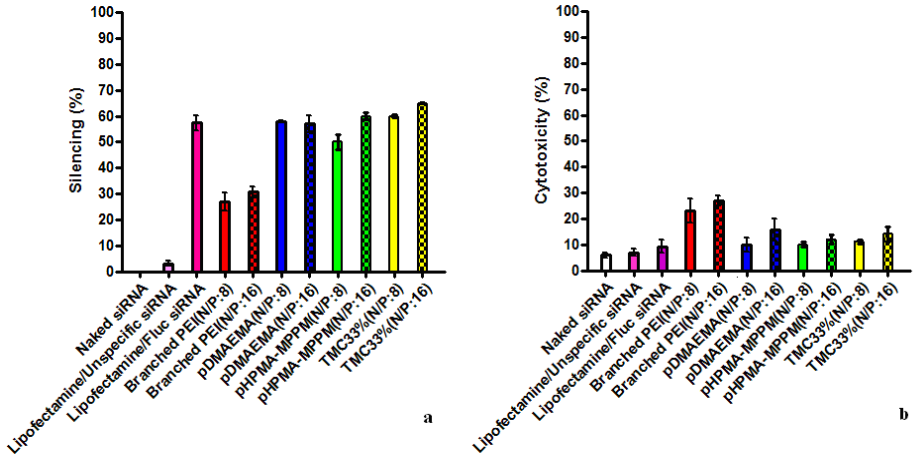

Figure S2. a. Effect of serum (10% FBS) on luciferase gene silencing of siRNA complexes in combination with PCI (compare with Figure 4a). b. Effect of serum (10% FBS) on cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in combination with PCI (compare with Figure 4b) (mean ± standard deviation (n=3)).

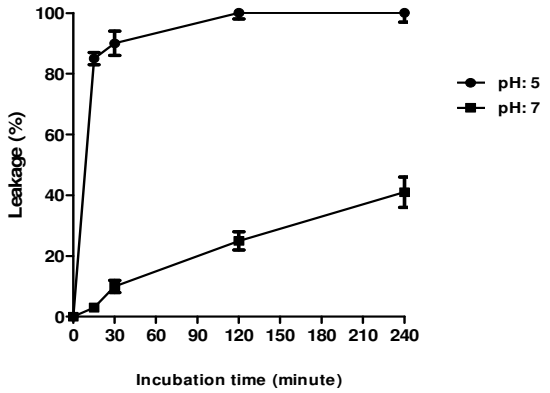

Figure S3. Liposome leakage assay. Leakage of calcein from liposomes after incubation with diINF-7 peptide (10 µg/ml) in pH 5 (125 mM citrate buffer).

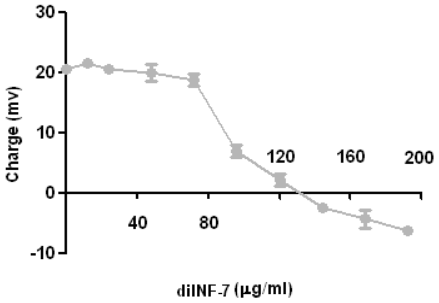

Figure S4. Zeta potential of pHPMA-MPPM/siRNA polyplexes after incubation with diINF-7 peptide.

Chapter 6

Gene Silencing Activity of siRNA Polyplexes Based on Thiolated N,N,N-trimethylated Chitosan

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Abstract

N,N,N-trimethylated chitosan (TMC) is a biodegradable polymer emerging as a promising nonviral vector for nucleic acid and protein delivery. In the present study, we investigated whether the introduction of thiol groups in TMC enhances the extracellular stability of the complexes based on this polymer and promotes the intracellular release of siRNA. The gene silencing activity and the cellular cytotoxicity of polyplexes based on thiolated TMC were compared with those based on the non-thiolated counterpart and the regularly used lipidic transfection agent Lipofectamine.
Incubation of H1299 human lung cancer cells expressing firefly luciferase with siRNA/thiolated TMC polyplexes resulted in 60-80% gene silencing activity, whereas complexes based on non-thiolated TMC showed less silencing (40%). The silencing activity of the complexes based on Lipofectamine 2000 was about 60-70%. Importantly, the TMC-SH polyplexes retained their silencing activity in the presence of hyaluronic acid, while non-thiolated TMC polyplexes hardly showed any silencing activity, demonstrating their stability against competing anionic macromolecules. Under the experimental conditions tested, the cytotoxicity of the thiolated and non-thiolated siRNA complexes was lower than those based on Lipofectamine. Given the good extracellular stability and good silencing activity, it is concluded that polyplexes based on TMC-SH are attractive systems for further *in vivo* evaluations.

Keywords: TMC-SH, siRNA, delivery, gene silencing, cytotoxicity

Introduction

Cationic polymers have been studied for nucleic acid delivery both *in vitro* and *in vivo* [1-4]. Due to their positive charge, these polymers are able to complex with anionic nucleic acid molecules to form polyplexes which are able to cross cellular barriers and reach their intracellular targets [5-8]. However, the complexes based on many of these cationic polymers are insufficiently stable in biological fluids and consequently do not protect the DNA/siRNA molecules against enzymatic degradation by nucleases [9,10]. Moreover, insufficient uptake by target cells, poor intracellular release properties and considerable cellular toxicity are other limiting factors for pharmaceutical and therapeutic application of these polymers. Therefore, the search for effective and non-toxic vectors is currently of prime interest [11-13].

N,N,N-trimethylated chitosan (TMC) is a biodegradable polymer emerging as a promising nonviral vector for nucleic acid and protein delivery [14-16]. This polymer is a partially quaternized chitosan derivative which, as compared to chitosan, has an improved solubility in aqueous solution at neutral pH, safety and effectiveness [17,18]. The beneficial ability of TMC to reversibly open tight junctions between epithelial cells, which facilitates the uptake of therapeutics, its muco-adhesive properties and relatively low toxicity, make TMC a valuable polymer for several drug delivery applications [19,20].

Introduction of thiol groups in several carrier systems has been shown to enhance their delivery properties. This is due to the formation of reducible disulfide bonds between thiol groups, leading to increased extracellular stability and improved intracellular release properties [21-23]. Therefore, introduction of thiol groups in TMC likely enhances the stability of its complexes with nucleic acids and also allows further chemical derivatization with targeting ligands and PEG making use of un-reacted SH groups [24-26]. Moreover, the thiol groups in TMC-SH can promote its muco-adhesive potential due to formation of disulfide bonds between the polymer and mucin glycoproteins on the cell membrane, which might lead to an enhanced cellular uptake [27,28].

Recently, considerable research efforts have been dedicated to the use of small interfering RNAs (siRNA) as novel biotherapeutics [29]. These double-stranded RNA molecules are rapidly degraded by nucleases present in biological fluids and are not able to enter the cytoplasm, where the RNA interference (RNAi) machinery is located [30,31]. Therefore, a

key challenge to the effective and widespread use of this new class of biotherapeutics is their cytosolic delivery [32-34].
Our group recently reported on thiolated TMC and the formation of nanoparticles based on this polymer [24]. In the present study, thiolated and non-thiolated TMC (Figure 1) were investigated for siRNA delivery and gene silencing. The silencing activity and the cellular toxicity of polyplexes based on these biodegradable polymers were compared with those based on the regularly used lipidic transfection agent Lipofectamine [35].

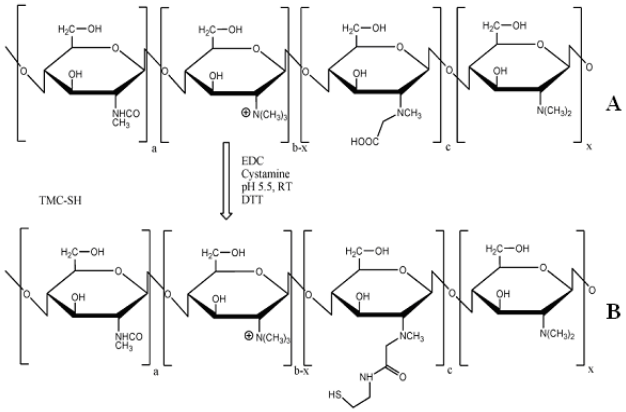

Figure 1. Structure of non-thiolated (A) and thiolated N,N,N-trimethylated chitosan (TMC) (B).

Materials and Methods

Materials

The double-stranded siRNA which specifically targets firefly luciferase (used against mRNA from pGL3; structure below), was obtained from Dharmacon bioscience (Lafayette,USA) and anti-EGFP siRNA as non-specific siRNA and Fluc-TYE563- labeled siRNA were obtained from Integrated DNA Technologies BVBA (Leuven, Belgium).
Sense strand: 5'- GAU UAU GUC CGG UUA UGU A UU
Antisense strand: UU CUA AUA CAG GCC AAU ACA U P-5'
Thiolated TMC (TMC-SH) with different extent of thiol modifications of 2, 5, 7% and molecular weights of 180, 215 and 144 kDa respectively and

non-thiolated TMC (MW=100 kDa) and thiolated HA (HA-SH, 40 kDa) with 21% thiol modification were synthesized and purified as described previously [24]. The degree of quaternization and acetylation of the studied TMCs were 25% and 17% respectively. Sodium heparin was obtained from Sigma Aldrich (Zwijndrecht, The Netherlands); and Lipofectamine 2000 and LysoTrackerR Green DND-26 were obtained from Invitrogen (Breda, The Netherlands). Luciferase assay reagent and reporter lysis buffer were obtained from Promega (Leiden, The Netherlands). DRAQ5™ was obtained from Biostatus Limited (Leicestershire, UK).

Physicochemical characterization of polyplexes

Polyplexes were prepared at different polymer to siRNA ratios (expressed as N/P ratios, where N is the moles of cationic nitrogens in the polymer and P is moles of phosphate in siRNA) between 0.5 - 14 by adding a siRNA solution (200 µl, 40 µg/ml) in HEPES (5 mM, pH 7.4) to polymer solutions (200 µl, various concentrations) in the same buffer. The resulting solutions were vortexed for 10 seconds. After 3 hours incubation at room temperature, Z- average diameters were measured with dynamic light scattering at 25 °C with a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern, UK). Viscosity and refractive index of water at 25 °C were used. Calibration was done with an aqueous dispersion of polystyrene particles with a diameter of 100 nm. Particle size distribution is characterized by the polydispersity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of the polyplexes prepared in HEPES (5 mM, pH 7.4) was determined at 25 °C in a DTS5001 cell using a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential. To study the formation of covalent disulfide bonds in the polyplexes based on TMC-SH and their effect on the stability of the complexes, in some experiments after adding the siRNA solution (200 µl, 40 µg/ml) in HEPES (5 mM, pH 7.4) to polymer solutions (200 µl, various concentrations) in the same buffer, immediately 50 µl of either thiolated (24) or non-thiolated hyaluronic acid (1 mg/ml) solution in HEPES (5 mM, pH 7.4) was added to the complex and incubated for 3 hours at room temperature.

Agarose gel electrophoresis

The complexation of siRNA with the thiolated and non-thiolated TMC as well as the L-glutathione/heparin-induced destabilization of the polyplexes was investigated using agarose gel electrophoresis. The agarose gels (NuSieve® GTG® Agarose, Lonza, Rockland, ME, USA) were made at a concentration of 4% (w/v) in Tris-Acetate-EDTA (TAE) running buffer and contained 0.5 µg/ml ethidium bromide. Polyplexes of TMC's with the siRNA and thiolated or non-thiolated HA made at N/P ratio of 8 were prepared as described above. Naked siRNA was used as control. Polyplex dispersions with or without heparin (1.5 USP/µg siRNA; molar ratio of amine groups of the polymer and sulfate groups of heparin was 0.5 (N/S)) were incubated for 5 minutes at room temperature. In another experiment, polyplex dispersions were incubated for 30 minutes at 37 °C with L-glutathione (5 mM) and subsequently incubated with or without heparin (N/S: 0.5) for 5 minutes at room temperature. Polyplexes (15 µl, corresponding to 15 pmol siRNA) were applied in the starting slot of the gel and electrophoresis was performed at 60 V for 50 minutes. The siRNA bands, stained with ethidium bromide, were detected on a UV transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

Gene silencing and cytotoxicity experiments

The human lung cancer cell line H1299 which expresses firefly luciferase was used to study the gene silencing activity of the TMC formulations. The cell line was maintained in RPMI 1640 medium with HEPES and L-glutamine (PAA laboratories GmbH, Pasching, Austria, catalog No. E15-842) completed with fetal bovine serum (FBS) (final concentration 10% v/v) and cultured at 37 °C at 5% CO_2 humidified atmosphere. The cells (8×10^3 cells/well) were seeded into 96-well plates and cultured overnight. The anti-Luciferase siRNA polyplexes were added to the cells in the presence or absence of serum in the medium and incubated at 37 °C for 24 hours. Then, the medium was removed, and fresh medium was added. Subsequently, the cells were incubated at 37 °C for 1 day, after which the luciferase protein expression was analyzed using Luciferase reporter gene assay (Promega). The cytotoxicity of the polyplexes was measured using the XTT colorimetric viability assay as previously described [36]. Complexes of either specific or non-specific siRNA with

Lipofectamine 2000 were prepared by gently mixing 50 µl siRNA (20 µg/ml) in HEPES (5 mM, pH 7.4) with 3.7 µl Lipofectamine 2000 in 50 µl HEPES (5 mM, pH 7.4) followed by 30 min incubation at room temperature and then were incubated with cells.

Determination of Luciferase activity

Luciferase activity was measured after removal of the growth medium and lysis of the cells by the addition of 100 µL reporter gene lysis buffer. After a freeze/thaw cycle at -80 °C/room temperature, 50 µl of luciferase assay reagent was added to 50 µl of the cell lysate and relative light units (RLU) were measured for 10 s at room temperature using a FLUOstar OPTIMA microplate based multi-detection reader with a microinjector.

Confocal laser scanning microscopy (CLSM)

The cellular uptake of polyplexes was investigated with Confocal Laser Scanning Microscopy (CLSM). In short, 8×10^3 cells were seeded in Fluorodish cell culture dish (WPI, Sarasota, US). The cells were cultured for 24 hours at 37 °C and subsequently incubated with Fluc TYE563-labeled siRNA lipo/polyplexes for 4 hours. Then, the cells were washed with PBS and incubated for 5 minutes with medium containing 40 µM DRAQ5™ for nuclear staining and 75 nM LysoTracker Green DND-26 for endosomal/lysosomal staining. The cells were subsequently analyzed on a Leica TCS-SP confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with three lasers: 488-nm argon, 568-nm krypton, and 647-nm HeNe laser.

Results and Discuaaion

Physicochemical characterization of polyplexes

The average diameter of the polyplexes prepared at different N/P ratios was measured by dynamic light scattering (Figure 2). Both thiolated and non-thiolated TMC were capable of forming complexes with siRNA over the whole N/P range of 0.5-14. The average size of the particles decreased with increasing N/P ratio, with small-sized nanoparticles of $\leq$ 200 nm being formed at N/P ratio $\geq$ 3. This can probably be ascribed to an increased polymer amount in the formulations which leads to tighter

complexes. There was no significant difference in size of the nanoparticles based on thiolated and non-thiolated TMCs. Furthermore, a polydispersity index (PDI) of ≤ 0.3 was observed for all polyplex formulations made at N/P ratio ≥ 3, which indicates a rather narrow size distribution.

At N/P of 0.5, the polyplexes had a negative zeta potential (about -10 to -25 mV), which can be explained by the excess of siRNA present in the complexes. At N/P ≥ 1.5, positively charged particles for the different TMC-SH based formulations (zeta potential +5 to +15 mV) and non-thiolated TMC (+5 to +10 mV) were formed (Figure 2). The average diameter and zeta potential of the particles based on TMC-SH and non-thiolated TMC (N/P: 8) in combination with thiolated hyaluronic acid (HA-SH) and the non-thiolated one (HA) were measured. The particles based on TMC-SH/siRNA/HA-SH had an average diameter 170 ±10 nm and an average zeta potential 10 ± 2 mV whereas the other formulations (with non-thiolated TMC instead of TMC-SH) led to formation of particles ≥1000 nm.

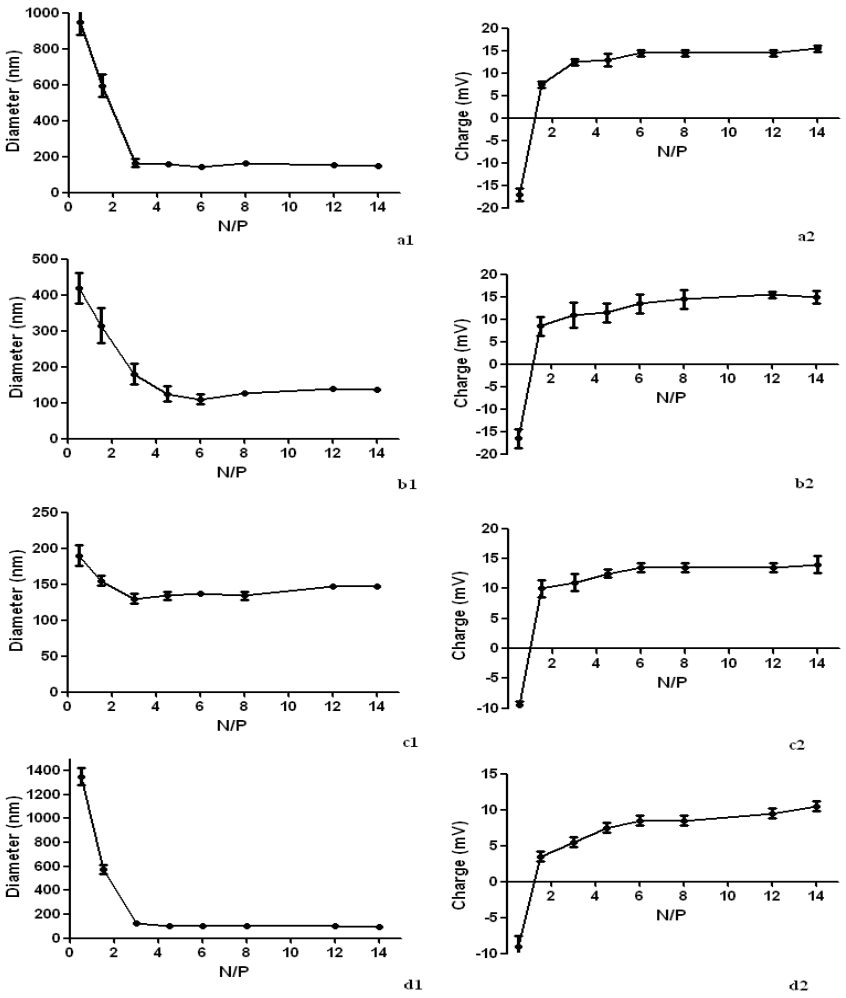

Figure 2. Average diameter and zeta potential of polyplexes prepared at different N/P ratios. a1, a2. TMC-SH 2%; b1, b2. TMC-SH 5%; c1, c2. TMC-SH 7%; d1, d2. Non-thiolated TMC (mean ± standard deviation (n=3)).

Agarose gel electrophoresis

Binding of TMC-SH to siRNA molecules and subsequent complex formation are essential for protection of siRNA against degradation by nucleases. Moreover, formation of covalent disulfide bonds between thiolated molecules likely increases the stability of the particles which

avoids the dissociation of the siRNA from the complex in the extracellular matrix by anionic macromolecules such as proteins and polysaccharides [37,38]. On the other hand, polyplex destabilization still needs to occur in the cytosol to achieve gene silencing due to released siRNA.
TMC-SH and HA-SH as another thiolated polymer were formulated with siRNA and the stability of the formed particles was studied. As control formulations, polyplexes base d on TMC-SH and non thiolated HA and additionally polyplexes based on non-thiolated TMC in combination with HA-SH were prepared.
Figure 3 shows that siRNA remains in the starting slots of the gel for all the polyplexes prepared at N/P=8, indicating that siRNA is stably associated with the cationic TMC. To monitor the sensitivity of these complexes for dissociation and release of siRNA, complexes were incubated with heparin, which is commonly used as a model anionic polyelectrolyte able to dissociate siRNA [39,40]. Figure 3 shows that after incubation of the complexes with heparin, siRNA is released from all complexes, except from those based on TMC-SH/siRNA/HA-SH, demonstrating that siRNA is stably encapsulated in these particles. SiRNA is released from these particles only after incubation with L-glutathione and heparin, which indicates that siRNA can only be released after the disulfide bonds in the particles are cleaved by L-glutathione. These observations indicate an enhanced stability of the polyplexes based on TMC-SH/HA-SH likely due to formation of disulfide bonds between TMC-SH and HA-SH molecules. As a result, release of the siRNA from these complexes is expected to occur in two steps: (1) after cellular internalization of the particles, cleavage of the disulfide bonds between thiolated molecules inside the complex occurs, due to the relatively high concentrations of reducing agenst in the cytosol, and subsequently followed by (2) dissociation of the complexes and release of siRNA is likely due to competition with the negatively charged macromolecules such as proteins and mRNA [41] present in the cytosol.

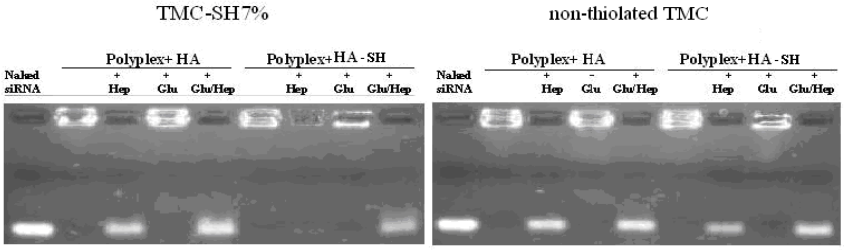

Figure 3. Agarose gel electrophoresis. Polyplexes of TMC-SH and non-thiolated TMC with siRNA made in 5 mM HEPES buffer (pH 7.4) at N/P ratio 8, containing either non-thiolated hyaluronic acid (HA) or HA-SH were applied on a 4% agarose gel. By adding heparin (N/S=0.5, N is the moles of cationic nitrogens in the polymer and S is moles of sulphate in siRNA) release of siRNA was observed for all complexes, except for those based on TMC-SH/siRNA/HA-SH, which is probably due to disulfide bond formation in the complex. Incubation of the complexes with L- glutathione (5 mM) for 30 minutes at 37° C did not lead to release of the siRNA Complexes based on TMC-SH/siRNA/HA-SH only showed release of the siRNA after incubation with L- glutathione and heparin sequentially.

Silencing activity and cell viability

Human lung cancer cells (H1299) expressing luciferase were used for gene silencing studies. SiRNA polyplexes based on thiolated and non-thiolated TMC polymers prepared at N/P ratios of 8 were selected for silencing studies, because these particles showed binding to the siRNA (Figure 3), and upon complexation had a positive mean zeta-potential and small average size (Figure 2). TMC-SH with highest degree of thiolation (7%) was selected for the silencing/cytotoxicity experiments, because the most stable particles can be expected using this polymer. The silencing activity and cytotoxicity of these complexes were compared to those based on Lipofectamine 2000 and in all cases the silencing results were corrected for cytotoxicity.

Figure 4a shows that increasing the dose of the TMC-SH polyplexes from 3 to 20 pmol siRNA content per well, resulted in a significant enhancement of the silencing activity from 10% to above 80%, whereas doses higher than 20 pmol siRNA led to a gradual decrease in silencing activity of the complexes due to the increased cytotoxicity (Figure 4b). In case of the non-thiolated TMC, by increasing the dose of the siRNA, a

gradual increase in silencing activity from 5% up to 40% was observed. In terms of silencing activity and cytotoxicity, the efficiency of TMC-SH polyplexes in gene silencing is substantially higher than those based on Lipofectamine 2000 (approximately 60% at a dose of 20 pmol siRNA) and non-thiolated TMC (approximately 30% at a dose of 20 pmol).
Figure 4b shows that the cytotoxicity of TMC-SH/siRNA formulation is lower than that of the standard transfection system Lipofectamine 2000. In addition, TMCs have substantially lower cytotoxicity than other cationic polymers [42,43]. Based on these results, a dose of 20 pmol siRNA per well was selected as dose for further gene silencing studies.

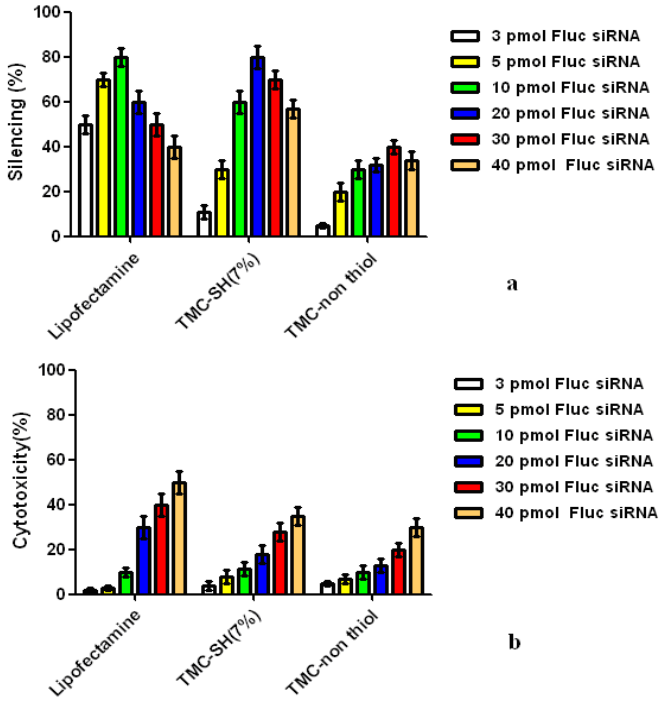

Figure 4. a. Dose response effect of the siRNA complexes in Luciferase gene silencing after incubation with H1299 cells for 24 h in serum-free medium. **b.** Cell viability as measured by XTT assay after incubation of H1299 cells for 24 h with siRNA complexes in serum-free medium (mean ± standard deviation (n=3)).

Figure 5a shows that in the absence of serum, incubation of cells with polyplexes based on TMC-SH polymers containing 2, 5 and 7% thiol groups, resulted in 60-80% silencing activity, whereas incubation with non-thiolated TMC complexes showed about 40% gene silencing. These results show that by introduction of thiol groups in TMC structure, the gene silencing activity of the polyplexes is substantially increased. This might be due to the increased stability of the polyplexes of thiolated TMC. The differences in gene silencing activity of the lipofectamine formulations in the absence and presence of serum are small (~60 and ~70%, respectively). It has been reported that the *in vitro* transfection activity of comyplexes increases once they aggregate in serum, due to more intense contact with cells [44]. In the presence of 10% FBS, gene silencing activity of the siRNA polyplexes based on TMC-SH polymers decreased to 45%, whereas the gene silencing activity of the non-thiolated TMC remained the same as serum free conditions. This reduction in silencing effect might be partly explained by the inhibitory effect of serum proteins on the uptake of nanoparticles (Figure 5a) [45].
Figure 5b shows low cytotoxicity of both thiolated and non-thiolated TMC/siRNA formulations made at an N/P ratio of 8 (<20%) compared to Lipofectamine with a cytotoxicity about 30%. Moreover, in the presence of serum, the cytotoxicity of the polyplexes was slightly lower than that of the complexes incubated with the cells in medium without serum. This effect is in line with the literature suggesting that serum proteins can mask the cytotoxicity of cationic polyplexes [46,47]. No significant differences in cytotoxicity of thiolated and non-thiolated TMCs were observed (Figures 4b and 5b).

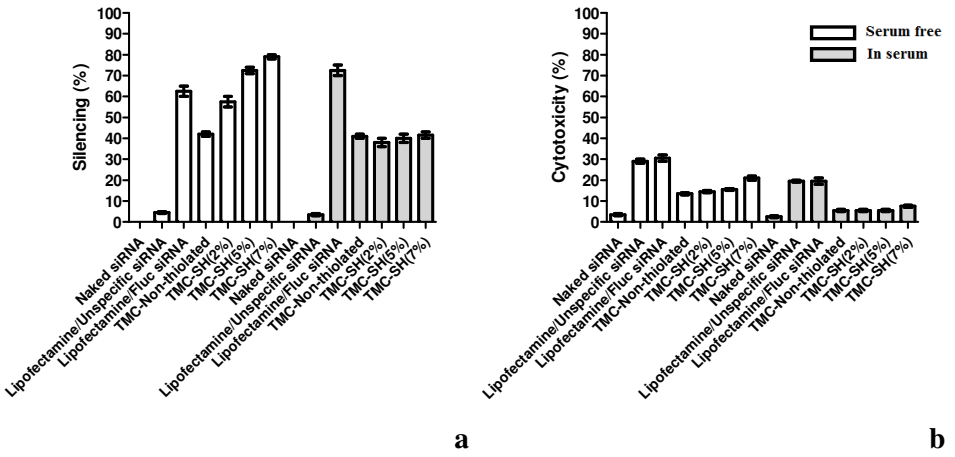

Figure 5. a. Luciferase gene silencing after incubation of H1299 cells with siRNA complexes in serum free medium and presence of serum. **b**. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum free medium and presence of serum (mean ± standard deviation (n=3)).

The gene silencing efficiency of the polyplexes based on thiolated and non-thiolated TMC polymers was further investigated in the presence of hyaluronic acid (HA) which is an polyanionic macromolecule present in biological fluids that has shown to inhibit the transfection activity of pDNA/cationic polymer nanoparticles [48,49].
In the presence of 0.5 mg/ml HA, TMC-SH/siRNA complexes showed up to 60% silencing activity, whereas the silencing activity of non-thiolated TMC polyplexes was less than 10%. In the presence of both HA and serum in the growth medium, the silencing activity of the TMC-SH polyplexes was decreased to 40%, whereas non-thiolated TMC polyplexes hardly showed any silencing (Figure 6a). These results illustrate the key role of disulfide bonds present in the TMC-SH polyplexes which enhance the stability of the complexes against competitor macromolecules present in biological fluids.

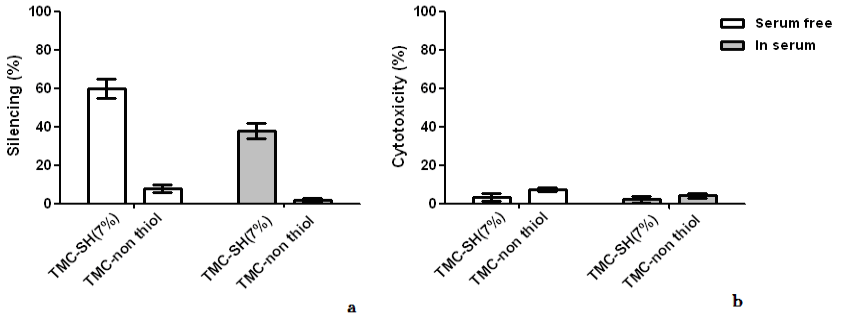

Figure 6. a. Effect of hyaluronic acid (HA) (0.5 mg/ml) on Luciferase gene silencing activity of siRNA complexes based on TMC-SH(7%) and non-thiolated TMC. **b**. Effect of HA on cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes (mean ± standard deviation (n=3)).

Furthermore, the silencing activity of the polyplexes based on TMC-SH and non-thiolated TMC in combination with either HA-SH or non-thiolated HA was studied. In the absence and presence of serum in the growth medium, siRNA complexes based on TMC-SH/HA-SH showed >50% gene silencing activity (Figure 7a) whereas the gene silencing activity of the other formulations is ≤ 20% (Figure 7a). These results are in line with Figure 3, indicating an better stability of the polyplexes based on TMC-SH/HA-SH due to formation of disulfide bonds between TMC-SH and HA-SH molecules which consequently results in a higher gene silencing activity of this formulation compared to the other formulations. The XTT cell viability assay showed low cytotoxicity of the studied complexes (Figure 7b).

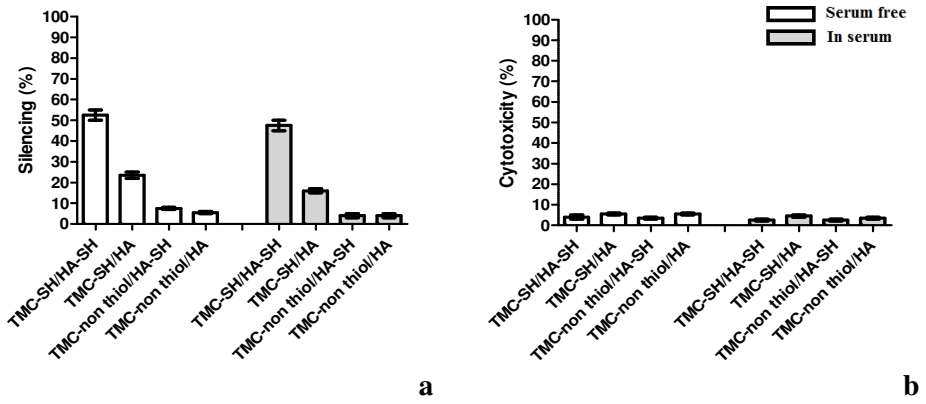

Figure 7. a. Luciferase gene silencing after incubation of H1299 cells for 24 h with siRNA complexes in serum free medium and presence of serum. **b**. Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum free medium and presence of serum (mean ± standard deviation (n=3)).

Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to study the cellular uptake and internalization of siRNA complexes using fluorescently labeled siRNA. Figure 8 shows that red spots originating from the labeled siRNA (images a and d) were intracellularly observed after incubation of the cells with the various siRNA formulations investigated in this study, demonstrating that the complexes were internalized. The images of cells treated with Lipofectamine/siRNA complexes do visualize presence of siRNA into the both cytosol and nucleus, in agreement with previous observations [23,50]. In the case of TMC-SH 7%- and TMC-SH 5%-based complexes treated cells, the intensity of the internalized siRNA (images a and d) is considerably higher than in case of the other groups, which is in agreement with the gene silencing results (Figure 5a). Furthermore, in the case of TMC-SH 7%, a diffuse pattern of the siRNA inside the cytosol is observed, while the lipofectamine and TMC-SH 5% treated cells mostly show a punctuate pattern of the siRNA. The yellow dots in panel d represent the co-localization of the siRNA with fluoresecent markers for endosomes/lysosomes. We speculate that these observations reflect the

enhanced level of cellular uptake for the complexes based on TMC-SH with higher extent of thiol modifications (5% and 7%) which might be related to their enhanced muco-adhesive properties [27,28] and stability in the extracellular environment.

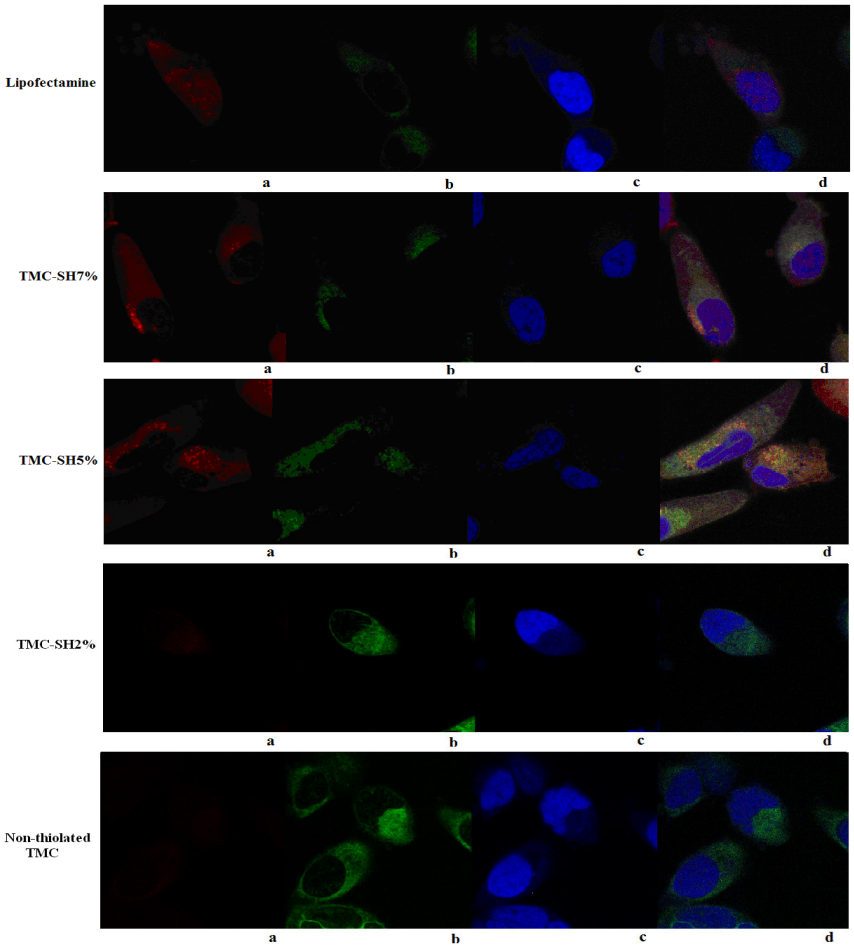

Figure 8. Confocal laser scanning microscopy images of H1299 cells incubated with complexes prepared with TYE563-labeled siRNA.
Images **a** : Pattern of intracellular TYE563-labeled siRNA,
Images **b**: Endosomes/Lysosomes stained with LysoTracker Green DND-26
Images **c**: Nuclei of cells stained with DRAQ5™,
Images **d**: Blue: Nuclei of cells, Green: Endosomes/Lysosomes, Red: Free or complexed siRNA inside the cytoplasm, Yellow: Free or complexed siRNA entrapped inside the endosomes/lysosomes.

Conclusion

This study reports on TMC-SH as a promising vector for siRNA delivery. As compared to the non-thiolated TMC and Lipofectamine-based complexes, the gene silencing activity of the siRNA complexes based on thiolated TMC was substantially higher. Importantly, the polyplexes based on TMC-SH retained their silencing activity in the presence of hyaluronic acid, while non-thiolated TMC polyplexes hardly showed any silencing activity. Furthermore, the cytotoxicity profile of TMC-SH and non-thiolated TMC were the same and more favorable compared to Lipofectamine. The results of this study suggest an enhanced stability of the TMC-SH polyplexes, which is likely due to formation of the reducible disulfide bonds in the complex. These features make TMC-SH an attractive candidate for further development and evaluation in animal studies.

Acknowledgment

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Chapter 7

A Preliminary Study on the Anti-inflammatory Properties of Polyplexes Based on Anti-TNF-α siRNA and Biodegradable Polymers in a Murine Rheumatoid Arthritis Model

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Abstract

In this chapter, initial findings are reported on the in vivo application of selected polyplex systems for the treatment of rheumatoid arthritis (RA). In the collagen antibody-induced arthritis (CAIA) mouse model, we observed that polyplexes containing siRNA, either specific to silence TNF-α or nonspecific, are able to diminish inflammation in the joints. However, further studies are needed to elucidate the mechanisms behind the observed effects.

Introduction

The large majority of the work presented in this thesis is concerned with the design and biopharmaceutical characterization of delivery complexes containing reporter nucleic acids. As a start to translate this work to therapeutic applications, this chapter reports on initial findings on the application of selected polyplex systems for the treatment of rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that may attack several organs and tissues, especially the synovial joints where inflammation of the synovium (synovitis) with increased synovial fluid and development of pannus in the synovium occurs [1,2]. Macrophages play a key role in RA. They are numerously present in the inflamed synovial membrane and at the cartilage-pannus junction and show clear signs of activation by over-expression of pro-inflammatory and regulatory cytokines and growth factors. Tumor necrosis factor-α (TNF-α), is one of the inflammatory cytokines produced by macrophages which is involved in synovial damage [3,4]. Therefore, local delivery of anti-TNF-α therapeutics represents a promising approach to achieve a reduction in joint inflammation.

Recently, considerable research efforts have been dedicated to the use of small interfering RNAs (siRNA) as novel biotherapeutics [5]. These double-stranded RNA molecules are rapidly degraded by nucleases present in biological fluids and are not able to enter the cytoplasm of target cells via passive diffusion, where the RNA interference (RNAi) machinery is located [6,7]. Therefore, a key challenge to the effective and widespread use of this new class of biotherapeutics is their cytosolic delivery [8,9,10].

Cationic polymers have been studied for nucleic acid delivery both *in vitro* and *in vivo* [11,12,13]. Due to their positive charge, these polymers are able to complex with anionic nucleic acid molecules to form so called polyplexes which are able to cross cellular barriers and reach their intracellular targets [14,15]. The search for effective and non-toxic delivery systems is currently of prime interest [16,17].

In the present study, 5 different polymers: thiolated N,N,N-trimethylated chitosan (TMC-SH) [18,19,20], pHPMA-DMAE and pHPMA-MPPM [21], PLGA [22,23] and dex-MA nanogels [24,25] have been investigated for *in vivo* anti-TNF-α siRNA delivery and associated anti-inflammatory effects in inflamed joints.

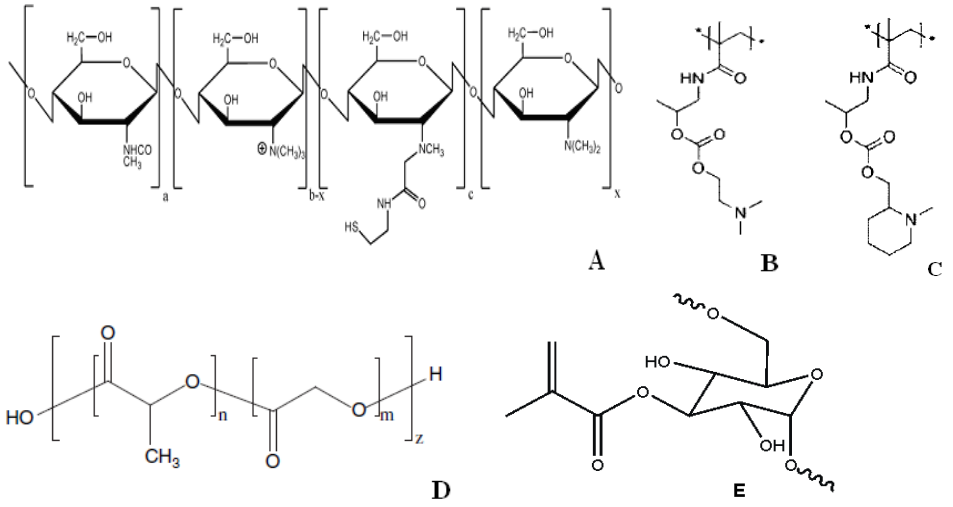

Figure 1. Structure of thiolated N,N,N-trimethylated chitosan (TMC) (A), pHPMA-DMAE (poly(2-(dimethylamino)ethyl (1-methacrylamidopropan-2-y1) carbonate)) (B), pHPMA-MPPM (poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol)) (C), PLGA (poly (lactic-co-glycolic acid)) (D) and dex-MA (dextran methacrylate) (E).

Materials and Methods

Materials

The double-stranded siRNA which specifically targets mouse TNF-α (structure below), and non-specific siRNA were obtained from Integrated DNA Technologies (IDT) (Germany).
Sense strand: 5´- pGUCUCAGCCUCUUCUCAUUCCUGct
Antisense strand: 3´- UACAGAGUCGGAGAAGAGUAAGGACGA
TMC-SH [18], pHPMA-MPPM, pHPMA-DMAE [21], PLGA nanoparticles [22,23] and PEGylated dextran nanogels [24,25] were synthesized, purified and characterized as described previously. ArthritoMab, a monoclonal antibody cocktail plus lipopolysaccharide (LPS) was obtained from MD Biosciences GmbH (Zurich, Switzerland). PLGA (lactide:glycolide molar ratio: 75:25, Mw: 20 kDa) was purchased from Wake Pure Chemical Industries, Ltd. (Osaka, Japan).

Polyvinylalcohol (PVA403) with 80.0% of degree of hydrolysis was provided by Kuraray (Osaka, Japan).

Preparation and physicochemical characterization of polyplexes

Polyplexes based on TMC-SH, pHPMA-MPPM and pHPMA-DMAE were prepared at a polymer to siRNA ratio of 12 (expressed as N/P ratio, where N is the moles of cationic nitrogens in the polymer and P is moles of phosphate in siRNA) in a total volume of 20 µl containing 10 µg siRNA. In brief, 10 µl siRNA solution (1 mg/ml) in HEPES buffer (5 mM, pH 7.4) was added to a polymer solution (10 µl) in the same buffer. The resulting dispersions were vortexed for 10 seconds and incubated at room temperature. The polyplexes were ready to use after 3 hours in case of the TMC-SH-based polyplexes and after 30 min in case of the pHPMA-DMAE- and pHPMA-MPPM- based polyplexes.
The PLGA nanoparticles were prepared by a double emulsion solvent evaporation (DESE) method as reported previously [22] with a few minor changes. In brief, 125 µl of 85.5 µM TNFα siRNA in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added to 250 µl of a DOTAP/PLGA solution in chloroform with a concentration of DOTAP and PLGA of 60 mg/ml and 15% (w/w), respectively. The mixture was sonicated for 90 s to obtain a water-in-oil (w_1/o) emulsion. A volume of 1 ml 2% (w/v) PVA in diethylpyrocarbonate (DEPC)-treated water was added to the emulsion and sonication of 60 s was performed, resulting in a water-in-oil-in-water (w_1/o/w_2) double emulsion. The double emulsion was subsequently diluted with 5 ml of 2% (w/v) PVA in DEPC-treated water and left with agitation overnight to evaporate the chloroform. The nanoparticles were collected by centrifugation at 18,000 x g for 12 min at 4 ºC. The supernatant was discarded, and the pellet containing the nanoparticles was re-dispersed in DEPC-treated water. Centrifugation and re-dispersion of the pelleted nanoparticles were repeated three times to ensure removal of the PVA. Next, the nanoparticles were freeze dried in a 200 mM trehalose solution employing parameters described previously [23]. To check the encapsulation efficiency of the particles, the siRNA was extracted from the PLGA/DOTAP nanoparticle matrix by dissolving a known fraction of the nanoparticle suspension, corresponding to app. 1 mg of nanoparticles, in 200 µl of chloroform followed by addition of 500 µl of TE buffer. The mixture was rotated for 90 min at room temperature to facilitate the extraction of siRNA from the organic phase and into the aqueous phase.

After incubation, the two phases were separated by centrifugation at 4 ºC and 18,000 x g for 20 min, and the aqueous supernatant was collected and incubated at 37 ºC for 5 min to allow for evaporation of residual chloroform. The concentration of siRNA in the supernatant was determined using the RiboGreen® RNA reagent according to the manufacturer's instructions applying a FLUOstar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Each sample was assayed in triplicate, and the result was corrected according to the total yield of nanoparticles resulting in a value for the encapsulation efficiency not affected by the yield of nanoparticles. The encapsulation efficiency (EE) of the tested formulation is described as the amount of encapsulated siRNA relative to the theoretical siRNA loading, corrected for any loss of nanoparticles during production and purification, according to Eq :

$$EE = \frac{\frac{m_{siRNA(encapsulated)}}{m_{siRNA(total)}} \times 100\%}{Yield}$$

For physicochemical characterization and injection into animals, the nanoparticles were dispersed in HEPES (5 mM, pH 7.4) to achieve a final siRNA concentration of 0.5 mg/ml.
Cationic dextran nanogels were prepared from dextran methacrylate (dex-MA), [2 (methacryloyloxy)ethyl]trimethylammonium chloride (TMAEMA) and 2-aminoethyl methacrylate hydrochloride (AEMA) using inverse mini-emulsion photopolymerization as described earlier [24,25]. The resulting cationic nanogels of about 200 nm were lyophilized and stored in a desiccator at room temperature. A weighed amount of the lyophilizate was dispersed in a known volume of 5 mM HEPES buffer at pH 7.4 and exposed to an ultrasound to disintegrate loose particle aggregates formed during lyophilization (Branson Tip Sonifier, 10 s, amplitude 10%). The nanogels were loaded by adding 10 nmol anti-TNF α siRNA per mg nanogel. This siRNA/nanogel ratio resulted in positively charged nanogels as described earlier [24,25]. The nanogels were consecutively PEGylated by adding 5 mg NHS-PEG/mg nanogel N-hydroxysuccinimidyl activated methoxypolyethylene glycol 5000 propionic acid (NHS-PEG, Sigma, Belgium) for 30 min.

Z-average diameter of the polyplexes was measured with dynamic light scattering at 25 °C with a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern, UK). Viscosity and refractive index values of water at 25 °C were used. Calibration was done with an aqueous dispersion of polystyrene particles with a diameter of 100 nm. Particle size distribution is characterized by the polydispersity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of the polyplexes prepared in HEPES (5 mM, pH 7.4) was determined at 25 °C in a DTS5001 cell using a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential.

Animal experiments

The collagen antibody-induced arthritis (CAIA) mouse model was used in this study [26]. Balb/c mice (5-7 weeks old) were obtained from Charles River (Maastricht, The Netherlands). The CAIA model was induced according to Khachigian et al [26], by IV injection of 200 µl ArthritoMab (day 0) and i.p. injection of 50 µg LPS in water (day 3). The animals were injected intra-articularly (2 elbows and 2 knees) with the polyplex formulations (containing 10µg siRNA) 2 times (at day 3 and day 5 after injection of LPS) (Table 1). In each group (n=8), 5 animals were treated with polyplexes containing anti-TNF-α siRNA and 3 animals treated with polyplexes containing nonspecific siRNA.

Table 1. In vivo study groups (n=8)

Group	Treatment
1	No treatment (only Antibody and LPS)
2	Free siRNA
3	SiRNA-pHPMA- DMAE nanoparticles
4	SiRNA-pHPMA- MPPM nanoparticles
5	SiRNA-TMC-SH nanoparticles
6	SiRNA-Nanogel nanoparticles
7	SiRNA-PLGA nanoparticles

For intra-articular injections, the animals were anaesthetized with 2.5% isoflurane and siRNA polyplexes were injected at a dose of 10 µg siRNA. At day 7, the therapeutic effects of the complexes in different treatment

groups were evaluated, using an arthritic scoring scale (Table 2) [26]. Based on this scale, the severity of arthritis in each limb is scored on a 0-4 scale. The final score of arthritis in each animal was reported as the sum of arthritis scores in 4 limbs (0 as minimum and 16 as maximum).
The data were analyzed using one-way Anova with a Dunnett's post-test. Afterwards, the animals were sacrificed with cervical dislocation and the limbs were cut and kept in formalin for future investigation.

Table 2. Scale for scoring severity of arthritis [26]

Severity of arthritis in each limb may be scored in a blinded manner on a 0-4 scale, as follows: 0 = normal 1 = mild redness, slight swelling of ankle or wrist 2 = moderate swelling of ankle or wrist 3 = severe swelling, including some digits, ankle and foot 4 = maximally inflamed

Results and Discussion

The average diameter and average zeta potential of the polyplexes were determind. All polymers used in this study were capable of forming complexes with siRNA with a mean size ranging from 150 to 250 nm and a positive zeta potential from 4 to 18 mV (Table 3).

Table 3. Mean size and zeta potential of the polyplexes (mean ± standard deviation (n=3))

polymer	mean size	zeta potential
pHPMA-DMAE	185 ± 8	16 ± 2
pHPMA-MPPM	170 ± 6	18 ± 2
TMC-SH	150 ± 4	14 ± 2
PLGA	254 ± 3	15 ± 1
Nanogel	182	+ 4

Figure 2 shows that the collagen antibody-induced arthritis (CAIA) in Balb-c mice resulted in a score of 6.8 ± 0.6 (out of 16) as maximum level of arthritis. After treatment of the mice with siRNA complexes, a

significantly lower arthritis score in treated mice in comparison to untreated ones was observed, which shows the efficacy of the siRNA complexes in diminishing the inflammation in the joints. The maximal reduction of the arthritis score (to a level between 0.5 - 2) was reached by treatment with siRNA formulations based on pHPMA-MPPM, PLGA and dextran nanogel. However, these effects are observed for both formulations containing specific and nonspecific siRNA. Remarkably, in this initial study, only treatment with free siRNA showed anti-inflammatory activity, whereas nonspecific free siRNA did not show significant anti-inflammatory activity. Therefore, the results suggest that nonspecific siRNA has significant anti-inflammatory activity only when it is used in complex with the carrier systems. Sequence-independent silencing activity of siRNA has been reported in several studies [27-29].

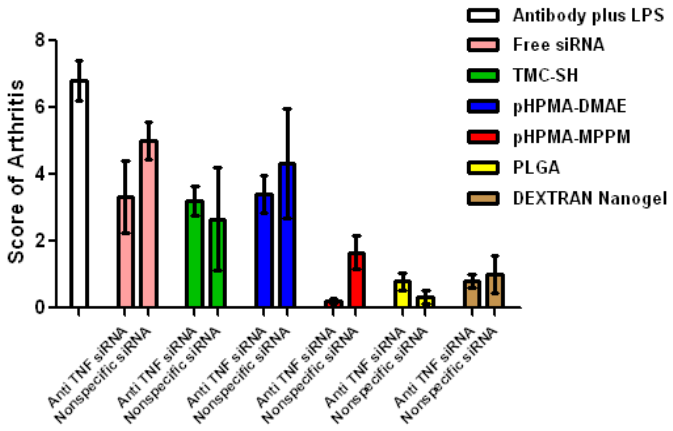

Figure 2. Scores of arthritis in collagen antibody-induced arthritis (CAIA) mice, after treatment with free siRNA or siRNA complexes. The score of RA for each limb ranges from 0 to 4 and the final score of arthritis in each animal has been reported as the sum of arthritis scores in 4 limbs (0 as minimum and 16 as maximum).

Therefore, it can be concluded that polyplexes containing siRNA, either specific to silence TNF-α or nonspecific, are able to diminish inflammation in joints of CAIA mice. However, further studies are needed to elucidate the mechanisms behind the observed effects. First of all, dose

response studies should be carried out. After performing the dose response studies, those polyplexes with superior anti-inflammatory properties can be identified. Secondly, also the question why nonspecific siRNA is anti-inflammatory in complexed form should be addressed. Then, when both these issues have been satisfactorily dealt with, the question why polyplexes are superior over free siRNA can be approached by determination of the targeting efficiency and pharmacokinetic properties *in vivo*.

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Chapter 8

Summary
and
Future Perspectives

Summary

Development of carrier systems with controllable physicochemical and delivery properties has opened up the possibility of nanomedicines containing nucleic acids. In the last decades, much effort has been dedicated to two exciting approaches in biomedicine, namely gene and RNA interference (RNAi)-based therapeutics. These two approaches have faced many hurdles as far as delivery issues are concerned, as an efficient cytosolic delivery of siRNA and nuclear delivery of plasmid DNA is necessary for RNAi and gene therapy, respectively [1]. Nowadays, considerable research is ongoing to utilize RNAi as a new therapeutic approach, using small interfering RNAs (siRNAs) which can be specifically which designed to reduce the expression of specific genes [2]. Since the RNAi machinery is located in the cytoplasm, siRNA molecules just need to be delivered into the cytoplasm which represents a substantial advantage, when compared to the requirement of nuclear delivery of plasmid DNA [3,4].

As described in **Chapter 1,** several viral and nonviral delivery vectors have been designed and used for nucleic acid delivery *in vitro* and *in vivo*. Viral vectors such as adenoviruses, retroviruses and adeno-associated viruses are efficient in gene delivery. However, due to limited loading capacity, potential of immunogenicity, oncogenesis, and other safety issues, synthetic nonviral vectors such as polymers, lipid carriers and carbon nanotubes (CNTs) are nowadays being developed. Figure 1 gives an overview of the chemical structures of the biodegradable and non-biodegradable carrier systems studied in this thesis.
As discussed in the literature study presented in **Chapter 2**, most of the biotherapeutics such as DNA and siRNA containing complexes enter cells via the endocytic pathway, a major uptake mechanism of cells. These complexes become entrapped in endosomes and subsequently are degraded by certain enzymes present in the lysosomes [5] limiting their efficacy. Therefore, different mechanisms of endosomal escape have been exploited to facilitate the endosomal release of the therapeutic complexes. The mechanisms of endosomal escape which have been introduced in chapter 2 are: 1. pore formation, 2. pH buffering effect (the proton sponge effect), 3. fusion with the endosomal membrane and 4. photochemical destabilization of the endosomal membrane. Based on these mechanisms, several approaches such as photochemical internalization (PCI) or the use

of endosomolytic peptides derived from different sources such as viruses, bacteria, humans and plants, as well as their synthetic biomimetic versions and also chemical agents have been applied to promote endosomal escape. PCI, which has been applied *in vitro* and *in vivo*, is an efficient established technique for endosomal escape and is based on using photosensitizers which destabilize the endosomal membrane after illumination, resulting in cytosolic release of the endosomal contents [6,7]. Most of the virus-derived peptides such as influenza-derived ones (diINF-7 and HA2) and adenoviral peptides (e.g. penton-base) destabilize the endosomal membrane through their fusogenic activity, while the bacteria-derived peptides such as listeriolysin O, Pseudomonas aeruginosa exotoxin A and Diphtheria toxin induce pore formation in the endosomal membrane [8,9]. In case of synthetic chemical compounds like cationic polymers, endosomal escape is mostly mediated by the so called proton sponge effect [10]. It should be realized though, that in many cases the exact mechanism of endosomal escape is unclear yet. In view of safety, synthetic biomimetic agents that mimic the viral/bacterial peptides are preferred to facilitate endosomal escape. They have also other advantages over those isolated from biological sources like possibility to control their amino-acid sequence, hydrophobicity and peptide length. Moreover, as fusion is a natural process in human cells, natural human fusogenic proteins/peptides (e.g. FGFR3) are also attractive candidates for promoting endosomal escape.

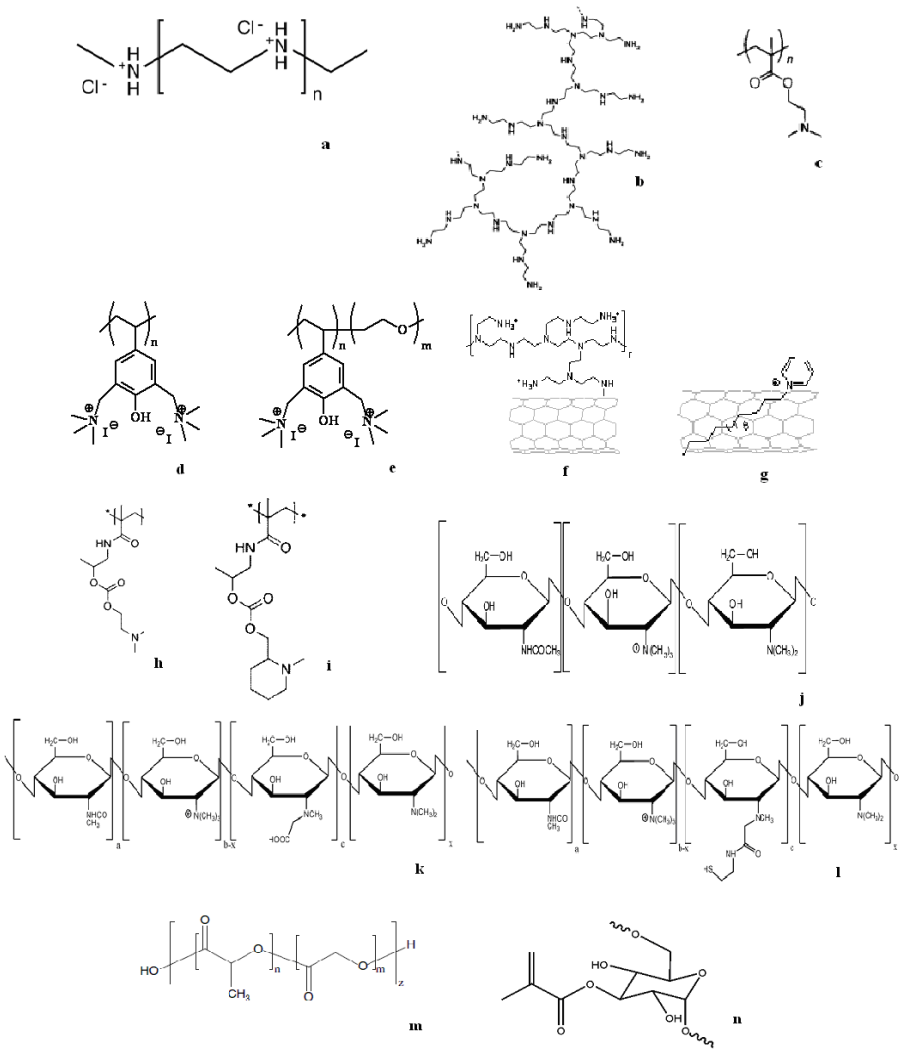

Figure 1. Chemical structures of the carriers used in this thesis. a. Linear PEI (poly(ethylenimine)), b. Branched PEI, c. pDMAEMA (poly(2-dimethylaminoethyl methacrylate)), d. QNPHOS (quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxy styrene]), e. QNPHOS-PEG block copolymer, f. Carbon Nanotube-PEI (CNT-PEI), g. CNT-pyridinium, h. pHPMA-DMAE (poly(2-(dimethylamino)ethyl (1-methacrylamidopropan-2-y1) carbonate)), i. pHPMA-MPPM (poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol)), j. TMC (N,N,N-trimethylated chitosan), k. carboxylated TMC (non-thiolated), l. TMC-SH (thiolated TMC), m. PLGA (poly (lactic-co-glycolic acid)), n. dex-MA (dextran methacrylate).

In **Chapter 3,** two functionalized carbon nanotubes (CNTs) were studied as delivery systems for siRNA delivery. Because of the nano-needle structure of CNTs, they have been proposed to cross the plasma membrane and to translocate directly into cytoplasm of target cells, utilizing an endocytosis-independent mechanism without inducing cell death [11,12]. If this interesting new mechanism is applicable to a great variety of target cell types, this endocytosis-independent cell entry mechanism of CNTs is a valuable advantage. However, there are indications for an endocytosis-dependent cell entry of CNTs as well. CNTs are insoluble in water but become dispersible after functionalization with positively charged groups at their surface which also enables them to bind negatively charged molecules such as siRNA or DNA through electrostatic interactions [13,14].
CNTs functionalized with the cationic polymer PEI (polyethylenimine) (CNT-PEI) and those functionalized with cationic pyridinium were both able to complex with negatively charged siRNA. The silencing activity and the cellular cytotoxicity of siRNA complexes based on these functionalized CNTs were compared with those based on the regularly used lipidic transfection agent Lipofectamine 2000 and the well-known polymeric transfectants PEI and pDMAEMA. In the absence of serum, incubation of cells with the siRNA complexes based on CNT-PEI showed up to 20% silencing activity, whereas incubation with PEI polyplexes showed a similar (20-30%) degree of gene silencing. The results of the XTT cell viability assay showed higher cytotoxicity of CNT-PEI-based complexes than those based on PEI, which suggests that CNT enhances the cytotoxicity of PEI. By increasing the dose of the siRNA complexes 3 fold, no significant enhancement in gene silencing activity of CNT-PEI and PEI-based complexes was observed whereas the cytotoxicity of the CNT-PEI based complexes increased up to 40%. It was observed that the gene silencing activity of the CNT-PEI based complexes (20%) was considerably lower than that of complexes based on Lipofectamine 2000 (60%) and pDMAEMA (50%). These results show that there is no added value of CNT-PEI over PEI and the reference transfectants Lipofectamine 2000 and pDMAEMA. In the absence of serum, incubation of cells with the CNT-pyridinium/siRNA complexes showed about 30% silencing activity a relatively high cytotoxicity of about 60%. By increasing the dose of the siRNA complexes from 10 to 30 pmol/well, no significant enhancement in the gene silencing activity of CNT-pyridinium based

complexes was observed whereas the cytotoxicity of the complexes increased up to 70%, which is probably due to the increased amount of the CNT-pyridinium in the growth medium. Cetylpyridinium in different concentrations (corresponding with its concentrations in siRNA complexes incubated with cells) showed ≤ 5% cytotoxicity. Obviously, the cytotoxicity of the CNT-pyridinium/siRNA complexes is substantially higher than that of the same dose of cetylpyridinium. These results reveal high cytotoxicity of the CNT-pyridinium/siRNA complexes and limited silencing activity, with no added value of CNT-pyridinium over Lipofectamine 2000, pDMAEMA and PEI.

In several studies the potential in vitro and in vivo toxicities of CNTs have been discussed and attributed to various factors such as, amongst others, length of the tubes, type of functionalization, dosage, duration of exposure, cell type, route of administration and tissue distribution. Still, most aspects regarding CNT toxicity remain uncertain [15-17]. Despite our disappointing results obtained with the two functionalized CNT types, other literature reports encourage further nucleic acid delivery studies with other types of functionalized CNTs. Probably, the type of functionalization of carbon nanotubes might be a key parameter to obtain an efficient and non-cytotoxic CNT-based delivery system. Nevertheless, in view of the present results and importantly also of the non-degradability of CNTs, preference should currently be given to designing biodegradable carriers which mimic the needle structure of CNTs.

Chapter 4 introduces a cationic polymer with two cationic sites per monomer unit (quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxy styrene] (QNPHOS). Also, its block copolymer with PEG was studied for nucleic acid delivery. The stability of the therapeutic complexes in extracellular matrices and biological fluids, such as blood, is an important issue [3,18]. We expected that the stronger the electrostatic interactions between cationic polymers and nucleic acids, the higher the stability of the polyplexes in biological fluids be. Therefore, in **Chapter 4**, the QNPHOS was used as a model polymer to investigate the hypothesis whether the presence of two charges per monomer unit can enhance nucleic acid binding and delivery properties in biological fluids. In this study, the nucleic acid binding capability of QNPHOS and QNPHOS-PEG, the gene silencing (with siRNA) and transfection (with plasmid DNA) activities of the formed polyplexes in comparison to the well-known transfectant pDMAEMA (poly(2-(dimethylamino)ethyl methacrylate)) were studied. The results showed the superior stability of

the siRNA/DNA polyplexes based on QNPHOS and QNPHOS-PEG over those based on pDMAEMA which might be as hypothesized due to the presence of two permanently charged nitrogen groups per monomer unit. It was shown that the QNPHOS- and QNPHOS-PEG-based polyplexes had a higher gene silencing activity and a substantially better cytocompatibility (toxicity ≤20%) than those based on pDMAEMA (toxicity up to 50%). It was concluded that formation of strong siRNA complexes is indeed advantageous, since it enhances the stability of polyplexes in biological fluids, contributing to an improved siRNA delivery resulting in a higher gene silencing activity when compared to pDMAEMA based systems. On the other hand, QNPHOS and QNPHOS-PEG formed too stable polyplexes with DNA with limited intracellular nucleic acid release, resulting in a lack of gene expression. Importantly, it was demonstrated that a high charge density of polymers is not necessarily accompanied by a higher cytotoxicity.

Considering the potential toxicity issues related to the use of non-degradable delivery systems, we switched in **Chapter 5** to biodegradable polymers for siRNA delivery. In this chapter, two biodegradable polymers were characterized and applied for siRNA delivery in vitro: 1. pHPMA-MPPM which contains a linker that is stable at pH 5 (pH in endo/lysosome) but that is degraded at pH 7. Further, TMC (O-methyl free N,N,N- trimethylated chitosan) which is a well defined and enzymatically degradable polymer [19,20] was investigated as carrier to deliver siRNA. These gene silcencing activity and toxicity of polyplexes based on these polymers were compared with those based on the well-known non-degradable polymeric transfectants pDMAEMA and branched PEI, and the cationic lipid Lipofectamine. All studied polymers were able to form small particles with an average size around 100-150 nm. In the absence of serum, incubation of cells with polyplexes based on pHPMA-MPPM and TMC polyplexes showed 30-40% gene silencing activity. Incubation with pDMAEMA-based polyplexes resulted in up to 50% silencing activity,whereas incubation with polyplexes based on branched PEI led to 25-30% silencing of luciferase. The activity of biodegradable polyplexes was comparable with the Lipofectamine formulation (60% silencing efficiency). The relatively low silencing activity of branched PEI/siRNA polyplexes can likely be explained by the strong binding of PEI to the siRNA which prevents the liberation of the siRNA. Importantly, the gene silencing activity of polyplexes was not affected by serum proteins. By application of photochemical internalization (PCI)

which is a technique for endosomal escape and described in chapter 2, the gene silencing activity of the pHPMA-MPPM and TMC polyplexes increased from 30-40% to 70-80%. This means that the endosomal escape properties of these polymers are limited. Application of PCI, only slightly increased the silencing efficiency of the pDMAEMA polyplexes which confirms previous findings that pDMAEMA polyplexes have intrinsic endosomal escape properties. Besides by PCI, endosomes can be destabilized by endosome-disruptive peptides. Therefore, we studied the effect of the endosome disruptive diINF-7 peptide which was introduced in chapter 2, on the gene silencing activity of pHPMA-MPPM polyplexes. At pH 7, diINF-7 is negatively charged and therefore binds via electrostatic interactions to the positively charged surface of the polyplexes. Incubation of cells with pHPMA-MPPM formulations containing 25-100 µg/ml diINF-7 peptide resulted in a gradual increase of the silencing activity from 25 to 50%, again indicating that the pHPMA-MPPM polyplexes have limited endosomal escape tendency. Moreover, low cytotoxicity of the Lipofectamine and polymer formulations (<15%) (except PEI formulation, ~25%) was observed in the absence of serum proteins, whereas in the presence of serum, the cytotoxicity of the polyplexes was slightly lower. These features made these polymers attractive candidates for further development and warrant application in animal studies.

In **Chapter 6**, thiolated N,N,N-trimethylated chitosan (TMC-SH) was investigated for siRNA delivery. Introduction of thiol groups in several carrier systems has been shown to enhance their delivery properties. This is due to the formation of reducible disulfide bonds between thiol groups, leading to good extracellular stability and intracellular release properties. Introduction of thiol groups in TMC enhances the colloidal stability of its complexes with nucleic acids and also allows further chemical derivatization with targeting ligands and PEG exploiting un-reacted SH groups. Moreover, the thiol groups in TMC-SH can promote muco-adhesive potential of polyplexes due to formation of disulfide bonds between the polymer and mucin glycoproteins present on the cell membrane, which might lead to an enhanced cellular uptake [21-25]. It was shown that incubation of the complexes based on non-thiolated TMC with heparin leads to release of the siRNA, while in case of the complexes based on thiolated TMC, release of the siRNA was observed only after incubation with both the disulfide bond reducing agent, L-glutathione, and heparin. These observations indicate that siRNA in TMC-SH complexes

can only be destabilized after the disulfide bonds in the particles are cleaved by L-glutathione. As a result, release of the siRNA from the complexes based on TMC-SH is expected to occur in two steps: (1) after cellular internalization of the particles, cleavage of the disulfide bonds between thiolated molecules inside the complex occurs, due to the relatively high concentrations of reducing agents in the cytosol, followed by (2) dissociation of the complexes and release of siRNA likely due to competition with negatively charged macromolecules such as proteins and mRNA present in the cytosol. It was shown that in the absence of serum, incubation of cells with polyplexes based on TMC-SH containing 2, 5 and 7% thiol groups, resulted in 60-80% silencing activity, whereas incubation with non-thiolated TMC complexes showed about 40% gene silencing. These results show that by introduction of thiol groups in TMC, the gene silencing activity of the polyplexes is substantially increased. This might be due to the increased stability of polyplexes based on thiolated TMC. In the presence of 10% FBS, gene silencing activity of the siRNA polyplexes based on TMC-SH decreased to 45%, whereas the gene silencing activity of the non-thiolated TMC remained the same as under serum-free conditions. This reduction in silencing effect can partly be explained by the inhibitory effect of serum proteins on the uptake of nanoparticles. Also, the results of cytotoxicity assays showed that both thiolated and non-thiolated TMC/siRNA formulations had a low cytotoxicity after 24 hours incubation with cells (<20%). Moreover, in the presence of serum, the cytotoxicity of the polyplexes was slightly lower than that of the complexes incubated with the cells in medium without serum. This effect is in line with the literature suggesting that serum proteins can mask the cytotoxicity of cationic polyplexes [26,27]. The gene silencing activity of the polyplexes based on thiolated and non-thiolated TMC polymers was further investigated in the presence of hyaluronic acid (HA), an anionic polyelectrolyte present in biological fluids that has shown to inhibit the transfection activity of pDNA/cationic polymer nanoparticles. In the presence of HA (0.5 mg/ml), TMC-SH/siRNA complexes showed up to 60% silencing activity, whereas the silencing activity of non-thiolated TMC polyplexes was less than 10%. In the presence of both HA and serum in the growth medium, the silencing activity of the TMC-SH polyplexes decreased to 40%, whereas non-thiolated TMC polyplexes hardly showed any silencing. These results again illustrate the key role of disulfide bonds present in the TMC-SH polyplexes which enhance the stability of the complexes against competitor negatively charged

macromolecules present in biological fluids. These features make TMC-SH an attractive candidate for further development and evaluation in animal studies.
The large majority of the work presented in this thesis is concerned with the design and biopharmaceutical characterization of delivery complexes containing reporter nucleic acids. As a start to translate this work to therapeutic applications, in **Chapter 7**, initial findings were reported on the *in vivo* application of selected polyplex systems for the treatment of rheumatoid arthritis (RA). In the collagen antibody-induced arthritis (CAIA) mouse model, we observed that polyplexes containing siRNA, either specific to silence TNF-α or nonspecific, are able to diminish inflammation in the joints. However, further studies are needed to elucidate the mechanisms behind the observed effects.

Future perspectives

Nonviral nucleic acid delivery has gained considerable interest within the pharmaceutical science field. After an intensive interest wave focusing particularly on plasmid DNA delivery, more recently the delivery of RNAi oligonucleotides has rapidly become a major focus in basic and applied research. The research presented in this thesis deals with the *in vitro* screening and biopharmaceutical characterization of various nanoparticulate systems with different characteristics to achieve efficient nucleic acid delivery.
The next step is obviously to translate the current *in vitro* results to the *in vivo* situation and finally to clinical applications. From a safety point of view, the biodegradable vectors such as TMC-SH and pHPMA-MPPM should be given priority for future development towards animal studies, as is also suggested by their relative low *in vitro* cytotoxicity when compared to the non-degradable vectors evaluated. Attaining the full therapeutic utility of nucleic acids requires proper understanding of the biological barriers that stand between initial administration of these new drugs and their final actions within cells. Of primary consideration in deciding on a nucleic acid delivery system of choice to be used *in vivo* is whether the intended disease target lends itself for local or systemic administration. Clearly, for many serious disease situations, i.v. administration is often needed. For this challenging but also most difficult route of administration, quite some design issues and key features need to be considered to obtain an effective nucleic acid nanoparticulate system.

However, it should be appreciated that even after local administration (e.g. in the tumor tissue), delivery issues are certainly not trivial. Some examples of key issues to be considered in case of i.v. administration are:

(1) The particle size should be roughly less than 150 nm, to allow for access to and mobility within tumors and other pathological sites.
(2) The particle surface charge should be around neutral, to avoid aggregation and nonspecific interactions and to achieve prolonged circulation in the bloodstream. Therefore, in this thesis steric stabilization using PEG conjugation has been employed.
(3) The particle should bear an appropriate targeting ligand, to provide selectivity for the target cells and to facilitate internalization via receptor-mediated endocytosis.
(4) It may be needed to exploit a specific endosomal escape mechanism, in order to enhance intracellular delivery efficiency. Therefore, in this thesis special attention was paid to this feature (e.g. use of PCI and fusogenic peptides).

RNAi is one of the most exciting findings in the medical and pharmaceutical sciences over the past 15 years, and the number of publications related to RNAi still is increasing exponentially. The clinical utility of siRNAs will strongly depend on the development of safe and efficacious delivery systems. Recently, the translation from design to clinic has been started with the clinical evaluation of a few siRNA formulations carefully designed for i.v. administration. Cationic lipoplexes termed 'Solid Nucleic Acid Lipid Particles (SNALPs)' that have been stabilized by PEGylation for improved pharmacokinetics have entered the clinical evaluation phase for targeting apolipoprotein B to treat hypercholesterolemia [28]. In this case, only key issues (1) and (2) have been taken into account to achieve targeted siRNA delivery to liver hepatocytes. Attachment of an 'active targeting' ligand is not used, as the desired therapeutic effects are expected to be realized by 'passive targeting' only. The second prominent example of a formulation that is reported to be in clinical trial deals with a targeted nanoparticulate formulation of siRNA, denoted as CALAA-01, which consists of a cyclodextrin-containing polymer, PEG as steric stabilization agent, and human transferrin (Tf) as a targeting ligand for binding to transferrin receptors (tfR) that are typically upregulated on cancer cells. The 4 component formulation is self-assembled into nanoparticles in the hospital pharmacy and is subsequently administered i.v. to patients [29]. As this delivery system does contain an active targeting ligand and meets the

design criteria (1), (2) and (3) listed above, the outcome of this study is eagerly awaited.

Despite this encouraging trend towards clinical application, it is also clear that multiple barriers still stand in the way of effective nucleic acid therapeutics. Still much has to be learnt about the extent of delivery of nucleic acids into target cells *in vivo*. While PEGylation or other surface modification techniques provide a partial solution, the reticuloendothelial system (RES) still manages to capture a large portion of the injected dose. The matter of effective intracellular delivery remains a key problem despite many clever attempts (e.g. PCI) to surmount it. Inefficient endosomal escape is likely to remain a problematic issue. Additional efforts should be devoted to find ways how to improve the intracellular tracking of nanoparticles and nucleic acids. Nevertheless, steady progress is being made on these aspects, and therefore it is expected that nucleic acid-based therapeutics will ultimately become a valued part of the pharmacological armamentarium.

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Appendices

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Nederlandse samenvatting

Voortschrijdende kennis op het gebied van biotechnologie en genetische modificatie heeft geresulteerd in het beschikbaar komen van een aanzienlijk aantal potente maar vaak in het lichaam instabiele macromoleculaire farmaca zoals eiwitten, peptiden en nucleïnezuren. Dit proefschrift richt zich op de ontwikkeling van geschikte toedieningsvormen voor het intracellulair afleveren van nucleïnezuren in relevante doelwit ('target') cellen. Dit is hard nodig omdat de fysisch-chemische eigenschappen van deze macromoleculen de opname door target cellen erg bemoeilijken. Nucleinezuren (zoals small silencing RNA (siRNA) en plasmide DNA) zijn sterk negatief geladen moleculen, en juist deze eigenschap van negatieve lading dient via de bereiding van een geschikt toedieningsvorm weggenomen te worden. Ook dient de toedieningsvorm de nucleïnezuren in het lichaam te beschermen tegen een te snelle afbraak.

Er zijn twee soorten toedieningsvormen voor de intracellulaire aflevering van nucleïnezuren te onderscheiden, namelijk virale en niet-virale dragersystemen. Virale systemen maken gebruik van virussen, die gespecialiseerd zijn in het binnendringen van cellen en het daar afleveren van hun genen. Echter aan het gebruik van virussen zitten potentiele nadelen met betrekking tot hun belading, veiligheid (mogelijk ernstige bijwerkingen) en grote-schaal productie. Daarom wordt ook onderzoek gedaan naar de ontwikkeling van niet-virale dragersystemen. Voorbeelden van niet-virale dragersystemen zijn liposomen (opgebouwd uit vet moleculen), polymeren, en recentelijk worden ook koolstof nanobuisjes onderzocht. Indien opgebouwd uit positief geladen eenheden, kunnen kleine nanodeeltjes worden gevormd met negatief geladen nucleïnezuren op basis van ladingsinteractie. Op deze manier worden de negatief geladen nucleïnezuur moleculen ook beschermd tegen afbraak in het lichaam en kunnen ze door target cellen worden opgenomen. De positieve lading van de deeltjes zorgt voor binding aan de celmembraan, waarna opname in de cel kan plaatsvinden via instulpingen in de membraan, een proces dat endocytose heet. De membraaninstulpingen vormen blaasjes, endosomen genaamd, in de cel. Na deze intracellulaire opname-stap moeten de deeltjes vrijkomen uit de endosomen, en in het cytoplasma belanden. Recentelijk is in de wetenschappelijke literatuur gerapporteerd dat koolstof nanobuisjes in staat zijn direct de celmembraan te kunnen penetreren, en op die manier in het cytoplasma terecht te komen zonder dat ontsnapping uit endosomen nodig is. Vandaar de keuze in dit proefschrift om ook koolstof nanobuisjes te onderzoeken. Eenmaal in het

cytoplasma gearriveerd, moeten vervolgens de nucleïnezuren uit de deeltjes vrijkomen en siRNA in het cytoplasma en plasmide DNA in de kern zijn werk gaan doen.
Het onderzoek gepresenteerd in dit proefschrift betreft de *in vitro* screening (dwz. met gekweekte target cellen) en biofarmaceutische karakterisering van verschillende nieuwe nanodeeltjes voor een efficiente intracellulaire afgifte van siRNA en plasmide DNA. Nadat in Hoofdstuk 1 een korte inleiding tot de materie van dit proefschrift is gegeven, wordt in Hoofdstuk 2 uitgebreid ingegaan op een van de belangrijkste intracellulaire barrières die de activiteit van nucleïnezuur-bevattende dragersystemen beperken, nl. het ontsnappen van deze deze systemen uit de endosomen om het cytoplasma te bereiken ('endomal escape'). Vervolgens worden in de Hoofdstukken 3-6 de fysisch-chemische karakterisering en de afleveringscapaciteit van diverse nieuwe dragersystemen beschreven. Een overzicht van de chemische basiselementen van deze systemen is gepresenteerd in Figuur 1 van het slothoofdstuk (Hoofdstuk 8). Bio-afbreekbare dragersystemen gebaseerd op de polymeren poly(hydroxypropyl methacrylamide-methylpiperidine methanol) (pHPMA-MPPM) en gethioleerd trimethyl chitosan (TMC-SH} lijken de voorkeur te verdienen voor verdere ontwikkeling, vooral vanwege hun relatief lage cytotoxiciteit ten opzichte van de geteste target cellen. De nieuwe nanobuis structuren bleken het minst geschikt voor effectieve nucleïnezuur aflevering aan de onderzochte cellen, hetgeen enigszins haaks staat op hun geclaimde vermogen efficiënt celmembranen te kunnen penetreren. Alhoewel het gros van het verrichte werk gebaseerd is op *in vitro* experimenten met gekweekte cellen, wordt in Hoofdstuk 7 een eerste poging gedaan om een vertaalslag te maken naar de *in vivo* situatie, met behulp van een muismodel van reumatoide artritis. Intraveneuze toediening van geselecteerde siRNA formuleringen resulteerde in anti-ontstekingsactiviteit, maar het mechanisme achter de waargenomen positieve effecten dient eerst in meer detail te worden bestudeerd alvorens definitieve conclusies kunnen worden getrokken. Tot slot geeft Hoofdstuk 8 nog een samenvatting van het in dit proefschrift beschreven onderzoek, en worden er suggesties gedaan hoe de verkregen *in vitro* resultaten te vertalen naar therapeutische toepassingen *in vivo*.

List of abbreviations

AMD	Age-related Macular Degeneration
AMP	Amphiphilic peptides
Bcl-2	B-cell CLL/lymphoma 2
bPrPp	bovine prion protein
CAIA	Collagen antibody-induced arthritis
CLSM	Confocal laser scanning microscopy
CNT	Carbon nanotubes
dex-MA	dextran methacrylate
DLS	Dynamic light scattering
DPc	dendrimer phthalocyanine
dsRNA	double-stranded RNA
DT	Diphtheria toxin
eiF-4E	eukaryotic translation initiation factor 4E
ETA	Exotoxin A
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FGFR	Fibroblast growth factors receptor
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
gpH	glycoprotein H
HA	Hyaluronic acid
HA	Haemagglutinin
hCT	human calcitonin
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1a	Hypoxiainducible factor 1 alpha
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus-1
i.p.	Intraperitoneal
i.v.	Intravenous
KSP	Kinesin spindle protein
LLO	Listeriolysin O
LMP2	Large multifunctional peptidase 2
LMP7	Large multifunctional peptidase 7
LNA	Locked nucleic acid
MECL1	proteasome subunit beta type-10
miRNA	microRNA
MPLSM	Multi-photon laser scanning microscopy
mRNA	messenger RNA
MWCNT	Multi-wall CNT
PAA	Poly(amidoamine)
PCI	Photochemical internalization
PDI	Polydispersity index

pDMAEMA	poly(2-dimethylaminoethyl methacrylate)
PEG	Polyethylene glycol
PEI	Poly(ethylenimine)
pHPMA-DMAE	Poly(2-(dimethylamino)ethyl(1-methacrylamidopropan-2-y1) carbonate)
pHPMA-MPPM	poly((2-hydroxypropyl)methacrylamide1-methyl-2-piperidine methanol)
PLGA	poly(lactic-co-glycolic acid)
PLK1	Polo-Like Kinase 1
PLL	Poly(L-Lysine)
PLO	Pneumococcal pneumolysin
PMO	Phosphorodiamidate morpholino oligomers
PNA	Peptide nucleic acids
PNK3	Protein Kinase N3
PPAA	Poly(propylacrylic acid)
proNGF	proform of nerve growth factor
PSCK9	Proprotein convertase Subtilisin/kexin
QNPHOS	quaternized poly[3,5-bis(dimethylaminomethylene)-p-hydroxy styrene]
RA	Rheumatoid Arthritis
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RSV	Respiratory syncytial virus
RTP801	DNA-damage-inducible transcript 4
SAP	Sweet arrow peptide
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLO	Streptococcal streptolysin O
SNALP	Stable nucleic acid-lipid particles
SWCNT	Single-wall CNT
TAR	Transactivating region
TAT	HIV-1 Trans-activator gene product
TMC	O-methyl free N,N,N-trimethylated chitosan
TMC-SH	thiolated TMC
VEGF	Vascular endothelial growth factor

List of publications

Amir K. Varkouhi, Rolf J. Verheul, Raymond M. Schiffelers, Twan Lammers, Gert Storm, Wim. E. Hennink. Gene Silencing Activity of siRNA Polyplexes Based on Thiolated N,N,N-trimethylated Chitosan. Bioconjugate Chemistry. 2010; 21 (12): 2339-2346

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Bernard C.M.te Boekhorst, Linda B. Jensen, **Amir K. Varkouhi**, Raymond Schiffelers, Twan Lammers, Gert Storm, Hanne M. Nielsen, Gustav J. Strijkers, Camilla Foged, Klaas Nicolay, Therapeutic effect of locally administered PLGA-nanocarriers loaded with anti-inflammatory siRNA on MRI parameters in murine collagen antibody-induced arthritis, submitted for publication.

Marina Talelli, Twan Lammers, Sabrina Oliveira, Cristianne J.F. Rijcken, Ebel H.E. Pieters, **Amir K. Varkouhi**, Tomas Etrych, Karel Ulbrich, Rene C.F van Nostrum, Gert Storm, Wim E. Hennink, Nanobody-targeted core-crosslinked polymeric micelles with covalently entrapped doxorubicin for tumor-targeted combination therapy, submitted for publication.

Selected abstracts

Amir K. Varkouhi, Rolf J. Verheul, Raymond M. Schiffelers, Twan Lammers, Gert Storm, Wim. E. Hennink. Gene Silencing Activity of siRNA Polyplexes Based on Thiolated N,N,N-trimethylated Chitosan. Nanomedicine: Reality Now and Soon - European Science Foundation (ESF)-UB Conference in Biomedicine. 23-28 October 2010, Sant Feliu de Guixols, Spain.

Amir K. Varkouhi, Twan Lammers, Raymond M. Schiffelers, Mies J. van Steenbergen, Wim. E. Hennink, Gert Storm. Gene silencing activity of siRNA polyplexes based on biodegradable polymers and Carbon nanotubes.11th European Workshop on Particulate Systems. 4-5 June 2010, Paris, France.

Amir K. Varkouhi, Twan Lammers, Raymond M. Schiffelers, Mies J. van Steenbergen, Wim. E. Hennink, Gert Storm.Photochemical internalization (PCI)-mediated enhancement of gene silencing efficiency of polymethacrylates and N,N,N-trimethylated chitosan (TMC) based siRNA polyplexes. 2nd European Science Fundation summer School in Nanomedicine, 12-16 June 2009, Lisbon, Portugal.

Curriculum vitae

Amir Khashayar Varkouhi was born on 13th February 1980 in Khorramabad, Iran. In June 1998, he obtained his high school diploma from National Organization for Development of Exceptional Talents (NODET) (SAMPAD), Khorramabad, Iran. Subsequently, he passed the National Academic Entrance Exam for Medical Sciences and started to study Medicine at Lorestan University of Medical Sciences. He obtained his MD degree on June 2005 and subsequently started to work as a medical doctor in few hospitals and medical centers in Lorestan province. In September 2006, he received a scholarship for a Topmaster position in "Medical and Pharmaceutical Drug Innovation (MPDI)" at Graduate University Institute for Drug Exploration (GUIDE), University of Groningen, The Netherlands. He worked on a project focused on "Construction of fusion proteins capable to escape the endosomal pathway for cancer gene therapy" at department of Therapeutic Gene Modulation (TGM), University of Groningen, under supervision of Prof. Dr. Hidde Haisma. After 1 year training, on September 2007, he started his Ph.D research project in department of Pharmaceutics, Utrecht University, under supervision of Prof. Dr. Gert Storm and Prof. Dr. Wim Hennink, resulting in a thesis entitled "Nanoparticulate systems for nucleic acid delivery". Currently he is working as a postdoctoral fellow at the department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands.

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Now after 4 years working on a PhD project in Biopharmacy of Utrecht, it is my turn to say good bye to all my dear friends and colleagues. I started my life in the Netherlands, in lovely 'Groningen' about 5 years ago. I started to work on a project with Prof. Hidde Haisma in cancer gene therapy, in a very nice and friendly group. After one year, I applied for a PhD position at Utrecht University and started my project on September 2007. Yes, time flies and now I am done….

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Amir Khashayar