STARCH BASED CATIONIC POLYMERS

FOR GENE DELIVERY

DISSERTATION

Zur Erlangung des Grades des Doktors der Naturwissenschaften

der Naturwissenschaftlich-Technischen Fakultät III

Chemie, Pharmazie, Bio- und Werkstoffwissenschaften

Von Hiroe Yamada

Saarbrücken 2014

Tag des Kolloquiums:	21. August 2014
Dekan:	Prof. Dr. Volkhard Helms
Vorsitzender:	Prof. Dr. Christian Ducho
Berichterstatter:	Prof. Dr. Claus-Michael Lehr (1. Gutachter)
	Prof. Dr. G Wenz (2. Gutachter)
Akad. Mitarbeiter:	Dr. Torsten Burkholz

Die vorliegende Dissertation entstand unter der Betreuung von

Prof. Dr. Claus-Michael Lehr

Dr. Brigitta Loretz

In der Fachrichtung Biopharmazie und Pharmazeutische Technologie der Universität der Saarlandes

Bei Herr Prof. Lehr und Frau Dr. Loretz möchte ich mich für die Überlassung des Themas und die wertvollen Anregungen und Diskussionen herzlich bedanken.

Table of contents

Short summary

Starch and starch derivatives are widely utilized pharmaceutical excipients, but so far have rarely been used for intracellular delivery of nucleotides. The concept of this thesis was to make use of starch as a biodegradable backbone and modify it with cationic side chains in order to achieve excellent transfection efficiency while maintaining biocompatibility and enzymatic biodegradability. A sufficiently controllable, reproducible and efficient side chain modification could be achieved via a water soluble intermediate of oxidized starch and an optimized protocol using the conjugation reagent, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride. Systematic variation of MW fraction of the starch backbone and the side chain type, polyethyleneimine (MW = 0.8 kDa), diethylenetriamine, tetraethylenepentamine and tris(2-aminoethyl)amine, and amount of cationic side chains (20 – 35 wt%) yielded a series of cationic starch derivatives. Following purification and chemical characterization by GPC, NMR and FT-IR, they were tested for enzymatic degradability by alpha amylase. Nano-scale complexes with plasmid DNA could be easily generated and were compared to standard 25 kDa branched polyethylene imine polyplexes regarding cytotoxicity and transfection efficiency. Some of the synthesized cationic starch derivatives were confirmed to show better properties than positive controls, namely, higher transfection efficiency, lower cytotoxicity and enzymatic biodegradability.

Kurzzusammenfassung

Stärke und Stärkederivate sind weit verbreitete Arzneistoffträger, die bisher kaum für Transfektion Verwendung finden. Das Konzept dieser Arbeit war die Verwendung von Stärke als biologisch abbaubares Grundgerüst. Durch die Kopplung von kationischen Seitenketten wurde eine Transfektionseffizienz erzielt, ohne dabei die Biokompatibilität und enzymatische Abbaubarkeit zu verlieren. Eine kontrollierbare, reproduzierbare und effektive Seitenkettenmodifikation konnte durch ein wasserlösliches Zwischenprodukt aus oxidierter Stärke und ein optimiertes Reaktionsprotokoll erreicht werden. Systematische Variation des molekularen Gewichtanteils von Haupt- und Seitenketten, namentlich unterschiedlicher Menge an, Polyethylenimin (MW = 0.8 kDa), Diethylentriamin, Tetraethylenpentamin oder Tris (2-aminoethyl) amin, sowie die Variation des MWs der Hauptkette lieferte einer Reihe von kationischen Stärkederivaten. Die gereinigten und chemisch charakterisierten Stärkederivate wurden hinsichtlich ihrer enzymatischen Abbaubarkeit mittels α-Amylase untersucht. Nanoskalige Komplexe mit Plasmid-DNA wurden hergestellt und mit 25 kDa Polyethylenimin Polyplexen hinsichtlich Zytotoxizität und Transfektionseffizienz verglichen. Einige der synthetisierten kationischen Stärkederivate zeigten bessere Eigenschaften im Vergleich zur Positivkontrolle, da sie höhere Transfektionseffektivität, geringere Zytotoxizität und zusätzlich enzymatische und hydrolytische Abbaubarkeit aufwiesen.

1 Introduction

A part of this chapter is included in the following published book as chapter 10.

Safety Aspects of Engineered Nanomaterials

Pan Stanford Publishing

Print ISBN: 9789814364850 / eBook ISBN: 9789814364867

Edited by: Axel Zweck and Wolfgang Luther

Chapter 10: *Chances of nanomaterials for pharmaceutical applications*

Loretz B., Ratnesh J., Dandekar P., Thiele C., **Yamada H**., Mostaghaci B., Lian Q,.

Lehr C. M.

The author of the thesis made the following contribution to the publication:

Interpreted and wrote sections of the manuscript concerning the challenges for developing future nanopharmaceuticals.

1.1 Gene delivery

The therapeutic possibilities of nucleotide drugs in the last two decades expanded notably. Nucleotides as drugs have some advantages in comparison to peptides/proteins. They are comparatively easy to produce. From the delivery point of view they are all quite similar molecules. This allows transferring the know-how and knowledge from one delivery system for a special siRNA or pDNA on others or even a mixture of several sequences[1]. In consequence a rather universal delivery platform for nucleotides should be easier feasible, while proteins differ in size and properties to an extent that favors tailored carrier synthesis. A view of gene therapy trials conducted so far show a majority of viral vectors. However, in research a shift toward non-viral delivery is apparent and numerous polymeric or lipid carriers have been developed. The report "Drug Delivery Technologies: Players, products & prospects to 2018"[2] sees significant opportunities for future commercial development, in particular the development of new polymers and biopolymers as carriers, the development of targeting strategies and the identification and delivery of polygenic genes in order to allow therapies of multi-gene based diseases. The key is an effective transport inside target cells and a controlled release at the target point. However, the history of gene delivery research evidences the high complexity factor of this task.

The plain nucleotide itself could not achieve the effective gene delivery due to the large molecular size, high negative charge and the prone to enzymatic degradation. Therefore, the ideal gene delivery vector should be able to bind and condense nucleotides molecules, neutralize the negative charges, provide a protection against the degradation. Besides, it should facilitate the cellular uptake, escape from endosome/lysosome into the cytoplasm, and in case of pDNA further transfer to the nucleus for the following transcription and translation.

1.1.1 Gene delivery pathway into the cells

In the development of gene delivery vector, the pathway of the delivery system should be always taken into account. The transgene/vector complex should overcome multiple barriers during the pathway and this is the first obstacle to solve. Further modifications or formulation strategies of transgene/vector complex to reach the target could be firstly undertaken after the successful cross of these barriers. In Figure 1, DNA delivery pathway is schematically shown and the important steps are briefly explained in the following section.

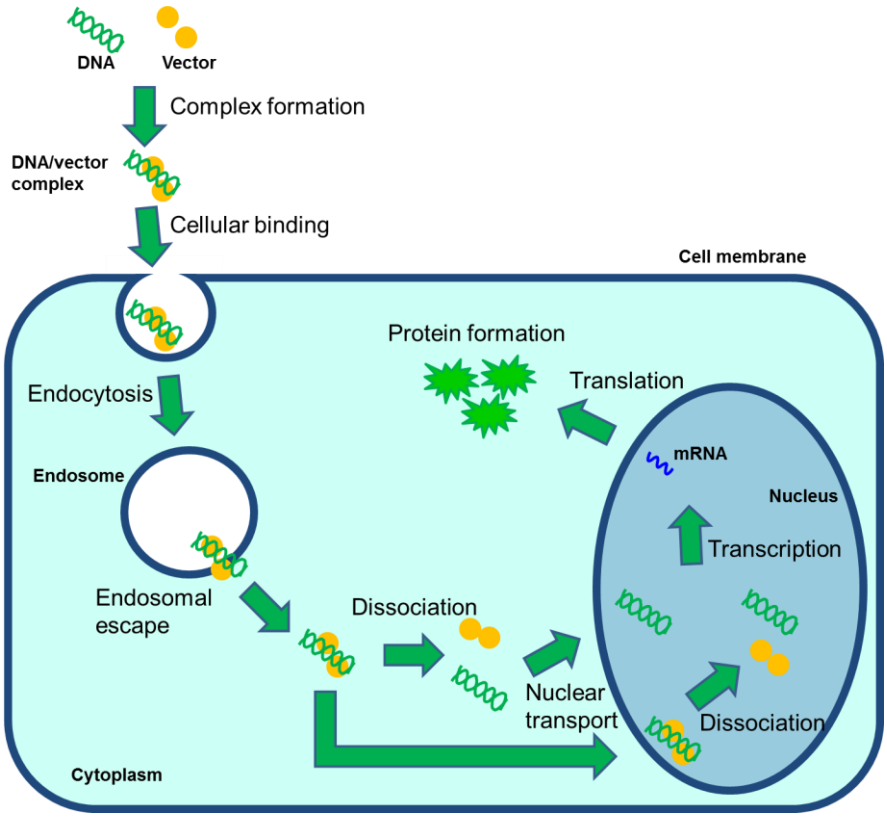


Figure 1: Gene (DNA) delivery pathways by non-viral vector

1) DNA/vector complex formation

There are three main purposes of the DNA complexation with a vector i) neutralization or masking of negatively charged DNA, ii) compaction of the large sized DNA and iii) protection of DNA from extra- and intracellular degradation (pH-dependent and

enzymatically). Most of the non-viral vectors have positive charges to make an electrostatic interaction with the negatively charged phosphate groups of DNA. These positive charges could neutralize the negatively charged DNA, and normally, complexes are formulated with a slight excess of positive charges to enhance the interaction with negatively charged cell surface. The size of the complexes can be also controlled depending on the vector type and cationic-anionic charge ratio. The size variations from 20 nm to 2 μm of the complexes were reported[3, 4].

2) Cellular uptake

Cellular uptake of DNA/vector complex is generally separated endocytotic- and non-endocytotic pathway (e.g. permeabilization, electroporation, fusion, penetration). However, the intensive studies of the cellular uptake exhibited that it mainly occurred by endocytotic pathways[5, 6, 7]. Thereby the resent focus is more on which endocytotic pathways various complexes can take and what complex properties are key to guide. There are two broad classes of endocytotic pathyways, phagocytosis, which involves the uptake of large particles (> 0.5 μm), and pinocytosis, which involves the uptake of fluid and solutes (Figure 2[8]).

Phagocytosis

Phagocytosis is an active and well regulated procedure that involves specific cell surface receptors and signaling cascades. It takes place in specific cells, such as macrophages, monocytes and neutrophils, which have a function to eliminate large pathogens (e.g. bacteria, parasites and large cell debris). Some viruses and miniviruses are known to use phagocytosis to enter cells[9, 10].

Pinocytosis

Major pinocytotic pathways with different morphological characteristics are introduced here: macropinocytosis, clathrin-mediated, caveolae-mediated, and clathrin/caveolae-independent endocytosis[8] and are also depicted in Figure 2. ***Macropinocytosis*** can be defined as a formation of primary large endocytotic vesicles of irregular size (< 5 μm) and shape, generated by actin-driven invaginations of the plasma membrane[11]. Since the sizes of macropinosomes are larger than other pinocytotic endosomes, macropinocytosis is regarded as an efficient route for the nonselective endocytosis of solute macromolecules. ***Clathrin-mediated endocytosis (CME)***, previously known as receptor-mediated endocytosis, is the major and best-characterized endocytotic pathway[12, 13]. The first step of internalization through CME is the strong binding of a ligand (e.g. transferrin, antibodies, low density of lipoprotein) to a specific cell surface receptor. This produces the clustering of the ligand-receptor complexes in coated pits on the plasma membrane, which is formed by the assembly of cytosolic coat proteins, the main assembly units being clathrin. Following, the coated pits invaginate and pinch off from the plasma membrane to form intracellular clathrin-coated vesicles (100 -150 nm in diameter), which would become the early endosome. ***Caveolae-mediated endocytosis;*** Caveolae are invaginated, flask-shaped pits (around 50 nm) of plasma membrane, which are especially enriched in cholesterol and sphingolipids. They are characterized by the presence of the integral membrane protein cavoelin. Though the pits are quite small, caveolae can internalize large molecular complexes, such as cholera toxin, and may serve as a portal of entry for certain viruses and bacteria. In this way, the internalized materials in caveolin coated vesicles could escape the delivery to lysosomes and digestion. Thus, caveolae-mediated endocytotic pathway could be advantageous in drug and gene delivery systems, as well as clathrin-mediated endocytotic pathway. ***Clathrin/Caveolae-independent endocytosis*** does not use a clathrin or caveolin coated vesicles, was reported by several researchers[14, 15]. These pathways should be further studied and defined by their dependency to various molecules such as cholesterol, small GTPases or tyrosine kinase.

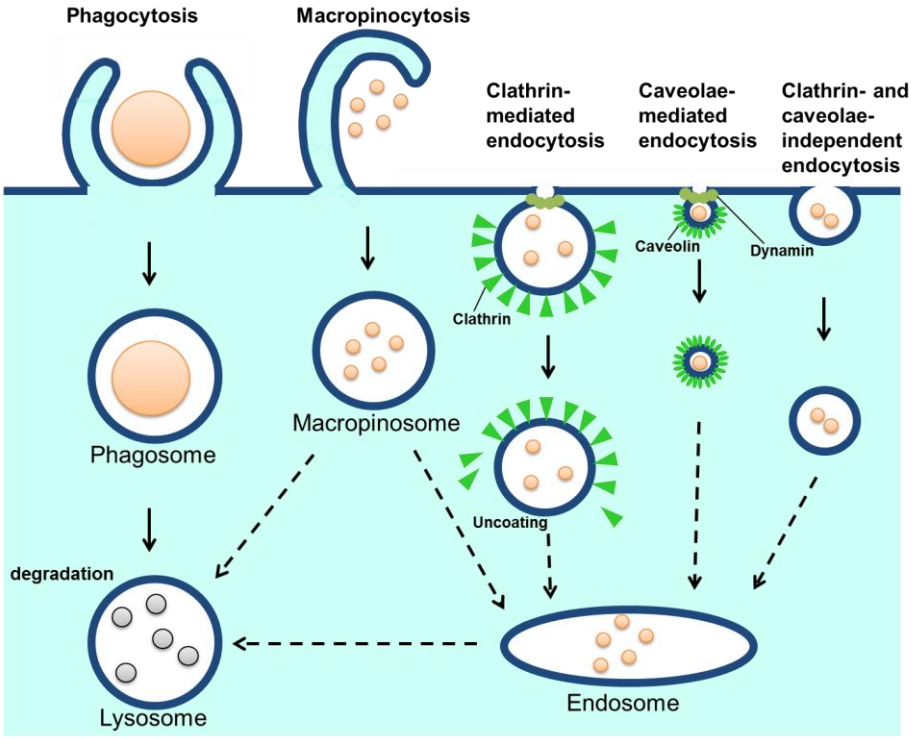


Figure 2: Different endocytotic pathways, modified from ref[8]

3) Endosomal escape

After the internalization through the endocytosis, the internalized molecules exist in endosomes and physically have no contact with cytosol or nucleus. These endosomes would either fuse with lysosomes for degradation or recycle their contents back to the cell surface. Therefore, the internalized complexes should escape from endosomes in time to exert their intended function. In the following, different procedures of endosomal escape are briefly introduced.

Pore formation

Basically, the pore formation in the endosomal lipid bilayer is mediated by a membrane tension that enlarges the pore, that caused by compounds which have high affinity to the rim of the pore. It has been shown that some cationic peptides could sufficiently bind the lipid bilayer and create pores[16].

Proton sponge effect[17] (pH-Buffering effect)

The proton sponge effect is mediated by vectors with high buffering capacity. With the decreasing endosomal pH to approximately 5.4, the protonation of the entrapped agents induces an extensive inflow of ions and water into the endosome. This would increase the osmotic pressure in the endosome. Subsequently, disruption of the endosomal membrane would be induced and entrapped materials would be released. The compounds with multiple amine groups, such as polyethyleneimine (PEI) and poly(amido amine)s, are known to show proton sponge effect because of their high buffering capacity.

Membrane fusion

Endosomal escape through the membrane fusion could only occur when the DNA is enveloped by a membrane (e.g. cationic liposomes). The fusion of the carrier membrane with the endosomal membrane could result in the escape of the nucleotides into the cytosol. A helper reagent, such as cholesterol[18], is sometimes incorporated into the liposomal particles to enhance the fusogenicity of the membranes.

Photochemical internalization (PCI)

PCI is a relatively new technology[19, 20] to release the endosomal entrapped compounds into cytoplasm by light-induced activation of endosomal localized photosensitizers (e.g. disulfonated aluminium phthalocyanine, meso-tetraphenylprophine with two sulfonate groups). The activated photosensitizers could induce photodynamic reactions which results in the destruction of the endosomal membrane mediated by reactive oxygen species. A number of successful gene delivery approaches was reported using this technology by incorporating photosensitizers into the transgene/vector complex[21, 22, 23]. Kloeckner et al.[22] established the PCI using PEI based polyplexes, and Nishiyama et al.[23] incorporated photosensitizers into dendrimer matrix and demonstrated the light-responsive supramolecular gene delivery.

4) Nuclear transport

In the nuclear membrane, there are small pores with a passive transport limit of 70 kDa of MW or 10 nm diameters. However DNA is much bigger than these pores even condensed by any vectors and simple passive transport through these pores would be difficult. *In vitro*, the most accepted nuclear transport mechanism is cell division. During the mitosis, the integrity of the nuclear membrane is temporary lost, which enables DNA (complexes) to enter the nucleus. As cell culture cells are often fast proliferating this step might be underestimated. Thus, *in vivo*, additional different mechanism have been suggested. It is known that many proteins are actively transported to the nucleus because of the presence of nuclear localization signals, which are short cationic peptide sequences that are recognized by importins[24]. Since many of the DNA/vector complexes have cationic charges, it has been considered that a similar mechanism could be used to enhance DNA (complex) transport into the nucleus.

5) Dissociation of DNA from vector

The DNA/vector complex should be dissociated either before or after the transport into the nucleus to enable transcription. In the ideal case the binding between vector and DNA should be broken and DNA released in the nucleus where the enzymatic protection is not needed any more. Still the binding strength should be controlled to keep the enable fast and efficient rules needed to achieve the successful translation.

1.2 Non-viral gene delivery vectors

The advantages and disadvantages of viral and non-viral gene delivery vectors have been often discussed in the history of gene delivery vector development. Viral vectors include viruses (e.g. retrovirus, adenovirus, adeno-associated virus), and generally they are very efficient gene delivery vectors. However fatal drawbacks limited their practical use. Viral vectors may elicit mutagenesis and carcinogenesis. Additionally, the repeated administration of viral vectors could induce an immune response which abolishes the transgene expression[25]. Therefore, researches are more and more shifted toward the non-viral vectors. Since the non-viral vectors are still rather inefficient in transfection, the recent researches generally focus on the improvement of the transfection efficiency, at the same time, retaining the advantages of non-viral vectors, being non-pathogenic and non-immunogenic.

1.2.1 Different types of non-viral gene delivery vectors

A large number of non-viral gene delivery vectors have been studied. In Table 1, different types of non-viral gene delivery vectors were summarized and are briefly explained in the following.

1.2.1.1 Ceramic based vectors

The successful gene deliveries were reported using ceramic based non-viral gene delivery vectors[26, 27, 28]. Silica (SiO_2), hydroxyapatite, zirconia (ZrO_2) and their modified materials are often studied. Mostaghaci et al.[28] synthesized amino-functionalized calcium phosphate nanoparticles and reported the successful pDNA transfection with negligible cytotoxicity. Generally, the advantages of the ceramic based vectors are the ease of the preparation, monodispersity of the vector and their water dispersability. On the other hand, the surface adsorption of the transgene instead of encapsulation could be a disadvantage, which may not effectively enough protect the nucleotides from degradation.

1.2.1.2 Metal based vectors

Gold, iron oxide, zinc oxide and their modified nanoparticles can be given as examples of the metal based gene delivery vectors[29, 30, 31]. The biggest advantage of the metal based vectors is the small and uniform particle size, less than 50 nm, which enlarge the surface area for the transgene - adsorption. Additionally, it is shown that superparamagnetic iron oxide nanoparticles provide a huge benefit as magnetic resonance imaging (MRI) contrast agents[32]. The availability of a universal MR marker to image gene expression is especially important to follow the delivery system and know where the transfection takes place. As such, iron oxide nanoparticle could confer a big opportunity. The disadvantage of metal based vectors would be, same as ceramic based vectors, surface adsorption of transgenes instead of encapsulation and also the toxicity of the materials.

Table 1: Different types of non-viral gene delivery vectors

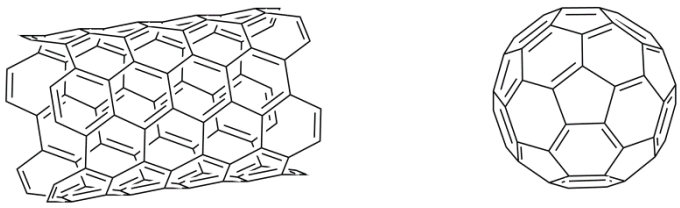
Material of non-viral gene delivery vector	**Chemical materials**	**Advantages**	**Limitations**
Ceramics	Silica, Hydroxyapatite, Zirconium dioxide, …	Easily prepared, Water dispersible	Surface adsorption instead of encapsulation
Metals	Gold, Iron oxide, Titanium dioxide, Zinc oxide, …	Small particles present a large surface area for surface decoration delivery	Toxicity of materials, time-consuming synthesis, surface adsorption instead of encupsulation
Liposomes	Phospholipids like DOTMA, DOTAP, DOGS, DOPE, …	Reduced systemic toxicity, increased circulation time	Some leakage of encapsulated agent, lack of colloidal stability
Carbon assemblies	CNTs, Fullerenes, Nanodiamonds	Large surface area allows multi-functionalization	Poor solubility in biological solution
Polymers	Natural polymers: Chitosan, Hyaluronic acid, cyclodextrin, dextran, … Synthetic polymers: PEI, PLL, PDMAEMA, PEG, …	Sustained localized drug delivery for weeks, Flexibility for chemical modification	Fixed functionality after sysnthesis may require new synthetic pathways for alternate surface functionalities

1.2.1.3 Liposome based vectors

A solution of cationic lipids is mixed with genes to form a positively charged complex, named lipoplex. Widely used commercial cationic lipids for gene delivery application are N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP), 3β(N-(N,N-dimethylaminoethane)-carbamoyl) cholesterol (DCChol), and dioctadecylamidoglycylspermine (DOGS)[33]. A natural lipid, Dioleoylphosphatidylethanolamine (DOPE), is often used with cationic lipids for the membrane destabilization at low pH intended for endosomal escape. The advantages of lipoplex are lack of immune response, low cost and diverse range of morphologies. As disadvantages, lack of colloidal stability and leakage of encapsulated transgenes are often mentioned.

1.2.1.4 Carbon assemblies based vectors

Carbon assemblies are a relatively new class of gene delivery vectors, such as carbon nanotubes (CNTs), fullerenes (C60) (Figure 3), nanodiamonds based materials[34, 35, 36, 37].



CNT **fullerene**

Figure 3: Structures of CNT and fullerene

Especially carbon nanotubes are intensively studied in last two decades[38, 39], not only in biomedical field but also many other field, such as sensing, material science, electronics, optics and gas storage etc. Cationic-functionalized CNTs are usually used to condensate the polynucleotides. CNTs own interesting structural, optical and electrical properties for drug and gene delivery system. The large surface area allows multi-functionalization of various

molecules to get suitable properties as gene delivery vectors and a number of successful gene deliveries were reported by using functionalized CNTs as vectors[40, 41, 42]. The disadvantage of the carbon assemblies would be the poor solubility in biological solution.

1.2.1.5 Polymer based vectors

Usually, positively charged complexes, so called polyplexes, are generated simply by mixing of the polymer solution with the transgene in an optimized ratio. This simple production without the need of complex processes or solvents is an advantage of such delivery systems. The cationic net charge and the impact of pH and ionic strength on the complex stability, however, are topics to be considered when such delivery systems aim for translation *in vivo*. Thus, not only polymers from various monomers but also many derivatives were synthesized in order to optimize their properties. Polymers are diverse vectors with respect to chemical components, as well as structures, e.g. linear polymers, branched polymers, homopolymers, di-, tri- block/random copolymers. More details of the major polymers in the use of gene delivery vectors are explained in the following section (1.2.2).

1.2.2 Most frequently used polymers in gene delivery

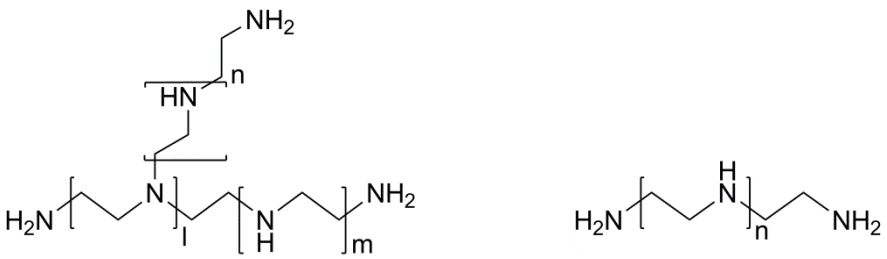
The first application of polymers as non-viral gene delivery vector was in 1965 by Vaheri and Pagano[43]. They used diethylaminoethyl functionalized dextran. Since then, a large number of polymers with different chemical structures and characteristics has been developed and studied for their ability as gene delivery vectors.

Regarding the chemical composition, polymers could be separated in synthetic and natural polymers. An advantage of the synthetic polymers is their potential for high reproducibility and homogeneity. The natural polymers on the other hand profit from their natural abundance and biodegradability. Within the group of synthetic polymers PEI and Poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) are the most prominent vector for gene

delivery. As natural polymers, poly(L-lysine) (PLL) and different carbohydrates are often studied as gene delivery vector. In the following sections, some of the most studied and focused polymers are categorized and briefly explained.

1.2.2.1 PEI

Since the first successful PEI mediated oligonucleotide transfer presented by Boussif et al.[44], PEI has been recognized as the gold standard polymer for gene transfection. The chemical structure of PEI contains nitrogen at every third atom, which results in a high cationic charge density. At a physiological pH, ca. 80 % of amine groups are unpronated, while less than 50 % amine groups are unprotonated at pH 5. This buffering capacity is the driving force to escape from the endosome (proton sponge effect), and this special feature is accepted as a one of the main reason of the high transfection efficiency of PEI. PEI exists as a branched structure, branched-PEI (b-PEI), and linear structure, linear-PEI (l-PEI) (Figure 4), and they showed different properties as gene delivery vectors. The degree of branching has been shown to have an influence to the polyplex formation and stability. Dunlap et al.[45] showed that l-PEI is less effective to condensate DNA compared to the similar MW b-PEI. Additionally, the more primary amine groups PEI has, the more stable polyplex were obtained. That makes b-PEI more suitable transfection vector than l-PEI[46].



b-PEI **l-PEI**

Figure 4: Two structures of PEI: b-PEI and l-PEI

The MW of the PEI is also known to have an effect on the transfection efficiency. Godbey et al.[47] showed that larger MW PEI could showed higher transfection efficiency in the range of 600 to 70 kDa, though the larger MW PEI showed the higher cytotoxicity[48]. In spite of its high ability to transfect oligo- and polynucleotides, PEI has critical drawbacks, cytotoxicity and non-biodegradability. Various groups have been working on the topic to overcome theese drawbacks. One of the most popular ways to reduce cytotoxicity is PEGylation[49, 50, 51, 52, 53]. Non-toxic and non-charged PEG could neutralize the positive surface charge of PEI, and result in a reduction of cytotoxicity. Linking small MW PEI with biodegradable chemical structure, such as disulfide and hydrolysable ester linkages has been studied to achieve a "biodegradable" PEI[54, 55, 56, 57, 58].

1.2.2.2 Poly(L-lysine) (PLL)

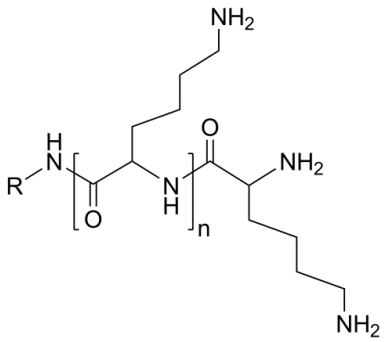
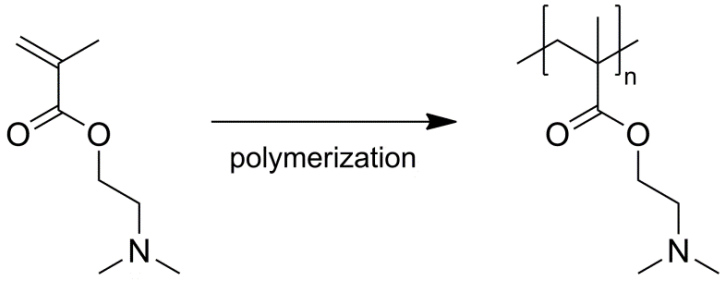


Figure 5: Chemical structure of PLL

PLL was recognized as a gene delivery vector since Laemmli et al. found its ability of gene condensation in 1975[59]. PLL, as well as PEI, contains amine groups in its structure and this primary amine groups could effectively condensate the oligo- and polynucleotide. Unlike PEI, all amine groups of PLL are protonated at physiological pH, resulting in the structure with no buggering capacity[60] to guide to endosomal escape. With the addition of chloroquine or membrane-active peptide, the endosomal escape was shown to be improved[61]. In general, PLL with MW > 3000 Da could effectively condense DNA to form stable polyplexes, though this

MW PLL showed relatively high cytotoxicity[62]. To overcome this cytotoxicity, several PLL derivates were synthesized containing biodegradable linkage, such as disulfide or hydrolysable ester functions. For instance, biodegradable poly(lactid-co-glycolid) acid (PLGA)-grafted PLL conjugates showed reduced cytotoxicity and the same level of transfection efficiency compared to PLL[63]. Another disadvantage of PLL/DNA polyplex is the tendency to aggregate and precipitate in the ionic biological solution. Conjugation with PEG and synthesize a PLL-PEG block copolymer is a common solution to avoid this aggregation and precipitation[64, 65]. Fukushima et al. additionally added poly((3-morpholinopropyl) aspartamide) chain to PLL-PEG and synthesized triblock copolymer[65]. This triblock copolymer showed improved buffering capacity and transfection efficiency compared to PLL-PEG.

1.2.2.3 Poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA)



DMAEMA monomer **pDMAEMA**

Figure 6: Chemical structure of DMAEMA and pDMAEMA

pDMAEMA is a polymethacrylate synthesized via radical polymerization of 2-(dimethylamino) ethyl methacrylate monomer (DMAEMA) (Figure 6). Since pDMAEMA has cationic charges in its repeating units, it shows good properties as gene delivery vector. The high potential of pDMAEMA as transfection reagent was reporter first in 1996 by Cherng et al.[66], with a 90 % efficiency compared to b-PEI 25 kDa. Since then pDMAEMA and its copolymers have been intensively studied. pDMAEMA with MW > 112 kDa is known to make small and stable polyplex and show efficient transfection, but at the same time, certain

cytotoxicity. To reduce the cytotoxicity without decreasing the transfection efficiency, the modification of pDMAEMA was studied. Also with pDMAEMA the PEGylation was studied and a pDMAEMA-PEG diblock copolymer was synthesized. The cytotoxicity of the pDMAEMA-PEG was improved, though the transfection was decreased[67]. Incorporation of hydrophobic block (polycarprolactone (PCL)) between pDMAEMA and PEG block, resulted in pDMAEMA-PCL-PEG triblock copolymer, showed 15 times higher transfection than pDMAEMA-PEG[68]. Another method is copolymerization with other hydrophobic or hydrophilic monomers with DMAEMA. Van de Wetering et al.[69] copolymerized N-vinylpyrrolidone (NVP) with DMAEMA, and found an increased transfection efficiency of the pDMAEMA-pNVP copolymer. Due to the improved technology of living radical polymerization, such as atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer (RAFT) polymerization, the pDMAEMA based gene delivery vector was further developed and investigated. Biodegradable pDMAEMA is one of the focused research area. Jiang et al.[70] synthesized biodegradable brush shaped copolymers composed from biodegradable poly(hydroxyethyl methacrylate) (pHEMA) backbone and pDMAEMA side chains via ATRP. The synthesized pHEMA-*graft*-pDMAEMA copolymer brush was shown to have lower cytotoxicity and higher transfection efficiency than high MW (M_W > 300 kDa) pDMAEMA homopolymer.

1.2.2.4 Dendrimer structured polymers

Dendrimers are well-defined chemical structures, which are composed of many branches and subbranches systematically radiating out from a central core (Figure 7). These unique molecular architectures provide interesting properties for the development of gene delivery vector, such as defined architecture and high ratio of multivalent surface moieties to molecular volume.

Figure 7: Schematic structure of a dendrimer.

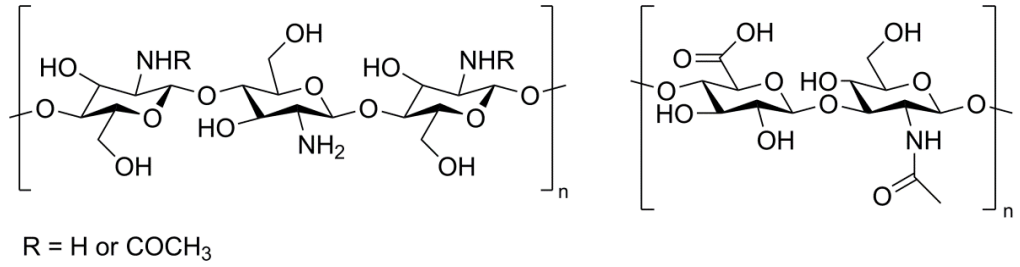
Because of the ease of synthesis and commercial availability, polyamidoamine (PAMAM) dendrimers have been the most utilized dendrimer based gene delivery vector. Due to the large number of secondary and tertiary amines on the polymer, PAMAM based polyplex show a proton sponge effect, helping them to escape from endosomes. Haensler and Szoka[71] reported that the sixth-generation PAMAM dendrimer showed the best properties within several other generation PAMAM dendrimers as gene delivery vector. "Generation" refers to the number of repeated branching units during the synthesis of dendrimers. The higher generation dendrimers have the more functional groups on the surface, which could show huge impact on the cytotoxicity and transfection efficiency. Except PAMAM, some amine functional polymers, such as Poly(propylenimine) (PPI) and PLL dendrimers are also investigated for gene transfection[72]. The possibility of multi-functionalization of the dendrimer surface was the most studied way to improve the transfection and lower the cytotoxicity.

1.2.2.5 Carbohydrate-based polymers

Carbohydrate-based polymers have been also investigated as gene delivery vectors[73]. In general, they have considerable advantages, such as a lack of toxicity and absence of antigenicity and immunogenicity. Some examples of intensively studied polymers are chitosan, hyaluronic acid, dextran and cyclodextrin-based polymers (Figure 8). Among them, chitosan is the most investigated polymer[74, 75, 76], because of its originated cationic charges in the structure.

Similar to the polymers introduced before, the MW of chitosan could give an impact to transfection efficiency. Huang et al.[77] demonstrated that with increasing MW of chitosan, the transfection efficiency was increased (213 kDa > 98 kDa > 48 kDa > 17 kDa). The deacetylation amount of chitosan is also important. Generally, increasing deacetylation amount improves transfection efficiency *in vitro* for various cell lines[78], though moderate deacetylation chitosan showed the best transfection efficiency *in vivo*[77]. These results emphasized the importance of optimal balance between protection/condensation of the DNA and facile release of DNA from the vectors. To improve the buffering capacity, chitosan modification with PEI and urocianic acid, which results in an imidazole containing chitosan, is often used[79, 80]. Both modifications could successfully improve the buffering capacity and resulted in the higher transfection efficiency. Since other polymers, hyaluronic acid, dextran and cyclodextrin do not have cationic charges, these polymers were often used after modification with cationic charges. Dandekar et al.[81] reported cationic polyrotaxane synthesized from cationic modified cyclodextrin and cationic axis used for the successful siRNA delivery. Cui et al.[82] modified dextran with various amine functionalized molecules and synthesized biodegradable cationic dextran derivatives and reported successful siRNA delivery.

(a) (b)



(c) (d)

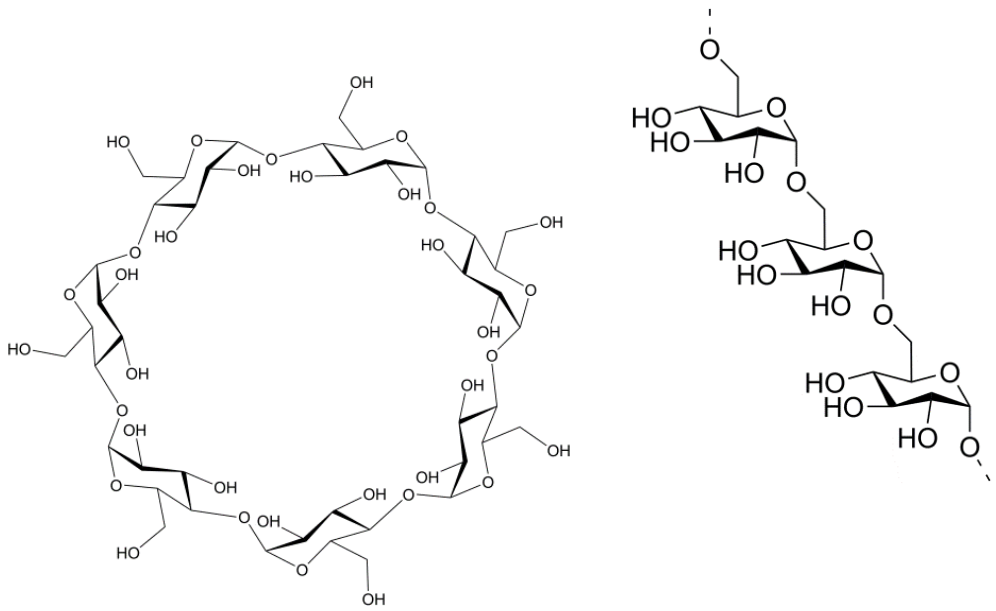
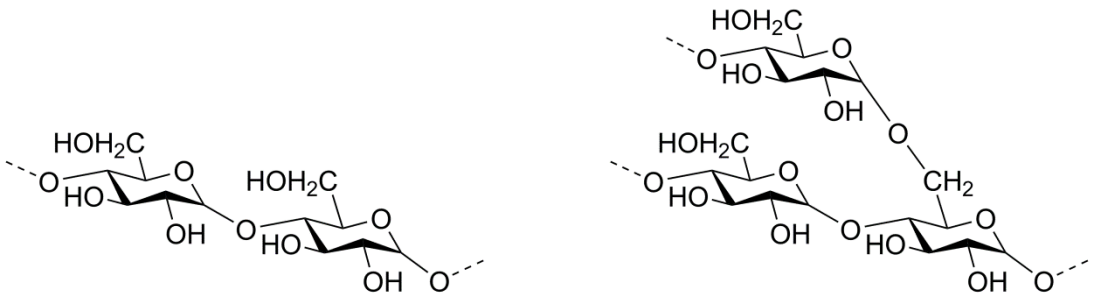


Figure 8: Structures of carbohydrate-based polymers

(a) chitosan, (b) hyaluronic acid, (c) cyclodextrin, (d) dextran

1.3 Starch

Starch is abundantly generated in nature as the main storage polysaccharide in higher plants. It exists as granules in the chloroplast of green leaves and the amyloplast of seeds, pulses and tubers[83]. From the chemical point of view, starches consist of two major components, amylose (10-25%) and amylopectin (75-90%). Both of them are polysaccharides from a number of glucose molecules linked together. The differences between them are the way of glucose linkages and the final structure, namely branching or no-branching. Amylose is a linear polysaccharide which glucose residues are alpha-1,4-linked, and the degree of polymerization is up to 6000. The amylose can easily form single or double helices, and is soluble in hot water. Amylopectin is a branched polysaccharide which glucose residues are alpha-1,4- and alpha-1,6- (5 %) linked, and the average degree of polymerization is, much higher than amylose, two million, thereby being one of the largest molecules in nature. Typical amylopectin has 20 to 25 glucose units between branching points. They are more difficult to solubilize than amylose, even in hot water. The chemical structures of amylose and amylopectin are shown in Figure 9.



Amylose **Amylopectin**

Figure 9: Chemical structures of amylose and amylopectin

1.3.1 Uses of starch

The biggest advantages of starch are the environmental acceptability, biocompatibility and biodegradability. Starch is a renewable raw material with huge prospectiveness, since the modification of starch could alter the characteristics of starch and give an additional value. Therefore, starches and modified starches are used for a wide range of products in different applications[84] (Table 2). The biggest consumers of non-food starch might be the paper and cardboard industry[85]. Starch and modified starches are used in the different processes in the production of paper and cardboard, not only as binders but also for the improvement of the product properties. For instance, the use of cationic starch can improve the mechanical strength of the resulting paper sheet[86].

Table 2: Industrial use of starch and modified starch[87]

Industry	**Use of starch and modified starch**
Food	Viscosity modifier, glazing agent
Paper and board	Binding, sizing, coating
Adhesive	Adhesive production
Textile	Sizing, finishing and printing, fire resistance
Plastics	Biodegradable thermoplastic materials
Detergent	Surfactants, builders, bleaching agents
Cosmetics	Face and talcum powders
Medical	Plasma extender/replacers, Transplant organ preservation
Pharmaceuticals	Diluent, binder, drug delivery

Additionally, starches are used as adhesives, detergents, and also thermoplastics. With the increasing needs and interests of environmental friendly products especially in food packaging industry, biodegradable thermoplastics/packaging materials are focused a lot[88]. López et al.[89] developed a starch based film for food packaging with a good heat sealing capacity. The high value-added applications of starch would be seen in cosmetic, medical and pharmaceutical industries. The use of starch for pharmaceutical application has been changed in the last few decades, with the emergence of drug delivery concept. High value-added starches could play a

cardinal role as substrate for tissue engineering[90] and as drug carriers. As such, their value is not the same anymore as simple diluents or binders.

1.3.2 Applications of starch derivatives in drug delivery and gene delivery

Recently an increasing number of researches is reported that applies starch derivatives in drug delivery. Starch was modified with different functional groups or polymers to improve its properties as a delivery vector. Micro- and nano sized particles, capsules and complexes of the drug and starch derivatives have been shown to possess high potential to overcome biological barriers and lead to successful drug delivery.

Balmayor et al.[91] presented biodegradable starch-poly-ε-caprolactone microparticles sized 5 to 900 µm as a drug delivery system for dexamethasone as a model drug. Different surface morphology (smooth and porous) was observed in the different formulations. The formulated microparticles showed excellent encapsulation efficiency up to 93 % and a reasonable release profiles, which would correspond to the degradation of the polymer.

Santander-Ortega et al.[92] used hydrophobic propyl-starches and form nanoparticles in a size range of 150 to 200 nm for transdermal drug delivery. These starch based nanoparticles could encapsulate a wide range of chemically different drugs (flufenamic acid, testosterone and caffeine) with a good efficiency, and showed a prominent enhancement of skin penetration for the flufenamic acid formulation.

Baier et al.[93] synthesized nanocarriers (170 to 300 nm) from crosslinked hydroxyethyl starch. The modification of the nanocarriers surface with folic acid dramatically increased the specific cellular uptake into HeLa cells. These results demonstrated a high potential of receptor-mediated targeting using starch based nanocarriers.

Zhang et al.[94] synthesized pH-responsible smart starch-*graft*-poly(L-glumamic acid) copolymer by click reaction. Their formulated starch-*graft*-poly(L-glumamic acid) nanoparticles changed their sizes depending on the pH, and proofed such an excellent pH-responsive property. Additionally, the insulin release profile was studied at different pH values. They showed that the insulin release was slower in low pH condition (artificial gastric juice,

pH = 1.2) than at higher pH condition (artificial intestinal liquid, pH = 6.8). These results indicated that these pH-responsible nanoparticles could protect the insulin in a gastric environment and prevent enzymatic degradation, followed by a controlled drug release in the intestine.

Through the increasing number of research regarding starch based vectors in drug delivery application, only two studies were found that worked on gene delivery and both were from same research group by Noga et al.[95, 96]. Hydroxyethyl starch (HES) instead of gold standard PEG, was used to shield the cationic charge of the DNA/l-PEI (22 kDa) polyplexes. Different from PEGylation, HESylation could be unshielded through the degradation of HES by the addition of α-amylase which leads to anincreased uptake of the polyplexes into the target cells. Their first paper[95] presented the increased transfection efficiency of HESylated PEI by the addition of α-amylase, though it was not the case for PEGylated PEI. In their second report[96], the optimal MW of HES and degree of HESylation were studied and the importance of the fine balance of these parameters became clear. The higher MW of HES could shield the cationic charges of polyplexes better. More hydroxymethyl groups on the starch reduced the biodegradability by α-amylase and leaded decreased transfection efficiency. In conclusion, HES with 70 kDa MW and 50 % hydroxyethyl groups against glucose unit showed the best results in an in vivo study. The large MW (70 kDa) HES effectively shielded the cationic charge of polyplexes and improved the stability, and the biodegradability of the shell at the same time allowed the same level of luciferase transfection efficiency in comparison to no-HESylated l-PEI (22 kDa), though it was not the case for PEGylated PEI.

2 Aim of this work

The main motivation of this thesis was to design and synthesize novel starch based polymers as a gene delivery vector which could achieve high transfection efficiency and safety. Though starch and starch derivatives are widely utilized pharmaceutical excipients, but so far have rarely been used for intracellular delivery of nucleotides. The concept of this study was to make use of starch as a biodegradable backbone and to modify it with different cationic side chains in order to achieve better transfection efficiency while maintaining biocompatibility and enzymatic biodegradability.

The major aims of this thesis were:

1) To establish the optimal reaction method of the starch and PEI conjugates as a suitable gene delivery vector (chapter 3).

2) To evaluate the synthesized starch and PEI conjugates as gene delivery vectors, focusing on the transfection efficiency and safety, and find out the specific characteristics of the polymer for the successful gene delivery (chapter 4).

3) To study the effect of the additional hydrophobic chains on the starch and PEI conjugates (chapter 5).

4) To study the effect of different cationic side chains (MW and structure) instead of PEI (chapter 6).

3 Synthesis of the Starch-*graft*-PEI polymers

The data presented in this chapter have been published in parts as a research article in:

Biomaromolecules **2014**, *15* (5), pp 1753–1761

Design of Starch-*graft*-PEI Polymers: An Effective and Biodegradable Gene Delivery Platform

Publication Date (Web): March 31, 2014 (Article)

DOI: 10.1021/bm500128k

Yamada H., Loretz B., Lehr C. M.

3.1 Introduction

The use of cationic polymers is well established in the gene delivery field[97] due to their capability to condense nucleic acids into compact polyplexes and thus protect them from degradation. The cationic charge of such polyplexes facilitates their cell surface attachment and endocytotic uptake, while their pH buffering capacity (known as the "proton sponge effect[17]") may allow for the possibility of endosomal escape. However, cationic polymers also have a crucial drawback, which is a high level of cytotoxicity. Therefore the amount and density of cationic charges in the polymer needs to be precisely controlled through careful polymer design, in order to maintain a balance between safety and successful gene delivery. A variety of cationic polymers including polyethylenimine, poly(dimethylaminoethyl methacrylate)[98,99], and chitosan[74, 75, 76] have been studied as gene delivery vectors. Branched-polyethylenimine (b-PEI, 25 kDa), a well-known cationic polymer often employed as a gene delivery vector, shows excellent transfection efficiency and is regarded as a "gold standard[44]" among polymers. However, b-PEI is also known to show certain cytotoxicity[100]. Many studies have therefore been conducted to develop improved PEI-based polymers which retain a high level of transfection efficiency, but exhibit a lower cytotoxicity. The shielding of cationic charges on the PEI surface with polyethylene glycol, so called "PEGylation", is one of the methods employed to reduce polymer cytotoxicity[49,50,51,52,53]. Petersen et al.[49] reported that PEGylated b-PEI showed lower cytotoxicity than b-PEI, and found the optimum PEG chain molecular weight (MW) and amount of b-PEI modification. Another drawback of PEI is its non-biodegradability. To overcome this obstacle, crosslinking of the low MW PEI with a biodegradable cross-linker has often been performed[54, 55, 56, 57, 58]. For example Gosselin et al.[54] cross-linked low MW PEI (0.8 kDa) with disulfide bonds in order to synthesize biodegradable b-PEI. These disulfide bonds can be cleaved by reducing agents like glutathione, but not by enzymes. The transfection efficiency of b-PEI cross-linked in this manner was not superior to b-PEI 25 kDa, however it was shown the advantages of a lower cytotoxicity and an improved degradability due to reduction of disulfide bonds was achieved.

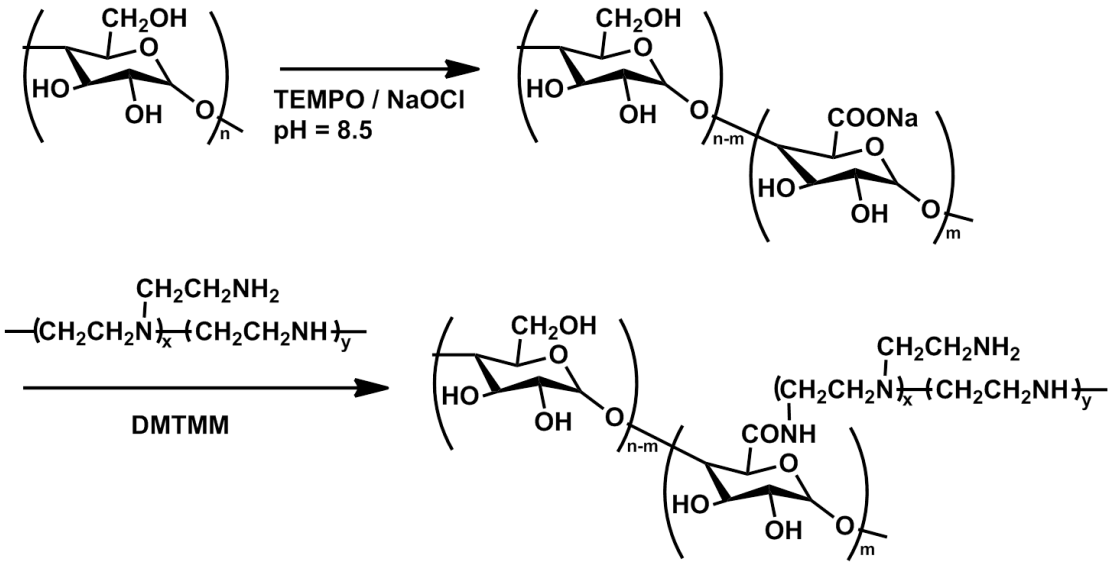
Several polysaccharide-based polymers have been studied as gene delivery vectors, mainly chitosan[74, 75, 76] and hyaluronic acid[101, 102]. Less of a focus has been placed to date on starch. Polysaccharides in general have considerable advantages for use as gene delivery vectors, including a lack of toxicity and absence of antigenicity and immunogenicity.

In this work, we propose the novel design of a well-defined and possibly biodegradable cationic copolymer composed of water-soluble potato starch and low MW PEI (s-PEI, 0.8 kDa). Starch is a biocompatible and biodegradable polymer, generated abundantly in nature as the main storage polysaccharide in higher plants. Starch, compared with other polysaccharides, has the additional advantages of a high cost performance and biodegradability not only by hydrolysis, but also by human enzymes, particularly α-amylase. It is well-known that α-amylase is found in the saliva, pancreatic juices, blood and urine. Some amounts of polysaccharides, including starch, are known to be degraded intracellularly by various enzymes[103, 104, 105]. In addition, the existence of amylase-producing tumors has been reported[106,107], which could be also a good target for α-amylase degradable starch-based delivery systems. As a further advantage, the OH groups of the glucose units of starch offer plenty of possibilities for functional substitution. The properties of the modified starch can therefore be greatly altered depending on the substitution groups and degree of substitution.

However, the modification of the glucose units of starch with larger or complex molecules is not an easy task because of the steric inaccessibility of the molecules to the reaction site of the starch. Therefore, no direct conjugation of PEI to a starch backbone was published so far. The researchers are using already modified starch (e.g. Hydroxyethyl starch[95]) and PEI, or opening the glucose ring and make the dialdehyde starch[108] to react with PEI. Zhou et al.[109] modified amylopectin in a conjugation with small cationic molecules and studied the transfection efficiency. Since the cationic side chains (consisting of ethylenediamine, diethylenetriamine and 3-(dimethylamino)-1-propylamine) of the produced modified amylopectins were quite small, it was deemed necessary to modify almost all OH groups of each glucose unit to get good transfection efficiency. While it was not investigated, such extensive modification could

potentially have led to a loss of the enzymatic biodegradability of these modified amylopectins, because of the specificity of the enzymatic degradation system.

The first aim of this thesis was to establish a method to modify starch with relatively large molecule PEI (0.8 kDa), while maintaining the structure and characteristics of the starch as much as possible. Once the optimal conjugation method was found, two parameters, MW of the starch and the modification amount of PEI, were varied in the synthesis reaction. The resulting starch-PEI conjugated polymers were analyzed to verify the altered parameters and used as candidate polymers for gene delivery vector.



Scheme 1: Total synthesis of the starch-*graft*-PEI polymers

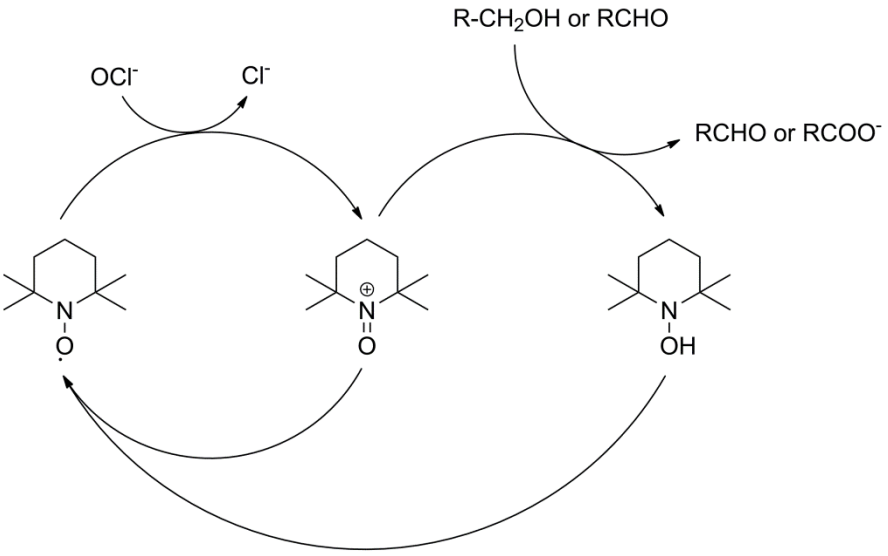
3.2 Experimental Methods

3.2.1 Materials

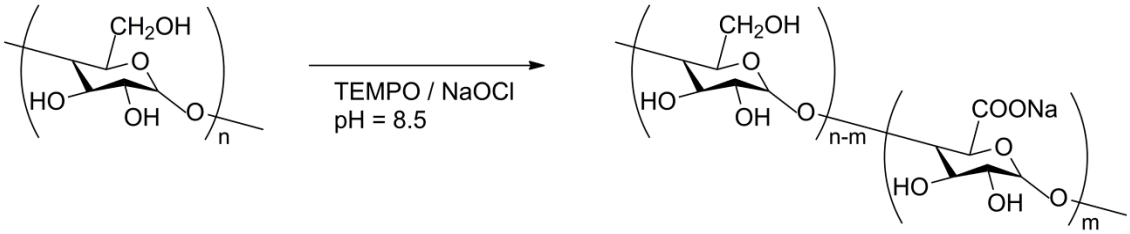
Partially hydrolyzed potato starch (MW was around 1,300,000 g/mol, amylose content was around 33%) was a kind gift from AVEBE (Veendam, NL). Two different molecular weight (MW) branched polyethylenimine, (b-PEI, 25 kDa) (s-PEI, 0.8 kDa) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used without further purification. 2,2,6,6-Tetramethyl-1-piperridinyloxy (TEMPO), Sodium hypochlorite solution (12%), 2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and 4-Methylmorpholine (NMM) were purchased from Sigma-Aldrich. Sodium borohydride was purchased from Merck. Purified water was produced by Milli-Q water purification system (Merk Millipore, Billerica, U.S.A.).

3.2.2 Oxidation of the starch

The water soluble oxidized starches were synthesized by TEMPO-mediated system[110] (Scheme 2). Briefly, 10 g of dried starch was suspended in 400 ml of purified water and heated at 95 °C for one hour. The suspension was cooled down to room temperature (R.T.) and TEMPO (4 mg/g starch) was added. The appropriate amount of sodium hypochlorite solution was added slowly over 2 h. During the addition of the sodium hypochlorite solution, pH of the reaction mixture was controlled to 8.5 by adding 1 M sodium hydroxide. After the addition of the sodium hypochlorite, sodium borohydride was slowly added (Scheme 3). The resulting solution was stirred overnight and then purified by ultrafiltration (Vivaflow200: 5 kDa Hydrosart membrane, Sartorius Stedim Biotech GmbH, Goettingen, Germany). Purified water was used for washing. The product was dried by lyophilization (Alpha 2-4, Martin Christ GmbH, Osterode, Germany).



Scheme 2: Mechanism of TEMPO-mediated oxidation

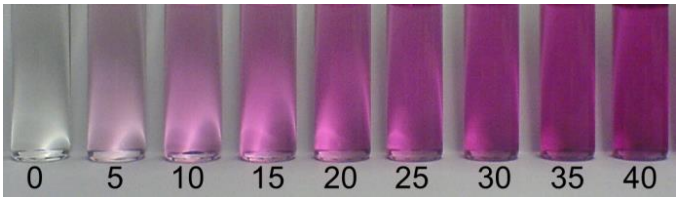


Scheme 3: Starch oxidation

3.2.3 Fractionation and characterization of the oxidized starch

Oxidized starch was further separated into three fractions depending on its MW using an Ultrafiltration system with purified water (Vivaflow200: 30 kDa Hydrosart membrane, 100 kDa Polyethersulfone membrane, Sartorius Stedim Biotech GmbH, Goetingen, Germany). Every fraction was collected, lyophilized and characterized by gel permeation chromatography (GPC, HLC-8320GPC, Tosoh, Japan), equipped with online viscometer (ETA-2010, PSS, Germany), on SUPREMA 1000 and 30 columns (PSS, Germany) at a flow rate 1 mL/min at 35 °C in 1 M Sodium nitrate. Pullulan standards were used for the universal calibration. The degree of substitution by sodium carboxylate (DS_{COONa}) was determined by a colorimetric assay according to Blumenkrantz et al.[111]. A Calibration curve was made using glucuronic acid (Figure 10) and used to calculate the DS_{COONa} of the each oxidized starch.

(a)



(b)

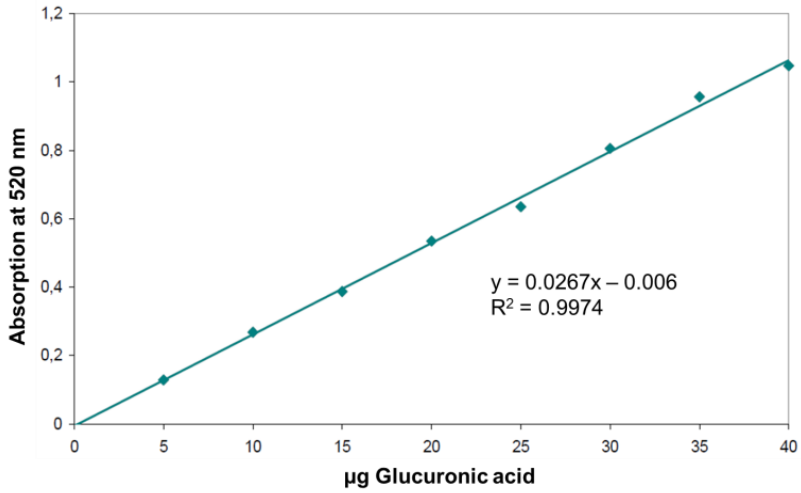


Figure 10: Calibration of the Blumenkrantz Assay, modified from ref.[112]

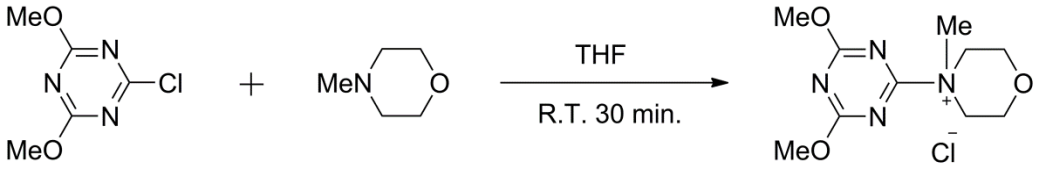
(a) Blumenkrantz solution containing different amount (µg) of glucuronic acid

(b) Calibration curve and its coefficient of determination (R^2)

3.2.4 Synthesis of 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4methyl-morpholinium Chloride (DMTMM)

The synthesis of DMTMM was done according to Kunishima et al.[113] (Scheme 4). Briefly, NMM (5.05 g, 50 mmol) was added to a solution of CDMT (9.65 g, 55 mmol) in THF (150 mL) at room temperature. A white solid appeared within several minutes. The solution was further stirred for 30 min and the white precipitation was collected by filtration. The product was washed with THF three times and dried under vacuum at 25 °C for 36 h.

The characterization of the DMTMM was done by ^{1}H-NMR. Bruker NMR Magnet System 400 MHz Ultra shield plus: in D_2O, δ_H DMTMM: 4.56 ppm (m, 2H), 4.09 ppm (m, 2H), 3.89 (m, 4H, N- C***H*****$_2$**-C***H*****$_2$**-O), 4.20 ppm (s, 6H, O- C***H*****$_3$**,), 3.56 ppm (s, 3H, N+-C***H*****$_3$**).

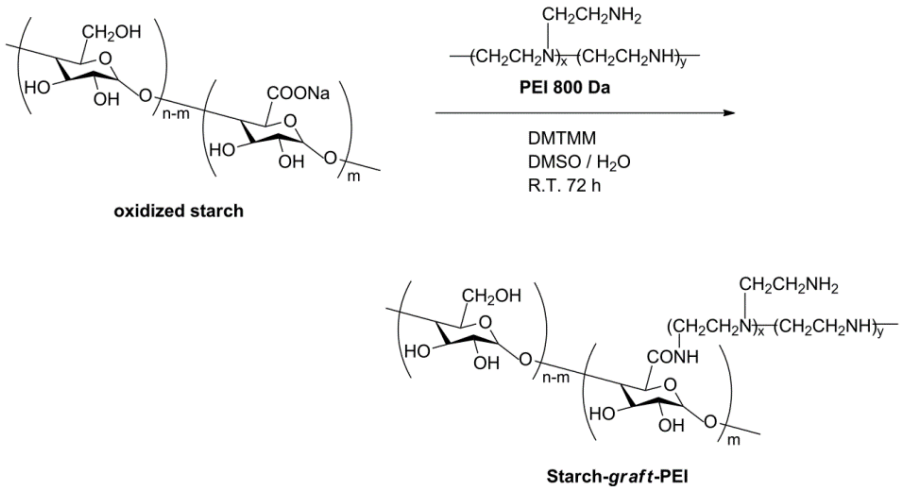


Scheme 4: Synthesis of DMTMM

3.2.5 Conjugation of oxidized starch and PEI (Starch-*graft*-PEI) (Scheme 5)

The reaction between sodium carboxylate groups of starch and amine groups of s-PEI was done according to Kunishima et al.[113]. Briefly, starch (500 mg, 0.57 mmol sodium carboxylate) was solubilized in 50 mL of solvent mixture (1 : 4 = purified water : DMSO) in a round-bottomed flask followed by the addition of 0.68 mmol conjugation reagent, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4methyl-morpholinium Chloride (DMTMM), while stirring. The reaction mixture was stirred for 30 min. in R.T. to activate the sodium carboxylate groups.

Subsequently, 17 mmol of s-PEI in 100 mL of solvent mixture (1 : 4 = purified water : DMSO) was slowly added. The reaction was proceeded with stirring for 72 h at R.T. and extensively dialyzed (3.5 kDa Mw cut-off, regenerated cellulose membrane, Fisher scientific) against purified water. The water insoluble white precipitation was removed by filtration and the reaction solution was collected and lyophilized. The complete purification (no-existence of unreacted s-PEI) was confirmed by GPC measurement. The amount of s-PEI modification (wt%) was determined by ^{1}H-NMR. Bruker NMR Magnet System 400 MHz Ultra shield plus: in D_2O, δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), PEI: (NH_2-C***H₂***-C***H₂***, 2.20 – 3.45 ppm). The purity of starch-*g*-PEI was examined by GPC measurement (HLC-8320GPC, Tosoh, Japan) on SUPREMA-MAX 1000 and 30 columns (PSS, Germany) at a flow rate 1 mL/min at 35 °C in 1 % formic arid.



Scheme 5: Conjugation reaction between starch and PEI 800 Da

3.3 Results and discussion

3.3.1 Oxidation of the starch

The oxidized water-soluble starch formed the precursor of the starch modification with s-PEI.The selective C6 position of COONa groups produced in this reaction, was later conjugated with amine groups of s-PEI (Scheme 1). Just after the oxidation procedure, the starch was polydisperse in nature and showed very broad peak in the gel permeation chromatography (GPC) measurement (Polydispersity: PD = 5 - 6). The oxidized starch was therefore separated into three different fractions, on the basis of MW, as this was one of the parameters selected for optimization in order to tailor the characteristics of the final product. The three fractions each showed a narrower PD than non-separated starch, proving that the separation worked successfully (Figure 11). Following starch oxidation and fraction separation, the degree of substitution (DS_{COONa}) was determined by the Blumenkrantz Assay[111]. The DS could be well controlled to the desired value via changing the amount of sodium hypochlorite used for the reaction. Three different DS_{COONa} were produced, of approximately 10 %, 20 % and 40 %. The MW, PD and DS_{COONa} of oxidized water-soluble starches before and after separation into three fractions are summarized in Table 3.

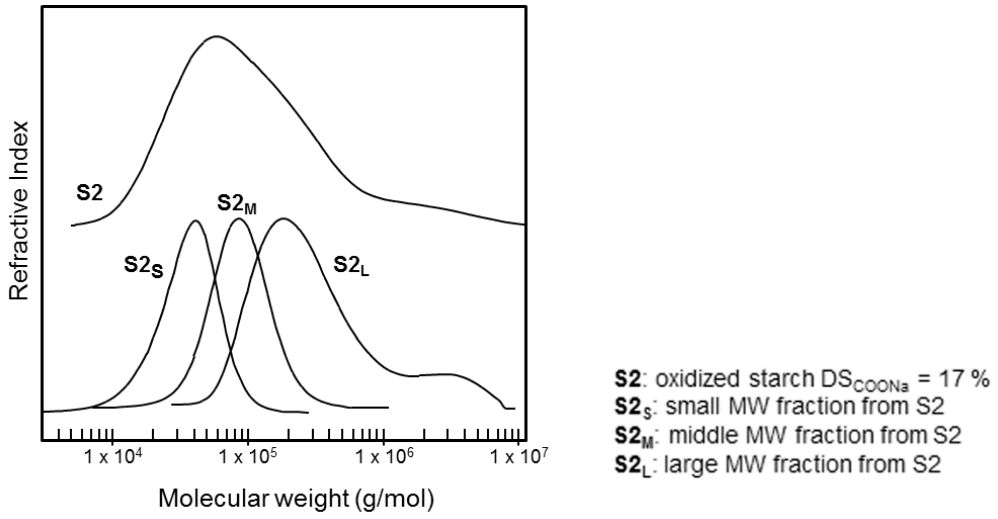


Figure 11: GPC spectra of oxidized starch and three different MW fractions

Table 3: Characteristics of oxidized starch and different MW fractions

Starch code	M_W (g/mol)	PD	DS_{COONa} (%)
S1	4.7×10^5	5.16	10.7
$S1_S$	3.0×10^4	1.75	13.5
$S1_M$	9.6×10^4	1.65	11.2
$S1_L$	7.6×10^5	3.14	11.5
S2	3.2×10^5	5.57	16.5
$S2_S$	4.1×10^4	1.38	18.9
$S2_M$	1.0×10^5	1.34	18.7
$S2_L$	6.9×10^5	3.24	17.0
S3	3.7×10^5	5.31	40.8
$S3_S$	5.1×10^4	1.13	40.6
$S3_M$	1.2×10^5	1.20	41.1
$S3_L$	5.8×10^5	2.37	43.9

M_w and PD were determined by GPC with universal calibration.
DS_{COONa} was determined by Blumenkrantz-assay.

3.3.2 Synthesis and characterization of the DMTMM

DMTMM is insoluble in THF while both CDMT and DMTM are soluble. This makes isolation of DMTMM simple when it is prepared in THF. The characterization of the DMTMM was done by ^{1}H-NMR in D_2O, δ_H DMTMM: 4.56 ppm (m, 2H), 4.09 ppm (m, 2H), 3.89 (m, 4H, N- C***H*****$_2$**-C***H*****$_2$**-O), 4.20 ppm (s, 6H, O- C***H*****$_3$**,), 3.56 ppm (s, 3H, N+-C***H*****$_3$**). The ^{1}H-NMR spectrum of Figure 12 showed the successful synthesis and purification of the DMTMM. Additional, the data of the elemental analysis also showed the successful synthesis of the DMTMM. Anal. Calcd. (%) for $C_{10}H_{17}ClN_4O_3$: C, 43.40; H, 6.19; N, 20.25. Found: C, 43.97; H, 5.95; N, 20.20.

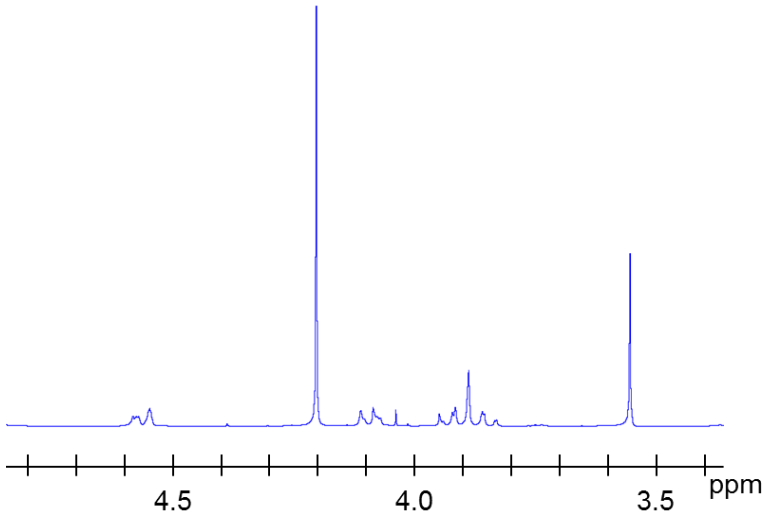


Figure 12: ^{1}H-NMR spectrum of the synthesized DMTMM
in D_2O, δ_H DMTMM: 4.56 ppm (m, 2H), 4.09 ppm (m, 2H), 3.89 (m, 4H, N- C***H*****$_2$**-C***H*****$_2$**-O), 4.20 ppm (s, 6H, O- C***H*****$_3$**,), 3.56 ppm (s, 3H, N+-C***H*****$_3$**).

3.3.3 Conjugation of oxidized starch and PEI (Starch-*graft*-PEI)

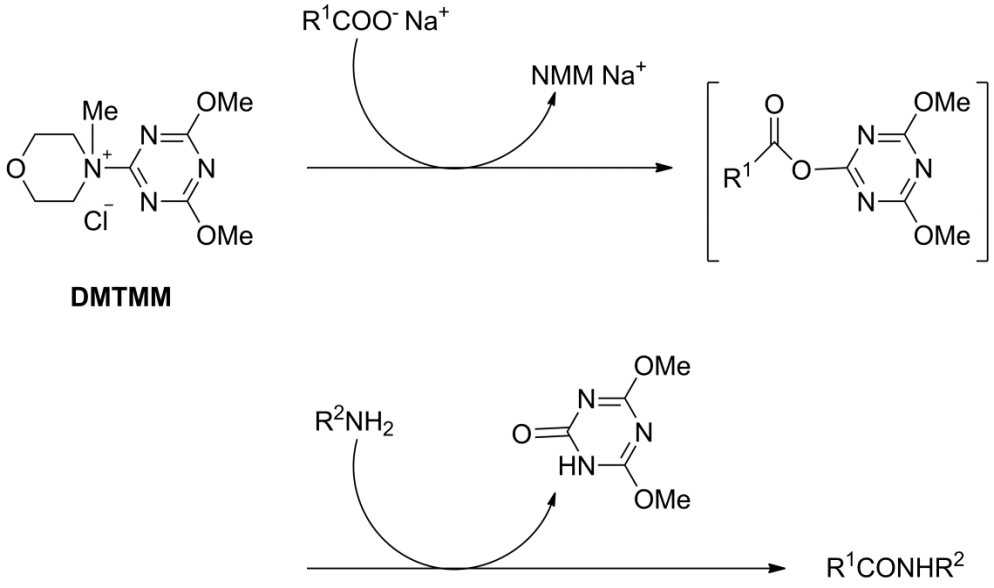
Modification of starch has generally been performed with small molecules such as acetic anhydride, in order to form acetylated starch[114]; ethylene oxide, to produce hydroxyethyl starch[115]; or, more recently, with a carbonyldiimidazole activation system, resulting in cationic

starch[109]. Modification with larger or more complex molecules is not an easy task because of the steric inaccessibility of the molecules to the reaction site of the starch. Therefore certain linkers[116,82] are often used as an intermediate product to introduce such molecules, in a process which needs at least two reaction steps. Cui et al.[82] used a biocleavable acetal linker to modify dextran with amine functionalized molecules and synthesized a smart gene delivery vector. Dialdehyde starch[108,117] is also often used as an intermediate product and has been reported to result in a successful modification. However, the drawback of using dialdehyde starch as an intermediate material is that the glucose ring would be opened and the key characteristic of the starch (e.g. enzymatic biodegradability) as a result would be lost. Since we aimed to synthesize an enzymatically-degradable starch-based polymer and to keep the original structure of the starch as much as possible, using dialdehyde starch was not the best option. Consequently, we aimed to conjugate the C6 position of COONa groups (which, as mentioned above, were made in the starch oxidation process) directly with amino groups of the s-PEI.

The conjugation reaction between carboxylic acid groups of starch (ion exchanged from COONa) and amine groups of s-PEI was first tried using the DCC/HOBt and EDC/NHS conjugation reagent. However, despite many trials of this reaction and altering reaction conditions such as used solvent, temperature and time, an effective reaction was not achieved. A successful conjugation product was however obtained using DMTMM as a reaction reagent in accordance with Kunishima et al.[113]. DMTMM is a water-soluble reaction reagent, which is easy to handle and is also suitable for carboxylate conjugation. It was synthesized by the reaction of CDMT and NMM (Scheme 4). The optimal reaction conditions for conjugation were found to be the use of 1.2 times excess of DMTMM against COONa groups, in the mixed solvent of water: DMSO = 1 : 4, at room temperature for 72 hours.

The reaction is thought to be initiated by addition of a carboxylate anion of starch to DMTMM to give an activated ester, which undergoes attack by an amine of s-PEI to give the amide bond (Scheme 6). The strength of this DMTMM conjugation reaction is; a) stronger activation system of carboxylate groups that is needed for the successful conjugation, b) DMTMM is easily removable after the reaction by washing with water, and c) through the mild reaction

condition at R.T. the side reaction possibly occurred in higher temperature (e.g. degradation of the starch that is occurred in case of DCC/HOBt at 50 °C for 72 h) can be avoided.



Scheme 6: Mechanism of conjugation reaction using DMTMM reaction reagent

3.3.4 Characterization of the synthesized Starch-*graft*-PEI polymers

The amount of s-PEI bound on the starch backbone and the total MW were calculated from the results of ^{1}H-NMR, based on the integration ratio of 1H of starch (5.1 – 5.6 ppm) and s-PEI protons (2.20 – 3.45 ppm). The higher the degree of modification with s-PEI, the higher was the observed s-PEI-derived proton peak at 2.2 – 3.45 ppm (Figure 13). Approximately 20, 30 or 35 wt% of s-PEI was introduced, depending on the DS_{COONa} of the starch backbone. In 35 wt%, every 9th glucose carries a s-PEI side chain, while in 20 wt% only every 19th glucose ring is modified.

The higher degree of modification with s-PEI was the more difficult to perform, because of the steric hindrance. Formation of a water-insoluble white precipitate was observed more frequently in the reaction involving the larger MW starch backbone with higher DS_{COONa}. This byproduct was therefore likely to be large MW conjugates of starch and s-PEI that became

insoluble in water, and as such, these were removed by centrifugation. As a result, the final yield of 30 wt% modified starch-g-PEI was 40 to 60 %.

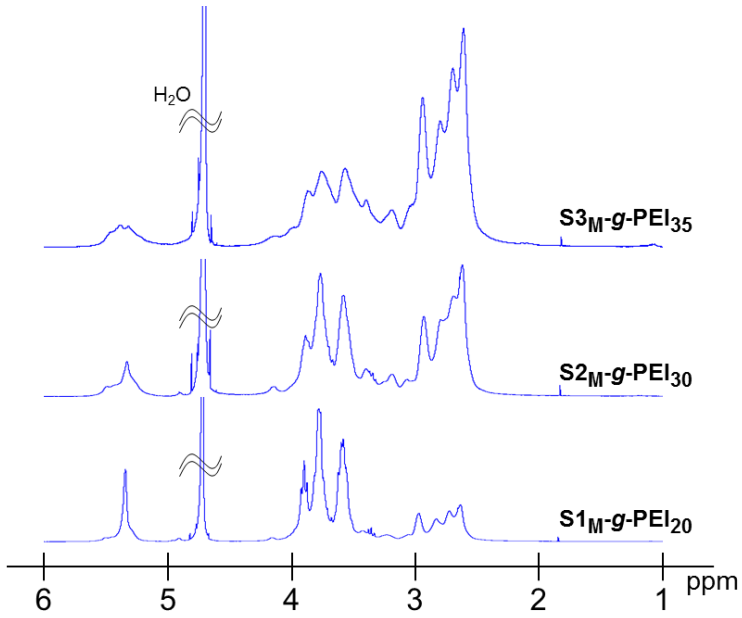


Figure 13: ^{1}H-NMR spectra of synthesized starch-*g*-PEI polymers in D_2O, δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), PEI: (NH_2-C***H*****$_2$**-C***H*****$_2$**, 2.20 – 3.45 ppm).

In the FT-IR measurement, no absorbance of COONa (1604 cm^{-1}) was observed in the starch-g-PEI, confirming that all the COONa groups had been converted (Figure 14). However, the amount of s-PEI calculated from the results of ^{1}H-NMR was lower than the expected value. This difference allows for estimation of the cross-linking of starch and s-PEI, possible as both species have several reactive sites of sodium carboxylate and amino groups, respectively. Calculated from the ^{1}H-NMR results, one s-PEI molecule reacted with 1.7 - 3.2 COONa groups on the starch backbone.

In the following, polymers are named with a systematic name, according to their DS_{COONa} (S1 = 10%; S2 = 20% and S3 = 40%) and the MW of starch backbone. The subscript letter S stands for small MW=30-50 kDa; M for medium = 100-120 kDa; L for large = 500-800 kDa.

The polymer without the subscript letter indicate the non-fractionated starch (MW=30-800 kDa). The subscript letter PEI stands for the modification amount (wt%) with s-PEI.

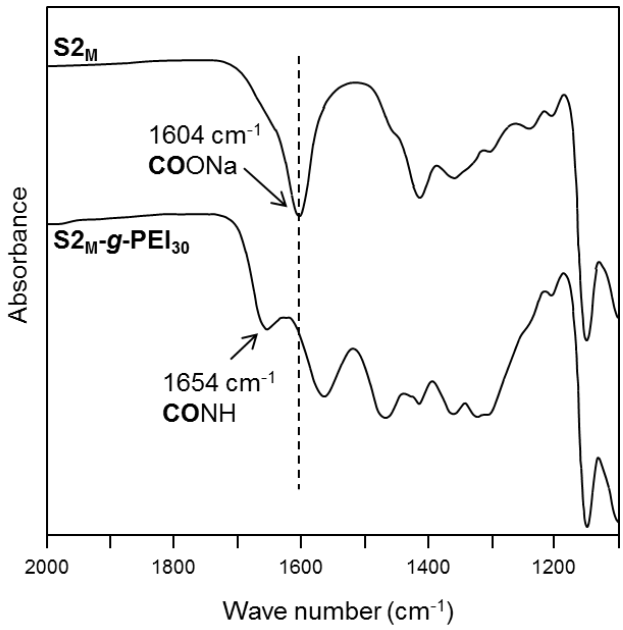


Figure 14: IR spectra of the starch: before and after the PEI modification

In the GPC measurement of starch-*g*-PEI polymers (Figure 15), the product peaks were observed to be mono-modal and PD values were low. These monomodal peaks and low PD values showed that the cross-linking was not intermolecular, but rather intramolecular. Intermolecular cross-linking might have also occurred, but it is reasonable to consider that the product of such intermolecular cross-linking would have an extremely large molecular weight; as such it was likely removed during the modification/purification process as part of the previously mentioned water-insoluble white precipitate.

Another important point of the GPC profile is that the product peaks were shifted in the direction of larger MW as compared to the pure s-PEI, which implies a successful conjugation reaction. Additionally, no peaks corresponding to the free s-PEI was observed in the conjugated products, which proves the successful purification and removal of excess s-PEI

from the reaction mixture. It can also be seen that each product has a different MW, depending on the MW of the starch backbone which was used. Therefore, the MW of the products can be controlled by changing the MW of the starch backbone.

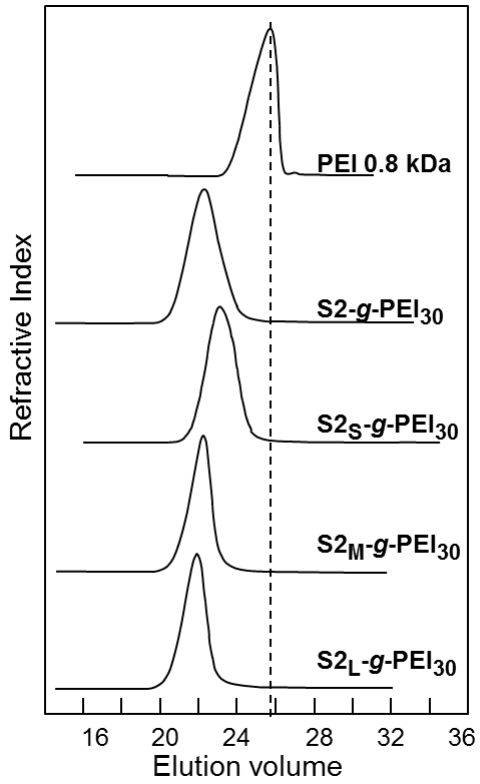


Figure 15: GPC spectra of the starch-*graft*-PEIs with different MW starch backbones and s-PEI

Summarizing the results of polymer synthesis, starch-*g*-PEIs were successfully synthesized by the precise control of two parameters: MW of the starch backbone and modification amount with s-PEI. The detailed characteristics of synthesized polymers are summarized in Table 4.

Table 4: Characteristics of the synthesized Starch-*g*-PEI polymers

Starch-*graft*-PEI	Total MW (g/mol)	PEI amount (wt.%)
S1-*g*-PEI_{20}	6.1×10^5	22.7
$S1_S$-*g*-PEI_{20}	3.8×10^4	20.4
$S1_M$-*g*-PEI_{20}	1.2×10^5	20.9
$S1_L$-*g*-PEI_{20}	9.6×10^5	19.2
S2-*g*-PEI_{30}	4.7×10^5	32.2
$S2_S$-*g*-PEI_{30}	6.0×10^4	32.0
$S2_M$-*g*-PEI_{30}	1.5×10^5	32.8
$S2_L$-*g*-PEI_{30}	9.5×10^5	27.4
S3-*g*-PEI_{35}	6.0×10^5	37.3
$S3_S$-*g*-PEI_{35}	8.0×10^4	35.7
$S3_M$-*g*-PEI_{35}	1.8×10^5	37.5
$S3_L$-*g*-PEI_{35}	8.3×10^5	30.4

Mw was determined by ^{1}H-NMR.

3.4 Conclusion

For the gene delivery application, as a non-viral gene delivery carrier, we have successfully synthesized cationic polymer composed from potato starch and s-PEI. The conjugation reaction of the starch and s-PEI was tested using different conjugation reagents, DCC/HOBt, EDC/NHS and DMTMM. Through the DCC/HOBt and EDC/NHS showed no conjugated product, DMTMM provided the conjugated product. The successful reaction was observed using the DMTMM reaction reagent. The optimal reaction conditions for conjugation were found to be the use of 1.2 times excess of DMTMM against COONa groups, in the mixed solvent of water: DMSO = 1 : 4, at room temperature for 72 hours. The optimized reaction condition was milder than the reaction condition of DCC/HOBt or EDC/NHS, and did not cause degradation of the starch backbone. Additionally the removal of the DMTMM after the reaction was easily possible by washing with water. By using DMTMM reaction reagent, we were successful in the introduction of effective and easy to handle reaction conditions.

Two parameters, the MW of the starch backbone and the modification amount of s-PEI, were closely controlled and a series of starch-*g*-PEIs with different characteristics was successfully obtained (Figure 16). The synthesized starch based cationic polymers with different characteristics are worth to study their ability as a non-viral gene delivery vector. A systematic study of all the polymers shown in Figure 16 as gene delivery carriers, could help to understand the impact of MW and amount of cationic charge on transfection efficacy and safety.

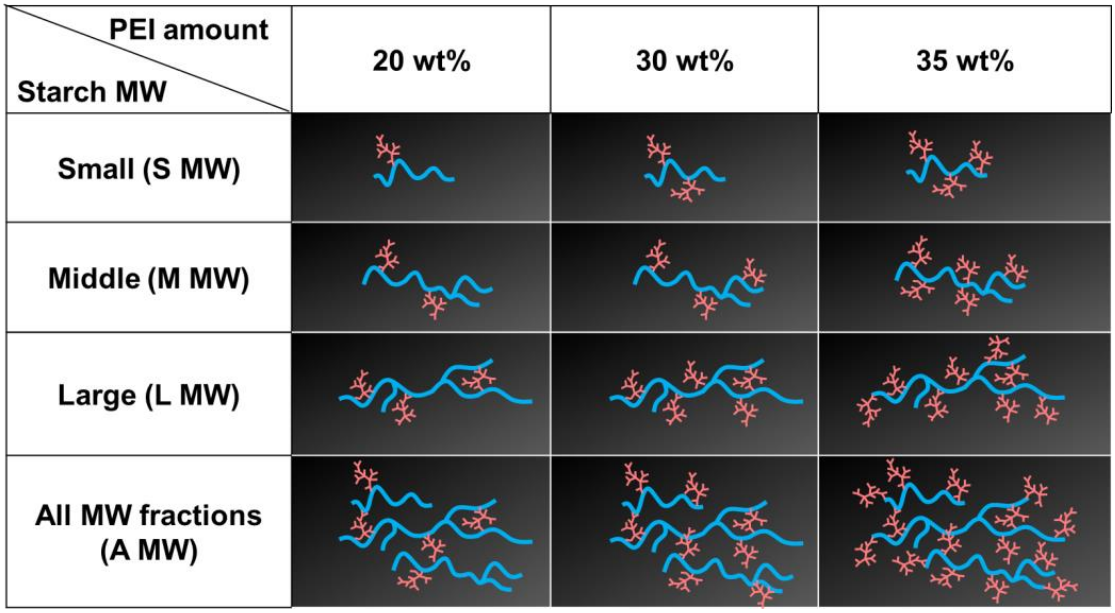


Figure 16: Scheme depicting the synthesized Starch-*g*-PEI polymer series

4 Evaluation of the Starch-*graft*-PEI polymers as gene delivery vector

The data presented in this chapter have been published in parts as a research article in:

Biomaromolecules **2014**, *15* (5), pp 1753–1761

Design of Starch-*graft*-PEI Polymers: An Effective and Biodegradable Gene Delivery Platform

Publication Date (Web): March 31, 2014 (Article)

DOI: 10.1021/bm500128k

Yamada H., Loretz B., Lehr C. M.

4.1 Introduction

The implementation of gene delivery in a clinical setting is an area of significant interest. This is due on the one hand to its many prospective applications. A large number of human diseases such as cancer, cardiac disorders and neurodegenerative diseases are attributable to a distinct genetic defect. Prophylactic (e.g. vaccination[118]) or therapeutic strategies which do not rely on complete transfection of target cells could also profit from transgene expression[119]. On the other hand, nucleic acid-based actives are becoming more and more advanced. The last decade brought programs for nucleotide sequence optimization, base modifications for improved stability and the use of mRNA for faster translation. As a result of such advances, the same delivery efficacy is now capable of producing a higher therapeutic effect. For gene delivery technologies this reduces the need for complete transfection. However, the safety of any such technology remains of utmost importance. As a consequence, recent research pays more attention to the safety and biodegradability of the transfection agents.

This chapter reports the study of the starch-*g*-PEI polymers synthesized in chapter 3 (Figure 16) as gene delivery vectors, focusing on their ability to show high transfection efficiency and safety. This study was driven by the hypothesis (Figure 17) that it should be possible to synthesize a safer (less cytotoxic and biodegradable) polymer which retains transfection efficiency by grafting of s-PEI to a suitable biopolymer.

Thus, our approach was to use water-soluble starch, oxidize it as a first step for more controlled conjugation, and subsequently modify it with just enough s-PEI (Scheme 1) to achieve a comparable transfection efficacy to b-PEI. The transfection-effective starch-*g*-PEI polymers were then tested for cytotoxicity and enzymatic degradability. By testing the starch-*g*-PEI polymer series with different MW of the starch and modification amount of s-PEI, we were able to identify some characteristics that the cationic polymer should possess as a superior gene delivery vector.

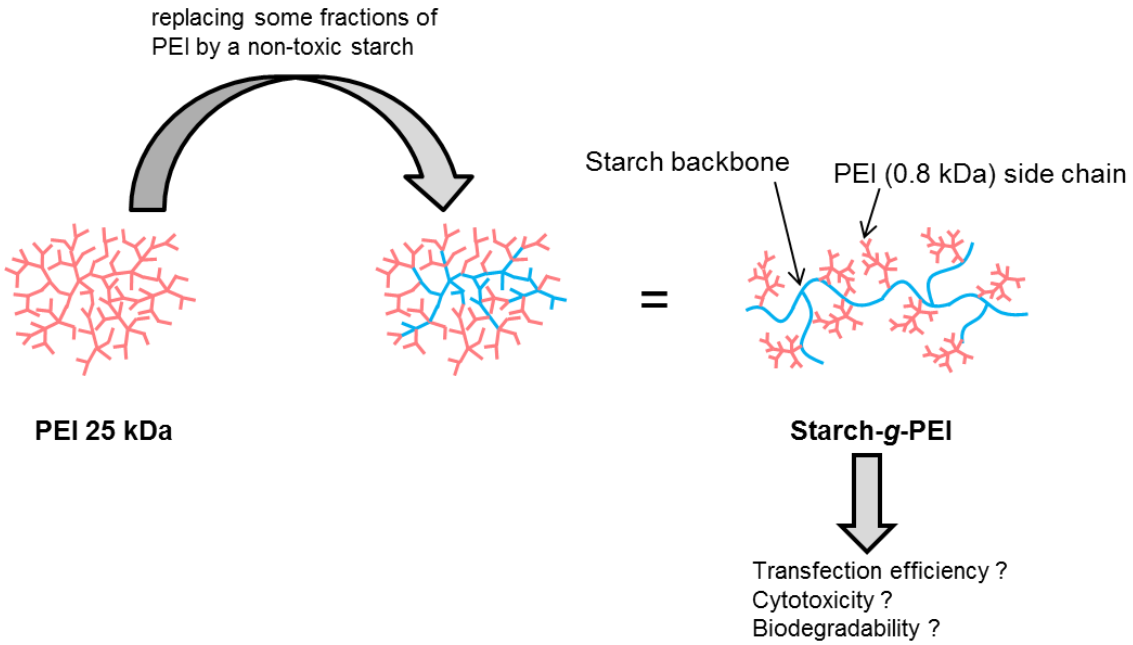


Figure 17: Scheme of the working hypothesis of replacing PEI partially to non-toxic starch backbone

4.2 Experimental Methods

4.2.1 Materials

A plasmid DNA (pDNA) encoding the modified firefly luciferase was bought from Promega (pGL3-basic vector), and another pDNA encoding the fluorescent protein AmCyan was bought from Clontech (pAmCyan1-C1). Plasmids were propagated in E. coli DH5α competent cells (Invitrogen), isolated and purified using Qiagen endotoxin-free plasmid Maxi kits (Qiagen) as per manufacturer's protocol. The quantity and quality of purified pDNA were evaluated by spectrophotometric analysis at 260 and 280 nm. Purified pDNA was suspended in purified water and stored at -20 °C until use. A transfection reagent, jetPRIME^{TM} was purchased from Polyplus transfection (Illkirch, France). A549 cells No ACC 107 were purchased from DSMZ GmbH (Braunschweig, Germany). Cell culture medium (RPMI 1640) was purchased from PAA Laboratories GmbH (Pasching, Austria) and fetal bovine serum (FBS) was purchased from Lonza (Basel, Switzerland). The Luciferase assay kit was purchased from Promega (WI, U.S.A.). The BCA Protein Assay Reagent Kit was purchased from Sigma-Aldrich.

4.2.2 Polyplex formation

The stem polymer solutions were made by solubilizing the polymers in purified water in the concentration of 1 mg/mL and stored at -20 °C until use. The stem aqueous solution of each polymer and pDNA were further diluted with purified water to the suitable concentration and incubated for 10 min at 50 °C. An appropriate amount of pDNA (N/P = 10, 12, 14 and 16: ratio between nitrogen of polymer and phosphate of pDNA) was added to polymer solution. The amount of N was calculated based on the weight of s-PEI side chain. If the X g of s-PEI side chain is contained in starch-*g*-PEI, the mol of N atom was calculated by X / 43. Here, 43 is the molecular weight of the PEI repeating units, $-CH_2CH_2NH-$. After the addition of pDNA, solution was immediately mixed for 10 seconds by vortex and incubated for 30 min at R.T.

4.2.3 Polyplex characterization

The characterization (size, particle homogeneity and zeta potential) of polyplexes was studied by Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He–Ne laser employing a wavelength of 633 nm and a backscattering angle of 173° at 25 °C. Polyplexes were analyzed as produced, without further dilution. The reported size is the z-average diameter (intensity based) of 3 measurements.

4.2.4 Transfection experiments

Transfection efficiency was evaluated with A549 cells. A549 cells were maintained in RPMI cell culture medium supplemented with 10 % (v/v) FBS at 37 °C in 5 % CO_2.

4.2.4.1 Luciferase transfection assay

A549 cells were seeded in a 24-well plate at a density of 25,000 cells per well in 500 µL of the cell culture medium. Polyplexes with luciferase pDNA (pGL3) in Hank's Balanced Salt Solution (HBSS buffer pH 7.4, 500 µL/ well) were added on the cells. In all the experiments the amount of pDNA was kept constant at 1 µg pDNA/well.). After 4h of incubation the polyplex solution was replaced by cell culture medium and the cells were further incubated for 48 h. Luciferase gene expression was evaluated using a Luciferase assay kit from Promega and the relative light units (RLU) were measured by Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany). Protein quantification was performed with BCA assay kit at 570 nm. The transgene expression was normalized for the protein content. All experiments were conducted in duplicate.

4.2.4.2 Flow cytometry

The amount of transfected cells was determined by flow cytometry. A549 cells were seeded in a 12-well plate at a density of 50,000 cells per well in 1 mL of the cell culture medium. Polyplexes with pDNA encoding the fluorescent protein AmCyan in HBSS buffer pH 7.4 were

added on the cells (2 µg pDNA in 1 mL HBSS buffer pH 7.4 / well), and incubated 4 h. After the transfection, the polyplex solution was replaced by cell culture medium and followed by further 48 h of incubation. The cells were washed three times with PBS, collected and centrifuged at 1,400 rpm for 4 min. The resulting cell pellet was suspended in 500 µL of cell culture medium and analyzed by flow cytometry, with excitation by argon laser at 488 nm and emission through the 515 – 545 nm filter. 10,000 cells per sample were analyzed using BD FACSCalibur™ (Becton-Dickinson, Heidelberg, Germany). The percentage of cell-associated fluorescence was determined using the computer program FlowJo (version 7.2.5, Tree Star, Stanford, CA). All experiments were conducted in duplicate.

4.2.4.3 CLSM observation

The images of the transfected A549 cells were taken using CLSM. A549 cells were seeded in a 24-well plate at a density of 25,000 cells per well in 500 µL of the cell culture medium. Polyplexes with pDNA encoding the fluorescent protein AmCyan in HBSS buffer pH 7.4 were added on the cells (1 µg pDNA in 1 mL HBSS buffer pH 7.4 / well), and incubated 4 h. Following the incubation, the polyplex solution was replaced by cell culture medium and incubated for further 48 h. The cells were washed with PBS, incubated for 10 min at 37 °C with rhodamine ricinus communis agglutinin solution (0.625 µg in 500 µL PBS buffer / well) for a red fluorescent staining of the cell membrane (emission: 550nm, excitation: 575nm). Then the cells were washed three times with PBS buffer and fixed by 4 wt% paraformaldehyde (in PBS buffer, for 10 min at R.T.). The cells were further washed three times with PBS and the cell nuclei were stained with DAPI (emission: 461 nm, excitation: 374 nm) by the incubation for 5 min. at R.T. Finally the cells were washed twice with PBS buffer and investigated by CLSM (LSM 510 META, Carl Zeiss, Oberkochen, Germany), equipped with an argon/neon laser and a 63× water immersion objective. Images were captured using DAPI channel (excitation 360 nm, band pass filter 390–465 nm), rhodamine channel (excitation 488 nm, band pass filter 500–530 nm), and AmCyan channel (excitation 543 nm,

band pass filter 560–615 nm). The measurements and image analysis were performed using the Zeiss LSM 510 software.

4.2.5 Cytotoxicity studies

LDH and MTT assay were performed to study the *in vitro* cytotoxicity of the starch-*g*-PEI polymers and their complexes. The cytotoxicity of starch-*g*-PEI polymers after a 3h incubation with α-amylase was also investigated. The A549 cells were seeded in a 96-well plate at a density of 10,000 cells per well in 200 µL of the cell culture medium. Once confluent, polymers or their polyplexes in HBSS buffer pH 7.4 were applied on the cells and incubated for 4 h at 37 °C with gently shaking. As reference, HBSS buffer (negative, 0%) and 0.1% (w/w) Triton X-100 solution (ICN, Eschwege, Germany) (positive, 100%) in HBSS buffer were applied. To ensure the no interference of polyplexes to the test system, polyplexes without any cells were also tested. The cytotoxicity calculation and determination of LD50 values were performed using Sigmaplot Version 12.0.

4.2.5.1 LDH assay

After 4 h incubation of the above-mentioned 96-well plate, the 100 µL of incubated HBSS solution was transferred to another 96-well plate. The 100 µL of LDH test solution (Roche Cytotoxicity LDH kit) was added to each well and the plate was incubated in dark for 5 min at R.T. The absorbance at 492 nm was read by Tecan microplate reader.

4.2.5.2 MTT assay

After the 4 h incubation of the above-mentioned 96-well plate, the test solution was removed and cells were washed once with HBSS buffer. The fresh HBSS buffer with 10 % (vol.) of MTT reagent (5 mg/mL) was added, and incubated further 4 h at 37 °C with gently shaking. The solution was removed and cells were dissolved in DMSO and incubated 10 min at 37 °C

with gently shaking and protected from light. The absorbance at 550 nm was read by Tecan microplate reader.

4.2.6 Biodegradability study by α-amylase

Biodegradability study of starch-*g*-PEI was performed according to Xiao et al.[120]. Briefly, 40 µL of polymer solution (2 mg/mL in purified water) was added into 96-well plate. Then, 40 µL of α-amylase solution (1288 µunit/mL in 0.1 M phosphate buffer pH 7) was added to each well. The plate was then incubated for 15, 30, 45, 60, 75 and 90 min at 37 °C and the digestion was stopped by adding 25 µL of 1 M hydrochloric acid. Subsequently, 100 µL of iodide color reagent was added and the absorbance at 580 nm was read by Tecan microplate reader. Calibration curves were made concurrently with experiments for each sample. As a comparative study, water soluble starch without any modification (Merck, Product number: 101253) was also tested. All the experiments were conducted in triplicate. The remaining starch amount in the each sample was calculated using the calibration curve and plotted against the incubation time with α-amylase. The degradation rates (nmol/min) and R^2 values were calculated from the linear regressions.

4.3 Results and discussion

4.3.1 Polyplex formation

A polyplex is an electrostatic complex of negatively charged nucleotides (here pDNA) and positively charged polymers. Using the synthesized starch-*g*-PEIs with different characteristics, polyplexes were formed with pDNA. A polyplex with b-PEI (25 kDa) was also produced as a control, in accordance with Boussif et al[44]. (Nitrogen atom / Phosphate atom = N/P = 10). The size and zeta potential of polyplexes were measured using a Zetasizer (Malvern Instruments, Worcestershire, UK) (Table 5). In general terms, the polyplexes formed from the starch-g-PEI with a low amount of s-PEI modification (S1 series, 20 wt%) were bigger than the others and not uniform in their size distribution, as indicated by a high Polydispersity index (PDI) value. A possible explanation for this could be that s-PEI side chains were non-homogenously and insufficiently distributed in such a structure, preventing the formation of stable polyplexes. Starting from 30 wt% modification, all polymers were able to form polyplexes with relatively uniform size distributions and with mean sizes of 70 - 100 nm. It has been shown that the size of polyplexes plays a very important role for cellular uptake mechanisms in many cell lines. Mintzer et al.[121] stated in their review article that the optimal size of polyplexes based on cationic polymer-DNA is between 70 to 90 nm. The polyplexes from S2 and S3 series mostly fit in this size range, and were therefore expected to show good cellular uptake.

Table 5: Characteristics of the generated polyplexes (N/P = 14) with starch-*g*-PEI polymers

Starch-*graft*-PEI			Polyplex		
	Total MW (g/mol)	PEI amount (wt.%)	Size (nm)	PDI	ζ-potential (mV)
S1-*g*-PEI_{20}	6.1×10^5	22.7	110 ± 2.3	0.23 ± 0.02	$+24.0 \pm 0.5$
$S1_S$-*g*-PEI_{20}	3.8×10^4	20.4	110 ± 2.2	0.19 ± 0.01	$+25.5 \pm 0.6$
$S1_M$-*g*-PEI_{20}	1.2×10^5	20.9	109 ± 2.4	0.28 ± 0.01	$+27.2 \pm 1.1$
$S1_L$-*g*-PEI_{20}	9.6×10^5	19.2	147 ± 2.5	0.46 ± 0.01	$+31.6 \pm 0.4$
S2-*g*-PEI_{30}	4.7×10^5	32.2	81 ± 1.5	0.15 ± 0.01	$+29.9 \pm 0.8$
$S2_S$-*g*-PEI_{30}	6.0×10^4	32.0	77 ± 1.6	0.12 ± 0.02	$+26.7 \pm 1.0$
$S2_M$-*g*-PEI_{30}	1.5×10^5	32.8	78 ± 1.7	0.13 ± 0.02	$+ 31.9 \pm 1.5$
$S2_L$-*g*-PEI_{30}	9.5×10^5	27.4	89 ± 2.5	0.16 ± 0.01	$+ 28.6 \pm 0.8$
S3-*g*-PEI_{35}	6.0×10^5	37.3	82 ± 1.2	0.17 ± 0.02	$+21.4 \pm 0.2$
$S3_S$-*g*-PEI_{35}	8.0×10^4	35.7	112 ± 1.3	0.06 ± 0.02	$+14.0 \pm 0.4$
$S3_M$-*g*-PEI_{35}	1.8×10^5	37.5	76 ± 1.4	0.16 ± 0.01	$+ 17.5 \pm 1.8$
$S3_L$-*g*-PEI_{35}	8.3×10^5	30.4	94 ± 2.0	0.22 ± 0.00	$+ 22.0 \pm 0.4$

Mw was determined by 1H-NMR.
Size and PDI is analyzed by zetasizer. PDI = Polydispersity Index (homogenity 0 – 1)

4.3.2 Transfection experiments

The transfection efficiency of the polyplexes from synthesized polymers was studied in A549 cells. This AT-II cancer cell line is obtained from the alveolar region of the human lung and widely used for *in vitro* transfection experiments.

4.3.2.1 Luciferase transfection assay

A luciferase assay was used for the screening of transfection efficiency because of the high throughput and sensitivity of this assay. First, the transfection efficiencies using all the polymers were compared with that of b-PEI (Figure 18(a)). N/P = 10 was used for all polyplex formulations according to Boussif et al.[44]. As is evident from Figure 18(a) there is a clear difference in transfection between the different s-PEI modification amounts. The 30 wt% s-PEI modified samples showed the best transfection efficiency. This result indicates that a modification of more than 30 wt% would not help to increase the transfection efficiency further. A possible explanation for this would be that the higher density of cationic charges in 35 wt% s-PEI modified polymer can interact very strongly with pDNA and fail to release it efficiently in the cells. In Figure 18(b), the N/P ratio was optimized for the best transfection efficiency using the 30 wt% modified samples. Polymers with N/P = 14 showed the best transfection, and $S2_L$-*g*-PEI_{30} showed an even better transfection efficiency than b-PEI. The MW of the starch backbone can also be seen to play an important role. The larger the MW of the bakcbone, the higher transfection was observed. This result fits with the findings of Bishop et al.[122], who studied the influence of MW of poly(β-amino ester)s on transfection efficiency.

Based on the results presented in Figure 18, the 30 wt% s-PEI modified polymers, $S2_L$-*g*-PEI_{30}, $S2_M$-*g*-PEI_{30} and $S2_L$-*g*-PEI_{30}, were chosen as the three best candidates for gene delivery vectors and were further studied.

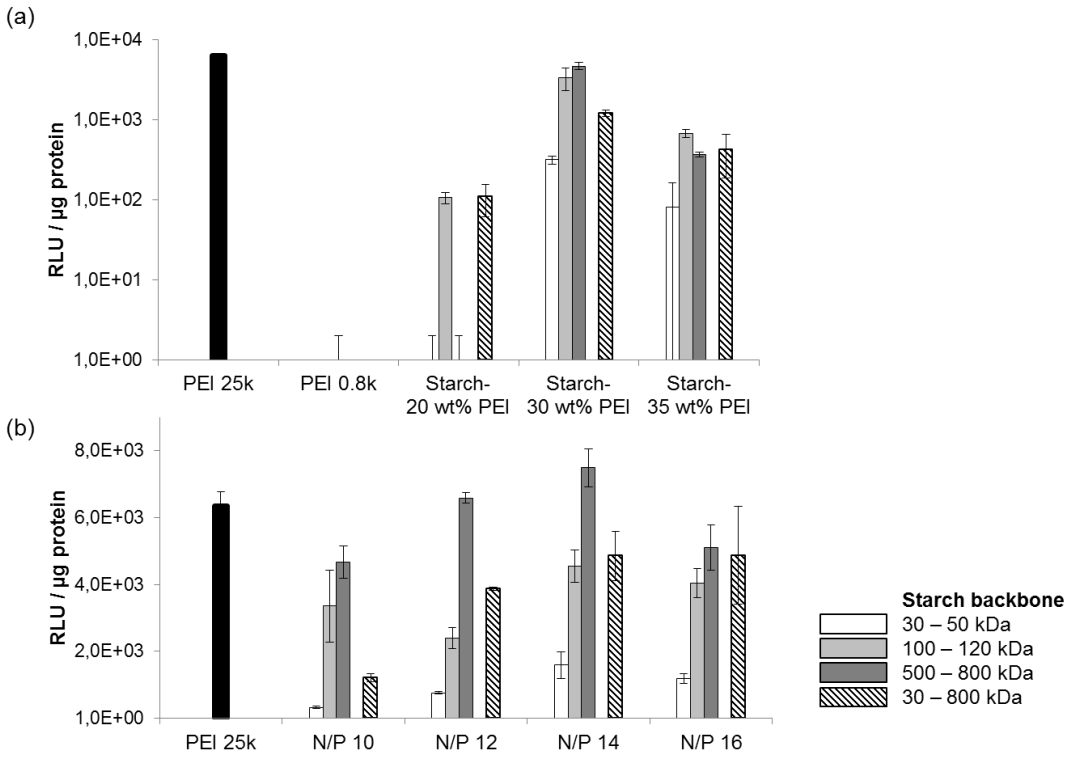


Figure 18: Results of the luciferase transfection assay.

(a) comparison of various wt% PEI modification amount against the b-PEI control; all at N/P = 10 ratio;

(b) optimization of N/P ratio with the 30 wt% series of starch-graft-PEI;

4.3.2.2 Flow cytometry

The amount of transfected cells was determined by flow cytometry (Figure 19). Compared with b-PEI (10.4 %), two starch-*g*-PEIs, $S2_M$-*g*-PEI_{30} (16.0 %) and $S2_L$-*g*-PEI_{30} (20.1 %), showed a superior number of transfected cells. The best tested sample, $S2_L$-*g*-PEI_{30} (20.1 %), showed almost double the percentage of transfected cells of b-PEI (10.4 %) and even gave a comparable result to that of a commercially-available positive control, jetPRIME™ (22.8 %). When comparing the three tested samples, the larger starch backbone showed the higher transfection efficiency (S2-*g*-PEI_{30} 6.0 %, $S2_M$-*g*-PEI_{30} 16.0 % and $S2_L$-*g*-PEI_{30} 20.1 %). This was the same tendency as observed in the luciferase transfection experiment, and verified that the MW of the starch backbone has an impact on the transfection efficiency. It can therefore be concluded that a starch backbone MW of approx. 100,000 g/mol is necessary for successful transfection.

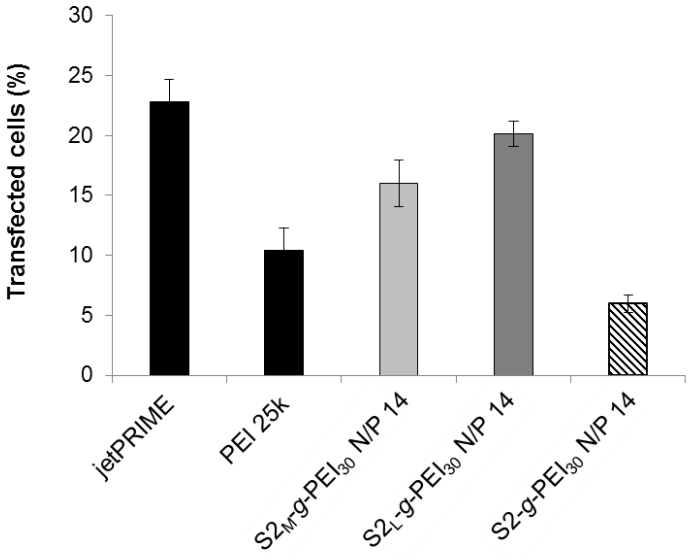


Figure 19: Results of the flow cytometry analysis of A549 cell transfection

4.3.2.3 CLSM observation

CLSM pictures were additionally taken for the optical confirmation of cellular transfection (Figure 20). The green fluorescence from successfully expressed pAmCyan proteins was observed in response to incubation of cells with the starch-*g*-PEI samples, as well as the positive controls, jetPRIME™ and b-PEI.

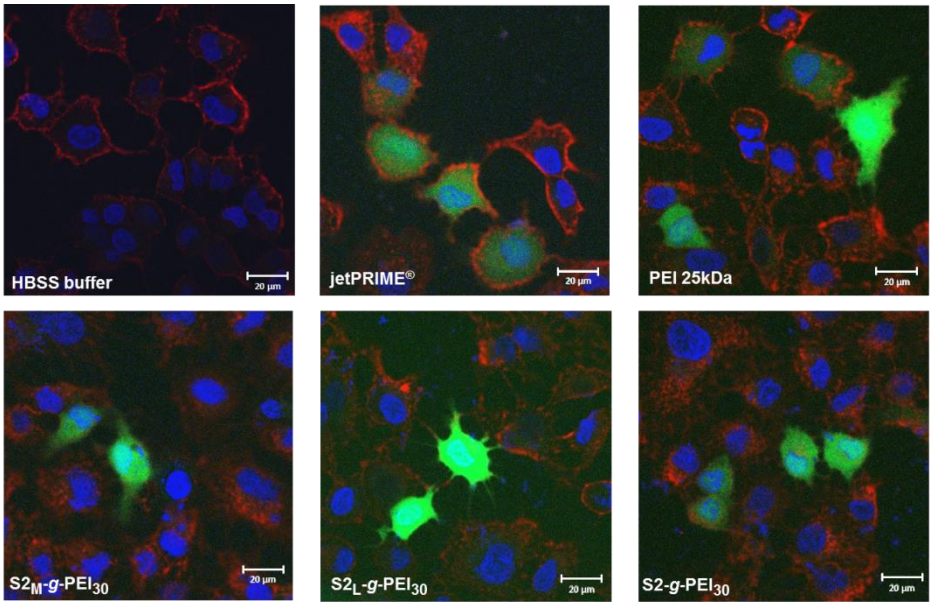


Figure 20: Confocal microscopy images of A549 cells transfected with pAmCyan using $S2_M$-*g*-PEI_{30}, $S2_L$-*g*-PEI_{30} and S2-*g*-PEI_{30} polymers.

green = AmCyan protein, red = RCA stained membrane, blue = DAPI stained nuclei.

4.3.3 Cytoxicity

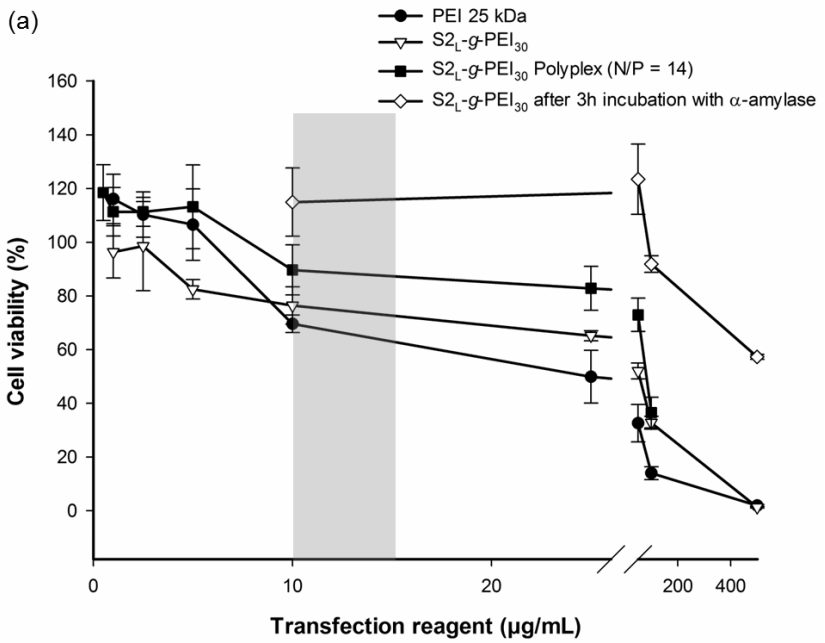
The cytotoxicity of the three best polymer candidates and their polyplexes was studied with MTT and LDH colorimetric assays. The MTT assay measure the metabolic activity of living cells by the formation of blue formazan crystals. The LDH assay, on the other hand, can determine cellular damage by measuring the amount of lactate dehydrogenase (LDH) released from cells that have a loss of membrane integrity.

In Figure 21 (a), the results of the MTT study of $S2_L$-*g*-PEI_{30} as a representative sample are shown. The gray area indicates the concentration needed for optimal transfection. In the case of all three polymers and their polyplexes, cell viability was more than 84 % at the concentration of successful transfection. From Figure 21 (a), it can be seen that the synthesized $S2_L$-*g*-PEI_{30} and its polyplex showed levels of cytotoxicity which were lower than PEI 25 kDa. The polyplex from $S2_L$-*g*-PEI_{30} showed less cytotoxicity than the pure polymer. This may be due to the decrease in cationic net charge in the polyplex as a result of the partial neutralization by polyplex formation with negatively charged pDNA. Furthermore, the most interesting observation is the greatly reduced cytotoxicity of $S2_L$-*g*-PEI_{30} after the 3 h incubation with α-amylase, which indicates degradation of the $S2_L$-*g*-PEI_{30}. It is supposed that, with the decreasing MW of the $S2_L$-*g*-PEI_{30} as a result of degradation, the multivalency of the polymer is also reduced. Correspondingly, the cytotoxicity greatly improves.

In Figure 21 (b), the calculated LD50 values of the three best starch-g-PEIs and b-PEI from the MTT and LDH study are summarized. The (A) values were calculated based on the exact weight of the polymer itself. Compared with the LD50 value of b-PEI, the starch-g-PEIs show a 3 - 6 fold lower level of cytotoxicity. Although, the (B) LD50 values, calculated based on the weight of the PEI part of polymers, should also be noted. As the synthesized polymers contain starch as well as s-PEI, (A) LD50 was normalized based on the s-PEI weight of the polymer to (B) LD50. Since the synthesized polymers have less cationic charges than b-PEI of the same weight, greater amounts (weights) of these polymers are required to get the best transfection efficiency. The polyplexes from starch-*g*-PEIs and b-PEI contain almost the same amount of PEI, and in that case there is almost no advantage in the cytotoxicity represented in (B) LD50.

The starch-*g*-PEIs only show an advantage with respect to cytotoxicity following the degradation of the starch backbone. Such degradation would result in small MW fragments of the glucose units and s-PEI; these are therefore the entities implied in the result of "$S2_L$-*g*-PEI_{30} after 3h incubation with α-amylase" in Figure 21 (a). A further biodegradability study of the synthesized polymers has been done in the following experiments.

(a)



(b)

LD50 (µg/mL)	MTT(A)	LDH(A)	MTT(B)	LDH(B)
$S2_M$-*g*-PEI_{30}	136	39	45	13
$S2_L$-*g*-PEI_{30}	112	62	31	17
S2-*g*-PEI_{30}	62	60	20	19
PEI 25 kDa	21	12	21	12

(A) Caliculated based on the weight of polymers
(B) Caliculated based on the weight of PEI part

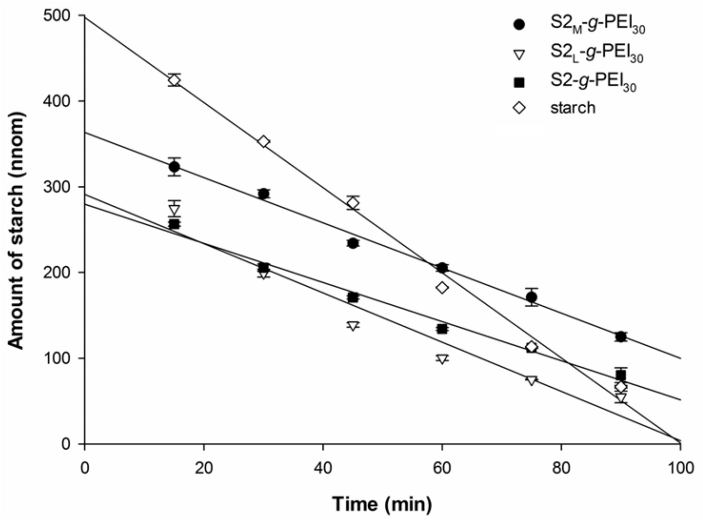
Figure 21: Results of the cytotoxicity tests (A549 cells)

(a) Results of MTT test with different transfection reagents (gray area shows the concentration needed for the best transfection efficiency). The cytotoxicity of $S2_L$-*g*-PEI_{30} is decreased after enzymatic degradation. (b) LD50s of S2 polymers and b-PEI

4.3.4 Biodegradability study by α-amylase

The biodegradability of the three best polymer candidates was studied by α-amylase using an iodine-based method[120]. As a comparison, water-soluble starch without any modification was also tested. It is known that α-amylase is less effective in degrading modified starches because of its molecular specificity. It was therefore of interest to test the degradability of our synthesized polymers, to determine whether they could still be degraded by the enzyme even after modification with s-PEI. In Figure 22 (a), the incubation time with α-amylase vs. the amount of remaining starch in nmol can be seen. Linear regression analysis of these profiles resulted in coefficient of determination (R^2) values close to 1 in all cases (Figure 22 (b)), confirming that the amount of starch in all samples declined in a linear manner over the 90 min experimental period. Thus, it is assumed that the enzyme was fully saturated and that degradation followed zero order kinetics. The calculated rates of the degradation of starch-*g*-PEIs were 1.7 – 2.2 times slower than that of unmodified starch, which is regarded as reasonable considering their modification with s-PEI side chains. Nevertheless, synthesized starch-*g*-PEIs do appear to be degraded by α-amylase, despite the fact that their degradation rate was slower than that of non-modified starch. This biodegradability of the synthesized polymers is a big advantage in terms of reducing cytotoxicity, as we have already observed in the MTT and LDH tests, since the starting materials of starch and s-PEI are virtually non-toxic. This benefit is expected to also be seen following repeated administration in vivo studies in the near future, since starch-*g*-PEIs can be degraded and excreted from the body.

(a)



(b)

	v (nmol/min)	R^2
$S2_M$-*g*-PEI_{30}	2.63 ± 0.02	0.9927
$S2_L$-*g*-PEI_{30}	2.87 ± 0.18	0.9390
$S2_M$-*g*-PEI_{30}	2.28 ± 0.09	0.9849
Starch	4.97 ± 0.07	0.9922

Figure 22: Biodegradability by α-Amylase over 90 min, at 37 °C

(a) incubation time with α-amylase vs. the amount of remaining starch in nmol

(b) rate and coefficient of determination of the starch-*g*-PEI degradation

4.4 Conclusion

The starch-*g*-PEIs, synthesized in Capter 3, could form nano-sized polyplexes with pDNA, some of which showed a very promising transfection efficiency in the A549 cell line. The successful transfection was observed both qualitatively and quantitatively, by luciferase assay and by flow cytometry and CLSM analysis of fluorescent protein-expressing cells. The transfection efficiency was shown to depend not only on the modified amount of s-PEI, but also on the MW of the starch backbone. The optimal combination was approximately 30 wt% of s-PEI modification onto a >100kDa MW starch backbone. The produced starch-graft-PEI polymers providing this combination, showed even higher transfection efficiency than b-PEI.

Furthermore, we demonstrated a lower cytotoxicity of the synthesized starch-*g*-PEI polymers than b-PEI. After the incubation process with α-amylase, the cytotoxicity of the starch-*g*-PEI polymer was dramatically decreased. It implied the starch-*g*-PEI polymer was degraded and became much less toxic small fragments. Additionally, the iodine-starch colorimetric assay proofed the biodegradability of the starch-*g*-PEI polymers by α-amylase more precisely.

In essence, our hypothesis, that replacing some fractions of b-PEI by a non-toxic starch would enhance the safety of the polymer while retaining the transfection efficiency, could thus be verified. The gained results demonstrate the advanced potential of newly developed starch-*g*-PEI polymers with superior characteristics as non-viral platform for gene delivery.

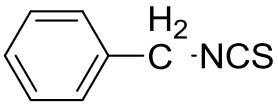
5 Hydrophobic modification of Starch-*graft*-PEI polymers

5.1 Introduction

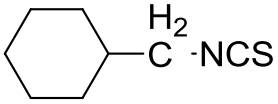
For the requirement to improve the transfection efficiency of non-viral gene delivery vector, the hydrophobic modification of PEI based polymer and other cationic polymers has been studied by several researchers[123]. However the effect of the hydrophobic chain to the transfection efficiency is still controversial. Some studies have shown that incorporation of hydrophobic chains can improve gene delivery efficiency[124,125,126], mainly explained by hydrophobic interaction conferred to the resulting amphiphilic polycation derivatives and by the enhanced cellular uptake by the hydrophobic chains via the lipophilic cell membrane. For example, Gabrielson et al.[126] acetylated PEI 25 kDa and obtained much higher (up to 58-fold) transfection efficiency in comparison to original PEI 25 kDa. With 57 % acetylation of primary amines of PEI gave the best transfection efficiency, though the buffering capacity of the hydrophobic modified PEI was decreased. On the other hand, some have shown the decreased transfection efficiency by introducing of the hydrophobic chains[127,128]. Masotti et al.[127] introduced many different hydrophobic chains (lauryl-, myristyl-, cetyl-, lauroyl-, myristoyl-, palmitoyl and poly-L-aspartic chains) onto PEI 25 kDa, and observed dramatically decreased transfection efficiency, though all the PEI derivatives were able to migrate into the cells. Doody et al.[129] also modified PEI (25 kDa) with acetate, butanoate and hexanoate and observed increasing and decreasing transfection efficiency compared to non-modified PEI. Over 25 mol% hydrophobic modification onto PEI caused decrease of the transfection efficiency. Overall, the different results (positive or negative) from the each study suggested that the gene delivery activity would be attributable to an appropriate balance of the hydrophobic groups and their introduced amount, which could alter important polymer characteristics as gene delivery vector, like buffering capacity and strength of polymer/DNA interactions.

In this chapter, we introduced different hydrophobic groups to the selected starch-*g*-PEI polymer, $S2_M$-*g*-PEI_{30} synthesized in chapter 3, and studied the effect of the hydrophobic modification on particle stability and transfection efficiency. Our hypothesis were; 1) balanced

amount of hydrophobic modification could help the stability and cellular uptake of the polyplexes, 2) excess of hydrophobic modification decreases the amine termini too much and have thus a negative effect. As the modification reaction, we have chosen the amine – isocyanate or isothiocyanate reaction. Isocynate groups have extremely high reactivity with amine groups through an exothermic reaction. At the same time, isocyanate group has a high reactivity with water. Since $S2_M$-*g*-PEI_{30} is soluble only in water, we needed to perform this reaction in the co-solvent system, H_2O/THF. Though isocyanate group has higher reactivity with amine group than water, this competing reaction would be a key factor of this modification. To avoid this competing reaction, isothiocyanate group was also used. Unlike isocyanate, isothiocyanate group is stable in water and it was expected that this stability could increase the reaction conversion with amine groups. As hydrophobic groups, we have chosen three different C7 structures. Benzyl-, cyclohexylmethyl and heptyl- iso(thio)cyanate (Figure 23) were selected as the coupling partner of the $S2_M$-*g*-PEI_{30}. Short and cyclic hydrophobic groups were chosen to avoid the steric hindrance and solubility problems with larger and aliphatic ones.



Benzyl isothiocyanate
(Benz-ITC)



Cyclohexylmethyl isothiocyanate
(CycHex-ITC)

Heptyl isocyanate
(Hep-IC)

Figure 23: Chemical structures of three different hydrophobic isothiocyanates and isocyanate

5.2 Experimental section

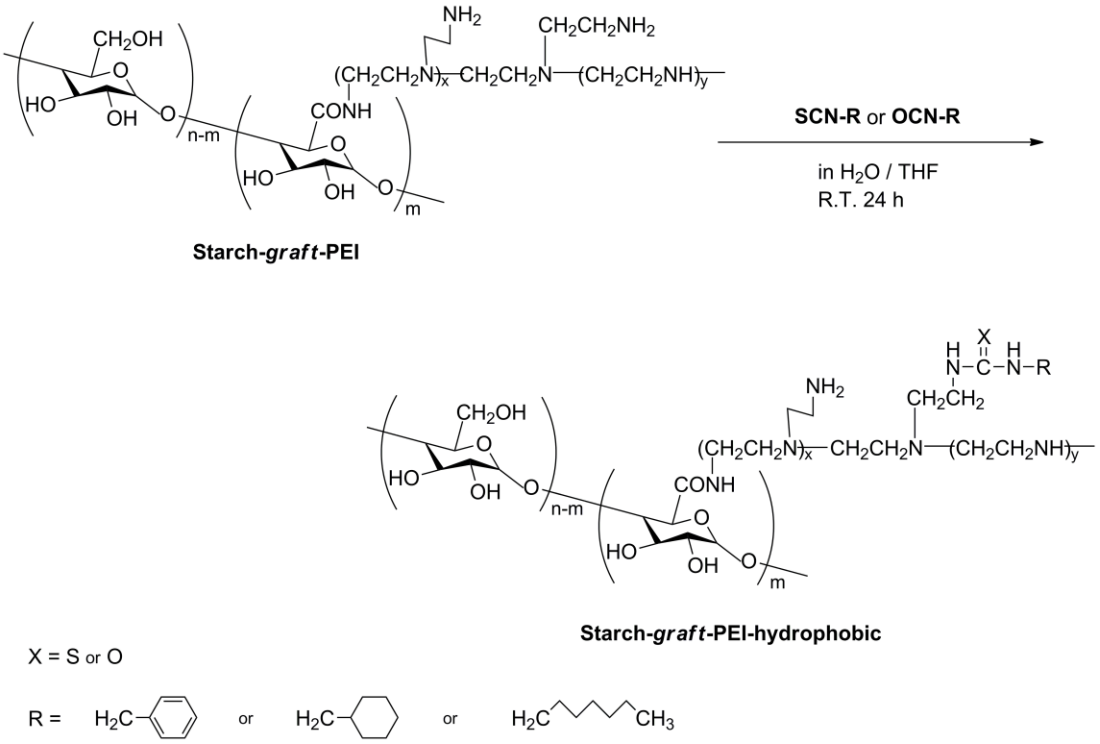
5.2.1 Materials

One of the most successful starch-*g*-PEI polymers from chapter 4, $S2_M$-*g*-PEI_{30} was used as the starting material of the hydrophobic modification. Three different hydrophobic iso(thio)cyanates, benzyl isothiocyanate (Benz-ITC), cyclohexylmethyl isothiocyanate (CycHex-ITC) and Heptyl isocyanate (Hep-IC) (Figure 23) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used without further purification. Agarose, ethdium bromide and the BCA Protein Assay Reagent Kit was purchased from Sigma-Aldrich, as well. Purified water is produced by Milli-Q water purification system (Merk Millipore, Billerica, U.S.A.). pGL3 pDNA encoding firefly luciferase was prepared and used as described in detail in chapter 4. 1 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7 was prepared and stored at 4 °C until use. A549 cells No ACC 107 were purchased from DSMZ GmbH (Braunschweig, Germany). Cell culture medium (RPMI 1640) was purchased from PAA Laboratories GmbH (Pasching, Austria) and fetal bovine serum (FBS) was purchased from Lonza (Basel, Switzerland). The Luciferase assay kit was purchased from Promega (WI, U.S.A.).

5.2.2 Hydrophobic modification of starch-*graft*-PEI polymer

Hydrophobic modification of $S2_M$-*g*-PEI_{30} was performed in co-solvent system, H_2O/THF. Briefly, 10 mg of $S2_M$-*g*-PEI_{30} (containing 3.38×10^{-3} mmol PEI) was solubilized in 400 µL of purified water and an appropriate amount of hydrophobic isothiocyanate or isocyanate in 100 µL of THF was quickly added to the $S2_M$-*g*-PEI_{30} aqueous solution with stirring. The reaction mixture was further stirred for 24 h at R.T. and purified by extraction with water / chloroform for five times. The water phase was collected and lyophilized. The resulting white polymers were characterized by FT-IR and ^{1}H-NMR. Bruker NMR Magnet System 400 MHz Ultra shield plus: in D_2O, δ_H Starch: (***1H***, 5.1 – 5.6 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), PEI: (NH_2-C***H$_2$***-C***H$_2$***, 2.20 – 3.45 ppm), benzyl group: (aromatic protons, 7.22 – 7.34 ppm),

cyclohexyl group (methyl protons, 0.68 – 2.00 ppm), heptyl group (metyl protons, 0.56 – 2.00 ppm).



Scheme 7: Hydrophobic modification of starch-*g*-PEI polymer:

Reaction with amine group and iso(thio)cyanate group

5.2.3 Polyplex formation and characterization

The stem polymer solutions were prepared by solubilizing the polymers into the purified water in the concentration of 1 mg/mL and stored at -20 °C until use. The stem aqueous solution of each polymer and pDNA were further diluted with purified water to the suitable concentration and incubated for 10 min at 50 °C. An appropriate amount of pDNA (N/P = 10, 12, 14) was added to the polymer solution. After the addition of pDNA, the solution was immediately mixed for 10 seconds by vortex and incubated for 30 min at R.T. The characterization (size, particle homogeneity and zeta potential) of polyplexes was studied by Zetasizer Nano-ZS

(Malvern Instruments, Worcestershire, UK) as described in chapter 4, page 51. Additionally, the stability of the polyplexes in HBSS buffer was studied to mimic the condition of transfection experiment. The polyplex solution was diluted with HBSS buffer 1 : 4 (polyplex solution: HBSS buffer) and particle size were measured after 2 and 4 h incubation in the buffer.

5.2.4 Luciferase transfection assay

Transfection efficiency was evaluated with A549 cells. A549 cells were maintained in RPMI cell culture medium supplemented with 10 % (v/v) FBS at 37 °C in 5 % CO_2. A549 cells were seeded in a 24-well plate at a density of 25,000 cells per well in 500 µL of the cell culture medium. Polyplexes with luciferase pDNA (pGL3) in Hank's Balanced Salt Solution (HBSS buffer) pH 7.4 were added on the cells (1 µg pDNA in 500 µL HBSS buffer pH 7.4 / well). After 4h of incubation, the polyplex solution was replaced by cell culture medium and further incubated for 48 h. Luciferase gene expression was evaluated using a Luciferase assay kit from Promega and the relative light units (RLU) were measured by Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany). Protein quantification was performed with BCA assay kit at 570 nm. The transgene expression was normalized for the protein content. All the experiments were conducted in duplicate.

5.2.5 Cytotoxicity studies

LDH and MTT assay were performed to study the *in vitro* cytotoxicity of the hydrophobic modified starch-*g*-PEI polymers. The A549 cells were seeded in a 96-well plate at a density of 10,000 cells per well in 200 µL of the cell culture medium. Once confluent, polymers in HBSS buffer pH 7.4 were applied on the cells and incubated for 4 h at 37 °C with gently shaking. As reference, HBSS buffer (negative, 0%) and 0.1% (w/w) Triton X-100 solution (ICN, Eschwege, Germany) (positive, 100%) in HBSS buffer were applied. To ensure the no interference of polyplexes to the test system, polyplexes without any cells were also tested.

5.2.5.1 LDH assay

After 4 h incubation of the above-mentioned 96-well plate, the 100 µL of the incubated HBSS solution was transferred to another 96-well plate. The 100 µL of LDH test solution (Roche Cytotoxicity LDH kit) was added to each well and the plate was incubated in dark for 5 min at R.T. The absorbance at 492 nm was read by Tecan microplate reader.

5.2.5.2 MTT assay

After the 4 h incubation of the above-mentioned 96-well plate, the test solution was removed and cells were washed once with HBSS buffer. The fresh HBSS buffer with 10 % (vol.) of MTT reagent (5 mg/mL) was added, and incubated further 4 h at 37 °C with gently shaking. The solution was removed and cells were dissolved in DMSO and incubated 10 min at 37 °C with gently shaking and protected from light. The absorbance at 550 nm was read by Tecan microplate reader.

5.2.6 Agarose gel electrophoresis

To study the DNA-binding/-release ability of the polyplexes, agarose gel electrophoresis was used. The polyplexes with appropriate N/P ratio were prepared as described above. 12 µL of polyplex (containing 0.12 µg pDNA) solution with/without 5 units of Heparin, were mixed with 2 µL of blue dye and loaded into a 0.7 wt% agarose gel containing 0.5 µg/mL ethidium bromide. Electrophoresis was run in 0.5 %TBE buffer at 50 V for 75 min. DNA retardation was analyzed using UV illuminator, Fusion FX7 imaging system (Peqlab, Erlangen, Germany), to show the band of the DNA.

5.2.7 Dye displacement assay using Heparin

For the precise study of the DNA-release ability by heparin competition, the Ethidium bromide dye displacement assay was used. The 50 µL of formulated polyplex, described above, was placed in the 96-well plate and add 100 µL of 1mM HEPES buffer (pH 7.0) containing 1µM Ethidium bromide. The fluorescence (ex: 544nm em: 590nm) of the fully complexed pDNA was measured by Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany) and set as the blank value. Titration was performed using heparin solution in purified water (40 unit/mL). After every addition of 2 µL of Heparin solution, the fluorescence increase by intercalation of Etbr into the released pDNA was measured and plotted against the amount of the added heparin.

5.3 Results and discussion

5.3.1 Synthesis and characterization of hydrophobic modified starch-*graft*-PEI polymers

The hydrophobic modification of the $S2_M$-*g*-PEI_{30} was done by the reaction of the amine-groups of the $S2_M$-*g*-PEI_{30} and isothiocyanate or isocyanate group of the hydrophobic chains (Benz-ITC, CycHex-ITC and Hep-IC, Scheme 7). The chemical structures of the resulting hydrophobic modification with three different hydrophobic groups are shown in Figure 24.

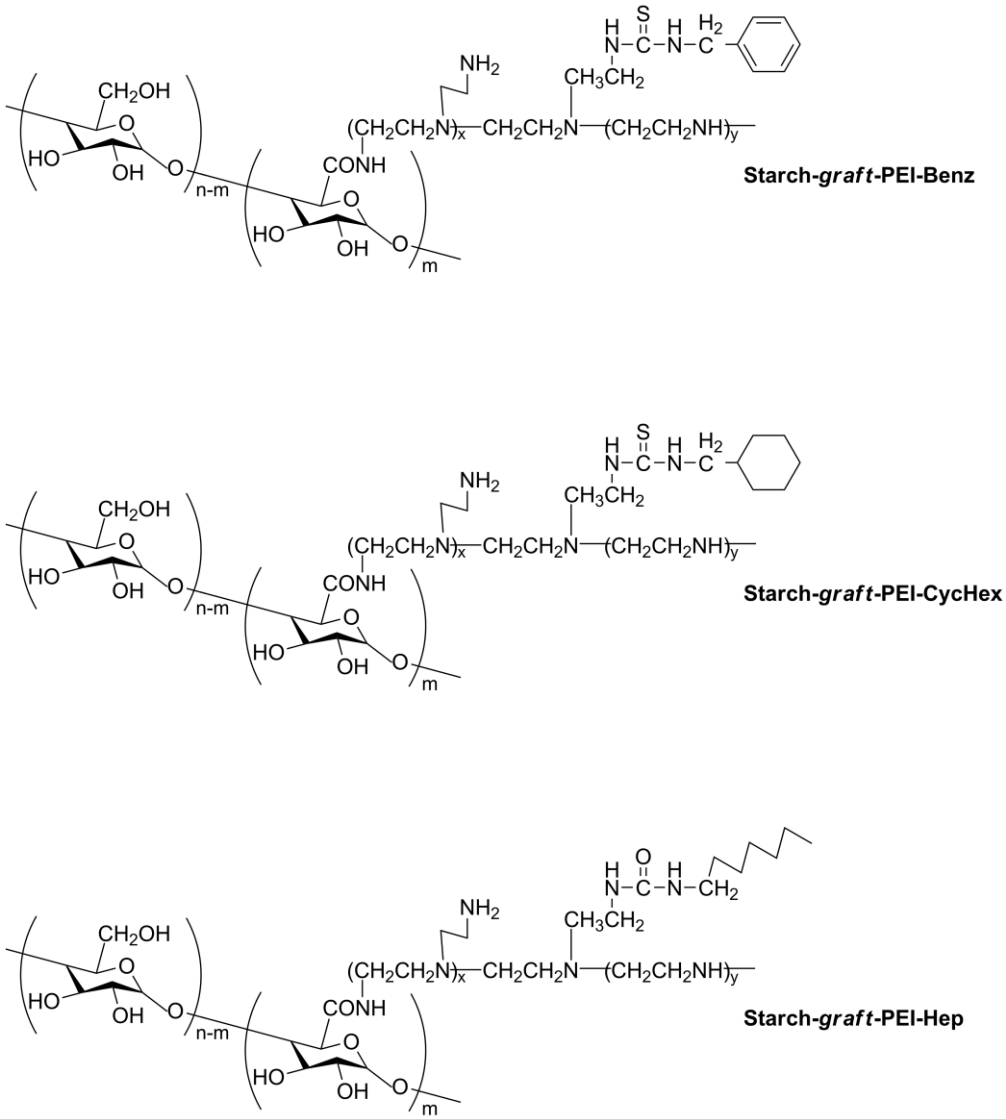


Figure 24: Structures of the three different hydrophobic modified starch-*g*-PEIs

The representative ^{1}H-NMR spectra of each hydrophobic modification are shown in Figure 25. Bruker NMR Magnet System 400 MHz Ultra shield plus: in D_2O, δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), PEI: (NH_2-C***H₂***-C***H₂***, 2.20 – 3.45 ppm), benzyl group: (aromatic protons, 7.22 – 7.34 ppm), cyclohexyl group (methyl protons, 0.68 – 2.00 ppm), heptyl group (metyl protons, 0.56 – 2.00 ppm).

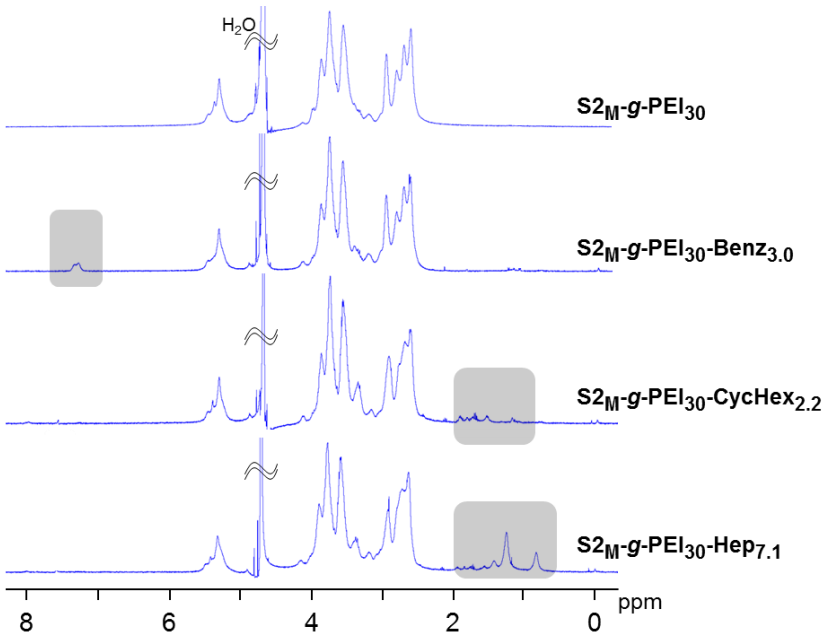


Figure 25: ^{1}H-NMR spectra of each hydrophobic modification in D_2O, δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), PEI: (NH_2-C***H₂***-C***H₂***, 2.20 – 3.45 ppm), benzyl group: (aromatic protons, 7.22 – 7.34 ppm), cyclohexyl group (methyl protons, 0.68 – 2.00 ppm), heptyl group (methyl protons, 056. – 2.00 ppm).

The amount of the hydrophobic chain introduced in the $S2_M$-*g*-PEI_{30} was calculated based on the ^{1}H-NMR results, the ratio between *1H* proton of the starch glucose units (5.12 – 5.58 ppm) and each hydrophobic groups´ protons, benzyl group: (aromatic protons, 7.22 – 7.34 ppm), cyclohexyl group (methyl protons, 0.68 – 2.00 ppm), heptyl group (methyl protons, 0.56 –

2.00 ppm), and summarized in Table 6. As is shown in Table 6, different amount of hydrophobic chain in the range of 10 - 200 (mol% against s-PEI side chains) was fed in the modification reaction. Thus, Table 6 shows the modification reaction efficacy when comparing the feed to the introduced amount (either as mol% against s-PEI side chains or wt% against the whole molecule) on the $S2_M$-*g*-PEI_{30}. The smallest modification amount of the Benz-ITC and CycHex-ITC were not detectable in ^{1}H-NMR. The conversions of the modification by Hep-IC were almost 100 %. We had concerns about the modification using isocyanate group because of the side reaction between water and isocyanate. However, the results were better than that of isothiocyanate. Because of the higher reactivity between isocyanate and amine, the side reaction between isocyanate and water did not occur and showed the successful reactions. On the other hand, the reactivity of the isothiocyanate group with amine was not high enough to achieve a 100 % conversion. Regarding the structures of hydrophobic groups, benzyl- cyclohexylmethyl- and heptyl group, the linear heptyl group modification leads to higher conversion than the other two. Since there is no big difference among these hydrophobic groups concerning the molecular size and hydrophobicity, the highest conversion of heptyl groups was most likely because of the efficient reaction of the isocyanate group compared to isothiocyanate group.

In the following, polymers are named systematically, according to their hydrophobic modification amount. The subscript number after each hydrophobic chain (Benz, CycHex and Hep) stands for the wt% of the hydrophobic modification against the whole molecule $S2_M$-*g*-PEI_{30}-hydrophobic polymer.

Table 6: Results of the hydrophobic modification with three different hydrophobic groups

Feed (mol% against PEI)	10	50	100	200	wt% against whole molecule			
Results								
$S2_M$-*g*-PEI_{30}-Benz	---	25	49	83	---	0.9	1.8	3.0
$S2_M$-*g*-PEI_{30}-CycHex	---	15	25	56	---	0.6	1.0	2.2
$S2_M$-*g*-PEI_{30}-Hep	12	54	102	187	0.5	2.1	4.0	7.1

The amount of the introduced hydrophobic chain was determined by ^{1}H-NMR.

FT-IR measurement was performed to confirm the purification. The typical and strong absorbance of the isothiocyanate and isocyanate, at 2076 and 2255 cm^{-1} respectively, was not observed in any of the product (Figure 26) that indicated the successful removal of the non-reacted hydrophobic molecules by the extraction procedure with water / chloroform.

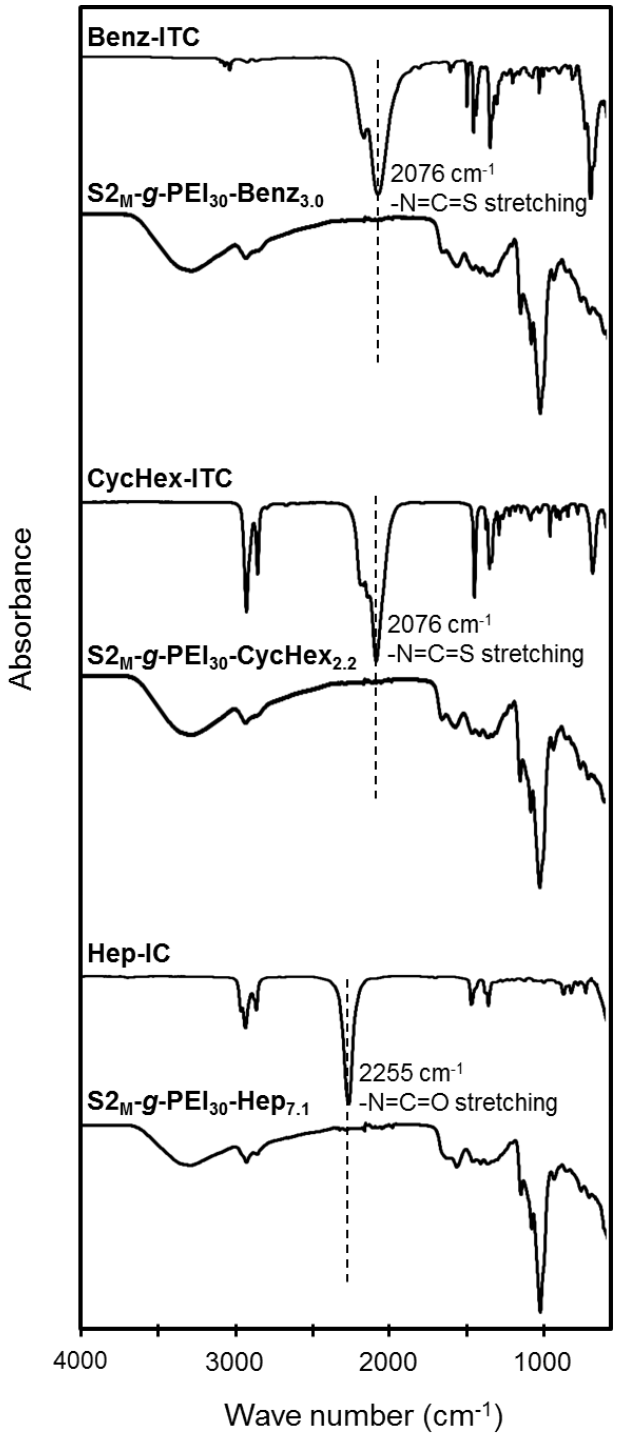


Figure 26: IR spectra of the hydrophobic molecules and starch-*g*-PEI-hydrophobic polymers

5.3.2 Polyplex formation and characterization

Using the synthesized starch-*g*-PEI-hydrophobic polymers, polyplexes were formed with pDNA. In order to see the influence of the hydrophobic modification, the polymer with the highest modification of each three hydrophobic chain was used, namely $S2_M$-*g*-PEI_{30}-$Benz_{3.0}$, $S2_M$-*g*-PEI_{30}-$CycHex_{2.2}$ and $S2_M$-*g*-PEI_{30}-$Hep_{7.1}$. The size and zeta potential of polyplexes were measured using a Zetasizer (Malvern Instruments, Worcestershire, UK) and summarized in Table 7. As a comparison, polyplex from $S2_M$-*g*-PEI_{30} without hydrophobic modification was also formulated and characterized. No big difference between the polyplexes, with and without hydrophobic modification, in size, PDI and zeta potential was observed.

The stability of the polyplexes was further studied in HBSS buffer which is used for transfection experiment. The polyplex solution was diluted with HBSS buffer five times and measured sizes using a Zetasizer. As is shown in Figure 27, the polyplex from $S2_M$-*g*-PEI_{30}-$Benz_{3.0}$ and $S2_M$-*g*-PEI_{30}-$CycHex_{2.2}$ showed a little decreased stability over the time in HBSS. On the other hand, $S2_M$-*g*-PEI_{30}-$Hep_{7.1}$ showed better stability, though having higher PDI, indicating a broader size distribution. However the difference was not so big and all polyplexes were stable enough for transfection, as they remained below 200 nm size after 4 h incubation in the buffer. Thus, no huge effect of the hydrophobic modification was found from these polyplex characterizations with Zetasizer.

Table 7: Characterization of the polyplexes from starch-*g*-PEI-hydrophobic polymers

Sample code	Size (nm)	PDI	ζ-potential (mV)
$S2_M$-*g*-PEI_{30}	97 ± 16.0	0.22 ± 0.03	$+27.4 \pm 2.0$
$S2_M$-*g*-PEI_{30}-$Benz_{3.0}$	97 ± 3.0	0.14 ± 0.01	$+32.2 \pm 0.3$
$S2_M$-*g*-PEI_{30}-$CycHex_{2.2}$	86 ± 1.2	0.23 ± 0.02	$+26.5 \pm 0.6$
$S2_M$-*g*-PEI_{30}-$Hep_{7.1}$	107 ± 1.8	0.19 ± 0.01	$+31.4 \pm 1.3$

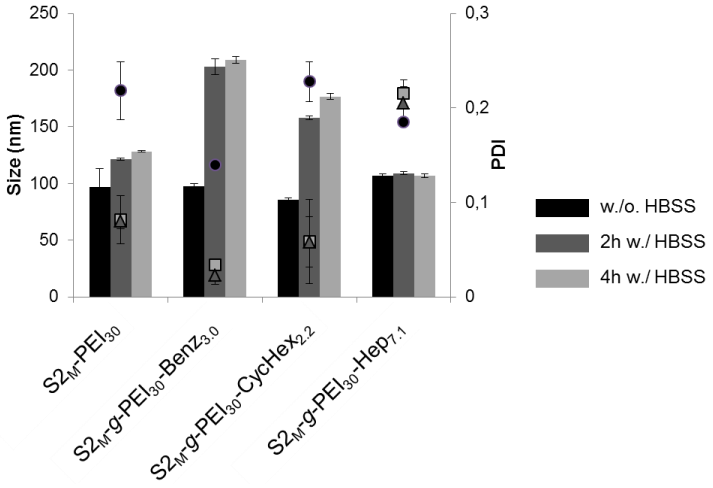


Figure 27: Polyplex stability in HBSS buffer

5.3.3 Luciferase transfection assay

Although, no apparent effect was found in the polyplexes between the absence or presence of the hydrophobic modification on the starch-*g*-PEI, the luciferase transfection assay was performed to elucidate any difference in the transfection efficacy.. The polyplexes were formulated as described before (N/P = 10, 12 and 14) and transfection efficiency was studied with A549 cells.

In Figure 28, the results of the luciferase transfection assay using all three different hydrophobic modifications are shown. As is easily recognized, the transfections from hydrophobic modified samples were decreased. The more hydrophobic modification, the less transfection was observed. The same tendency was seen in all three hydrophobic chain modified samples. It became clear that, the hydrophobic modification on the starch-*g*-PEI, unfortunately, did not increase but decrease the transfection efficiency. Focusing on the $S2_M$-*g*-PEI_{30}-Hep series (Figure 28(c)), the amount of the heptyl group to the whole molecular weight is 0.5, 2.1, 4.0 and 7.1 wt%. Taking this amount into account, the effect of the hydrophobic chain on the transfection efficiency is really huge. Only 0.5 wt% of heptyl groups

did already decrease the transfection efficiency, and 4.0 and 7.1 wt% heptyl group modified samples resulted in almost no transfection any more.

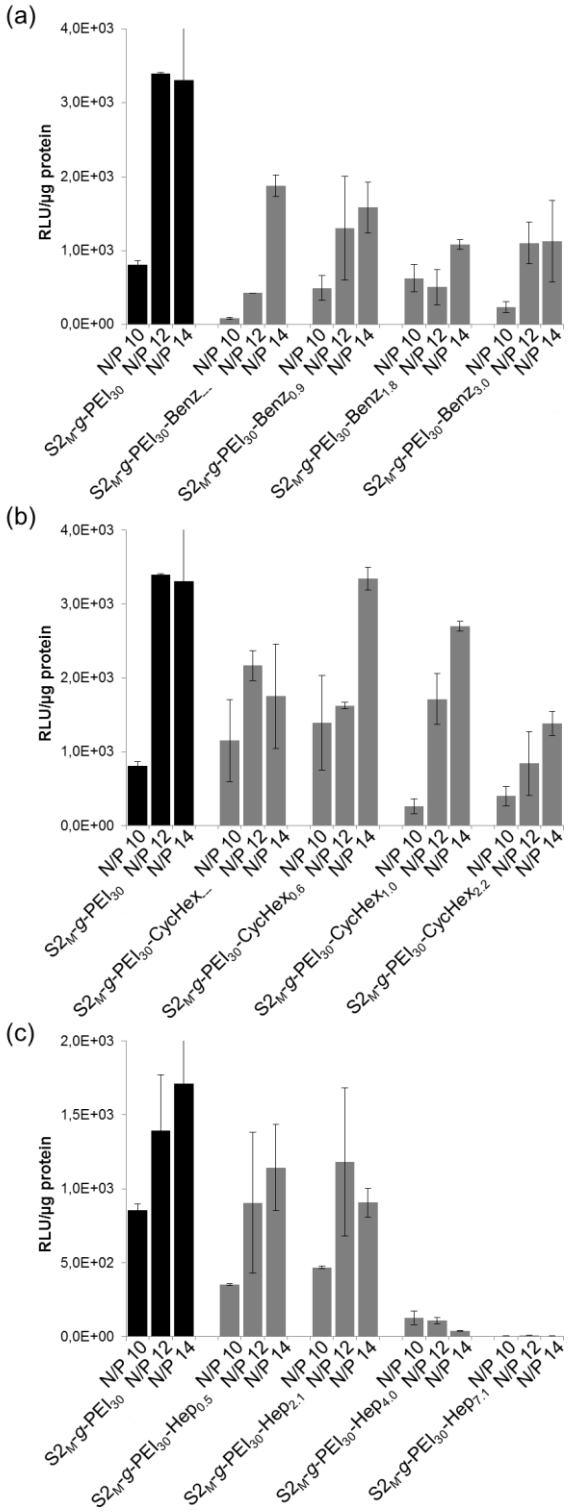


Figure 28: Results of luciferase transfection assay with different hydrophobic modification (a) $S2_M$-*g*-PEI_{30}-Benz, (b) $S2_M$-*g*-PEI_{30}-CycHex, (c) $S2_M$-*g*-PEI_{30}-Hep

5.3.4 Cytotoxicity studies

The negative effect of the hydrophobic chain on the transfection efficiency became clear. To find out the cause of this decrease in transfection efficiency, cytotoxicity studies were performed. Only the highest hydrophobic modification of each hydrophobic group ($S2_M$-*g*-PEI_{30}-$Benz_{3.0}$, $S2_M$-*g*-PEI_{30}-$CycHex_{2.2}$ and $S2_M$-*g*-PEI_{30}-$Hep_{7.1}$) was tested and compared with the non-hydrophobic modified $S2_M$-PEI_{30} and PEI 25 kDa. In LDH assay, no big difference between hydrophobic modified and unmodified polymers were observed at lower concentrations (< 50 µg/mL), however in higher concentrations (> 100 µg/mL) the benzyl and heptyl group modified polymer showed obviously higher cytotoxicity than $S2_M$-PEI_{30} (Figure 29 (a)). The higher cytotoxicity of hydrophobic modified polymers than non-hydrophobic modified polymer was more easily recognized in MTT assay (Figure 29 (b)). However, in comparison to PEI 25 kDa, all the hydrophobic modified polymers showed still lower cytotoxicity. Therefore, one of the reasons of the decreased transfection efficiency would be the increased cytotoxicity though it would not be the only reason, since the cytotoxicity of the hydrophobic modified polymers were not as critical as that of PEI 25 kDa (Figure 29).

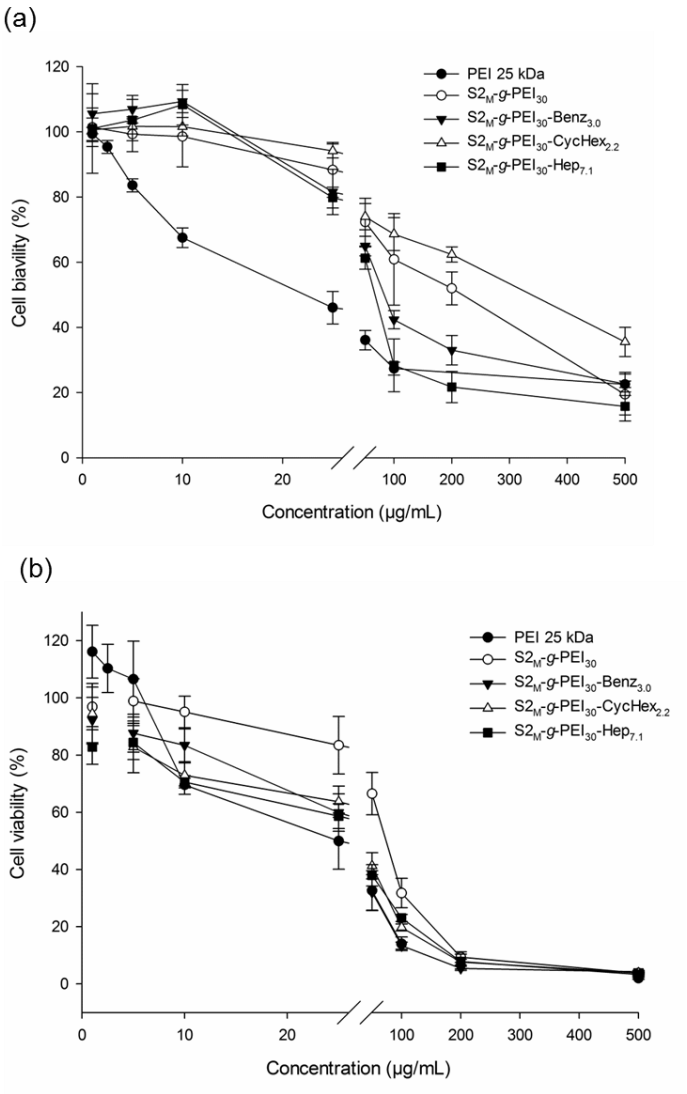


Figure 29: Cytotoxicity study, (a) LDH and (b) MTT assay

5.3.5 Agarose gel electrophoresis

The DNA-binding ability of the polyplexes was tested using agarose gel electrophoresis to see the difference between hydrophobic modified/unmodified starch-*g*-PEI polymers. At the same time, DNA releasing ability was tested by adding the strongly negative charged molecule heparin to the polyplexes and run an electrophoresis.

In Figure 30, several small differences between the samples with and without hydrophobic modifications were observed. All polymers could make nice polyplexes and no free, uncomplexed pDNA was observed without the addition of the heparin (Figure 30 a, b, c and d).

However, the fluorescence of pDNAs with hydrophobic modified samples (b, c, and d) were brighter than without hydrophobic modification (a). That indicated the ethidium bromide could intercalate into pDNA stronger in the polyplexes with hydrophobic chains. In other words, the starch-*g*-PEI without hydrophobic chain has stronger binding ability with pDNA than the polymers with hydrophobic chains. By the addition of the heparin (Figure 30, alphabets with apostrophe), pDNA-polymer complexes were broken and free pDNA ran through the agarose gel. The clear pDNA band was visible by means of ethidium bromide. Polyplex from $S2_M$-*g*-PEI_{30}-$Hep_{7.1}$ (d)´ showed the less pDNA release than the other samples (a)´, (b)´ and (c)´, which would fit with the results of HBSS-stability measurement (Figure 27).

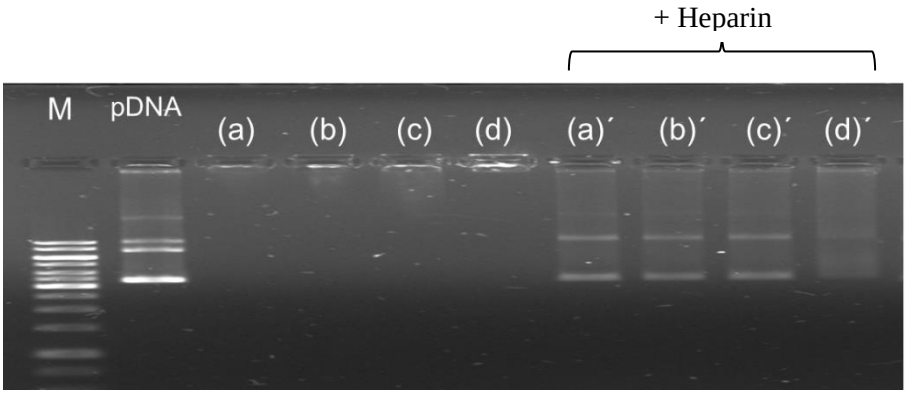


Figure 30: Agarose gel electrophoresis with and without hydrophobic modification: M: marker, pDNA: not complexed, free pDNA (a) $S2_M$-*g*-PEI_{30}, (b) $S2_M$-*g*-PEI_{30}-$Benz_{3.0,}$ (c) $S2_M$-*g*-PEI_{30}-$CycHex_{2.2}$, (d) $S2_M$-*g*-PEI_{30}-$Hep_{7.1}$, Alphabets with apostrophe were after the addition of 5 unit heparin

5.3.6 Dye displacement assay using Heparin

For the precise study of the DNA release by heparin competition, the dye displacement assay was used. The titration was performed with the addition of heparin (each 0.08 unit) and

fluorescence measurement (ex: 544nm em: 590nm) by a microplate reader. In Figure 31, the fluorescence intensity was plotted against the added amount of the heparin (unit). The release of pDNA from all three hydrophobic modified polyplexes were faster than that of $S2_M$-*g*-PEI_{30} polyplex. That means negatively charged heparin could more easily destroy the polyplexes from hydrophobic modified polymers. Hence, it is conceivable that the hydrophobic modification weakened the interaction between pDNA, and the DNA-binding ability of the hydrophobic modified samples decreased in comparison to that of $S2_M$-*g*-PEI_{30}. This slight difference between the hydrophobic modified polyplexes and non-hydrophobic modified polyplex was difficult to see in polyplex characteristics in size, PDI and zeta-potential by means of Zetasizer, but could be observed in this titration study and also the agarose gel electrophoresis. Comparing these three hydrophobic samples, the $S2_M$-g-PEI_{30}-$Hep_{7.1}$ carried more hydrophobic groups (less free amine groups left), however showed not much faster in pDNA release. Thus, it would be reasonable to conclude that there was still a small stabilization effect of the Hep-chains that was acting against the decreased number of primary amines. The dramatic decrease of the transfection efficiency in luciferase assay is most likely a combined effect caused by the slight increase in cytotoxicity (compared to $S2_M$-gPEI_{30}), the increased complex stability in HBSS, which leads to a lower amount of complexes getting in contact with the cell in the transfection experiment and the faster release of pDNA due to the decreased number of primary amines.

Our expectation before this study was that the hydrophobic chain would increase the polyplex stability because of the micelle effect (Figure 32 (a)) and increase the transfection efficiency. Yet the effects were unlike. A possible explanation for this could be that the chosen hydrophobic chains are not long enough to allow self-assembly of the amphipilic molecules in stable structures (Figure 32 (a)), though heptyl modification seems to be more efficient than the cyclic ones. The complex branched structure of the starch-g-PEI-hydrophobic polymers could also be a cause. It is known that the stiffness of branched polymers, which are composed of a main chain and side chains, would increase by increasing density and length of the side chains[130]. Those polymers do not behave as worm-like chain any more in a good solvent, and

repulsions between the main chain and side chains, and between neighboring side chains makes the high stiffness of this branched polymer. Since both of the starch and s-PEI have branched structures, the starch-*g*-PEI was expected to have certain stiffness. Though the density of the side chains (amount of the modification) is not so high, every 12th glucose carries a s-PEI side chain, the size of the s-PEI (800 Da) was expected enough large to increase the stiffness of starch. Thereby, the introduced hydrophobic chains did not have a freedom to move and could not exist central part of the polyplex but dispersed all of the polyplex structure (Figure 32 (b)). The dispersed hydrophobic chain could interfere the compact and tight electrostatic interaction between amine groups and pDNA, and decrease the DNA-binding ability. In that context, the hydrophobic group deemed to a defect. Thus, the more hydrophobic chain caused the more defect, weaken the DNA-binding ability, unstabilize the polyplex and decrease the transfection efficiency.

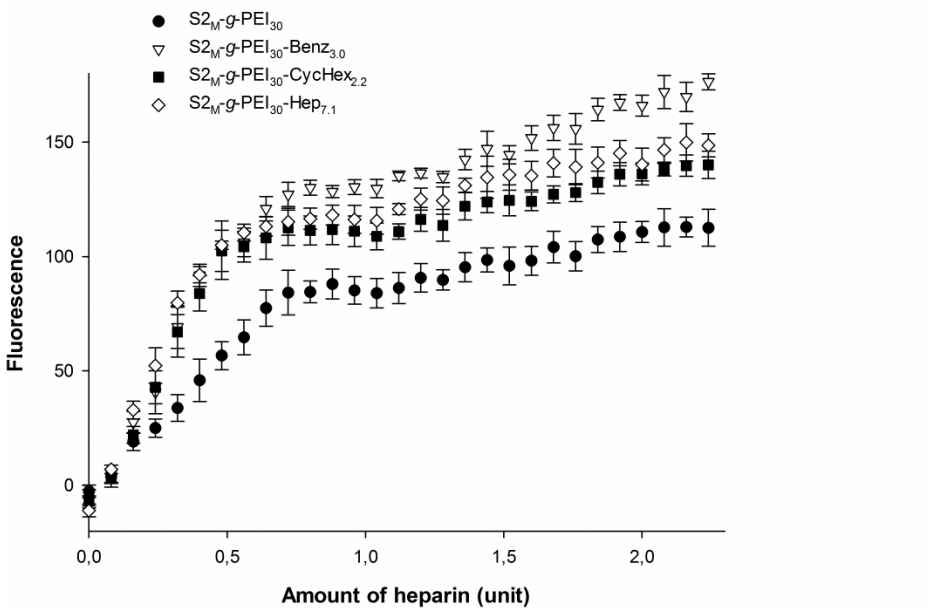


Figure 31: Titration curves for DNA release

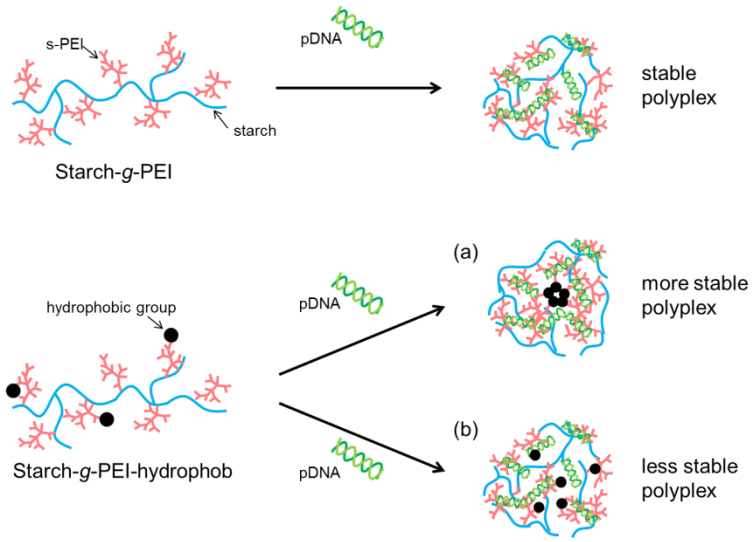


Figure 32: Polyplex structure, with and without the hydrophobic modification

Another explanation of the decreased transfection would be the loss of the primary amine groups because of the reaction with isothiocyanate or isocyanate groups of the hydrophobic groups. One s-PEI side chain (800 Da) has around 5 - 1 = 4 primary (1 primary amine group was already used in the reaction with starch backbone), 10 secondary and 5 tertiary amine groups. If one of the primary amine groups of the each s-PEI side chain is reacted with iso(thio)cyanate, the amount of the primary amine group of the whole molecule becomes 75 %. The amount of the primary amine groups of the each hydrophobic modified polymer compared with $S2_M$-*g*-PEI_{30} is, $S2_M$-*g*-PEI_{30}-$Benz_{3.0}$ = 79 %, $S2_M$-*g*-PEI_{30}-$CycHex_{2.2}$ = 86 %, $S2_M$-*g*-PEI_{30}-$Hep_{7.1}$ = 53 %. This loss of the primary amine group could also cause the decrease of the binding ability with pDNA and polymer and decrease the transfection efficiency. This result fit with other reported studies[131,132] which demonstrated the decreased transfection efficiency caused by the reduced number of primary amine groups. In conclusion, the true cause of decreased transfection efficiency was not one but combination of the whole explained matters.

5.4 Conclusion

The successful hydrophobic modification of the $S2_M$-*g*-PEI_{30} has been done using three different hydrophobic molecules, Benz-ITC, CycHex-ITC and Hep-IC. The conversion of the hydrophobic modification with isocyanate group were around 100 % and worked better than that of isothiocyanate groups (conversion = around 25 – 50 %).

Polyplexes were formulated using the hydrophobic modified polymers. Compared with the polyplex of $S2_M$-*g*-PEI_{30}, no significant difference was observed in size, PDI, zeta-potential, but a slightly altered stability in HBSS buffer.

However hydrophobic modification gave the remarkable effect to the transfection efficiency. The more hydrophobic modification, the less the luciferase transfection efficiency was observed. Only few weight percent of the hydrophobic groups were enough to decrease the transfection efficiency.

To study the cause of this decreased transfection efficiency, pDNA-binding and release ability was investigated by agarose gel electrophoresis and dye displacement assay using ethidium bromide and heparin. It became clear that the DNA-binding ability of the hydrophobic modified polymers were decreased compared to $S2_M$-*g*-PEI_{30}. The release of the pDNA in hydrophobic modified polyplexes was faster than $S2_M$-*g*-PEI_{30}. The reason of this weakened DNA-binding ability were assumed to be two points; 1) the interference of the DNA-amine electrostatic interaction by hydrophobic chain (Figure 32 (b)), and 2) the loss of the primary amine groups which should have electrostatic interaction with pDNA.

6 Starch-*graft*-cationic polymers: Comparison of PEI side chain and small MW side chains

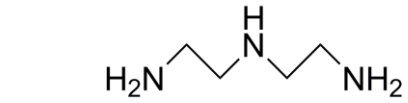
6.1 Introduction

In their attempts to develop improved gene delivery vectors, many researchers have synthesized cationic polymers, which aimed to mimic PEI 25 kDa as model structure. For that purpose, oligoamines are occasionally used[133, 134, 135, 136]. Lin et al.[134] introduced different linear oligoamines, namely, ethylenediamine (EDA), dietylenetriamine (DETA), triethylenetetramine (TETA), tetraethylenepentamine (TEPA), dipropylenetriamine and spermine onto the disulfide-amide polymer backbone and synthesized bioreducible poly(amido amine)s. The synthesized poly(amido amine)s showed different characteristics depending on the side chains. The increased alkyl spacer from ethylene to propylene between the amino units in side chains resulted in significantly lower transfection efficiency. Srinivasachari et al.[135] also used linear oligoamines, either DETA, TETA, TEPA, or pentaethylenehexamine (PEHA) to modify β-cyctodextrine. All synthesized polymers showed good transfection efficiency and low cytotoxicity, without significant differences from the different side chain. Wei et al.[136] synthesized poly-(glycidyl methacrylate) by RAFT polymerization and decorated with three different types of oligoamines, i.e., TEPA, PEHA, and tris(2-aminoethyl)amine (TREN), respectively. With respect to the transfection efficiency and cytotoxicity, they concluded that the TEPA is the best side chain for this polymer. Some works of the peptide modification with oligoamines were also published[137, 138]. Salcher et al.[138] used three different oligoamines, TETA, TEPA and PEHA to modify peptide (oligoamino acid) and proved a clear preference of the oligoamines, in the order of PEHA>TEPA>>TETA for pDNA compaction and transfection efficiency. The modification of carbohydrate backbone was also studied by some researchers[109, 139]. Amylopectin modification[109] and chitosan modification[139] were done with several oligoamines. The synthesized polymers showed good cytotoxic properties, however the transfection efficiency was not more than PEI 25 kDa.

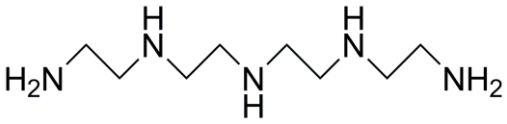
In this chapter, we studied the effect of different oligoamines side chains on the starch backbone. The intention was to replace the s-PEI (0.8 kDa) side chains of starch-*g*-PEI from chapter 4 with smaller MW cationic side chains in order to achieve lower cytotoxicity and, if

possible, maintain a similar level of transfection efficiency. Figure 33 depicts the used oligoamines, namely, dietylenetriamine (DETA), tetraethylenepentamine (TEPA) and tris(2-aminoethyl)amine (TREN).

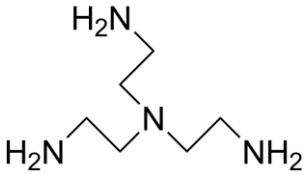
The question to be answered by this study was which cationic side chain can best combine the two main properties of low cytotoxicity and high transfection efficiency, and how this compares with the starch-*g*-PEI from chapter 4. For a systematical study of different size and structure of the side chain, we selected three different oligoamines, DETA, TEPA and TREN. These three molecules have same repeating units to PEI, $-CH_2CH_2NH-$, which is accounted for the "proton sponge" effect to enable endosomal escape. Two of them, DETA and TEPA are linear structure which MW are 103.17 g/mol and 189.30 g/mol respectively, and TREN has branched structure as well as s-PEI with MW of 146.23 g/mol (Figure 33). Through the comparative study of these three different molecules and s-PEI, we aimed to learn important structural characteristics of successful side chains.



DETA (MW = 103.17 g/mol)



TEPA (MW = 189.30 g/mol)



TREN (MW = 146.23 g/mol)

Figure 33: Chemical structures and MW of three different oligoamine side chains

Since the results in chapter 4 showed the superior potential of the larger MW starch backbone, only large MW (> 290,000 g/mol) of the oxidized starch backbones were used for the study in this chapter, and modified with three different oligoamines. The ability of the resulting three new starch-g-polymers as a gene delivery vector was then, compared with PEI 25 kDa, a commercial transfection reagent and the best polymer in chapter 4, namely $S2_L$-*g*-PEI_{30} as benchmarks.

6.2 Experimental section

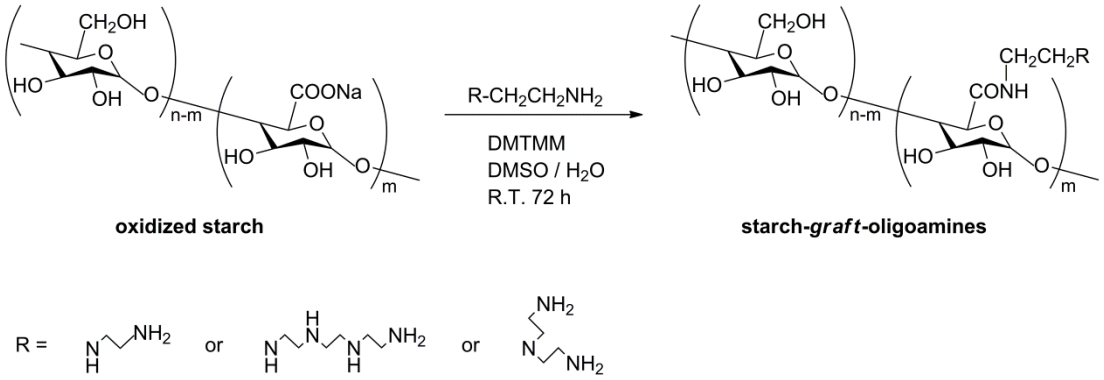
6.2.1 Materials

Large molecular weight (MW > 290,000 g/mol) of the partially oxidized potato starch, synthesized according to chapter 3, was used as the backbone of the cationic polymers. Two different MW branched polyethylenimine, (b-PEI, 25 kDa) (s-PEI, 0.8 kDa) and three different small cationic side chains, dietylenetriamine (DETA), tetraethylenepentamine (TEPA), tris(2-aminoethyl)amine (TREN) were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used without further purification. 2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and 4-Methylmorpholine (NMM) were purchased from Sigma-Aldrich Co. Purified water is produced by Milli-Q water purification system (Merk Millipore, Billerica, U.S.A.). pGL3 pDNA encoding firefly luciferase and another pDNA encoding fluorescent protein AmCyan were prepared and used as detailed in chapter 4. A transfection reagent, jetPRIME^{TM} was purchased from Polyplus transfection (Illkirch, France). A549 cells No ACC 107 were purchased from DSMZ GmbH (Braunschweig, Germany). Cell culture medium (RPMI 1640) was purchased from PAA Laboratories GmbH (Pasching, Austria) and fetal bovine serum (FBS) was purchased from Lonza (Basel, *Switzerland*). The Luciferase assay kit was purchased from Promega (WI, U.S.A.). The BCA Protein Assay Reagent Kit was purchased from Sigma-Aldrich.

6.2.2 Synthesis and characteristics of the starch based cationic polymers

The reaction between sodium carboxylate groups of Starch and amine groups of DETA, TEPA or TREN were done according to Kunishima et al.[113] (Scheme 8). Briefly, Large MW of the oxidized starch from chapter 3 (500 mg, 1.66 mmol sodium carboxylate) was solubilized in 50 mL of solvent mixture (1 : 4 = purified water : DMSO) in a round-bottomed flask followed by the addition of 1.99 mmol conjugation reagent, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4methyl-morpholinium Chloride (DMTMM), while stirring. The reaction mixture was stirred for 30 min. in R.T. to activate the sodium carboxylate groups. Subsequently, 166 mmol of amino

functionalized compound, either DETA, TEPA or TREN in 100 mL of solvent mixture (1 : 4 = purified water : DMSO) was slowly added. The reaction was proceeded with stirring for 72 h at R.T. and extensively dialyzed (3.5 kDa Mw cutoff, regenerated cellulose membrane, Fisher scientific) against purified water. The water insoluble white precipitation was removed by filtration and the reaction solution was collected and lyophilized. The amount of cationic side chains (wt%) on the starch backbone was determined by ^{1}H-NMR. Bruker NMR Magnet System 400 MHz Ultra shield plus: in D_2O, δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), DETA, TEPA, TREN: (NH_2-C***H₂***-C***H₂***, 2.29 – 3.46 ppm). The purity of starch based cationic polymers were examined by GPC measurement (HLC-8320GPC, Tosoh, Japan) on SUPREMA-MAX 1000 and 30 columns (PSS) at a flow rate 1 mL/min at 35 °C in 1 % formic arid.



Scheme 8: Synthesis of the starch based cationic polymers using different cationic side chains

6.2.3 Polyplex formation

The stem polymer solutions were made by solubilizing the polymers into the purified water in the concentration of 1 mg/mL and stored at -20 °C until use. The stem aqueous solution of each polymer and pDNA were further diluted with HEPES buffer (pH 7 with 5 wt% glucose) to the suitable concentration and incubated for 10 min at 50 °C. An appropriate amount of

pDNA (N/P = 2 - 40 : ratio between nitrogen of polymer and phosphate of pDNA) was added to polymer solution. After the addition of pDNA, the solution was immediately mixed for 10 seconds by vortex and incubated for 30 min at R.T.

6.2.4 Polyplex characterization

The characterization (size, particle homogeneity and zeta potential) of polyplexes was studied by Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) as described in chapter 4, page 51.

6.2.5 Transfection experiments

Transfection efficiency was evaluated with A549 cells. A549 cells were maintained in RPMI cell culture medium supplemented with 10 % (v/v) FBS at 37 °C in 5 % CO_2.

6.2.5.1 Luciferase transfection assay

A luciferase assay was used for the screening of transfection efficiency because of the high throughput and sensitivity of the assay. A549 cells were seeded in a 24-well plate at a density of 25,000 cells per well in 500 µL of the cell culture medium. Polyplexes with luciferase pDNA (pGL3) in Hank's Balanced Salt Solution (HBSS buffer) pH 7.4 were added on the cells (1 µg pDNA in 500 µL HBSS buffer pH 7.4 / well). After 4h of incubation the polyplex solution was replaced by cell culture medium and further incubated for 48 h. Luciferase gene expression was evaluated using a Luciferase assay kit from Promega and the relative light units (RLU) were measured by Tecan microplate reader (Tecan Deutschland GmbH, Crailsheim, Germany). Protein quantification was performed with BCA assay kit at 570 nm. The transgene expression was normalized for the protein content. All the experiments were conducted in duplicate. The results were depicted as mean value ± SD. Significance among the mean values was calculated using Student’s t-test. Probability value $p < 0.01$ was considered significant.

6.2.5.2 Flow cytometry

The amount of transfected cell was determined by flow cytometry. A549 cells were seeded in a 12-well plate at a density of 20,000 cells per well in 1 mL of the cell culture medium. Polyplexes with pDNA encoding fluorescent protein AmCyan in HBSS buffer pH 7.4 were added on the cells (2 µg pDNA in 1 mL HBSS buffer pH 7.4 / well), and incubated 4 h. After the incubation, the polyplex solution was replaced by cell culture medium and incubated for further 48 h. The cells were washed three times with PBS, collected and centrifuged at 1,400 rpm for 4 min. The resulting cell pellet was suspended in 500 µL of cell culture medium and analyzed by flow cytometry, with excitation by argon laser at 488 nm and emission through the 515 – 545 nm filter. 10,000 cells per sample were analyzed using BD FACSCalibur™ (Becton-Dickinson, Heidelberg, Germany). The percentage of cell-associated fluorescence was determined using the computer program FlowJo (version 7.2.5, Tree Star, Stanford, CA). All the experiments were conducted in duplicate. The results were depicted as mean value ± SD. Significance among the mean values was calculated using Student's t-test. Probability value $p < 0.01$ was considered significant.

6.2.6 Agarose gel electrophoresis

To study the DNA-binding and –release ability of the polyplexes, agarose gel electrophoresis was used. The polyplexes with appropriate N/P ratio were prepared as described above. 12 µL of polyplex (containing 0.12 µg pDNA) solution with/without 5 units of Heparin, were mixed with 2 µL of blue dye and loaded into a 0.7 wt% agarose gel containing 0.5 µg/mL ethidium bromide. Electrophoresis was run in 0.5 %TBE buffer at 50 V for 75 min. DNA retardation was analyzed using UV illuminator, Fusion FX7 imaging system (Peqlab, Erlangen, Germany), to show the band of the DNA.

6.2.7 Endosome buffering capacity measurements

The endosome buffering capacities of three different side chains and synthesized polymers were measured by the acid-base titration according to Kim et al.[140]. As a comparison, the buffering capacities of two different MW PEIs, (25 kDa and 0.8 kDa) were also measured.

10 mg of each material was dissolved in 10 mL of 0.1 M NaCl solution and adjust pH 11 with 1 M NaOH. Then the solution was titrated to pH 3 with 0.1 M HCl. After each addition (50 or 100 µL) of HCl, the pH was measured by CG 843 P pH meter (Schott Geräte GmbH, Hofheim am Taunus, Germany) and plotted against the amount of the added HCl. Endosome buffering capacity was calculated as percentage of amine groups becoming protonated within the pH-range from 7.4 to 5.1, according to the following equation:

$$Buffering\ capacity\ (\%) = \frac{\Delta HCl\ \times 0.1\ M}{N\ mol} \times 100\ (\%)$$

ΔHCl is the volume of HCl solution (0.1 M) which was needed for the titration from pH 7.4 to 5.1. N mol is the total moles of protonable amine groups in 10 mg of each sample.

6.2.8 Cytotoxicity studies

MTT assay was performed to study the *in vitro* cytotoxicity of the starch-*g*-PEI polymers and their complexes. The cytotoxicity of starch based cationic polymers after the 3h incubation with α-amylase was also investigated. The A549 cells were seeded in a 96-well plate at a density of 10,000 cells per well in 200 µL of the cell culture medium. Once confluent, polymers in HBSS buffer pH 7.4 were applied on the cells and incubated for 4 h at 37 °C with gently shaking. After the incubation, the test solution was removed and cells were washed once with HBSS buffer. The fresh HBSS buffer with 10 % (vol.) of MTT reagent (5 mg/mL) was added, and incubated further 4 h at 37 °C with gently shaking. The solution was removed and cells were dissolved in DMSO and incubated 10 min at 37 °C with gently shaking. The absorbance at 550 nm was read by Tecan microplate reader. As reference, HBSS buffer

(negative, 0%) and 0.1% (w/w) Triton X-100 solution (ICN, Eschwege, Germany) (positive, 100%) in HBSS buffer were applied. To ensure the no interference of polymer to the test system, polymers without any cells were also tested.

6.2.9 Biodegradability study by α-amylase

6.2.9.1 Starch-iodine colorimetric assay

Biodegradability study of synthesized starch based cationic polymers was performed according to Xiao et al.[120]. Briefly, 40 µL of polymer solution (2 mg/mL in purified water) was added into 96-well plate. Then, 40 µL of α-amylase solution (25 – 125 µunit/mL in 0.1 M phosphate buffer pH 7) was added to each well. The plate was then incubated for 15, 30, 45, 60, 75 and 90 min at 37 °C and the digestion was stopped by adding 20 µL of 1 M hydrochloric acid. Subsequently, 100 µL of iodide color reagent was added and the absorbance at 580 nm was read by Tecan microplate reader. Calibration curves were made concurrently with experiments for each sample. All the experiments were conducted in triplicate. The remaining starch amount in the each sample was calculated using the calibration curve and plotted against the incubation time with α-amylase.

6.3 Results and discussion

6.3.1 Synthesis and characteristics of the starch based cationic polymers

The conjugation reaction between sodium carboxylate groups of the oxidized starch and amino groups of DETA, TEPA or TREN were done with the established method in chapter 3 using DMTMM conjugation reagent (Scheme 8). The chemical structures using three different cationic side chains are shown in Figure 34.

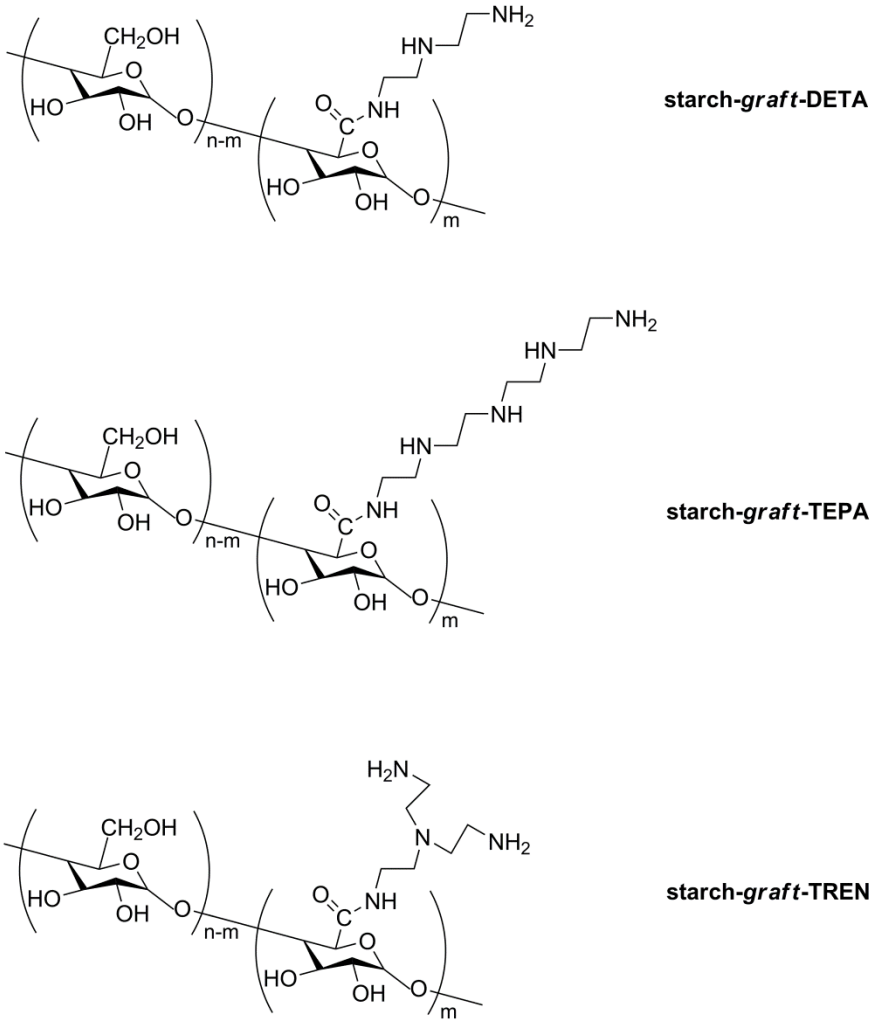


Figure 34: Starch based cationic polymers with different cationic side chains

The M_w and DS_{COONa} of the oxidized starch, which are used for the conjugation reaction, were summarized in Table 8.

Table 8: Characteristics of the large MW starch backbones for the conjugation reaction

Starch	M_W (g/mol)	PD	DS_{COONa} (%)
$S4_L$	5.8×10^5	2.37	43.9
$S5_L$	2.9×10^5	1.83	55.6

M_w and PD were determined by GPC with universal calibration.
DS_{COONa} was determined by Blumenkrantz-assay.

By using two starch backbones with different DS_{COONa} (Table 8), several polymers with different amount of the cationic side chain modification were synthesized. The modified amount of cationic side chains (wt%) on the starch backbone was determined by ^{1}H-NMR. Bruker NMR Magnet System 400 MHz Ultra shield plus: in D_2O, δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), DETA, TEPA, TREN: (NH_2-C***H₂***-C***H₂***, 2.29 – 3.46 ppm). In Figure 35, ^{1}H-NMR spectra of different amine grafted starches are shown. The peaks in the region of 2.29 – 3.46 ppm corresponding to the amine modification, NH_2-C***H₂***-C***H₂***, were observed in all polymers. In the following, polymers are named with a systematic name, according to the used starch backbone ($S4_L$ or $S5_L$), their side chains and modification amount. The subscript number after each oligoamine side chain (DETA, TEPA and TREN) stands for the wt% of the side chain against the whole molecule. The purity of starch based cationic polymers were examined by GPC measurement (HLC-8320GPC, Tosoh, Japan) on SUPREMA-MAX 1000 and 30 columns (PSS) at a flow rate 1 mL/min at 35 °C in 1 % formic arid.

In Figure 36, the GPC curves of pure TEPA and synthesized $S5_L$-*g*-$TEPA_{28}$, as representative samples were shown. Since there is no peak of free TEPA observed in $S5_L$-*g*-$TEPA_{28}$, it became clear the purification of excess amount of TEPA by dialysis worked successfully. The

GPC curves of all starch-*graft*-oligoamines showed monomodal peak with polydispersity of 1.39 to 1.52, which indicated the successful conjugation reaction between oligoamine side chains and the starch backbone.

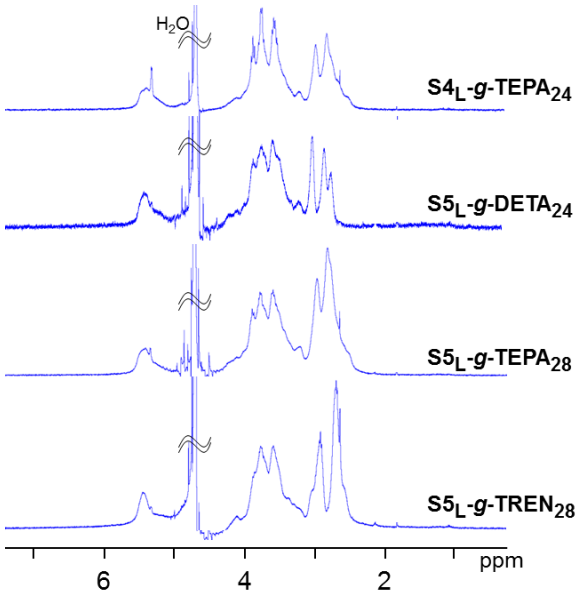


Figure 35: ^{1}H-NMR spectra of the synthesized cationic polymers

δ_H Starch: (***1H***, 5.12 – 5.58 ppm), (***2H***, ***3H***, ***4H***, ***5H***, ***6H***, 3.45 – 4.30 ppm), DETA, TEPA, TREN: (NH_2-C***H***$_2$-C***H***$_2$, 2.29 – 3.46 ppm).

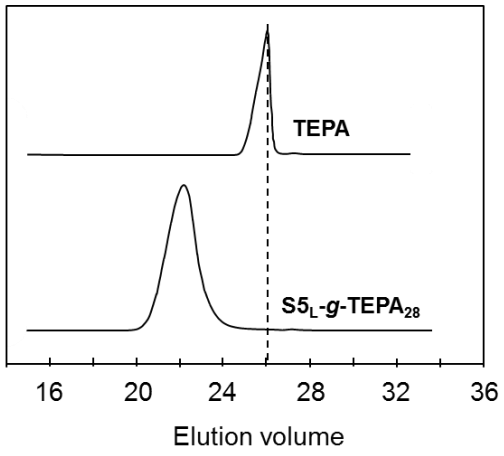


Figure 36: GPC spectra of pure TEPA and $S5_L$-*g*-$TEPA_{28}$

In Table 9, the characterization results of the synthesized starch based cationic polymers are summarized. Three different cationic modifications have been done based on the $S5_L$ starch. By using the TEPA and TREN side chains, a modification of approximately 30 wt% was achieved, which was the target value, since starch-*graft*-PEI with 30 wt% modification showed the best transfection ability (compare chapter 4). However the modification with DETA was only 24.4 wt%. This is due to the smaller MW of DETA (MW = 103.17 g/mol) compared to other two side chains (MW = 189.30, 146.23 g/mol). Additionally the modification of S4, the starch with lower DS_{COONa}, was done with TEPA, and resulted in a modification amount of 23.6 wt%. Every 3.7th glucose of $S4_L$-*g*-$TEPA_{24}$, every 1.9th glucose of $S5_L$-*g*-$DETA_{24}$, every 2.9th glucose of $S5_L$-*g*-$TEPA_{28}$ and every 2.2th glucose of $S5_L$-*g*-$TREN_{28}$ has a conjugated cationic side chain. All four polymers showed a monomodal peak in GPC and the PDs were around 1.5, which were even smaller than that of starch-*graft*-PEI polymers, indicated the better homogeneity of the starch-*graft*-oligoamine polymers. The successful transfer of the synthesis method developed for PEI to the oligoamines was proofed. The time needed for the purification became shorter, since the oligoamine side chains were smaller and easier to remove through the dialysis membrane. The obtained starch-*graft*-oligoamines were white powder and well soluble in water, those were the same properties that obtained starch-*graft*-PEI polymers.

Table 9: Characterization of the synthesized starch based cationic polymers

Starch-*g*-cationic side chains			
	Total MW (g/mol)	PD	Side chain (wt.%)
$S4_L$-*g*-$TEPA_{24}$	7.6×10^5	1.47	23.6
$S5_L$-*g*-$DETA_{24}$	3.8×10^5	1.52	24.4
$S5_L$-*g*-$TEPA_{28}$	4.1×10^5	1.39	28.4
$S5_L$-*g*-$TREN_{28}$	4.0×10^5	1.48	28.3

MW and amount of side chain was determined by ^{1}H-NMR.
PD was determined by GPC.

6.3.2 Polyplex formation

Using the synthesized four different starch based cationic polymers, polyplexes were formulated with pDNA (N/P = 18). The size and zeta potential of polyplexes were measured using a Zetasizer (Malvern Instruments, Worcestershire, UK) and summarized in Table 10.

The largest polyplex, 164nm, was observed from $S5_L$-*g*-$DETA_{24}$, which has the smallest side chain MW and less modification (24 wt%). In general, larger side chains and higher modification lead to smaller polyplexes with higher zeta potential. $S4_L$-*g*-$TEPA_{24}$ showed a little larger size and lower zeta potential polyplex (87 nm, +26 mV) than $S5_L$-*g*-$DETA_{24}$ (78 nm, + 35 mV), which has its reason most likely in the larger starch backbone with higher PD. The polplexes from $S5_L$-*g*-$TEPA_{28}$ and $S5_L$-*g*-$tirsAm_{28}$ showed almost same characteristics, the smallest sizes, 70 to 80 nm and highest zeta potentials around + 35 mV. These polyplex characteristics of $S4_L$-*g*-$TEPA_{24}$, $S5_L$-*g*-$TEPA_{28}$ and $S5_L$-*g*-$tirsAm_{28}$ were comparable to that of $S2_L$-*g*-PEI_{30} polymer, which showed the best transfection efficiency in chapter 4. The polyplex characterisics also fit the properties reported for good cellular uptake[121].

Table 10: Characterization of the polyplexes from synthesized starch based cationic polymers (N/P = 18)

Starch-*g*-cationic side chains			Polyplex		
	Total MW (g/mol)	Side chain amount (wt.%)	Size (nm)	PDI	ζ-potential (mV)
$S4_L$-*g*-$TEPA_{24}$	7.6×10^5	23.6	87 ± 0.9	0.27 ± 0.01	$+25.8 \pm 0.3$
$S5_L$-*g*-$DETA_{24}$	3.8×10^5	24.4	164 ± 0.3	0.11 ± 0.01	$+13.7 \pm 0.8$
$S5_L$-*g*-$TEPA_{28}$	4.1×10^5	28.4	78 ± 0.1	0.18 ± 0.01	$+34.5 \pm 0.6$
$S5_L$-*g*-$TREN_{28}$	4.0×10^5	28.3	72 ± 0.3	0.23 ± 0.01	$+34.2 \pm 1.0$
$S2_L$-*g*-PEI_{30}	9.5×10^5	27.4	89 ± 2.5	0.16 ± 0.01	$+28.6 \pm 0.8$

6.3.3 Transfection experiments

The transfection efficiency of the polyplexes from synthesized polymers was studied in the A549 cell line.

6.3.3.1 Luciferase transfection assay

As first transfection study, the luciferase transfection assay has been performed. From each of the four polymers, polyplexes in the N/P range 2 to 40 were formulated and tested for their transfection efficiency. PEI 25 kDa (N/P = 10) according to Boussif et al.[44] were always used as positive control.

In Figure 37, a clear difference between the four polymers was observed. The transfection efficiency using the polymers with TEPA side chains (a and c) were much higher than that of DETA or TREN (b and d). Though the amount of the cationic modification is almost equal in (a) 23.6 % of TEPA side chains and (b) 24.4 % of DETA side chains, the transfection results showed a huge difference (Figure 37 (a) (b)). It was assumed that, the characteristic of different side chain would bring this huge difference. Both TEPA and DETA are linear structures, and their difference could be seen in their MWs, 189.30 and 103.17 g/mol, respectively (Figure 33). From this result, it could be demonstrated that the MW of the side chain is one of the key factors for the ability as gene delivery vectors. Regarding the Figure 37 (c) and (d), a similar discussion can be applied. The amount of the cationic modification is almost equal, (c) 28.4 % of TEPA side chains and (d) 28.3 % of TREN side chains, and the MWs of these two starch based cationic polymers are also the same. Therefore, the cause of the huge difference in transfection efficiency was related to the side chains. Here we have two different molecular structures, a linear structure in TEPA and a branched structure in TREN. Additionally the MW of the both molecules is a little different, 189.30 and 146.23 g/mol, respectively (Figure 33). Hence, the difference in transfection efficiency could be caused by the MW difference and structural difference of the side chains. From these results of the luciferase transfections using three different oligoamine side chains, we could learn; 1) the DETA is too short to mediate efficient transfection, and 2) the branched structure, more precisely, tertiary amine group is not essential for the good transfection efficiency.

The two cationic starches with very promising results, $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$, were further studied to find out the optimal N/P ratio in the range of N/P = 16 to 30. We included two additional positive controls, the commercial transfection reagent jetPRIME™ and the best starch-*g*-PEI polymer from chapter 4, $S2_L$-*g*-PEI_{30} (N/P = 14).

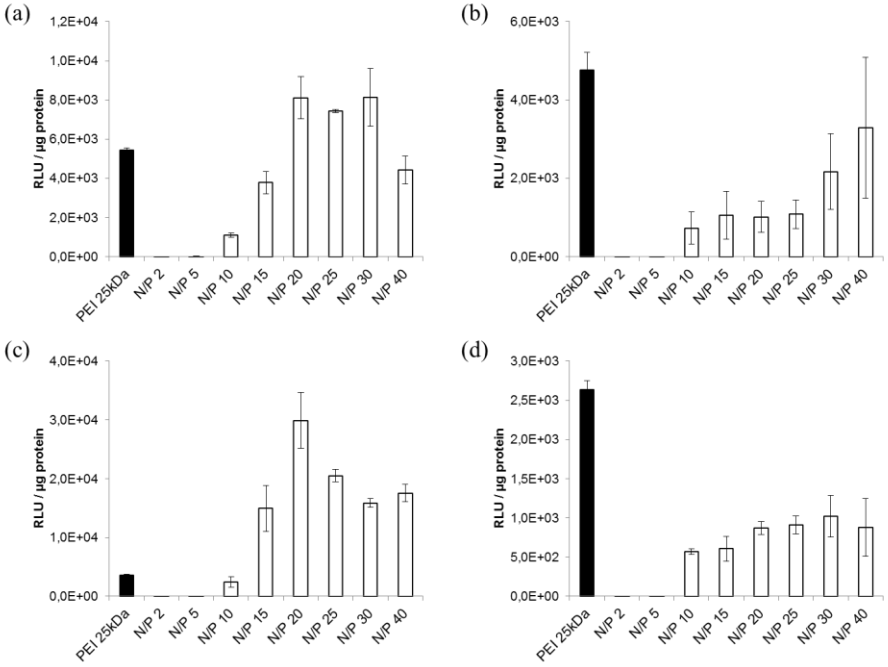


Figure 37: Results of luciferase transfection assay:

(a) $S4_L$-*g*-$TEAP_{24}$, (b) $S5_L$-*g*-$DETA_{24}$, (c) $S5_L$-*g*-$TEPA_{28}$, (d) $S5_L$-*g*-$TREN_{28}$

As is evident from Figure 38, the optimal N/P ratio for the best luciferase transfection efficiency was 18 for both polymers. In optimized conditions, both polymers showed higher transfection efficiency than the all three positive controls, jetPRIME™, PEI 25 kDa and $S2_L$-*g*-PEI_{30}. These excellent results of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ indicated that TEPA is more suitable as a side chain than s-PEI. As we discussed, the MW of the cationic side chain is an essential factor as a good gene delivery vector. The MWs of DETA and TREN were not large enough, however too large MW side chain, s-PEI (800 Da), was also not perfect, and it

should be somewhere in between, in this case TEPA (MW = 189.30) was the best. At the same time, the existence of the tertiary amine groups is not a prerequisite, since not only TREN but also s-PEI with a lot of tertiary amine groups showed less transfection efficiency than TEPA.

Based on the results presented in the luciferase transfection assay (Figure 37 and Figure 38), the TEPA modified starch, $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$, were chosen as the two best candidates for gene delivery vectors and were further studied by flow cytometry.

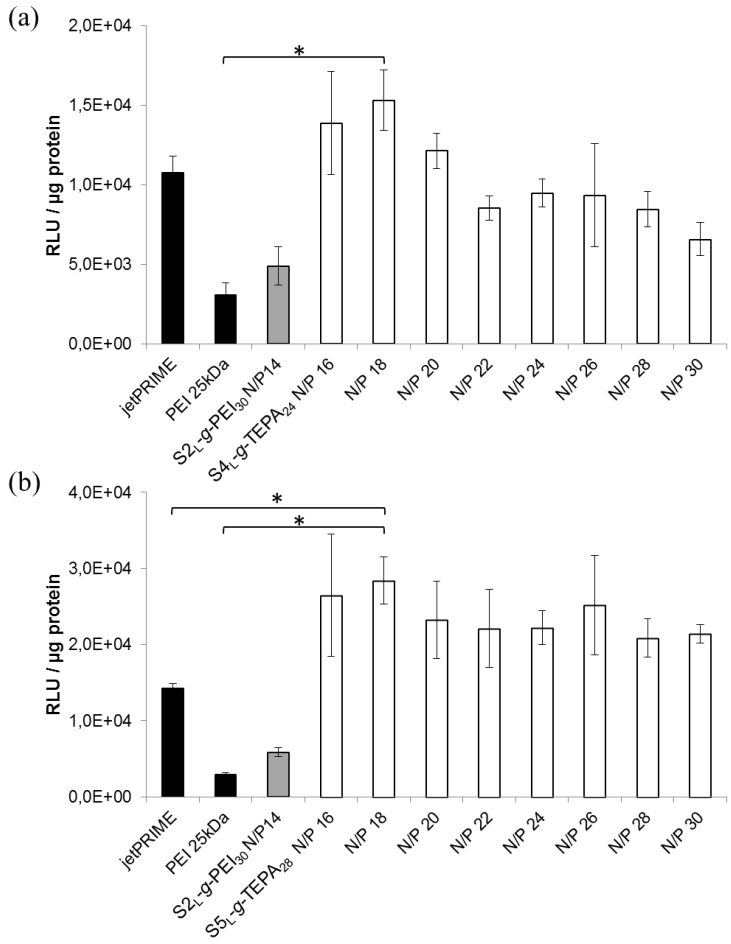


Figure 38: Results of luciferase transfection assay (N/P = 16 to 30);

(a) $S4_L$-*g*-$TEPA_{24}$, (b) $S5_L$-*g*-$TEPA_{28}$

Values are the mean + SD from n = 4 measurements.

Statistical significance is indicated as follows: * ($p < 0.01$)

6.3.3.2 Flow cytometry

The transfection efficiency of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ with N/P = 18 was further studied with flow cytometry, to determine the percentage of transfected cells (Figure 39).

The highest amount of transfected cells, 33 % and 27 %, were observed from the TEPA modified starches, $S5_L$-*g*-$TEPA_{28}$ and $S4_L$-*g*-$TEPA_{24}$. They transfected more cells than any other positive controls, jetPRIME (24 %), PEI 25 kDa (13 %), and $S2_L$-*g*-PEI_{30} (16 %) (Figure 39). This was the same tendency as observed in the luciferase transfection experiment, and thus verification that TEPA was the superior side chain within all tested candidates with respect to the transfection efficiency.

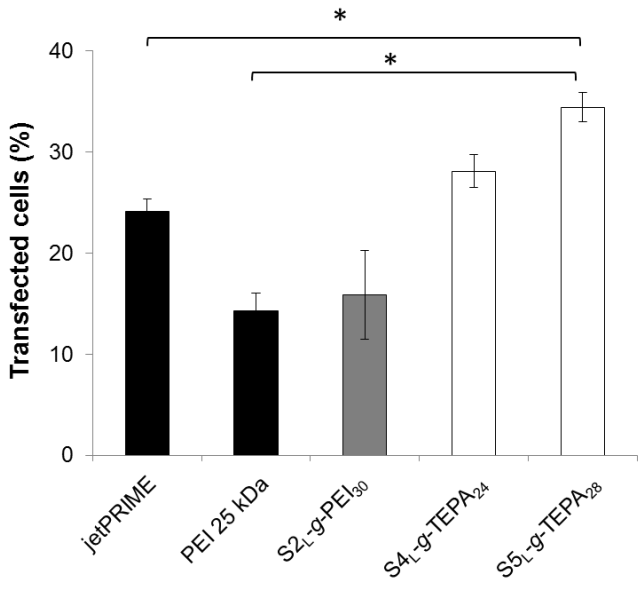


Figure 39: Results of Flow cytometry

Values are the mean + SD from n = 4 measurements.

Statistical significance is indicated as follows: * ($p < 0.01$).

In Figure 40, Flow cytometry results are shown in the histograms of cell counts against fluorescence intensity to compare the different transfection characteristics between s-PEI modified starch ($S2_L$-*g*-PEI_{30}) and TEPA modified starch ($S5_L$-*g*-$TEPA_{28}$). The white peak

showed the negative control without any pDNA application and the gray peak showed the cells with polyplex application. The difference between the transfection of $S2_L$-*g*-PEI_{30} (Figure 40(a)) and $S5_L$-*g*-$TEPA_{28}$ (Figure 40 (b)) is not only the number of the transfected cells but also the fluorescence intensity of the transfected cells. The transfected cells by $S5_L$-*g*-$TEPA_{28}$ polyplex showed much higher fluorescence intensity and a stronger peak shift to the direction of higher fluorescence intensity (Figure 40(b)). In fact, there were almost no cells which showed fluorescence intensity of 1,000 in the $S2_L$-*g*-PEI_{30} polyplex applied cells, on the other hand, there was even a small peak observed over the fluorescence intensity of 1,000 in the $S5_L$-*g*-$TEPA_{28}$ polyplex treated cells (Figure 40(c)). Taken together, the $S5_L$-*g*-$TEPA_{28}$ has advantages in transfection not only the number of transfected cells but also in the total amount of the produced proteins.

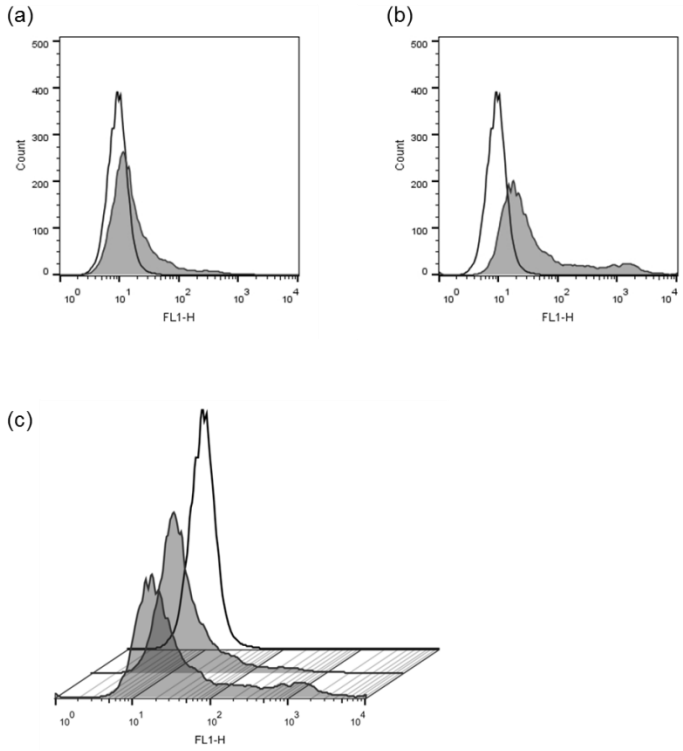


Figure 40: Histograms from Flow cytometry experiment in A549 cells. White peak: negative control. Gray peaks: polyplexes applied cells. (a) $S2_L$-*g*-PEI_{30} polyplex, (b) $S5_L$-*g*-$TEPA_{28}$ polyplex, (c) stagger offset of $S2_L$-*g*-PEI_{30} (middle) and $S5_L$-*g*-$TEPA_{28}$ (front) polyplex

6.3.4 Agarose gel electrophoresis

To study the DNA binding and condensation ability of each polyplex, agarose gel electrophoresis was done. In Figure 41, no pDNA could run through the gels without the addition of heparin (a, b, c, d and e), that indicated the perfect polyplexes formation without any free pDNA remaining. By the addition of an excess of heparin (samples marked with an apostrophe), all the pDNA-polymer complexes were broken and the released pDNA ran through the agarose gel. The pDNA band was made visible by ethidium bromide staining and it was thus possible to estimate the amount of pDNA in each polyplex was equal.

Though all the polyplexes contained the same amount of pDNA, fluorescence intensity from the various polyplexes was different. The fluorescence intensity was attributed to the condensing, i.e. the tightness of the packing. (c) $S5_L$-*g*-$DETA_{24}$ showed the strongest fluorescence intensity, which represented the weakest DNA condensation ability of these polyplexes. This weak DNA condensation ability resulting in larger polyplex sizes, as is shown in Table 10, and it could be one of the reasons of the poor transfection efficiency of this sample. The strongest DNA-condensing (tight packing) ability was observed in (a) $S2_L$-*g*-PEI_{30} and (e) $S5_L$-*g*-$TREN_{28}$, and the middle were (b) $S4_L$-*g*-$TEPA_{24}$ and (d) $S5_L$-*g*-$TEPA_{28}$. TREN seemed to condense pDNA very strong, this result again fit very well with small size of the polyplex shown in Table 10.

As is obvious from the results of transfection experiment and agarose gel electrophoresis, it is not always the case that the higher DNA condensation ability leads to higher transfection. Polymers with too strong DNA condensation ability might have disadvantage to release the DNA once inside the cytoplasma.

Another interesting finding was the similarity of the two polyplexes from (a) $S2_L$-*g*-PEI_{30} and (e) $S5_L$-*g*-$TREN_{28}$. The size, zeta-potential, and also the DNA condensation ability of these polyplexes were quite similar, though $S2_L$-*g*-PEI_{30} showed good and $S5_L$-*g*-$TREN_{28}$ showed poor transfection efficiency. To find the dissimilarity of these two polyplexes, further studies have been done in the following part.

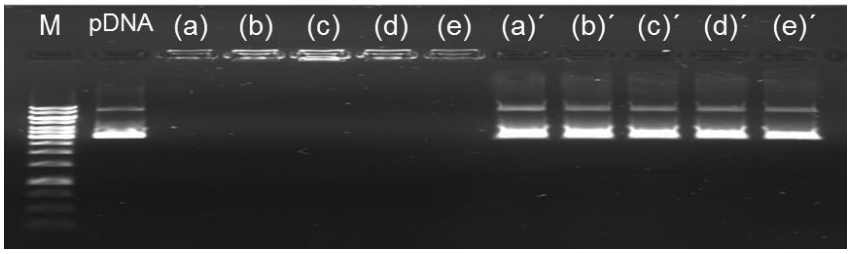


Figure 41: Agarose gel electrophoresis of polyplexes from different polymers

M: size marker, pDNA: non-complexed plasmid (a) $S2_L$-*g*-PEI_{30} N/P14, (b) $S4_L$-*g*-$TEPA_{24}$ N/P18, (c) $S5_L$-*g*-$DETA_{24}$ N/P18, (d) $S5_L$-*g*-$TEPA_{28}$ N/P18, (e) $S5_L$-*g*-$TREN_{28}$ N/P18, Character with apostrophe indicate the sample after the addition of 5 unit heparin

6.3.5 Endosome buffering capacity measurements

Endosome buffering capacity is one of the most important parameters of gene delivery vectors since it indicates the possibility of efficient endosomal escape. For example, b-PEI is well known to have high buffering capacity at acidic endosomal pH, known as "proton sponge effect"[17].

Through the acid-base titration, the endosome buffering capacities of different oligoamine side chains and b-PEI was studied. In Figure 42, the acid-base titration curves of each material are shown. The gray area is the endosomal pH-range, 7.4 to 5.1. Additionally, the amount of HCl needed for titration from pH 7.4 to 5.1 and the calculated buffering capacities were summarized in Table 11. As is obvious from Figure 42, the three oligoamine side chains showed quite different acid-base titration curves. The titration curve of TEPA is most similar to those of s-PEI and b-PEI. The pH change of the TEPA in the pH-range of 7.4 to 5.1 is the slowest of the three oligoamine side chains, which corresponds to the higher buffering capacity (15 %) compared to the other two oligoamines (Table 11).

This difference of buffering capacity between TEPA and the other two side chains could explain the difference in transfection efficiency. Especially, the characteristics of two polyplexes from $S5_L$-*g*-$TEPA_{28}$ and $S5_L$-*g*-$TREN_{28}$ were quite similar (Table 10), though showed completely different transfection efficiency (Figure 37). It is likely that, the characteristics of these polyplexes (size, zeta-potential and PDI) were preferable for the uptake, however the lower buffering capacity of TREN in the endosomal pH did not allow the same efficient endosomal escape. In combination with lower release by higher condensation that induced the much lower transfection efficiency compared to the TEPA grafted starch. In conclusion, TEPA is the most competent side chain of the three small MW side chains from the view point of buffering capacity and following endosomal escape. This low buffering capacity of TREN could also explain the difference in transfection efficiency compared to $S2_L$-g-PEI_{30}, though polyplex from $S2_L$-g-PEI_{30} and $S5_L$-*g*-$TREN_{28}$ showed the similar size, PDI, zeta potential and DNA-binding ability.

Despite the lower buffering capacity of TEPA than s-PEI, $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ showed higher transfection efficiency than $S2_L$-*g*-PEI_{30}. This fact indicated that the buffering capacity of TEPA (15 %) is enough to escape from endosome, and not only the high buffering capacity is the required parameter for high transfection efficiency. Gabrielson et al.[126] claimed that, the gene delivery activity could be attributable to an appropriate balance between polymer buffering capacity and strength of polymer/DNA interactions. Referring that, $S2_L$-*g*-PEI_{30} was assumed to have good buffering capacity, but not to have appropriate strength of polymer/DNA interactions. Since s-PEI has many amine groups, the interaction between polymer/DNA was extremely strong, and could not release the DNA effectively compared to the $S5_L$-*g*-$TEPA_{28}$. This strong DNA-binding ability of $S2_L$-*g*-PEI_{30} was also observed in the agarose gel electrophoresis (Figure 41). The $S5_L$-*g*-$TEPA_{28}$ is assumed to have an appropriate balance of the buffering capacity and binding ability to the DNA, thus showed the superior transfection efficiency than $S2_L$-*g*-PEI_{30}.

The buffering capacities of the synthesized polymers were also studied and summarized in Table 11. All polymers showed the increased buffering capacities compared to their side

chains by the conjugation with starch backbone, though the sequence of the buffering capacity was exactly equivalent to that was observed in the side chains. Starch-*graft*-TEPA showed its advantage in buffering capacity compared to other side chains, though it is still lower than starch-*graft*-PEI. This result fit with the discussion before in the buffering capacity of the side chains; not only is the high buffering capacity the required parameter for high transfection efficiency.

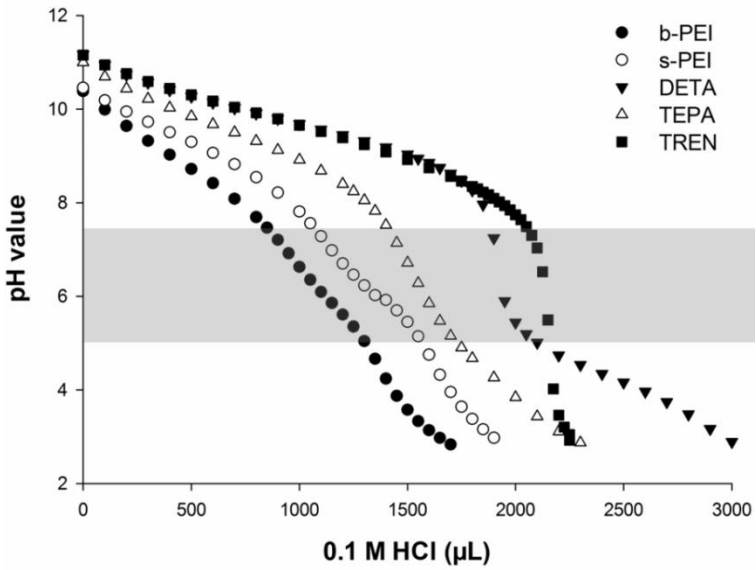


Figure 42: Acid-base titration of different side chains and b-PEI. The gray area is pH-range of 7.4 to 5.1.

Table 11: Calculated buffering capacities of each side chains, synthesized polymers and b-PEI, in the pH-range of 7.4 to 5.1

Side chains	ΔHCl (µL)	Buffering capacity	Polymers	Buffering capacity
b-PEI	442	19 %	b-PEI	19 %
s-PEI	458	19 %	$S2_L$-*g*-PEI_{30}	29 %
DETA	331	11 %	$S5_L$-*g*-$DETA_{24}$	18 %
TEPA	401	15 %	$S4_L$-*g*-$TEPA_{24}$	21 %
			$S5_L$-*g*-$TEPA_{28}$	22 %
TREN	159	6 %	$S5_L$-*g*-$TREN_{28}$	15 %

6.3.6 Cytoxicity

The cytotoxicity of the $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ and was studied with MTT assay. As is shown in Figure 43 (a) $S4_L$-*g*-$TEPA_{24}$ and (b) $S5_L$-*g*-$TEPA_{28}$, both polymers showed almost no cytotoxicity (almost 100 % cell viability), in the concentration needed for optimal transfection (gray area). As a comparison, the cytotoxicity of PEI 25 kDa and $S2_L$-*g*-PEI_{30} is also shown in the same graph. The cytotoxicity of $S2_L$-*g*-PEI_{30} was lower than PEI 25 kDa, but higher than the both polymers with TEPA side chains, $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$. These results are a clear evidence of the superior competency of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ to $S2_L$-*g*-PEI_{30} and PEI 25 kDa as gene delivery vectors, in terms of gene delivery efficiency and also safety.

Surprisingly, the cytotoxicity of the $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ were further decreased after the 3 h incubation with α-amylase, as is observed with $S2_L$-*g*-PEI_{30} in chapter 4. The decreasing of the cytotoxicity was not expected, since the starch backbone of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ were highly modified with TEPA side chains compared to the $S2_L$-*g*-PEI_{30}. However, this decreased cytotoxicity obviously indicated the degradation of those polymers by α-amylase. Thus a further biodegradability study of the synthesized polymers has been done in the following experiments.

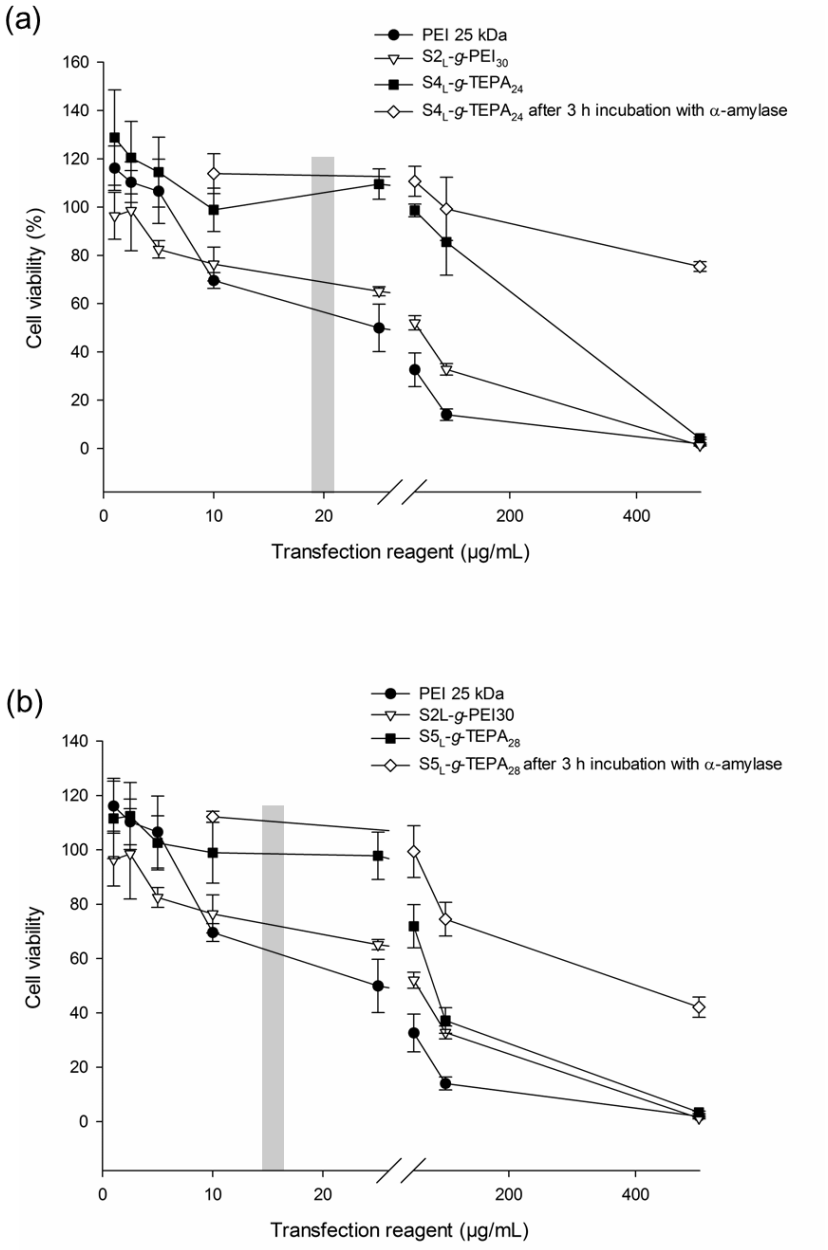


Figure 43: Results of the MTT cytotoxicity tests (A549 cells).

The gray area shows the concentration needed for the best transfection efficiency.

(a) $S4_L$-*g*-$TEPA_{24}$ transfection reagents (b) $S5_L$-*g*-$TEPA_{28}$ transfection reagents

6.3.7 Biodegradability study by α-amylase

The biodegradability of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ was studied by α-amylase using an iodine-based method. In Figure 44, incubation time with α-amylase vs. the absorbance at 580 nm from starch-iodine complex can be seen. The amount of the α-amylase was adjusted for each sample in order to see the time dependent degradation. 10 µunit of α-amylase for $S4_L$-*g*-$TEPA_{24}$ and 1 µunit for $S5_L$-*g*-$TEPA_{28}$ was used for each well.

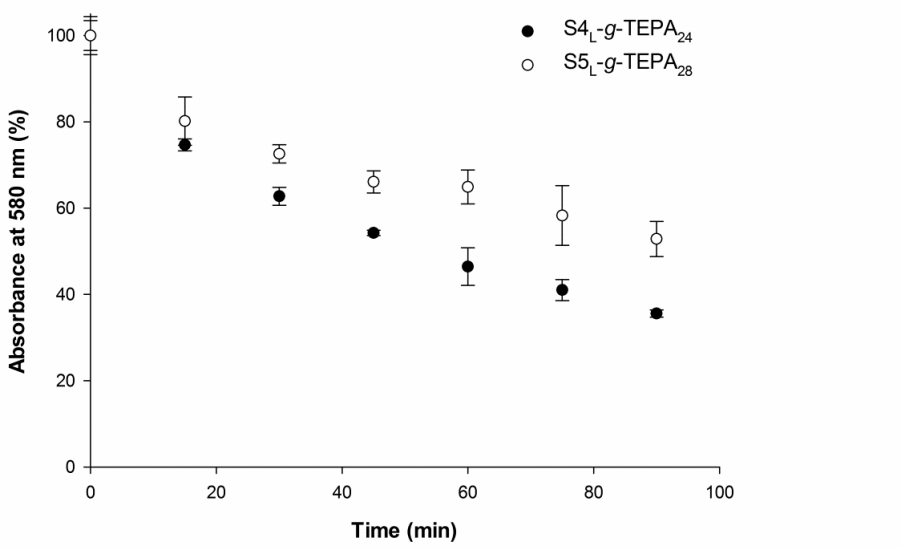


Figure 44: Biodegradability by α-Amylase over 90 min, 37 °C

It is known that α-amylase is less effective in degrading modified starches because of the molecular specificity of the enzymatic reaction. It was therefore not expected that the synthesized cationic starch polymers with high modification amount maintained enzymatic degradability. Compared with $S2_L$-*g*-PEI_{30}, $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ were much more modified with side chains. Every 3.7th glucose of $S4_L$-*g*-$TEPA_{24}$ and every 2.9th glucose of $S5_L$-*g*-$TEPA_{28}$ carries one TEPA side chain, on the other hand, every 12.6th glucose of $S2_L$-*g*-PEI_{30} carries one s-PEI side chain.

In Figure 44, contrary to what we expected, the absorbance at 580 nm which corresponds to the amount of the starch-iodine complex was decreased over 90 min by incubation with α-amylase. These results are the clear evidence of the degradation of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ by α-amylase. And these results were also supported by the reduced cytotoxicity of the MTT test (Figure 43). Unfortunately, the degradation rate cannot be compared to original starch or starch-*g*-PEI, even between $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$, since the amount of α-amylase was optimized for each sample. However the fact of remaining biodegradability should be a huge benefit in respect of the safety issue as gene delivery vector.

6.4 Conclusion

The starch based cationic polymers modified with oligoamine side chains could form nano-sized polyplexes with pDNA, some of which showed a very promising transfection efficiency in the A549 cell line. Successful transfection was observed both qualitatively and quantitatively, by the luciferase assay and by flow cytometry analysis of fluorescent protein-expressing cells. The transfection efficiency was seen to depend on the MW and the structure of the side chains. The best transfection efficiency was observed from the starch based cationic polymer which had 28 wt% of TEPA side chains. The observed transfection efficiency was much higher than all three positive controls, commercial transfection reagent jetPRIME^{TM} used as recommended in the manufacturer's protocol, PEI 25 kDa, and our best starch-*g*-PEI, $S2_L$-*g*-PEI_{30}.

Furthermore, we demonstrated a lower cytotoxicity of $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ than $S2_L$-*g*-PEI_{30} and PEI 25 kDa. After the incubation with α-amylase, the cytotoxicity of synthesized starch-g-TEPA polymer was even further decreased. This implied that the starch backbone of the modified polymer was degraded and became almost non-toxic small fragments. By iodine-starch assay, the degradation of the $S4_L$-*g*-$TEPA_{24}$ and $S5_L$-*g*-$TEPA_{28}$ was confirmed.

In conclusion, we have successfully developed the starch based cationic polymers, which could achieve a superior transfection efficiency and safety (low cytotoxicity and biodegradability).

7 Summary and outlook

The aim of presented work was to design biodegradable starch based cationic polymers and evaluate their potential as non-viral gene delivery vector. In this context, it was demonstrated that a simple and reproducible method to synthesize starch based cationic polymers with various kinds of side chains was successfully established. By use of DMTMM reaction reagent, the conjugation reaction proceeded satisfactorily in co-solvent H_2O / DMSO. The advantages of the DMTMM reagent are; 1) it works in the presence of water, 2) the mild reaction condition in R.T., 3) easy handling (white powder) and 4) easy purification method by washing with water. (chapter 3).

Additionally, it was shown that several synthesized polymers had promising potential as gene delivery vectors with respect to efficiency and safety by precisely controlled polymer design. Namely, it was shown that starch-*g*-PEI polymer required around 30 wt% PEI modification and large MW (> 550,000 g/mol) for optimal transfection efficiency. These well designed starch-*g*-PEI polymers showed higher transfection efficiency and lower cytotoxicity than positive control PEI 25 kDa, and retaining the biodegradability by α-amylase. (chapter 4)

Hydrophobic modification onto the starch-*g*-PEI polymer had been done in chapter 5. Due to the introduction of hydrophobic groups, the DNA-binding ability decreased slightly, that brought the significant decrease of transfection efficiency, though polyplex characteristics (size, PDI and zeta-potential) were almost same. The presence of only few hydrophobic groups was enough to show this effect. These results demonstrated the great importance of the precise design of polymers, and only a small change on the polymer could change dramatically the ability of the polymer as gene delivery vector.

Furthermore, it was demonstrated that the TEPA cationic side chain brought even more advantage than s-PEI side chain. Starch-*g*-TEPA with around 30 wt% side chain modification showed the similar polyplex characteristics (size, PDI and zeta-potential) as that of starch-*g*-PEI, but superior results in transfection efficiency and cytotoxicity, at the same time, retaining the biodegradability by α-amylase. (chapter 6)

In summary, these results underline that well designed starch based cationic polymers would be promising candidates as non-viral gene delivery vector, and only a little modification could drastically alter their ability. For realizing an ideal polymer, suitable valance of every property would be required, for instance, MW, amount of cationic charges, DNA-binding/-release ability, and endosome buffering capacity.

However, it should be emphasized that here presented works are just early-stage studies of starch based cationic polymers as gene delivery vector. Although this thesis as a piece might contribute to the establishment of a simple and reproducible starch modification method and better understanding on the essential properties of polymers, still several open questions are raised.

- Regarding the hydrophobic modifications, the increased cytotoxicity and decreased DNA-binding ability was a critical factor in chapter 5. Yet, several researchers reported the increasing transfection efficiency by the introduction of hydrophobic groups. Thus, it would be a great of interest to find out the key parameter of the hydrophobic groups to give a positive or negative effect to gene delivery systems.
- In this thesis, four different cationic side chains were tested to modify starch backbone and studied the effect of the different side chains to the starch-*graft*-cationic polymers as gene delivery vectors (Chapter 4 and Chapter 6). However, there are much more possible cationic side chains that could provide satisfactory properties to starch backbone. It would

be definitely interesting to do a screening test of the buffering capacity of different cationic molecules to find out the promising candidates as cationic side chains.

- With respect to the practical applications, *in vivo* experiments are prerequisite. All studies performed in this thesis were based on *in vitro* and further experiments using different type of cells are needed to find out the appropriate practical applications and *in vivo* studies.

List of abbreviations

Benz-ITC	benzyl isothiocyanate
b-PEI	branched-polyethyleneimine
CD	cyclodextrine
CDMT	2-chloro-4,6-dimethoxy-1,3,5-triazine
CLSM	confocal laser scanning microscopy
CME	clathrin-dependent endocytosis
CNT	carbon nanotubes
CycHex-ITC	cyclohexylmethyl isothiocyanate
DCC	*N,N'*-dicyclohexylcarbodiimide
DCChol	3β(N-(N,N-dimethylaminoethane)-carbamoyl) cholesterol
DETA	dietylenetriamine
DMAEMA	2-(dimethylamino)ethyl methacrylate
DMSO	dimethyl sulfoxide
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4methyl-morpholinium chloride
DOGS	dioctadecylamidoglycylspermine
DOPE	Dioleoylphosphatidylethanolamine
DOTAP	1,2-bis(oleoyloxy)-3-(trimethylammonio)propane
DOTMA	N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride
DS	degree of substitution
EDA	ethylenediamine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGMA	ethylene glycol methacrylate
EPR effect	Enhanced Permeation and Retention effect

FBS	fetal bovine serum
FT-IR	fourier transform infrared spectroscopy
-g-	*-graft-*
GPC	gel permeation chromatography
GTP	guanosine triphosphate
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hep-IC	heptyl isocyanate
HES	hydroxyethyl starch
HOBt	hydroxybenzotriazole
IC	isocyanate
ITC	isothiocyanate
LDH	lactate dehydrogenase
l-PEI	linear-polyethyleneimine
MMA	methyl methacrylate
MR	magnetic resonance
MRI	magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
MW	molecular weight
NMM	4-methylmorpholine
NMR	nuclear magnetic resonance
NHS	*N*-hydroxysuccinimide
NVP	N-vinylpyrrolidone
PAMAM	polyamidoamine
PBS	phosphate buffered saline
PCL	polycarprolactone
PCI	Photochemical internalization
PD	polydispersity

PDI	polydispersity index
pDNA	plasmid DNA
PEG	polyethylene glycol
PEHA	pentaethylenehexamine
PEI	polyethyleneimine
PLGA	Poly(lactid-co-glycolid) acid
PLL	Poly(L-lysine)
PPI	Poly(propylenimine)
RAFT	reversible addition−fragmentation chain transfer
RGD	arginine-glycine-aspartic acid
RLU	relative light units
TBE	Tris/Borate/EDTA
TEMPO	2,2,6,6-tetramethyl-1-piperridinyloxy
TEPA	tetraethylenepentamine
TETA	triethylenetetramine
THF	tetrahydrofuran
TREN	tris(2-aminoethyl)amine

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Scientific Output

Publications

Paper

Biomacromolecules

"Design of Starch-*graft*-PEI polymers: an effective and biodegradable gene delivery platform"

Hiroe Yamada, Brigitta Loretz, Claus-Michael Lehr

Book chapter

ISBN: 9789814364850

Safety Aspects of Engineered Nanomaterials, *Pan Stanford Publishing Pte. Ltd.*

Chap.10: Chances of Nanomaterials for Pharmaceutical Applications

Brigitta Loretz, Ratnesh Jain, Parajakta Dandekar, Carolin Thiele,

Hiroe Yamada, Babak Mostaghaci, Qiong Lian, Claus-Michael Lehr

Presentations

06/2011: ESF Research Conferences: 3rd European Science Foundation Summer School. Nanomedicine 2011, Wittenberg, Germany

Poster presentation:

Synthesis of biodegradable starch derivatives for gene delivery

02/2013: CRS - Germany local chapter 2013, Ludwigshafen Germany

Oral presentation:

Novel starch based biodegradable polymers for gene delivery

10/2013 DPhG annual conference 2013

Freiburg, Germany

Poster presentation:

Synthesis of biodegradable cationic starch derivatives for gene delivery

11/2013 1. Doktorandentag der Naturwissenschaftlich-Technischen Fakultät III

Saarbruecken, Germany

Poster presentation:

Synthesis of biodegradable cationic starch derivatives for gene delivery

02/2014 10th International Conference and Workshop on BIOLOGICAL BARRIERS

Saarbruecken, Germany

Poster presentation:

Synthesis of cationic starch derivatives for gene delivery

06/2014 4th International HIPS-Symposium

Saarbruecken, Germany

Poster presentation:

Novel Polysaccharide-based Polymers for Antiinfective Drug and Gene Delivery

07/2014 41st Annual Meeting & Exposition of the Controlled Release Society

Chicago, USA

Poster presentation:

Biodegradable starch derivatives for gene delivery

Acknowledgments

First of all, I would like to express my special appreciation and thanks to Prof. Dr. Claus-Michael Lehr for giving me this great opportunity to challenge my doctoral research in this department. You gave me not only excellent scientific ideas, facilities and financial support, but also a huge encouragement to broaden my knowledge and to develop my scientific skills. I am really grateful for your heartfelt support during last years.

I owe my deepest gratitude to Dr. Brigitta Loretz as my supervisor. I would like to thank for your huge support and trust throughout good and bad times during the last years. You always have great ideas and gave me an exact advice which I needed. I would like to thank not only for your scientific support, but also for your kindness and care to me. You always made time for me, even you were in the busiest situation. This work would not be done without your greatest support. You are a tremendous mentor for me!

I would especially like to thank Prof. Dr. Gerhard Wenz as my second professor. You are always kind and open for discussion. Your professional advice and comments in polymer chemistry have been a great help for the synthesis and analysis of new developed polymers. Additionally, I greatly thank for your kind permission to use the lab facilities in the beginning days of my doctoral research. I would also like to thank Dr. Carolin Thiele for her kind introduction of preparative starch chemistry.

My special thanks go to all of the technicians who helped me with different lab works. Heike, Petra, Leon, Chris, Marijas, Sarina, Chiara, Birgit and Peter, thank you very much for your big support in cell culture and by using different equipments. You always helped me and solved a lot of problems in the lab.

Additionally, I thank to my colleagues in drug delivery team, Claudia, Ratnesh, Prajakta, Qiong, Carolin, Niko, Christina, Babak, Tine, Tijana, Emad, Sarah, Nicole, Elise and Brigitta, for the intensive discussion and helpful advice in our group meeting on every Friday morning. Special thanks to Babak, performing experiments with you were always fun. I do not forget our first pDNA extraction, and our surprise of the complicated procedure in CLMS & FACS.

I want to thank my office colleagues, Christine, Claudia, Fransisca, Michael, Jurian, Christian, Biethe, Simon, Sarah, Stefanie, Nicole and Jenny. Thank you for your patience to talk to me in German. Because of you guys, I think, *ich habe mein Deutsch viel verbessert*! I really loved the cheerful atmosphere of our office!

I also thank all my colleagues in our department for all the support and friendship inside and outside of the lab. I have a lot of good memories during the last years, conferences, cycling, beach volleyball, dancing, concerts, cooking international dishes, barbecue, etc… It was really great to get to know all of you.

I would like to thank also my friends who supported my private life a lot. Masayuki and Robert, you guys gave me a motivation to come to Germany and get Ph.D., and always took care about me and my future, thank you very much. Yumi, thanks being my best friend. Even we are living such far away each other, you have always taken care of me. You have visited me every year in Europe. I do not forget our precious time and moments in different countries. Sibylle, Ulrike and Jing, because you supported me in Germany, I do not feel alone. Thank you for your friendship. Impi, you are always nice and gave me a lot of advice. I could not overcome many difficulties without your support. Thank you very much.

At the end, I would like to thank my parents for allowing me to come to Germany, such a far place from Japan, and to challenge getting a Ph.D. Words cannot express how grateful I am to my mother and my father for all of the ungrudging support and unending love.