Doctoral Thesis

Calcium Phosphate Nanoparticles and Polyion Complex Micelles for Nucleic Acid Delivery

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Calcium Phosphate Nanoparticles and Polyion Complex Micelles for Nucleic Acid Delivery

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Calcium Phosphate Nanoparticles and Polyion Complex Micelles for Nucleic Acid Delivery

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# Table of Contents

Summary................................................................................................................................................... 1

Zusammenfassung....................................................................................................................................... 3

1. **Background and Purpose** .................................................................................................................. 5  
   1.1. Gene therapy ................................................................................................................................... 5  
   1.2. Types of nucleic acids .................................................................................................................... 6  
   1.3. Viral gene delivery systems ........................................................................................................... 8  
   1.4. Non-viral gene delivery systems ..................................................................................................... 9  
   1.5. Calcium phosphate nanoparticles .............................................................................................. 11  
   1.6. Polyion complex micelles ............................................................................................................. 13

2. **Well-Defined Multivalent Ligands for Hepatocytes Targeting via Asialoglycoprotein Receptor – a Review** ........................................................................................................................................................ 15  
   2.1. Introduction ..................................................................................................................................... 16  
   2.2. Asialoglycoprotein receptor (ASGP-R) ......................................................................................... 16  
   2.3. Well-defined multivalent ligands for ASGP-R ............................................................................. 19  
   2.4. Summary and outlook ..................................................................................................................... 32

3. **Characterization of Calcium Phosphate Nanoparticles Based on PEGylated Chelators for Gene Delivery** ........................................................................................................................................... 33  
   3.1. Introduction ..................................................................................................................................... 34  
   3.2. Materials and Methods .................................................................................................................. 36  
   3.3. Results and discussion .................................................................................................................... 46  
   3.4. Conclusion ...................................................................................................................................... 65

4. **Preparation of Polyion Complex Micelles for the Hepatocytes-Targeted Delivery of Nucleic Acids** ........................................................................................................................................... 67  
   4.1. Introduction ..................................................................................................................................... 68  
   4.2. Materials and Methods ................................................................................................................... 69
Summary

Gene therapy holds the potential to provide treatments for diseases that were previously considered untreatable or for which only suboptimal therapies are available. However, a major challenge for the clinical success of gene therapy remains the safe and efficient delivery of nucleic acid drugs to target cells. To address this problem, numerous delivery vehicles have been developed in the last decades. Initially, viral delivery systems were widely investigated owing to their high transfection efficiency, but they suffer from safety issues. Non-viral delivery systems partially solve these safety issues, but are plagued by low transfection efficiency. However, recent developments in material science, nanotechnology and nucleic acid chemistry have resulted in large transfection efficiency improvements. In this Ph.D. thesis, two non-viral delivery systems were developed to deliver nucleic acid drugs to liver cells. The first system is based on calcium phosphate (CaP) nanoparticles, and the second system is based on polyion complex micelles.

Chapter 1 describes the history and current status of gene therapy. The different types of nucleic acids, their mechanisms, and the viral and non-viral gene delivery systems are presented.

Chapter 2 provides an in depth discussion on the design of synthetic multivalent ligands for hepatocyte targeting via the asialoglycoprotein receptor (ASGP-R). Since well-defined multivalent ligands can normally achieve an optimal targeting effect, important properties such as linker length, hydrophilic-hydrophobic balance and spatial geometry are discussed in detail. In addition, the different synthesis routes of the ligands are presented and compared in terms of synthetic complexity.

Chapter 3 discusses the characterization of PEGylated chelator-stabilized calcium phosphate nanoparticles for gene delivery. Here, new synthesis routes involving click chemistry were developed to prepare the PEGylated chelators PEG-inositol 1,3,4,5,6-pentakisphosphate (PEG-IP5) and PEG-bisphosphonate (PEG-BP) that can coat and stabilize CaP nanoparticles. Two methods (1 and 2) differing in the time of addition of the PEGylated chelator were employed to produce PEG-BP/IP5 stabilized particles. Surprisingly, distinct particles were obtained by both methods. PEG-IP5 stabilized nanoparticles prepared by method 1 were internalized with significantly higher efficiency in HepG2 cells than those prepared by method 2, and the uptake was dramatically influenced by the reaction time of Ca²⁺ and PO₄³⁻ and sedimentation of the particles. Morphological transformations were observed for both types of particles after different storage times, but this barely influenced their in vitro cellular uptake. This study provides a better understanding of the properties (e.g. size, morphology and crystallinity) of PEGylated CaP nanoparticles and how these influence the particles’ in vitro uptake and transfection efficiency.

Chapter 4 describes the preparation of polyion complex micelles (PICMs) for hepatocyte-targeted delivery of nucleic acids. A trivalent N-acetyl galactosamine (T-GalNAc) ligand was designed
and prepared for ASGP-R targeting, and properties such as sugar type, linker length, hydrophilic-hydrophobic balance and spatial geometry of the scaffold were taken into consideration. The T-GalNAc was coupled to anionic block copolymer to produce T-GalNAc-PICMs. An acetyl group was also coupled to produce Ac-GalNAc-PICMs (control micelles). In uptake experiments on HepG2 cells, the T-GalNAc-PICMs showed enhanced uptake efficiency compared to Ac-GalNAc-PICMs, which was nearly completely inhibited by the co-incubation with excess free GalNAc. The efficient targeted delivery of nucleic acid drugs to hepatocytes holds great promise for the treatment of various liver diseases.

Chapter 5 provides a general conclusion and outlook. The major achievements of this Ph.D. thesis are presented and also the limitations and future possibility of improvement of the present work are presented.
Zusammenfassung


Kapitel 1 beschreibt die Geschichte und den aktuellen Stand der Gentherapie. Die wichtigsten Konzepte, deren Mechanismen, sowie virale und nichtvirale Transfektionssysteme werden erklärt.

Kapitel 2 diskutiert die Konstruktion multivalenter Liganden, die mit Hilfe des Asiaglycoprotein Rezeptors (ASGP-R) auf Hepatozyten wirken. Klardefinierte, multivalente Liganden können dies für gewöhnlich am besten, weshalb wichtige Eigenschaften wie die Länge des Linkers, hydrophobe-hydrophile Balance sowie Molekulgeometrie im Detail erörtert werden. Zusätzlich werden die verschiedenen Synthesewege des Liganden verglichen.


Kapitel 5 fasst die Ergebnisse zusammen und liefert einen Ausblick. Es werden sowohl die wesentlichen Leistungen der Arbeit, als auch die Grenzen und Möglichkeiten zur Optimierung aufgezeigt.
Chapter 1

1. Background and Purpose

1.1. Gene therapy

Gene therapy involves the delivery of therapeutic nucleic acids, ideally to specific cells. Both inherited as well as acquired diseases can be targeted by gene therapy. More importantly, gene therapy has the potential to provide treatments for diseases that are currently not treatable with conventional medications. The basic process of gene therapy includes the replacement of a mutated gene with a healthy gene, the silencing of a gene, or the introduction of a new gene.

The first officially approved clinical trial involving the introduction of a foreign gene into humans was performed in 1989, not to seek an actual therapeutic effect, but rather to track the movements of gene-modified tumor-infiltrating lymphocytes in cancer patients after infusion. In 1990, for the first time a gene therapy trial for therapeutic purpose was approved by the U.S. Food and Drug Administration (FDA). In this case, the idea was to treat 2 children suffering from adenosine deaminase-deficient severe combined immune deficiency (ADA-SCID) with gene-modified blood cells that were able to express ADA normally. Only one patient showed a temporary response, however, a poly(ethylene glycol)-conjugated ADA (PEG-ADA) had to be administered in addition to the gene therapy. Despite the mitigated results of these first studies, gene therapy has been widely investigated since then. A major setback for gene therapy occurred in 1999 with the tragic death of 18-year-old Jesse Gelsinger, who took part in a gene therapy trial for ornithine transcarboxylase deficiency. He passed away 4 days after the treatment due to multiple organ failure that was believed to be triggered by a severe immune response to the gene carrier-adenovirus. Finally, in 2000, a clinical trial for the treatment of two patients suffering from severe combined immunodeficiency (SCID-X1) achieved the first clinical success employing gene therapy.

In 2003, the first commercial gene therapy medicine Gendicine™ was approved by the Chinese State Food and Drug Administration (SFDA). Gendicine™ was developed by SiBiono GeneTech Co. and consists of an adenovirus vector containing a human p53 tumor-suppressor gene for the treatment of head and neck squamous cell carcinoma. In 2012, Glybera became the first gene therapy product recommended by the European Medicines Agency (EMA) for approval in the European Union. Glybera is a product of UniQure and is an adeno-associated viral vector encoding the lipoprotein lipase gene for the treatment of lipoprotein lipase deficiency (LPLD).

To date, more than 2300 clinical trials of gene therapy for a broad range of diseases have been conducted or are still ongoing. Among these trials, cancer is the most common target representing more than 60% of all trials. However, the clinical success of gene therapy depends on the
development of safe and effective delivery vectors. Currently, both viral and non-viral vectors are widely used in clinical trials for the systemic delivery of various exogenous nucleic acids.

1.2. Types of nucleic acids

The types of nucleic acids used for gene therapy include DNA, mRNA, small interfering RNA (siRNA), microRNA (miRNA) or antisense oligonucleotides (AON). The mechanisms for nucleic acid therapy based on non-viral delivery systems are described in Figure 1.1. The mechanisms for viral delivery systems are slightly different and will be discussed only briefly in this thesis.

The common challenges for the delivery of all nucleic acid therapeutics are selective accumulation at the target tissues, cellular internalization and endosomal escape. Furthermore, the delivery of DNA faces an addition challenge since it requires transportation into the nucleus to reach the transcriptional machinery (Figure 1.1a). To this end, plasmid vectors are routinely used for the expression of DNA due to their ease of construction and amplification, as well as their episomal and non-integrating properties which lowered the risk of insertional mutagenesis. The expression of plasmid DNA was found to diminish rapidly upon hydrodynamic infusion (a technique involving the rapid injection of a large volume of DNA solution into the animal) into the mouse liver. This situation was largely improved by the use of minicircle DNA which gave more persistent and 100 to 1000 times higher expression than plasmid DNA. The minicircle DNA vectors consisting of a circular expression cassette without the bacterial plasmid DNA backbone and will probably prevail in gene therapy trials.

mRNA is not as robust as DNA, but its delivery for gene therapy is more straightforward since it does not need nuclear localization for expression. Additionally, mRNA does not imply a potential hazard in terms of mutagenesis caused by genomic integration. Once the mRNA is loaded into the ribosome, the target protein will be expressed (Figure 1.1b). So far, mRNA therapeutics have not been intensively investigated owing to their immunogenicity and instability.

Since the discovery of the mechanism of RNA interference (RNAi) in 1998, it has been intensively investigated for therapeutic applications. RNAi is an endogenous pathway for post-transcriptional gene silencing which is triggered by double-stranded RNA, including siRNA and miRNA. siRNAs are double-stranded RNAs with a typical length of 21-23 base pairs (bp) that mimic the cleavage products of the enzyme Dicer. Upon introduction into the cytoplasm, the synthetic siRNA is loaded into the RNA-induced silencing complex (RISC) followed by the degradation of the sense strand. The remaining guide strand in the RISC complex binds to the target mRNA strand (of complementary sequence) and eventually cleaves it (Figure 1.1c). Theoretically, siRNA can efficiently and specifically silence the expression of almost any gene, including traditionally ‘undruggable’ targets. miRNAs are double-stranded RNAs with a typical length of 19-25 bp, which shares the similar RNAi pathway with siRNA. The mRNA is degraded by the miRNA when the
complementarity between them is high, while the protein translation is inhibited if this complementarity is low (**Figure 1.1c**). Since miRNA is similar to siRNA in terms of structure, size and charge, the systems used for siRNA delivery are probably also suitable for miRNA delivery.

AON are synthetic single-stranded strings of nucleic acids that bind to RNA. The first investigation on AON was carried out by Zamecnik and Stephenson in 1978. Since then, numerous AON drugs have been developed. Nowadays, a typical therapeutic AON is about 20 nucleotides long and chemically modified. Once AON reach the cytoplasm, they suppress the gene expression via two pathways. In one pathway, the binding of AON to the target mRNA strand sterically hinders ribosome binding, thus blocking the translation of mRNA into the protein. In another pathway, AON bind to the target mRNA strand and the AON-mRNA hybrid is subsequently recognized by the enzyme RNase H, which degrades the mRNA transcript and enables the AON to be recycled (**Figure 1.1d**).

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**Figure 1.1** Mechanism for nucleic acids therapy (non-viral delivery). (a) DNA is transported to the nucleus where mRNA is produced by transcription, the mRNA is then translated to protein by ribosome. (b) The mRNA is loaded into ribosome for protein translation. (c) siRNA or miRNA are loaded into the RNA-induced silencing complex (RISC) complex, which binds to the target mRNA after the degradation of the sense strand. The RISC-siRNA complex cleaves the mRNA while the resulting RISC-miRNA complex either causes the cleavage of the mRNA or inhibits its translation. (d) AON binding to the mRNA forms a complex which either inhibits the ribosome translation or being degraded by the enzyme RNase H.
1.3. Viral gene delivery systems

Viruses can encapsulate and deliver their nucleic materials to human cells in a very efficient way. For this reason, viruses can constitute very effective vectors for gene therapy. To this end, they have to be genetically modified to remove disease-causing genes and encode therapeutic ones instead. Due to the high in vivo transfection efficiency, viruses are the preferred gene delivery system in clinical trials. In fact, about 70% of the current gene therapy clinical trials use modified viruses as vectors, among which retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAVs) are the most common ones. Other viruses such as vaccinia virus, poxvirus and herpes simplex virus are also used in current clinical trials.

1.3.1. Retroviral vectors

Retroviruses were the first viral vectors investigated for gene therapy. These viruses save their genetic information in the form of RNA and integrate it into the host cells’ genome by producing a DNA copy. The main limitations of retroviral vectors are their low transfection efficiency compared to other viral vectors and the risk of insertional mutagenesis. In addition, most of the retroviruses can only infect dividing cells, hindering their application in tissues such as brain, eye, lungs and pancreas.

1.3.2. Lentiviral vectors

Lentiviruses belong to the retrovirus family, although they have acquired the ability to infect non-dividing cells unlike other retroviruses. This property largely extends the range of cell types that they can be employed on for gene therapy. Lentiviral vectors show stable and robust transgene expression, lower immunogenicity and are able to accommodate transgene as large as 8 kilobases (kb) in size.

1.3.3. Adeno-associated viral vectors

AVVs are members of the parvovirus family, which keep their genetic information in the form of single-stranded DNA. AVVs are non-pathogenic and are not associated with any disease in humans. Another special property of AAVs is their ability to insert into a specific site on chromosome 19 with close to 100% certainty. However, a major drawback of these vectors is that the size of therapeutic genes insert is restricted to just over 4 kb.

1.3.4. Adenoviral vectors

Adenoviruses keep their genetic material in the form of double-stranded DNA and can infect dividing as well as non-dividing cells. It is possible to insert large therapeutic sequences into adenoviral vectors due to their large 36-kb genomes. Moreover, they vector can be easily purified to high titres. One challenge of adenoviral vectors is the low persistence of transgene expression, as their genome remains episomal.
1.4. Non-viral gene delivery systems

Although around 70% of the current gene therapy clinical trials rely on viral gene delivery systems, these still suffer from several limitations such as insertional mutagenesis, immunogenicity, low gene-packaging capacity and complexity of vector production. Interestingly, most of these limitations could be addressed by non-viral gene delivery systems. Besides having reduced pathogenicity and low capacity for insertional mutagenesis, non-viral vectors also have no size limits on their genetic payloads and are easy to produce in large scales. Previously, poor transfection efficiencies held back the application of non-viral vectors in clinical trials. However, recent advances in material sciences, nanotechnology and nucleic acid chemistry have yielded promising non-viral vectors with transfection efficiencies comparable to those of viral vectors.

Non-viral gene delivery systems are based on physical methods or chemical vectors. Amongst the physical methods, naked DNA injection, gene gun, electroporation, sonoporation and magnetofection are the most prominent ones. Many of these techniques are not readily applicable to gene delivery in humans and are therefore not as common as chemical systems in clinical trials. In the following sections, chemical non-viral delivery systems will be discussed.

1.4.1. Lipid-based vectors

Lipid-based particles (LNPs) are widely used for non-viral gene delivery in vivo. Cationic lipids can naturally interact with the negatively charged nucleic acids, an essential feature for the encapsulation of nucleic acids into the LNPs. Cationic lipids structurally consist of a cationic head group, a hydrophobic tail and a linker connecting both domains. To enhance transfection efficiency and particle stability, neutral lipids like the fusogenic phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) or the membrane component cholesterol are introduced into the LNPs. Finally, the use of PEG as surface coating layer further enhances particle stability and minimizes clearance. One type of LNPs that is commonly used in ongoing clinical trials is the stable nucleic acid lipid particle (SNALP). Produced by a controlled step-wise dilution technique, SNALPs have high gene-encapsulation efficiency, and a small and uniform size. Currently, the SNALP formulation ALN-TTR02 (Patisiran) developed by Alnylam Pharmaceuticals for the suppression of mutant transthyretin (TTR) in TTR-mediated amyloidosis is in phase 3 clinical trial (#NCT01960348).

1.4.2. Polymer-based vectors

Cationic polymers are an alternative class of non-viral vectors for gene delivery. Due to their immense chemical diversity and their potential for functionalization, cationic polymers have great potential for gene delivery in humans. Poly(L-lysine) (PLL), a homopolypeptide consisting of the amino acid lysine, is one of the earliest polymer-based vectors developed for gene delivery. However, PLL has poor transfection efficiency and showed obvious cytotoxicity in vitro. In order to overcome these drawbacks, various modifications of PLL have been explored, including its PEGylation. Polyethylenimine (PEI) is the most widely used polymer-based vector for gene delivery. PEI has a
high density of positively charged groups, which might facilitate the condensation of genetic material and mediate endosomal escape.44 The structure (linear or branched)45 as well as the molecular weight46 of PEI have a strong influence on its transfection efficiency and cytotoxicity. PEI has been used as gene carrier for local treatment of different types of cancer (#NCT00595088). However, similar to PLL, PEI is also limited by its cytotoxicity.47 Modified PEI such as PEGylated PEI shows improved biocompatibility and a PEG-PEI-cholesterol lipopolymer-based vector is currently being used in clinical trials for the treatment of ovarian and colorectal cancer (#NCT01118052, NCT01300858).

In addition to the two classic polymers described above, various new classes of polymers are being investigated for safe and efficient gene delivery in vitro and in vivo.37 One of these new classes, cyclodextrin-based polycations (CDP), has shown very low toxicity. CDP-based nanoparticles have been used for the delivery of siRNA to solid tumors in a clinical trial (#NCT00689065), where they induced a decrease in the level of the target mRNA ribonucleotide reductase M2 (RRM2).48

1.4.3. Conjugate-based vectors

Synthetic siRNAs are chemically synthesized double-stranded RNAs with specific chemical modifications that can prevent degradation by endonucleases and activation of the innate immune system upon administration.49 Several conjugate-based vectors have been developed by conjugating ‘helpful elements’ to the siRNA such as targeting ligands. The most advanced clinical candidates so far are the dynamic polyconjugates (DPC) and N-acetylgalactosamine (GalNAc) conjugates.

DPC aims at treating liver diseases through targeting to hepatocytes. It contains a membrane-disrupting polymer (polyconjugate of butyl and amino vinyl ethers, PBAVE), the shielding polymer PEG, the targeting ligand GalNAc and the therapeutic siRNA. A DPC-based nucleic acid drug termed ARC-520, developed by Arrowhead Research Corporation for the treatment of Hepatitis B disease, is currently in clinical trials (#NCT02065336). Another conjugate-based vector for the targeting of hepatocytes is the GalNAc conjugate, which contains a chemically modified therapeutic siRNA that is covalently attached to a trivalent GalNAc ligand. So far, at least 7 drug candidates based on this platform have been developed at Alnylam Pharmaceuticals. Among these, ALN-TTRsc is the most clinically advanced one, which is developed for the treatment of transthyretin (TTR)-mediated amyloidosis by suppressing the mutant TTR (#NCT01981837).

1.4.4. Inorganic particle-based vectors

Inorganic particle-based vectors are also quite common for applications in gene delivery.50 Despite the fact that very often organic particles display better transfection efficiency, inorganic particles present other interesting features such as that they can be easily prepared and surface-functionalized, they are usually quite robust and show very good storage stability. In the last decades, numerous inorganic materials have emerged in gene delivery, amongst which calcium phosphate
Inorganic particles consisting of silica or gold can not condense or load genes directly, therefore, certain surface modifications are required in order to use them as gene carriers. Radu et al. modified mesoporous silica nanoparticles by covalently attaching poly(amidoamine) (PAMAM) dendrimers to their surface. The modified silica particles were able to complex plasmid DNA and successfully transfected several cell lines with high transfection efficiency. Klibanov et al. reported a gold nanoparticle gene delivery system in which PEI of 2 k was conjugated to the surface of the particles. These PEI-decorated gold particles displayed 15-fold increase of transfection efficiency compared to non-modified PEI (without gold nanoparticles). Other inorganic particles like carbon nanotubes, magnetite, quantum dots, and double hydroxides have also shown promising transfection ability in vitro and in vivo.

**Figure 1.2** Schematic illustration showing the main non-viral gene delivery systems, including lipid particles, polymeric particles, gene conjugates and inorganic particles.

### 1.5. Calcium phosphate nanoparticles

Calcium phosphate (CaP) is the inorganic component of biological hard tissues such as bones and teeth, where it exists in the form of hydroxyapatite. Thus the use CaP materials *in vitro* or *in vivo* does not elicit toxicity issues. CaP was one of the earliest inorganic materials to be used in gene delivery, originally being developed by Graham and van der Eb in 1973. The gene material is incorporated into the CaP particles through a co-precipitation process and incubated with cells immediately to achieve cell transfection. Although this method has shown promising transfection efficiency *in vitro*, the transfection efficiency is quite sensitive to the preparation conditions of the CaP particles. Parameters such as pH value, concentrations of calcium, phosphate and DNA, temperature, and the time interval between particle preparation and transfection can have a considerable impact on
Chapter 1 - Background and purpose

the transfection efficiency.58 One major drawback of this method is the rapid CaP particles size increase after preparation, which hinders their in vivo application. During the last decades, various strategies have been developed to control the growth of CaP particles, including polymer stabilization,59, 60 and lipid61-63 or DNA/siRNA64, 65 coatings. Kataoka and coworkers66, 67 have developed a simple method to prepare CaP/polyanion hybrid nanoparticles by mixing synthetic polyanion and other components (Ca²⁺, PO₄³⁻ and gene) in solution, which upon systemic administration resulted in VEGF gene silencing and a therapeutic effect on pancreatic cancer.

The Leroux group has previously described a simple PEGylated chelator coating approach to produce CaP nanoparticles. Both PEGylated bisphosphonate (PEG-BP) and PEGylated inositol 1,3,4,5,6-pentakisphosphate (PEG-IP5) were used to stabilize CaP-siRNA/DNA nanoparticles of 190-220 nm in size.68, 69 Inositol phosphates are ubiquitous metabolites in eukaryotes.70 Highly phosphorylated inositols such as IP5 and inositol hexakisphosphate (IP6) are the most abundant ones and display high binding affinity to calcium and hydroxyapatite.71, 72 Previous data from the Leroux group showed improved stabilization of CaP nanoparticles by PEG-IP5 compared to PEG-BP.69 Based on this, a systematic study was carried out on the impact that the chelator and preparation method employed can have on the particles’ size, morphology and crystallinity, and ultimately on their in vitro cellular uptake and transfection efficiency (chapter 3). The PEGylated chelators PEG-IP5 and PEG-BP were prepared via new synthesis routes. Two different methods were employed to produce PEG-IP5 and PEG-BP stabilized particles which were intensively characterized. Finally, the interactions of PEG-IP5 stabilized nanoparticles with cells were investigated in vitro and the effects exerted by the distinct particle characteristics on their cellular uptake and transfection efficiency were studied (Figure 1.3).

Figure 1.3 Schematic illustration showing the preparation of CaP nanoparticles. CaP nanoparticles were prepared by two methods. Method 1, addition of Ca²⁺ solution to PO₄³⁻ solution resulted in growing particles which were subsequently stabilized by introducing the PEGylated agent in a time-
dependent manner. **Method 2**, PEGylated agent and \( \text{PO}_4^{3-} \) solution were premixed and then added to \( \text{Ca}^{2+} \) solution to produce stabilized particles.

### 1.6. Polyion complex micelles

In the last decades, polyion complex micelles (PICMs) have drawn particular interest in the field of nanomedicine. PICMs are typically obtained through the electrostatic interaction between the nucleic acid and the cationic copolymer. A hydrophilic neutral segment, for instance PEG, is covalently attached to the copolymer to give the complexes a hydrophilic shell.\(^{73}\) In recent years, an optimized approach for the preparation of PICMs for gene delivery has been developed in our laboratory. This approach takes advantage of the combined efficiency of cationic condensing agents and of pH-responsive anionic diblock copolymers. PAMAM dendrimers are used as cationic condensing agents due to their intrinsic endosomolytic activity, which facilitates the escape of the delivered gene from the endosomes of the transfected cells into their cytosol.\(^{74}\) Amphipathic polymers bearing carboxylate groups are chosen as pH-responsive anionic diblock copolymers since the transition pH of polycarboxylates (4-6) is in a physiologically relevant range. We then introduced to this robust PICM-based gene delivery system a synthetic ligand targeting the asialoglycoprotein receptor (ASGP-R), aiming to achieve an efficient delivery of nucleic acids to the hepatocytes.

The ASGP-R was the first discovered C-type lectin to be discovered, and is an endocytic receptor that is predominantly expressed on the surface of mammalian hepatocytes.\(^{75,76}\) The extracellular domain of the receptor specifically recognizes and multivalently interacts with galactose (Gal) or N-acetylgalactosamine (GalNAc) residue-containing ligands, which subsequently triggers clathrin-mediated internalization of the glycoprotein.\(^{77}\) Furthermore, ASGP-R shows much higher binding affinity to a glycoprotein containing multivalent GalNAc epitopes than to monosaccharides.\(^{78-81}\) Such epitopes could be taken up by cells via receptor-mediated endocytosis, and were widely employed as targeting ligands for hepatic cell uptake of various nanoparticles including liposomes,\(^{82}\) cyclodextrin,\(^{83,84}\) polyconjugated systems,\(^{85}\) and inorganic nanoparticles.\(^{63,81}\) The design and synthesis of multivalent ligands for ASGP-R targeting are reviewed in depth in **chapter 2** of this thesis.

In **chapter 4**, we describe the design and synthesis of a trivalent GalNAc (T-GalNAc) ligand and the preparation of T-GalNAc decorated PICMs for the targeted delivery of nucleic acids to hepatocytes. To achieve an optimal targeting effect, the properties of the ligand scaffold such as linker length, amphiphilicity and spatial conformation were well defined. The obtained T-GalNAc ligand was successfully conjugated to PICMs, and the targeting effect was evaluated on HepG2 hepatocytes (Figure 1.4).
Figure 1.4 Schematic illustration showing the formation of T-GalNAc-decorated PICMs through the self-assembly of anionic diblock copolymer with cationic PAMAM dendrimers and nucleic acid.
Chapter 2

2. Well-Defined Multivalent Ligands for Hepatocytes Targeting *via* Asialoglycoprotein Receptor – a Review
2.1. Introduction

The liver’s role in the metabolism of toxic substances implies that liver cells are frequently exposed to drugs, microbes, and toxic materials that can lead to various liver diseases. Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world and the third most mortal cancer.\(^{86, 87}\) Two main types of hepatitis viruses, namely hepatitis viruses B and C (HBV and HCV), are associated with the development of HCC. HBV infects approximately 2 billion people worldwide and is estimated to result in 320,000 deaths annually,\(^{88}\) while HCV infection affects about 170 million people worldwide.\(^{89, 90}\) Since HCC and other liver diseases such as fibrosis and cirrhosis\(^{91}\) mainly affect the hepatocytes, the targeted delivery of therapeutic agents to these cells is an attractive approach. In order to design hepatocyte-directed delivery system, a target molecule that is abundantly expressed on the surface of hepatocytes but minimally expressed by extrahepatic cells needs to be identified. The asialoglycoprotein receptor (ASGP-R) fits this expression pattern, positioning itself as an ideal target for the delivery of therapeutic agents to hepatocytes.\(^{92}\)

Monovalent and multivalent ligands have been proposed to target ASGP-R. The present review discusses the design of synthetic multivalent ligands for ASGPR-mediated hepatocyte delivery. Since only the well-defined multivalent ligands can achieve an optimal targeting effect, important properties such as linker length, hydrophilic-hydrophobic balance and spatial geometry are discussed in detail. In addition, the different synthesis routes of the ligands are presented and compared in terms of synthetic complexity. The monovalent or natural ligand for ASGP-R has been recently reviewed by D’Souza et al.\(^{93}\) and Ahmed et al.\(^{94}\) and has thus not been included here.

2.2. Asialoglycoprotein receptor (ASGP-R)

2.2.1. General characteristics

The ASGP-R was the first mammalian lectin to be identified\(^{95}\) and was originally discovered by Ashwell, Morell and their co-workers in the 1960s during their studies on the metabolism of plasma glycoproteins in mammals.\(^{96-98}\) The main function of ASGP-R is to maintain the homeostasis of serum glycoproteins by mediating the recognition and endocytosis of a broad range of desialylated glycoproteins that carry terminal galactose (Gal) or N-acetyl galactosamine (GalNAc) residues.\(^{99}\) These desialylated glycoproteins are endocytosed \textit{via} clathrin-coated pits, and once in the acidic endosomal compartment they dissociate from the receptor, followed by trafficking to the lysosomes for degradation. Notably, ASGP-R belongs to the recycling receptors group and is endocytosed and recycled constitutively about every 15 minutes, with or without the ligands.\(^{100-103}\) The ASGP-R is predominantly expressed on the surface of liver parenchymal cells, which contain 1-5 \(\times\) 10\(^5\) binding sites per cell.\(^{99, 104, 105}\) In addition, ASGP-R would play a role in infectious diseases since viruses like hepatitis A and B, as well as Marburg virus are shown to bind to it, thus facilitating hepatic infections.\(^{106-109}\)
2.2.2. Structure

The mammalian ASGP-R is composed of two homologous major and minor subunits that are encoded by two distinct genes. In humans, the major subunit H1 and the minor subunit H2 are 46 and 50 kDa in size respectively, and the combination of various ratios of the subunits forms functional homo- and hetero-oligomers with different receptor configurations. Among all the identified configurations of ASGP-R, the trimer composed by two H1 and one H2 is the most abundant one and shows the highest binding affinity to the ligand asialoorosomucoid (ASOR) (Figure 2.1). Both H1 and H2 are type II single-spanning membrane proteins with 58% sequence identity, and the most relevant difference between them is an 18-amino acid insert included only in the cytoplasmic domain of H2. The general structure of H1 and H2 subunits includes a 40-amino acid N-terminal cytoplasmic domain, a ~20-amino acid single-pass transmembrane domain, an ~80-amino acid extracellular stalk region, and an ~140-amino acid functional calcium-dependent carbohydrate recognition domain (CRD) (Figure 2.1). The stalk region, composed of heptad repeats characteristic of α-helical coiled-coil structure, mediates the protein oligomerization. Importantly, cell lines expressing only H1 or H2 are unable to bind or internalize the ligand ASOR since the co-expression of both subunits is required for endocytosis of the ligand.

![Figure 2.1](image.png)

Figure 2.1. Schematic representation of the ASGP-R, illustrating the hetero-oligomer composed of two H1 and one H2 subunit, each of them containing four domains. The geometric relationship of the ASGP-R binding sites is also indicated. Figure adapted from Ernst et al.

In addition to human, ASGP-R has also been isolated from hepatocytes of other mammals including rabbit, mouse and rat, although the size and number of subunits that constitute the receptor vary slightly among species. Despite the species-dependent composition of the receptor, the protein sequences for a given subunit are highly conserved and probably originate from the same ancestral gene. For example, there is 80% homology between the human H1 and the rat hepatic lectin 1 (RHL1), and 62% between the H2 and the RHL2.
2.2.3. Carbohydrate recognition domain (CRD)

The carbohydrate recognition domain (CRD) of the ASGP-R subunits belongs to the C-type (Ca\(^{2+}\)-dependent) superfamily.\(^{133}\) Most C-type CRDs preferentially bind to D-mannose (Man), D-glucose (Glu) and their derivatives (Man-type ligands), or to D-Gal and its derivatives (Gal-type ligands). The monosaccharide ligands bind to these CRDs by direct coordination to a calcium ion.\(^{134}\) The first study involving the CRD of ASGP-R was performed by Weis and Kolatkar,\(^{135}\) where the active site of the CRD was mutated from a Man-binding protein (MBP) to that of ASGP-R. The mutated protein termed QPDWG was obtained with the mutations Glu\(^{185} \rightarrow\) Gln\(^{185}\), Asn\(^{187} \rightarrow\) Asp\(^{187}\), and His\(^{189} \rightarrow\) Trp\(^{189}\) and the insertion of a Gly-rich loop. The binding specificity for Man was converted to Gal with an affinity almost identical to that of the CRD of ASGP-R. Moreover, NMR measurements revealed similar modes of Gal binding by mutant QPDWG and ASGP-R, confirming that the mutant QPDWG could be used as a model to investigate ligand binding to ASGP-R.\(^{136}\) This QPDWG mutant was a good model for the binding mode of Gal to ASGP-R, but it could not explain the fact that ASGP-R always displays preferential binding to GalNAc compared to Gal. Therefore, further mutations were performed on QPDWG (Thr\(^{202} \rightarrow\) His\(^{202}\)) to create the mutant QPDWGH for the selective binding of GalNAc.\(^{137}\) The molecular basis of selective GalNAc recognition by CRD was revealed by the crystal structure of QPDWGH complexed with GalNAc (Figure 2.2a). The Ca\(^{2+}\) binds to the CRD by the coordination to residues Gln\(^{185}\), Asp\(^{187}\), Glu\(^{198}\), Asn\(^{210}\) and Asp\(^{211}\), and GalNAc binds directly to the Ca\(^{2+}\) with its 3-OH and 4-OH groups, replacing two water molecules. In addition, the sugar also forms hydrogen bonds with the residues Gln\(^{185}\), Asp\(^{187}\), Glu\(^{198}\), Asn\(^{210}\) and Asp\(^{211}\). Furthermore, the apolar face (C3, C4, C5 and C6) of the sugar exhibits hydrophobic interaction with the plane of the side-chain of Trp189. The histidine residue (His\(^{202}\)) plays a key role by direct coordination with the 2-acetamido methyl group of GalNAc (Figure 2.2b). This crystallographic analysis of GalNAc bound to the CRD of QPDWGH provides a model for the binding mode of GalNAc to ASGP-R.
Figure 2.2. (a) The QPDWGH mutant of MBP co-crystallized with GalNAc (PDB: 1BCH\textsuperscript{137}), calcium is represented by green spheres and GalNAc by the ball and stick model in grey and red. Figure are prepared by NGL Viewer\textsuperscript{138, 139}. (b) GalNAc binds to the CRD of the QPDWGH mutant of MBP. Coordination and hydrogen bonds are represented by blue and green dashed lines, while the hydrophobic interaction is represented by red dashed lines. Apolar face of GalNAc is depicted in orange\textsuperscript{137}. (c) Crystal structure of the H1-CRD, the ligand GalNAc binds to calcium ion from calcium-binding site 2 (PDB: 1DV8\textsuperscript{140}). Calcium is represented by the green spheres.

The first crystal structure of the H1 subunit of the CRD of ASGP-R was determined by Meier et al\textsuperscript{140} (Figure 2.2c). The CRD contains several calcium-binding sites and the binding site 2 is essential for sugar binding. Although the CRD was co-crystallized with lactose, no lactose ligand was visible at the sugar binding site. Therefore, the straightforward data such as crystal structure of Gal binding to the CRD of ASGP-R are still missing. However, at the sugar binding site, the Ca\textsuperscript{2+} is coordinated to five residues, Gln\textsuperscript{239}, Asp\textsuperscript{241}, Glu\textsuperscript{252}, Asp\textsuperscript{265} and Asn\textsuperscript{264}, which are the same residues mediating Ca\textsuperscript{2+} binding to the mutant MBP. Hence, it is reasonable to assume that Gal binding to the CRD of ASGP-R and to the CRD of mutant MBP are likely to happen in an analogous fashion.

2.3. Well-defined multivalent ligands for ASGP-R

In contrast to a monovalent ligand, multivalent ligands show dramatically enhanced binding affinity towards the ASGP-R. The binding hierarchy of multivalent ligands to the rabbit ASGP-R was tetra- > tri- >> bi- >> monoantennary according to the half-maximum inhibitory concentration (IC\textsubscript{50}) of 10\textsuperscript{-9}, 5×10\textsuperscript{-9}, 10\textsuperscript{-6} and 10\textsuperscript{-3} M, respectively\textsuperscript{141, 142}. The addition of sugar beyond triantennary only gave a modest increase of the binding affinity. This is probably related to the fact that the trimer with three sugar binding sites is the most abundant receptor conformation, as mentioned above.

In the last two decades, multivalent ligands based on different scaffolds have been developed with the aim of achieving optimal ASGP-R targeting\textsuperscript{143-145}. Since the sugar binding sites of the receptor are estimated to be 15-25 Å apart in a triangle spatial geometry (Figure 2.1)\textsuperscript{146}, properties like linker length, hydrophilic-hydrophobic balance of the linker and spatial geometry of the scaffold are important factors to consider in the design of multivalent ligands.
2.3.1. Bivalent glycoclusters (BG)

2.3.1.1. Length of linker

Despite the fact that the most prevalent presentation of the ASGP-R is the trivalent one, simple divalent targeting ligands have been reported, such as the BG1 based on a benzaldehyde scaffold (Figure 2.3a). A BG1-oligonucleotide conjugate was prepared for the targeted delivery of the transcription factor nuclear erythroid 2-related factor 2 (Nrf2) to the liver. In this conjugate, BG1 enhanced the uptake of oligonucleotides that were bound to the transcription factor, and a hydrophobic membrane-disruptive chain helped the complex escape the endosomes. Indeed, the BG1-oligonucleotide conjugate enhanced the delivery efficiency of the transcription factor to the liver; however, the contribution of BG1 was proved to be low since BG1-oligonucleotide conjugate without the hydrophobic membrane-disruptive chain was unable to deliver Nrf2 in vivo. The generally low targeting effect of BG1 should be ascribed to the short linker. With a linker length of 11 Å, BG1 displays its sugars 17 Å apart from each other, a distance that would seem too short for bivalent binding to ASGP-R (Figure 2.3b). One advantage of this benzaldehyde scaffold is that the acid-labile acetal linkage between the linker and the backbone can be cleaved at the acidic endosomal pH, exposing the hydrophobic membrane-disruptive domain only upon endocytosis, which was demonstrated to be critical for the delivery efficiency.

To achieve bivalent binding to ASGP-R, BG2 was prepared based on a dimethylglutamate scaffold using a longer linker than the one employed with BG1 (Figure 2.3a). The length of the linkers of BG2 were 15 and 12 Å respectively, which separated the sugars 22 Å apart from each other and allowed bivalent binding to ASGP-R (Figure 2.3b). Indeed, BG2 displayed 10-fold higher binding affinity for the ASGP-R on HepG2 cells compared to a monovalent ligand (IC₅₀, 0.47 mM vs. > 4 mM). The administration of ⁹⁹mTc-labeled BG2 to hepatic fibrosis mice resulted in significantly different pharmacokinetic parameters (derived from microSPECT/CT imaging) compared to the normal mice, whereas no difference was observed for mice injected with ⁹⁹mTc-labeled monovalent ligand.

Another example with an appropriate linker length that achieved bivalent binding effect was BG3, which was based on a lysine scaffold (Figure 2.3a). BG3 showed high binding affinity (Kᵢ = 30 nM) to ASGP-R in rat hepatocytes, as determined by a competition assay with ¹²⁵I-ASOR. The high binding affinity of BG3 is likely due to the fact that the distance between the sugars of 21 Å is appropriate for a bivalent binding mode (Figure 2.3b). The BG3 was further conjugated to several systems for targeted delivery of the drug antiviral nucleoside phosphonate 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and the antisense peptide nucleic acid (asPNA) to the liver of mice and rats.
Chapter 2 - Multivalent ligands for ASGP-R - a review

2.3.1.2. Spatial geometry of scaffold

The effect of the scaffold’s spatial geometry on the targeting efficacy to ASGP-R was investigated in a coiled-coil scaffold platform aimed at determining the distances between the sugar-binding sites of ASGP-R. The coiled-coil folding motif was tailored by attaching ligands at specific sites along the axis. Glycopeptides that differed in the number of displayed ligands, their position on the peptide sequence, and the distance between peptide backbone and ligands were prepared. Among the different glycopeptides prepared, BG4 (Figure 2.4) showed the best targeting potential according to an uptake experiment in HepG2 cells. BG4 had an 14-Å distance between peptide backbone and Gal, and 13 Å in between the functionalized amino acids on the peptide chain. Altering the distance between the peptide backbone and Gal to 0 Å or to 30 Å resulted in a dramatic loss of uptake efficiency. Furthermore, adding a third Gal moiety to BG4 barely changed the uptake efficiency, which was probably due to the fact that the rigid coiled-coil backbone displayed the three Gal in a brush-like geometry that was not matching that of ASGP-R. This study showed that the scaffold’s geometry should be taken into consideration in the design of multivalent ligands to achieve maximum binding to ASGP-R.
Figure 2.4. (a) Peptide sequence was first produced by solid phase peptide synthesis (SPPS). BG4 was obtained by coupling Gal to the side chains of lysines (at position 3 and position 10) of a peptide sequence, via glutaric anhydride in 3 steps.  
(b) Geometry of the Gal moieties.

2.3.2. Trivalent glyoclusters (TG)

2.3.2.1. Length of linker
An elegant study about the influence of the linker length of trivalent glyoclusters on the targeting efficacy to ASGP-R was first conducted by Biessen et al. In this study, a series of trivalent glyoclusters based on Tris scaffold was prepared with various linker lengths for ASGP-R targeting. In a competition assay against $^{125}$I-ASOR for binding to rat parenchymal liver cells, the TG1 with a 20-Å linker (Figure 2.5) showed the strongest binding affinity ($K_i = 200$ nM) to ASGP-R. This was at least 2000-fold higher than the TG with a 4-Å linker and 6-fold higher than the TG with a 10-Å linker. This study demonstrated that including a linker of appropriate length between the Gal residues and the branching point of the trivalent glyoclusters was necessary to obtain a high binding affinity to ASGP-R. The TG1 with a linker length of 20 Å displayed its Gal moieties at a distance of 32 Å, thus allowing trivalent binding to ASGP-R. A TG1-cholesterol conjugate was shown to directly associate with both low-density lipoprotein (LDL) and high-density lipoprotein (HDL), and its introduction into $^{125}$I-lipoprotein dramatically accelerated the serum decay and enhanced the liver uptake of these lipoproteins in rats. The enhancement of hepatic uptake was probably mediated by ASGP-R, since the uptake decreased by about 80% in the presence of free GalNAc.

A nitro-triacid scaffold-based conjugate (TG2-ASO, Figure 2.5) was also optimized in terms of linker length, showing a quite promising binding affinity ($K_i = 6.2$ nM) to primary mouse hepatocytes. One advantage of TG2 was that the use of nitro-triacid scaffold reduced significantly the synthetic complexity. Notably, when the linker length of TG2 was varied in the range of 13-21 Å, the measured binding affinity remained high ($K_i = 6.2-10.3$ nM), while decreasing it to 10 Å resulted in more than a 2-fold decrease of binding affinity ($K_i = 26$ nM).

The studies cited above showed that there was a minimal linker length required for multivalent binding of trivalent glyoclusters. However, the existence of a maximal linker length remained to be investigated. A lysine scaffold-based TG3 was prepared for targeted delivery of magnetic nanoparticles to HepG2 cancer cells (Figure 2.5) with one linker of 12 Å and two linkers of 13 Å.
Given that TG3 had at least two sugar moieties displayed at a distance of 21 Å, bivalent or trivalent binding to ASGP-R was expected. The uptake of TG3-magnetic nanoparticles by HepG2 was attributed to their interaction with ASGP-R based on the observation that HeLa cells, which do not express ASGP-R, did not internalize the particles. In addition, since the uptake of the TG3-magnetic nanoparticles by HepG2 cells was more efficient than the uptake of particles decorated with monovalent Gal, TG3 was suggested to bind to ASGP-R in a multivalent mode. Prolonging the linker length of TG3 from 13 Å to 19 Å caused significant loss of the uptake efficiency. TG3 was also used for targeting delivery of the anticancer drug paclitaxel (PTX) to the HepG2 cells. However, in another study where Gal was replaced by GalNAc, the prolongation of the linker from 13 Å to 16 Å by introducing a glycine lead to a 12-fold increase of the binding affinity to rat hepatocytes. It is possible that a linker length of 19 Å but not of 16 Å would separate the sugars too much from each other, hindering the ligand’s multivalent binding to ASGP-R. Therefore, it can be concluded that there is a maximal linker length for multivalent binding of trivalent glycoclusters, which for this asymmetric lysine scaffold-based ligand should be below 19 Å.

One delivery system that benefited from an optimal linker length was a triacid scaffold-based TG4 that was prepared with the aim of specifically decreasing the copper concentration in hepatic cells. Upon its internalization into the hepatic cells via ASGP-R-mediated uptake, TG4 was converted by the reducing agent glutathione (GSH) into an efficient CuI chelator, which ultimately decreased the free copper concentration in the hepatic cells (Figure 2.5). A dye-labeled TG4 was taken up by the ASGP-R-expressing hepatic cells HepG2 and WIF-B9 but not by the ASGP-R-lacking HeLa cells, suggesting an ASGP-R-mediated uptake mechanism. Incubation of TG4 with the WIF-B9 cells containing high intracellular level of copper ions resulted in dramatic decrease of the copper concentration. To achieve an optimal ASGP-R recognition, the linker of TG4 was designed to be 18 Å in length. Indeed, this length was able to present the three GalNAc moieties approximately 29 Å apart from each other. Monestier et al. further investigated the impact of ligand valency of the chelator on its uptake by HepG2 cells and found that the uptake of the multivalent chelator was significant higher than the monovalent one, revealing the multivalent binding mode between the trivalent ligand and ASGP-R. A pentaerythritol scaffold was also used for the preparation of trivalent glycoclusters, but in this case multivalent binding was not expected due to the short linker length.
2.3.2.2. Hydrophilic-hydrophobic balance of linker

The influence of the linker’s hydrophilic-hydrophobic balance on the targeting effect to ASGP-R was studied by Biessen and coworkers by introducing two important modifications to the linker of TG1. The methylene acetals connecting the linker to Tris were replaced by acid stable ether bonds, and an alkyl chain containing two amide bonds was used for the linker instead of the oligo(ethylene glycol). In a competition experiment with $^{125}$I-ASOR binding to rat parenchymal liver cells, the newly obtained TG5 (Figure 2.6) showed slightly higher binding affinity ($K_i = 93$ nM).
compared to TG1 ($K_i = 200$ nM). Replacing the linker of TG5 with a new linker (linker 1, Figure 2.6) did not change the binding affinity. Moreover, liposomes with 5% (w/w) of TG5-lipid conjugate showed efficient liver uptake in mice, and since preinjection with asialofetuin nearly completely inhibited the uptake, the liposomes were proposed to be recognized by ASGP-R. Therefore, trivalent glyoclusters containing a hydrophilic-hydrophobic balanced-linker, such as an alkyl chain with two amides, bind stronger to ASGP-R than trivalent glyoclusters containing very hydrophilic linkers like oligo(ethylene glycol). Different reasons could account for this. For instance, it is possible that the hydrophobic linker would enhance the interactions between the linker and the protein surface. Alternatively, the hydrophilic linker could form aggregates and therefore suppress the multivalent binding.161-163

Early work established that the binding affinity of trivalent glyoclusters towards ASGP-R could be greatly improved by up to 60-fold in rat hepatocytes by replacing the Gal moieties for GalNAc.164 Based on these findings, Biessen and coworkers165 modified TG5 and generated a new GalNAc-terminated TG6 (Figure 2.6) that displayed 50-fold higher binding affinity ($K_i = 2$ nM) to ASGP-R compared to TG5. The linker of TG6 contained a short oligo(ethylene glycol) chain and an amide bond, which provided a new option for preparing the hydrophilic-hydrophobic balanced linker. A TG6-lipid conjugate was able to associate with plasma lipoproteins, and intravenous injection of this glycolipid containing lipoproteins lead to an efficient dose-dependent uptake of lipoproteins by the liver. The involvement of ASGP-R in the uptake was confirmed by competition experiments performed with preinjection of asialoorosomucoid, which significantly inhibited the uptake of TG6-lipid conjugate. In addition, intravenous injection of the TG6-lipid conjugate to hyperlipidemic low density lipoprotein receptor-deficient mice resulted in an efficient cholesterol-lowering effect.

Another attempt to optimize the hydrophilic-hydrophobic balance of the linker was carried out by Ernst and coworkers,128 who replaced the more frequently used ethylene glycol-based linker by a propylene glycol-based linker regarded as a good combination of flexibility and hydrophilic-hydrophobic balance. The obtained TG7 (Figure 2.6) had linker length of 18 Å and distance between the sugars of 29 Å. The fluorescently labeled TG7 was selectively internalized by HepG2 via ASGP-R, as demonstrated by fluorescence microscopy and flow cytometry analysis. However, it is difficult to compare TG7 with other ligands since its binding affinity to ASGP-R was not measured.

Finally, Alnylam Pharmaceuticals developed a new TG8 which was described in a 2012 patent166 and was quite similar to TG5 except for the inclusion of GalNAc instead of Gal (Figure 2.6).167 The binding affinity of TG8 to ASGP-R on freshly isolated mouse hepatocytes was quite strong ($K_d = 2.3$ nM), as determined by flow cytometry.168 Its efficacy was first demonstrated using TG8-decorated ionizable lipid nanoparticles.82
In addition to the TG8-lipid nanoparticles, a TG8-siRNA conjugate is also under investigation at Alnylam Pharmaceuticals. The conjugation of TG8 to siRNA remarkably increased the uptake of siRNA by primary mouse hepatocytes. This enhanced uptake was largely inhibited in the presence of free TG8 or the calcium binder EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), indicating that the uptake was mediated by specific binding of the TG8 ligand to ASGP-R. The ability of the TG8-siRNA to silence gene expression was further evaluated in vivo using a siRNA that specifically targeted rodent TTR. Upon subcutaneous administration of a single 25 mg/kg dose to mice, more than 80% TTR mRNA in the liver was suppressed after 24 h. Chronic weekly administration of the conjugates over 9 months resulted in sustained dose-dependent gene silencing without any adverse effect in rodents. Base on TG8-siRNA conjugate, a nucleic acid drug under the name of ALN-PCSsc for the treatment of hypercholesterolemia showed promising results in phase 1 and phase 2 clinical trials (#NCT02314442, NCT02963311). In addition to the conjugate and targeted nanoparticle formulation described above, a series of TG8-based nucleic acid drugs are currently under development at Alnylam Pharmaceuticals for the treatment of rare diseases, cardiovascular and metabolic diseases, and hepatic infectious diseases. Thus, the TG8 is currently the most advanced trivalent ligand for ASGP-R targeting.

Due to the robust ASGP-R-targeting effect of TG8, the ligand was also used for the delivery of short interfering ribonucleic neutrals (siRNN), antisense oligonucleotides (ASO), and anti-microRNA therapeutics to the liver of mice and humans. Interestingly, altering the linker length of a TG8-ASO conjugate in the range of 12-20 Å showed a high binding affinity ($K_i = 6.2-8.0$ nM), while introducing a longer hydrophobic alkyl chain (27 Å) resulted in almost a 3-fold decrease of binding affinity ($K_i = 23$ nM) (Figure 2.6). This loss of binding affinity could be ascribed to the hydrophobicity of the scaffold, which would disrupt the hydrophilic-hydrophobic balance.
Figure 2.6. (a) TG5: (Ac)TG-NH$_2$ was obtained from Tris and Bz-Gal in 16 steps; the coupling of trivalent glycoclusters to the lipid gave the final TG5-lipid conjugates.$^{160}$ TG6: (Ac)TG-NH$_2$ was obtained from Tris and Ac-Gal in 11 steps. The coupling of trivalent glycoclusters to the lipid gave TG6-lipid conjugates.$^{79}$ TG7: (Ac)TG-NH$_2$ was obtained from Tris and Ac-GalNAc in 17 steps, coupling the trivalent glycoclusters to the dye gave the final fluorescently labeled TG7.$^{128}$ TG8: (Ac)TG-NH$_2$ was obtained from Tris and Ac-GalNAc in 11 steps, coupling the trivalent glycoclusters to siRNA produced the final TG8-siRNA conjugates.$^{167}$ The binding affinity of TG8-ASO conjugates with various linker lengths for ASGP-R is reported in the inset table.$^{145}$ (b) Geometry of the sugar moieties.

The effect of the linker’s hydrophilic-hydrophobic balance on the targeting effect of trivalent glycoclusters was also studied in a lysine scaffold-based system. TG9 (Figure 2.7) showed very strong binding affinity ($K_i = 4$ nM) to ASGP-R on rat hepatocytes, as determined by a competition assay with $^{125}$I-ASOR. In this system, the replacement of GalNAc by Gal also caused a significant decrease of the binding affinity ($K_i = 650$ nM).$^{149}$ In an early study, Gal and oligo(ethylene glycol) linker were used instead of GalNAc and alkyl linker, but this early version of TG9 only showed micromolar binding affinity,$^{172, 173}$ demonstrating once again that a more hydrophobic linker resulted in enhanced affinity.
2.3.2.3. Spatial geometry of scaffold

The scaffolds of trivalent glycoclusters need to have an appropriate spatial geometry that matches the one of ASGP-R in order to achieve trivalent binding. This was well illustrated by the BG4 ligand discussed before, where the addition of a third Gal moiety barely changed the uptake efficiency due to the brush-like geometry that did not match that of ASGP-R. In 1987, Lee and coworkers\textsuperscript{174} used a glutamic acid-based scaffold to produce the multivalent ligand TG10 for ASGP-R (Figure 2.8). The binding affinity of TG10 to ASGP-R of rat hepatocytes was in the subnanomolar level (IC\textsubscript{50} = 0.2 nM) as determined by an inhibition assay with \textsuperscript{125}I-ASOR. The removal of one GalNAc residue from TG10 caused a 15-fold decrease of the binding affinity to ASGP-R, suggesting a trivalent binding mode for TG10. In a later study, a lower binding affinity was obtained for TG10 (IC\textsubscript{50} = 10 nM), according to a modified inhibition assay in which Eu-labeled ASOR was employed instead of \textsuperscript{125}I-ASOR and surface-immobilized hepatocytes were used instead of suspended ones.\textsuperscript{155} The binding affinity of TG10 was 10-fold lower than that of the new version of TG3 (GalNAc, 16 Å), which was likely related to the brush-like geometry of the scaffold that did not fit the triangle geometry of ASGP-R, therefore hinders TG10 from reaching the maximal binding affinity to ASGP-R. TG10 was then used for targeting delivery of several nucleic acid drugs to hepatocytes.\textsuperscript{143, 175-177} Lee and coworkers also made an attempt to replace the glutamic acid scaffold with other amino acids such as aspartic acid. The new aspartic acid scaffold-based trivalent glycocluster displayed a similar binding affinity to the TG10,\textsuperscript{178} which is not surprising since the spatial geometry was in fact the same for both ligands.

More recently, Alnylam Pharmaceuticals attempted to produce several ligands based on TG8 with brush-like spatial geometry, but unfortunately none of them could further improve the binding of TG8. Hydroxyprolinol scaffold-based TG11 was one of these ligands produced. A TG11-siRNA conjugate was prepared for hepatocyte-specific delivery in which GalNAc was conjugated to the 3’-end of the sense strand of the siRNA by a phosphodiester linkage (Figure 2.8).\textsuperscript{179} Compared to the previously reported TG8-siRNA conjugates (optimal range of linker length: 12-20 Å), TG11-siRNA had the same optimal sugar valency and was in the same optimal range of linker length, but required fewer synthetic steps (10 steps vs. 15 steps). In a fluorescence-based assay, the binding affinity of
TG11-siRNA ($K_i = 37.6$ nM) to primary mouse hepatocytes was demonstrated to be 2-fold lower than that of the TG8-siRNA ($K_i = 18.9$ nM). In conclusion, although TG11 implied a lower synthetic complexity, it also displayed lower binding affinity to ASGP-R compared to TG8, which was probably because the brush-like geometry of the scaffold did not fit that of ASGP-R. Similarly, trivalent glycoclusters based on nucleosides also failed to outperform TG8 in terms of binding affinity. These trivalent glycoclusters displayed GalNAc in a row along the scaffold with a similar spatial geometry as TG11. Therefore, the brush-like geometry of the ligand scaffold would not be the ideal one, and optimal binding effect was usually achieved by ligands with the triangular geometry.

**Figure 2.8.** (a) TG10: TG10 was obtained from $\gamma$-L-glutamyl-L-glutamic acid and Ac-GalNAc in 7 steps. TG11: TG11-siRNA conjugates were obtained from hydroxyprolinol and Ac-GalNAc in 10 steps. (b) Geometry of the sugar moieties.

### 2.3.3. Tetravalent glycoclusters (TeG)

#### 2.3.3.1. Valency

The effect of valency on the binding affinity of multivalent ligands to ASGP-R was first studied by Lee et al. in an inhibition experiment, which demonstrated that the binding affinity to rabbit ASGP-R increased dramatically from monovalent to trivalent ligand, but only slightly from trivalent to tetravalent ligand. More recently, the effect of valency was studied in a cyclic peptide scaffold-based system. TeG1 was prepared with the aim of selectively decreasing copper concentration in hepatocytes (Figure 2.9), and it displayed a controlled conformation with the upper face presenting a cluster of ligands for the ASGP-R targeting and the lower face presenting thiolates for copper complexation. Experiments in hepatic cell lines demonstrated that TeG1 was internalized by the HepG2 and WIF-B9 cells after as early as 2 h incubation time, and that it was able to lower the free intracellular copper concentration. The removal of one sugar moiety from TeG1 only resulted in
less than 2-fold decrease of binding affinity to ASGP-R.\textsuperscript{182} Therefore, the contribution of the 4\textsuperscript{th} sugar moiety of tetravalent glycoclusters on the binding affinity was very low, which would be expected given the fact that ASGP-R with 3 binding sites is the most prevalent configuration.

Figure 2.9. (a) The cyclic peptide was first prepared by multi-steps of SPPS from amino acids (AA), then the TeG1 was obtained by coupling GalNAc to the side chains of lysines.\textsuperscript{181} In the presence of glutathione (GSH), the cleavage of the disulfide bonds of TeG1 released the functional Cu\textsuperscript{II} chelator. (b) Geometry of the GalNAc moieties.

2.3.3.2. Anomeric carbon configuration of sugar

The impact of the anomeric carbon configuration (α and β) of the sugar on the uptake by hepatic cells was also studied by Monestier \textit{et al.}\textsuperscript{182} (Figure 2.9), revealing that no significant difference was observed between the uptake efficiency of tetravalent glycoclusters bearing β-GalNAc and α-GalNAc.

2.3.4. Other multivalent glycoclusters (MG)

2.3.4.1. Multivalency

Dendrimer\textsuperscript{103,104} and polymer-based scaffolds have been widely used to prepare multivalent ligands to target ASGP-R. These ligands with high valency display enhanced binding activity compared to the corresponding monovalent ligands, a phenomenon termed “cluster glycoside effect”.\textsuperscript{161} MG1-β-cyclodextrin (βCD) conjugate is a dendrimer scaffold-based platform for targeted delivery of drugs to hepatocytes (Figure 2.10a).\textsuperscript{84} In an uptake experiment with HepG2 cells, the rhodamine B-loaded MG1-βCD displayed significantly enhanced uptake efficiency compared to the Man-functionalized βCD control. From a structural point of view, MG1 can be considered as a combination of 3 trivalent glycoclusters with a 12 Å-long linker that can separate the sugars 19 Å apart, enabling a multivalent binding mode (Figure 2.10b). However, it was not clear whether the targeting effect resulted from single trivalent glycoclusters or from the synergistic effect of 3 trivalent glycoclusters. Therefore, it would be interesting to compare the uptake of MG1-βCD with a single trivalent glycocluster-βCD conjugate to investigate the impact of multivalency.\textsuperscript{183,184}
One of the most clinically advanced polymer scaffold-based systems for ASGP-R targeting is the dynamic polyconjugate MG2 (Figure 2.10), which was prepared with the aim of treating liver diseases by targeting hepatocytes with GalNAc ligands. The MG2 contained a membrane-disrupting polymer PBAVE (polyconjugate of butyl and amino vinyl ethers), a shielding polymer PEG, a targeting ligand GalNAc and a therapeutic siRNA, which were reversibly conjugated to the backbone polymer PBAVE. Upon GalNAc-mediated cellular uptake and subsequent exposure to the low pH of endosomes and the reducing conditions of cytosol, the reversible bonds in the polyconjugate would break, releasing the siRNA into the cytoplasm of the target cell. MG2 proved to be able to deliver siRNA to hepatocytes in vitro and in mice, probably thanks to the cluster glycoside effect since MG2 has high ligand valency. In this case, it will be very interesting to investigate the binding affinity of MG2 to ASGP-R, so that it can be compared with the well-defined trivalent ligands which are already at the nanomolar level. A drug candidate ARC-520 based on a new generation of MG2 is being developed by Arrowhead Research Corporation for the treatment of Hepatitis B disease, and is currently in Phase 2 clinical trials (#NCT02065336). In this new generation of MG2, two important chemical modifications were introduced, the original backbone PBAVE was replaced with melittin-like peptide (MLP), siRNA cargo was modified with cholesterol but not covalently linked to the backbone. In the end, the MLP-modified MG2 and cholesterol-modified siRNA were co-injected to the animals or humans.185

Figure 2.10. (a) MG1: The Ac-Gal dendrimer was prepared from Tris and Ac-Gal in 15 steps, then MG1-β-cyclodextrin (βCD) conjugate was obtained by coupling the dendrimer to βCD.84 MG2: Dynamic polyconjugates MG2. The synthesis route of DPC has not been described in detail in the literature, probably because this system is proprietary technology.10 (b) Geometry of the sugar moieties.
2.4. Summary and outlook

The type of sugar used in the multivalent ligands was found to play an important role in the binding to ASGPR. The monovalent GalNAc showed a 60-fold stronger binding affinity to ASGP-R than Gal,\textsuperscript{164} similarly to what was observed for multivalent ligands where replacing the Gal with GalNAc remarkably enhanced the binding affinity. Interestingly, although the impact of the sugar’s anomeric carbon configuration on the interaction with ASGP-R was rarely reported, the $\beta$ configuration was predominantly used for multivalent ligands. However, a recent study found that this was not a significant parameter. In contrast, the length of the linker was found to be the most decisive factor for the binding affinity of the multivalent ligands to ASGP-R. Since the sugar binding sites of ASGP-R are estimated to be 15-25 Å apart, the ideal linker should display the ligands within this range of distance to prevent the loss of binding affinity. Indeed, numerous published studies confirmed that optimal binding affinity was obtained when the distance between the sugars was 19-32 Å. Another property that has been deeply investigated is the hydrophilic-hydrophobic balance of the linker. In early studies, hydrophilic linkers such as oligo(ethylene glycol) were often chosen, but these were deemed suboptimal after more recent studies revealed that ligands containing linkers with appropriate hydrophilic-hydrophobic balance displayed better binding affinity to ASGP-R. In addition, the compatibility between the scaffold’s spatial geometry and that of the ASGP-R was found to be an important factor in achieving a strong binding (Figure 2.1). This would explain why many attempts to make brush-like geometry of the sugar ligands failed to obtain optimal binding affinities. So far, the best fitting geometry of the sugar ligands proved to be the symmetric or asymmetric triangle with a side length between 19 and 32 Å.

The parameters that were highlighted in this review article are of crucial importance for the design of a multivalent ligand for efficient ASGP-R targeting. It is very encouraging to see that several gene delivery systems associated with the multivalent ligands for liver diseases targeting are undergoing clinical trials. The success of these systems will prompt the scientific community to generate multivalent ligands, with increasingly higher binding affinity and less synthetic complexity. The experience and knowledge obtained from the design and preparation of the multivalent ligands for ASGP-R should provide a guide for the synthesis of multivalent ligand for other receptors.
Chapter 3

3. Characterization of Calcium Phosphate Nanoparticles Based on PEGylated Chelators for Gene Delivery
Chapter 3 - CaP nanoparticles for gene delivery

3.1. Introduction

Non-viral gene delivery systems have attracted much attention during the past decades due to their low immunotoxicity, low cost, and ease of production. Cationic lipid and polymer-based nanoparticles represent the most common non-viral gene delivery systems. Both of them can form complexes with DNA, protecting it from degradation by nucleases and facilitating its cellular uptake. However, nearly all synthetic gene delivery vectors present toxicity issues given their polycationic nature, which hinders their translation into the clinic.

Calcium phosphate (CaP) is a naturally-occurring inorganic material found in bones and teeth. The CaP-DNA co-precipitation was developed as a transfection method for plasmid DNA in the early 1970s, and has been successfully employed in a wide variety of cell lines. The method is attractive because CaP is bioresorbable, generally non-toxic, easy to prepare, inexpensive and presents high gene loading efficacy. In addition, the dissolution of CaP in mildly acidic environments facilitates the endosomal escape of the cargo. However, the rapid growth of CaP particles after preparation prevents the in vivo application of this method. To solve this problem, several strategies have been implemented to control the growth of the CaP nanoparticles, including polymer stabilization, lipid or DNA/siRNA coatings. Li et al. and Hu et al. developed a microemulsion method to produce lipid-coated CaP nanoparticles for the delivery of siRNA or DNA to tumor cells, and although systemic delivery was achieved, potentially toxic organic solvents and surfactants were employed to prepare the microemulsions. Pittella et al. reported a relatively simple strategy to prepare CaP/polyanion hybrid nanoparticles by mixing solutions containing synthetic polyanion and other components, which upon systemic administration resulted in Vascular endothelial growth factor (VEGF) gene silencing and therapeutic effect of pancreatic cancer.

Recently, we developed a solvent- and surfactant-free PEGylated chelator coating strategy for the preparation of PEGylated CaP nanoparticles by simply mixing a PEGylated chelator solution with other components. Briefly, a PEGylated bisphosphonate (PEG-BP) and PEGylated 1,3,4,5,6-pentakisphosphate (PEG-IP5) were used to stabilize CaP-siRNA/DNA nanoparticles of 190-220 nm in size. Despite the method’s simplicity, smaller particle sizes (< 100 nm) could not be obtained and the formation mechanism of these particles was not studied. Developing a method to produce smaller CaP particles would be attractive as nanoparticles with a diameter of 10-100 nm are suitable for tumor targeting via the enhanced permeability and retention (EPR) effect. We previously observed that PEG-IP5 can stabilize CaP nanoparticles better than PEG-BP, but did not investigate these particles further.

Here, we present a systematic study of the impact that the chelator and preparation method employed can have on the particles’ size, morphology and crystallinity, and ultimately on their in vitro cellular uptake and transfection efficiency. Firstly, the PEGylated chelators PEG-IP5 and PEG-BP
were prepared, for which new synthesis routes involving click chemistry were developed. Two different methods were employed to produce PEG-IP5 and PEG-BP stabilized particles. The structure of these nanoparticles was characterized by different methods. Finally, the impact of each preparation method on the cellular uptake and transfection efficiency of PEG-IP5 was studied.

**Figure 3.1.** Schematic illustration showing the preparation of CaP nanoparticles and their cellular uptake. CaP nanoparticles were prepared by two methods. **Method 1**, adding Ca\(^{2+}\) solution to PO\(_4^{3-}\) solution resulted in growing particles which were subsequently stabilized by introducing the PEGylated agent in a time-dependent manner. **Method 2**, PEGylated agent and PO\(_4^{3-}\) solution were premixed and then added to Ca\(^{2+}\) solution to produce stabilized particles. The obtained CaP nanoparticles were incubated with HepG2 cells, followed by internalization into the cells, dissolution in endosomal pH and the release of DNA which was ultimately transferred to the nucleus for active transcription.
3.2. Materials and Methods

3.2.1. Materials

Methoxy-PEG-azide (MeO-PEG-N$_3$, 5000 g/mol) was obtained from JenKem (Allen, Texas). All other chemicals were purchased from Sigma Aldrich (Buchs, Switzerland). Size exclusion PD-10 columns (Sephadex G-25 M, containing 0.15% Kathon CG as preservative) was from GE Healthcare life science (Chalfont St Giles, UK). Hydroxyapatite (DNA-grade Bio-Gel HTP) was from Bio-Rad Laboratories (Hercules, CA). HepG2 (hepatocellular carcinoma) cell line was purchased from ATCC (American Type Culture Collection) (Manassas, VA). Minimum essential medium (MEM) with GlutaMax™, MEM without phenol red, penicillin/streptomycin, fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin-EDTA, and Lipofectamine™ were bought from Thermo Fisher Scientific (Carlsbad, CA). CellTiter® 96 Aqueous Non-Radioactive Cell Proliferation Assay was from Promega (Dübendorf, Switzerland). Label IT® Fluorescein Plasmid Delivery Control was purchased from Mirus (Madison, WI). Enhanced Green fluorescent protein plasmid (pEGFP-DNA) was generated by cloning the green fluorescent protein gene into the pcDNA3 vector that carries an ampicillin-resistance gene (Addgene), inserted into Escherichia coli BL21(DE3) and purified using the HiSpeed® Plasmid Maxi Kit (Qiagen, Hilden, Germany).

3.2.2. Analysis equipment

NMR spectra were acquired on a Bruker AV400 spectrometer (Bruker BioSpin, Fällanden, Switzerland) operating at 400 MHz for protons. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) was performed using Bruker’s UltraFlex II system with Compass 1.5 control software.

3.2.3. Synthesis of PEGylated chelator PEG-IP5/BP

3.2.3.1. Synthesis of compound 3.1

![Synthesis of myo-inositol monoorthoformate](image)

myo-Inositol (10.00 g, 55.5 mmol), trimethylorthoformate (14 mL, 127.7 mmol), and p-toluenesulfonic acid monohydrate ($p$TsOH·H$_2$O) (0.42 g, 2.2 mmol) were dissolved in $N,N$-dimethylformamide (DMF) (100 mL) and heated to 110 °C for 4.5 h. Then the mixture was cooled to rt and stirred for 16 h. It was quenched with sat. aq. NaHCO$_3$ (30 mL) and stirred at rt for 45 min. The precipitation was filtered off and the filtrate was concentrated under reduced pressure at 60 °C.
Purification by flash column chromatography (silica gel, acetonitrile (ACN)) was carried out. The colorless solid myo-inositol monoorthoformate was isolated after washing with EtOAc (4.81 g, 46% yield), NMR spectrum was identical to that reported in literature.\textsuperscript{200}

**Synthesis of compound 3.1**

NaH (242 mg, 6 mmol, 60% in mineral oil) was added to a solution of *myo-inositol monoorthoformate* (500 mg, 2.5 mmol) in anhydrous DMF (10 mL) at 0 °C. After stirring for 20 min, 4-methoxybenzyl chloride (PMBCl) (0.74 mL, 5.5 mmol) was added and the solution was stirred at rt for 2 h. The mixture was quenched with H\textsubscript{2}O (5 mL) and concentrated under vacuum. The crude product was partitioned between H\textsubscript{2}O (5 mL) and dichloromethane (DCM) (10 mL). The combined organic layers were washed with brine (5 mL) and dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. After concentration under vacuum, it was purified by flash column chromatography (silica gel, 1:1 ethyl acetate (EtOAc)/hexane). Compound 3.1 (464 mg, 41% yield) was isolated. NMR spectrum was identical to that reported in literature.\textsuperscript{201}

**3.2.3.2. Synthesis of compound 3.7**

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (430 mg, 2.2 mmol) was added to a stirred solution of 4-pentynoic acid (200 mg, 2.0 mmol) and *N*-hydroxysuccinimide (NHS) (258 mg, 2.2 mmol) in DCM (10 mL). The resulting mixture was stirred overnight at room temperature. The mixture was concentrated under vacuum and the resulting residue was purified by flash column chromatography (silica gel, EtOAc/hexane 30% → 40%) to afford 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (alkyne-NHS ester) (305 mg, 77% yield). NMR spectrum was identical to that reported in literature.\textsuperscript{202}

**Synthesis of compound 3.7**

To a solution of sodium alendronate trihydrate (131 mg, 0.40 mmol) in PBS (pH 7.0, 100 mM, 3 mL), 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (alkyne-NHS ester) (100 mg, 0.51 mmol) in acetonitrile (3 mL) was added, and reaction mixture was stirred for 8 h at rt. The resulted solution was concentrated and precipitated in ethanol three times. The white solid product was obtained (50 mg, 31% yield). NMR spectrum was identical to that reported in literature.\textsuperscript{203}
3.2.3.3. Synthesis of compound 3.2

NaH (30.8 mg, 0.77 mmol, 60% in mineral oil) was added to a solution of compound 3.1 (221 mg, 0.51 mmol) in dry DMF (5 mL) at 0 °C. After 10 min, propargyl bromide (66.3 µL, 0.77 mmol) was added and the mixture was stirred for 16 h at rt. The mixture was quenched with methanol (MeOH), evaporated to dryness and partitioned between H₂O and EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. Purification with flash column chromatography (silica gel, EtOAc/hexane 1:3 −> 1:1) yielded compound 3.2 (203 mg, 85% yield). ¹H NMR (400 MHz, CDCl₃), δ: 7.23-7.18 (m, 4H, oPh-PMB), 6.86-6.80 (m, 4H, mPh-PMB), 5.50 (d, J = 1.3 Hz, 1H, H-C7), 4.60 (d, J = 11.2 Hz, 2H, Bn), 4.51 (d, J = 11.2 Hz, 2H, Bn), 4.42-4.38 (m, 1H, H-C5), 4.36-4.32 (m, 4H, H-C1, H-C3, H-C4 and H-C6), 4.20 (d, J = 1.5 Hz, 1H, H-C2), 3.81 (s, 6H, OCH₃), 2.43 (t, J = 2.4 Hz, 1H, H-C10).

¹³C NMR (101 MHz, CDCl₃), δ: 159.41 (pPh-PMB), 129.87 (Ph-PMB), 129.41 (oPh-PMB), 113.85 (mPh-PMB), 76.79 (C9), 75.17 (C10), 73.86 (C4 and C6), 71.55 (Bn), 70.56 (C1 and C3), 68.36 (C5), 67.66 (C2), 56.84 (C8), 55.43 (OCH₃). ESI-MS: m/z [M+H]^+ calcd 469.1857, found 469.1852.

3.2.3.4. Synthesis of compound 3.3

Compound 3.2 (220 mg, 0.47 mmol) was refluxed in ethanol (EtOH) (6 mL) and 1 N HCl (3 mL) at 90 °C for 5 h. After concentration of the reaction mixture, it was precipitated in Et₂O. Compound 3.3 (54.4 mg, 53% yield) was isolated. ¹H NMR (400 MHz, Methanol-d₄), δ: 4.51 (d, J = 2.4 Hz, 2H, H-C7), 3.95 (t, J = 2.7 Hz, 1H, H-C2), 3.57 (t, J = 9.5 Hz, 2H, H-C4 and H-C6), 3.41 (dd, J = 9.9, 2.7 Hz, 2H, H-C1 and H-C3), 3.37 (t, J = 9.2 Hz 1H, H-C5), 2.83 (t, J = 2.4 Hz, 1H, H-C9).

¹³C NMR (101 MHz, Methanol-d₄), δ: 80.09 (C8), 79.87 (C2), 75.71 (C9), 74.37 (C5), 72.50 (C4 and C6), 71.21 (C1 and C3), 60.51 (C7). ESI-MS: m/z [M+Na]^+ calcd 241.0683, found 241.0680.
3.2.3.5. Synthesis of compound 3.4

Compound 3.3 (20 mg, 0.09 mmol) was dried under vacuum for 24 h. Then dry ACN was added and the mixture was sonicated until a fine suspension resulted. Tetrazole solution (0.45 M in ACN, 5.1 mL, 2.29 mmol) and phosphorylating reagent o-xylylene-N,N-diethylphosphoramidite (215 mg, 0.9 mmol) were added and the solution was stirred for 2 d at rt. The reaction mixture was cooled to -10 °C (ice bath with acetone), treated with meta-chloroperoxybenzoic acid (mCPBA) (70-77%, 244 mg, 0.99 mmol) and stirred for 15 min. After diluting the crude mixture with DCM, it was treated with solution of aqueous NaSO₃, washed with NaHCO₃ (sat. aq.) and brine. The combined organic layers were dried over anhydrous Na₂SO₄, concentrated under reduced pressure and purified with flash column chromatography (silica gel, MeOH/DCM 0 → 3:97). After precipitation in Et₂O (2×), compound 3.4 (69.9 mg, 69% yield) was isolated. Rf = 0.2 (MeOH/DCM 3:97); ¹H NMR (400 MHz, CDCl₃), δ: 7.43-7.20 (m, 20 H, Ph), 5.67-4.91 (m, 23H, H-C4, H-C5, H-C6, and Bn), 4.79-4.69 (m, 3H, H-C1, H-C2 and H-C3), 4.52 (d, J = 2.4 Hz, 2H, H-C7) 2.42 (t, J = 2.4 Hz, 1H, H-C9). ¹³C NMR (101 MHz, CDCl₃), δ: 135.78, 135.71, 135.39, 135.08, 129.41, 129.28, 129.22, 129.16, 128.96, 128.87, 77.19, 76.57, 76.19, 65.60, 69.53, 69.47, 69.43, 69.38, 69.36, 69.23, 69.16, 69.09, 61.62, 60.92 (splitting of the signals is caused by the phosphorous contained in the molecule). ³¹P NMR (162 MHz, CDCl₃), δ: -1.06, -4.37, -4.89. ESI-MS: m/z [M+H]⁺ calcd 1129.1527, found 1129.1525.

3.2.3.6. Synthesis of compound 3.5

Compound 3.4 (20 mg, 0.018 mmol) and mPEG5k-Azide (78 mg, 0.015 mmol) were dissolved in 400 µL DCM, CuI (0.113 mg, 0.591 µmol), N,N-diisopropylethylamine (DIPEA) (0.206 µL, 1.182 µmol) in 100 µL DCM and Acetic acid (AcOH) (0.068 µL, 1.182 µmol) in 100 µL DCM were added to the solution. The reaction mixture was stirred at rt for 20 h under inert atmosphere. After purification by flash column chromatography (silica gel, MeOH/DCM 5% → 20%), compound 3.5 (48 mg, 50%) was isolated. ¹H NMR (400 MHz, CDCl₃), δ: 7.90 (s, 1H, H-C9), 7.43-7.20 (m, 20H, Ph), 5.63-4.88 (m, 26H, H-C4, H-C5, H-C6, H-C7 and Bn), 4.82-4.68 (m, 3H, H-C1, H-C2 and H-C3), 4.41 (t, J = 5.5 Hz, 2H, H-C10), 3.85-3.42 (m, H-C11 and PEG), 3.37 (s, 3H, OCH₃). ³¹P NMR
(162 MHz, CDCl$_3$-d), δ: -1.34, -4.24, -4.55. MALDI-TOF-MS: Centered in $m/z = 6312$ g/mol (mPEG5k-Azide had a $m/z = 5158$ g/mol) was found.

3.2.3.7. Synthesis of PEG-IP5 (3.6)

![Diagram of PEG-IP5](image)

Compound 3.5 (48 mg, 7.49 µmol) was dissolved in MeOH (3.0 mL) and H$_2$O (1.0 mL) and set under inert atmosphere. Excess Pd/C was added and the reaction mixture was purged with H$_2$. It was stirred at rt for 16 h and then quenched with 1.0 M NaOH until a slightly basic solution was obtained. The crude was filtered over a small amount of celite in a syringe and concentrated to 1.0 mL of solvent and was directly loaded on a PD-10 column. After lyophilization, the product was passed through a resin column (Dowex, 50WX8, H$^+$) and was neutralized with NH$_4$OH and concentrated. PEG-IP5 (3.6) (27 mg, 59% yield) was obtained. $^1$H NMR (400 MHz, D$_2$O), δ: 8.25 (s, 1H, H-C9), 5.05 (s, H-C7), 4.66 (t, $J = 5.3$ Hz, 2H, H-C10), 4.54-4.39 (m, 3H, H-C1, H-C3 and H-C5), 4.18-4.27 (m, 3H, H-C2, H-C4 and H-C6), 4.03 (t, $J = 5.4$ Hz, 2H, H-C11), 3.85-3.60 (m, PEG), 3.40 (s, 3H, OCH$_3$). $^{31}$P NMR (162 MHz, D$_2$O), δ: 0.91, 0.50, -0.31. MALDI-TOF-MS: Centered in $m/z = 5992$ g/mol (mPEG5k-Azide had a $m/z = 5158$ g/mol) was found.

3.2.3.8. Synthesis of PEG-BP (3.8)

![Diagram of PEG-BP](image)

Compound 3.7 (10 mg, 0.025 mmol) and mPEG5k-Azide (112 mg, 0.021 mmol) were dissolved in an ethanol-water solution (1/1, 600 µL). Copper sulfate (0.338 mg, 2.115 µmol) and sodium ascorbate (4.19 mg, 0.021 mmol) were then added separately under argon. The reaction mixture was stirred at rt for 3 days. After removal of the solvent, the product was acidified by 0.1 M HCl and purified with a PD-10 column with water as the eluent. PEG-BP (3.8) (56 mg, 46% yield) was obtained. $^1$H NMR (400 MHz, D$_2$O), δ: 7.76 (s, 1H, H-C6), 4.57-4.46 (m, 2H, H-C7), 3.91-3.85 (m, 2H, H-C8), 3.66-3.57 (m, PEG), 3.31 (s, 3H, CH$_3$), 3.09 (t, $J = 6.8$ Hz, 2H, H-C3), 2.93 (t, $J = 7.4$ Hz, 2H, H-C5), 2.53 (t, $J = 7.4$ Hz, 2H, H-C4), 1.90-1.80 (m, 2H, H-C2), 1.71 (dd, $J = 6.8, 3.0$ Hz, 2H, H-C1). $^{31}$P NMR (162 MHz, D$_2$O), δ: 18.15 (s). MALDI-TOF-MS: Centered in $m/z = 5608$ g/mol (mPEG5k-Azide had a $m/z = 5158$ g/mol) was found.
3.2.3.9. *Synthesis of PEG-IP5/BP (PEG 2 k)*

PEG-IP5/BP with PEG 2 k were synthesized in the same way as described above.

3.2.4. *Comparison of the binding affinity of PEG-IP5/BP to hydroxyapatite (HA)*

Hydroxyapatite is the final crystalline state of CaP precipitation, therefore, we used hydroxyapatite powder as a model to test the binding affinity of PEG-IP5/BP (PEG 2 k was used here). Briefly, both of the coating materials were mixed with hydroxyapatite in the amino acid 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer (pH 7.4). After equilibrating overnight, the mixtures were centrifuged and filtered, and the filtrate was be analyzed by $^1$H NMR using a calibration curve based on PEG integration to determine the binding isotherm.

3.2.5. *Preparation of CaP nanoparticles.*

Two methods were used to produce the blank CaP nanoparticles. Method 1: 150 µL of HEPES buffer pH 7.4 (140 mM NaCl, 1.5 mM Na$_2$HPO$_4$ and 50 mM HEPES) was mixed with 150 µL of CaCl$_2$ solution (15 µL of 2.5 M CaCl$_2$, 135 µL of 10 mM Tris with pH 7.4) under stirring, after certain reaction time, 150 µL of 5 or 10 µM chelator solution (PEG-IP5/BP) in 10 mM Tris with pH 7.4 was added, and the mixture was stirred for 15 s to produce the final CaP nanoparticles. Method 2: 150 µL of HEPES buffer pH 7.4 (140 mM NaCl, 6.0 mM Na$_2$HPO$_4$ and 50 mM HEPES) was mixed with 150 µL of 30 or 40 µM chelator solution (PEG-IP5/BP) in 10 mM Tris with pH 7.4 first, then 150 µL of CaCl$_2$ solution (15 µL of 2.5 M CaCl$_2$, 135 µL of 10 mM Tris with pH 7.4) was added and the mixture was vortexed for 15 s to produce the final CaP nanoparticles.

Gene-loaded CaP nanoparticles were also prepared with the two methods described before. Method 1: 150 µL of HEPES buffer pH 7.4 (140 mM NaCl, 1.5 mM Na$_2$HPO$_4$ and 50 mM HEPES) was mixed with 150 µL of CaCl$_2$ solution (2.8 µL of 1.09 µg/µL pEGFP-DNA, 15 µL of 2.5 M CaCl$_2$, 132.2 µL of 10 mM Tris with pH 7.4) under stirring, after certain reaction time, 150 µL of 5 or 10 µM chelator solution (PEG-IP5) in 10 mM Tris with pH 7.4 was added, and the mixture was stirred for 15 s to produce the final CaP nanoparticles. Method 2: 150 µL of HEPES buffer pH 7.4 (140 mM NaCl, 6.0 mM Na$_2$HPO$_4$ and 50 mM HEPES) was mixed with 150 µL of 30 or 40 µM chelator solution (PEG-IP5) in 10 mM Tris with pH 7.4 first, then 150 µL of CaCl$_2$ solution (2.8 µL of 1.09 µg/µL pEGFP-DNA, 15 µL of 2.5 M CaCl$_2$, 132.2 µL of 10 mM Tris with pH 7.4) was added and the mixture was vortexed for 15 s to produce the final CaP nanoparticles.

3.2.6. *Particle size and zeta potential determination*

Size and zeta potential measurements were performed by dynamic light scattering (DLS) and Doppler laser anemometry, respectively, using a DelsaNano C Particle Analyzer (Beckman Coulter, Krefeld, Germany). All the size measurements were carried out at a fixed scattering angle of 90°. The
CONTIN method included in the software of the instrument was used to calculate the intensity-average hydrodynamic diameter of the nanoparticles.

3.2.6.1. Influence of reaction time (method 1)

The size of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5, 5 µM) with different reaction time (1-7 s) was monitored by DLS over time at 25 °C.

3.2.6.2. The screening of optimal PEG-IP5 and phosphate concentrations (method 2)

The size of CaP nanoparticles prepared by method 2 with various PEG-IP5 and phosphate concentrations was monitored by DLS over time at 25 °C.

3.2.6.3. Influence of PEG concentration (method 1)

The size of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 5-40 µM) was monitored by DLS over time at 25 °C.

3.2.6.4. Long-term stability of CaP nanoparticles

The stability of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5/BP 5 µM or 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5/BP 30 µM or 40 µM) was assessed by monitoring changes in size over 1 month. The size was determined by DLS at 25 °C as described before.

3.2.6.5. Stability of CaP nanoparticles in different media and pH

The stability of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5, 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5, 40 µM) in different media was monitored by DLS for 6 h at 37 °C. The particles were prepared one day before. The size of CaP nanoparticles in a cell culture medium (MEM, 10% FBS, 1% penicillin-streptomycin) and PBS/FBS (1:1, v/v) solution was evaluated after dilution of samples with both media respectively (1:7, v/v). After 6 h, the suspension in PBS/FBS was acidified to pH 6.5 (to mimic the slightly acid environment of endosome) with 1 N HCl, and the size was determined by DLS at 37 °C.

3.2.6.6. Size and zeta potential of DNA loaded CaP nanoparticles

The DNA loaded CaP nanoparticles were prepared as described above, EGFP-DNA was used here and a final concentration of 6.66 µg/mL was obtained. The size and zeta potential of the DNA loaded CaP nanoparticles was determined by DLS at 25 °C.
3.2.6.7. Influence of PEG length

The size of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5, 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5, 40 µM) with PEG length of 2 k and 5 k was monitored by DLS over time at 25 °C.

3.2.7. X-ray diffraction (XRD)

XRD measurements of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5, 10 µM) with 0 and 1 day storage were performed in transmission mode with sample dispersed between two mylar-foils on a Stoe STADIP powder diffractometer (curved Germanium monochromator, CuKα1-radiation, λ = 1.540598 Å) equipped with a Mythen silicon strip detector (Dectris AG, Baden, Switzerland).

3.2.8. Electron microscopy

3.2.8.1. Transmission electron microscopy (TEM)

The TEM experiments of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5/BP 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5/BP 40 µM) were carried out on a Philips CM12 (tungsten cathode) transmission electron microscope (FEI, Hillsboro, OR). The particles were prepared one day before. The sample (3.5 µL) was loaded on a glow discharged carbon-coated copper grid for 1 min, washed once with double-distilled water, drained excess moisture along the periphery using filter paper. The dried samples were examined at 200 kV, and images were recorded with a Gatan CCD 794 camera (Gatan Inc., Pleasanton, CA).

3.2.8.2. Scanning electron microscopy (SEM)

The SEM experiments of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5/BP 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5/BP 40 µM) were carried out on a field-emission scanning electron microscope (Leo 1530, Zeiss/LEO, Oberkochen, Germany). The particles were prepared 1 day before. The carbon/formvar® coated 200 mesh Cu-grid was glow-discharged (Emitech K 100 X, Quoram Technologies, Newhaven, UK) for 45 s, then the sample (3.5 µL) was loaded on it for 1 min, washed once with double-distilled water, drained excess moisture along the periphery using filter paper. Dried grids were sputter-coated with 2 nm of platinum in a high-vacuum sputtering device (MED 010, Bal-Tec, Balzers, Liechtenstein). The coated samples were examined at an acceleration voltage of 5 kV using the in-lens secondary electron detector.

3.2.8.3. Cryogenic Transmission Electron Microscopy (Cryo-TEM)

The cryo-TEM experiments of CaP nanoparticles prepared by method 2 (phosphate 6.0 mM, PEG-IP5, 40 µM, with 20 min and 24 h storage after preparation) were carried out on a FEI Tecnai
F20 (FEI Company, Eindhoven, The Netherlands). Lacey carbon 300 mesh Cu-grid (EMS, Hatfield, PA) was glow discharged (Emitech K 100 X, Quorum Technologies, Newhaven, UK) for 45 s, the sample dispersion (2.5 µL) was applied onto the grid and excess of liquid was removed by manual blotting to produce a thin liquid layer (around 100 nm thick) followed by plunging the grid into melting ethane cooled to -175 °C. The vitrified samples were transferred on a Gatan cryo-holder into the microscope and continuously cooled during the imaging process at -180 °C. Micrographs were recorded under low dose conditions (< 500 e−/nm²) using a 4k × 4k Gatan CCD camera operating the microscope at 200 kV acceleration voltage in bright field mode and Low dose selected area electron diffraction (LDSAED) was performed in diffraction mode.

3.2.9. Encapsulation efficiency

The encapsulation efficiency of DNA was evaluated by a fluorescence assay. The fluorescein DNA-loaded CaP nanoparticles (180 µL, DNA 6.66 µg/mL) were prepared as described above. The particles suspensions were centrifuged at 4 °C and 20,000 × g for 30 min to precipitate the particles. DLS measurement was carried out to make sure no particles remaining in the supernatant. Free DNA was determined by measuring the fluorescence intensity (Ex: 495 nm, Em: 518 nm) with a plate reader (Infinite M-200, Tecan, Männedorf, Switzerland). Encapsulation efficiency was identified by subtracting the free DNA concentration in the supernatant from the total concentration.

3.2.10. Cell culture

HepG2 cells were cultivated in minimum essential medium (MEM GlutaMAX, ThermoFisher, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in humidified environment of 5% CO₂. Subcultivation was done every 3 days, and 60%-80% confluence of the cells was obtained before harvesting for uptake and transfection experiment. Cells were confirmed to be free of mycoplasma (MycoAlert, Lonza, Rockland, ME).

3.2.11. Cell uptake studies

The uptake of different CaP nanoparticles formulations were assessed in HepG2 cells, lipofectamine was used as a positive control. To track the CaP nanoparticles, a fluorescently-labeled Label IT® Fluorescein Plasmid Delivery Control was used. The cells were seeded at a density of 6 × 10⁴ cells/well into 24-well plates 24 h before uptake. The medium was replaced by fresh Opti-MEM medium containing 10% FBS, the CaP nanoparticles were added to the cells at a final DNA concentration of 0.5 µg/well, and incubated for 4 h. The cells were then washed twice with PBS, detached with trypsin 0.05% for 5 min, followed by the addition of complete medium to stop trypsinization. Then, the cells were collected by centrifugation (10 min, 300 × g, 4 °C) and re-suspended with FACS buffer (PBS, 2 mM EDTA, 0.5% BSA). Data for 5,000 cells were collected on a BD FACSCanto™ Flow Cytometer (BD Biosciences, San Jose, CA) and were analyzed using the
FlowJo software (Tree Star, Inc., Ashland, OR). Uptake efficiency was determined by normalizing the mean fluorescence intensity to the control cells.

3.2.12. In vitro transfection

The transfection efficiency of different CaP nanoparticles formulations were also assessed in HepG2 cells. The cells were seeded at a density of $3 \times 10^4$ cells/well into 24-well plates 24 h before transfection. The medium was replaced by fresh Opti-MEM medium containing 10% FBS, the CaP nanoparticles were added to the cells at a final DNA concentration of 0.5 µg/well or 2.0 µg/well, and incubated for 5 h. Then, the medium was removed and fresh complete medium was added and incubated for 48 h. The cells were analyzed by flow cytometry as described above and the transfection efficiency was defined as percentage of GFP-positive cells.

3.2.13. Cytotoxicity experiments

The viability of cells exposed to the CaP nanoparticles was determined by the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt] (CellTiter® 96 Aqueous Non-Radioactive Cell Proliferation Assay) according to the manufacturer’s protocol. HepG2 cells were seeded in a 96-well plate at a density of $5 \times 10^3$ cells/well in 100 µL complete medium and cultured for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was then replaced by 100 µL/well Opti-MEM with 10% FBS and different amounts of CaP nanoparticles were added to the wells. After 5 h incubation, the medium was removed and cells were washed twice with PBS, then fresh complete medium was added and incubation for 48 h. Then, the medium was replaced with 100 µL MEM medium without phenol red plus 20 µL CellTiter 96® Aqueous One Reagent containing the tetrazolium compound MTS for about 2 h. MTS was reduced by cells into a formazan compound. The absorbance of formazan was measured at 490 nm with a plate reader (Infinite M-200, Tecan, Männedorf, Switzerland). Cell viability was determined according to the following equation (eq. 1):

\[
\text{Cell viability [%]} = \left( \frac{\text{OD}_{490} \text{ sample}}{\text{OD}_{490} \text{ control}} \right) \times 100
\]

Where OD$_{490}$ sample represents the optical density of the wells treated with CaP nanoparticles and OD$_{490}$ control represents the wells treated with growth medium only.

3.2.14. Statistical Analysis

The one-way ANOVA test combined with Tukey’s post-hoc test was applied for the pairwise comparison between multiple groups. $p < 0.05$ was considered statistically significant in all analyses.
3.3. Results and discussion

3.3.1. Synthesis of the PEGylated chelators.

PEG-IP5 chelator (Figure 3.1) was synthesized from a known partially protected myo-inositol intermediate (Figure 3.2A). Briefly, an alkyne was introduced by alkylation of the 2-hydroxyl group of orthoformate-protected myo-inositol (1), followed by deprotection of the remaining hydroxyls, which were then phosphorylated to produce compound (4). The PEG chain was introduced by a copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction, followed by hydrogenolysis to give the final PEG-IP5. The clickable alkyne group was introduced in order to allow straightforward conjugation of the chelator to various polymers at a later stage, thus avoiding complicated purification. Similarly, a commercial bisphosphonate (BP, alendronate) was conjugated by click chemistry to PEG to obtain PEG-BP (Figure 3.1, Figure 3.2B).

Figure 3.2. Synthesis of (A) PEG-IP5 (3.6) and (B) PEG-BP (3.8). Reagents and conditions: (a) Propargyl bromide, NaH, DMF, rt; (b) 1 N HCl, EtOH, 90 °C; (c) o-xyylene-N,N-diethylphosphoramidite, tetrazole, DCM, mCPBA, rt; (d) mPEG-azide, CuI, DIPEA, AcOH, DCM, rt; (e) H2, Pd/C, MeOH, H2O; (f) mPEG-azide, sodium ascorbate, CuSO4, EtOH, H2O, rt;
3.3.2. Comparison of the binding affinity of PEG-IP5 and PEG-BP to hydroxyapatite (HA).

We previously showed that PEG-IP5 could stabilize CaP nanoparticles better than PEG-BP.\(^69\) In order to verify whether binding affinities play a role in this effect, we studied the binding isotherm of both chelators to HA powder, the final crystalline state of CaP.\(^207-209\) The adsorption isotherms of the two chelators on HA were determined by \(^1\)H NMR spectroscopy. Their affinity and maximum binding towards HA were determined by non-linear curve fitting of a Langmuir adsorption isotherm (eq. 2) to the binding data,\(^210, 211\)

\[
q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \tag{2}
\]

where \(C_e\) is the concentration of PEG-BP/IP5 in solution in equilibrium with the HA particles (M), \(q_e\) is the amount of PEG-BP/IP5 bound to the surface per gram of HA (mol/g HA), \(q_m\) is the maximum binding of PEG-BP/IP5 to the surface per gram of HA (mol/g HA), and \(K_L\) is the Langmuir constant representing the inverse of the bulk solution concentration of PEG-BP/IP5 required to achieve half of the maximum surface binding (L mol\(^{-1}\)).

The maximum binding to HA was slightly higher for PEG-BP (\(q_m = 41.8 \pm 1.1 \mu\text{mol/g HA}\)) than for PEG-IP5 (\(q_m = 31.5 \pm 1.6 \mu\text{mol/g HA}\)) (Figures 3.5A, B), which is likely due to the larger molecular footprint of the latter (IP5 with five phosphate groups occupies more space than BP with only two phosphate groups). The affinity (Langmuir) constants for both chelators were very close with values of \(1.29 \pm 0.17 \times 10^5\) L mol\(^{-1}\) and \(3.17 \pm 0.69 \times 10^5\) L mol\(^{-1}\) for PEG-BP and PEG-IP5 respectively. Therefore, the better stabilizing effect of PEG-IP5 could be mainly ascribed to other factors, such as the difference in the charges of the chelator. A previous report\(^212\) showed that the binding affinity of free alendronate (the BP used here) to HA was \(K_L = 29.4 \pm 2.4 \times 10^5\) L mol\(^{-1}\) which was higher than what we obtained for PEG-BP, probably due to the bulk of the long PEG chain.

3.3.3. Preparation of CaP nanoparticles.

To prepare stable CaP nanoparticles, a PEGylation agent can be introduced to the system either before\(^67, 196\) or after\(^68, 69\) the formation of the particles. In this work, we used both methods to produce CaP nanoparticles. In method 1, a calcium solution containing plasmid DNA was added to a phosphate solution. Due to the strong precipitation propensity of CaP, rapidly-growing nanoparticles formed immediately. The PEGylated chelator solution was added after different reaction times in order to stop the growth and obtain stable CaP nanoparticles (Figure 3.1). We previously reported that stable CaP nanoparticles could be obtained with 1.5 mM phosphate and a minimum of 5 µM of PEGylation chelator.\(^69\) In this work, we used these concentrations and specifically investigated the influence of
reaction time of Ca$^{2+}$ and PO$_4^{3-}$ on the size of CaP nanoparticles by dynamic light scattering (DLS). Not surprisingly, the particle size increased with longer reaction times, and the relationship between them was found to be linear (Figure 3.3). After 4 s of reaction time, the particle size was around 220 nm and the gene loading efficiency was as high as 97.3 ± 1.3% (Table 3.1). Based on the particle yield and sizes obtained, we chose 4 s as optimal time for subsequent studies.

**Figure 3.3.** Effects of reaction time on size and scattering intensity of CaP nanoparticles. Particles prepared by method 1 with reaction time 1-7 s, PEG-IP5 was 5 µM and phosphate was 1.5 mM in concentration. The size showed linear relationships with the reaction time ($R^2 = 0.998$). Data are presented as the means ± SD (n = 3).

**Table 3.1.** Encapsulation efficiency of pDNA to CaP nanoparticles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Encapsulation efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method 1</td>
<td>97.3 ± 1.3</td>
</tr>
<tr>
<td>Method 2</td>
<td>80.0 ± 2.0</td>
</tr>
</tbody>
</table>

*Formulations prepared by method 1 (phosphate 1.5 mM, PEG-IP5 10 µM), and by method 2 (phosphate 6.0 mM, PEG-IP5 40 µM). Data are presented as the means ± SD (n = 3).

In method 2, the PEGylated chelator and phosphate were premixed and then added to a solution of calcium containing DNA (Figure 3.1). To determine the optimal conditions for CaP nanoparticles production by this method, various PEGylated chelator and phosphate concentrations were screened (Figure 3.4). While particles were not obtained with the concentrations used in method 1, particles with a size of around 40 nm (measured by DLS) were produced at a phosphate concentration of 6.0 mM and a PEGylated chelator concentration of 30 or 40 µM. We therefore chose these as optimal conditions for subsequent studies. The particles prepared under these conditions had a gene loading efficiency of 80.0 ± 2.0% at a PEG-IP5 concentration of 40 µM (Table 3.1). The particles were not further purified since both purified and non-purified CaP nanoparticles previously showed very similar gene silencing efficiency *in vitro* without any toxicity issues.205
Figure 3.4. The screening of optimal PEG-IP5 and phosphate concentrations for CaP nanoparticles prepared by method 2. Particles with the size over 200 nm only showed the parts below 200 nm due to the limit of Y axis.

3.3.4. Colloidal stability of CaP nanoparticles.

We then investigated the stabilization effect of the IP5- and BP-based PEGylated chelators on CaP nanoparticles. Particles prepared by method 1 with PEG-IP5 concentrations of 5 µM and 10 µM had sizes between 180-230 nm and were stable for at least one month (Figure 3.5C). When PEG-BP chelator was used instead, the particles had similar sizes but were only stable at the higher chelator concentration (10 µM) since particles prepared with the lower concentration (5 µM) increased in size after 1 week. Notably, increasing the PEGylated chelator concentration above 10 µM did not influence the size or scattering intensity of CaP nanoparticles (Figure 3.7). Particles prepared by method 2 with 30 µM and 40 µM PEGylated chelator exhibited much smaller sizes than particles prepared with method 1 (Figure 3.5D). Surprisingly, their initial size (measured by DLS) of about 40 nm increased to around 80 nm after one day and remained stable for one month thereafter, suggesting particle transformation after a storage period (vide infra).
**Figure 3.5.** Binding affinity of PEG-BP/IP5 to HA and stability of PEG-BP/IP5 coated CaP nanoparticles. Binding affinity of (A) PEG-BP and (B) PEG-IP5 to HA. The binding was carried out in HEPES buffer (pH 7.4, 50 mM) at rt. Free ligand was measured by $^1$H NMR integration of the PEG signal (Figure 3.6). Maximum Binding ($q_m$) and Langmuir constants ($K_L$) were obtained from non-linear curve fitting of a Langmuir adsorption isotherm (eq. 2) to the binding data (Prism 6.0 Software). Data are presented as the means ± SD (n = 3). Size measurement by DLS of particles prepared by (C) method 1 using PEG-BP/IP5 at 5 µM or 10 µM and phosphate at 1.5 mM, reaction time 4 s and (D) method 2 using PEG-BP/IP5 at 30 µM or 40 µM and phosphate at 6.0 mM. Data are presented as the means ± SD (n = 3).
3.3.5. Structure and morphology.

Figure 3.6. PEG calibration curve based on PEG integration of $^1$H NMR (1, 3, 5-Trioxane was used as inner standard).

Figure 3.7. The effects of PEG concentration on the CaP nanoparticles. Particles were prepared by method 1 (phosphate 1.5 mM, PEG-IP5 5-40 µM). Data are presented as the means + SD (n = 3).

We then compared the morphology of the different CaP nanoparticles using scanning electron microscopy (SEM). Particles prepared by method 1 (Figures 3.8A, B) had aggregated spheres morphology, with single spheres measuring about 50 nm in diameter. Considering the size measurement obtained by DLS (about 220 nm), the nanoparticles probably consisted of a small number of aggregated spheres in solution (Figure 3.8E). In contrast, particles prepared by method 2 (Figures 3.8C, D) had elongated and irregular morphology. A single particle measured about 40 nm in width and 100 nm in length, which is consistent with the size obtained by DLS (about 80 nm), suggesting that these nanoparticles existed in suspension as single particles (Figure 3.8F). In general, the nature of the PEGylated chelator did not influence the morphology as the particles prepared with either PEG-IP5 or PEG-BP displayed similar morphology by SEM.
Figure 3.8. Morphology of PEG-BP/IP5 coated CaP nanoparticles. Scanning electron micrographs (SEM) of particles prepared by method 1 with phosphate 1.5 mM and (A) PEG-BP 10 µM (B) PEG-IP5 10 µM or by method 2 with phosphate 6.0 mM and (C) PEG-BP 40 µM (D) PEG-IP5 40 µM. Schematic illustration of the structure of particles prepared by method 1 (E) or method 2 (F), $D_h$ is hydrodynamic diameter.

The morphology of the CaP nanoparticles was also investigated by transmission electron microscopy (TEM), which corroborated the structural information obtained by SEM. In addition, the aggregated particles prepared by method 1 (Figures 3.9A, B) were found to be amorphous CaP by X-ray powder diffraction (XRD) analysis (Figure 3.10). Similar amorphous aggregated spheres structures were also observed in previous reports. Meanwhile, the particles prepared by method 2 (Figures 3.9C, D) were in fact needle bundle-like nanostructures, which were quite similar to the morphology of nanocrystals reported before. This type of particles was studied in more detail as described below.
Figure 3.9. Morphology and morphological transformations of PEG-BP/IP5 coated CaP nanoparticles. Transmission electron micrographs (TEM) of particles prepared by method 1 with phosphate 1.5 mM and (A) PEG-BP 10 µM (B) PEG-IP5 10 µM or by method 2 with phosphate 6.0 mM and (C) PEG-BP 40 µM (D) PEG-IP5 40 µM. Cryo-TEM images of CaP nanoparticles prepared by method 2 after storage times of (E) 20 min and (G) 24 h, and their corresponding low-dose selected-area electron diffraction (LDSAED) pattern (F) and (H), respectively.

Figure 3.10. XRD pattern of the CaP nanoparticles. Particles prepared by method 1 (phosphate 1.5 mM, PEG-IP5, 10 µM) with 0 and 1 day storage respectively. All the peaks belong to NaCl which was come from the buffer used here. No obvious characteristic peaks of hydroxyapatite were observed.

3.3.6. Characterization of the morphology transformation.

We further investigated the transformation of nanoparticles prepared by method 2 that led to a size change from about 40 nm to around 80 nm 24 h after their preparation (Figure 3.5D). Time-resolved DLS size measurements revealed that the change in fact occurred between 4 to 8 h after particle preparation (Figure 3.11). In order to obtain precise morphology and size information of the
particles as they underwent transformation, we used cryogenic TEM (cryo-TEM), an in situ analysis technique, to study the particles at various time points under their native hydrated state. Low dose condition was used to avoid radiation and thermal damage to the frozen specimen. The size of the particles was about 25 nm after 20 min storage and was about 40 nm in width and 100 nm in length after 24 h storage (Figures 3.9E, G), this increase of the size was consistent with the DLS data. Simultaneously, a morphological transformation from sphere assemblies to needle bundles was observed, as shown by the cryo-TEM images at different magnifications (Figure 3.12). The particles obtained after 20 min storage are in fact loose aggregates of nanometer-sized clusters (Figure 3.12 inset). As the precipitation of CaP eventually results in crystalline HA, we further studied the crystallinity of these particles by low-dose selected-area electron diffraction (LDSEAD). Not surprisingly, no crystallinity was detected in the sphere assembly-like sample (20 min) according to the diffuse band in the LDSEAD (Figure 3.9F). The white diffraction spots in the LDSEAD of the needle bundle-like sample (24 h) demonstrated that these CaP nanoparticles were at least partially crystalline (Figure 3.9H). For LDSEAD, a clear diffraction pattern normally requires a high density of nanocrystals in the selected area. Therefore, the relatively weak diffraction observed here was probably due to the low density of particles in the selected area (Figure 3.9G). Interestingly, after a longer one-week storage period a morphological transformation was also observed for the particles prepared by method 1 (Figures 3.13, 3.8A, B), whose spherical shape turned to an elongated shape with irregular surface. This is important for a future in vivo application since the surface and shape of nanoparticles has been linked to distribution, uptake efficiency and circulation time in vivo. Our results suggest that the morphology of CaP particles should be investigated after different time point to have an accurate picture.

![Figure 3.11. Size development of CaP nanoparticles over time. Particles were prepared by Method 2 (phosphate 6.0 mM, PEG-IP5 40 µM), the size of the particles was measured by DLS at fixed timepoints. Data are presented as the means ± SD (n = 3).](image)
Figure 3.12. Cryo-TEM images of CaP nanoparticles with different storage time at lower magnification condition. (A) 20 min. Inset: higher magnification of the marked area. (B) 24 h. Particles prepared by method 2 (phosphate 6.0 mM, PEG-IP5 40 µM).
3.3.7. Particle formation mechanism.

The formation mechanism for CaP particles without PEGylation was widely reported previously, but was rarely investigated for PEGylated CaP particles. Here, a formation mechanism for both types of PEGylated CaP nanoparticles is proposed by integrating the data obtained here with the elegant previous studies (Figure 3.14).

For method 1, pre-nucleation clusters would form first when calcium and phosphate ion solutions are mixed (stage 1a), and then these pre-nucleation clusters lead to loosely aggregated networks by reaction-limited cluster aggregation (RLCA) (stage 1b). In a subsequent step, these networks would transform into densely aggregated spheres by taking up ions from solution, after which they would grow rapidly (stage 1c) until they are stabilized by the PEGylated chelator (stage 1d). These aggregated spheres were also observed in previous literatures and the formation mechanism was described. A morphological transformation would occur after one week of storage, yielding elongated PEGylated aggregated spheres with irregular surface (stage 1e).

For method 2, pre-nucleation clusters would form similarly as described in method 1 (stage 2a) with the difference that the loosely aggregated networks would not be obtained due to the presence
of PEGylated chelators. Instead, only small PEGylated assemblies would form (stage 2b), which are in fact loose aggregates of nanometer-sized clusters, as observed by others during the precipitation of CaP in the presence of poly(aspartic acid).\textsuperscript{214} In a subsequent step, these PEGylated assemblies would transform into PEGylated needle bundles and experience a dramatic increase of size (stage 2c). It is notable that the presence of the PEGylated chelator in the very early stage of CaP precipitation has an impact on the size of particles.

In another experiment, varying the PEG chain length on the chelator (2000 vs. 5000 g/mol) did not influence the size of particles prepared by method 1, but it significantly changed the size of particles prepared by method 2 (Figure 3.15). This phenomenon is due to the fundamental difference between the two methods. For method 1, PEG is not involved in the particle formation process, therefore, PEG length shows no influence on the particle size. In method 2, PEG is present before the formation of particles and involved in the precipitation process, therefore, PEG length influences the particle size.

**Figure 3.14.** Schematic illustration showing the formation mechanism of CaP nanoparticles. Particles prepared by **method 1.** Stage 1a: pre-nucleation clusters. Stage 1b: loosely aggregated networks, formed by reaction-limited cluster aggregation (RLCA). Stage 1c: aggregated spheres. Stage 1d: PEGylated aggregated spheres. Stage 1e: PEGylated aggregated elongated spheres with irregular surface. Particles prepared by **method 2.** Stage 2a: pre-nucleation clusters. Stage 2b: PEGylated sphere assemblies. Stage 2c: PEGylated needle bundles.
Figure 3.15. Effects of PEG length on size of CaP nanoparticles. Particles were prepared by method 1 (phosphate 1.5 mM, PEG-IP5, 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5 40 µM) with PEG length of 2 k and 5 k. Data are presented as the means ± SD (n = 3). *p < 0.001

3.3.8. Stability in biological media and dissolution at acidic pH.

Since PEG-IP5 was found to be a superior stabilizer for CaP nanoparticles compared to PEG-BP, we selected it for further cellular uptake and transfection studies. Thus, the stability of the PEG-IP5-coated CaP nanoparticles was studied by DLS in different biological media. Nanoparticles prepared by both methods were stable in cell culture medium and 50% fetal bovine serum (FBS) solution for at least 6 h (Figure 3.16). In view of their application as transfection agents, we mimicked the acidic environment present in early endosomes and studied the particles stability. Upon acidification of the suspension of particles in FBS solution to pH 6.5, the size of the particles for method 1 dropped to ~20 nm (the size of serum proteins), while the size of the particles for method 2 decreased to ~30 nm, which indicated some dissolution of the CaP nanoparticles.61
Figure 3.16. Stability of CaP nanoparticles over time in different media. Particles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 10 µM) and by method 2 (phosphate 6.0 mM, PEG-IP5 40 µM) were diluted 1:7 (v/v) in cell medium and FBS/PBS at 37 °C. Particles in FBS/PBS were acidified to pH 6.5 at timepoint 6 h. Data are presented as the means ± SD (n = 3).

3.3.9. Cellular uptake.

To study their cellular uptake, PEG-IP5-stabilized CaP nanoparticles were loaded with fluorescently-labeled DNA and incubated for 4 h with HepG2 cells in Opti-MEM medium containing 10% FBS, after which the cells were washed twice with PBS and collected for their analysis by flow cytometry. Since the preparation of CaP nanoparticles by method 1 was time-dependent, we first investigated the effect of reaction time of Ca$^{2+}$ and PO$_4^{3-}$ (1 s, 4 s, 7 s) on the cellular uptake (vide infra). The latter increased as the reaction time was prolonged (Figure 3.17A), which was probably a consequence of the increasing particle size (150, 220, 290 nm) (Figure 3.3) that in turn augmented the sedimentation on the cell surface and ultimately facilitated their uptake. In addition, the size increase might result in decreasing PEG density on the surface of the particles since the total PEG amount is fixed. This variation in surface chemistry could be another reason for different uptake efficiency. We then studied the effect of the two preparation methods (Figure 3.17B). For particles prepared by method 1, the uptake was quite high and comparable to lipofectamine. In fact, only about 50% of the lipofectamine-treated cells internalized particles, while more than 80% of the cells incubated with CaP nanoparticles from method 1 contained particles (Figure 3.18). For particles prepared by method 2 however, the uptake was much lower, which may be associated with their smaller size and higher PEGylation density. As expected, the uptake of these particles was found to be DNA dose-dependent (Figure 3.19). To further understand the difference in uptake efficiency between these two sets of particles and specifically analyze the potential contribution of particle sedimentation, we carried out the experiments also in inverted cell culture conditions. For particles prepared by method 1, the uptake efficiency decreased dramatically under inverted conditions, which indicated that this type of particles had a strong sedimentation propensity that contributed significantly to their uptake efficiency (Figure 3.17C), as previously observed with a number of different particulate-based transfection agents. In contrast, the uptake efficiency of particles prepared by method 2 was similar under upright and inverted conditions, indicating the effect of sedimentation was probably negligible in this case. We then investigated the impact of the morphological changes that occur after 1 week and 8 h in particles prepared by methods 1 and 2, respectively, on their uptake efficiency. For all the particles tested, the uptake remained unaffected by the morphological transformation, and even after 7 days of storage the nanoparticles could be taken up by the cells as efficiently as the freshly prepared ones (Figure 3.17D).
Figure 3.17. Cellular uptake of PEG-IP5 stabilized fluorescently-labeled DNA/CaP nanoparticles by HepG2 cells. The mean fluorescence intensity (MFI) was determined by flow cytometry after 4 h incubation, the presented values were normalized to control cells (Normalized MFI). 0.5 µg DNA per well was used based on feeding amount. (A) Uptake of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM and PEG-IP5 10 µM) as a function of reaction time of Ca$^{2+}$ and PO$_4^{3-}$. (B) Uptake of CaP nanoparticles prepared by method 1 (PEG-IP5 5 µM or 10 µM, reaction time 4 s) and method 2 (phosphate 6.0 mM, PEG-IP5 30 µM or 40 µM), lipofectamine was used as a positive control. (C) Uptake of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 10 µM, reaction time 4 s) and method 2 (phosphate 6.0 mM, PEG-IP5 40 µM), incubated with cells in upright or inverted conditions. (D) Uptake of CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 10 µM, reaction time 4 s) or method 2 (phosphate 6.0 mM, PEG-IP5 40 µM) and stored at rt for different periods of time prior to the cell experiments. Data are presented as means + SD (n = 3). *p < 0.05.
Figure 3.18. Uptake of fluorescently-labeled DNA/CaP nanoparticles by HepG2 cells after an incubation time of 4 h as determined by flow cytometry. Particles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 5 µM or 10 µM, reaction time 4 s) and method 2 (phosphate 6.0 mM, PEG-IP5 30 µM or 40 µM), lipofectamine was used as a positive control. (A) Flow cytometry histogram of cells treated with CaP nanoparticles and lipofectamine, (B) Uptake shown as percentage of DNA containing cells. Data are presented as means ± SD (n = 3). *p < 0.01.
Chapter 3 - CaP nanoparticles for gene delivery

Figure 3.19. Uptake of fluorescently-labeled DNA/CaP nanoparticles by HepG2 cells after an incubation time of 4 h as determined by flow cytometry. CaP nanoparticles prepared by method 2 (phosphate 6.0 mM, PEG-IP5 40 µM) containing 0.5 µg DNA/well and 1.0 µg DNA/well. (A) Flow cytometry histogram of cells treated with CaP nanoparticles (B) Uptake shown as normalized mean fluorescence intensity (vs. control). Data are presented as means ± SD (n = 3). *p < 0.03.

3.3.10. In vitro transfection in HepG2 cells.

Enhanced Green Fluorescent Protein plasmid (pEGFP-DNA) was loaded in the two sets of PEG-IP5-stabilized CaP nanoparticles with a final DNA concentration of 6.66 µg/mL. Compared to blank particles, the incorporation of pEGFP-DNA increased the particles size from 193 ± 3 nm to 257 ± 8 nm and 40 ± 1 nm to 82 ± 1 nm for the set of particles prepared by method 1 and 2 respectively (Table 3.2). The zeta potential of the nanoparticles of method 2 was -5.4 ± 2.2 mV, and was closer to neutral than the nanoparticles prepared by method 1 (-11.2 ± 1.5 mV), which could correspond to a higher PEGylation density in the former.
Table 3.2. Size and zeta potential of pEGFP-DNA loaded CaP nanoparticles.

<table>
<thead>
<tr>
<th>Method</th>
<th>PEG-IP5 (µM)</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>254 ± 10</td>
<td>-14.6 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>257 ± 8</td>
<td>-11.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>84 ± 3</td>
<td>-3.4 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>82 ± 1</td>
<td>-5.4 ± 2.2</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SD (n = 3).

The transfection ability of the PEG-IP5-stabilized CaP nanoparticles prepared by both methods was examined in HepG2 cells by flow cytometry. Cells were incubated for 5 h with pEGFP-DNA loaded particles in FBS-supplemented Opti-MEM medium, washed and further incubated for 48 h in fresh, particle-free, FBS-supplemented minimal essential medium (MEM) to allow for EGFP expression. The transfection efficiency of particles prepared by method 1 with either 5 or 10 µM of PEG was similar, and increasing the DNA amounts from 0.5 µg/well to 2.0 µg/well did not lead to a significant enhancement of the transfection efficiency (Figure 3.20). The transfection efficiency of particles prepared by method 2 was quite low in all conditions tested, which was expected given their lower cellular internalization. For particles prepared by this method, a targeting agent could be used to increase specific uptake in the future.
Figure 3.20. Transfection of pEGFP-DNA/CaP nanoparticles in HepG2 cells. Cells were incubated with the particles for 5 h, after which they were incubated further for 48 h in fresh particle-free medium to allow gene expression. Transfection efficiency was determined as the percentage of GFP-positive cells within the total of cells analyzed by flow cytometry upon treatment with CaP nanoparticles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 5 µM or 10 µM), method 2 (phosphate 6.0 mM, PEG-IP5 30 µM or 40 µM) or lipofectamine which was used as a positive control. Two different amounts of pEGFP-DNA were tested in each case (0.5 µg or 2.0 µg per well based on feeding amount). Data are presented as means + SD (n = 3). *p < 0.05.

Finally, the toxicity of PEG-IP5 coated CaP nanoparticles was assessed on HepG2 cells using the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) assay. None of the formulations of CaP nanoparticles tested induced any detectable toxicity to the cells, probably due to the generally non-toxic nature of CaP (Figure 3.21).
Figure 3.21. Cytotoxicity of EGFP-DNA/CaP nanoparticles in HepG2 cells. Particles were removed after 5 h incubation with the cells and the cells were incubated for 48 h in fresh complete medium. Particles prepared by method 1 (phosphate 1.5 mM, PEG-IP5 10 µM) and method 2 (phosphate 6.0 mM, PEG-IP5 40 µM), lipofectamine was used as a positive control, DNA concentration corresponding the concentration used in transfection assay (0.5 µg/mL). Data are presented as means ± SD (n = 3)

3.4. Conclusion

This study provided a better understanding of the characteristics of PEGylated CaP nanoparticles that influence their in vitro uptake and transfection efficiency. We demonstrated that inositol phosphate-based chelator PEG-IP5 was a better stabilization agent than a BP-based chelator PEG-BP. The morphology, size and crystallinity were intimately linked to the particle preparation method, but only some of these properties had an influence on the uptake in vitro, and hence the transfection efficiency. It remains to be investigated what impact the morphology and crystallinity could have in vivo. Furthermore, we demonstrated again that in vitro uptake can be artificially enhanced by particle sedimentation, a property that would not be desired for an eventual in vivo application.
Chapter 4

4. Preparation of Polyion Complex Micelles for the Hepatocytes-Targeted Delivery of Nucleic Acids
4.1. Introduction

As described in chapter 3, CaP nanoparticles smaller than 100 nm could be obtained by method 2. However, the uptake of these particles was relatively low probably due to a high PEG density. One strategy to solve this problem is to decorate the particles with a targeting ligand. Therefore, a well-defined ligand against ASGP-R (T-GalNAc) was designed and synthesized according to the principles described in chapter 2 (Figure 4.1). The T-GalNAc was coupled to amino-PEG-IP5 to produce T-GalNAc-CaP nanoparticles (Figure 4.2). The specific uptake of T-GalNAc-CaP nanoparticles was then evaluated on HepG2 cells.

![Figure 4.1](image1)

**Figure 4.1.** Schematic illustrations showing the structure and spatial geometry of the trivalent ligand T-GalNAc. The spatial geometry was obtained using the ChemBio3D 12.0 software, with which the extend length of the linker was estimated through an MM2 molecular mechanics energy minimization and the angle between the two linkers was obtained. The distance between sugars was then calculated by applying the cosine rule to triangles.

In addition, the newly synthesized targeting ligand was also evaluated using a different type of nanoparticles. PICMs have been previously used as carriers and are typically prepared from cooperative electrostatic interactions between the nucleic acid material and a cationic diblock copolymer.73, 219 The Leroux group previously reported pH-responsive ternary PICMs prepared from the diblock copolymer PEG-b-poly(propyl methacrylate-co-methacrylic acid) (PEG-b-P(PrMA-co-MAA)) and poly(amido amine) (PAMAM) dendrimers, which could encapsulate AON and siRNA and protect them from degradation. These PICMs were further functionalized with fragment antigen-binding (Fab′) for specific targeting via the transferrin receptor, and efficient silencing effect was achieved in vitro in PC-3 cells.220, 221 Here, the T-GalNAc was coupled to amino-PEG-b-P(PrMA-co-MAA) to produce T-GalNAc-PICMs (Figure 4.2). The specific uptake of T-GalNAc-PICMs was then evaluated on HepG2 cells.
Figure 4.2. Schematic illustrations showing the formation of T-GalNAc-CaP nanoparticles and T-GalNAc-PICMs.

4.2. Materials and Methods

4.2.1. Materials

Heterobifunctional PEG (HO-PEG\textsubscript{169}-NH\textsubscript{2}, \(M_n = 7500\) g/mol) was obtained from Jenkem Technology (Beijing, China). Methoxy-PEG\textsubscript{115}-OH (\(M_n = 5000\) g/mol), PAMAM dendrimers (G5) and accutase solution were purchased from Sigma Aldrich (Buchs, Switzerland). Propyl methacrylate (PrMA), tert-butyl methacrylate (tBMA) and triethylamine (TEA) were obtained from ABCR-Chemicals (Karlsruhe, Germany) and were distilled before use. All other chemicals were purchased from Sigma Aldrich. HPLC column (XBridge Prep BEH130 C18 5µm) was purchased from Waters Assoc. (Milford, MA). HepG2 (hepatocellular carcinoma) cell line was purchased from ATCC (American Type Culture Collection) (Manassas, VA). Dulbecco’s Modified Eagle Medium (DMEM, low glucose, GlutaMAX), penicillin/streptomycin, fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin-EDTA, and Lipofectamine\textsuperscript{TM} were bought from Thermo Fisher Scientific (Carlsbad, CA). CellTiter\textsuperscript{®} 96 Aqueous Non-Radioactive Cell Proliferation Assay was obtained from Promega (Dübendorf, Switzerland). Cy3-bcl-2-siRNA (sense strand 5’-(Cy3)GCA UGC GGC CUC
UGU UUG A UU-3’, antisense 3’-UU CGU ACG CCG GAG ACA AAC U-5’) was obtained from Microsynth AG (Balgach, Switzerland).

4.2.2. Analysis equipment

NMR spectra were acquired on a Bruker AV400 spectrometer (Bruker BioSpin, Fällanden, Switzerland) operating at 400 MHz for protons. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) was performed using Bruker’s UltraFlex II system with Compass 1.5 control software.

4.2.3. Methods

4.2.3.1. Synthesis of T-GalNAc-NHS

4.2.3.1.1. Synthesis of compound 4.2

A solution of tetrafluorophenyl trifluoroacetate (TFP-OTFA) (70 μL, 0.424 mmol) in anhydrous DMF (1.0 mL) was added dropwise to a solution of tetraacid (4.1) (60.0 mg, 0.141 mmol) and TEA (59.0 μL, 0.424 mmol) in anhydrous DMF (1.0 mL) at 0 °C. The mixture was stirred in an ice bath for 1 h. The solvent was removed under high vacuum, and then the crude was purified by flash column chromatography (silica gel, ACN/DCM 0 to 1:9, with 0.5% AcOH). Compound 4.2 (30.8 mg, 25% yield) was isolated as pale green oil. \( R_f = 0.45 \) (ACN/DCM 1:9, with 0.5% AcOH); \(^1\)H NMR (400 MHz, CDCl\(_3\)), \( \delta \): 6.99 (tt, \( J = 9.9, 7.1 \) Hz, 3H, H-C3), 3.78 (t, \( J = 6.0 \) Hz, 6H, H-C9), 3.67 (t, \( J = 6.0 \) Hz, 2H, H-C13), 3.47 (d, \( J = 4.4 \) Hz, 8H, H-C12 and H-C10), 2.90 (t, \( J = 6.0 \) Hz, 6H, H-C8), 2.61 (t, \( J = 6.0 \) Hz, 2H, H-C14). \(^13\)C NMR (101 MHz, CDCl\(_3\)), \( \delta \): 174.48 (C15), 167.64 (C7), 147.61-146.82 (C2), 145.17-144.32 (C4), 142.17-141.47 (C5), 139.66-139.00 (C6), 103.43, 103.20, 102.98 (C1 and C3), 70.18 (C12), 69.80 (C10), 66.31 (C13), 66.16 (C9), 45.09 (C11), 34.58 (C14), 34.34 (C8). ESI/MALDI-MS: \( m/z \) [M+Na]+ calcd 891.1281, found 891.1282.

4.2.3.1.2. Synthesis of compound 4.4

Carboxybenzyl chloride (CbzCl) (1.14 mL, 7.99 mmol), 4-(dimethylamino) pyridine (DMAP) (32.5 mg, 0.266 mmol) and TEA (1.07 mL, 7.72 mmol) were added to a solution of 3-aminopropan-1-ol (4.3) (0.5 g, 6.66 mmol) in 1,4-dioxane/water (2:1, 7.5 mL). The reaction mixture was stirred overnight at rt. The solvents were removed under reduced pressure to give a thick solution, which was diluted with DCM (7.5 mL). The solution was washed with 1.0 M solution of HCl (4.0 mL) and the
aqueous layer was back-extracted with DCM (2 × 7.5 mL). The pooled organic layers were dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The residue was further purified by silica gel column chromatography (EtOAc, $R_f = 0.50$). Compound 4.4 (0.42 g, 30.2% yield) was isolated as white crystals. NMR spectrum is identical to that reported in literature.$^{222}$ $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.31-7.24 (m, 5H, ph), 5.04 (s, 2H, OCH$_2$Ph), 4.93 (s, 1H, NH), 3.61 (dd, $J = 11.6, 5.9$ Hz, 2H, H-C1), 3.30 (dd, $J = 12.4, 6.2$ Hz, 2H, H-C3), 2.40 (t, $J = 5.9$ Hz, 1H, OH), 1.68-1.59 (m, 2H, H-C2).

4.2.3.1.3. Synthesis of compound 4.6

![Diagram of compound 4.6]

D-galactosamine pentaacetate (2.0 g, 5.14 mmol) and compound 4.4 (1.182 g, 5.65 mmol) were dissolved in 20 mL anhydrous DCM under argon. The mixture was warmed to a gentle reflux at 40 °C for 24 h after the addition of trifluoromethanesulfonic acid (TfOH) at rt. After cooling, the mixture was diluted by DCM and washed once with saturated aq. NaHCO$_3$, dried with anhydrous Na$_2$SO$_4$, concentrated and purified by column chromatography (EtOAc) to obtain compound 4.6 (1.934 g, 69.9% yield) as a white powder. $R_f = 0.45$ (EtOAc); $^1$H NMR (400 MHz, CDCl$_3$), $\delta$: 7.46-7.29 (m, 5H, Ph), 6.25 (d, $J = 8.7$ Hz, 1H, NHAc), 5.31 (d, $J = 2.8$ Hz, 1H, H-C4), 5.10 (q, $J = 12.3$ Hz, 2H, Bn), 4.99 (dd, $J = 11.0, 3.1$ Hz, 2H, H-C3 and NHCbz), 4.33 (d, $J = 8.5$ Hz, 1H, H-C1), 4.23-4.05 (m, 3H, H-C2, H-C6a and H-C6b), 4.01-3.92 (m, 1H, H-C7a), 3.77 (t, $J = 6.6$ Hz, 1H, H-C5), 3.63-3.48 (m, 1H, H-C9a), 3.40 (dd, $J = 9.9, 7.0$ Hz, 1H, H-C7b), 3.08 (m, 1H, H-C9b), 2.14, 20.5, 2.00 (3 × s, 9H, CH$_3$, OAc), 1.94 (s, 3H, CH$_3$, NHAc), 1.81 (ddd, $J = 14.4, 9.5, 4.6$ Hz, 1H, H-C8a), 1.64 (d, $J = 15.3$ Hz, 1H, H-C8b). $^{13}$C NMR (101 MHz, CDCl$_3$), $\delta$: 170.77, 170.66, 170.42, 170.33 (C, O), 128.60, 128.22, 128.14 (Ph), 101.85 (C1), 71.06, 70.72 (C3, C5), 66.85, 66.73 (double intensity) (C4, Bn, C7), 61.49 (C6), 50.79 (C2), 37.12 (C9), 29.85 (C8), 23.30 (CH$_3$, NHAc), 20.80-20.66 (CH$_3$, OAc). ESI-MS: $m/z$ [M+H]$^+$ calcld 539.2235, found 539.2230.

4.2.3.1.4. Synthesis of compound 4.7

![Diagram of compound 4.7]

A solution of compound 4.6 (500 mg, 0.928 mmol) in MeOH (15 mL) was treated with sodium methoxide (MeONa) (2.5 mg, 46 μmol) at rt for 6 h. The mixture was then neutralized by passing through an acid resin column (Dowex, 50WX8, H$^+$), filtered and concentrated. Compound 4.7 (314 mg, 82% yield) was obtained. $^1$H NMR (400 MHz, D$_2$O), $\delta$: 7.55-7.34 (m, 5H, Ph), 5.13 (s, 2H, Bn), 4.39 (d, $J = 8.5$ Hz, 1H, H-C1), 3.98-3.83 (m, 3H, H-C4, H-C7a and H-C2), 3.82-3.74 (m, 2H, H-
Chapter 4 - T-GalNAc-PICMs for hepatocytes targeting

C6a and H-C6b), 3.72-3.57 (m, 3H, H-C3, H-C5 and H-C7b), 3.27-3.10 (m, 2H, H-C9), 2.03 (s, 3H, CH3, Ac), 1.76 (p, J = 6.6 Hz, 2H, H-C8). 13C NMR (101 MHz, D2O), δ: 174.97-174.50 (CO), 128.77, 128.33, 127.63 (Ph), 101.62 (C1), 75.06 (C5), 71.01 (C3), 67.78, 67.51, 66.78 (C4, Bn and C7), 60.94 (C6), 52.41 (C2), 37.20 (C9), 28.75 (C8), 22.14 (CH3, Ac). 

ESI-MS: m/z [M+Na]+ calcd 435.1738, found 435.1740.

4.2.3.1.5. Synthesis of compound 4.8

Compound 4.7 (314 mg, 0.761 mmol) was dissolved in MeOH (4 mL) and H2O (6 mL) and set under inert atmosphere. Excess Pd/C was added and the reaction mixture was purged with H2 and stirred at rt for 16 h. The crude was filtered over a small celite plug. After the removal of the solvent, compound 4.8 (207 mg, 98% yield) was obtained. 1H NMR (400 MHz, D2O), δ: 4.44 (d, J = 8.4 Hz, 1H, H-C1), 4.08-3.98 (m, 1H, H-C7a), 3.96–3.86 (m, 2H, H-C4, H-C2), 3.84-3.76 (m, 2H, H-C6a and H-C6b), 3.71 (ddd, J = 11.2, 9.2, 4.0 Hz, 3H, H-C3, H-C7b and H-C5), 3.00 (t, J = 6.9 Hz, 2H, H-C9), 2.05 (s, 3H, CH3, Ac), 1.94-1.84 (m, 2H, H-C8). 13C NMR (101 MHz, D2O), δ: 174.93 (CO), 101.71 (C1), 75.09 (C5), 70.80 (C3), 68.02, 67.72 (C4 and C7), 61.00 (C6), 52.38 (C2), 37.69 (C9), 27.76 (C8), 22.16 (CH3, Ac). ESI-MS: m/z [M+H]+ calcd 279.1551, found 279.1557.

4.2.3.1.6. Synthesis of compound 4.9

A solution, containing compound 4.8 (42.6 mg, 0.109 mmol), TEA (38 µL, 0.272 mmol), and anhydrous DMF (0.85 mL), was added dropwise to a solution of compound 4.2 (23.6 mg, 0.027 mmol) in anhydrous DMF (0.85 mL). The reaction was stirred at rt overnight. The solvent was removed under high vacuum, and then the crude product was dissolved in water and purified by reverse phase HPLC (A: ACN, B: water, A: 0% => 10%). Compound 4.9 (17.3 mg, 53%) was obtained as a lyophilizate. 1H NMR (400 MHz, D2O), δ: 4.46 (d, J = 8.4 Hz, 3H, H-C1), 3.99-3.86 (m, 2H, H-C4, H-C7a and H-C2), 3.85-3.77 (m, 6H, H-C6a and H-C6b), 3.76-3.61 (m, 17H, H-C15, H-C11, H-C5 and H-C7b), 3.38 (d, J = 6.9 Hz, 8H, H-C14 and H-C12), 3.25 (ddd, J = 28.7, 13.6,
4.2.3.1.7. Synthesis of compound 4.10

To a solution of compound 4.9 (15 mg, 12 µmol) in 0.5 mL of DMF, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI) (14.3 mg, 75 µmol) and N-hydroxysuccinimide (NHS) (8.59 mg, 75 µmol) were added. The reaction mixture was stirred at rt for 24 h and HPLC showed the reaction was completed. The crude was precipitated in Et2O (14 mL) once, dried by N2 flow and high vacuum. The crude was then dissolved in 0.5 mL of DMF and purified by reverse phase HPLC (A: ACN, B: water, A: 0% => 10%). Compound 4.10 (3.4 mg, 21% yield) was obtained as a lyophilizate. The product was confirmed by 1H NMR and was used directly for the next step without further characterization. Selected 1H NMR (400 MHz, D2O), δ: 2.88 (s, 4H, H-C1).

4.2.3.2. Synthesis of T-GalNAc-PEG-IP5 (4.17)

4.2.3.2.1. Synthesis of compound 4.12

DMAP (1.25 mg, 10.27 µmol), CbzCl (44.0 µL, 0.30 mmol) and TEA (41.0 µL, 0.298 mmol) were added to a solution of N3-PEG5k-NH2-TFA (0.5 g, 5.61 mmol) in 1,4-dioxane/water (2:1, 7.5 mL), and the reaction mixture was stirred overnight at rt. The solution was concentrated and insoluble salts were removed by filtration through glass wool, followed by precipitation in Et2O. The precipitate was collected by filtration and dried under vacuum, after which, it was precipitated three times in 400 mL of EtOH (absolute). For this purpose, the precipitate was warmed to 37 °C until dissolved, cooled in ice-water bath to precipitate, and finally collected by centrifugation at 1 °C. The precipitate was then dissolved in DCM and re-precipitated twice in cold Et2O and dried under vacuum. Compound
4.12 (17.37 g, 35%) was isolated as white solid powder. \[^1^H\text{NMR}\ (400\ \text{MHz, CDCl}_3) \delta \ 7.42-7.32\ (\text{m, 5H}),\ 5.12\ (\text{s, 2H, OCH}_2\text{Ph}),\ 3.66\ (\text{s, 443H, PEG}),\ 3.41\ (\text{s, 4H, Cbz-CH}_3,\ CH_2-N_3)\].

4.2.3.2.2. Synthesis of compound 4.15

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\text{Compound 4.14 (32 mg, 0.028 mmol) and compound 4.12 (95 mg, 0.019 mmol) were dissolved in 400 µL DCM. CuI (0.144 mg, 0.757 µmol), N,N-diisopropylethylamine (DIPEA) (1.321 µL, 7.57 µmol) in 100 µL DCM and Acetic acid (AcOH) (0.433 µL, 7.57 µmol) in 100 µL DCM were added to the solution. The reaction mixture was stirred at rt for 20 h under inert atmosphere. After purification by flash column chromatography (silica gel, MeOH/DCM 1:19 \rightarrow 1:4), compound 4.15 (104 mg, 90% yield) was isolated.}^{1}\text{H NMR (400 MHz, CDCl}_3-d,d) \delta: 7.89\ (s, 1H, H-C9), 7.41-7.20\ (m, 25H, Ph), 5.61-4.91\ (m, 28H, H-C4, H-C5, H-C6, H-C7 and Bn), 4.80-4.67\ (m, 3H, H-C3, H-C2, H-C1), 4.40\ (t, J = 5.5 Hz, 2H, H-C10), 4.39-3.42\ (m, H-C11 and PEG), 3.40-3.32\ (m, 2H, CH\_2NHCbz).}^{31}\text{P NMR (162 MHz, CDCl}_3-d,d) \delta: -1.34, -4.27, -4.58.}\text{MALDI-TOF-MS: Centered in m/z = 5942 g/mol (NH\_2-PEG5k-Azide had a m/z = 4890 g/mol) was found.}}\text{Synthesis of compound 4.16}

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\text{Compound 4.15 (104 mg, 0.017 mmol) was dissolved in MeOH (8.0 mL) and H\_2O (2.67 mL) and set under inert atmosphere. Excess Pd/C was added and the reaction mixture was purged with H\_2. It was stirred at rt for 16 h and then quenched with 1.0 M NaOH until a slightly basic solution was obtained. The crude was filtered over a small amount of celite in a syringe and concentrated to 1.0 mL of solvent and was directly loaded on a PD-10 column. After lyophilization, the product was passed through a resin column (Dowex, 50WX8, H\textsuperscript{+}) and was neutralized with NH\textsubscript{4}OH\textsubscript{aq} and concentrated. Compound 4.16 (70 mg, 70% yield) was obtained.}^{1}\text{H NMR (400 MHz, D}_2\text{O), \delta: 8.27\ (s, 1H, H-C9), 5.06\ (s, 2H, H-C7), 4.67\ (t, J = 5.3 Hz, 2H, H-C10), 4.48-4.36\ (m, 3H, H-C1, H-C3 and H-C5 ), 4.10-4.27\ (m, 3H, H-C2, H-C4 and H-C6), 4.03\ (t, J = 5.3 Hz, 2H, H-C11), 3.84-3.59\ (m, PEG), 3.23\ (s, 2H, CH\textsubscript{2}NH\textsubscript{2}).}^{31}\text{P NMR (162 MHz, D}_2\text{O), \delta: 1.48, 0.98, -0.03.}\text{MALDI-TOF-MS: Centered in m/z = 5535 g/mol was found.}}

74
4.2.3.2.4. Synthesis of T-GalNAc-PEG-IP5 (4.17)

![Chemical Structure](image)

Compound 4.9 (12.6 mg, 10.45 μmol), compound 4.16 (24.5 mg, 4.27 μmol) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (3.25 mg, 8.54 μmol) were dissolved in an ACN/DMF/H2O (960 μL, 12.5:4.5:1) in a 5 mL flask followed by the addition of DIPEA (11.2 μL, 0.064 mmol). The reaction mixture was stirred at rt for 24 h, after which most of the solvent was removed under high vacuum, re-dissolved in H2O and lyophilized. Subsequently, the crude product was purified by size exclusion column PD-10 (G-25) and ultrafiltration unit Vivaspin 20 (cutoff 5000 g/mol), then passed through a resin column (Dowex, 50WX8, H+) and the solution was basified by NH4OH followed by the removal of solvent under rotovap. The solid was re-dissolved in H2O and lyophilized to give T-GalNAc-PEG-IP5 (4.17) as a white powder (18.8 mg, 64% yield). $^1$H NMR (400 MHz, D$_2$O), δ: 8.25 (s, 1H, H-C1), 5.04 (d, J = 4.8 Hz, 2H, H-C20), 4.66 (t, J = 5.2 Hz, 2H, H18), 4.56-4.38 (m, 6H, H-C2’, H-C4’, H-C6’, 3H-C1), 4.34-4.12 (m, 3H, H-C1’, H-C3’, H-C5’), 4.02 (t, J = 5.2 Hz, 2H, H-C17), 3.97-3.50 (m, 3 × H-C4, 3 × H-C7a, 3 × H-C2, 3 × H-C6a, 3 × H-C6b, 3 × H-C3, H-C15, 3 × H-C11, 3 × H-C5, 3 × H-C7b, PEG), 3.42 (t, J = 5.5 Hz, 2H, NHCH2PEG), 3.36 (s, 8H, 3 × H-C12, H-C14), 3.32-3.15 (m, 6H, 3 × H-C9), 2.49 (dt, J = 11.7, 5.9 Hz, 8H, 3 × H-C10, H-C16), 2.05 (s, 9H, CH3, Ac), 1.85-1.73 (m, 6H, 3 × H-C8). $^{31}$P NMR (162 MHz, D$_2$O), δ: δ -0.08 (s), 0.37 (s), -0.37 (s). MALDI-MS: Centered in m/z = 5869 g/mol was found.

4.2.3.3. Synthesis of T-GalNAc-PEG$_{169}$b-P(PrMA$_{17}$-co-MAA$_{47}$) (4.19)

4.2.3.3.1. Synthesis of non-targeted copolymer

![Chemical Structure](image)
The non-targeted copolymer mPEG$_{113}$-b-P(PrMA$_{24}$-co-MAA$_{56}$) was synthesized by the reported procedures.$^{220}$ $^1$H NMR (400 MHz, D$_2$O), δ: 4.10-3.92 (m, OCH$_2$ in PrMA), 3.84-3.61 (m, PEG), 2.23-1.49 (br, CH$_2$ in the PrMA and polymer backbone), 1.30-0.67 (br, CH$_3$ in PrMA and MAA).

4.2.3.3.2. Synthesis of compound 4.18

![Chemical Structure](image)

The NH$_2$-PEG$_{169}$-b-P(PrMA$_{17}$-co-MAA$_{47}$) (4.18) was obtained by the reported procedures.$^{220}$ $^1$H NMR (400 MHz, D$_2$O), δ: 4.07-3.92 (m, OCH$_2$ in PrMA), 3.84-3.62 (m, PEG), 2.21-1.61 (br, CH$_2$ in the PrMA and polymer backbone), 1.27-0.74 (br, CH$_3$ in PrMA and MAA).

4.2.3.3.3. Synthesis of compound 4.19

![Chemical Structure](image)

To a solution of the activated ester compound 4.10 (2.6 mg, 2.0 µmol) in 150 µL of mixed solvent DMF/H$_2$O (4/1), amine-copolymer compound 4.18 (10.5 mg, 0.67 µmol) was added, followed by the addition of 15 µL of TEA. The reaction mixture was stirred at rt for 24 h and purified by Amicon ultrafiltration (cutoff 10 k, 15 mL). Compound 4.19 (6.6 mg, 59% yield) was obtained as a lyophilizate. The final ligand content of the polymer was 69% as determined by $^1$H NMR spectroscopy. Selected $^1$H NMR (400 MHz, D$_2$O), δ: 3.74-3.51 (m, 678 H, PEG), 3.24-3.06 (m, 6H, H-C1).

4.2.3.3.4. Synthesis of compound 4.20

![Chemical Structure](image)
To a solution of the activated ester \( N \)-succinimidyl acetate (0.9 mg, 5.8 µmol) in 150 µL of mixed solvent DMF/H2O (4/1), amine-copolymer compound 4.18 (9.2 mg, 0.58 µmol) was added, followed by the addition of 15 µL of TEA. The reaction mixture was stirred at rt for 24 h and purified by Amicon ultrafiltration (cutoff 10 k, 15 mL). Compound 4.20 (2.9 mg, 31% yield) was obtained as a lyophilizate. Large excess of \( N \)-succinimidyl acetate was used to ensure the full conversion of amine groups. The product was used directly for micelle preparation without further characterization.

4.2.3.4. NMR spectroscopy analysis and molecular weight determination

\(^1\)H NMR spectra were obtained by a Bruker Av400 spectrometer (Bruker BioSpin, Fällanden, Switzerland). Gel permeation chromatography (GPC) measurements were performed in Tris-HCl buffer (pH 8.0, 10 mM), using a Viscotek TDAmax system (Viscotek, Houston, TX) equipped with a differential refractive index and light scattering detectors.

4.2.3.5. Preparation of CaP nanoparticles

Fluorescently-labeled DNA/CaP nanoparticles were prepared by method 2 as described in chapter 3. Briefly, 150 µL of HEPES buffer pH 7.4 (140 mM NaCl, 6.0 mM Na₂HPO₄ and 50 mM HEPES) were mixed with 150 µL of 30 µM chelator solution (T-GalNAc-PEG-IP5/PEG-IP5) in 10 mM Tris pH 7.4. Subsequently, then 150 µL of CaCl₂ solution (250 mM CaCl₂) containing DNA were added and the mixture was vortexed for 15 s to produce the final CaP nanoparticles. The feeding amounts of T-Gal-PEG-IP5 were 10 and 30 mol%.

4.2.3.6. Preparation of PICMs

PICMs were prepared according to a previously reported protocol. Briefly, negatively charged micelle components PEG\(_{113}\)-b-P(PrMA\(_{24}\)-co-MAA\(_{56}\)), T-GalNAc-PEG\(_{169}\)-b-P(PrMA\(_{17}\)-co-MAA\(_{47}\)) (40 mol%) and siRNA were combined in Tris buffer and mixed with PAMAM dendrimer at increasing N/(P+COOH) molar ratios. N corresponds to the number of primary amine groups of the PAMAM, while P and COOH account for the phosphate and carboxylate groups of the nucleic acid and MAA copolymer, respectively. The samples were gently stirred for 20 min at rt to allow micelle formation. PICMs were prepared with the concentration of MAA copolymer of 0.5 mg/mL, which was diluted to 0.1 mg/mL for further experiments.

4.2.3.7. Particle size and zeta potential determination

Size and zeta potential measurements were performed by dynamic light scattering (DLS) and Doppler laser anemometry, respectively, using a DelsaNano C Particle Analyzer (Beckman Coulter, Krefeld, Germany). All the size measurements were carried out at a fixed scattering angle of 90°. The CONTIN method included in the software of the instrument was used to calculate the intensity-average hydrodynamic diameter of the nanoparticles.
4.2.3.8. Stability of PICMs in different media and pH

The stability of PICMs in different media was monitored by DLS for 5 h at 37 °C. The size and scattering intensity of PICMs were evaluated in cell culture medium Dulbecco's Modified Eagle Medium GlutaMAX low glucose (DMEM) with 2% BSA and a solution of PBS and fetal bovine serum (FBS) (1:1, v/v) after dilution of the samples (1:4, v/v). After 5 h, the suspension in PBS/FBS was acidified to pH 5.5 (to mimic the slightly acidic environment of the late endosomes\textsuperscript{205}) with 1 N HCl, and the size and scattering intensity were determined by DLS at 37 °C.

4.2.3.9. Cell culture

HepG2 cells were cultured in DMEM supplemented with 10% FBS (complete medium) and 1% penicillin-streptomycin at 37 °C in humidified environment of 5% CO\textsubscript{2}. Cells were subcultured every 3 days and were allowed to reach 80%-90% confluence before harvesting them for uptake and transfection experiments. Cells were confirmed to be free of mycoplasma (MycoAlert, Lonza, Rockland, ME).

4.2.3.10. Cell uptake studies of CaP nanoparticles

The uptake of different CaP nanoparticles formulations was assessed in HepG2 cells. The cells were seeded at a density of \(1.5 \times 10^5\) cells/well into 24-well plates 24 h before uptake. The medium was replaced by fresh Opti-MEM medium containing 10% FBS, the CaP nanoparticles were then added to the cells at a final DNA concentration of 0.5 μg/well and were incubated for 4 h. The cells were then washed twice with PBS, detached with trypsin 0.05% for 5 min, followed by the addition of complete medium to stop trypsinization. Then, the cells were collected by centrifugation (10 min, \(300 \times g\), 4 °C) and re-suspended with FACS buffer (PBS, 2 mM EDTA, 0.5% BSA). Data for 5000 cells were collected on a BD FACSCanto™ Flow Cytometer (BD Biosciences, San Jose, CA) and were analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR). Uptake efficiency was determined by normalizing the mean fluorescence intensity of nanoparticle-treated cells to the background intensity of untreated cells.

4.2.3.11. Cell uptake studies of PICMs

The uptake of different PICM formulations was assessed in HepG2 cells. To track the PICMs, a fluorescently-labeled Cy3-Bcl2-siRNA was used. The cells were seeded at a density of \(2 \times 10^6\) cells/well into 24-well plates and incubated for 48 h. The medium was replaced by fresh DMEM (low glucose) containing 2% BSA, the PICMs were added to the cells at a final siRNA concentration of 400 nM and were incubated for 3 h. The cells were then washed twice with PBS, detached with accutase for 10 min, followed by the addition of complete medium to disperse the cells. Then, the cells were collected by centrifugation (5 min, \(300 \times g\), 4 °C), washed once with FACS buffer and re-suspended in
it. Data for 10,000 cells were collected on a BD FACSCanto™ Flow Cytometer and were analyzed using the FlowJo software. Uptake efficiency was determined by normalizing the mean fluorescence intensity of PICMs-treated cells to the background intensity of the untreated cells.

4.2.3.12. Cytotoxicity experiments

The viability of cells exposed to the PICMs was determined by the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt] (CellTiter® 96 Aqueous Non-Radioactive Cell Proliferation Assay) according to the manufacturer’s protocol. HepG2 cells were seeded in a 96-well plate at a density of $5 \times 10^3$ cells/well in 100 μL complete medium and cultured for 24 h at 37 °C in a humidified atmosphere containing 5% CO2. The medium was then removed and various concentrations of PEG$_{113}$-b-P(PrMA$_{24}$-co-MAA$_{56}$) and T-GalNAc-PEG$_{169}$-b-P(PrMA$_{17}$-co-MAA$_{47}$) in 100 μL of DMEM with 2% BSA were added to the wells. After 5 h incubation, the medium was removed, the cells were washed twice with PBS, and were incubated further for 24 h with fresh complete medium. Then, the medium was replaced by 100 μL medium without phenol red plus 20 μL CellTiter 96® Aqueous One Reagent containing the tetrazolium compound MTS for about 2 h. MTS was reduced by cells into a formazan compound. The absorbance of formazan was measured at 490 nm with a plate reader (Infinite M-200, Tecan, Männedorf, Switzerland). Cell viability was determined according to the following equation (eq. 1):

$$\text{Cell viability [%]} = \left( \frac{\text{OD}_{490 \, \text{sample}}}{\text{OD}_{490 \, \text{control}}} \right) \times 100$$  

Where OD$_{490 \, \text{sample}}$ represents the optical density of the wells treated with PICMs and OD$_{490 \, \text{control}}$ represents the wells treated with growth medium only.

4.2.3.13. Statistical Analysis

The one-way ANOVA test combined with Tukey’s post-hoc test was applied for the pairwise comparison between multiple groups. $p < 0.05$ was considered statistically significant in all analyses.
4.3. Results and discussion

4.3.1. Synthesis of trivalent ligand T-GalNAc-NHS

A trivalent ligand (T-GalNAc) was designed and prepared for ASGP-R targeting (Figure 4.3). The most abundant configuration of ASGP-R displays its ligand-binding sites with a triangular spatial geometry. To fit this receptor geometry, a symmetric tetraacid (4.1) was chosen as the scaffold of the trivalent ligand. Another advantage of tetraacid scaffold is that the fourth carboxyl group can be activated by N-hydroxysuccinimide (NHS), facilitating the conjugation of the ligand to any systems containing amine groups. To couple the sugar to the tetraacid scaffold, three out of four acid moieties were activated by tetrafluorophenyl trifluoroacetate (TFP-OTFA) via stoichiometric control. Owing to the hydrophobic aromatic ring, the TFP-activated tetraacid (4.2) can be easily purified by silica gel chromatography without the necessity of protecting the free carboxyl groups. The next step was to choose an appropriate chain to prolong the linker of the ligand. Here, 3-amino-1-propanol (4.3) was employed for two considerations. First, this chain results in a linker of the ligand with a length of 13 Å, which should be able to display the sugar 21 Å apart, an optimal distance as discussed in chapter 2 (Figure 4.1). Second, this chain enhances the hydrophobicity of the linker since a hydrophilic linker was proved to be suboptimal. GalNAc was used in this work since it was shown to display a 60-fold higher binding affinity compared to Gal toward the ASGP-R of rat hepatocytes. The carboxybenzyl (Cbz)-protected linker (4.4) was then introduced to the acetyl protected GalNAc (4.5), and amino-GalNAc (4.8) was obtained after global deprotection. The NHS-activated trivalent ligand T-GalNAc-NHS (4.10) was produced by coupling the sugar (4.8) to the activated scaffold (4.2), followed by the activation with NHS. It should be noted that the trivalent ligands for ASGP-R previously reported normally require large number of synthesis steps, whereas here, only 7 steps were required to produce a NHS-activated T-GalNAc (4.10). This ligand is ready to couple to any systems containing an amine group.
Figure 4.3. Synthesis of T-GalNAc-NHS (4.10). Reagents and conditions: (a) TFP-OTFA, TEA, DMF, 0 °C; (b) CbzCl, TEA, DMAP, 1,4-dioxane, H2O, rt; (c) TFOH, DCM, reflux; (d) NaOMe, MeOH; rt; (e) H2, Pd/C, MeOH, H2O, rt; (f) TEA, DMF, rt; (g) NHS, EDCI, DMF, rt.

4.3.2. Synthesis of targeted chelator T-GalNAc-PEG-IP5 (4.17)

Compound 4.12 was obtained by previously reported procedures (Figure 4.4). The synthesis of compound 4.14 was described in chapter 3. Compound 4.16, which displays a primary amine for coupling, was obtained via the “click” reaction between compounds 4.12 and 4.14, followed by hydrogenolysis. Coupling the NHS-activated trivalent ligand T-GalNAc-NHS (4.10) yielded the final T-GalNAc-PEG-IP5 (4.17)
4.1.4

**4.15**

**4.16**

**4.17**

**Figure 4.4.** Synthesis of T-GalNAc-PEG-IP5 (4.17). Reagents and conditions: (a) CbzCl, TEA, DMAP, 1,4-dioxane, H2O, rt; (b) CuI, DIPEA, AcOH, DCM, rt; (c) H2, Pd/C, MeOH, H2O; (d) HATU, DIPEA, ACN/DMF/H2O (12.5:4.5:1), rt.

4.3.3. Cellular uptake of CaP nanoparticles

The T-GalNAc-CaP nanoparticles were prepared by method 2 as described chapter 3, using 10 or 30 mol% of T-GalNAc-PEG-IP5. These particles were then assessed for their uptake by HepG2 cells. Unfortunately, the ligand-decorated CaP nanoparticles displayed no significant enhanced uptake upon incubation for 4 h in Opti-MEM medium with 10% FBS (Figure 4.5). The lack of effect of the ligand could be due to the generally low stability of the system. The particles can slowly dissolve over time and the ligand might detach from their surface. Thus, we decided to investigate this ligand in the context of PICMs, which had been previously shown by the Leroux group to be internalized by cells specifically when decorated with antibody fragments targeting the transferrin receptor.\(^\text{220}\)
Chapter 4 - T-GalNAc-PICMs for hepatocytes targeting

Figure 4.5. Uptake of fluorescently-labeled DNA/PICMs by HepG2 cells after an incubation time of 4 h as determined by flow cytometry. The feeding amount of T-GalNAc/Ac-copolymer was 10 and 30 mol%, and the final amount of DNA was 0.5 µg/well. Uptake is shown as normalized mean fluorescence intensity (vs. untreated cells). Data are presented as means ± SD (n = 3). N.S.: not significant.

4.3.4. Synthesis of targeted copolymer.

In order to assess the targeting ability of the ligand, targeted PICMs were prepared (Figure 4.6). First, a non-targeted copolymer mPEG₁₁₃₋b₋P(PrMA₂₄₋co-MAA₅₆) was synthesized by ATRP as previously described (Table 4.1). Targeted copolymer T-GalNAc-PEG₁₆₉₋b₋P(PrMA₁₇₋co-MAA₄₇) (4.19) was synthesized by coupling T-GalNAc-NHS (4.10) to a previously reported copolymer H₂N-PEG₁₆₉₋b₋P(PrMA₁₇₋co-MAA₄₇) (4.18). An acetyl group was also coupled to the copolymer (4.18) and Ac-PEG₁₆₉₋b₋P(PrMA₁₇₋co-MAA₄₇) (4.20) was obtained and used as a control. Similarly to Fab'-functionalized PICM, the accessibility of the targeting ligand on the micelle surface was ensured by using a longer PEG chain than the non-targeted copolymer (7.5 vs. 5 kDa).
4.18

4.19

4.20

Figure 4.6. Synthesis of T-GalNAc-PEG_{169-b-P(PrMA_{17-co}-MAA_{47})} (4.19) and Ac-PEG_{169-b-P(PrMA_{17-co}-MAA_{47})} (4.20). Reagents and conditions: (a) TEA, DMF/H_2O (4:1), rt; (b) N-succinimidy acetate, TEA, DMF/H_2O (4:1), rt.

Table 4.1. Characteristics of PEG-b-P(PrMA-co-MAA)s.

<table>
<thead>
<tr>
<th>Diblock copolymer</th>
<th>M_n [a]</th>
<th>M_w [b]</th>
<th>M_w/M_n [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPEG_{113-b-P(PrMA_{24-co}-MAA_{56})}</td>
<td>16,440</td>
<td>13,390</td>
<td>1.23</td>
</tr>
<tr>
<td>H_2N-PEG_{169-b-P(PrMA_{17-co}-MAA_{47})}</td>
<td>15,760</td>
<td>14,310</td>
<td>1.10</td>
</tr>
</tbody>
</table>

[a] Determined by ^1^H NMR spectroscopy.

[b] Determined by GPC.

4.3.5. Preparation and characterization of PICMs

PICMs were prepared according to a previously reported protocol. Briefly, mixing the copolymers with PAMAM G5 and siRNA resulted in charge neutralization, and PICMs with size typically below 100 nm were obtained (Figure 4.2). PICMs prepared at different N/(P+COOH) ratios were characterized by DLS. N corresponds to the primary amine groups of the PAMAM while P and COOH represent the phosphate and carboxylate groups of the nucleic acid and MAA copolymer, respectively. No micelles were observed at a ratio of 0.5, whereas micelles with size of about 50 nm were obtained at the ratios of 0.75 to 1.5. Eventually, a ratio of 1.0 was selected for preparing PICMs for further experiments owing to the maximal and reproducible scattering intensity obtained under this condition (Figure 4.7).
Figure 4.7. Size and scattering intensity of PICMs prepared at different N/(P+C) molar ratios.
The feeding amount of T-GalNAc-copolymer was 40 mol%, and the final concentration of Cy3-siRNA was 100 nM. Data are presented as the means ± SD (n = 3).

4.3.6. Stability in biological media and pH sensitivity

To achieve a successful delivery to the target cells, the PICMs need to be sufficiently stable in the biological media to protect the nucleic acids from degradation by nucleases. Thus, the stability of PICMs was studied by DLS in different biological media. PICMs were stable in cell culture medium (DMEM + 2% BSA) and 50% FBS/PBS solution for at least 5 h (Figure 4.8). The original diameter of PICMs in serum-free buffer was around 50 nm; this size was smaller in the biological media due to the abundant presence of the serum proteins (around 10 and 25 nm). One advantage of the copolymer is its ability to dissociate from the micelle PAMAM/nucleic acid core upon protonation of MAA units at endosomal pH. The disassembly of the PICM facilitates the endosomal escape of the genetic material.221 To mimic the acidic environment present in endosomes, a suspension of micelles in FBS solution was acidified to pH 5.5. Immediately after this, the size of PICMs dramatically increased to ~400 nm, indicating the destabilization of PICMs at low pH.
Chapter 4 - T-GalNAc-PICMs for hepatocytes targeting

Figure 4.8. Stability of PICMs over time in different media. PICMs were diluted 1:4 (v/v) in DMEM/BSA and FBS/PBS at 37 °C. Micelles in FBS/PBS were acidified to pH 5.5 at timepoint 5 h. The feeding amount of T-GalNAc-copolymer was 40 mol%, and the final concentration of Cy3-siRNA was 100 nM. Data are presented as the means ± SD (n = 3). *p < 0.001.

4.3.7. Cellular uptake of PICMs

The uptake of T-GalNAc-PICMs was evaluated in the human hepatic cell line HepG2 (Figure 4.9). Upon incubation for 3 h in DMEM with 2% BSA, the targeted micelles T-GalNAc-PICMs showed small but significant enhanced uptake compared to the control micelles Ac-PICMs. Moreover, preincubating the cells for 30 min with 60 mM free GalNAc resulted in the decrease of the uptake of T-GalNAc-PICMs to the same level of Ac-PICMs, but showed no influence to the Ac-PICMs. This suggested that T-GalNAc-PICMs were partially taken up via the ASGP-R, which is abundantly expressed on the surface of mammalian hepatocytes. Here, only a small enhancement of uptake was observed, similar to what has been reported in the literature using HepG2 cells. This could be explained by the fact that the ASGP-R expression level is lower in cultured cell lines than the primary hepatocytes. Isolated primary hepatocytes are expected to show better uptake results than HepG2, but the isolation process is complex and the expression level of ASGP-R is affected by factors such as digestion enzyme and temperature, hence showing a wide variation from lab to lab. While the specific uptake is modest, HepG2 have been used as a simple model to evaluate the targeting systems, and higher targeting efficacy may be expected in vivo owing the higher expression of the receptor.

The carbohydrate recognition domain (CRD) of the ASGP-R subunits belongs to the C-type (Ca²⁺-dependent) superfamily, making Ca²⁺ crucial for binding of the ligand to the receptor. The T-GalNAc-PICMs should be internalized and not just bound to the receptors on the cell surface since cells were washed with PBS buffer containing EDTA (Ca²⁺ binder) prior to flow cytometry analysis. The control micelles also showed some uptake, which is probably due to unspecific non-receptor
mediated endocytosis. Overall, these data show that uptake of T-GalNAc-PICMs into HepG2 is in part mediated by specific binding of T-GalNAc ligand to the ASGP-R.

![Graph](image)

**Figure 4.9.** Uptake of Cy3-siRNA/PICMs by HepG2 cells after an incubation time of 3 h as determined by flow cytometry. The feeding amount of T-GalNAc/Ac-copolymer was 40 mol%, and the final concentration of Cy3-siRNA was 400 nM. (A) Flow cytometry histogram of cells treated with targeted micelles T-Gal-PICMs and control micelles Ac-PICMs, in presence or absence of 60 mM GalNAc. (B) Uptake is shown as normalized mean fluorescence intensity (vs. untreated cells). Data are presented as means ± SD (n = 3). *p < 0.01.

4.3.8. Cell viability assay

The toxicity of the targeted and non-targeted copolymers was assessed in HepG2 (Figure 4.10). It has been previously shown that the complexation of PAMAMs to non-targeted copolymers displayed no measurable cytotoxicity up to 0.5 mg/mL. Here, we focused on the newly synthesized targeted copolymers, since their cytotoxicity had not been tested before. Not surprisingly, both the targeted and non-targeted copolymers showed no sign of cytotoxicity at concentrations as high as 0.5 mg/mL (the working concentration of the copolymer for the uptake experiments was 0.1 mg/mL).
Chapter 4 - T-GalNAc-PICMs for hepatocytes targeting

**Figure 4.10.** Cell viability of HepG2 cells exposed to different concentrations of copolymer mPEG$_{113}$-$b$-P(PrMA$_{24}$-co-MAA$_{56}$) and T-GalNAc-copolymer T-GalNAc-PEG$_{169}$-$b$-P(PrMA$_{17}$-co-MAA$_{47}$). Data are presented as means ± SD (n = 3).

### 4.4. Conclusion

A well-defined trivalent ligand T-GalNAc was designed and prepared for ASGP-R targeting. The introduction of this ligand to PICMs improved their uptake efficiency in HepG2 cells, indicating a binding interaction between the ligand and the ASGP-R of HepG2 cells. In addition, the targeted micelles were destabilized immediately at endosomal pH (5.5), which is needed to facilitate the endosomal escape of nucleic acids. The T-GalNAc-PICMs are a promising gene delivery system for hepatocytes *in vitro*. Their potential for liver targeting *in vivo* should be investigated in future studies.
Chapter 5

5. General Conclusion and Outlook

One major challenge for the clinical application of gene therapy is the safe and efficient delivery of nucleic acid drugs to target cells. So far, viruses and synthetic complexes are the most common delivery systems employed to address this problem. Non-viral vehicles are often preferred since in principle they would display better safety profiles than viral delivery systems. The present Ph.D. thesis was aimed at developing two different types of non-viral vectors (inorganic and organic nanocarriers) for nucleic acids.

In Chapter 3, inorganic nanoparticles based on PEGylated chelator-stabilized CaP nanoparticles were developed and characterized for nucleic acid delivery (Figure 5.1).

CaP nanoparticles are promising carriers due to their bioresorbability, ease of preparation, high gene loading efficacy and endosomal escape properties. However, rapid particle aggregation hinders their potential application in vivo. Recently, the Leroux lab developed a solvent- and surfactant-free PEGylated chelator coating strategy for the preparation of PEGylated CaP nanoparticles by simply mixing a PEGylated chelator solution with other components (Ca$^{2+}$, PO$_4^{3-}$ and gene). CaP-siRNA/DNA nanoparticles with sizes of 190-220 nm were obtained by PEG-BP and PEG-IP5. In the present work, new synthesis routes involving click chemistry were implemented to prepare the PEGylated chelators PEG-IP5 and PEG-BP. The introduction of the clickable alkyne group allows straightforward conjugation of the chelator to various polymers at a later stage, thus avoiding complicated purification steps.

Although CaP nanoparticles with a size of about 200 nm could be easily prepared by a method previously established in the Leroux lab, smaller particle sizes (< 100 nm) could not be obtained. Developing a method to produce smaller CaP particles would be desirable as nanoparticles with a diameter of 10-100 nm are suitable for tumor targeting via the EPR effect. Therefore, in addition to the published method (1), a new protocol (2) was developed with the aim of producing particles with the size below 100 nm. The difference of these two methods depended on the time of addition of the PEGylated chelator. Method 1 yielded amorphous aggregated spheres with a particle size of about 200 nm, whereas method 2 generated 40-nm amorphous loose aggregates of clusters, which were quickly turned into needle bundle-like crystals of about 80 nm in a few hours.

Surprisingly, CaP particles prepared by these two methods exhibited distinct properties such as size, morphology and crystallinity. Since these CaP nanoparticles were designed and prepared for nucleic acid delivery, it is important to better understand the relationship between CaP nanoparticle properties and their interactions with cells. In the in vitro uptake experiments, PEG-IP5 stabilized
nanoparticles prepared by method 1 were internalized more in HepG2 cells than those prepared by method 2. The uptake was dramatically influenced by the reaction time of Ca\(^{2+}\) and PO\(_4^{3-}\) and sedimentation of the particles. Interestingly, morphological transformations were observed for both types of particles after different storage times, but this barely affected their \textit{in vitro} cellular uptake. The transfection efficiency of the particles prepared by method 1 was significantly higher, and none of the formulations tested showed signs of cytotoxicity at the screened concentrations.

The present study provided a better understanding of the structure (e.g. size, morphology and crystallinity) of PEGylated CaP nanoparticles and showed how some of the particles’ properties could influence \textit{in vitro} uptake and transfection efficiency. However, several issues and future work related to this study have to be mentioned.

The stability of CaP particles in buffer with low concentrations of calcium and phosphate ions is an important issue, due to the dynamic exchange of calcium and phosphate ions between the solution and the CaP particles. One strategy to tackle this problem is to increase the crystallinity. A very common approach is using hydrothermal treatment, after which CaP particles normally show very high stability.\(^{230}\) However, cargos such as nucleic acids are not suitable with this treatment since they might degrade at high temperature. Another issue is the retention of the PEG coating on the particle surface. One strategy to improve the stability of the coating could be to interconnect the chelator groups with covalent bonds in order to increase the binding affinity between the chelators to CaP particles. Some studies using polymeric chelators have achieved relatively stable CaP particles.\(^{196, 205, 231, 232}\) Finally, the steric stability of the particles could be further increased by augmenting the PEG-chelator concentration during the preparation process. Indeed, in the present study, particles prepared by method 2 possessed high PEG density and were more stable than particles prepared by method 1, as suggested in pilot dilution and purification experiments (data not shown).

In chapter 4, organic carriers based on PICMs were developed for the targeted delivery of nucleic acids to hepatocytes (Figure 5.1).

Targeted delivery of therapeutic agents to hepatocytes is a particularly attractive strategy for the treatment of hepatocellular carcinoma and other liver diseases.\(^{91}\) The ASGP-R is abundantly expressed on hepatocytes and minimally expressed on extrahepatic cells, making it an ideal entry gateway for hepatocyte-targeted delivery.\(^{92}\) Therefore, a T-GalNAc ligand was designed and prepared for ASGP-R targeting, and properties such as sugar type, linker length, hydrophilic-hydrophobic balance and spatial geometry of the scaffold were taken into consideration.

In fact, this newly synthesized ligand was firstly introduced to the CaP nanoparticles system in order to improve the uptake efficiency. Unfortunately, no significant enhanced uptake was observed. This could be due to the generally low stability of this system. Although it was shown that the particles were stable for at least 6 h in different media, the ligand might still detach from their surface.
Therefore, this ligand was applied to another more robust gene delivery system which is well-established in the Leroux lab.

![Figure 5.1](image)

**Figure 5.1.** Schematic illustration showing the main achievements and development of this Ph.D. thesis. Stage 1: preparation and characterization of CaP nanoparticles based on PEGylated chelators for gene delivery. Stage 2: design and synthesis of a well-defined trivalent ligand for ASGP-R targeting. Stage 3: attempts to introduce the trivalent ligand to CaP nanoparticles failed. Stage 4: introduction of trivalent ligand to PICMs showed enhanced uptake in HepG2 cells.

It was previously reported that pH-responsive PICMs prepared from a diblock copolymer PEG-b-P(PrMA-co-MAA) and PAMAM dendrimers could encapsulate AON and siRNAs and protect them from degradation. These PICMs were further functionalized with Fab’ and an efficient silencing
effect was achieved \textit{in vitro}.\textsuperscript{220, 221} In the present work, the T-GalNAc was coupled to amino-PEG-\textit{b}-P(PrMA-\textit{co}-MAA) to produce T-GalNAc-PICMs for the targeted delivery of nucleic acids to hepatocytes. In uptake experiments carried out on HepG2 cells, the T-GalNAc-PICMs showed enhanced uptake efficiency compared to Ac-GalNAc-PICMs (control micelles), which was nearly completely inhibited by the co-incubation with an excess of GalNAc. The efficient targeted delivery of nucleic acid drugs to hepatocytes holds great promise for the treatment of various liver diseases.

However, PICMs are also unstable under certain conditions. For example, a preliminary experiment showed that the size of PICMs continued to increase upon the addition of 150 mM NaCl solution (to reach the osmolarity desirable for injection \textit{in vivo}), indicating the destabilization of the PICMs (data not shown). This could be due to the fact that the PICMs are formed \textit{via} electrostatic interactions which are deeply related to the ion strength. One solution to this problem could be using a disulfide crosslinker to crosslink the PICMs, which has been shown to enhance the stability of PICMs.\textsuperscript{233}

Overall, the present Ph.D. thesis described two different types of non-viral gene delivery vectors namely inorganic nanoparticles based on PEGylated chelators-stabilized CaP nanoparticles and organic nanocarriers based on PICMs. CaP nanoparticles with different size, morphology, crystallinity, stability could be obtained by varying the preparation protocol. Thus, the appropriate properties of the particles could be tailored according to the application. The T-GalNAc decorated PICMs showed targeting effect to hepatocytes \textit{in vitro}, and further research should focus on their capability of delivering nucleic acid drugs to the liver \textit{in vivo}. 
6. Appendix

6.1. NMR and mass spectra of chapter 3

6.1.1. NMR and mass spectra of compound 3.2
6.1.2. NMR and mass spectra of compound 3.3
6.1.3. NMR and mass spectra of compound 3.4

$^1$H NMR, 400 MHz, CDCl$_3$

$^{13}$C NMR, 101 MHz, CDCl$_3$
Appendix

$^{31}P$ NMR, 162 MHz, CDCl$_3$
6.1.4. NMR and mass spectra of compound 3.5
6.1.5. NMR and mass spectra of compound PEG-IP5 (3.6)
6.1.6. NMR and mass spectra of compound PEG-BP (3.8)

\[ \text{\textsuperscript{1}H NMR, 400 MHz, D}_2\text{O} \]

\[ \text{\textsuperscript{31}P NMR, 162 MHz, D}_2\text{O} \]
6.2. NMR and mass spectra of chapter 4

6.2.1. NMR and mass spectra of compound 4.2
6.2.2. NMR and mass spectra of compound 4.6

**$^1$H NMR, 400 MHz, CDCl$_3$**

**$^{13}$C NMR, 101 MHz, CDCl$_3$**
6.2.3. NMR and mass spectra of compound 4.7

\[ \text{\textsuperscript{1}H NMR, 400 MHz, D}_2\text{O} \]

\[ \text{\textsuperscript{13}C NMR, 101 MHz, D}_2\text{O} \]
6.2.4. NMR and mass spectra of compound 4.8
6.2.5. NMR and mass spectra of compound 4.9
6.2.6. NMR of compound 4.10

\[ ^1H \text{ NMR, 400 MHz, D}_2O \]
6.2.7. NMR of compound 4.15

\[ \text{H NMR, 400 MHz, CDCl}_3 \]

\[ \text{^31P NMR, 162 MHz, CDCl}_3 \]
6.2.8. NMR of compound 4.16

$^1$H NMR, 400 MHz, D$_2$O

$^{31}$P NMR, 182 MHz, D$_2$O
6.2.9. NMR of compound 4.17
6.2.10. NMR of compound 4.19

\[\text{H NMR, 400 MHz, D}_2\text{O}\]
References


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<th>Reference</th>
<th>Details</th>
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References


References


<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ASGP-R</td>
<td>asialoglycoprotein receptor</td>
</tr>
<tr>
<td>Ac-GalNAc</td>
<td>acetyl-GalNAc</td>
</tr>
<tr>
<td>ADA-SCID</td>
<td>adenosine deaminase-deficient severe combined immune deficiency</td>
</tr>
<tr>
<td>AON</td>
<td>antisense oligonucleotides</td>
</tr>
<tr>
<td>AAVs</td>
<td>adeno-associated viruses</td>
</tr>
<tr>
<td>ASOR</td>
<td>asialoorosomucoid</td>
</tr>
<tr>
<td>asPNA</td>
<td>antisense peptide nucleic acid</td>
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<td>AA</td>
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<td>ATRP</td>
<td>atom transfer radical polymerization</td>
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<td>bp</td>
<td>base pair</td>
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<td>BG</td>
<td>bivalent glycoclusters</td>
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<tr>
<td>CaP</td>
<td>calcium phosphate</td>
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<tr>
<td>CDP</td>
<td>cyclodextrin polymer</td>
</tr>
<tr>
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<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>βCD</td>
<td>β-cyclodextrin</td>
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<td>Cryo-TEM</td>
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<tr>
<td>CuAAC</td>
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<td>Cbz</td>
<td>carboxybenzyl</td>
</tr>
<tr>
<td>CFDA</td>
<td>China Food and Drug Administration</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
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<tr>
<td>DPC</td>
<td>dynamic polyconjugates</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
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<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeability and retention</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<tr>
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<td>N-acetyl galactosamine</td>
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<tr>
<td>Glu</td>
<td>D-glucose</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HBV and HCV</td>
<td>hepatitis viruses B and C</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>amino acid 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
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<td>HA</td>
<td>hydroxyapatite</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LPLD</td>
<td>lipoprotein lipase deficiency</td>
</tr>
<tr>
<td>LNPs</td>
<td>lipid-based particles</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LDSEAD</td>
<td>low-dose selected-area electron diffraction</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>Man</td>
<td>D-mannose</td>
</tr>
<tr>
<td>MBP</td>
<td>man-binding protein</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>------------</td>
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<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>MG</td>
<td>multivalent glycoclusters</td>
</tr>
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<td>Nrf2</td>
<td>nuclear erythroid 2-related factor 2</td>
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<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<td>PEG-IP5</td>
<td>PEG-inositol 1,3,4,5,6-pentakisphosphate</td>
</tr>
<tr>
<td>PEG-BP</td>
<td>PEG-bisphosphonate</td>
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<td>PICMs</td>
<td>polyion complex micelles</td>
</tr>
<tr>
<td>PrMA</td>
<td>propyl methacrylate</td>
</tr>
<tr>
<td>PMAA</td>
<td>poly(methacrylic acid)</td>
</tr>
<tr>
<td>PLL</td>
<td>poly(l-lysine)</td>
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<tr>
<td>PEI</td>
<td>polyethylenimine</td>
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<td>PBAVE</td>
<td>polyconjugate of butyl and amino vinyl ethers</td>
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<td>PAMAM</td>
<td>poly(amidoamine)</td>
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<td>PTX</td>
<td>paclitaxel</td>
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<tr>
<td>pEGFP-DNA</td>
<td>enhanced green fluorescent protein plasmid</td>
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<td>PMEA</td>
<td>9-(2-phosphonyl-methoxyethyl)adenine</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td>RNA-induced silencing complex</td>
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<td>rat hepatic lectin 1</td>
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<td>reaction-limited cluster aggregation</td>
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<td>SCID-X1</td>
<td>severe combined immunodeficiency</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SNALP</td>
<td>stable nucleic acid lipid particle</td>
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<td>solid phase peptide synthesis</td>
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<td>interfering ribonucleic neutrals</td>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>TFP-OTFA</td>
<td>tetrafluorophenyl trifluoroacetate</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
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