Doctoral Thesis

Stabilization of calcium phosphate nanoparticles for transfection with nucleic acid drugs

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STABILIZATION OF CALCIUM PHOSPHATE NANOPARTICLES
FOR TRANSFECTION WITH NUCLEIC ACID DRUGS

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Summary

Gene therapy is a powerful approach that can theoretically be used for the treatment of any disease by inserting genes to substitute defective ones or by silencing undesired mRNA. Clinical trials on gene therapy have started more than 20 years ago and have often shown promising results. However, only two drugs have been approved thus far: Gendicine® (tumor suppressor gene p53 incorporated into an adenovirus) in China and Vitravene® (Fomivirsen, an antisense oligonucleotide for the treatment of cytomegalovirus retinitis in AIDS patients) in the US and Europe. However, the latter was withdrawn for commercial reasons. More recently, a formulation consisting of an adeno-associated virus expressing a human lipoprotein lipase has been submitted to the European Medicine Agency (EMA) for approval for treatment of lipoprotein lipase deficiency.

One of the main challenges associated with gene therapy is to successfully deliver nucleic acids to their targets. Free nucleic acids are prone to rapid clearance, enzymatic degradation and poor membrane permeation. To achieve high transfection efficiencies in vivo, they generally have to be applied locally and in high doses.

Different delivery vehicles have been investigated for the transport of nucleic acids. Most of them are based on viruses. While these systems show high transfection efficiency, their viral envelope raises safety issues. Non-viral gene delivery systems such as lipoplexes and nanoparticles are therefore attractive alternatives due to their generally better safety profile, though transfection efficiencies are usually low. Approaches aimed at increasing the transfection efficiency of non-viral gene delivery systems are being widely investigated.

Calcium phosphate co-precipitate has been used in vitro for transfection for more than 35 years. Its combined capacity to condense nucleic acids, innocuousness, and transfection efficiency is attractive for its use as de-
Summary

livery system. Unfortunately, calcium phosphate particles are unstable and increase in size immediately after preparation, making long-term storage or \textit{in vivo} use impossible. This PhD work investigates bisphosphonates as stabilizing agents for calcium phosphate particles and their use for the delivery of plasmid DNA and siRNA. More specifically, bisphosphonates bind with high affinity to hydroxyapatite (a crystalline form of calcium phosphate) by chelation. Thereby they inhibit particle growth. The goal of this thesis was to prove that increasing the stability of these particles by bisphosphonates would make them suitable transfection agents for eventual \textit{in vivo} application.

In Chapter 2 of this thesis, the diverse applications of bisphosphonates are reviewed and discussed. Bisphosphonates are used in the clinic for the treatment of bone diseases such as osteoporosis and Paget’s disease. They have been extensively employed for the last twenty years and became first-line medications. Exogenously administered bisphosphonates bind to bone. This process is reversible in the resorption space between the bone and the osteoclasts due to the acidity of this environment. The released bisphosphonates accumulate in this area, are taken up by osteoclasts, and inhibit osteoclast function. The mechanism is dependent on the bisphosphonate structure: nitrogen-containing bisphosphonates inhibit bone resorption by the mevalonate pathway while non-nitrogen-containing bisphosphonates achieve the same inhibition by metabolism into a cytotoxic analog of ATP. Thereby bone degradation is arrested and fractures due to weak bones are prevented. Side-effects of bisphosphonates are usually mild. Their most severe observed side-effect is osteonecrosis of the jaw. Its incidence depends on co-morbidities and is between 1:10$^5$ and 1:10. However, the connection between bisphosphonates and osteonecrosis of the jaw is not always clearly proven.

The bioavailability of bisphosphonates is poor and is typically only 1–3\% after oral administration. To increase their bioavailability and to alter their biodistribution, bisphosphonates have been incorporated into different delivery systems such as liposomes and nanoparticles. Additionally, due to their high affinity for hydroxyapatite, which is unique to bone and teeth in the human body, they have been investigated as targeting ligands to transport different drugs to bone tissue. Furthermore, bisphosphonates play a role as linkers between specific targeting agents and materials like iron oxide nanoparticles, for which bisphosphonates
also have a high affinity.

In Chapter 3 the affinity of bisphosphonates for inorganic surfaces was exploited to stabilize calcium phosphate particles. Two commercially available bisphosphonates were chosen to stabilize calcium phosphate particles for the delivery of plasmid DNA. The first bisphosphonate was covalently linked to a linear chain of polyethylene glycol (PEG; Mw 750 g/mol) to prevent enzymatic degradation of the incorporated nucleic acids and for colloidal stability. The second bisphosphonate had an ammonium group (Amm-bp), which should facilitate cellular uptake of the particles due to the positive charge. Unlike unstabilized calcium phosphate co-precipitates, calcium phosphate nanoparticles without incorporated nucleic acids coated with either bisphosphonate were stable for 72 h with a size of \( \sim 150 \) nm. The size of the particles increased by encapsulation of plasmid DNA (to \( \sim 220 \) nm) and remained stable for 48 h. The polydispersity of all preparations was low. Higher concentrations of bisphosphonate increased the particle stability (but decreased uptake of the particles \textit{in vitro}). The zeta potential of particles containing plasmid DNA was more negative than empty ones. As expected, the uptake of ammonium-bisphosphonate coated nanoparticles was higher compared to particles bearing PEG chains due to the positive charge of the ammonium group. Nevertheless, transfection efficiencies of \( \sim 60\% \) were achieved for both types of nanoparticles. In conclusion, stable calcium phosphate nanoparticles loaded with plasmid DNA could be prepared and their transfection efficiency remained high.

As for many other plasmid delivery vehicles, calcium phosphate nanoparticles can also be used for siRNA delivery. siRNA therapeutics are advantageous in comparison to other nucleic acid drugs as siRNA does not need to reach the nucleus to exert its therapeutic activity. In Chapter 4 of this thesis, the incorporation of siRNA in calcium phosphate nanoparticles was explored, and their transfection efficiency examined. In comparison to the work described in Chapter 3, a longer PEG-chain was chosen (Mw = 2000 \textit{vs.} 750 g/mol) and covalently linked to alendronate (a bisphosphonate commonly used in the clinic), to produce a conjugate, PEG-ALE. In addition, an alternative new stabilizer PEG inositol pentakisphosphate (PEG-IP5) with stronger affinity for calcium was synthesized, to examine whether additional stability could be obtained. Calcium phosphate nanoparticles containing siRNA stabilized
with either chelator displayed high stability over a one month period, with the particles coated with PEG-IP5 being slightly more stable. The evaluation of PEG-IP5 was not further pursued due to poor particle uptake. PEG-ALE stabilized particles containing siRNA could efficiently silence the model oncprotein Bcl-2 \textit{in vitro}. Furthermore, the uptake mechanism of PEG-ALE-stabilized nanoparticles was investigated. Using endocytotic inhibitors, it was shown that nanoparticles were predominantly taken up \textit{via} clathrin-dependent endocytosis. This was confirmed by observation of co-localization of nanoparticles with endocytotic markers such as Rab5 and Rab7. Further monitoring the intracellular fate of nanoparticles showed that acidification in the endosomes/lysosomes was necessary for successful release of the nucleic acid cargo into the cytosol.

It was found that the stabilizing bisphosphonates had an effect on the mevalonate pathway, which is influenced by nitrogen-containing bisphosphonates. This led to the assumption that alendronate was at least partly released from PEG-ALE, possibly due to hydrolases.

In conclusion, this work presents a stable, inexpensive, and relatively non-toxic system for nucleic acids. The system showed high transfection efficiency and appears promising for delivery of nucleic acid drugs \textit{in vivo}. Due to their use in a wide variety of diseases, successful application of nucleic acid drugs would be a major breakthrough in modern medicine. The system described in this thesis has the drawback of being taken up unspecifically and local application would be necessary \textit{in vivo} to reach the target cells. Eventually, targeted particles could be produced using an antibody fragment and investigated for specific uptake. Also, this system could then be suitable for systemic administration.
Zusammenfassung


Calciumphosphat-Co-Präzipitate werden seit mehr als 35 Jahren für in vitro Transfektionen verwendet. Calciumphosphat kann Nukleinsäuren effizient kondensieren und besitzt neben der hohen biologischen Sicher-
heit auch gute Transfektionseffizienz. Nachteilig erweist sich die physikalische Instabilität von Calciumphosphat-Partikeln, die unmittelbar nach Herstellung zu wachsen beginnen, wodurch langfristige Lagerung oder *in vivo* Gebrauch verunmöglicht wird.


Die orale Bioverfügbarkeit von Bisphosphonaten ist niedrig und liegt normalerweise zwischen 1 und 3%. Um diese zu erhöhen und die Biodistri-
bution zu verändern wurden Bisphosphonate in verschiedene Transportsysteme wie Liposomen und Nanopartikel verpackt. Andererseits wurden Bisphosphonate dank ihrer hohen Affinität zu Hydroxyapatit, welches im menschlichen Körper nur in Knochen und Zähnen vorkommt, selbst auch als Liganden für den zielgerichteten Transport (targeting) von verschiedenen Arzneistoffen zum Knochen erschlossen. Auch spielen Bisphosphonate eine Rolle als Verbindungsmolekül zwischen spezifischen Liganden und Materialien wie Eisonoxid-Nanopartikel, für welche Bisphosphonate ebenfalls eine hohe Affinität haben.


Wie viele andere Plasmid-DNA Transportsysteme können Calciumphosphat-Nanopartikel auch für den Transport von siRNA verwendet werden. Der Vorteil von siRNA gegenüber DNA liegt darin, dass die siRNA nicht in den Zellkern transportiert werden muss, um einen biolo-

Nicht nur die Partikel sondern auch die stabilisierenden Bisphosphonate haben einen zellulären Effekt. Sie beeinflussen den Mevalonatweg. Dies führte zur Annahme, dass Alendronat zumindest teilweise von PEG getrennt wird, möglicherweise durch Hydrolasen.

mit Erkennungsmolekülen (z.B. Antikörper oder Antikörperfragmente) für Oberflächenstrukturen von spezifischen Zellen ausgestattet werden. Diese optimierten Partikel könnten dann auch systemisch verabreicht werden.