Nano-Particulate Calcium Phosphate as a Gene Delivery System

Babak Mostaghaci¹,², Arash Hanifi³, Brigitta Loretz¹ and Claus-Michael Lehr¹,⁴

¹Dept. of Drug Delivery (DDEL), Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infectious Research (HZI)
Saarland University, Saarbruecken

²Biomaterials Group, Materials Engineering Department
Isfahan University of Technology, Isfahan

³Tissue Imaging and Spectroscopy Lab, Bioengineering Department
Temple University, Philadelphia, PA

⁴Department of Biopharmaceutics and Pharmaceutical Technology
Saarland University, Saarbruecken

¹,⁴Germany
²Iran
³USA

1. Introduction

Four decades ago, calcium phosphate systems were introduced for in-vitro gene delivery applications. Recently, many studies have been conducted regarding the different applications of these systems in delivering genes to different cell types for therapeutic purposes. Although there are important limitations of using calcium phosphates in gene delivery, there is a high interest in using this type of gene delivery system. This is because of the significant biocompatibility of calcium phosphates, easy synthesis methods of this system, and intrinsic characteristics of calcium phosphates that increase the transfection efficiency. The combination of these properties are rarely seen in other gene delivery systems.

This chapter aims to localise calcium phosphate nanoparticles among the most common non-viral gene delivery systems. It also reviews the history of using calcium phosphates in gene delivery applications and the efforts made to make this system suitable for further clinical applications.

1.1 Non-viral gene delivery

The application of non-viral systems increased considerably after it was shown that using viral systems can result in several problems including difficulty in production, limited opportunity for repeated administrations due to acute inflammatory response, and delayed humoral or cellular immune responses. Insertional mutagenesis is also a potential issue for
some viral systems that integrate foreign DNA into the genome (Al-Dosari & Gao, 2009). Although viral systems such as retrovirus, adenovirus, and adeno-associated virus are potentially efficient, non-viral systems have some advantages in that they are less toxic, less immunogenic, and easier to prepare (Nishikawa & Huang, 2001).

A lot of research has been conducted to find suitable non-viral systems. An ideal gene delivery method needs to meet 3 major criteria:

i. It should protect the transgene against degradation by nucleases in intercellular matrices.

ii. It should be able to carry the transgene across the cell membrane and into the nucleus of targeted cells.

iii. It should have no detrimental effects (Gao et al., 2007).

Recently, various materials have been introduced as potential gene delivery systems. Three groups of substances are more advantageous in this application. These three groups are:

i. Cationic polymers (like polyethylenimine (Kichler et al., 2001; Kircheis et al., 2001; Wightman et al., 2001), dendrimers (Tang et al., 1996; Zinselmeyer et al., 2002; Dufes et al., 2005), chitosan (Lee et al., 1998; Koping-Hoggard et al., 2001; Loretz & Bernkop-Schnurch, 2006) and poly-L-lysine (Trubetskoy et al., 1992; Benns et al., 2000));

ii. Lipids (like liposomes (Alton et al., 1993; Templeton et al., 1997; Templeton & Lasic, 1999));

iii. Inorganic materials (like calcium phosphates (Liu et al., 2005) and silica nanoparticles (Kneuer et al., 2000; Csogor et al., 2003; Sameti et al., 2003)).

However, some limitations accompany the use of most of these systems including cell toxicity, immune response and low transfection efficiency.

1.2 Inorganic vectors

Inorganic systems have been used in in-vitro gene delivery for many years, but their clinical application has been developed mostly in the last decade when amino-functionalized silica was introduced. Researchers at Saarland University showed that amino-functionalized silica exhibits good gene transfection efficiency in addition to its suitable biocompatibility (Kneuer et al., 2000; Csogor et al., 2003; Sameti et al., 2003). Because of this, several studies have been conducted on using amino-functionalized silica as a gene delivery system (Bharali et al., 2005; Roy et al., 2005; Klejbor et al., 2007; Choi et al., 2008). Research was also conducted on using silica in combination with other polymers for gene delivery. Results demonstrated that making composites of certain polymers with silica nanoparticles could enhance transfection efficiency due to the dense nature of silica nanoparticles (Luo et al., 2004).

There is an increasing interest in mesoporous silica for drug/gene delivery applications because of their higher capacity and of the potential for tailored release of the active molecule. Some studies have been conducted on functionalized or non-functionalized mesoporous silica but the research on using this type of inorganic systems is still ongoing (Park et al., 2008; Slowing et al., 2008).

Some studies have been done on using functionalized gold nanoparticles as a gene delivery system. The results demonstrated the feasibility of using this approach, but further research is needed in this new area (Liang et al., 2010; Niidome et al., 2011).

In addition to calcium phosphate, (their gene delivery application is reviewed in this chapter), other inorganic systems have also been studied regarding in-vitro gene delivery to
targeted cells. Silica nanotubes (Namgung et al., 2011), zirconia (Tan et al., 2007), carbon nanotubes (Pantarotto et al., 2004) and layered double hydroxides (Choy et al., 2008) are some examples of these inorganic systems. However, their low transfection efficacy limits their use. Table 1 summarizes inorganic nanoparticles properties.

The following sections discuss calcium phosphates; one of the most important groups of inorganic non-viral gene delivery systems.

2. Calcium phosphate

The work of Graham and Van Der Eb completed in 1973 shows the first application of calcium phosphate in condensation of genetic materials. The brilliant results of their research were that calcium phosphate could condense DNA and increase the transfection efficiency with a relatively simple procedure (Graham & Van Der EB, 1973a). This first research led to vast application of this technology in in-vitro gene delivery because of the demonstrated easy preparation method and proper results.

In order to have a better understanding of calcium phosphate gene delivery properties, first we shall have a look at the structure and characteristics of the calcium phosphate family.

2.1 Calcium phosphates family

Calcium phosphate-based bioceramics have been used in medicine and dentistry for decades. Applications include dental implants, percutaneous devices, periodontal treatment, alveolar ridge augmentation, orthopedics, maxillofacial surgery, otolaryngology and spinal surgery (Hench, 1991).

Bone is a natural nano-composite composed of organic (40%) and inorganic (60%) components. The inorganic constituent of bone is made up of biological apatites, which provide strength to the skeleton and act as a storehouse for calcium, phosphorus, sodium, and magnesium. These biological apatites are structurally similar, though not identical, to the mineral apatite hydroxyapatite (HAp, Ca₁₀(PO₄)₆(OH)₂). Hydroxyapatite is the most ubiquitous and well-known phase of calcium phosphate. It has the Ca/P ratio of 1.67 (Narayan et al., 2004). Different phases of calcium phosphate ceramics are used depending upon whether a resorbable or bioactive material is desired. The stable phases of calcium phosphate ceramics depend considerably upon temperature and the presence of water, either during processing or use in the environment (See Fig. 1) (Hench, 1991).

Going through aforementioned properties, it can be realized that the calcium phosphates family includes several members with different characteristics. Calcium phosphate ratio, Ca/P, has been found as the best way to distinguish among these members. In table 2 these members are shown based on their Ca/P ratio.

2.2 Properties

Calcium phosphates being light in weight, chemically stable and compositionally similar to the mineral phase of the bone are preferred as bone graft materials in hard tissue engineering. They are composed of ions commonly found in physiological environment, which make them highly biocompatible. Many research works demonstrated the biocompatibility of calcium phosphates in-vitro and in-vivo. In addition, these bioceramics are also resistant to microbial attack, pH changes, and solvent conditions (Thamaraiselvi & Rajeswari, 2004; Kalita et al., 2007). Degradation properties are very important, especially in the application of calcium phosphates related to drug delivery. It has been shown that
<table>
<thead>
<tr>
<th>Kind of nanoparticle</th>
<th>Chemical Composition</th>
<th>Typical Size Range</th>
<th>Solubility in μg L⁻¹</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium Sulfide</td>
<td>CdS</td>
<td>2–5 nm</td>
<td>0.69 ng L⁻¹</td>
<td>toxic, fluorescent, semiconducting</td>
</tr>
<tr>
<td>Calcium Phosphate</td>
<td>Ca₁₀(PO₄)₆(OH)₂ (hydroxyapatite)</td>
<td>10–100 nm</td>
<td>6.1 mg L⁻¹</td>
<td>biodegradable, biocompatible; may be made fluorescent by incorporation of lanthanides; cations and anions may be substituted</td>
</tr>
<tr>
<td>Carbon Nanotubes</td>
<td>Cₙ</td>
<td>diameter of a few nm and length of a few mm</td>
<td>0</td>
<td>Not biodegradable, hollow; may be covalently functionalized to improve solubility and may be loaded with molecules</td>
</tr>
<tr>
<td>Cobalt-Platinum</td>
<td>CoPt₃</td>
<td>3–10 nm</td>
<td>≈ 0</td>
<td>ferromagnetic or superparamagnetic; toxic in uncoated form</td>
</tr>
<tr>
<td>Gold</td>
<td>Au</td>
<td>1–50 nm</td>
<td>≈ 0</td>
<td>easily covalently functionalized, for example, with thiols</td>
</tr>
<tr>
<td>Iron Oxide (Magnetite)</td>
<td>Fe₃O₄</td>
<td>5–20 nm</td>
<td>≈ 0</td>
<td>ferromagnetic or superparamagnetic; harmful for cells in uncoated form; solubility increases with falling pH</td>
</tr>
<tr>
<td>Layered Double Hydroxide</td>
<td>Mg₆Al₂(CO₃)(OH)₁₆·₄H₂O (hydrotalcite)</td>
<td>50–200 nm</td>
<td>moderate, increases below pH 5–6</td>
<td>high selective anion exchange capacity; biodegradable in slightly acidic environment; cations can be substituted</td>
</tr>
<tr>
<td>Nickel</td>
<td>Ni</td>
<td>5–100 nm</td>
<td>≈ 0</td>
<td>immunogenic, toxic</td>
</tr>
<tr>
<td>Silica</td>
<td>SiO₂·nH₂O</td>
<td>3–100 nm</td>
<td>ca. 120 mg SiO₂ L⁻¹ (for silica particles)</td>
<td>Biodegradable; available also in micro- or mesoporous form (e.g., zeolites); easily functionalizable, for example, by chlorosilanes</td>
</tr>
<tr>
<td>Silver</td>
<td>Ag</td>
<td>5–100 nm</td>
<td>≈ 0</td>
<td>Bactericidal; dissolution product (Ag⁺) potentially harmful for cells</td>
</tr>
<tr>
<td>Zinc Oxide</td>
<td>ZnO</td>
<td>3–60 nm</td>
<td>1.6 to 5 mg L⁻¹</td>
<td>fluorescent, semiconducting</td>
</tr>
<tr>
<td>Zinc Sulfide</td>
<td>ZnS</td>
<td>3–50 nm</td>
<td>67 ng L⁻¹</td>
<td>fluorescent, semiconducting</td>
</tr>
</tbody>
</table>

Table 1. Some key properties of inorganic nanoparticles which are used for transfection in cell biology (Reprinted from (Sokolova & Epple, 2008)).
different crystalline phases of calcium phosphate present different degradation properties.
Table 3 summarizes the solubility properties and stability pH range of calcium phosphate.

![Calcium phosphate phase equilibrium diagram with 500 mmHg partial pressure of water. Shaded area is the processing range to yield HAp (Hench, 1991).](image)

<table>
<thead>
<tr>
<th>Ca/P</th>
<th>Name</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Tetracalcium phosphate</td>
<td>Ca₂O(PO₄)₂</td>
</tr>
<tr>
<td>1.67</td>
<td>Hydroxyapatite</td>
<td>Ca₁₀(OH)(PO₄)₆</td>
</tr>
<tr>
<td>N/A'</td>
<td>Amorphous calcium phosphate</td>
<td>Ca₉₋ₓHₓₙ(PO₄)ₙ(OH)₂</td>
</tr>
<tr>
<td>1.50</td>
<td>Tricalcium phosphate (α, β, γ)</td>
<td>Ca₃(PO₄)₂</td>
</tr>
<tr>
<td>1.33</td>
<td>Octacalcium phosphate</td>
<td>Ca₉H₃(PO₄)₆.5H₂O</td>
</tr>
<tr>
<td>1</td>
<td>Dicalcium phosphate dihydrate</td>
<td>CaHPO₄.2H₂O</td>
</tr>
<tr>
<td>1</td>
<td>Dicalcium phosphate</td>
<td>CaHPO₄</td>
</tr>
<tr>
<td>1</td>
<td>Calcium pyrophosphate (α, β, γ)</td>
<td>Ca₂P₂O₇</td>
</tr>
<tr>
<td>1</td>
<td>Calcium pyrophosphate dihydrate</td>
<td>Ca₃P₂O₇.2H₂O</td>
</tr>
<tr>
<td>0.7</td>
<td>Heptacalcium phosphate</td>
<td>Ca₇(P₅O₁₆)₂</td>
</tr>
<tr>
<td>0.67</td>
<td>Tetracalcium dihydrogen phosphate</td>
<td>Ca₃H₂P₁₀O₂₀</td>
</tr>
<tr>
<td>0.5</td>
<td>Monocalcium phosphate monohydrate</td>
<td>Ca(H₃PO₄)₂.2H₂O</td>
</tr>
<tr>
<td>0.5</td>
<td>Calcium metaphosphate (α, β, γ)</td>
<td>Ca(PO₃)₂</td>
</tr>
</tbody>
</table>

*N/A = not applicable

Table 2. Various calcium phosphates with their respective Ca/P atomic ratios (Reprinted from (Vallet-Regi & Gonzalez-Calbet, 2004)).
### Table 3. Solubility and pH stability of different phases of calcium phosphates (Reprinted from (Kalita et al., 2007)).

<table>
<thead>
<tr>
<th>Phases</th>
<th>Solubility at 25 °C, -log (K_{sp})</th>
<th>pH Stability Range in aqueous solution at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyapatite (HAp)</td>
<td>116.8</td>
<td>9.5-12</td>
</tr>
<tr>
<td>β-Tricalcium Phosphate (β-TCP)</td>
<td>28.9</td>
<td>Cannot be precipitated from aqueous solutions.</td>
</tr>
<tr>
<td>α-Tricalcium Phosphate (α-TCP)</td>
<td>25.5</td>
<td>Cannot be precipitated from aqueous solutions.</td>
</tr>
<tr>
<td>Tetracalcium Phosphate (TTCP)</td>
<td>38-44</td>
<td>Cannot be precipitated from aqueous solutions.</td>
</tr>
<tr>
<td>Dicalcium Phosphate Dehydrate (DCPD)</td>
<td>6.59</td>
<td>2.0 – 6.0</td>
</tr>
<tr>
<td>Dicalcium Phosphate Anhydrate (DCPA)</td>
<td>6.90</td>
<td>Stable at temperatures above 100 °C</td>
</tr>
<tr>
<td>Amorphous Calcium Phosphate (ACP)</td>
<td>Cannot be measured precisely. However, the following values were reported: 25.7 ± 0.1 (pH 7.40), 29.9 ± 0.1 (pH 6.00), 32.7 ± 0.1 (pH 5.28)</td>
<td>Always metastable. The composition of a precipitate depends on the solution pH value and composition.</td>
</tr>
<tr>
<td>Calcium-deficient Hydroxyapatite (CDHA)</td>
<td>≤ 85.1</td>
<td>6.5-9.5</td>
</tr>
</tbody>
</table>

2.3 Calcium phosphate nanoparticles

With the introduction of smaller calcium phosphate particles, it has become possible to use them in advanced fields of biomedicine. Calcium phosphate nanoparticles, with a size about 100 nm, are highly biocompatible. These particles are able to penetrate the outer membrane of cells and bacteria. Calcium phosphate nanoparticles could be utilized in different fields of biomedicine such as drug delivery, gene delivery, and imaging (Epple et al., 2010). Also, to produce high quality HAp bioceramics for artificial bone substitution, ultrafine HAp powder is usually employed. Nano-HAp powder results in easy handling, casting, and sintering leading to an excellent sintered body in the bioceramics preparation process (Cao et al., 2005).

3. Calcium phosphate nanoparticles as gene delivery vector

3.1 Historical view

Previously, it is mentioned that the first use of calcium phosphate in gene delivery application was conducted by Graham and Van Der EB in 1973. In this study, calcium phosphate was used for transfecting cells with Adenovirus 5 DNA to assay infectivity. (Graham & Van Der EB, 1973a). They diluted Adenovirus 5 DNA in a buffer containing Na₄HPO₄. Then, calcium chloride was added and the mixture was incubated with KB Cells. Using labeled DNA they concluded that by adding the calcium precursor in the experiment, the uptake of DNA increased and DNA showed a better stability against enzymatic degradation (Fig. 2). It was reported that this technique gave a 100 fold increase in efficiency over the DEAE-dextran method for human adenovirus DNA.
Fig. 2. Effect of CaCl$_2$ on adsorption of $^{14}$C-Ad5 DNA to KB cells. KB cells were exposed to MEM-Tris containing DNA plus CaCl$_2$ at various concentrations. The curves represent the fraction of radioactivity recovered in the medium (●), in the DNase digest (○), or in the SDS lysate of the cells (▲) (Graham & Van Der EB, 1973a).

With the same methodology, this group conducted another study to transform rat kidney cells with the DNA of human adenovirus 5. In Fig. 3 the transfected area is clearly visible as contained small, round, densely packed cells characteristic of adenovirus transformation. This work claimed that the “calcium technique” was a suitable system to study transformation by adenovirus DNA and the efficiency of transformation, though not high, appeared to be reasonably reproducible (Graham & Van Der EB, 1973b).

In another study, Graham, Veldhuisen and Wilkie used the aforementioned technique to investigate the infectivity of herpes simplex virus type I (HSV-I) (Graham et al., 1973). In 1975, Abrahams and Van Der EB made a transformation of rat kidney cells and mouse 3T3 cells by DNA from Simian Virus 40 using “calcium technique”. They stated that this technique for in-vitro transformation was reproducible (Abrahams & Van Der Eb, 1975). Later, Van Der EB and Graham successfully used “calcium technique” to determine the ability to transform primary baby rat kidney (BRK) cells with specific fragments of human adenoviruses 2 and 5 DNAs (Van Der EB et al., 1977).

In 1976, Stow and Wilkie reported that treatment of cells with dimethyl sulphoxide (DMSO) after injection with “Herpes Simplex Virus DNA”/calcium phosphate complex could lead to a significant increase in the number of plaques obtained. These researchers proposed that DMSO could initiate the plaque formation. It was interesting that in other method (DEAE-dextran) using DMSO did not exhibit that significant enhancement (Fig. 4) (Stow & Wilkie, 1976).
During the 1980's, the calcium phosphate method for in-vitro gene delivery had become a common method. In 1981, some of the parameters that affect the transformation procedure by calcium phosphate system had been investigated by Corsaro and Pearson (Corsaro & Pearson, 1981). First, to confirm the work of Stow and Wilkie in 1976, they performed a study on the effect of rinsing the complex of DNA/calcium phosphate with DMSO. They also added an additional variable to this experiment which was the exposure time of DNA/calcium phosphate complex to cells. They claimed that when suboptimal DNA exposure time is applied (e.g. 4-12 hours), the DMSO rinse increases the transformation frequency. However, rinsing with DMSO had no effect when the optimal condition was utilized. They concluded that exposure to DMSO offers no significant advantage.

Fig. 4. The effect of DMSO concentration on the enhancement of HSV-I DNA infectivity. Varying concentration of DMSO dissolved in HeBS (●) or eagle's medium (■) (Stow & Wilkie, 1976).
Regarding the formation of DNA/calcium phosphate precipitates, they found that it is critical to add the solution of DNA/\(\text{CaCl}_2\) to the HEPES-phosphate buffer rather than in the reverse order. Also, they claimed that it is important to add the solution drop-wise, rather than directly (Corsaro & Pearson, 1981).

In 1982, a research group at Yale University conducted research on the mechanism for entry of DNA/calcium phosphate complex into mammalian cells by electron microscopy and fluorescent dyes (Loyter et al., 1982a; Loyter et al., 1982b). Electron microscopy and filter hybridization studies revealed that most of the DNA strands enter by phagocytosis. The effect of different drugs and respiratory inhibitors on the entry of DNA was also investigated (Table 4, Fig. 5). Results showed phagocytosis of DNA is inhibited both by respiratory inhibitors and drugs, such as Colcemid, which disassemble microtubules. They concluded that the uptake of DNA/calcium phosphate resembles "receptor mediated" phagocytosis. Also it was seen that ATP-depleted and cold treated cells were not able to adsorb the complex. Thus the authors claimed that the phagocytosis of DNA/calcium phosphate complex is an energy- and temperature-dependent process (Loyter et al., 1982a).

![Fig. 5. Effect of increasing concentration of Colcemid on the entry of DAPI-stained DNA/calcium phosphate complexes into Ltk- cells (Loyter et al., 1982a).](www.intechopen.com)

These researchers also claimed that the pH of the formation of the DNA/calcium phosphate complexes is crucial for successful gene transfer. Studies on the effect of pH and DNA concentration on the entry of fluorescent dye-labeled DNA into cells showed that only during the calcium phosphate complexes formation in the pH range of 7.1 to 7.5 could fluorescent spots be visualized in the cytoplasm of recipient cells. For the complexes formed above pH = 7.5 no entry to cells could be detected (Fig. 6A). On the other hand, the DNA/calcium phosphate ratio is important on the adsorption of the complexes. When higher concentrations of DNA was utilized with the constant concentration of calcium phosphate, adsorption was not affected, whereas the appearance of cytoplasmic fluorescence was drastically reduced (Fig. 6B) (Loyter et al., 1982b).
Table 4. The effect of various drugs and respiratory inhibitors on introduction of DNA into *Ltk*⁻*Aprt* cells (Reproduced from (Loyter et al., 1982a)).

<table>
<thead>
<tr>
<th>System</th>
<th>Effect of DNA Entry</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drugs</strong></td>
<td></td>
</tr>
<tr>
<td>Cytochalasin B (1-4 μg/ml)</td>
<td>No effect</td>
</tr>
<tr>
<td>Colcemid (5 μg/ml)</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td>DMSO (10%, 10-30 min)</td>
<td>No effect</td>
</tr>
<tr>
<td><strong>Respiratory inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>2 deoxyglucose</td>
<td>Partial inhibition</td>
</tr>
<tr>
<td>NaN₃</td>
<td>Partial inhibition</td>
</tr>
<tr>
<td>NaF</td>
<td>Partial inhibition</td>
</tr>
<tr>
<td>NaF + 2 deoxyglucose</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td>NaN₃ + 2 deoxyglucose</td>
<td>Complete inhibition</td>
</tr>
</tbody>
</table>

Fig. 6. Adsorption (cells containing adsorbed fluorescent dots) and uptake (cells containing more than 10 intracellular fluorescent dots) of DAPI-stained DNA as a function of the pH of the DNA/calcium phosphate complex (A) and DNA concentration in DNA/calcium phosphate complex (B) (Loyter et al., 1982b).

One of the limitations of calcium phosphate systems in gene delivery applications is that most of the input DNA is degraded before it reaches the nucleus of the cell, where gene expression and DNA replication take place. In 1983, Luthman and Magnusson conducted research on increasing the efficiency of transfection by inhibiting the lysosomal degradation using Chloroquine as a lysosomotropic compound. For this purpose they used a conventional procedure for transfection with calcium phosphate, but they added Chloroquine to the growth medium of the cells. In Fig. 7 the effect of Chloroquine...
concentration on transfection efficiency using DNA/calcium phosphate complexes can be seen. The authors concluded that when Chloroquine treatment was effective, it increased the fraction of cells that could be successfully transfected. They claimed that this conclusion was supported by the results of experiments in which cells were transfected with linear forms of viral DNA. In that case, in Chloroquine treated cells, the number of DNA molecules which had re-circularized and were able to replicate was much larger than untreated cells (Luthman & Magnusson, 1983). With the same approach, in 1984 a research group in Norway used different inhibitors of intracellular degradation (such as 3-methyl adenine, NH$_4$Cl, FCPP and etc.) and claimed that the frequency of transformation was increased due to increasing the cytoplasmic level of exogenous DNA (Table 5) (Ege et al., 1984).

In 1987, Chen and Okayama introduced a new method for gene delivery with calcium phosphate systems. The aim of their work was the formation of DNA/calcium phosphate complexes gradually in medium during incubation with cells. They found that in this method the crucial factors that affect the transfection efficiency are the pH of the buffer used for calcium phosphate precipitation (optimized pH was 6.95) and the CO$_2$ level during the incubation of DNA with cells. They also found that the amount and the form of DNA are important factors. It was observed that circular DNA has better efficiency than linear DNA but, the reason for this phenomenon was not clear at that time. The authors claimed that the efficiency of their method is comparable to the efficiency of other common transfection systems of that time (Chen & Okayama, 1987).

In 1990 Orrantia and Chang investigated the intracellular distribution of DNA after the DNA/calcium phosphate complexes move into the cells. Results showed that only a small fraction of internalized DNA could be found in the nucleus, the target place for gene delivery. In the enriched nuclear fraction, the mouse cells retained 6.4% of internalized DNA while the human cells retained only 2.2% (Fig. 8).

![Graph](Fig. 7. Effect of Chloroquine concentration on transfection efficiency. Rat-1 cultures were transfected by co-precipitating calcium phosphate and polyoma DNA, 20 ng (■) and 100 ng (○) (Luthman & Magnusson, 1983).)
Table 5. Effect of different compounds on the transformation frequency of rat 2 \( tk \) cells transfected with pAGO DNA 6 hours after incubation of the indicated compounds with the cells (reproduced from (Ege et al., 1984)).

The authors concluded that transfection with DNA/calcium phosphate is a procedure with low efficiency partly because most of the endocytosed DNA is quickly degraded and excreted to the cytosol (Orrantia & Chang, 1990).

In 1994, O’Mahoney and Adams modified the calcium phosphate transfection procedure described by Chen and Okayama in 1987 and claimed that they reached a reliable and reproducible method with high transfection efficiency. They claimed that the critical factor in this method is the standing time of the DNA/CaCl\(_2\)/BES-buffered saline prior to addition to cultured cells. They concluded that in the optimal condition it is possible to reach 100% efficiency (Omahoney & Adams, 1994).

![Fig. 8. Distribution of internalized DNA in subcellular fractions from human and mouse cells. Cultured Cells were transfected with \(^{32}\)P-labeled high-molecular-weight DNA/calcium phosphate for 4 h. ■: Human primary fibroblast cells, □: Transformed mouse Ltk- cells (Orrantia & Chang, 1990).](www.intechopen.com)
In 1996, a research group in Taiwan conducted some research works on electrochemical properties of DNA/calcium phosphate complexes. The study focused on the variation of zeta potential with changes in pH for calcium phosphate and DNA/calcium phosphate complexes. The point of zero charge (pzc) and isoelectric point (iep) were found to be at pH 7.09 and 7.0, respectively. With addition of plasmid DNA, both pzc and iep points shifted to higher values of 7.18 and 7.15, respectively (Yang & Yang, 1996a).

In their other research on this topic, they revealed that the pH of the formation of DNA/calcium phosphate complexes and the concentration of DNA within the complexes were the crucial factor for the entry of these complexes to cells. The results of their study showed that optimum transfection efficiency occurred in the region close to the iep of DNA-calcium phosphate co-precipitates of pH 7.15 and close to the maximum flocculation of this colloidal system. The enhanced cell transformation efficiency occurred at pH 7.01. The zeta potentials of the DNA co-precipitates prepared in the absence of DMEM and calf serum were determined to lie between 11 and 21 mV. Preparation within these limits resulted in an efficient internalization of the DNA/calcium phosphate complexes, and for endocytosis to occur (Yang & Yang, 1996b).

In 2004, Jordan and Wurm investigated the methods that were applied previously for gene delivery with calcium phosphate particles by different authors. They stated that all of the numerous variations of the protocol found in the literature are based on the same principle—a spontaneous precipitation that occurs in supersaturated solutions. Although a wide range of conditions will lead to precipitates, high transfection efficiencies are only obtained within a narrow range of optimized parameters that assure certain properties of the precipitate. Finally, they concluded that despite a rapidly growing choice of efficient transfection reagents, this method remains highly attractive due to its highly biocompatible nature (Jordan & Wurm, 2004).

### 3.2 Current studies

Research on using calcium phosphate nanoparticles for gene delivery application is still continuing. Researchers perform a lot of new experiments to optimize the parameters involved in gene delivery with calcium phosphate nanoparticles. We have tried to review some of these studies in this chapter.

A research group in the University of Duisburg-Essen, proposed a method to prepare multi-shell calcium phosphate/DNA particles. They utilized a simple method to prepare multi-shell calcium phosphate as illustrated in Fig. 9.

They prepared different nanoparticles and showed that with multi-shell calcium phosphate/DNA nanoparticles the transfection efficiency is increased due to the protection of DNA against nuclease enzymes (Fig. 10). Moreover, the authors claimed that in contrast with conventional calcium phosphate, these particles could be stored for weeks without loss of their transfection efficiency (Sokolova et al., 2006).

They also showed that the standard calcium phosphate method selectively unbalanced intracellular calcium homeostasis while it remained at low control levels after transfection using nanoparticles. They concluded that with using DNA-functionalized calcium phosphate nanoparticles, cells are able to cope with the associated calcium uptake and therefore proved their method to be a superior transfection method (Neumann et al., 2009).

Hanifi et al. conducted some research on the feasibility of using strontium and magnesium substituted calcium phosphate in gene delivery applications. They prepared the particles via a simple sol-gel route. They obtained some particles with nano-size structure, high specific
surface area, and a high dissolution rate (Fig. 11). The zeta potential (Table 6) was increased in comparison with simple calcium phosphate. They concluded that due to increased surface charge and solubility, these novel systems could increase the gene transfection efficiency (Hanifi et al., 2010a; Hanifi et al., 2010b).

Fig. 9. Schematic set-up of the apparatus used for preparation of DNA-functionalized calcium phosphate nanoparticles. Calcium nitrate and diammonium hydrogen phosphate solutions are mixed in a vessel to form a precipitate. A part of the dispersion is taken with a syringe and mixed with DNA solution in an Eppendorf tube (Sokolova et al., 2006).

Fig. 10. Comparison of the transfection efficiency of multi-shell calcium phosphate/DNA by different methods. There are significant differences between single-shell and triple-shell (P<0.01) and triple-shell and the standard calcium phosphate methods (P<0.05) (Sokolova et al., 2006).
Recently there has been an approach to incorporate other agents or materials with calcium phosphate to improve its function as a gene delivery system. Stabilizing with bisphosphonate (Giger et al., 2011), coating with lipids (Zhou et al., 2010), incorporating in alginate hydrogel (Krebs et al., 2010) and association with Adenovirus (Toyoda et al., 2000) are some examples for this approach.

Fig. 11. Concentration of Ca\(^{++}\) ions in SBF solution after predicted period of time. A: Sr-CaP, B: Mg-CaP (Not Published).
<table>
<thead>
<tr>
<th>Sample composition</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sr-Substituted CaP</td>
<td>0.0Sr-CaP: 4.5±0.1</td>
</tr>
<tr>
<td></td>
<td>0.5Sr-CaP: 5.0±0.2</td>
</tr>
<tr>
<td></td>
<td>1.0Sr-CaP: 6.1±0.1</td>
</tr>
<tr>
<td></td>
<td>5.0Sr-CaP: 7.3±0.3</td>
</tr>
<tr>
<td></td>
<td>10.0Sr-CaP: 7.8±0.2</td>
</tr>
<tr>
<td>Mg-Substituted CaP</td>
<td>0.0Mg-CaP: 3.2±0.5</td>
</tr>
<tr>
<td></td>
<td>0.25Mg-CaP: 6.7±0.4</td>
</tr>
<tr>
<td></td>
<td>0.50Mg-CaP: 7.5±1</td>
</tr>
<tr>
<td></td>
<td>1.0Mg-CaP: 8±0.8</td>
</tr>
</tbody>
</table>

Table 6. Surface charge of Sr and Mg substituted calcium phosphate nanoparticles (Reproduce from (Hanifi et al., 2010a; Hanifi et al., 2010b)).

4. Conclusion

Nano-particulate calcium phosphate has shown several interesting advantages in biomedical applications because of its biocompatibility and easy preparation process. The DNA condensation characteristic of nano-particulate calcium phosphate makes it a potential choice for gene therapy system applications. Nano-particulate calcium phosphates are able to condense DNA strands, carry them in the blood, deliver the genetic material to target cells, and move them into cells resulting in reasonable transcription. Therefore, there is a common agreement among most of the works regarding gene delivery application on utilizing the calcium phosphate to deliver the gene into the nucleus; the final target of gene therapy methods. Because of the advantages of the DNA/calcium phosphate complex, it is one of the highly appealing systems currently studied, although it has been used in in-vitro gene delivery for many years already. The translation of its application into clinical therapy methods requires more work.

Researchers need to solve the instability of calcium phosphate in physiological conditions. If calcium phosphate/DNA complexes degrade in the blood circuit, it cannot be used in most of the clinical gene delivery applications. The other problem is the low transfection efficiency, which currently limits the application of the system. There are controversial reports about the transfection efficiency of calcium phosphate/DNA system, mostly because of instability and the complicated nature of calcium phosphate in solution. Once these problems are overcome by adequate novel technologies, the excellent biocompatibility and biodegradability of calcium phosphate remains as a major advantage.

5. References


Nano-Particulate Calcium Phosphate as a Gene Delivery System


www.intechopen.com


This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:
