1.115. Calcium Phosphates for Cell Transfection

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1.115.1. Introduction

Transfection is the process of introducing nucleic acids into cells by nonviral methods, whereas transduction is a term generally used to describe virus-mediated DNA transfer. Naked DNA is not able to significantly transfect cells ex vivo, although some limited gene transfer can be detected when injected into muscles. This is the reason why materials and protocols have been developed in order to increase the efficiency of transfection for therapeutic use.

Today, any gene can be artificially synthesized and made readily available for gene therapy. Thus, the challenge is to get a good vector to deliver this gene in the right cell type. Since the first protocol of gene therapy used in humans was approved in September 1990, the number of available vectors has dramatically increased while the ideal vector is still to be found.

Viral vectors have been widely used for clinical trials due to the natural ability of viruses to deliver genetic material into the cell nucleus. They represent the most effective way to deliver DNA, but show limitations regarding cell type targeting, size of carried DNA, production and packaging problems. Furthermore, immunological reactions, cytotoxicity, and hazardous integration events (when using retroviruses as vectors) led to secondary effects and even the death of several patients in clinical trials making viral vectors difficult to use for large-scale clinical research (Table 1).

Besides gene delivery methods based on recombinant viruses, there are various alternative methods of introducing foreign DNA into eukaryotic cells. Physical methods have been developed since the permeability of cellular membranes is increased by electric or heat shocks, magnetic force or ultrasound. Although efficient in vitro, these physical methods are difficult to adapt for in vivo. Finally, many materials have been used as carriers for DNA transfection, including hydrogel polymers, polycationic lipids, polylysine, polyornithine, dendrimers, histones, and other chromosomal proteins, and precipitated calcium phosphate. These different carriers apply the theory and methods of advanced particulate drug delivery to introduce DNA in selective somatic cells. In these systems, DNA is condensed to form nanoparticles which are up taken in the endocytic pathway. Functional molecules can be incorporated in these DNA nanoparticles in order to target a

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<td>HA</td>
<td>Hydroxylapatite</td>
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<tr>
<td>HLA-DR</td>
<td>Major histocompatibility complex (class II)</td>
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<tr>
<td>Lac-Z</td>
<td>Bacterial enzyme β-galactosidase</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
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<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
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Liposomes are lipids that are observed to form bilayers, similar to those in biological membranes. They are easy to prepare and consist of one or several lipid bilayers that separate intravascular space from an external medium. Within these lipid bilayers, liposomes can carry different kinds of surface molecules such as viral glycoproteins, giving them some specificity for an organ or a place where they can be trapped in the liver, spleen, and lung. Liposomes are phagocytosed by macrophages and are rapidly degraded in the lysosomal space.

In addition, liposomes must exhibit membrane-fusion promoting molecules at their surface so that they can escape the lysosomal degradation. DNA vaccination consists in transfection of subcutaneous or muscular cells with a gene coding for a viral or a bacterial protein that will be recognized by the adaptive immune system as a foreign antigen. When transiently expressed by transfected cells, this foreign antigen will trigger an antibody response that provides long-term immunity against the corresponding pathogen. Furthermore, the mechanical properties of powdered bioceramics also allow their spray with a gene gun.

Among these transfection agents, liposomes are particularly interesting. Liposomes are spherical vesicles with a diameter ranging from 20 nm to a few thousands nm. These vesicles consist of one or several lipid bilayers that separate an intravascular space from an external medium. Within these lipid bilayers, liposomes are phagocytosed by macrophages and are rapidly trapped in the liver, spleen, and lung. In addition, liposomes must exhibit membrane-fusion promoting molecules at their surface so that they can escape the lysosomal degradation of DNA. They can also carry different kinds of surface molecules such as viral glycoproteins, giving them some specificity for an organ or a defined tissue.

The synthetic vectors are applicable in vitro with isolated cells, but in vivo applications are greatly limited by their toxicity or because physicochemical conditions necessary for the transfection are difficult to reach in an open medium. Transfection agents are much less efficient than viral vectors with regard to the transfection yield but they can be used with almost all gene size at the opposite of virus.

DNA vaccination is a technique that involves the transfection of subcutaneous or muscular cells with a gene coding for a viral or a bacterial protein that will be recognized by the adaptive immune system as a foreign antigen. When transiently expressed by transfected cells, this foreign antigen will trigger an antibody response that provides long-term immunity against the corresponding pathogen. Furthermore, the mechanical properties of powdered bioceramics also allow their spray with a gene gun.

Maybe the most important argument for using this material would be to obtain a DNA vaccine that recruits and activates dendritic cells (DC). In this field, preliminary results suggest that HA particles could both mediate DC transfection and induce their maturation as particulate bioceramics were proven to act as a vaccine adjuvant. DNA vaccination has been highly effective in mice, while less immunogenic in different primates including humans. The efficiency of DNA vaccination depends on appropriate interactions between the foreign antigen, lymphocytes, and antigen-presenting cells (APCs). B lymphocytes need helper signals from T lymphocytes to produce antigen-specific antibodies, but the efficient activation of antigen-specific T lymphocytes critically relies on three kinds of biological signals provided by APCs. The APCs must (1) express MHC molecules to present antigen-derived peptidic fragments to T lymphocytes, (2) express suitable costimulatory molecules at their surface like CD80 and CD86, and (3) secrete costimulatory cytokines in their microenvironment such as IL-12. Only DC that have received a maturation signal can express these different signals at optimal levels to mediate T lymphocyte activation.

We have demonstrated that when human immature dendritic cells were grown in vitro with hydroxylapatite particles, they matured within 48 h. The presence of the particles induced the expression of CD40, 83, 86, and HLA-DR by the cells. It means that the HA bioceramic particles trigger in the APCs a series of events necessary for the priming of T lymphocytes. Interestingly, HA-induced activation of APCs critically relies on TLR4, a membrane receptor well-characterized for its role in the detection of pathogen-associated molecular patterns like bacterial lipopolysaccharides and viral glycoproteins.

1.115.2. Applications of Bioceramic Cell Transfection

Many clinical assays have been carried out in order to correct gene expression deficiencies. However, transfection protocols based on bioceramics could be more useful for two particular applications, DNA vaccination and bone tissue engineering that do not require the integration and long-term expression of the transgene (Table 3).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biological characteristics of viral and nonviral vectors</th>
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<tr>
<td><strong>Viral</strong></td>
<td><strong>Nonviral</strong></td>
</tr>
<tr>
<td>Cell specificity</td>
<td>No limit on plasmid size</td>
</tr>
<tr>
<td>High transfection efficiency</td>
<td>Toxicity predictable</td>
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<tr>
<td>Transportation mechanism of DNA into nucleus</td>
<td>Low transfection efficacy</td>
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<tr>
<td>Immune reaction against viral proteins</td>
<td>No integration</td>
</tr>
<tr>
<td>Virus infectivity</td>
<td>Low transfection efficacy</td>
</tr>
<tr>
<td>Limitation on gene size</td>
<td>Not always efficient in vivo</td>
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<td>Random chromosomal insertion: possibility of proto-oncogene activation</td>
<td>Unclear nuclear transport</td>
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<th>Table 2</th>
<th>Barriers in gene delivery</th>
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<tr>
<td><strong>Extracellular</strong></td>
<td><strong>Intracellular</strong></td>
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<tr>
<td>Plasma nuclease</td>
<td>Endosomal escape</td>
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<td>Uptake by reticuloendothelial system</td>
<td>Lysosomal degradation</td>
</tr>
<tr>
<td>Opsonization</td>
<td>Cytoplasmic nuclease</td>
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<tr>
<td>Complement activation</td>
<td>Translocation to nucleus</td>
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<tr>
<th>Table 3</th>
<th>Diseases and conditions of the musculoskeletal system that may be amenable to gene therapy</th>
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<tr>
<td><strong>Inherited</strong></td>
<td><strong>Acquired</strong></td>
</tr>
<tr>
<td>Osteogenesis imperfecta</td>
<td>Arthritis</td>
</tr>
<tr>
<td>Familial osteoarthritis</td>
<td>Arthritis</td>
</tr>
<tr>
<td>Osteopetrosis</td>
<td>Delayed unions (pseudarthrosis)</td>
</tr>
<tr>
<td>Certain crystal-deposition arthropathis</td>
<td>Osteosarcomas, chondrosarcomas, bone metastasis</td>
</tr>
<tr>
<td>Certain form of osteoporosis</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>Inherited abnormalities of the growth plate</td>
<td>Rheumatic disease</td>
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<tr>
<td>Ehler–Danlos syndrome and other connective-tissue diseases</td>
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pDNA to make efficient vaccine in human beings without the need to bring additional signals to trigger the adaptive immune response.

Calcium phosphate bioceramics were used for decades for bone reconstruction and for vectorization of bone cell progenitors. In this field, this technology can be used to induce the differentiation of cells grown at the material surface. Differentiation of Mesenchymal Stem Cells (MSCs) in osteoblasts for example using pDNA bearing BMPs genes could be achieved using porous ceramics with high surface area on which the pDNA is immobilized. MSCs are generally dedifferentiated after a culture period even when they are phosphatase alkaline positive, and the presence of morphogenetic proteins at the surface of the material would permit to induce osteoprogenitor cell differentiation and maintain cells in this differentiated state in culture. This type of material would also be useful for direct implantation into connective tissue such as pseudarthrosis fibrous tissue in order to transfect the cells of the surrounding tissue in order to modify their differentiation by transfection.

1.115.3. The Role of Bioceramic Characteristics for their Applications

Bioceramics show biological and physicochemical characteristics, which make possible their use in special applications.

1.115.3.1. Bioactivity

Bioceramics are bioactive materials interacting with bone tissue when implanted inside bone to be totally integrated in several stages and replaced by the neoformed bone. This property makes these materials particularly adapted to the transient transfection of bone cells, in particular osteoblasts and/or osteoclasts which are functionally deficient in some genetic diseases like osteogenesis imperfecta or aging diseases like osteoporosis. There is a number of genetic and acquired bone diseases clearly identified which could benefit from bioceramic transient transfection (Table 3).

1.115.3.2. Degradability

Bioceramics are degradable and get replaced by bone following a process of resorption/reconstruction identical to that of natural bone. The ceramic grain boundaries are first resorbed. This degradation of the material allows the release of micro- or even nanoparticles that trigger biological reactions which can be useful for special applications. A good example of this is the transient induction of a foreign body reaction around the microparticles released by the calcium phosphate ceramics (Figure 2). The foreign body reaction is constituted by cells of monocytic origin involved in the immune response and in particular antigen presentation to the lymphocytes. Antigen expression...
in antigen presenting cells might enhance immune response. The degradability can thus amplify the foreign body and innate immune reaction.

1.115.3.3. Physicochemical Properties

The biological activity of bioceramics is linked to their physical characteristics, and in particular properties of their surface. We have demonstrated that the toxicity and the inflammatory power of hydroxylapatite (HA) – particles were related to their physical properties, while their chemical composition was the same. In the field of vaccination for example, the physical properties of ceramic HA particles, in particular their size and structure of their surface, show a major influence on the synthesis of interleukins and TNF that determine the adjuvant properties of the particles.

Bioceramics can be powdered, porous, or under a bulk material form. Compared to the other transfection tools, the solid state of bioceramics which are implanted in a specific site allows a kind of cell specificity as the transfected cells must come into contact with the material surface. For example, it is of interest for tumor cell transfection since the material can be implanted directly in the tumor.

1.115.4. Traditional Use of Calcium Phosphate for Cell Transfection

Calcium phosphate has been one of the earliest transfection agent under the form of nanoparticles coprecipitated with pDNA. These nanoparticles are obtained by precipitation, through the incubation of pDNA in a supersaturated calcium and phosphate solution. The particles precipitated are nanosized and formed by a coprecipitate of DNA and a poorly characterized calcium phosphate. Transfection is achieved when this coprecipitate is contacting a cell monolayer.

This method has a poor transfection yield, with only a few percents of transfected cells being obtained. Above all, this method is only tractable in vitro in a closed environment as in vivo in an open medium the supersaturation cannot be reached. For this reason, the use of this method for in vivo cell transfection must be made in two times. The cells must be first transfected in culture and then injected in the tissue. It is also possible to isolate the nanoparticles from the solution after their formation in vitro and inject it in the tissue to be transfected. However, the migration risk is very high.

Higher transfection yields (60%) can be obtained when the precipitation factors are better controlled. Temperature, DNA concentration, and reaction time were shown to be crucial for better results over standard procedure. Almost 100 000 plasmid molecules could be delivered into individual cells.

Cell cycle is also a key factor for transfection efficiency, and the percentage of cells in S phase when adding the transfection agent correlates with the percentage of transfected cells. The nuclear membrane is dismantled during mitosis, and this probably increases the number of pDNA molecules accessing the nuclear environment.

However, the transfection mechanism is unclear. It seems that the entrapped DNA in the nanoparticles is protected at least partially from the degradation by cellular nucleases, and released by the calcium phosphate degradation in the low pH environment of the endosomes and lysosomes. The way of migration from these cellular compartments into the nucleus is highly speculative.

1.115.5. Hydroxylapatite Ceramics for Cell Transfection

We have developed new hydroxylapatite ceramics for transfection. They have different characteristics from the traditional calcium phosphate nanoparticles obtained by precipitation.

1.115.5.1. Physicochemistry

The calcium phosphate ceramics are different from the nanoparticles obtained by coprecipitation. The ceramics are made of calcium phosphate grains joined by grain boundaries due to the sintering process, with a porosity between the grains that depends on the sintering temperature. This structure makes the material surface area much lower than that of coprecipitates and less reactive. The degradation of the calcium phosphate ceramic is well documented. The degradation of the grain boundaries triggers the release of grains or grain aggregates that are endo- or phagocytosed depending on their size. The internalized particles are then solved inside the low pH cell compartments. Interestingly, we have shown the presence of multinucleated giant cells that contribute actively to the dissolution of the material at the cell–material interface.

DNA is a negatively charged molecule. Calcium phosphate ceramics used in our experiments also show a negatively charged surface. However, we have been able to immobilize pDNA at the surface of calcium ceramics, suggesting that pDNA binding to HA particles is not electrostatic. Numerous theories have been published to describe the interaction between organic molecules and HA. None of them however takes into account the linked water or the modification of surface occurring at the surface when the ceramics are immersed into a saline solution. The epitaxial growth of carbonated apatite at the surface of HA ceramics has been widely described. It is suggested that DNA could be coprecipitated at the surface of the ceramic with the carbonated apatite. The protocol to immobilize pDNA at the ceramic
surface includes the maceration of the powder in a saline solution containing the DNA. Precipitation is then visible at the ceramic surface.40

1.115.5.2. Transfection of Isolated Cells

Isolated cells, established or primary cell lines grown in monolayer, were transfected using pDNA loaded ceramic powder. The powder must be introduced into the culture flask and let in contact with the cell layer to transfect for various periods of time. Transfection is generally time dependent. With a constant amount of powder, the percentage of cells expressing the reporter gene is dependent on the time of incubation. The yield of transfection is also dependent on the mass of powder used for the transfection (Figures 3 and 4).

1.115.5.3. Transfection of Tissue Culture

Different tissues can be maintained in culture and used to test their behavior in contact with some biomaterials. The interactions between the different cell types is relatively conserved on such biopsies and their differentiated functions maintained, allowing a better understanding of their relationships with the transfection material than when working with isolated cells.

We grew small fragments of bone tissue from rats in contact with HA-beads loaded with pDNA containing a galactosidase reporter gene for two periods of time, 8 and 21 days. The grown bone was made of calvaria, cancellous bone, and growth plate cartilage. The histology of the bone fragments showed that almost no cells were positive at 8 days while all cells expressed the LacZ reporter gene at 21 days. It was significantly different from the control made of pDNA in PBS.

The calvaria histology demonstrated that the HA beads were covered by layers of proliferating cells coming from the periostium of the immature bone formed by a direct ossification process. Some multinucleated cells from monocytic origin were also present at the contact of the HA particles. The cells clearly expressed the LacZ gene whatever their distance from the beads (Figure 5).

The same results were observed with other types of bone. The cells were stained in blue whether they are proliferating like the mesenchymal cells located inside the pores of the cancellous bone or not as it was shown for the chondrocytes.

1.115.5.4. In Vivo Transfection

The same HA particles implanted in a connective tissue such as rabbit bone tissue trigger a foreign body reaction before integration within newly formed bone. Within 3 weeks, the most of the cells of the foreign body reaction at the particle contact expressed the LacZ gene (Figures 6 and 7). Other cells of monocytic origin were found positive for galactosidase staining. Osteoclasts in Howship’s lacunae in the cancellous bone at remote location from the implantation zone were found to express the transgene. Some of these cells were found among the connective tissue present inside the bone pores. Other cells were labeled in this region, including some perivascular and mesenchymal cells, but also some cells of the dental ligament.

The cells labeled at 3 months are different. Fibroblasts of connective tissue and even odontoblasts expressed the galactosidase reporter gene.

1.115.6. Perspectives and Material Evolution

At the opposite of other transfection agents, bioceramics can be shaped. They can even be put in suspension to make solutions of bioceramic powder that can be injected or delivered by spray. Some interesting results were obtained using carboxymethyl cellulose without altering the powder transfection properties. The shaping of the bioceramics will allow their use in the various applications evoked in this chapter.

**Figure 3** Established murine gliosarcoma cell line grown a few hours in the presence of HA particles showing that many particles were phagocytosed.

**Figure 4** Transfection of a HEK293T cell line with different concentration of HA particles at 24 h. The particles were suspended at different concentrations in 1 ml of culture medium. The percentage of the transfected cells increases with the powder concentration.
Two other characteristics need to be improved in order to use such particles for *in vivo* transfection. First, particles injected in the blood stream are recognized by the innate immune system and are rapidly cleared by the spleen, the liver, and to a lower extent by the lungs. Thus, it is very important to minimize their recognition by macrophages so that the material is able to reach and enter targeted organs or tissues. Second, in spite of many works that have been published in the last 30 years, it is impossible to achieve transfection of a specific cell type by addressing particles into a specific tissue. This needs to be improved. Associated to a better biocompatibility of the material, humanized antibodies immobilized at the material surface should provide a way to target specific cells. In contrast to their nonhumanized counterparts, these molecules have proved to be able to target specific cells before they get eliminated by the immune system. The chemical molecules bridging these molecules to the material surface should also be eliminated or hidden in the body to minimize that their recognition by the immune system.

References