Calcium Based Non-viral Gene Delivery: An Overview of Methodology and Applications

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Abstract- Application of therapeutic gene transfer in the treatment of genetic diseases is a notable progress but there are some disadvantages and limitations in it. The process of overcoming these barriers is a drastic change in gene delivery. Recently, calcium phosphate nanoparticles alone, or in combination with viral and nonviral vectors, were found to have a positive effect on gene transfer especially when incorporated in the colloidal particulate systems. This review elaborates on various successful methods of using calcium phosphate nanoparticles in gene delivery, which are considered an advancing approach to gene delivery.
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Introduction

A fundamental goal in various scientific areas including biotechnology and nanotherapy is the ability of safe and efficient transfer of foreign DNA into cells. Recently, rapid advances have been made in DNA stability and transport. Non-viral vectors are being developed as alternative carriers of viral vehicles, because of some advantages including relative ease of production and scale up, higher DNA carrying capacity and lower immunogenicity and cytotoxicity (1-3). Among the non-viral vectors, complexes of nucleic acids and organised lipid and/or phospholipid molecules (lipoplexes and nanolipoplexes) are the most applied (4). The effectiveness of expression of foreign gene depends on several factors including stability of DNA molecules, unpackaging of the DNA-vector complex, intracellular release of DNA and its targeting to the nucleus. The toxicity and biocompatibility of these delivery systems along with their economical feasibilities should also be taken into consideration. A potential candidate for efficient nucleic acid delivery is the use of divalent cations or their combination with other vectors. Inorganic calcium has been used for the transportation of various nucleic acid molecules via complex formation with nucleic acid backbone (5-7). Calcium phosphate confers stability, targetability (8), and transportation across the cell membrane due to increasing membrane permeability (9). However, it suffers from poor reproducibility in precipitation and transfection (9, 10) because of various experimental variables and non-standardised procedures for transfection, such as pH, DNA concentration, temperature, time between precipitation, and the type of cells to be transfected. In this paper the role of the calcium in nucleic acid transportation, particularly with respect to the improvement of transfection efficiency of nanolipoplexes is reviewed.

Role of calcium phosphate in gene transfer

Divalent cations are an important class of carriers for bioactive molecules and their advantages include no microbial attack, storage stability and low cost (11). They have various biomedical applications in vaccination, drug delivery and as gene therapy vectors (12, 13). Calcium salts have been extensively developed and studied as a delivery system due to its biocompatibility and being physiologically acceptable. Calcium phosphate is considered to be the model compound for the inorganic constituent of bone and teeth. Calcium phosphate is used for manufacturing various forms of implants due to its excellent biocompatibility (14) when compared to iron oxide (magnetite) (15) or silica (16). Calcium phosphate

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particles are biocompatible, biodegradable (17) and do not cause adverse reactions at the site of injection when administered parenterally (18). Calcium plays an important role in endocytosis (as the major route of material internalisation) and has the advantage of being readily absorbed and high binding affinity for a variety of molecules (19). Therefore, calcium phosphate is being used as a delivery vehicle for various molecules and bioactive agents e. g. insulin (oral delivery) (20), growth factors (21), antibiotics and contraceptives (22, 23).

Furthermore, calcium phosphate precipitation is widely used as a routine laboratory procedure for the delivery of oligonucleotides (24) and plasmid DNA (pDNA) (25). The main advantages of calcium phosphate precipitation are simplicity, low cost and applicability to a wide variety of cell types. The mechanism is formation of ionic complexes with the helical phosphates of DNA and, may impart a stabilising function to certain DNA structures (9).

The transfection efficiency by this widely applicable inorganic particle depends on many experimental parameters including cell type, morphology of the precipitate, concentration, pH, precipitation time, type of DNA, the experimentalist (25-28, 10), cell line, laboratory setting, and particle size (13, 29, 30). The transfection solutions cannot be stored because the calcium phosphate nanocrystals grow with time into ineffective microcrystals.

A brief chemical shock (usually 10–25% glycerol or dimethylsulfoxide, or by the diethylaminoethyl-dextran) (31-33) in many cell types also require increases transfection efficiency. Chemical shock is not necessarily in slow formation of the calcium phosphate–DNA precipitation occurs in alkaline culture medium (25, 34). It has been reported that inclusion of glycerol in the culture medium during calcium phosphate transfection also improves transgene expression in several cell lines, obviating the need for additional chemical shock (35, 36). Recently Mozafari and co-workers developed a nanolipoplex formulation containing calcium and glycerol exhibiting reduced cytotoxicity and improved transfection properties (37). Glycerol, a physiologically acceptable chemical, was used in this formulation in place of potentially toxic organic solvents generally employed to prepare liposomes. Critical factors which affect the efficiency of DNA transfer and expression efficiency include the pH of the precipitate-forming solution and the culture medium, the buffer used for precipitate formation, the exact concentrations of calcium and phosphate employed, the time of precipitation, and the presence or absence of serum in the culture medium (25, 34, 38, 39). The time between precipitation and transfection is important since a longer time leads to a decrease in transfection efficiency due to bulk precipitation of calcium phosphate, which leads to varying particle sizes of the calcium phosphate-DNA complex (34, 40). Also DNase I degradation and interactions with serum proteins increase particle size due to aggregation and decrease in vivo efficiency (41).

Applications of calcium phosphate nanoparticles in gene transfer

As mentioned above, the standard calcium phosphate method strongly depends on the experimental parameters. The transfection solutions cannot be stored because the calcium phosphate nano-crystals grow with time into ineffective microcrystals. To overcome these disadvantages, simple and reproducible methods have been developed by adsorbing DNA onto calcium phosphate nanoparticles, preventing further particle size growth. Organic and inorganic additives have been used to inhibit their further growth and preserve the small size of them. Chowdhury et al. (41) showed that the addition of magnesium (in calcium phosphate nanoparticle preparations), inhibited the particle grow and increased transfection efficiency. Kakizawa et al. (42) prepared nanoparticles consisting of calcium phosphate, DNA and block-copolymers. A small size of the particles and good colloidal stability was achieved by the steric effect of a poly(ethylene glycol) (PEG) layer surrounding the calcium phosphate core (16, 42). Calcium phosphate nanoparticles are especially well suited for gene transfer because an in situ precipitation of the inorganic salt in the presence of DNA results in the formation of nanoparticles which cells can immediately take up. This has been the subject of extensive investigations (5, 7, 10, 16, 26, 30, 34, 43-46). The method is very easy and inexpensive, but the transfection efficiency is inferior to commercially available transfection agents, which are based on liposomes (47, 48) or polymers (49).

Welzel et al showed that custom made nanoparticles of calcium phosphate can be efficiently coated and colloidally stabilised by DNA and then used for cell transfection (44). The advantage of this method in comparison to the other conventional *in situ* precipitation approaches is a much better control over size and composition of particles. However, transfection efficiency could not be increased further by changing precipitation conditions (the amount of DNA and the nature of the nanoparticles), because several physical

and chemical barriers e.g. intracellular degradation in lysosomes should be overcomed before DNA entrance to nucleus (50-52).

A calcium phosphate nanoparticle served as core which was then coated with DNA for colloidal stabilisation. The efficiency of transfection could be considerably increased by adding another layer of calcium phosphate on the surface, thereby incorporating DNA into the particle and preventing its degradation within the cell by lysosomes. A subsequent outermost layer of DNA on the calcium phosphate gave a colloidal stabilisation. Such shells help to protect DNA from the nucleases digestion inside the cells. By a comparatively easy and well reproducible process (53) prepared calcium phosphate/DNA-nanoparticles with a calcium phosphate core and DNA/calcium phosphate shells (53) and showed that the transfection efficiency was significantly higher than that of simple DNA-coated calcium phosphate nanoparticles. The dispersions were stable and could be used for transfection after 2 weeks of storage at 41°C without loss of efficiency. Sokolova et al. (53) found that the DNA is still intact in the single- and multi-shell particles as shown by the high transfection efficiency.

Reverse emulsion technique is another method for the preparation of ultra low-size calcium phosphate nanoparticles encapsulating DNA molecules. Using microemulsion as the microreactor (30) prepared calcium phosphate nanoparticles possessing 85% DNA entrapment efficiency and an average particle size of 80 nm. Bisht et al. (54) showed that by further modification of the procedure, the calcium phosphate nanoparticles prepared showed 99% entrapment and a reduction of particle size from 80 to around 30 to 40nm. By using polycarboxylate segment of poly (ethylene glycol)-b-poly (aspartic acid) (PEG-b-PAA), the crystal growth is suppressed because of adsorption on the crystal surface to compensate for the increased interfacial free energy. The PAA segment of PEG-b-PAA is adsorbed on the crystal surface, leading to the formation of core-shell particles with a hybrid core of calcium phosphate crystal and pDNA surrounded by a PEG shell. The concentration of PEG-b-PAA influences the particle size of the complex, where an increased concentration causes reduction of particle size (16). These nanoparticles showed enhanced DNA stability, higher DNA entrapment, protection from nuclease and higher gene expression due to calcium mediated endocytosis. Diffusion of the active form of DNA into the nucleus through the nuclear pore is due to the dissolution of calcium phosphate nanoparticles in the cytoplasm, which has significantly lowered calcium and higher phosphate ion concentration compared with the extracellular environment, releasing free DNA (55). The dissolution of nanoparticles at endosomal pH (5.0 – 5.5) also causes osmotic imbalance and disruption of the endosomal compartment, leading to plasmid release into cytosol (54). Calcium phosphate-DNA nanoparticles showed no toxicity and better gene expression in vivo compared with standard precipitation method (56). Cellular uptake of these nanoparticles can be improved by combination with other vectors (27) or by incorporating specific ligands on the outer surface of the shell.

These experimental nanoparticle preparation procedures provide stronger control over precipitation, higher biocompatibility, biodegradation, stability and is a reproducible method indicating the feasibility of calcium phosphate nanoparticles as efficient nonviral vectors. By surface coating of these nanoparticles (with adhesive polymers e. g. chitosan or polyacrylic acid, chemically conjugating polyethyleneglycol (PEG) or a suitable targeting ligand) targeting to specific cells and tissues and long blood circulation can be achieved (57). Some reports are focused on PEG coated (pegylated) calcium phosphate nanoparticles encapsulating pDNA targeted delivery to tumors (58, 59).

The calcium phosphate (CaPi)-mediated gene transfer method has been modified by several groups of scientists in an attempt to improve the cell transformation efficiency. Transfection can be carried out either in the presence or absence of DMEM and calf serum 133Z11V'2. Calcium phosphates should be advantageous due to their high biocompatibility and good biodegradability compared to other types of nanoparticles used for cell transfection such as iron oxide (magnetite) or silica. In contrast to the classical calcium phosphate method, the particle/DNA dispersions can be stored for weeks without loss of their transfection efficiency. The DNA is still intact in the single- and multi-shell particles as shown by the high transfection efficiency.

Role of divalent cations in cationic nanolipoplex gene delivery

Complexes of cationic liposomes and DNA molecules i.e. cationic lipoplexes are widely used as vectors for in vitro and in vivo transfection (60-62). Physical characteristics of the liposomes (membrane fluidity, charge and size), and the nature of the target cell influence the mode of liposome interaction with the target cell membrane.

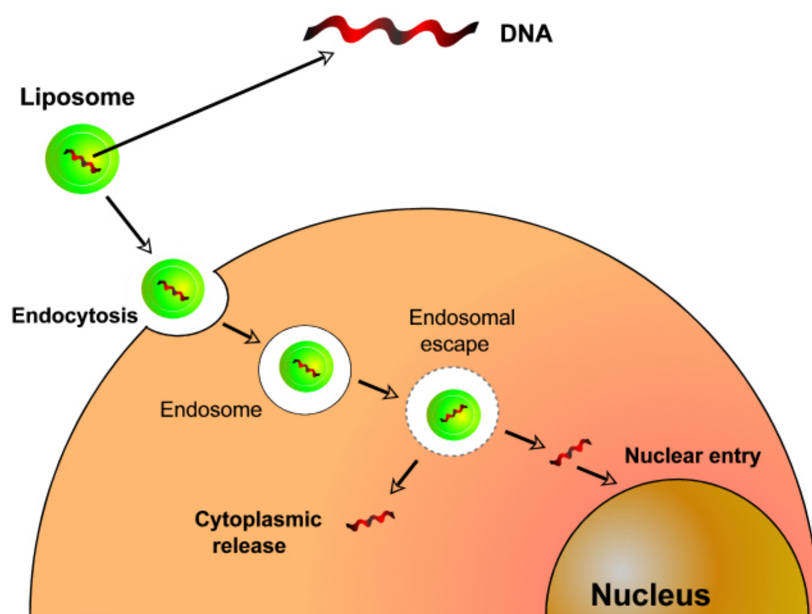

Figure 1. Schematic representation of endocytosis as a main mechanism of liposomal gene delivery into cells.

Endocytosis is the main mechanism of the interaction of liposome-DNA complexes with cells. After entrance of the lipid-DNA complex into the cells, it destabilises the endosomal lipidic layer components (63). Endosomal anionic lipids laterally diffuse into the lipid-DNA complex and form a charge neutral ion pair with the liposomal cationic lipids (6). This results in displacement of the DNA from the cationic lipid and release of the DNA into cytosol (Figure 1).

Serum affects the stability of liposomes and their ability to interact with cells (64). Furthermore, the transfection efficiency of cationic lipoplexes decreases in the presence of serum (65). It has been shown that serum proteins have an affinity to positively charged liposomes more than anionic ones (66). Consequently, transfection by cationic lipoplexes is a relatively ineffective process due to higher leakage by serum proteins, inefficient endosome escape during endocytosis, intracellular nuclease degradation of DNA, poor nucleus entry (67) and toxicity concerns (68, 69). Various efforts have been made to improve the transfection efficiency of cationic liposomes by using helper lipids, fusogenic peptides, DNA-condensing agents and nuclear localization signals as targeting ligands (70-74). Calcium phosphate is also used to enhance in vitro transfection efficiency of cationic lipoplexes by facilitating endocytosis and its entry to the nucleus (50, 75, 76). The improved transfection efficiency is due to a higher gene delivery to a variety of cell lines mediated by calcium and phosphate molecules. These results were specific to transfection with calcium ions and not with other divalent metals ions (77).

Role of divalent cations in non-cationic nanolipoplex gene transfer

The major limitation of some cationic liposomes in clinical applications is their toxicity. Cationic amphiphiles or detergents (78) are being inactivated in serum and have shown cytotoxic effects leading to cell death both *in vitro* (79) and *in vivo* (68). The origin of the toxicity caused by cationic liposomes has not been completely elucidated. It has been suggested that intermixing of the cationic lipids and the anionic lipids of cell organelle membranes, such as mitochondrial membranes, is responsible for cytotoxicity (6). Another postulated mechanism for cationic lipid-mediated toxicity in the lung is the involvement of reactive oxygen intermediates.

To improve the transfection efficiency of cationic liposomes and to reduce (or preferably eliminate) their toxicities, researchers have focused on the synthesis of new cationic lipids and the preparation of better formulations (70, 80). An alternative to cationic liposome protocols is employment of zwitterionic or

anionic lipid vesicles as gene transfer vectors. These species are much safer to the target cells and have longer circulating times and varied clearance profiles (81, 82). Repulsive electrostatic interaction between the anionic or zwitterionic lipids and negatively charged DNA (83, 84) results in unfavourable electrostatic interaction between these molecules; therefore, making them susceptible to low DNA molecule entrapment (85) and poor transfection. Low entrapment of DNA in anionic or zwitterionic liposomes was resolved by the addition of calcium, which neutralises the phosphate group on DNA (86). A method of incorporating DNA molecules, by the mediation of divalent cations, to anionic liposomes has been reported and the structure of the ternary complexes of liposome/Ca^{2+}/DNA has been characterised morphologically (87, 88). In addition, the mechanism of calcium induced DNA interaction with liposomes containing zwitterionic lipids, as well as those containing anionic lipids, has been studied (89). Calcium may also serve as a bridge between the phosphate head group of the lipid and the phosphate group of DNA. Another postulated mechanism is the interaction of calcium with the phosphate head group at the ratio of 1:2 (Ca^{2+}: phosphatidylcholine), leaving the head group of phosphatidylcholine with a positively charged amine, which interacts with the negatively charged DNA (90, 91). Calcium ions not only condense DNA molecules, but also complex with the nitrogen and oxygen at positions 7 and 6, respectively, of the guanine component. Readily release of free DNA from the Ca^{2+}–DNA complexes at high concentration of sodium; broader concentration range of Ca^{2+} for DNA condensation with low cell toxicity; and increased DNA entrapment in the liposome by Ca^{2+} mediating DNA-liposomes binding are also other advantages of using calcium ions.

The DNA aggregation and the free fusion of liposomes should occur simultaneously to achieve higher entrapment efficiency (92). Measuring the change in distance between the lamella repeat of liposomes vesicles due to calcium interactions with zwitterionic lipids in the presence of DNA was elucidated by differential scanning calorimetry and small-angle X-ray scattering (90). Not much research has been devoted to the effect of divalent cations on anionic lipoplex transfection. A bio-distribution study showed that one-third of the liposome-calcium DNA complex was still remaining in the circulation 1 h after administration. The highest amount of pDNA was found in the circulation (the majority remaining in the liver, spleen, and lungs) indicating that DNA is protected from serum nuclease. *In vivo* results demonstrate higher stability of neutral liposomes-calcium complexes as a DNA carrier in contrast with cationic liposomes (92). Although transfection efficiency of anionic liposome-DNA complex was enhanced by sevenfold without cytotoxic effect (unlike calcium liposomes), but in the presence of serum transfection efficiency was reduced. Therefore, anionic liposome would be useful for the *ex vivo* and *in vitro* application of DNA transfer into cell lines sensitive to cationic lipids (93). Tissue-specific targeting and overcoming the barrier of cell transfection by neutral liposome-calcium-DNA complexes would be possible with further improvement in this technique, thereby offering an important alternative nonviral gene delivery system.

The morphological changes of calcium phosphate in the transfection

Different modifications of this method have been proposed in the past to improve the transformation efficiency (5, 6, 25, 94). Although the precise function of CaPi remains unclear and the direct components participating in CaPi-DNA complex formation are undefined, it has been shown that the pH, DNA concentration and aging time of the CaPi suspension are critical factors for optimal transfection efficiency (25, 38). The kinetics of the formation of CaPi particles are controlled by the number of ionic species in solution through the dissociation of $CaCl_2$ and Na_2HPO_4 present in HEPES-buffered saline (HBS). The precipitation reaction is very complex, involving the formation of non-apatitic calcium phosphate phases during the early stages of nucleation (95). The experimental evidence of the effects of several environmental factors on the transformation efficiency suggests the possibility that alterations of the CaPi compositions and morphological changes had taken place during maturation of the precipitates in the CaPi-DNA colloidal dispersions. Relatively less attention has been given to the maturation and primary precipitates formed in the CaPi reaction solution. Yang et al has examined the morphological changes of the preparation of CaPi precipitates that occur during the DNA transfection process and studied the dependence of the conversion process of CaPi on the solution environment, including the effects of pH of the HBS and other ingredients in the culture medium, such as plasmid DNA, Dulbecco's modified Eagle medium (DMEM) and calf serum (7). The presented experimental evidence have showed the observations which was obtained from transmission electron microscopy (TEM) of the products in the CaPi-

DNA precipitation reactions, illustrating the conversion steps in this system and the dependence of the CaPi maturation process on several components in the preparations (7).

TEM was employed to investigate the reaction products of CaPi precipitates prepared in the presence or absence of plasmid DNA, Dulbecco's modified Eagle medium and calf serum. The effect of pH of the HEPES-buffered saline (HBS) in which CaPi was formed was also examined. The results showed that CaPi precipitates underwent several steps of conversion in the presence of other components in the cell culture medium. The pH of the HBS in which CaPi precipitates were formed also exerts a profound effect on the morphology of the CaPi solid phase, indicating that the associated compositional and structural changes occurred during the maturation of CaPi in the DNA transfection medium.

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