Expert Opinion

Role of calcium in gene delivery

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The treatment of genetic diseases using therapeutic gene transfer is considered to be a significant development. This development has brought with it certain limitations, and the process of overcoming these barriers has seen a drastic change in gene delivery. Many metal ions such as Mg^{2+}, Mn^{2+}, Ba^{2+} and, most importantly, Ca^{2+} have been demonstrated to have significant roles in gene delivery. Recently, calcium phosphate alone, or in combination with viral and nonviral vectors, was found to exert a positive effect on gene transfer when incorporated in the colloidal particulate system, which is an advancing approach to gene delivery. This review elaborates on various successful methods of using calcium in gene delivery.

Keywords: calcium phosphate, co-precipitate, liposomes, nanoparticles, nonviral vectors, plasmid, transfection, viral vectors

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1. Introduction

The modern era of molecular medicine has been highlighted by revolutionary accomplishments in genetics, genomics and human molecular biology. Therapeutic gene transfer is not a new concept as it was first developed more than two decades before the first gene transfer took place in the clinical setting [1]. The treatment of human disease by gene transfer was originally envisioned as a means to treat diseases arising from single gene defects. Inherited diseases encompass a wide range of disorders, wherein a defective gene leads to the failure to synthesise a particular protein or to the synthesis of an abnormal protein. In either event, the absence of the normal protein can lead to a variety of clinical manifestations, depending on the structural or enzymatic role that the protein normally plays in the cell. Such conditions range from mild disorders that require no treatment to life-threatening diseases [2]. Furthermore, the management of these genetic diseases by conventional pharmacological means are only partially effective in ameliorating the manifestations of the disease and are accompanied by significant complications. The size, complexity and cellular inaccessibility of proteins make their delivery impossible [3]. Conceptually, gene therapy can overcome these barriers by the selective introduction of recombinant DNA to the target site so that the biologically active protein can be synthesised within the cell whose function is altered. Treatment of these diseases at the genetic level seems to be a certainty due to the revolutionary advances in cell and molecular biology [4]. Therapeutic gene transfer is regarded as the employment of polynucleotide sequences as medicines to cure disease, or is more specifically referred to as nucleic acid delivery for subsequent clinical benefits [5]. Current programmes are applying gene delivery technology across a broader spectrum of disease conditions such as:

- replacing missing or defective genes,
- delivering genes that catalyse the destruction of cancer cells or cause cancer cells to revert back to normal tissue,
- delivering viral or bacterial genes for vaccination,

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- delivering genes that promote the growth of new tissue or stimulate regeneration of damaged tissue.

Nonviral delivery systems for gene therapy have been increasingly proposed as safer alternatives to viral vectors, as they have the potential to be administered repeatedly with minimal host immune response, they are targetable, are stable in storage and are easy to produce in large quantities [6]. In general, DNA is packed compactly into native genomes, and the manner of this packaging can be expected to influence the mechanism of gene expression. Various chemical agents, including polyamines, metal cations, neutral polymers and cationic peptides, can induce the *in vitro* compaction of DNA in aqueous solution [7,8].

Various nonviral vectors for gene delivery have been developed and they are classified as [9]:

- Mechanical: microinjection, particle bombardment, pressure;
- Electrical: electroporation (high and low voltage), iontophoresis;
- Chemical: diethylaminoethyl–dextran, calcium phosphate, artificial lipids (lipoplexes);
- Polycations or proteins (polyplexes), cyclodextrins, dendrimers;
- Other polymers (including controlled-release polymers).

1.1 Calcium particles as delivery vehicle

Inorganic particles are an emerging area for entrapping biomolecules; they have potential application in material science as their advantages include no microbial attack, excellent storage stability and low cost. These inorganic compounds have various biomedical applications in the vaccination process, drug delivery and as a gene therapy vector [10,11]. Among the various inorganic particles, calcium salts have been extensively developed and studied as a delivery system. Calcium phosphate is considered to be the model compound for the inorganic constituent of bone and teeth. Blood serum is considered to be an aqueous solution, supersaturated with respect to a calcium phosphate. Due to the presence of proteins and enzymes in biological fluid, extensive complexation of free calcium takes place, thus reducing the actual supersaturation [12]. Calcium plays an important role in endocytosis, which is the major route for cellular internalisation (Figure 1). Calcium phosphate is used for manufacturing various forms of implants due to its excellent biocompatibility [13]. It has the advantage of being readily absorbed and a high binding affinity with a variety of molecules [14], and has been used as a delivery vehicle for various proteins, for example, as oral insulin delivery [15], sustained release of growth factors [16], and as a carrier material for antibiotics, macromolecules and contraceptives [17,18]. Among the various nonviral gene-delivery techniques, calcium phosphate co-precipitation is widely used as a routine laboratory procedure for the transfection of plasmid DNA (pDNA) [19] and oligonucleotide [20]. The main advantages of the calcium phosphate method are its simplicity, low cost and its applicability to a wide variety of cell types. This method relies heavily on the fact that calcium, as well as other divalent metal cations such as Mg^{2+}, Mn^{2+} and Ba^{2+}, can form ionic complexes with the helical phosphates of DNA and, thus, may impart a stabilising function to certain DNA structures [21]. Calcium phosphate particles are biocompatible, biodegradable, nontoxic and do not cause adverse reactions at the site of injection when administered parenterally [22]. The toxic effect of calcium phosphate and other vectors has been studied in rat and human mesangial cells. The major toxic changes were cytoplasmic vacuolisation and cell shrinking, followed by detachment of the cell from the culture vessel; however, calcium phosphate showed low toxicity compared with other vectors on mesangial cells [23].

2. Types of particulate delivery systems employing calcium transfection studies

The role of calcium as a vector in the preparation of a stable and effective gene delivery vehicle has been extensively used for its various advantages in the transfection of genetic material. Several researchers have worked on calcium phosphate in various delivery systems and have found it to be a promising candidate for clinical application in the future. The detailed studies carried out on calcium in various delivery systems are described below. The aim of this review is to provide an overview of formulation development and delivery issues of calcium-mediated gene delivery. Only a cursory mention will be given to the effects of the delivery route, and with more focus on its formulation aspects.

2.1 Calcium phosphate co-precipitate

The most widely used technique for the delivery of DNA to mammalian cells is calcium phosphate co-precipitation of DNA [24,25]. Macromolecules, when co-deposited on cell culture surface with inorganic minerals, form bioactive nanocomposites through the process of biomineralisation. This allows the macromolecules to be concentrated at a close proximity to be taken up by the cells. DNA and phosphate buffer precipitates extremely small, insoluble particles of calcium phosphate containing condensed DNA [26]. Although the mechanism of this type of transfection has not been characterised in detail, it is presumed that the calcium phosphate–DNA complexes adhere to the cell membranes leading to phagocytosis [27]. The conditions for transfection with calcium phosphate–DNA complexes were studied by optimising the calcium and phosphate solution (accomplished by monitoring calcium phosphate formation) [28]. Calcium phosphate-mediated transfection can be applied successfully for transient and stable transfection experiments to most adherent cells and some cells in suspension [29], with high copy-number transfer analysed by flow cytometry [30]. Furthermore, the addition of glycerol in the culture media with calcium phosphate–DNA precipitation mixture resulted in a transfection efficiency 50 – 100% greater than those obtained following shock with glycerol after removing the

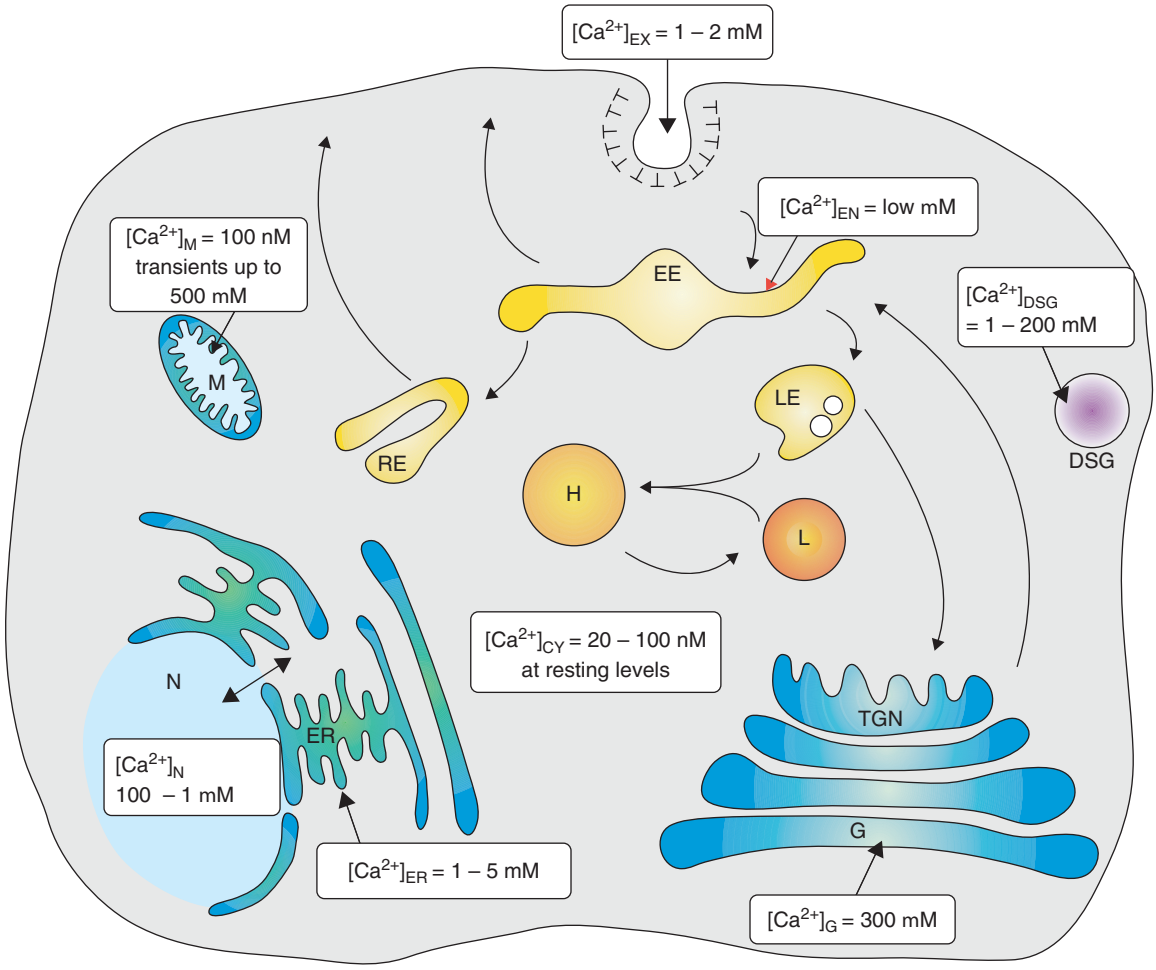

Figure 1. Endocytosis at the plasma membrane results in the uptake of millimolar free Ca^{2+} into endocytic vesicles. Endocytic-vesicle acidification releases Ca^{2+} into the cytosol. Cytosolic free Ca^{2+} is kept at low concentrations, relative to the extracellular medium, by the actions of calcium pumps at the plasma membrane, in the ER, the Golgi and the mitochondria. The free Ca^{2+} concentration in the endocytic vesicles remains higher than that of the cytosol. It is released before membrane fusion and is required for the reformation of lysosomes from late endosome/lysosome hybrid organelles. The calcium concentration within the DSG represents total calcium. Reprinted with permission from PRYOR PR, BUSS F, LUZIO JP: Calcium, calmodulin and the endocytic pathway, Figure 1. *ELSO Gazette* (2000) **2**.

CY: Cytoplasmic; DSG: Dense-core secretory granule; EE: Early endosome; ER: Endoplasmic reticulum; EN: Endoplasmic; EX: Exoplasmic; G: Golgi cisternae; H: Late endosome/lysosome hybrid organelle; L: Lysosome; LE: Late endosome; M: Mitochondrion; N: Nucleus; RE: Recycling endosome; TGN: Trans-Golgi network.

DNA mixture [31]. The effect of composition of calcium- and magnesium-containing mineral solutions on the efficiency of gene transfer was compared, wherein DNA/calcium phosphate nanocomposites showed enhanced transfection efficiency than Mg^{2+}, and with low toxicity, suggesting that these nanocomposites will be promising candidates for clinical application in the future [32].

2.2 Calcium phosphate nanoparticles

Calcium phosphate forms complexes with the nucleic acid backbone, thereby imparting stability and potential targeting ability [33], as well as helping in the transportation of the complex across the cell membrane by enhancing endocytosis of the membrane-bound DNA complex, or by enhancing plasma membrane permeability [21]. However, it suffers from poor reproducibility in precipitation and transfection [34,35] due to various experimental parameters and nonstandardised procedures for transfection, such as pH, DNA concentration, temperature, time between precipitation, and the kinds of cells to be transfected. The time between precipitation and transfection is also critical; 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 [9,36]. In addition, the calcium phosphate procedure has limited application for *in vivo* delivery because of DNase I degradation, and interaction with

protein present in the serum, which leads to an increase in particle size due to aggregation [37].

To overcome these disadvantages, simple and reproducible methods have been tried by adsorbing DNA onto calcium phosphate nanoparticles, preventing further particle size growth. Another method for the preparation of ultra low-size calcium phosphate nanoparticles encapsulating DNA molecules is by the reverse emulsion technique using microemulsion as the microreactor, resulting in 85% entrapment and an average particle size of 80 nm [38]. After further modification of the procedure, the calcium phosphate nanoparticles prepared showed 99% entrapment and a reduction of particle size from 80 to 30 – 40 nm [39]. Inhibition of uncontrollable growth was also controlled by using polycarboxylate segment: poly(ethylene glycol)-*b*-poly(aspartic acid) (PEG-*b*-PAA), which prevents the crystal growth because of its 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 particle size of the complex varied with the concentration of PEG-*b*-PAA. Increasing its concentration reduced the particle size of the complex [40]. These calcium phosphate nanoparticles showed better DNA stability, higher DNA entrapment, protection from nuclease and higher gene expression due to endocytosis mediated by calcium ions. 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 [41]. 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 [39]. Calcium phosphate-DNA nanoparticles showed better gene expression *in vivo* than the standard calcium phosphate-precipitate method and naked DNA, with no toxic effect on the cells [33]. These calcium phosphate nanoparticles, in combination with other vectors [42] or by incorporating specific ligands on the outer surface of the shell to improving cellular uptake [43], may exert a positive effect on gene transfer. 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 nonviral vectors in this developing field of gene therapy.

2.3 PLGA-loaded calcium phosphate DNA co-precipitates

Poly(D,L-lactide-co-glycolide) (PLGA) microparticles have been used as a carrier system for drug and DNA delivery. Varying the molecular weight of the lactide and glycolide components of PLGA can control the rate and duration of the release of entrapped DNA. Microparticles can also protect the DNA from nuclease degradation and facilitate DNA uptake [44-46]. The stability of pDNA during microencapsulation depends on the processing parameters to avoid the conversion of the supercoiled form to the linear and open circular form, as the supercoiled form is thought to give an improved gene expression [47,48]. Calcium phosphate co-precipitated DNA (CaPi-DNA) was prepared and loaded into PLGA microparticles of 2 – 5 µm with 30 – 50% entrapment efficiency. Calcium phosphate–DNA increases the stability of pDNA in PLGA microparticles and also ensures constant slow release. pDNA in the calcium phosphate co-precipitate was protected during the processing of microencapsulation [49]. Further improvement in this novel nonviral gene delivery system combining CaPi-DNA and PLGA biodegradable matrices involved the absorption of the CaPi-DNA co-precipitate onto two-dimensional PLGA matrices with 100% adsorption, and onto three-dimensional PLGA matrices with 59% adsorption. The PLGA matrix provides a surface for cell attachment, growth and organisation for gene delivery. Controlled gene delivery was observed for 42 days with improved loading efficiency of pDNA without DNA damage, nuclease protection or enhanced cell endocytosis [3].

2.4 Calcium phosphate-mediated transfection of brain neurons

Postnatal neurons are generally not transfected by conventional methods because they have already completed their cell division. Only viral vectors have shown success in transfecting these cells [50,51]. The modified calcium phosphate method has been shown to transfect postnatal cortical [52] and hippocampal neurons [53]. Transfection of the substantial nigra pars compacta, which is the major brain nucleus containing dopaminergic neurons, was carried out by calcium phosphate. These calcium phosphate precipitates were applied for a short period of time in serum-free media with successful transfection, and with no deleterious side effects on postnatal neurons compared with the lipofectamine method [26,54]. Due to the vigorous endocytic activity of cultured neurons, young cultures were transfected more successfully. The transfection capability of a primary-cultured neuron by modified calcium phosphate is a convenient tool for further investigation.

2.5 Stabilisation of DNA using calcium and alcohol

The stability of naked DNA is the prime concern during formulation and long-term storage, which is susceptible to chemical and processing degradation [55,56] such as mixing, filtration, lyophilisation, spray drying and transport. Condensation of DNA not only reduces the particle size but also stabilises the DNA. Various condensing agents such as cationic lipid, peptide and polymers have been used in transfection studies, but proved less efficient in *in vivo* experiments due to their cytotoxicity [57] and complement activation [58]. Condensing DNA with inorganic polycations [59] reduces the repulsion between DNA segments and increases the attractive force based on fluctuations of the correlated counter ions [60-62]. Calcium chloride showed

robust DNA condensation in tertiary butanol rather than other metal salts with two forms of particles: toroid and rod-like forms. These formed particles protect DNA from stress induced by sonication. DNA retains its supercoiled- and open-circular form of plasmid. The specificity of condensation in the presence of calcium salt over other divalent metals is based on coulombic interactions [63]. In DNA grooves, a smaller ion can better fit due to electrostatic mechanisms that induce condensation and bending [64]. Calcium has the largest radius compared with zinc and magnesium in crystal form, but has the smallest hydrodynamic radius in aqueous solution and, therefore, has the highest charge to surface area [65]. The negative charge of the DNA is not fully neutralised by the divalent metal ions in the alcohol solvent system; therefore, the condensed DNA has a negative charge. Stability of DNA condensed by calcium was equivalent to peptide-condensed DNA formulation [63].

2.6 Calcium enhanced transfection of DNA–cationic lipid particulate complex

Cationic liposome–DNA complexes are widely used as transfection agents for *in vitro* and *in vivo* application [66-68]. However, transfection by cationic liposome–DNA complex is a relatively ineffective process due to inefficient endosome escape during endocytosis, intracellular nuclease degradation of plasmid by DNase, and poor nucleus entry [69]. Various efforts have been made to improve the transfection efficiency of cationic liposomes by using helper lipids, fusogenic peptides, DNA-condensing agent and nuclear localisation signals as targeting ligands [70-74]. Calcium phosphate is used to enhance *in vitro* transfection by facilitating the uptake of DNA, which enters the cell by endocytosis, and the nucleus after endosomal escape [75-77]. Therefore, to overcome this problem, calcium was used together with DNA–cationic liposome complex for the transfection studies. The DNA–cationic lipid complex showed an increase in transgene expression in the presence of calcium without any cell toxicity. Similarly, calcium stimulates the transfection potency of stabilised plasmid-lipid particles *in vitro* without destabilising the particles, indicating that no free plasmid is released for the particulate system; this calcium effect occurs in the presence of phosphate ions [78]. The higher transfection efficiency is due to a higher gene delivery to the variety of cell lines. These results were specific to transfection with calcium ions and not with other divalent metal ions [79]. The DNA–cationic lipid complex offers a simple procedure for enhancing transfection.

2.7 Enhanced encapsulation and transfection efficiency of DNA in anionic and zwitterionic lipoplex induced by calcium

Although cationic liposomes are relatively efficient in delivering DNA into the cell, they have the disadvantage of not being natural products. They are cationic amphiphiles or detergents [80], which are inactivated in serum and have shown cytotoxic effects leading to cell death both *in vitro* [81] and *in vivo* [82]. Cationic lipid particles are charged and, therefore, interact with serum proteins leading to rapid clearance without systemic effects. Intravenous administration of cationic liposomes predominately transfects the endothelium of the lungs [83-85], which may be further associated with the pulmonary toxicity-induced by-production of reactive oxygen intermediates [86]. Current efforts to improve transfection efficiency of cationic liposomes focus on the synthesis of new cationic lipids and the preparation of better formulations [70], or to retarget lipid–DNA to other tissues to reduce lung-associated toxicity [87]. A possible alternative to the toxic cationic lipids is the employment of zwitterionic or anionic liposomes, which have been used in earlier work for the incorporation of DNA and RNA by techniques of passive entrapment or reverse phase evaporation [88,89]. These species have longer circulating times, varied clearance profiles and are much safer to the target cells [90,91]. Repulsive electrostatic interaction between these anionic lipids and negatively charged DNA [92,93] have no direct electrostatic interaction between DNA and zwitterionic lipids, therefore, making them susceptible to low DNA molecule entrapment [94] and poor transfection. Low entrapment of DNA in anionic or zwitterionic liposomes was overcome by the addition of calcium, which neutralises the phosphate group on DNA [59]. Anionic liposome-plasmid particles were developed in the presence of calcium by a heating technique, and characterised by scanning tunnelling microscopy and fluorescence microscopy interaction assay [95,96]. Calcium may also serve as a bridge between the phosphate head group of the lipid and the phosphate of DNA. Another postulated mechanism is the interaction of calcium with the phosphate head group of the lipid 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 (Figure 2) [97,98]. 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. The advantages of using calcium ions are:

- the calcium–DNA complexes easily release free DNA at high concentration of sodium,
- there is a broader concentration range of calcium ions for DNA condensation with low cell toxicity,
- calcium increases DNA entrapment in the liposome by mediating DNA–liposomes binding.

The aggregation of DNA and the fusion of liposomes should occur simultaneously to achieve a higher entrapment efficiency [99]. The interaction of calcium with zwitterionic lipid in the presence of DNA was elucidated by differential scanning calorimetry and small-angle X-ray scattering for measuring the change in distance between the lamella repeat of liposome vesicles due to calcium interaction [97].

A biodistribution study showed that one-third of the liposome–calcium DNA complex was still remaining in the circulation 1 h after administration; the majority remaining in

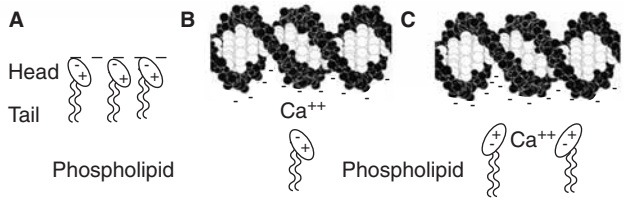

Figure 2. A. Schematic diagram of the orientation of phosphatidylcholine in water. B. Option 1: the binding of phosphatidylcholine to DNA via calcium. The calcium ion bridges the phosphate of the lipid to the phosphate of the DNA. **C. Option 2:** the calcium ion bridges two adjacent phosphatidylcholine molecules, leaving a net positive charge on each head group of the phospholipids, which then binds to the negatively charged DNA.

the liver and spleen, and a little in the lungs. The highest amount of pDNA was found in the circulation indicating that DNA is protected from serum nuclease. *In vivo* results demonstrate higher stability of neutral liposomes–calcium complex as a DNA carrier in contrast with cationic liposomes [99]. Transfection efficiency of anionic liposome–DNA complex was enhanced by about sevenfold without cytotoxic effect (unlike cationic liposomes), but transfection efficiency was reduced in the presence of serum; therefore, anionic liposome would be useful for the *ex vivo* and *in vitro* application of DNA transfer into cell lines sensitive to cationic lipids [100]. Tissue-specific targeting and overcoming the barrier of cell transfection by neutral liposome–calcium–DNA complex would be possible with further improvement in this technique, thereby offering an important alternative nonviral gene delivery system.

2.8 Calcium ions as cofactor for polycation-mediated gene delivery

Due to their DNA packing ability and nuclear localisation signal, a number of DNA–protein complexes were shown to be transported to the nucleus with high efficiency. Among the proteins used, histone H1 was an efficient carrier of DNA in the presence of calcium ions [77,101,102]. The H1–DNA complex transfection that was inhibited in the presence of serum was overcome by the presence of calcium ions in the cell culture medium [103]. Partial replacement of histone by polylysine also produced transfection of the cell [104]. The addition of calcium in the form of calcium phosphate precipitate or soluble calcium ($CaCl_2$) as cofactor, demonstrated a fivefold increase in transfection efficiency when added to the cell culture medium during transfection and at postincubation (after transfection); the same effect was not observed with magnesium ions. In the absence of calcium, the complexes are taken up by the cells but remain arrested at an unknown cellular site. Improved transfection occurred as a result of enhanced cell entry of the protein–DNA complex, endosomal/lysosomal escape and nuclear transport, or the enhanced transcription/translation of introduced transgene by calcium [105]. Endosomal/lysosomal vesicular transport facilitated by calcium phosphate precipitate could be due to the fusion between biological membranes, leading to the local solubilisation of phospholipid bilayers. Calcium phosphate also induces mitogenic effects on cells, and thereby helps to overcome the nuclear barrier by inducing mitosis and promoting nuclear transport of the H1–DNA complexes [106]. Further research on immunohistochemical and electron microscopic data of polycation–DNA complexes and calcium in combination would provide more information on the exact mechanism of enhanced transfection.

2.9 Gene transfer by DNA–gelatin nanospheres with calcium

Positively charged gelatin has been tested as a novel gene-delivery vector by the coacervation technique to form gelatin-DNA nanospheres [21]. In the presence of exogenously added calcium phosphate, transferrin-coated gelatin–DNA nanospheres produced higher transfection due to calcium-enhanced permeability of the plasma membrane [42], and presumably because of transferrin acting as a ligand to the corresponding cell surface receptor, thereby enhancing the binding and uptake of the nanospheres. The same effect was observed when gelatin–DNA nanospheres were loaded with calcium due to the extracellular release of calcium from the nanospheres, which resulted in the formation of DNA–calcium phosphate complexes (formed with phosphate in the medium), leading to enhanced endocytosis. These results suggest that DNA–gelatin nanospheres with calcium could be attractive vehicles for DNA delivery.

2.10 Calcium phosphate enhanced adeno-associated virus mediated gene therapy

Adeno-associated virus (AAV) is also a potential vector for gene therapy due to its target integrating capacity, broad host range and lack of immunogenicity [107]. Various attempts have been made to improve transfection efficiency by complexation of the viral vector with polycation polymers and cationic lipid [108,109]. Incorporation of calcium phosphate with adenovirus enhances transfection efficiency of airway epithelia *in vitro* and *in vivo* [110-113]. The enhancement in transfection efficiency by calcium phosphate is due to the increase in binding of the virus to the cell surface, thus increasing local virus concentration, and also facilitating endosome escape to reach the nucleus. The study by Yang *et al.* reported that the administration of AAV–DNA–calcium phosphate complexes into the liver of diabetic mice significantly reduced blood glucose levels of the mice, demonstrating the enhancement effect of calcium phosphate on AAV-mediated gene transfer *in vivo* [114]. The mechanism was attributed to the increased cell binding, the subsequent uptake by endocytosis and, due to a fall in pH in the endosome, the calcium phosphate precipitate dissociates, releasing the virus [115]. It is also possible that calcium release from the endosome may activate signal transduction

pathways that facilitates gene transfer [114]. Delivery of the insulin gene or the cystic fibrosis transmembrane regulator and receptor gene by recombinant AAV–calcium phosphate complexes *in vivo* can be specific through receptor targeting with specific antibodies or ligands, which may have future applications for the treatment of diabetes and cystic fibrosis with a lower cost of treatment.

3. Expert opinion and conclusion

In the last decade, therapeutic gene transfer received a boost with the possibility of gene delivery in a clinical setting. The success of the research was also associated with various barriers in cellular transfection that needed to be solved. A number of remedies were tested to overcome these barriers, including calcium particles, which have been found to be an effective means of gene transfection. Further research on elucidating a detailed mechanism of enhanced transfection efficiency in the presence of calcium as a delivery vehicle or cofactor with other nonviral vectors in gene delivery needs to be explored. The disadvantages associated with *in vivo* transfection in the presence of serum can be overcome by improvisation in the formulation parameters. In addition, targeting these viral/nonviral vectors would further aid in lowering the side effects associated with the present delivery vectors. Capitalising on the ability of calcium for endosomal escape, transfection of a variety of cells would offer an important alternative to viral/nonviral vectors for gene delivery. The potential of calcium would assist in developing novel gene delivery vectors for the treatment of various genetic diseases. The use of calcium has seen enormous improvements in transfection efficiency; from calcium phosphate DNA co-precipitates to calcium phosphate nanoparticles, in addition to other particulate systems. These formulations have given promising results in both *in vitro* and *in vivo* studies. This is evident in the application of calcium phosphate particles in the transfection of brain cells, wherein the other conventional methods have failed. Another significant achievement has been the reduction of glucose levels by insulin gene delivery, using AAV–calcium phosphate complexes for the treatment of diabetes with minimum toxicity. These preclinical results have created an optimal base for clinical trials. In this advent, calcium salts may open a new chapter in the treatment of various genetic diseases.

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