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New Challenges in Gene Delivery *in vivo*

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SUMMARY

Adverse effects of viral vectors, instability of naked DNA, cytotoxicity and low transfection of cationic lipids, cationic polymers and other synthetic vectors are severe limitations in gene therapy now-a-days. An ideal non-viral vector, in addition to targeting to specific cell type, must manifest an efficient endosomal escape, render sufficient protection of DNA in cytosol and help make an easy passage of cytosolic DNA to nucleus. Virus-like size calcium phosphate nanoparticles have been found to succeed many of these limitations in delivering genes to the nucleus of specific cells. This review article has focused some applications of DNA loaded calcium phosphate nanoparticles system as non-viral vector in gene delivery and its potential use in gene therapy as well as highlighted the mechanistic studies to probe the reason for high transfection efficiency of the vector. It has been demonstrated that calcium ions play an important role in endosomal escape, cytosolic stability and enhanced nuclear uptake of DNA through nuclear pore complexes. The special role of exogenous calcium ions to overcome obstacles in practical realization of this field suggests that calcium phosphate nanoparticles is not a "me too" synthetic vector and can be designated as second generation non-viral vector for gene therapy.

INTRODUCTION

Gene therapy is an alternative therapeutic procedure in molecular medicine which can be defined in a most simplified way as a technique by which therapeutic gene can be introduced into the nucleus of a somatic cell and the product of which is responsible for curing or retarding an inherited or acquired disease. The concept of gene therapy was originally developed in early seventies (1). Prolonged studies based on the basic concept of gene therapy and output of some promising data from *in vitro* and animal models studies and through clinical trials have culminated to the birth of several proof-of-the concept results which led the scientists to have high expectations for the practical implementation of the gene therapy technology in the clinic. But unfortunately it has still not come up as a successful tool for the treatment of human diseases. With the promise of specificity and low toxicity it is anticipated that such technology will have a lasting impact on medicine and biotechnology.

The first approved protocol of gene therapy used for human being was in September 1990 (2). During the span of more

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than 14 years since then about 600 gene therapy protocols have been approved and nearly 5000 human beings have been subjected to carry genetically engineered cells into their body (3). The outcome from these trials has established that gene therapy has potential to make revolution in medicine for treating broad array of human diseases and the procedure appears to have very low risk of adverse side reaction unlike drug therapy.

Despite growing body of knowledge regarding the chemistry, biology and pharmacology of genes, the widespread application of DNA based therapeutic remains a daunting challenge. Practical implementation has been more difficult than originally expected. Efficient expression of gene to get therapeutic product is still a major obstacle in further advancement of this field of research. Due to large size, highly anionic in nature and easy degradability by the nucleases, naked DNA results in quite low levels of transduced gene expression (4). In the beginning of gene delivery research in early eighties (5), scientists thought of exploiting natural ability of viruses of delivering genetic materials for the delivery of therapeutic transgenes. Till now this is the most popular way of delivering genes used in gene therapy protocol. However, the inadequacy of the current methodology based on virus as carrier for DNA is attributable to the limitations including immunogenicity, cytotoxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems, recombination and high cost. Most important concerns among them are the stimulation of strong immune responses and the potential for oncogenic transformations (6). These major limitations and eventual clinical failures of viral vectors have led to a shift in focus on synthetic non-viral gene delivery systems. Synthetic vectors, although currently orders of magnitude less efficient than biological vectors, are increasingly being considered as possible alternatives to viral vectors. Unfortunately, the low *in vivo* efficiency often, precludes their use for gene therapeutic purposes; yet, their favourable 'inertness' justifies further exploration of non-viral alternatives (7). Non-viral synthetic carriers such as polymers or lipids are attractive owing to flexibility in design ability to be chemically or biochemically functionalized and tunable toxicity properties. However, the gene expressions using these synthetic carriers are still very inefficient compared to viral vectors. Non-Viral vectors are synthetic vehicles for the introduction of foreign DNA sequences into target cells. Various methods for non-viral gene therapy have been proposed. Some approaches are aimed at developing an artificial virus that attempt to mimic the process of viral infusion using synthetic materials. Other approaches apply the theory and methods of advanced particulate drug delivery to deliver DNA to selected somatic targets. These approaches employ DNA complexes containing lipid, proteins, peptide or polymeric carriers as well as ligands capable of targeting the DNA complex to cell-surface receptors on the target cells and ligands for directing the intracellular trafficking of DNA to the nucleus. These non-viral vectors rely on the basics of supramolecular chemistry in which anionic DNA molecules are condensed into compact, ordered nanoparticles that are 50-200 nm in diameter by complexing DNA with an appropriately designed cationic molecule (8). The polycations reduce the size of the com-

plex, and confer excess cationic charge to the complex, thereby enhancing their cellular uptake by an endocytosis pathway. Since very large DNA molecules can be condensed into compact particles, non-viral vectors permit the incorporation of the gene regulatory regions that may afford better control of gene expression. DNA molecules as large as 2.3 mega base-pair (Mb) can be condensed into compact particles and these particles are suitable for gene delivery (9). Once DNA condensation has been established with synthetic vectors, it is possible to incorporate functional molecules or groups into the carrier entity either by physical entrapment or by chemical conjugation so that cell-specific targeting, cytoplasmic release as well as nuclear localization of DNA can be facilitated (10).

Lipid-based carriers (11), polycationic lipids (12), polylysine (13), polyornithine (14), histones and other chromosomal proteins (15), hydrogel polymers (16), all of which can ionically condense DNA and bind to the cell surface, are found to be ideal candidates for these vector types. But in the use of different types of cationic liposomes, cationic polymers and dendrimers as non-viral vectors for delivery of genes, it has been observed that in addition to cytotoxicity, these carriers do not lead to satisfactory amount of gene expression in the cells. *In vitro* transfection of cultured cells with plasmid DNA is a crucial indication towards the success of DNA as pharmaceutical. Because of the unsuccessfulness of the existing non-viral vectors for providing satisfactory and efficient transfection, these studies also, albeit for different reasons, could not bring the gene therapy technology at the level of clinical practice.

We have recently developed some inorganic phosphate nanoparticles like calcium phosphate, magnesium phosphate, manganese phosphate nanoparticles as safe and efficient carriers of plasmid DNA which manifested quite high degree of transfection efficiencies compared to liposomes and other non-viral vectors (17-19). Calcium phosphate as such is not a new candidate for facilitating gene transfer and subsequent gene expression. One of the early methods of gene transfer in cells in culture involved co-precipitating DNA with calcium phosphate (20). When added to a cell monolayer this insoluble precipitate is taken up by the cell through endosomal pathway and the endosome is broken down in a calcium ion dependent manner, thereby releasing the DNA into the cytosol, which, under suitable circumstances, can be incorporated into the host cell genome. The process by which this transfer of DNA occurs is poorly understood. But it is clear that the division of the recipient cell within 24 hours to 48 hours of gene transfer is important. This method is found to be simple, effective and has been widely used. However, the main drawback of the method of using precipitated calcium phosphate is the extremely low transfection efficiency (10-15%) as compared to that of viral vectors and the difficulty of applying it to *in vivo* studies (21). Moreover, the method also suffers from variations in calcium phosphate-DNA particle sizes, which causes variation among experiments. The transfection efficiency has been found to be dependent on a large number of factors, such as the cell type used, the morphology of the precipitate, pH of the buffers and the time of contact between precipitate and cells. Summing up the results of various studies, it is clearly understood that one of the major impediments of using calcium phosphate precipitate as carrier for nuclear delivery of DNA, like other particulate carriers, is the large

particle size (22) which resulted in the slow transfer of the materials across the cell membranes. Calcium phosphate nanoparticles of average size greater than 300 nm diameters have also been reported to serve, as non-toxic, biocompatible adjuvant for vaccination but these particles are too large to use as carrier for effective DNA delivery (23-24).

Therefore, these two wrongfully conceived myths of low transfection efficiency and inability to apply calcium phosphate precipitates *in vivo* condition have arisen due to large particle size of calcium phosphate and can be overcome by preparing the virus-like size calcium phosphate nanoparticles i.e. diameter of 100nm or less, in which DNA molecules are encapsulated to completely protect the nucleic acid from external hostile environment. It is envisaged that if these two impediments are overcome, the use of calcium phosphate and probably other biocompatible inorganic nanoparticles as gene carriers can become a novel technology in the area of gene therapy and are advantageous over the other viral and non viral vectors in the sense that these vectors are not only safe as well as cost effective but also may overcome many limitations of the other vector types.

With the prospect of using calcium phosphate nanoparticles as carriers, we have involved the use of the aqueous core of the water-in-oil microemulsion as nanoreactor for the preparation of controlled tailor-made size and plasmid DNA encapsulated calcium phosphate nanoparticles (18). These pDNA-loaded nanoparticles have been prepared by forcing the DNA-calcium phosphate co-precipitation inside the aqueous core of the reverse micelles to obtain a highly monodispersed nanoparticles with a narrow size distribution which have been further characterized by their crystal structure, surface charge, aggregational behaviour and pH dependent pDNA release. Upto an extent of 20% w/w of

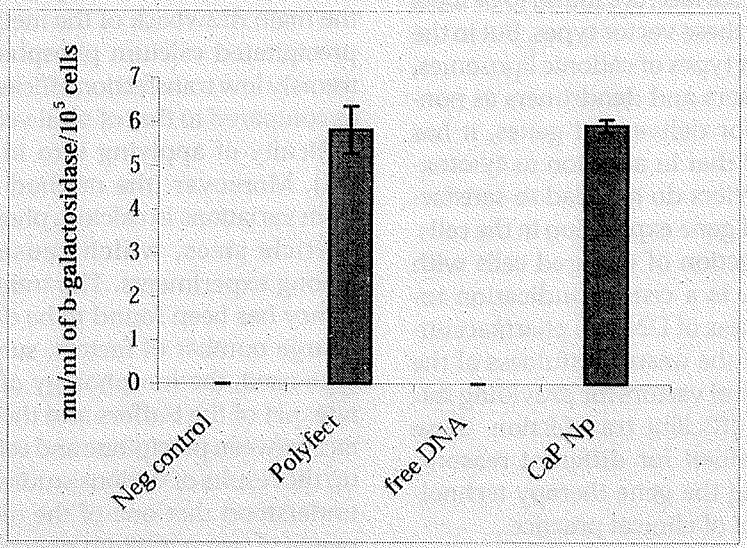

Figure 1 : *In vitro* transfection of pSVβgal plasmid in *HeLa* cell line (vide ref 18). Polyfect as positive control (vide ref 44).

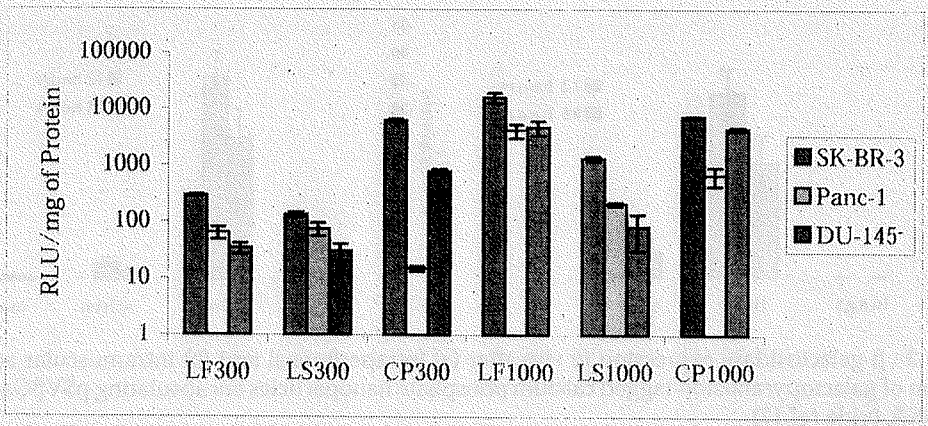

Figure 2 : Luciferase expression in different cell lines using lipofectamine (LF), Liposome (LS) and Calcium Phosphate nanoparticles (CP) as non-viral vectors in presence of transferrin (DNA : Tf = 1:12.5 w/w). DNA quantity 300ng and 1000ng respectively (vide ref 25).

pSVβgal plasmid in calcium phosphate nanoparticles could be loaded. Moreover, these polymer capped nanoparticles can be stored easily in the form of a dry lyophilized powder, which can be readily redispersed in aqueous buffer. It was also demonstrated (18) that the transfection efficiency of these particles in HeLa cell line using pSVβgal as a marker plasmid was found to be more than 100% to that of the polyfect used as control (Figure 1). In DU145 cell lines the transfection efficiency of DNA loaded calcium phosphate nanoparticles was found to be about 1000 times higher compared to that in liposomal system (Figure 2) (25).

One of the important aspects of a gene therapy vector is that it should be targeted to specific cell types. It is possible to attach the liver targeting ligands such as asialoglycoprotein, lactose, galactose etc. on the surface of the nanoparticles by chemically conjugating on the particle surface. It has been demonstrated (17) that the surface modification of pSVβgal loaded calcium phosphate nanoparticles and coated with polyacrylic acid through chemical conjugation of ligand such as p-aminophenyl-1-thio-β-D-galactopyranoside (PAG) are specifically targeted to liver paranchymal cells in vivo (Figure 3a&b) resulting in an enhanced expression of βgalactosidase in the liver.

Calcium Phosphate nanoparticles have been applied as non-viral vector for gene therapy of experimental autoimmune hepatitis (26). Development of disease model was established from significantly elevated levels of IgG, presence of antinuclear antibodies and necroinflammatory changes in the liver tissues and significant depletion of IL-2. Nanoparticles of Calcium phosphate encapsulating the plasmid expression vector of IL-2 (pUMVC3-mIL-2) were synthesized using reverse micelles method (18). More than 60-fold increase in IgG level was observed in disease animal which have been significantly reduced when the animals were treated with pUMVC3-mIL-2 encapsulated calcium phosphate nanoparticles (Figure 4). Fluoresceinated nuclei seen in the liver tissue section of diseased animal indicating the presence of

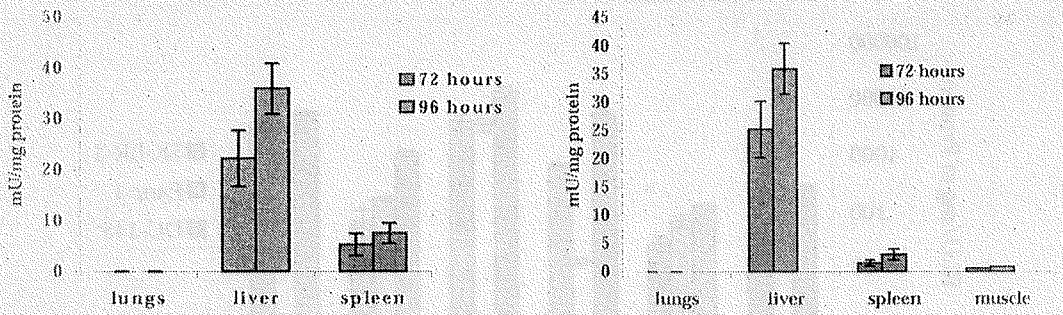

Figure 3 : β-galactosidase expression *in vivo* after (a) intraperitoneal and (b) intramuscular administration of galactopyranoside-tagged calcium phosphate nanoparticles encapsulating pSVβGal plasmid DNA (vide ref 17)

antinuclear antibodies in the serum have been totally eliminated when these animals were treated with pDNA loaded calcium phosphate nanoparticles (result not shown). Histopathological observations of liver sections also indicated a mark regression of inflammatory condition of the tissues. From the results, we have conclusively established the effective therapeutic use of calcium phosphate nanoparticles as non-viral vector *in vivo* for pDNA expression of IL-2.

addition to antibodies because of the increasing recognition of the role and need for CTL in such vaccines. Efforts are also being made to develop vaccines that can induce specific types of T helper responses; Th1 or Th2. DNA vaccines have the potential as new vaccines because of their ability to elicit both humoral and cellular immunity. DNA vaccines are also thought to be potentially safer than traditional vaccines (28). Whilst safety was demonstrated and

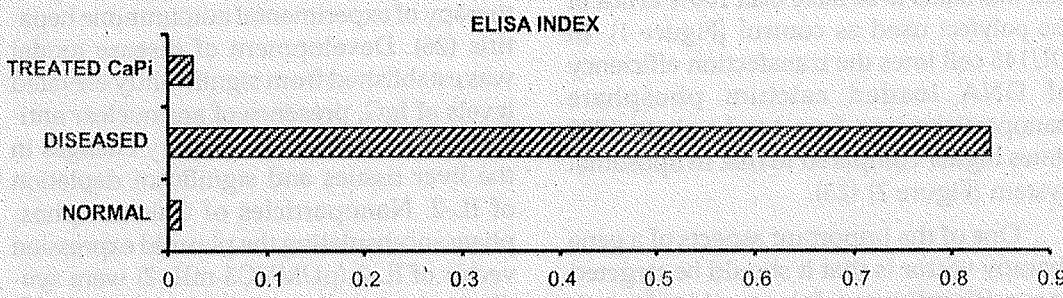

Figure 4 : Elisa index of Anti-liver surface protein antibody in the serum (vide ref 26).

Another interesting application of DNA loaded calcium phosphate nanoparticles reported recently is in DNA vaccines (27). New efforts to develop vaccines emphasized on inducing CD8+ cytolytic T lymphocytes (CTL) responses in

immune responses were generated in many DNA vaccine formulations, overall the potency has been found to be disappointing. A variety of approaches are under evolution to increase the potency of DNA vaccines whilst still retaining their attractive

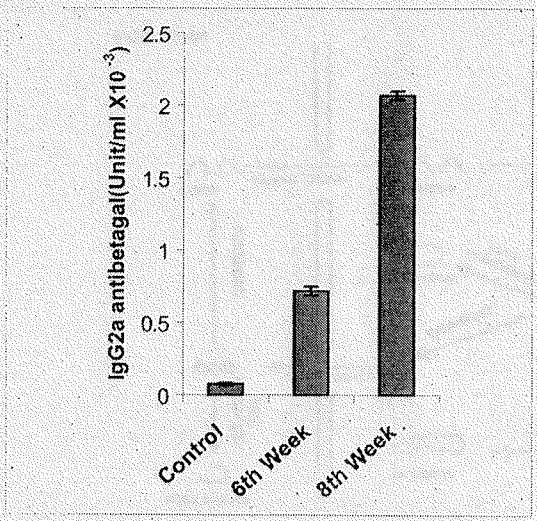

Figure 5(a) : Immunoglobulin (IgG2a) assay in serum of mice injected intraperitoneally with 0.9 μg of pDNA entrapped in calcium phosphate (vide ref 27)

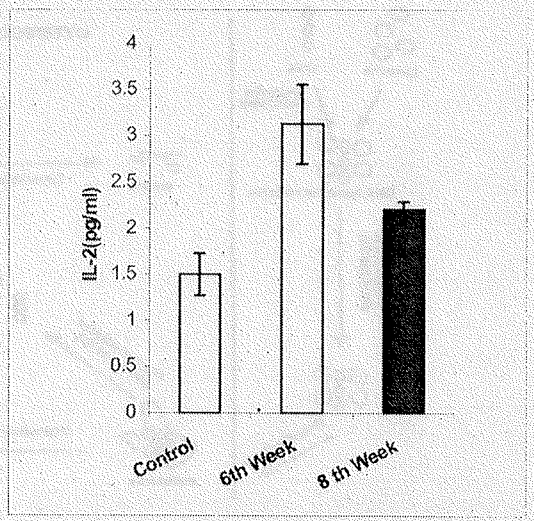

Figure 5(b) : Lymphokine proliferation in Splenocytes of mice *in vitro* (Vide ref 27)

features. Based on the observation of high transfection efficiency by using calcium phosphate nanoparticles as non-viral vector it was thought promising to use these nanoparticles as carriers for DNA vaccines. As a preliminary step an investigation was conducted using pSVβgal plasmid as marker DNA encapsulated in calcium phosphate nanoparticles in murine model. A 10-fold increase of antibody levels in the 6th week post immunization predominated by IgG2a along with immunological synapse formation and lymphocyte proliferation were manifested (Fig. 5). Thus, it is presumed that these calcium phosphate nanoparticles entrapping pDNA have a high potential and a well-defined role in DNA delivery as well as in transfection that can also be used as a further advantage in DNA vaccination.

Now the question is: why calcium phosphate nanoparticles should be preferred to other non-viral vectors? Why calcium phosphate nanoparticles exhibit such a high transfection efficieny? What is mechanism of intracellular trafficking of DNA from the cell surface to the nucleus? According to the current state of knowledge, most of these non-viral DNA delivery systems operate at one of the three general levels: DNA condensation, and complexation, endocytosis and nuclear targeting/ entry (Figure 6).

Negatively charged DNA molecules are usually condensed with different cationic vectors before the entry. Due to its excess positive charge, the vector-DNA complex binds to the negatively charged cell membrane and is subsequently taken up by endocytosis. After endocytosis, the DNA containing particles are largely retained in perinuclear endosomes/ lysosomes. The entrapment of the carrier within these vesicles is one of the major barriers for transfection. The extremely low pH and enzymes within endosomes and

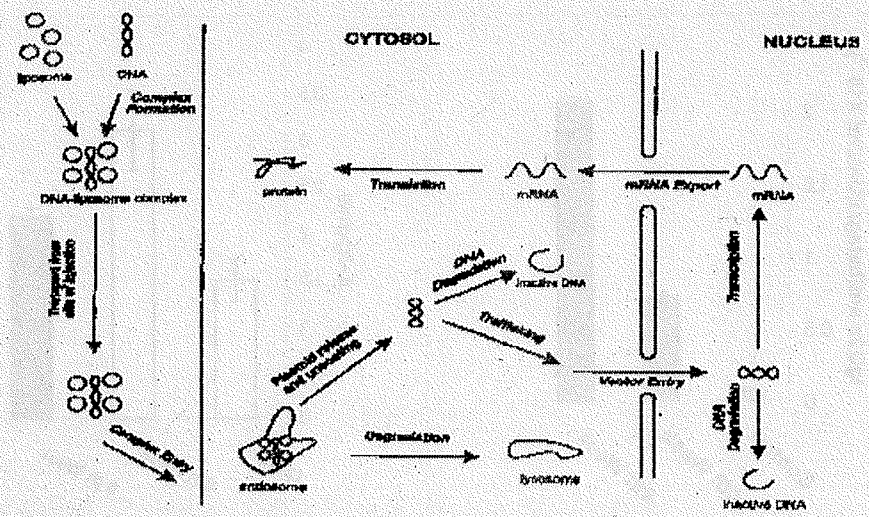

Figure 6 : Schematic illustration of the process involved in gene expression

lysosomes usually bring about the degradation of entrapped DNA inside the carriers. So, endosomal escape is one of the most crucial steps. Ideally, the entire delivered vector should escape from the endosome before degradation along the trafficking route, either by vector fusion with the endosomal membrane or through endosome disruption, in either event, ultimately resulting in the release of the nucleic acid (with or without the protective vehicle) into the cytosol (29).

Several approaches have been used in an attempt to facilitate the cytoplasmic release of the DNA. Ca^{2+} in the form of nascent calcium phosphate microprecipitates (29) and other lysosomolytical agents such as chloroquine, facilitate endosomal/lysosomal release by their fusiogenic and membranolytic activity (30).

In the case of calcium phosphate nanoparticles as delivery system, use of additional compound to disrupt the endosome is not required. This is because, upon internalization, endosomal compartments undergo continuous acidification from the initial cell-surface pH (~7) to an acidic pH of about 5.0.As a result, the dissolution of calcium phosphate nanoparticles in such an acidic endosomal compartment takes place (18) which ultimately leads to an osmotic disbalance and consequent disruption of the endosomal compartment thereby facilitating the release of entrapped DNA into the cytosol.

Exit from the endo-lysolytic pathway however, does not always result in high transfection efficiency. Once the DNA is released in cytoplasm, it must reach the nucleus for expression. There is considerable evidence suggesting that the nuclear membrane presents a barrier to the entry of free or carrier-associated DNA from the cytoplasm (31-33)1. The nucleus of a cell is separated from the cytoplasm by concentric bilayers that form a double membrane structure known as nuclear envelope (34). The nuclear envelope is an integral part of the structural framework of the nucleus (Figure 7), and is involved in organizing

intranuclear events. It serves as a selective barrier, actively transporting proteins required for normal nuclear function and exporting RNA. The movement of molecules across the nuclear envelope is critical for cellular homeostasis, and it allows cells to respond to external events. The outer bilayer of nuclear membrane is continuous with the endoplasmic reticulum. The inner bilayer renders the nuclear structural stability through protein filaments. The inner and outer bilayers fuse at sites throughout the nuclear envelope where large protein pores known as nuclear pore complexes (NPC), are located. The NPCs are the only known pores spanning both bilayers of the nuclear envelope and, as such, represent the most direct pathway into or out of the nucleus. The intermediate space between the two bilayers of nuclear envelope is called cisterna where Ca^{2+} is stored and is released to regulate the passage of molecules through NPC.

After the DNA is released into the cytosol, it may enter the nucleus through the nuclear pore directly or by the support of the nuclear localization signals. The transport of the DNA across the nuclear envelope occurs through the nuclear pores (35). Once within the nucleus, DNA is transcribed into the mRNA, which is then processed and translated into a therapeutic gene product. Nucleocytoplasmic transport from cytoplasm along the NPC proceeds through three mechanisms – (i) unregulated passive diffusion, which is limited to spe-

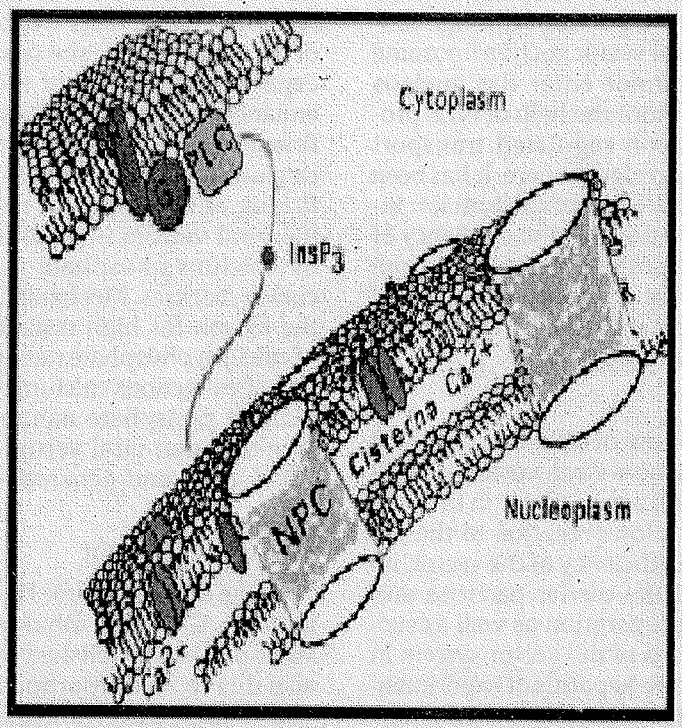

Figure 7 : Schematic of the components of the nuclear membrane involved in CA^{2+} regulated transport through the nuclear pore complex (NPC) (vide ref 34)

cies of less than 10 kDa; (ii) active transport, which is operable for large (> 70 kDa) molecules and requires the presence of a nuclear localization signal (NLS) proteins and the hydrolysis of ATP as an energy source; and (iii) Ca^{2+} regulated transport, which involves intermediate sized molecules (10-70 kDa range) and does not require a NLS or the evolution of ATP. Several experimental evidences suggest that the 'plug on' and 'plug off' of the NPC are always associated with the Ca^{2+} ion dependent mechanism (34). In presence of excessive calcium ions in the cytosol (such as from calcium phosphate nanoparticles), the $InsP_3$ is inactivated through calcium ion mediated complexation so that the $InsP_3$-receptor assisted drainage of cisternal calcium ion to the cytoplasm does not happen. DNA-Ca^{2+} compact complex, under such circumstances, can easily enter the nucleus through NPC. DNA, delivered through conventional non-viral vector such as liposome or a polymer, cannot enter the nucleus through NPC without the help of NLS proteins as calcium ion regulated transport across the NPC is irrelevant here. It has been shown (36) that addition of calcium ion increases the *in vitro* transfection efficiency of pDNA-cationic liposome complexes from 3 to 20 fold and the effect can be inhibited by the presence of EGTA, a calcium complexing chelating ligand.

Conclusion

Although the use of calcium phosphate nanoparticles as non-viral vector is very promising, it is still a new comer in the field of gene therapy. However, due to the the high transfection efficiency of the vector, no adverse effect of the carrier particles and easy method of preparation as well as successful applications of the vector system in curing autoimmune hepatitis of experimental animal models and in DNA vaccine formulation, the areas and the questions that have to be addressed are now being defined. An ideal non-viral vector must manifest an efficient endosomal escape, renders sufficient protection of DNA in cytosol and easy passage of cytosolic DNA to nucleus. Because of the dissolution of calcium phosphate in the low acidic medium of endosomal compartment, the calcium phosphate nanoparticles are dissolved in the endosome and destabilize it through osmotic disbalance and finally deliver the genetic material out in the cytosol. The electrostatic interaction of Ca^{2+} with negatively charged DNA makes the DNA stable against nuclease attack so that DNA can slowly enter the nucleus without any degradation. The efficient nuclear uptake of DNA from the cytosol is also assisted by Ca^{2+} ion through the deactivation of $InsP_3$ so that the nuclear pore complex (NPC) remain wide open thereby allowing DNA-Ca^{2+} complex to pass through it. We discuss here two major areas that foreseeably will be the focal point of investigations in the near future: further experimental proofs of the mechanism of enhanced nuclear uptake so as to make refinement of our understanding the role of calcium ions in the whole process of trafficking of DNA and an attempt to replace the viral vectors in gene therapy protocol by calcium phosphate nanoparticles for curing diseases. Mechanistic study to probe the reason for high transfection efficiency of calcium phosphate nanoparticles and the role of exogenous calcium ions indicate that calcium phosphate nanoparticles is not a "me too" non-viral vector and can be classified as second generation non-viral vector.

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