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# Gene Delivery by Hydroxyapatite and Calcium Phosphate Nanoparticles: A Review of Novel and Recent Applications

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## Abstract

Gene therapy is a targeted therapy which can be used in the treatment of various acquired and inherited diseases. Inhabitation of a gene function, restoring or improving a gene, or gaining a new function can be achieved by gene therapy strategies. The most crucial step in this therapy is delivering the therapeutic material to the target. Nanosized calcium phosphates (CaPs) have been considered as promising carriers due to their excellent biocompatibility. In this chapter, the delivery of DNA, siRNA, and miRNA by using CaP nanocarriers were compiled in detail and the main parameters which can affect the carrier properties and thus the gene transfer efficiency were also discussed.

**Keywords:** calcium phosphate nanoparticles, non-viral vector, hydroxyapatite, gene therapy, gene silencing

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## 1. Introduction

Recently, targeted therapies are becoming more attractive since they do not harm healthy cells and have high selectivity. Gene therapy is the modification of patients' cells genetically for curing or making the health state better. With gene therapy strategies, one gene function can be inhibited, restored or improved or a new function can be gained. For an improvement in functional disorders induced by the mutation or to inhibit the expression of an inadequate gene, a therapeutic gene material, and its related regulatory components are delivered to the nucleus to treat the diseases. In most cases, owing to their negative charge, naked oligonucleotides cannot penetrate the cell; therefore, an appropriate carrying agent is needed. The most crucial issue in gene therapy is delivering the therapeutic gene, which first must overcome the extracellular

barriers and, subsequently, the cellular barriers. The carrier used for loading them is called “a vector,” while the vectors recently been used for gene therapy are roughly sorted as viral vectors and non-viral vectors. However, viral vectors’ side effects, potential cytotoxicity of the carriers, and poor transfection efficiency of non-viral vectors have currently limited the accomplishment of gene therapy. As the use of nanoparticles has been a remarkable methodology in the solution of a variety of problems, they can be employed as non-viral delivery vehicles for oligonucleotides in molecular biology and medicine [1]. With having excellent biocompatibility and high chemical affinity toward DNA and RNA, calcium phosphate nanoparticles can participate in the delivery systems which can limit most of the problems in delivering the therapeutic molecules to the nucleus of target cells. Further, calcium phosphates can overcome the extracellular barriers and then dissolve in the acidic pH within endosomes and lysosomes which lead the release of nucleic acids in the targeted region of the cell [2].

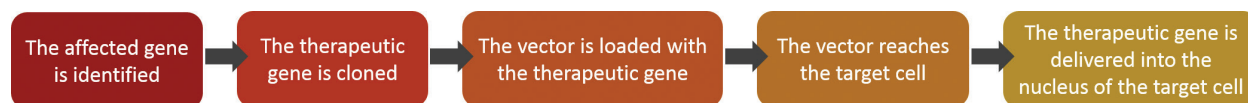
This review focuses on the potential employment of hydroxyapatite and other calcium phosphate nanoparticles as non-viral vectors in gene therapy and gene silencing as well as emphasizing the recent studies to expose the benefits for using such vectors.

## 2. What is gene therapy?

The genes on the chromosomes are the smallest genetic units that are effective in producing vital proteins for the cells. Any mutation that occurs in the genome can cause the disease because it changes the protein functions. Gene therapy is basically described as the transfer of genetic material with therapeutic effect to specific targeted cells or tissues with minimal toxicity to provide clinical benefits in the treatment of genetic and infectious diseases. Inhabitation of a gene function, restoring or improving a gene, or gaining a new function can be achieved by gene transfer strategies.

Gene therapy has been quite important in treating the various acquired and inherited diseases (e.g., AIDS, cancer, and other genetic disorders). As a theory, if the sequence of a mutant gene which is defined as an oncogene is recognized, gene therapy can be used in the treatment of any cancer disease. Moreover, its execution in the cure of genetic disorders such as muscular dystrophy, cystic fibrosis and severe combined immunodeficiency (SCID) has attracted attention due to three main reasons: (a) being well-characterized, (b) knowing the mutations responsible for the disease, and (c) existing no other satisfactorily efficient treatment [3].

Independent of the type of applied gene therapy method, the therapy first identifies the mutant gene that causes the disease. Following that, the identical healthy gene (e.g., therapeutic gene or transgene) is cloned. The therapeutic gene is designed according to the augmenting or suppressing or repairing needs. Since the penetration of “naked” oligonucleotides into cells is not probable, an appropriate carrier must be used in loading the produced therapeutic gene [4, 5]. This carrier is called vector and it is responsible for transporting the therapeutic gene to the patient’s target cell successfully. In this sense, a vector needs to come through the extracellular barriers and, then, the cellular barriers [6, 7]. Consecutive stages in gene therapy are given in **Figure 1**. The biggest challenge in a successful gene therapy is the issue of

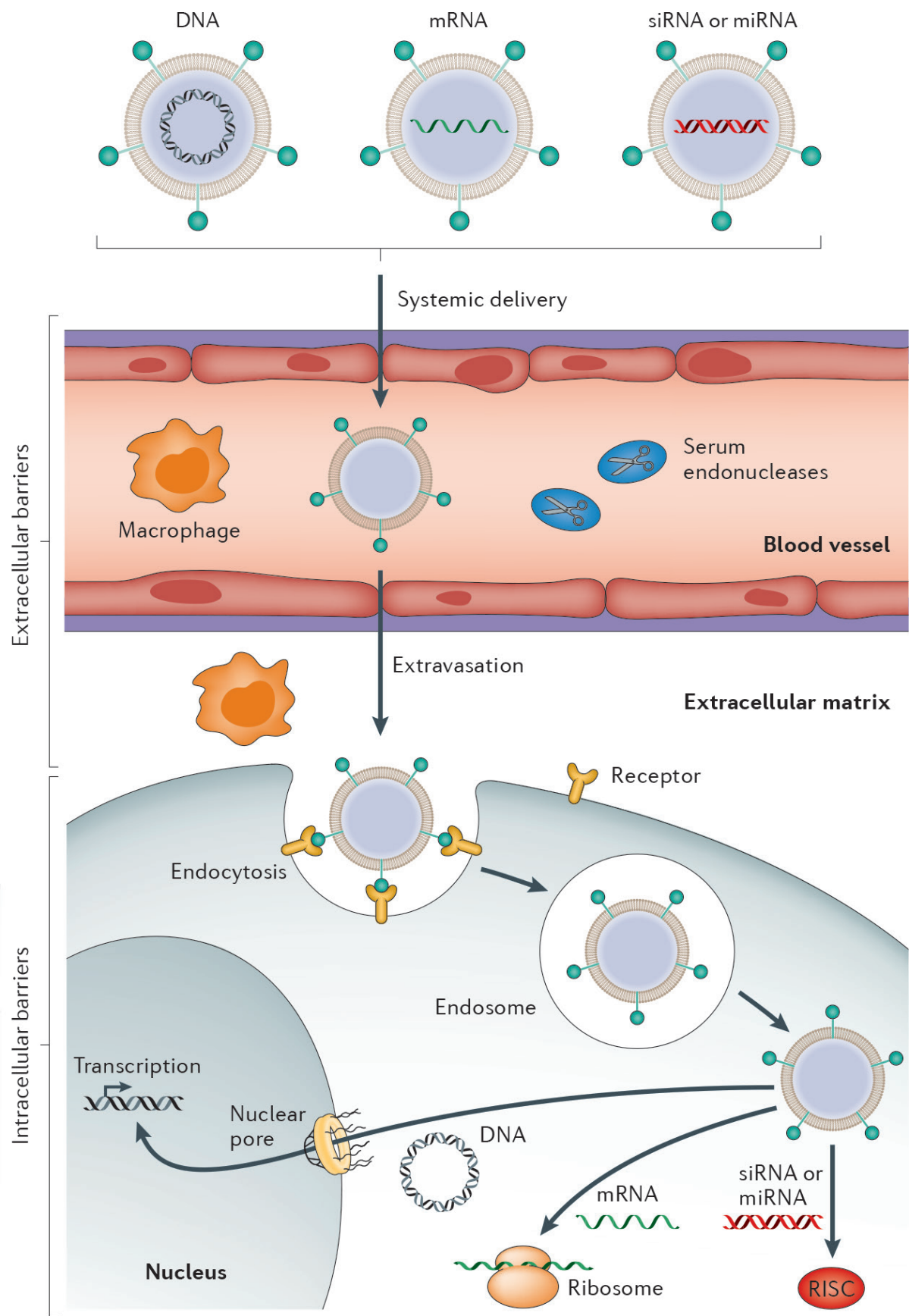


**Figure 1.** Schematic illustration of consecutive stages in gene therapy.

delivery. The optimal vector and the delivery system depend on the target cells and its characteristics, duration of expression, and the size of the genetic material to be incorporated in the vector [8]. Therefore, an optimal delivery vector needs to be safe, particular, and efficient.

There are two main sorts of vectors used in the gene therapy: viral vectors and non-viral (or synthetic) vectors. Viral vectors are viruses that have been genetically mutated for transporting normal human DNA. Viruses have developed a way of encapsulating and transferring their genes to human cells in a pathogenic manner. This capability can be used in the manipulation of the viral genome to replace disease-causing genes with the therapeutic ones [9]. Among all vectors, viral vectors exhibit higher efficiencies for transfecting host cells. However, the immunogenicity and the cytotoxicity are the two major drawbacks of using these carriers [10]. The first failure in the clinical trial of the related gene therapy was identified with the inflammatory response to the viral vector (Adenovirus). An additional and very important reason for the concerns over the employment of the viral vectors is the formation of insertional mutagenesis in which an exogenous DNA sequence integrates into the genome of a host organism. This can trigger the proliferation of oncogenes which leads to the malignant transformation of cells [8]. Therefore, non-viral vectors have the safety advantage over the viral vectors due to the demonstrated reduced pathogenicity. However, due to low delivery efficiency, thus, poor expression of their transgenes, the applications of non-viral gene transfer were previously disregarded [8]. Since non-viral vectors have less immunotoxicity, they have been applied in clinical trials from 2004 to 2013, whereas the use of viral vectors in the same treatments decreased significantly, then. Applications of a variety of non-viral vectors entering clinical trials, increased because of the advances in their performance, selectivity, gene expression period, and safety characteristics [8]. For therapeutic purposes; DNA, mRNA and short double-stranded RNA, including small interfering RNA (siRNA) and microRNA (miRNA) mimics, can be delivered by using various non-viral vectors; however, there might be some drawbacks in the use of non-viral vectors in clinical trials. **Figure 2** presents the barriers to achieve a successful in vivo delivery of nucleic acids using non-viral vectors [11]. The degradation caused by serum endonucleases, the immune detection, the renal clearance from the blood, and the emerging non-specific interactions are some of these handicaps and they need to be considered and avoided for a successful delivery. Furthermore, the carrier needs to extravasate from the bloodstream in order to reach the tissue targeted. Within the target tissue, it should mediate the cell entry and endosomal escape, as siRNA and miRNA mimics must be loaded into the RNA-induced silencing complex (RISC), whereas mRNA must bind to the translational machinery. DNA should be further transported to the nucleus to implement its activity [11].

Owing to their easy preparation and surface-functionalization, inorganic nanoparticles are promising non-viral vectors for gene therapy applications. If the nanoparticles are small enough (<100 nm), different nanoparticle materials such as calcium phosphates, polymers,



**Figure 2.** The barriers to success in vivo delivery of nucleic acids using non-viral vectors. Reprinted by permission from Macmillan Publishers Ltd.: [Nature Reviews Genetics] (13), copyright (2014).



liposomes, silica, magnetite, carbon, clay, and gold can be employed as non-viral vectors [8]. Compared to rest of all nanoparticles, calcium phosphate (CaP) has several advantages because of its easy preparation [12], high chemical affinity toward DNA and RNA [13, 14], high biocompatibility, and good biodegradability properties in biological systems [12]. Further, CaP particles have the ability to enter the cell and breakdown in the cell, also addressing them as an appealing candidate among all other gene delivery vectors [1, 2].

### 3. Calcium phosphates

CaP is one of the major mineral constituents of all mammalian calcified tissues and it is biocompatible, biodegradable, non-toxic, and non-immunogenic [14], therefore, CaP has widespread use in nanomedicine, including tissue engineering, imaging, and drug/gene delivery [14]. Furthermore, it can degrade into ions inside the early lysosomes of the cells, which makes it a convenient and safe intracellular delivery agent for the therapeutic small molecules and genes [14, 15]. Therefore, CaP nanoparticles have been widely used as non-viral vectors since the 1970s [16]. Negatively charged nucleic acids can bind to CaP nanoparticles with high affinity via calcium ion chelation and are consequently protected from degradation by endonucleases [14].

Hydroxyapatite (HA), with the formula  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , has a Ca/P molar ratio of 1.67 and it is the most stable and least soluble phase of all calcium orthophosphates. Pure HA never forms in biological systems. Nevertheless, because of its excellent biocompatible, bioactive, and osteoconductive properties, HA is widely used in medical and dental applications, such as hard tissue repair, bone defect filling, bone regeneration, coatings of metal prostheses, and tumor surgery applications, etc. [17]. HA nanoparticles have recently been used in medical applications as carriers for growth factors, antibiotics, cancer drugs, antigens, and nucleic acids [18]. Moreover, HA columns are used for protein chromatography [19]. Several techniques, such as solid state, precipitation, sol-gel, hydrothermal, multiple emulsion, and biomimetic deposition have been used in the synthesis of HA nanoparticles [20]. However, some variability in the structure and morphology may occur depending on the synthesis method [21].

The non-stoichiometric apatites are of biological importance because they resemble mineral component of bone, enamel, dentin, and cementum. Bone mineral is essentially calcium-deficient hydroxyapatite (CDHA,  $\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ ) with a Ca/P ratio of approximately 1.5. While CDHA is chemically and compositionally similar to beta-tricalcium phosphate ( $\beta$ -TCP), it exhibits structural similarity to stoichiometric HA [14]. CDHA exhibits high solubility in water or body fluids while high crystalline stoichiometric HA has poor solubility. Over a broad range of concentration, pH, and temperature, non-stoichiometric apatites can be synthesized by various precipitation techniques [21].

#### 3.1. The use of calcium phosphate nanoparticles for gene therapy

Although several studies have focused on the direct injection of naked DNA into various tissues such as skeletal muscle, liver, thyroid, heart muscle, brain, and urological organs, significantly (respectively) low transfection efficiencies were gained [22]. The literature indicates

that there are three general levels that non-viral CaP-DNA delivery systems operate: (a) DNA condensation and complexation, (b) endocytosis, and (c) nuclear targeting or entry [23]. Before introducing into the cell, negatively charged DNA is complexed with CaP nanoparticles. Because of its positive charge, CaP-DNA complex gets bound to the negatively charged cell membrane and is consequently received by the cells. For the uptake of macromolecules or nanoparticles, endocytosis as a frequent methodology can internalize non-viral gene delivery vectors [24]. Endocytosis can be defined as the penetration of the cell membrane which is followed by the incorporation into an intracellular vesicle [25]. After endocytosis, the vector-DNA complex is mostly kept in perinuclear endosome/lysosomes. In case, DNA does not escape from the endosome, endosomal degradation of DNA might happen during endocytosis. The acidic pH (5–5.5) of endosomes and lysosomes usually causes the decomposition of DNA captured inside the vectors [23]. For this reason, DNA transfection requires endosomal escape. The degradation of DNA by particular enzymes (nucleases) depends on an efficient release of DNA into the cytoplasm. There are some barriers needed to overcome for having a successful entry of DNA into the nucleus; hence, protection from the nucleases is required. In the next step, the DNA is introduced into the nucleus. Nuclear pore complexes (NPCs) make the transfer of molecules into the nucleus [10]. Even though NPCs are very permeable to small molecules, they restrict the movement of larger molecules across the nuclear envelope. For the purpose of eliminating this obstacle; first, macromolecules carrying a nuclear localization sequence (NLS) are recognized by importins, and next they are actively transported through the pore into the nucleus [10]. Furthermore, Ca<sup>2+</sup> regulated transport involves intermediate-sized molecules (10–70 kDa) and it does not entail an NLS [23]. Under conditions in which Ca is present in the cytosol (such as from CaP), the central plug is located well below the cytoplasmic ring of the NPC, and the pore can allow the diffusion of even medium-sized molecules. Under such circumstances, DNA-Ca complexes can easily enter the nucleus through the NPC.

The biochemistry field has been using the standard calcium phosphate transfection method, since it was first introduced by Graham and van der Eb in 1973 [26]. In many studies, calcium and phosphate solutions are mixed in the presence of DNA and coprecipitated with DNA to form the transfection complex [27]. However, this technique allows for the formation of a heterogeneous size distribution of CaP-DNA complexes and induces a large deviation in the transfection efficiency. The control of the growth and thus the size of the CaP-DNA complex are the two important factors for developing DNA delivery systems [28]. The size of the complex can be regulated by adding organic or inorganic modifiers during the precipitation process. Kakizawa et al. [28] developed monodisperse CaP nanoparticles surrounded by hydrophilic polymer layer through complexation with poly(ethylene glycol)-block-poly(aspartic acid) (PEG-PAA) via basic mixing of calcium/DNA and phosphate/PEG-PAA solutions. It was shown that the use of PEG and PAA is beneficial to obtain a narrow size distribution. The efficiency of the complex was determined by measuring the amount of DNA incorporated into the particles by using gel permeation chromatography and fluorescence measurements. Similarly, HA nanorods with a length of 100 nm and a diameter of 25 nm were synthesized by using a stabilizing block copolymer (PLGA-mPEG) and the DNA loading capacity was investigated [29]. In this study, first, HA nanorods were synthesized and then, the HA-DNA complex was obtained by quick mixing them together in the presence of CaCl<sub>2</sub>.

Plasmid DNA and salmon sperm DNA were used to test the DNA loading capacity and a huge enhancement of DNA binding capacity was observed. The strategy of using block copolymer and post-binding of HA nanorods with DNA make them remarkably efficient and safe gene vector [29]. Zhu et al. [30] synthesized short HA nanorods with a length of 40–60 nm and then mixed them with EGFP-N1 pDNA to form the nanoparticle-DNA complex. The agarose gel electrophoresis showed that the HA nanorods potentially adsorb EGFP-N1 pDNA under acidic (pH = 2) and neutral (pH = 7) conditions. HA-EGFP-N1 pDNA complex was transfected to the SGC-7901 cells in vitro with the efficiency about 80%. Two weeks after tail vein injection into mice, no acute toxicity was revealed. The liver, the kidney, and the brain organ tissue specimen of the sacrificed mice were investigated via TEM after receiving the injection of pDNA complex. EGFP green fluorescence expression was observed, which suggested that the EGFP-N1-pDNA did effectively transport on the HA nanoparticles into these organ tissues and the pDNA did integrate into the cell genome and ultimately successfully expressed there.

In another study, CaP nanoparticles with varying calcium (Ca) to phosphate ion (P) ratios were synthesized and the effect of the Ca/P stoichiometry on controlling the particle size and the plasmid DNA binding efficiency was investigated [31]. The results indicated that the determination of the Ca/P ratio which optimizes both DNA binding and condensation can overcome many barriers associated with transporting the DNA into the nucleus and thus improve transfection efficiencies. The reported optimal size of the complex is about 25–50 nm which performed efficiently at both binding and condensing the plasmid DNA.

To obtain a better capturing of the DNA, substitute ions, such as  $Mg^{2+}$ ,  $CO_2^{3-}$ ,  $K^+$ , and  $Al^{3+}$ , can be added in CaP crystal lattice [32]. The studies concerning the effect of the substitute ions on the precipitation and the crystallization of HA and other CaPs are present in literature. It was reported that the integration of  $Mg^{2+}$  into the CaP lattice limits the particle growth and this leads to the formation of smaller sizes of CaP/DNA or CaP/siRNA precipitates, which resulted in remarkably enhanced cellular uptake. Chawdory et al. [33] reported that an increase in the dose of  $Mg^{2+}$  dramatically reduced the particle diameters and remarkably higher cellular uptake of DNA and its subsequent expression (N10-fold) was obtained compared with classical calcium phosphate coprecipitation [33].

Another strategy for improving the transfection efficiency is producing a multi-shell CaP-DNA complex, in which calcium phosphate core was coated with DNA, followed by calcium phosphate coating, and finally a second DNA coating [5, 34]. In this way, DNA can be better protected from enzymatic degradation and intracellular attack and therefore, a considerable increase in transfection efficiency can be obtained compared to single-shell CaP-DNA complex. It should be considered that the reaction temperature, the way of mixing, reagent concentration, pH, precipitation time, and the time between precipitation and transfection are important for the efficiency of cell transfection [1, 5, 34].

To protect the therapeutic agent from the biological environment, CaP-DNA can be encapsulated within a synthetic polymer such as polyethylene glycol, poly(methacrylic acid) or polyethyleneimine. However, synthetic polymers such as polyethyleneimine (PEI) are cytotoxic and not suitable for the in vivo delivery of nucleic acids. The toxicity of PEI partly comes from



its limited biodegradability [35]. Klesing et al. [36] functionalized commercial hydroxyapatite nanorods with polyethyleneimine (PEI) and an average particle size of 190 nm without any agglomerates was obtained. In order to track the adsorption capacity of the HA-PEI nanorods, varying amounts of DNA was added to the dispersion and the dispersion stability was monitored by dynamic light scattering. Varying amounts of EGFP encoding DNA were added to the HA-PEI nanorods for the transfection of HeLa and MG-63 cells. As the addition of DNA reduces the surface zeta potential of the cationic HA-PEI delivery system, the nanorods which have small amounts of DNA exhibit higher positive zeta potential and thus better cellular uptake by the negatively charged cell membrane.

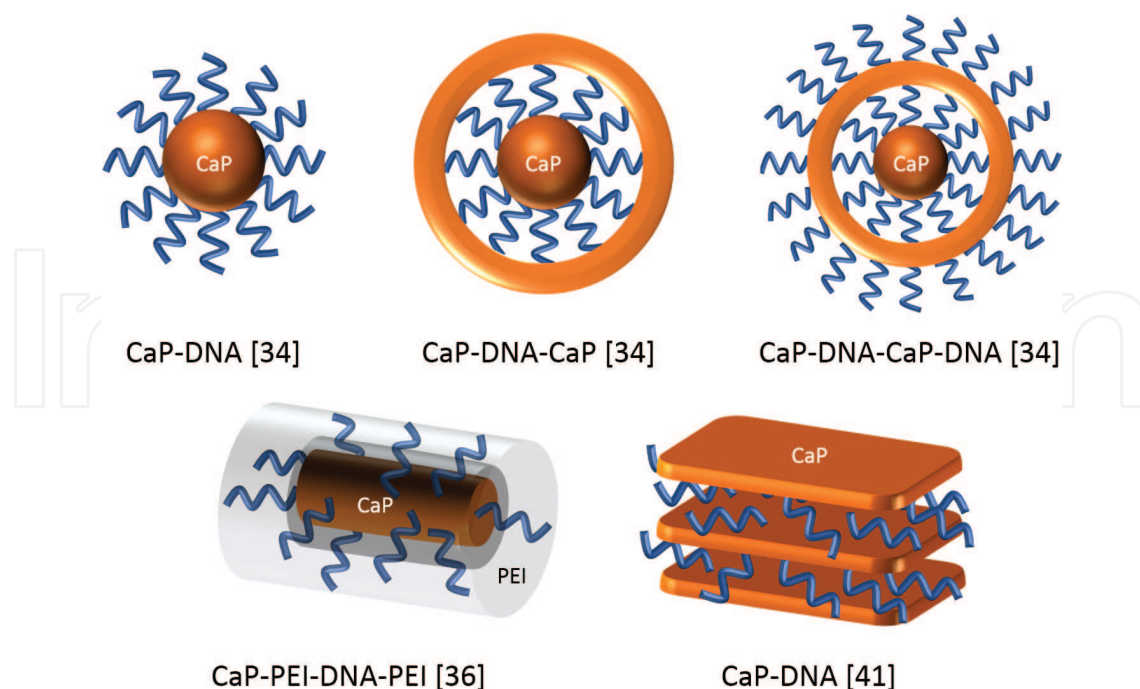
Since the cell membrane is negatively charged, positively charged nanoparticles lead to the higher degree of internalization due to the ionic interactions occurring between positively charged particles and cell membranes [37, 38]. Furthermore, it was reported that the positively charged nanoparticles might escape from lysosomes if they are internalized and show perinuclear localization, while the negatively and neutrally charged nanoparticles colocalized with lysosomes [39, 40]. The surface modification can strongly alter some characteristics of nanoparticles (e.g., hydrophilicity, hydrophobicity, and surface charge). Therefore, conjugating the nanoparticles with special functional groups can enhance the cellular uptake and thus the transfection efficiency of the delivery system. Recent research has revealed that arginine with guanidyl group can facilitate the cellular uptake of covalently conjugated particles, although the uptake mechanism is still controversial [37]. Wang et al. [37] synthesized rod-like arginine functionalized HA (HA/Arg) nanoparticles with an average length of 50–90 nm and the DNA complex was prepared by mixing the HAp/Arg with plasmid DNA (pEGFP-N1). Transfection efficiency was tested on the HeLa cells which were treated with pEGFP-N1-HA/Arg complex and the transfection efficiency increased after arginine modification.

In another study, it was reported that if two-dimensional layered materials are used as gene delivery materials, they exhibit desired functionalities [41]. Zuo et al. [41] prepared lamellar HA (L-HA) nanoplates with varying size and shape by changing the amount of the templating agent and precursor solutions. pDNA was added dropwise into L-HA suspensions with varying concentrations for the complex formation. It was suggested that pDNA loading is not only limited by the adsorption of the HA surface but also intercalation of pDNA into HA layers. Thus, a mouse fibroblast L929 cell line was used to investigate the gene transfection of pDNA/L-HA complexes. As the L-HA showed much better DNA loading capacity and transfection yield, the great potential of the nanoplates with good lamellar structure was reported.

In summary, different strategies were developed for improving the gene transfection efficiency by using CaP nanoparticles as non-viral vectors and some of the reported models are illustrated in **Figure 3**. Most of the approaches were focused on controlling the size and the shape by utilizing special agents during CaP synthesis or encapsulating the particle-DNA complex mostly in a proper layer of polymer to obtain positive surface charges.

### 3.2. The use of calcium phosphate nanoparticles for gene silencing

Small interfering RNA (siRNA) is a class of short, double-stranded RNA molecules of about 21–25 nucleotides, which can mobilize the RNA interference (RNAi) pathway [14, 42]. RNA



**Figure 3.** Some of the CaP-DNA complex strategies reported in the literature.

interference (RNAi) effect is a sequence-specific gene-silencing process which is effectively applied for functional genomics, in vivo target validation, and gene-specific medicines. Similar to the antisense and ribozyme strategies, RNAi depends on complementarity between the RNA and its target mRNA for the destruction of the target gene [42].

When compared to conventional drugs or small molecules with a limited range of protein targets, siRNA may perhaps be used to interfere with the expression of nearly any gene transcript in a specific manner. Although siRNA-based RNAi technology has been launched as an effective methodology in treating several genetic diseases, this technology is limited owing to the degradation of siRNA in the nucleases [14]. Thus, a biocompatible carrier approach is necessary to overcome the barriers for the delivery of siRNAs for successful therapeutic applications [14].

Although both plasmid DNA and siRNA are similar double-stranded nucleic acids having anionic phosphodiester backbones and the same negative charge to nucleotide ratio, the use of DNA requires some different major aspects regarding its complex formation and its intracellular delivery [39]. For being transcribed, plasmid DNA needs to cross the cytosol and reach the nucleus. Therefore, the delivery of plasmid DNA by using chemical delivery vectors is sometimes ineffective on non-dividing quiescent cells due to the cell nucleus envelope barrier [42]. Further, the siRNA only requires to be delivered to the cytosol for initiating the silencing activity and the therapeutic potential of siRNAs has prompted a revival and strong research and development effort in this field.

In some studies, CaP is precipitated with siRNA to form the gene silencing complex. Sokolova et al. [5] investigated the efficiency of single-shell (the core of CaP, the shell of siRNA) and multi-shell (the core of CaP, a first shell of siRNA, the second shell of CaP, the third and outermost

shell of siRNA) nanoparticles to inhibit the expression of intercellular enhanced green fluorescent protein (EGFP) in HeLa cells. Both single-shell and multi-shell nanoparticles have a spherical shape with a diameter of about 100–200 nm. The gene silencing efficiency is proportional to the extent of EGFP inhibition in the cells (cells which show no green fluorescence anymore) and determined by transmission light microscopy and fluorescence microscopy. Not only the concentration of oligonucleotides but also the nature of oligonucleotides (single- or double-stranded) can affect the stability of the colloidal delivery system. Monodisperse colloids can be prepared with siRNA at a concentration of 45 mM. The relative gene silencing efficiencies of different delivery systems were investigated and high gene silencing efficiency was obtained for multi-shell nanoparticles in which siRNA is protected from intracellular degradation.

Although the use of CaP for siRNA delivery has performed a lot of promising progresses, the application of CaP-siRNA in clinical therapy is still far from satisfaction [31]. To improve the physical stability and the transfection efficiency of CaP nanoparticles, different strategies have been evaluated for the modification of CaP carriers. One of the most crucial factors of a successful siRNA therapy is to control the size and the surface charge of the siRNA complex for ensuring the delivery and release of the therapeutic material into the target gene [31]. For this aim, some surface modifications including encapsulating the CaP-siRNA complex with different polymers or adding a stable polymer outer layer were reported [43]. In order to obtain better colloidal stability and to reduce protein adsorption, Polyethylene glycol (PEG), a neutral and hydrophilic polymer, could be used [44]. It was reported that, compared with some commercially available transfection reagents, CaP-PEG nanoparticles reveals better serum tolerability. Owing to the decomposition of CaP nanoparticles under a cytosolic environment, calcium ion concentration increases. However, by using PEG as a shell on the CaP core, much lower calcium ion concentrations can be maintained and thus significant biological effects can be reached in cultured cell lines. Giger et al. [44] modified CaP nanoparticles with PEG-alendronate (alendronate is a bisphosphonate used to treat osteoporosis) for siRNA delivery and it was reported that PEG-ALE could stabilize the nanoparticles better. The transfection efficiency of the nanoparticles was assessed with a model siRNA targeting the Bcl-2 protein which inhibits apoptosis and is overexpressed in several cancers. PEG-ALE-CaP-siRNA exhibited a strong silencing effect *in vitro* at both the mRNA and protein levels. The cellular trafficking study showed that PEG-ALE-CaP-siRNA internalized into cells relied largely on the clathrin-dependent endocytosis. They further used PEG-functionalized bisphosphonate (PEG-bp) to prepare bp-stabilized CaP nanoparticles with the size of 200 nm for gene delivery [45]. PEG-bp-CaP showed effectively and sustained transfection ability to cells *in vitro* with low toxicity. Zhang et al. [18] developed CaP nanomicellar carriers which can efficiently keep siRNA in their CaP inner core by using PEG for regulating the size of the nanoparticles, which facilitated the internalization of siRNA by cells. The nanoparticles exhibited a well-defined spherical shape with a diameter of 100 nm and the thickness of PEG shell is about 10 nm. To evaluate the gene-silencing efficiency, dual luciferase assay was applied to the nanoparticles having different PEG shell thicknesses. Compared to 15 kDa PEG, the samples prepared with 12-kDa PEG showed better gene silencing efficiency. So, the difference in gene knockdown may stem from the difference in cellular uptake of the nanoparticles with different PEG shell thicknesses. Further, compared to the samples prepared at pH 8.0, the samples prepared at pH 7.5 showed higher gene knockdown levels under the same conditions.

Polyethyleneimine (PEI), another hydrophilic polymer, can also be used as an outer layer of the CaP-siRNA complex. However, it should be noted the potential cytotoxicity may limit its gene or drug delivery-based clinical applications [35]. Although PEI is an efficient transfection agent for DNA due to its great endosomal escaping capability, PEI/siRNA complexes are highly unstable [46]. Nevertheless, some successfully in vitro siRNA delivery applications were reported by using PEI as a surface modifier. Klesing et al. [36] developed a cationic nucleic acid delivery system based on hydroxyapatite nanorods functionalized with a layer of PEI. For gene silencing, HeLa-EGFP cell line (a genetically modified transformed cervix epithelial cell line expressing the enhanced green fluorescent protein, EGFP) was used. Commercial carriers, Lipofectamine™ and PolyFect®, were also used for comparing the transfection efficiency of the CaP-PEI system. Gene silencing efficiency of hydroxyapatite-PEI-siRNA nanoparticles on HeLa-EGFP cells was determined approximately 48–72 h after transfection by transmission light microscopy and fluorescence microscopy. The gene silencing efficiency of HeLa-EGFP cells was systematically determined for increasing amounts of added siRNA, up to a charge reversal by the anionic siRNA. The efficiency of gene silencing increased with higher amounts of siRNA, however, minimized at the point where particles agglomerated. By adding a PEI layer around the hydroxyapatite/PEI/siRNA, the charge reversed back to positive, resulting in even higher silencing rates.

To enhance the silencing efficiency, Neuhaus et al. [47] generated triple-shell CaP nanoparticles (CaP-siRNA-CaP-PEI) with an outer shell of PEI. PEI was used for the colloidal stabilization and to give the particles a positive charge. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), which is a pro-inflammatory cytokine, was silenced by treating LPS-stimulated MODE-K cells with triple-shell CaP nanoparticles. MODE-K cells are a murine epithelial cell line from the intestine and serve as a model for intestinal epithelial cell function. In this study, the gene silencing efficiency was first demonstrated with HeLa-EGFP cells by three different methods: cell counting by using fluorescence microscopy, fluorescence-activated cell sorting (FACS) analysis, and quantitative real-time PCR (qPCR). The silencing efficiency by fluorescence microscopy was difficult to interpret. More accurate results were obtained by FACS analysis and qPCR. qPCR results show enhanced silencing effects with the increasing amount of siRNA. By introducing triple-shell CaP nanoparticles into MODE-K cells, the expression of TNF- $\alpha$  was strongly downregulated. Gene silencing efficiencies close to results with the commercial agent Lipofectamine were achieved. Thus, CaP nanoparticles are well suited to reduce inflammatory reactions by silencing the corresponding cytokines, e.g., TNF- $\alpha$ .

In most of the studies, siRNA was precipitated together with calcium and phosphor precursor solutions for obtaining core-shell delivery complexes. Therefore, the particles were only characterized in terms of particle size and surface charges, while it was not determined if the particles were hydroxyapatite or other CaP phases. However, according to the current state of knowledge; particle size, particle shape, surface charge, and crystallinity may have an effect on the siRNA binding capacity and thus the efficiency of cellular uptake. For this reason, Bakan et al. [14] synthesized spherical-like hydroxyapatite (HA-s), needle-like hydroxyapatite (HA-n), and calcium deficient hydroxyapatite (CDHA), and the particle characteristics were investigated in detail. The effects of particle size, particle shape, crystallinity, and surface charge



on the binding capacity of two different siRNAs were investigated. Arginine functionalized needle-like HA nanoparticles with a diameter of 15 nm and a length of 100–150 nm provided the best binding behavior for siRNA among the others, due to the high positive zeta potential and the geometry factor of the particles. Needle-like HA nanoparticles have also a much larger surface to volume ratio, hence, the surface area of the  $\text{Ca}^{2+}$  rich plane, which is considered to be positively charged, is higher than the others. Therefore, the binding efficiency of HA-n is higher than HA-s. Although CDHA has similar geometry to HA-n with almost the same dimensions, the binding potential of CDHA is lower than HA-n due to the deficiency of positive charged  $\text{Ca}^{2+}$ . Additionally, the nanoparticles without any functionalization also exhibited considerable affinity to the siRNAs.

### 3.3. The use of calcium phosphate nanoparticles for in vivo gene silencing

The success of in vitro siRNA therapy has been demonstrated by inhibiting the expression of many different genes and in many different cell types [48]. However, siRNA delivery to animal tissues requires considering some physical, chemical, and biological complicated processes [48]. There are some important weaknesses that limit the extensive application of several delivery systems such as the mutagenesis or oncogenesis potential, host immune responses, and high cost. As the immune response toward the non-viral siRNA delivery systems is very few, this yields an advantage in drug target validation and permits the therapeutic applications of siRNA [49].

$\text{CaCl}_2$ -modified HA nanoparticles were used as a non-viral vector in the in vivo delivery of si-Stat3 plasmid-based siRNA for suppressing the mouse prostate tumor growth and promoting apoptosis of cancer cells [49]. Stat3 is a transcription factor and has been found in an activated state in numerous primary tumors and the Stat3 signaling pathway can be used as a potential target for antitumor therapy. The levels of Stat3 mRNA and protein were downregulated in cancer tissues after si-Stat3 treatment, indicating that HA can deliver Stat3-specific siRNA into cancer cells, resulting in the inhibition of Stat3 expression.

The effect of HA-siRNA complex on chronic inflammatory pain was investigated in vivo in mice [50]. HA nanorods with a length of 40–50 nm were synthesized by a chemical precipitation-hydrothermal technique for the delivery of siRNA. NR2B, the target gene, is a NMDA receptor subunit which plays an important role in the adjustment of chronic pain. To obtain HA-NR2B-siRNA complex, HA nanoparticle suspension was mixed with siRNA under different pH conditions. The results show that HA nanoparticles can bind to siRNA effectively in the acidic or neutral condition at an HA: siRNA mass ratio of 35:1 or higher. In addition, HA-NR2B-siRNA complex remains stable in the physiological condition. HA-siRNA complex was injected into subarachnoid space of each mice. On the seventh day after injection, 1% formalin solution was injected into the plantar surface of the right hind paw for the formalin test. The contralateral paw served as control. Pain scores were given and the Time-Score Curves were plotted. Via intrathecal injection, HA-siRNA complex can significantly reduce formalin-induced nociception in the tonic phase in mice. Therefore, HA may have a potential as an effective siRNA carrier even in in vivo.



In another study, multi-shell CaP nanoparticles (CaP-siRNA) encapsulated into poly(D,L-lactide-co-glycolide) (PLGA) and a final layer of polyethyleneimine (PEI) for the local therapeutic treatment of colonic inflammation [51]. The synthesis of nanoparticles was performed via rapid mixing of equal amounts of calcium-L-lactate and diammonium hydrogen phosphate aqueous solutions. Right after mixing, the CaP dispersion was mixed with siRNA and then encapsulated into PLGA. The freeze-dried nanoparticles were afterward resuspended in an aqueous solution of PEI. PLGA allows for a controllable releasing behavior which can be affected by the geometry and the molecular weight of the nanoparticles [51]. PEI was employed as a shell layer to provide a positive surface charge to the encapsulated nanoparticles. siRNA-loaded nanoparticles could be effectively delivered into the cytoplasm of epithelial cells and immune cells of mice *in vivo* and thus induce active gene silencing. Such a treatment induced a significant reduction of the target genes (TNF- $\alpha$ , IP-10, and KC) in colonic biopsies and mesenteric lymph nodes when they were accompanied with a distinct amelioration of intestinal inflammation. Thus, this study provides a promising approach for the treatment of intestinal inflammation via CaP/PLGA-siRNA nanoparticles.

### 3.4. Calcium phosphate nanoparticles for miRNA delivery

MicroRNAs (miRNAs; miRs) are single-stranded, non-coding RNA molecules which are biochemically and functionally indistinguishable from siRNAs [52]. It is known that miRNA can bind to the target mRNA and inhibits its expression [52]. Although the functions of miRNAs have not quite known yet, the role of miRNA in the regulation of gene expression by controlling the various cellular and metabolic pathways was revealed. When a single miRNA is able to regulate several mRNA targets, multiple miRNAs are cooperatively able to regulate a single mRNA target [53].

As similar to siRNA applications, the major challenge of miRNA therapy is delivering the miRNA to the target tissue efficiently [54]. The nucleases (e.g., serum RNase A-type nucleases in the blood) can degrade naked miRNAs within seconds, therefore, employing a sophisticated carrier is crucial to enhance the intracellular delivery and provide an endosomal escape [54]. Current non-viral technologies used Lipofectamine®2000, PEG or PEI as a carrier for the protecting of miRNAs [55]. However, CaPs are a promising alternative to polymers or synthetic lipids with having better biocompatibility and the ease of use [55]. Manipulation of gene expression by using microRNAs (miRNAs) offers remarkable and promising potential for the field of tissue engineering [55]. Nevertheless, the deficiency of adequate site-specific and bioactive carriers has limited the clinical translation of miRNA-based therapies. For this purpose, Castano et al. [55] developed a novel non-viral delivery complex by combining HA nanoparticles with reporter miRNAs (nanomiRs). To investigate the transfection efficiency of this complex, human mesenchymal stem cells (hMSCs) which are defined as a particularly hard cell type to efficient transfection was used. The results demonstrate that HA nanoparticles combined with miR-mimics and antagomiRs resulted in high cellular internalization in monolayer hMSCs with limited cytotoxicity and both nanomiR-mimics and nanoantagomiRs provided continuous interference of greater than 90% in monolayer over 7 days. Although the efficient intracellular internalization generally requires positively charged particles of less

than 200 nm, negatively charged HA-miRNA complexes with approximately 300 nm size provided high cellular internalization in accordance with the related literature [55].

Mauro et al. [56] investigated the potential of negatively charged CaP nanoparticles as a therapeutic system for the intracellular delivery of miRNAs to the cardiac tissue. In this study, CaP nanoparticles were synthesized using citrate as a stabilizing agent and crystal growth regulator. During the CaP synthesis, different amounts of synthetic unmodified and unprotected miRNA duplexes (ranging from 1 to 10  $\mu\text{g ml}^{-1}$ ) were added to the reaction medium. Although the size of CaP-miRNAs increased with the increasing amount of miRNA, the PDI values and surface charges stayed close to those of miRNA-free nanoparticles. To evaluate the efficiency of miRNA delivery, HL-1 cells were exposed to CaP-miRNAs both in vivo and in vitro and the intracellular levels of miRNAs were measured at different times after the transaction. The results show that CaP nanoparticles can be successfully penetrated into cardiomyocytes without increasing toxicity or mediating with any functional properties and synthetic microRNAs can be successfully encapsulated within CaP nanoparticles.

In summary, it was revealed that both positively and negatively charged HA nanoparticles have the potential to complex with both miR-mimics and antagomiRs for forming nanomiRs, and they have a tremendous capability to maintain a successful internalization. Therefore, HA nanoparticles can be employed as a delivery system for the clinical translation of miRNA-based therapies.

## 4. Conclusion

The yield of gene therapy generally relies on the performance of the carrier vector. Because of the high efficiency of viral vectors in gene therapy, their use has been prevailing so far in the clinical trials. However, their potential immunogenicity, cytotoxicity, and mutagenesis risks are the major drawbacks of using them. Therefore, the use of a variety of non-viral vectors entering clinical trials is because of the advances in their efficiency, specificity, gene expression duration, and safety characteristics. In order to design bio-adaptable non-viral vectors, mechanisms of transfection, from complex formation to intracellular delivery, have to be well-understood. In the gene delivery process through of non-viral vectors, there have been potential rate-limiting stages such as, cell membrane interaction efficiency, internalization, endosomal escape, gene release from intracellular compartments and the transcription into the nucleus. Each of the aforementioned stages has to be determined by examining the characteristics at the molecular level for coming through the sorted limitations. Clinical achievement is still beyond the ideal conditions despite the fact that non-viral vectors can be applied efficiently for in vitro trials. Promisingly, advances in novel technologies for the development of these carriers allow many research groups for studying in vitro and in vivo behaviors of non-viral vectors for gene therapy.

Calcium phosphates (CaPs) are the main mineral constituents of biological hard tissues. Except for enamel, they are always found as nanoparticles. Due to their excellent features, they have widespread use as a bionanomaterial in the fields of nanomedicine, including tissue engineering, imaging, and drug/gene delivery. They are biocompatible, biodegradable,

non-toxic, and non-immunogenic. Moreover, its characteristic degradation into ions is present in all cells and extracellular fluids make it a safe and suitable intracellular delivery system for therapeutic small molecules and genes. CaP nanoparticles have been widely used in non-viral gene delivery systems since the 1970s. Negatively charged nucleic acids can bind to CaP nanoparticles with high affinity by calcium ion chelation and are consequently protected from degradation by endonucleases. During internalization, continuous acidification in the lysosomes causes degradation of CaP particles and the therapeutic molecules can be released. Nevertheless, the cytotoxicity may slightly increase owing to the rise in the level of intracellular calcium which is caused by this degradation. In the literature, it has been reported that a large spectrum of CaP nanoparticles has been synthesized and employed as a carrier for DNA, siRNA, and miRNA. In most of the studies, CaP-nucleic acid complex was obtained by quick mixing of calcium precursor solution with DNA/siRNA which is followed by adding the phosphate solution. Since the nucleation and crystal growth can be influenced by the precipitation parameters such as pH, concentrations of each solution and temperature and also, cell transfection efficiency may be drastically affected by similar conditions. The time slot between precipitation and transfection also influences the transfection efficiency in terms of colloidal stability of the complex. The efficiency of transfection has been found to be cell-type dependent and intercellular trafficking pathways. If DNA/siRNA is surrounded by an additional shell of calcium phosphate, it can be better protected from enzymatic degradation and intracellular attack by nucleases. Core-shell structure leads to a significant enhancement in the transfection or silencing efficiencies compared to single-shell calcium phosphate nanoparticles. It should also be noted that the surface charge of the complex has a significant effect on the penetration through the cell membrane. Due to the surface-exposed phospholipids, the cell membrane is negatively charged and positively charged particles are taken up by the membrane at a higher rate compared to negatively charged ones. For having positively charged CaPs, surface modification strategies including amino acid functionalization, surface coating with cationic or neutral polymers were carried out. Recent research has indicated that the modification of CaP nanoparticles by mostly coating a suitable polymer, provides a better colloidal stability and thus improves not only *in vitro* but also *in vivo* gene transfection or silencing efficacy compared to using bare CaP nanoparticles. The amount of the therapeutic materials largely depends on the carrier adsorption potential and this drastically affects the surface charge of the complex; therefore, the higher the amount of DNA or siRNA does not bring about the higher transfection or silencing efficiency. To have a better understanding of the effect of carrier shape on cellular uptake and cytotoxicity, various studies have been carried out and reported in the literature. It was found that the cytotoxicity of the particles not only depends on the particle shape but also it can be cell-type dependent and the mechanisms that induce cytotoxicity through particle-cell association, whether arising from ROS generation, cytokine production or cellular uptake, are still not clear.

The effects of chemical, physical, and/or biological aspects generally make *in vivo* DNA or siRNA delivery complicated for animal tissues. The limitations in the use of most delivery systems *in vivo* can be summarized as responses of host immune, the risk of mutagenesis or oncogenesis and high costs. Considering CaP nanoparticles as non-viral vectors are still to be questioned for clinical trials. The increase in the level of the solution's ion strength, for instance, might cause a decrease in the electrostatic repulsion between nanoparticles and this

might result in agglomeration. In the presence of proteins, hydrophilicity, surface chemistry, and the charge of the nanoparticles can be altered due to adsorption of proteins on their surface. Accordingly, the behavior of a functionalized nanoparticle will be different in the tissue or the bloodstream compared to aqueous medium or physiologic solutions. This case leads to complications which are difficult to handle in the clinical trials. Nanoparticles have completely different biological properties when compared to their bulk properties and therefore all aspects are to be considered carefully for maintaining appropriate colloid stability and cellular interaction behavior. In the modified nanoparticles, the physicochemical state becomes more complex and this leads to less predictable biological responses and the cytotoxicity of the modified systems are also to be considered.

Bringing together all outcomes of the recent research, it can be concluded that the use of CaPs as non-viral vectors prevails among other non-viral vectors owing to their low-cost and safety behavior, as well as they exhibit better performance in overcoming the limitations that have been observed in other vectors. Providing that the size of the delivery complex is small enough, the surface charge of the complex permits maximum cellular internalization and the therapeutic molecules are protected through the cellular uptake; a successful transfection can be achieved and CaP nanoparticles can be defined as “second generation non-viral vectors” as Maitra [23] denoted.

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