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Review Article

The prospective applications and challenges of calcium phosphate nanoparticles

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Abstract

The use of metal-based nanoparticles in biological system is associated with various well-known shortcomings. So, the role of inorganic calcium phosphate nanoparticles in drug delivery, imaging, bone and tooth augmentation and repair is increasing due to their bioactivity, biocompatibility, no immunogenicity and less toxicity. They are used as scaffold, cement, coating material and nanoparticles. However, their use as nanoparticles in drug delivery and imaging is appreciable in biological system. They are emerging as a second-generation non-viral vector for efficient delivery and stabilization of nucleic acids. The current appraisal is an update about the recent advancements in drug delivery, imaging, orthopedic and dental fields. The review will also discuss about the shortcomings and related strategies to overcome the problems with calcium phosphate particles.

Keywords: Calcium phosphate nanoparticles, Drug delivery, Nanoparticle toxicity

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1. Introduction

The application of nanotechnology to the medical science promises advances in the therapeutic measures. Recently, many nanoparticles (NPs) are being tested for their therapeutic potential, but their biological fate to target pathological condition is minimal. The main reason for this meager application of nanotechnology is the cellular toxicity induced by them. Now-a-days, it is well known that nanoparticles instigate toxicity via a mechanism implicating oxidative stress, inflammation, apoptosis and finally cell death [1-3]. Moreover, NPs are also known to cause genotoxicity, fibrosis, carcinogenesis etc. [4-5]. The root cause for the NPs toxicity is the production of reactive oxygen species (ROS), which are known to orchestrate a series of pathological response like inflammation. The inflammatory cells like neutrophils and macrophages have been shown to generate ROS by some NPs [6-8]. Some NPs like Ar, Be, Co, Ni were ascertained to activate free-radical generating, MAPK and NF-kB pathways. An *in vivo* study showed that exposure with SiO_2 and quartz NPs increased reactive nitrogen species [9-10]. Several other metal NPs including Cu, Ti and Si impaired mitochondrial function via generating ROS [11-13]. Overall, one can easily speculate that metal-based NPs are not suitable for biological applications. All aforementioned limitations of the metal-based NPs diverted the interest of scientists to biologically compatible, calcium phosphate nanoparticles (CaPNP). These nanoparticles are of great interest in medicine, biology and materials sciences. The main applications along with examples are shown in figure 1. Albeit, they are used as Nanoparticles, Scaffolds, Cement and Coating but the review will focus mainly on the applications of CaPNP, its uses, limitation and strategies to overcome its current constraints especially in the field of drug delivery, imaging, orthopedic and dental. Further, the major side effects and associated strategies will also be discussed.

Types of Calcium Phosphate

The calcium and phosphate combine together to form various compounds depending on the availability of water, temperature and any other element. The two phases of calcium phosphate (amorphous or crystalline) and particle shape is temperature and time dependent feature; the reaction at low temperature (4^0C) and/or for less duration, is associated with the formation ofamorphous and round shaped particles and higher temperature (37^0C) and/or longer duration with crystalline and spindle shaped particles [14]. The input of chemistry techniques like X-rays has let to the formation and characterization of various compounds of calcium phosphate like monocalcium phosphate, dicalcium phosphate, tricalcium phosphate, octacalcium phosphate, hydroxyl apatite etc. The details with chemical formula and subsequent Ca/P

ratio are mentioned in the table 1.

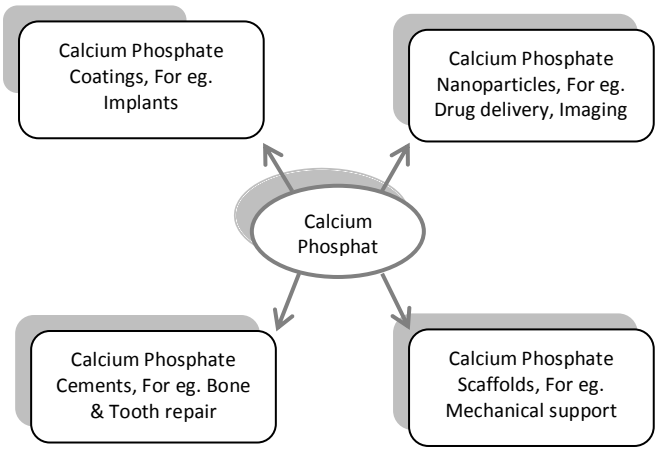

Figure 1: The application of calcium phosphate

Calcium Phosphate nanoparticles in drug delivery

Now days, the scientists are focusing on CaPNP[15-16], because of their better stability and biocompatibility with the biological tissue. Moreover, CaPNP are emerging as a second-generation vector for efficient delivery and stabilization of nucleic acids inside cells. Although, their application in cell biology as a DNA delivery agent started almost two decades before [17], and as compared to a commercially available gene transfecting reagent Polyfect, CaPNP were found to have higher transfection efficiency of plasmid DNA [18]. Peeping in to the interaction chemistry between CaPNP and nucleic acids, Banik and Basu [19] and found a binding constant of the order of 10^4M^{-1} with approximately 1:3.3 stoichiometry of interaction amid CaPNP.

And Salmon testis DNA. The use of CaPNP to transfect platelet-derived growth factor plasmids into fibroblast was found to be efficient and biocompatible [20].The therapeutic potential of nucleic acids is diverse in diseases pertaining to genetic ailments, viral diseases, cancer etc., and CaPNP being stable and biocompatible, considered as a potential suitable carrier for gene delivery as compared to the viral gene delivery system. However, the lack of tissue specificity and uncontrollable crystal growth, their clinical use is restricted. The uncontrolled growth of CaPNP overcome by coating of dopa (3,4-dihydroxy-L-phenylalanine) modified chitosan (Chi) on the surface of calcium phosphate particles. The conjugate CAP/pDNA/dopa-Chi significantly improved transfection efficiency and stability of pDNA [21]. Hence, now days, research focus is to innovate combinations of CaPNP with other molecules to make them tissue specific. One such combination of the CaPNP particles with 3,4-dihydroxy-l-phenylalanine and hyaluronic acid (HA) was produced and studied, where hyaluronic acid encapsulated DOPA and CaPNP-siRNA conjugate, to target tumors and deliver siRNA [22].The calcium phosphate hybrid NPs used to deliver VEGF siRNA showed promising therapy against pancreatic cancer [23]. The stem cells also showed biocompatibility with CaPNP and accepted CAP encapsulated plasmid DNA efficiently [24]. The role of CaPNP as an anti cancer drug carrier is also increasing. The two separate studies showed therapeutic efficacy of anti-cancerous drugs-doxorubicin [25] and methotrexate [26].

Table 1: Diverse combinations of calcium phosphate mineral phase

Chemical Name	Mineral Name	Chemical Formula	Ca/P ratio
Monocalcium phosphate monohydrate		$Ca(H_2PO_4)_2.H_2O$	0.5
Monocalcium phosphate		$Ca(H_2PO_4)_2$	0.5
Dicalcium phosphate dihydrate	Brushite	$CaHPO_4.2H_2O$	1.0
Dicalcium phosphate anhydrous	Monetite	$CaHPO_4$	1.0
α-Tricalcium Phopshate		α-$Ca_3(PO_4)_2$,	1.5
β-Tricalcium phosphate	Whitlockitte	β-$Ca_3(PO_4)_2$,	1.5
Tetracalcium Phopshate	Hilgenstockite	$Ca_4O(PO_4)_2$	2
Octacalcium phosphate		$Ca_8H_2(PO_4)_6.5H_2O$	1.33
Carbonated Apatite	Dahlite	$Ca_{10}(PO_4)_6(CO_3)(H_2O)$	1.67
Apatite	Hydroxyapetite Fluoroapetite Chloroapetite Oxyapetite	$Ca_{10}(PO_4)_6(OH)_2$ $Ca_{10}(PO_4)_6(F)_2$ $Ca_{10}(PO_4)_6(Cl)_2$ $Ca_{10}(PO_4)_6O$	1.67

Were significantly improved using CaPNP. Calcium phosphate hybrid nanoparticles used as a nanocarrier for anticancer drug docetaxel also showed higher drug-loading capacity and high anticancer effect [27].Recently, the platinum pharmacophore cis-4-diaquadiamine platinum (II), folic acid and rhodamine isothiocyanate conjugated on CaPNP and used for platinum delivery for inducing cytotoxicity to cancerous He La Cells [28]. The CaPNP stabilized with polyethylene glycol and used as a carrier system for oral insulin delivery [29].

The use of CaPNP as a potential vaccine adjuvant is growing. In one study, the CaPNP loaded with FMDV "O" P1-3CD DNA vaccine was found to induce humoral and cell mediated immune response significantly in mice and guinea pigs [30]. The coating with S-layer protein (of Aeromonashydrophila) over nano sized CaPNP and usedfor immunization, elicited both innate and adaptive immune response [31]. In another study, the CaPNP were synthesized in β-cyclodextrin medium, conjugated with amino propyl triethoxy silane, coupled with Newcastle disease virus (NDV) and proved as a possible way of enhancing immunogenicity against virulent NDV challenge in chicken [32]. To increase the transfection efficiency, the CaPNP nanoformulation, prepared using iron oxide as a core and under applied magnetic field these magnetic CaPNP reported to increase transfection efficiency by 30% [33]. Another nanocomposite silkfibroin/ Calciumphosphate/ PLGA scaffold was used

as delivery vehicle for vascular endothelial growth factor and reported as an effective scaffold for bone tissue engineering applications [34]. The stability of a drug Timolol was enhanced with calcium phosphate nanoparticles and the experimental healthy rabbits were hypotensive after injection [35]. Although CaPNP have wide range of applications in biomedical system and presently emerging as an efficient moiety for drug delivery but still, various studies, including our own has showed significant toxicity of CaPNP. Additionally, the most common use of calcium phosphate nano-crystals in gene delivery is challenging due to uncontrolable growth of particles [21].

Calcium Phosphate nanoparticles in bones and teeth

The principle mineral phase component of bones and teeth is hydroxyapatite [$(Ca_{10}(PO4)_6(OH)_2$] [36] and the same can be synthesized *in vitro* in the chemistry labproposed by Bouyer [37] in the following reaction-

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O$$

The biocompatibility with skin, muscles, gums etc. make hydroxyapatite an ideal choice in the form of implanting material for orthopedic and dental implants. It possess superior osteoconductive and osteoinductive properties and slow biodegradability, however, the pure hydroxyapatite has some issues like low mechanical support, so in order to provide ample strength and enhance adhesion with tissue, certain elements are mixed to improvise the acceptance of the implants. The nanohydroxyapetite coated implants are reported to possess more mechanical strength [38]. The application of this material is not limited to-but also used for bone augmentation, repair hard tissues, fillers of bone and teeth and coating of implants [39-45]. The nanophasic hydroxyapatite ceramics have showed superiority towards biomechanical properties. The round shaped CaPNP and rice shaped CaPNP demonstrated a range of different parameters related to osteoblast viability and activity [46].

Recently, a novel Gelatin-CaP colloidal gel has created a hope in the direction of regenerative medicine. The gel is reported to stimulate proliferation and boost attachments in cultured stem cells[47]. The nanocrystaline hydroxyapatite with additional coating of silica material was used for bone repair and regeneration [48]. The different ratios of hydroxyapatite and β-tricalcium phosphate are being explored for use as scaffolds in bone tissue reconstruction [49]and complete regeneration of the damaged region was observed *in vivo* when mesenchymal stem cells derived from placenta were used with nanoceramic (Ca/P ratio 1.58) in the experimental defect created in the femur of Wistar rats. To strengthen the graft of hydroxyapatite and β-tricalcium phosphate, treatment with 24% EDTA showed improved retention [50]. The hydroxyapatite stipulated stability to a hydrogel-based engineered cartilage construct, provides good integration between bone and cartilage [51]. The hydroxyapatite nanoparticles coating changed the surface chemistry of non-compatible implants and induced rapid healing with better attachment to the bone [52]. As a new therapeutic approach, the antibacterial dimethylaminododecyl methacrylate medicine together with synthesized amorphous calcium phosphate reported to augment the healing of the dentin-pulp complex [53]. The nanoparticles of amorphous calcium phosphate were found to be effective in reducing caries in a human *in situ* model [54]. The calcium phosphate particles coating over the silver incorporated titanium nanotubes provided smooth surface, biocompatibility, strength and suggested to be used in biomedical implants [55]. The fabrication with CaPNP improved radial strength and biocompatibility of metal-based stents [33].

Calcium Phosphate nanoparticles in Imaging

The CaPNP are not only biocompatible and bioactive but they are also not luminescent and that property furnishes another promising application i.e. the use of fluoroprobes-labelled CaPNPin diagnosis. On the other hand, the fluoroprobes are usually not biocompatible and cleared off very fast. Therefore, the doping of CaPNP with such fluoroprobes is aprime choice for increasing their stability and bioavailability. A luminescent probe of lanthanide series i.e. Europium (Eu^{3+}) that gave red luminescence with visible excitation when coupled with CaPNP was found to be suitable candidate for live cell imaging [36]. A good image results from good contrast and this is the major limitation of good quality imaging. Recently, a suspension of calcium phosphate nanoparticles powder with colloidal gold and super paramagnetic iron oxide particles were used to enhance contrast for imaging techniques [56]. In one more study, a contrasting agent, diethylenetriamine pentaacetic-acid gadolinium (III) was incorporated in calcium phosphate nanoparticles/ PEGylated shell composite and it showed promising potential as a contrasting agent for magnetic resonance imaging (MRI) for diagnosis of solid non-invasive tumors [57]. A formulation of calcium phosphate/lipids /fluorescent material delivered to lymph nodes was found to be extremely useful in examination of lymph node metastasis [58]. Recently, calcium phosphate nanoparticles were coupled with contrasting agents indocyanine green and Gadolinium, labeled with 99m-Technetium-methylene diphosphonate and used successfully for optical, magnetic and nuclear imaging [59]. Tumor specificity was enhanced by Chlorine e6-loaded with calcium phosphate nanoparticles rather than free chlorine e6 [60].

Calcium Phosphate nanoparticles in oxidative stress and calcification

Hydroxyapatite (a form of calcium phosphate) nanoparticles have been discovered to exert cytotoxicity and induce apoptosis. This ability of CaPNP makes them suitable candidate for cancer treatment. Recently, a report by Turkez et al, [61] showed that Calcium phosphate (especially Hydroxyapatite) particles induce oxidative damage, genotoxicity and cytotoxicity in human blood cells in a dose-dependent manner. Still the mechanism of

interaction of cells with particles is not clearly understood. The increased cytoplasmic calcium load is likely to be the cause of cell death by the particle exposure [62]. Hydroxyapatite nanoparticles have ability to elude the phagocytic pathway and even enter the nuclei through nuclear pores. Recently, Hunter et al.[63] incubated human serum isolated calcium phosphate nanoparticles (210nm) with VSMCs *in vitro* and found CaPNP to be associated with apoptosis, mineralization and VSMCs calcification[63].

Futuristic Strategies

For all the aforementioned applications, the CaPNP are usually introduced in the living being, hereby, the size of CaPNP is an important prerequisite factor to minimize any toxic side effects. As per an *in vivo* study conducted by Adair et al [64] the size of CaPNP between 10-200 nm is appropriate for drug delivery with minimal toxicity. Further, even a marginal increase in size up to 210 nm is shown to cause apoptosis is VSMCs [63]. Still there is a need to critically analyze relationship between particle size versus toxicity and therefore enough data is required to validate above statement. The maintenance of size after conjugation and/or absorption is quite difficult. The storage of CaPNP is also not advisable, as they grow bigger in size, consequently transfection efficiency hampers. Thus, there is need of new approaches to control the size of particles during synthesis. A study showed method to control the shape and size (5-60 nm) of the CaPNPusing polyacrylic acid [65]. Additionally, CaPNP lacks tissue specificity, few conjugates are available for tumor specificity, but delivery of drugs to normal tissue is still under research. However, the coupling of CaPNP with some molecules has shown better transfection efficiency. For example, multi layer coatings of transfect have shown greater transfection efficiency and at the same time less lysosomal degradation of the conjugate [66].

The existing toxicity can be reduced by adding/replacing harmful element with the CaPNP. Likewise, fluoroapetite-hydroxyapetite complexes are observed to be nontoxic and reported to have better treatment abilities for hard tissue engineering [67]. Another study reported better non-viral gene delivery efficacy of magnesium substituted calcium phosphate nanoparticles [68]. Further Calciprotein particles, which are still not explored for drug delivery holds future of CaPNP drug delivery system. These particles have natural proteins incorporated with them like, fetuin and albumin, thus they become more biocompatible and non-toxic [69]. Additionally, proteins are lipid molecules can be loaded in these particles and then used to transfect specifically at required location.

Concluding remarks

The biological compatibility, bioactivity, bio-stability, no immunogenicity, non fluorescence, less toxicity etc. are the excellent factors for using CaPNP as a carrier of a drug/other molecule, thus making it a suitable candidate for various applications like drug delivery, imaging, bone& teeth augmentation and repair etc. On the other hand, toxicity, uncontrolled size growth, lack of tissue specificity etc., are the important points require serious consideration. Some studies have already showed promises to abide the mentioned problems and various others are under progress but still there is need to generate more data and expedite on concerned issues using new concepts to overcome the existing limitation.

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