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## Biodegradable Calcium Phosphate Nanoparticles for Cancer Therapy

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### Abstract

Calcium phosphate is the inorganic mineral of hard tissues such as bone and teeth. Due to their similarities to the natural bone, calcium phosphates are highly biocompatible and biodegradable materials that have found numerous applications in dental and orthopedic implants and bone tissue engineering. In the form of nanoparticles, calcium phosphate nanoparticles (CaP's) can also be used as effective delivery vehicles to transfer therapeutic agents such as nucleic acids, drugs, proteins and enzymes into tumor cells. In addition, facile preparation and functionalization of CaP's, together with their inherent properties such as pH-dependent solubility provide advantages in delivery and release of these bioactive agents using CaP's as nanocarriers. In this review, the challenges and achievements in the intracellular delivery of these agents to tumor cells are discussed. Also, the most important issues in the design and potential applications of CaP-based biomaterials are addressed with more focus on their biodegradability in tumor microenvironment.

### Keywords

Calcium phosphate; nanoparticles; degradable; cancer; drug delivery; intracellular delivery; gene delivery

## 1. Introduction

Developing a wide range of therapeutic and diagnostic agents by recent advancements in nanotechnology has drawn an increasing attention toward their intracellular delivery. Most

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front-line cancer drugs are water insoluble, untargeted and have dose-limiting side effects [1]. In addition, several groups of these therapeutic agents (*e.g.*, DNA or siRNA) are both charged and large molecules and it is difficult for them to cross the cell membrane to elicit their desired effects. Therefore, an appropriate delivery system is required to assist their trafficking and cellular uptake. Although, recombinant viral vectors demonstrated high efficacy for treatment of human diseases, their major drawbacks and eventual clinical failure have led to quick shift toward synthetic non-viral carriers [2, 3].

To overcome limitations of drug delivery techniques, several formulations of nanoparticles have been proposed, including biodegradable polymers [4], gold [5], iron oxide [6, 7], liposomes [8], silica [9], dendrimers [10] and calcium phosphate based mineral systems [11, 12]. An ideal drug carrier should be able to incorporate potential bioactive agents either physically or chemically and protect them in the bloodstream. Furthermore, the carrier complex should de-assemble gradually and provide sustained drug release over prolonged period of time to increase therapeutic efficiency [13]. In addition, it should provide a feasible mechanism to specifically bind to target cells or tissues, in order to reduce their off-target effects and enhance the on-site drug concentration. Among different synthetic vectors calcium phosphate nanoparticles (CaP's) have shown promising results toward the abovementioned criteria.

Synthetic CaP's in the form of carbonate apatite are similar to inorganic mineral of natural bone, which are highly biocompatible and biodegradable materials, extensively used in orthopedic implants and bone tissue engineering [14]. Furthermore, CaP's hold outstanding bioactivity and tailorable biodegradability that distinguish them from the other biominerals and make them an excellent option for drug delivery in nanomedicine applications, orthopedics and dentistry [15–18] or as adjuvants in different vaccines [19–22]. Moreover, their innate properties such as pH-dependent solubility along with facile and straightforward preparation and functionalization make them useful in diverse therapeutic applications, including cancer therapy [23]. Biodegradable nanoparticles are generally preferred for cancer therapy since their predictable body clearance pathways and mechanisms makes them safer candidates for clinical applications [24].

Finally, site-specific cellular entry plays an important role in the bioactivity and bioavailability of the delivered biomolecules. Studies have shown that physical and chemical characteristics of CaP nanoparticles including size, charge, morphology, composition and surface chemistry are the critical parameters that determine the route of internalization of nanomedicines [25–28]. Other essential factors such as dose of bio-agents and their functionalities depend on the cellular entry pathway and the final location inside the cells [29]. The cell has a high number of organelles thus, it is important that the delivery system releases its payload in the specific site in order to induce the desired effect. Insights into how the cell passes its components to the specific cellular compartments could improve drug design for better management of the tumors.

This paper summarizes recent achievements in preparation and cancer therapy applications of CaP nanoparticles and reviews opportunities and difficulties associated with various available strategies to overcome their cellular barriers. In addition, their potential

applications as carriers of bio-agents (*e.g.*, cancer drugs and nucleic acids) and their required properties are discussed. Moreover, the critical roles of their intrinsic characteristics such as pH-dependent solubility for facilitated delivery and release of bioactive agents are addressed in details. Compared with previous publications reviewing CaP design and applications in nanomedicine (*e.g.*, [30–32]), our review provides a new materials science perspective, focusing more on biodegradability of different crystallographic phases of CaP's designed as cancer therapeutics. This is a major safety concern hindering clinical translation of different formulations of nanomedicines developed for cancer therapy.

## 2. CaP Biomaterials for Intracellular Delivery to Cancer Cells

### 2.1. Tuning CaP's Physicochemical Properties for Cancer Therapy

**Size and Morphology:** Binding to specific cell membrane receptors, trafficking inside the cells, intracellular flow and pharmacokinetics, extravasation and clearance mechanisms depend on nanoparticles size and morphology [25, 27, 33–35]. Among various techniques for preparation of cancer therapeutic CaP's [36], synthesis in the presence of nucleic acids (*e.g.*, DNA, siRNA and oligonucleotides) has been reported as a facile and straightforward method [22, 37, 38]. This method usually results in particles with larger sizes (~100–200 nm). However, recent approaches such as laser ablation enabled synthesis of CaP-based particles as small as 3 nm [39]. Larger nanoparticles usually have a short blood circulation time due to rapid accumulation in reticuloendothelial system (RES), while ultrasmall nanoparticles smaller than 8–10 nm have a fast renal clearance from the blood. Therefore, synthesis of CaP nanoparticles should be optimized to tune their sizes within the range of 10–80 nm, as also recommended for other types of nanoparticles, in order to achieve longest blood circulation time and highest amount of uptake by tumors [24].

As one of the pioneering studies, more than three decades ago, Graham and Van Der Eb discovered that entrapment of biomolecules such as nucleic acids into the CaP's can be used for tuning nanoparticles physicochemical properties such as size and morphology [40]. Their approach involved mixing of a calcium chloride solution, containing these biomolecules (*e.g.*, DNA), with phosphate-buffered saline (PBS) for *in situ* co-precipitation of CaP nano- and microparticles (Figure 1). Although this approach had notable advantages such as low cost and facile preparation method, the rapid growth of precipitates resulted in large particle sizes and therefore low transfection efficiency [41].

Later studies showed that controlling synthesis parameters such as temperature, concentration of calcium and phosphate ions, pH, and reaction time are critical to achieve smaller size precipitates [42–44]. Since, the reaction between calcium and phosphate ions in the precursor solutions is spontaneous, adjusting their concentrations and selection of their precursors play important roles in producing a consistent particle size and morphology [45, 46]. After initial burst of nucleation, there is a high affinity for rapid aggregation of particles, due to the van der Waal's and secondary surface interaction between particles. Particle aggregation is a common phenomenon in colloidal suspensions and is much stronger in the nano-sized range, due to the intrinsic surface reactivity of the nanoparticles. It is reported that using dilute solutions of initial precursors of calcium and phosphate ions can minimize these aggregations by controlling the kinetics of precipitation reaction [47]. However, lower

concentration of calcium ions can also reduce the CaP precipitation rates and therefore optimized conditions should be found for each type of mineralization solution and biomolecule. For example, it is known that addition of magnesium to mineralization solution can help to obtain CaP nanoparticles with smaller sizes by inhibiting their crystal growth [48]. This lower growth rate is attributed to distortion of atomic structure of the CaP particles, since some  $\text{Ca}^{2+}$  ions are substituted with smaller  $\text{Mg}^{2+}$  cations.

Incorporation of block copolymers with a polycarboxylate section, such as poly (ethylene glycol)-block-poly (aspartic acid) (PEG-PAA) can also suppress the crystal growth of CaP nanoparticles. This approach involves mixing of a HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing phosphate and PEG-PAA with a Tris buffer containing  $\text{Ca}^{2+}$  and biomolecules (*e.g.*, DNA). It has been known that PAA gets adsorbed on CaP crystals during the growth and compensates the increased interfacial energy. Utilizing this strategy resulted in formation of monodispersed nanoparticles with a core-shell structure. For example, when DNA was added to the  $\text{Ca}^{2+}$ -containing Tris buffer, the core of the resulted nanoparticles consisted of DNA-incorporated CaP crystals that were surrounded by hydrophilic palisades of PEG shell [49, 50]. It was shown that small size and stable nanoparticles could form only above certain value of PEG-PAA concentrations and the particles size could be tuned by modifying the PEG-PAA concentration. In another study, poly (ethylene glycol)-block-poly (methacrylic acid) or PEG-PMA was used to stabilize CaP crystals. The resulted nanoparticles were in the range of several hundreds of nanometers with a considerable colloidal stability, due to the steric hindrance effect of PEG palisade. The PMA segment of the nanocarrier underwent a conformational change at pH 4–6 and formed a more hydrophobic coating as compared to physiological pH. This pH-dependent conformational transition enhanced the interaction of nanoparticles with the membrane of endosomes and facilitated endosomal escape [51]. Pre-mixing of the precursor solutions containing phosphate ions with PEG and then addition of  $\text{Ca}^{2+}$ -containing solutions was also reported as an efficient method to generate stable CaP nanoparticles, due to chelating effect of the PEG molecules with phosphates [52].

As it is shown in Table 1, most of the recent CaP-based *in vivo* cancer therapy studies have used spherical calcium phosphate nanoparticles or shells for delivery of the therapeutic agents to cancer tissues. This is mainly because of mild mineralization conditions used for preparation of the CaP's, which usually results in formation of spherical and round morphologies that are more stable thermodynamically. Rod-shape morphologies have also been prepared by relatively higher temperature synthesis methods. For example, a recent study by Sun *et al.* [53] discussed synthesis and efficacy of hydroxyapatite nanorods for targeting mitochondria of the cancer cells to inhibit growth of lung cancer xenografts in mice. However, spherical nanoparticles are generally preferred for therapeutic delivery applications, since they provide highest possible specific surface area for loading drugs or nucleic acid molecules. Other CaP morphologies such as nano-clusters [54] and porous structures [55, 56] have also been investigated as high-loading capacity candidates for cancer therapy applications, but the relatively larger size of these particles and multiple steps involved in their synthesis are considered as the major drawback hindering their potential applications in clinical trials.

Despite the extensive use of CaP-based vectors for gene and drug delivery during the last 30 years, there are still concerns regarding inconsistencies in transfection or drug delivery efficacy of the synthesized CaP's in different studies. Recent studies have indicated that such inconsistencies can be minimized by tuning the stoichiometry (Ca/P ratio) and crystallographic phase of the CaP particles, addition of cations such as strontium or zinc, using optimized mode of mixing, and selecting proper stabilizing macromolecules (*i.e.*, nucleic acids, oligonucleotides and synthetic polymers) with specific molecular structures [22, 37, 47, 57]. Therefore, accurate controlling of these elaborated synthesis parameters should be considered for reproduction of the nano-sized CaP particles with efficient condensation and bounding of the desired macromolecules.

**Biodegradability and Crystallographic Phase:** Degradability of the cancer therapeutic CaP's in acidic tumor microenvironment or endolysosomal organelles is the other major characteristic that should be considered and designed properly for their clinical translational applications. In fact, most of the nanoparticles studied for nanomedicine are not degradable in body and this has been considered as one of the major safety concerns delaying their translation to human applications. There are a wide range of calcium phosphates with different Ca:P ratios and crystallographic phases (Table 2), and among these different phases, amorphous calcium phosphates and hydroxyapatite are the most common types of CaP's used for cancer therapy. Degradation of the CaP's is pH-dependent and changes with their crystallographic structures, and Ca/P ratio [78–80]. Generally, degradability of different CaP phases in acidic buffers is sorted as follows [32] (“>>” denotes much greater solubility.):

Amorphous Calcium Phosphates >>  $\alpha$ -tricalcium phosphates >>  $\beta$ -tricalcium phosphates > Calcium-deficient hydroxyapatite >> hydroxyapatite > fluorapatite

Amorphous calcium phosphates are expected to degrade faster, since there is no long-range crystallographic bonding in their atomic structure, which results in easier detachment of the atomic layers by consuming less thermodynamic energy. Liu *et al.* [72] designed nanoparticles that were coated with amorphous CaP coatings. Their results showed that this amorphous CaP layer could be degraded in weakly acidic microenvironment of the tumors, to enhance release of the chemotherapeutic drugs that were entrapped inside them. Also, a recent *in vitro* study showed similar results for apoptosis of breast cancer cells, using degradable and drug-loaded amorphous CaP nanoparticles [81]. CaP's in the form of calcium-deficient hydroxyapatite and tricalcium phosphate have also shown promising degradability, especially at lower pH values, which makes them favorable candidates. Kang *et al.* [76] coated drug-loaded silica nanoparticles with calcium-deficient hydroxyapatite and showed that the hydroxyapatite layer could be degraded in low-pH environment of the tumors to release chemotherapy agents. Calcium-deficient hydroxyapatites formed by substitution of  $\text{Ca}^{2+}$  with other ions such as  $\text{Fe}^{2+}$  and  $\text{Mg}^{2+}$  have also been developed for cancer therapy [82]. For example, iron-doped magnetic hydroxyapatite nanoparticles were developed and used for treatment of liver [83] and colon [84] cancers using hyperthermia. Mg-doped hydroxyapatites have also been used to induce apoptosis in cancer cells, due to release of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions as the result of nanoparticles degradation [85]. Additionally, calcium-deficient hydroxyapatite nanoparticles formed by doping hydroxyapatite with small

drug molecules (*e.g.*, 5-Fluorouracil) can also be used as effective cancer chemotherapy agents [86]. Also, it is possible to accelerate degradation of the CaP's by applying external stimuli such as near infra-red laser irradiation. For example, Xu *et al.* [58] doped ultrasmall CaP's into copper sulfide (CuS) hollow nanoparticles and showed that irradiation of the core-shell particles with near infra-red laser can cause disintegration of the CaP's, as a result of photothermal interactions between CuS and light. Their *in vivo* studies showed that released Ca<sup>2+</sup> ions could enhance apoptosis rate of the cancer cells. Safety and biocompatibility of the degradation by-products are also important factors that should be carefully considered in future design of the biodegradable CaP's for cancer therapy. More studies are required to investigate the performance and degradability of different phases of CaP's such as amorphous calcium phosphates, tricalcium phosphates and calcium-deficient hydroxyapatites in low-pH environment of the tumors or endolysosomes. However, *in situ* characterization of the CaP's inside the cells or tumors is a challenging procedure that needs sophisticated materials science expertise. Such characterizations become even more challenging, considering transition of different CaP phases to each other (*e.g.*, transition of calcium-deficient hydroxyapatite to tricalcium phosphate [87]) at low pH environments of the tumors.

## 2.2. Surface Functionalizing and In vivo Cancer Targeting

Surface functionalization of the cancer therapeutic CaP's is necessary to prolong their circulation in blood or adding targeting molecules (*e.g.*, peptides and antibodies) to their surface. These two phenomena enable CaP's enhanced permeation and retention (EPR) in tumors, leading to higher therapeutic efficacy with lower dosage of the administered nanoparticles. As it is shown in Table 1, various methods have been used for surface functionalization of the CaP's, using covalent or non-covalent (electrostatic) conjugation of macromolecules (*e.g.*, polymers, peptides and nucleic acids) to their surface. Overall, these methods can be categorized into four different strategies. The procedure descriptions, and major advantages and disadvantages of each procedure are summarized in Table 3. Selection of the surface functionalization procedure depends on type of targeted tumors and approaches used for their treatment.

Surface functionalization usually increases the hydrodynamic size of the CaP's, due to addition of large molecules to their surface. As mentioned before, CaP's with larger hydrodynamic sizes usually have shorter blood circulation time and therefore less uptake by tumors. However, it is known that addition of polyethylene glycol (PEG) to the surface of the CaP's can help increasing the blood circulation time, due to steric hinderance and less protein adsorption of the PEG molecules. Some recent *in vivo* studies based on using PEG or PEG-derivative molecules for surface modification of the cancer therapeutic CaP's are listed in Table 1.

Surface functionalization with different types of cationic or anionic molecules can also be used for tuning the surface charge of the CaP's, which is a crucial characteristic determining adhesion of CaP's to cancer cells. In addition, electrostatic repulsive forces between charged CaP's, helps to prevent their aggregation and therefore increases the chances of their uptake by tumors. Klesing *et al.* [28] functionalized CaP's with different surface charges by adding

a mixture of anionic carboxymethyl cellulose (CMC) polymer and photoactive porphyrin dye to the CaP's. The negative charge of these particles was then inverted by adding a layer of cationic poly (ethyleneimine) (PEI). Then, they assessed the effect of CaP's surface charge on their ability to pass through the cell membrane. Their results showed that CaP's coated with positively-charged PEI molecules had higher uptake and therefore, more therapeutic efficacy.

Targeted delivery of CaP nanoparticles to tumors is also critical in order to increase their therapeutic efficacy and diminish their undesirable off-target effects. Covalent or non-covalent binding of peptides [70] or antibodies [88] to the surface of CaP's have been reported as effective methods to improve their specific targeting. Type of the targeting molecules that should be conjugated to CaP's depends on cancer cells and receptors available on their membranes. An extensive list of these targeting molecules and methods for loading them to the surface of CaP's are provided in Tables 1 and 3 and some of them will be discussed below.

Folic acid is one of the common molecules used for targeting folate-receptors on cancer cells such as breast and colon cancers and has been used in several recent CaP-based *in vivo* studies [23, 55, 65]. Folic acid is a small molecule and its conjugation to CaP's does not increase their hydrodynamic size significantly. Therefore, it is a suitable targeting molecule that can be used for specific delivery of the therapeutic molecules to tumors using CaP nanoparticles. Also, CaP's can be functionalized with conjugates of folic acid and PEG (*i.e.*, FA-PEG) for increasing their specific delivery to tumors, as a result of synergistic targeting effect of folic acid and steric hindrance of the PEG molecules [65]. Hyaluronic acid (a sugar-based polymer) has also been used for targeted delivery of CaP's to tumors with CD44 receptors. Negative charge of hyaluronic acid facilitate its electrostatic adsorption to calcium ions ( $\text{Ca}^{2+}$ ) on the surface of CaP's through a simple mixing reaction. In two recent studies, CaP's were functionalized by electrostatic adsorption of hyaluronic acid and were used for targeted siRNA delivery to melanoma and lung cancer [59, 64]. A modified procedure has also been developed by Kang *et al.* [76] for covalent bonding of hyaluronic acid to CaP's for targeted drug delivery to breast tumors.

Conjugation of antibodies to CaP's can also be used for their targeted delivery to cancers. For example, Tang *et al.* [23] conjugated EGFR-specific single chain fragment antibody (ABX-EGF scFv) to lipid-coated CaP's and used the nanoparticles for specific siRNA delivery to breast tumor in mice. In a separate study, a bispecific antibody (BsAb) was loaded to the surface of lipid-coated CaP's by non-covalent adsorption and the resulted nanoparticles were used for *in vivo* siRNA delivery and inhibition of breast tumors [61]. While the extensive list of available antibodies offer different opportunities for targeting various cancers, their major drawback in nanomedicine is their large sizes, which also increases the hydrodynamic size of the nanoparticles inevitably. These larger size nanoparticles have shorter blood circulation half-life and therefore show less uptake by tumors. Unlike antibodies, cancer targeting peptides with smaller sizes can be designed and prepared for specific delivery of the nanoparticles to tumors. For example, Zhang *et al.* [70] mineralized CaP's in the presence of a synthetic peptide (A54) and used them for targeted drug delivery to hepatic cancers. Recent studies showed that conjugation of PEG molecules

to peptides such as arginyl glycy l aspartic acid (RGD) and urokinase plasminogen activator analogues peptide (uPA) can help increasing the blood circulation half-life and tumor uptake of the CaP's [60, 68].

### 2.3. Endocytosis of CaP's in Cancer Cells

Cell membrane, a flexible lipid bilayer, separates the intracellular milieu from the environment and plays a vital role in internalization of therapeutic molecules such as nucleic acids, proteins and drugs. Since most cancer therapeutic agents have large sizes and are hydrophobic, they cannot cross the cell membrane easily and require nanoscale carriers that can facilitate their intracellular delivery. Among different types of nanoparticles, CaP's have been extensively used for this purpose, mainly due to their biocompatibility, degradability and facile synthesis methods. However, efficient delivery of these agents to different types of cancer cells requires a thorough understanding of their nanostructures, crystallographic phases, biofunctionalities and the mechanisms by which they get internalized inside each specific type of target cells.

Generally, the uptake pathways of synthetic nanoparticles can be divided into two major categories: endocytic uptake pathway and nonendocytic delivery [89–91]. Endocytosis is defined as the vesicular uptake of the nanoparticles by invagination and pinching of the part of cell membrane [92, 93]. Endocytic routes are divided into several subgroups (Figure 2) including phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and nonclathrin- and noncaveolae-mediated endocytosis [94]. Clathrin mechanism is a multi-step endocytosis process, which is activated by binding of ligands on the surface of the nanoparticles to their specific receptors on cell membrane. Also, caveolae endocytosis is characterized as a small invagination of the cell membrane to internalize the nanoparticles [95]. Pinocytosis, on the other hand, refers to the hydrophobic or electrostatic interaction of nanoparticles with the plasma membrane and their internalization following a mechanism similar to uptake of fluid-phases. It should be noted that endocytic mechanisms are not simply limited to internalizing the nanoparticles and include intracellular transport of different types of macromolecules and nutrients. In fact, they are involved in more complex levels of cellular master plan that control and regulate the spatial and temporal dynamics of cell signaling circuitry [96]. Therefore, cancer therapeutic efficacy of the nanoparticles is closely linked to their uptake mechanisms and subsequent biological responses of the targeted cells.

Endocytosis has been reported as the main mechanism for cellular uptake of the CaP's in different cancer therapeutic studies (Table 1). Through endocytosis pathway macromolecules assembled on the surface of the CaP's (*e.g.*, coating molecules, targeting peptides or antibodies) interact with the receptors on the plasma membrane of the cancer cells. Various receptors on the cell membrane can be targeted by selection or engineering of functional groups or ligands on the surface of the CaP's, as listed in Table 1 (also see Section 2.2). High-resolution visualization techniques such as electronic microscopies, have shown that multiple simultaneous mechanisms are often involved in internalization of nanoparticle-based endocytic cargos [97, 98]. For example, clathrin and caveolae-mediated pathways have been identified as the main routes for polymeric [99–101] and lipid-based [99] nano-



delivery systems. Most of the cancer therapeutic CaP's are also coated and functionalized with polymers and lipids and therefore similar mechanisms are usually involved in their endocytosis.

Almost 20 years ago, it was first thought that intracellular trafficking of CaP's has been only toward the clathrin-mediated endocytosis [102]. However, a decade later, studies by Olton *et al.* indicated that cellular uptake mechanism of CaP's might be facilitated by both clathrin- and caveolae-dependent endocytosis [26]. It is currently known that the exact endocytosis mechanisms of the CaP's depends on their surface coating molecules, size, morphology and type of the targeted cells. For example, Sun *et al.* [53] used *in vitro* studies to delineate endocytosis mechanism of the CaP's (10 nm × 50 nm hydroxyapatite nanorods, without any specific surface coating) in a lung cancer cell line. They inhibited caveolae-mediated pathway using a biochemical agent (methyl β-cyclodextrin) and found that uptake of the CaP's was blocked, showing their uptake was through the caveolae-mediated mechanism. Conversely, when they blocked the clathrin-mediated pathway using a different inhibiting agent (chlorpromazine), the uptake of the CaP's did not change, showing that clathrin was not involved in uptake of their rod-shape nanoparticles. Similar assays were used by Zhou *et al.* [59] to evaluate endocytosis mechanism of spherical-shape hyaluronic acid-coated CaP's (hydrodynamic size ~ 150 nm) in melanoma. Their results indicated that these CaP's entered cancer cells through a combination of micropinocytosis, and caveolae- or clathrin-mediated pathways. Qiu *et al.* [64], however, evaluated endocytosis of almost similar CaP's (hyaluronic acid-coated, size ~ 170 nm) in a lung cancer cell line and showed that only caveolae- or clathrin-mediated pathways were actively involved in internalization of their nanoparticles, suggesting dependency of the endocytosis mechanism on type of the cells. Later, it was shown that caveolae-mediated mechanism was the main pathway for internalization of smaller size CaP's (~ 20 nm) that were coated with PEG and a liver cancer targeting peptide (Arginyl glycyl aspartic acid or RGD) [60]. However, a separate study using HeLa (cervical cancer) cells reported clathrin pathway as the major endocytosis mechanism for 140 nm CaP's that were coated with a co-polymer of PEG and poly(benzoxaborole) [103]. Overall, these examples together suggest that endocytosis mechanism of the CaP's is dependent on type of the cancer cells, and nanoparticles physiochemical properties such as size, coating molecules, and morphology. Therefore, accurate investigations are necessary to delineate and tune the exact endocytosis pathway for each specific type of CaP's and targeted cells.

#### 2.4. The Endosomal Escape Phenomenon

After endocytosis, CaP's tend to be localized inside the vesicles (*i.e.*, early endosomes) with no access to the cytosol. These early endosomes either mature to the late endosomes and fuse with lysosome for degradation or recycle their contents outside the cell membrane (*i.e.*, exocytosis) [104]. This uptake mechanism usually facilitates targeting of the endolysosomal compartments by the bio-agents loaded on CaP's [105]. However, entrapment inside these vesicles and subsequent degradation of the therapeutic bio-agents by lysosomes is undesirable when targeting of the other cellular organelles such as nucleus or cytoplasm is required. Therefore, escaping from endosome microenvironment is an essential requirement

for trafficking of nucleic acids, proteins, drugs and other molecules that function outside the endosome compartment.

Several surface coating strategies have been employed to facilitate CaP's endosomal escape. One of the most common approaches is based on incorporating fusogenic lipids such as dioleoylphosphatidylethanolamine (DOPE) on the surface of CaP's. DOPE has the ability to undergo structural change from a stable lipid bilayer at physiological pH 7 to an inverted hexagonal structure at low pH environments. This conformational transition causes DOPE to fuse with endosomal membrane and destabilizes it [89]. Another well-known method for escaping from endosomal network is by coating CaP's with polymers that show significant buffering capacity at pH values ranging from 5.2 to 7. For example, polycations such as polyethyleneimine (PEI) mediate this escape through a process called proton-sponge effect [106]. At low endosomal pH, these polymers become more protonated, leading to influx of chloride ions and protons. This process follows by influx of water molecules that eventually induces osmotic swelling and rupture of the endosome membrane, leading to release of its content within the cytoplasm (Figure 3) [107].

It is worth mentioning, that the use of PEI coating is usually associated with toxicity that bounds their application. To overcome this issue, Lee *et al.* [108] proposed new hybrid nanocomposite particles consisting of PEI-PEG (polyethylene glycol) copolymer grafted to nanoparticles. This system showed safe and non-toxic delivery functionality, by inducing a proton-sponge effect and taking the advantage of electrostatic and steric interaction of the polymer palisade. Similar studies showed that coating of CaP nanoparticles with PEI significantly increased their transfection efficiency and protein expression [109]. PEI coating provides electro-steric stabilization and necessary positive charge to the CaP's and increases their uptake through the electrostatic interaction with negatively charged plasma membrane. However, an optimum concentration of low molecular weight PEI or its copolymers (*e.g.*, PEI-PEG) should be used to avoid the undesirable cytotoxicity [110, 111]. After escaping from endosome environment, the biomolecules loaded on CaP's should be localized at the target organelles to induce their proposed functions. For instance, nucleic acids such as DNA should reach to nucleus for expression [57], while others such as siRNA can function within cytoplasm [64]. Likewise, cancer therapeutic drugs and proteins have different sites of action and their proper intracellular trafficking is required for effective treatment [16, 112, 113]. Delivery of nucleic acids (*i.e.*, DNA and siRNA) and chemotherapeutic drugs by CaP's will be discussed more in Sections 3.1 and 3.2.

In addition to the mechanisms discussed above, CaP's that are fully or partially degradable in acidic pH of the endosomes are also ideal candidates to enable endosomal escape as a result of releasing calcium ions and other lysosomolytical molecules. However, this mechanism is highly dependent on solubility of the CaP's at lower pH values, which is correlated to their crystallographic structure (Table 2). Different *in vitro* studies have been used to simulate endosomal degradation (partial or full degradation) of CaP's and subsequent release of their cargos. For example, Bisht *et al.* [114] studied the pH-dependent release profile of plasmid DNA (*pSVβgal*) from hydroxyapatite CaP's that were degradable at low pH values. They used agarose gel electrophoresis to quantify *pSVβgal* release at two different buffers with pH values of 5 and 8 during various incubation intervals (0, 1, 2, 4 and

24h). No *pSVβgal* was released from CaP's at pH=5 and at zero hour. However, consistent *pSVβgal* release was observed at later incubation times (2, 4 and 24h). There was no indication of *pSVβgal* release from these particles even after 24h, when the pH of the incubating buffer was increased to 8. This indicates pH-dependency of the *pSVβgal* release as a result of CaP degradation.

CaP's (Ca:P ~ 0.98) encapsulating different organic fluorophores were also developed and used to verify the effect of endosomal pH on dissolution rate of these nanoparticles. Figure 4 shows the diffusion coefficients and hydrodynamic radii of these nanoparticles at pH values of 4 and 7. The hydrodynamic sizes of the CaP's incubated at pH 4 was almost similar to the values observed for a solution containing free Cy3 fluorophore molecules, indicating that CaP nanoparticles were fully dissolved at such a low pH. In addition, the diffusion coefficient shifted to significantly higher values when the pH was decreased from 7 to 4, further confirming CaP's dissolution and subsequent release of their encapsulated contents within low pH environments, similar to that of endolysosomes [115]. *In vitro* evaluation using bovine aortic endothelial cells also indicated that cells were effectively stained with these nanoparticles, which further validated the dissolution of nanoparticles in the endosome. Several other studies used similar methods and reported full or partial dissolution of CaP-based nanoparticles (*e.g.*, amorphous calcium phosphates [81, 116], hydroxyapatite [70, 117] or calcium-deficient hydroxyapatite [118]) at lower pH values for controlled release of different drugs (*e.g.*, doxorubicin [70, 119] and cisplatin [117]) or proteins (*e.g.*, bovine serum albumin or BSA, and green fluorescent protein or GFP [116]).

Overall, among different types of CaP's, hydroxyapatite-based nanoparticles are preferred candidates for intracellular delivery of the therapeutics through endosomal escape pathway. This is mainly because of their less degradability at physiological pH, compared to their relatively higher degradability at acidic pH of the endosomes (See Section 2.1 for details). Hydroxyapatite is one of the most stable phases of CaP's at physiological pH. This characteristic facilitates its synthesis and drug-loading, as a wide range of drug molecules are only stable at physiological pH. Also, less degradability at physiological pH helps to prolong shelf-life of the hydroxyapatite nanoparticles before their *in vivo* administration. These properties enable hydroxyapatite nanoparticles to protect therapeutic agents at physiological pH conditions and release them in the targeted intracellular organelle.

## 2.5. Releasing Calcium Ions: An Implication for Cancer Therapy

The normal concentration of calcium and phosphate ions in the cells and blood are in the range of millimolar [121]. It has been shown that any change in concentration of calcium ions can trigger a variety of physiological responses and eventually lead to apoptosis [122–124]. Accordingly, several studies indicated the potential toxicity and inhibition of proliferation in various cell lines, due to the different characteristics of CaP's [125–127]. CaP nanoparticles have high surface area to volume ratio, which differs them from the bulk material that they are derived from. Due to this property their cellular toxicity is different from their bulk counterparts.

Motskin *et al.* [120] used human macrophage cell lines to evaluate and compare *in vitro* cytotoxicity of different hydroxyapatite nanoparticles with spray dried calcium phosphate

microparticles. They showed that preparation methods significantly influence the nanoparticles physiochemical properties (Figure 5) and therefore their toxicity level. Nanoparticles generated by sol-gel method showed the greatest toxicity. In addition, microparticles that were prepared through spray drying showed significantly lower cytotoxicity, due to their less macrophage uptake. As it can be seen in Figure 6, the toxicity level of these different types of nanoparticles was associated with the amount of uptake, in which the higher the amount of internalized particles, the greater the cytotoxicity. It is reported that this toxicity enhancement is due to release of calcium ions from these internalized particles and subsequent interference with the intracellular calcium ions homeostasis [58].

In a separate study, Neumann *et al.* [128] used standard calcium phosphate transfection method (addition of a mixture of DNA and  $\text{Ca}^{2+}$  solutions to the cells [129, 130]) and two types of synthesized CaP/DNA nanoparticles to compare variation of the intracellular calcium level and related toxicity in T24 cells (human bladder carcinoma) as a result of transfection. Their observations indicated that when standard calcium method was used, intracellular calcium level rapidly raised to more than one hundred times higher than the normal level, whereas the CaP/DNA nanoparticles only resulted in minor changes (Figure 7). Such a low calcium disturbance was associated with the moderately small nanoparticle-mediated calcium uptake and efficient calcium extrusion mechanism which helped to balance the intracellular calcium concentration. In contrast, the irregular calcium spiking in response to standard calcium phosphate transfection was due to rapid dissolution of large particles after endocytosis. The uptake of these particles dramatically increased the intracellular calcium concentration and this excessive amount of calcium ions could not be pumped out of cells instantly. Instead, they were gradually extruded into the cell culture medium, up to 48 h after transfecting the cells. In addition, it was shown that transfection using standard calcium phosphate method could increase the overall concentration of calcium ions in the cells, whereas calcium variation because of transfecting with CaP/DNA nanoparticles was only limited to subcellular sections.

It is worth noting that different cells have various calcium sensitivities and each type of CaP nanoparticle has its own specific degradation and  $\text{Ca}^{2+}$  release kinetics [132]. Recently, Wu *et al.* [69] showed that intracellular  $\text{Ca}^{2+}$  release depends on CaP's size, morphology, crystallinity, specific surface area (SSA) and synthesis method. First, they generated five types of hydroxyapatite nanoparticles with varying characteristics (Figures 8.A and B) using different synthesis methods (*i.e.*, different combination of air drying, hydrothermal treatment and calcination). Then, using *in vitro* and *in vivo* studies, they evaluated efficacy of these different CaP's for melanoma treatment, based on their  $\text{Ca}^{2+}$  release (Figure 8.C). Their *in vitro* studies showed that, granular nanoparticles with smaller size, higher SSA, and lower crystallinity (HA-A) were the most effective nanoparticles in inhibiting the viability of A375 melanoma cells (~ 34.90% viability at day 3). This was mainly because of higher degradation rate and  $\text{Ca}^{2+}$  release from these relatively amorphous nanoparticles, as discussed in Section 2.1. Also, while all samples were able to induce apoptosis, HA-A and HA-B showed highest apoptosis rates (20.10% for HA-A and 19.41% for HA-B, at day 3). Interestingly, none of these nanoparticles caused any apoptosis in normal human epidermal fibroblasts (HSF) after incubation in similar conditions, suggesting the tumor specificity of

their therapeutic effects. Their mice studies showed that HA-A and HA-C were more effective in limiting the growth of melanoma tumor xenografts, compared with other samples, and HA-A therapeutic efficacy was more than HA-C (49.1% vs. 34.0 at day 23).

Several other studies have reported tumor-specificity of this toxicity effect. For example, Sun *et al.* [53], synthesized hydroxyapatite nanorods (width ~10 nm and length ~ 50 nm, without any surface functionalization or drug loading) and incubated them with human lung cancer (A459) and normal bronchial epithelial cells (16HBE, control samples). Their results showed that these nanorods could selectively cause apoptosis in cancer cells, without any noticeable effect on epithelial cells. This was because degradation of nanorods consistently increased the amount of  $\text{Ca}^{2+}$  in cancer cells, while the  $\text{Ca}^{2+}$  elevation in normal control cells was transient. Their mice studies resulted in 40% tumor growth inhibition, confirming the *in vitro* results. Drug-loaded CaP's can also be designed for simultaneous release of  $\text{Ca}^{2+}$  and chemotherapeutic drugs, for enhanced cancer therapy. Xiaoyu *et al.* [54] generated Doxorubicin-loaded CaP's (spherical, 30–40 nm, clusters of hydroxyapatite nanoparticles with low crystallinity) that could degrade after cell internalization, releasing the drug and increasing the intracellular  $\text{Ca}^{2+}$  simultaneously for more effective therapy of gastric adenocarcinoma.

Intracellular  $\text{Ca}^{2+}$  release can also be enhanced using external stimuli, such as laser irradiation. Recently, Xu *et al.* [58] synthesized spherical nanoparticles that were coated with a CuS shell (final size ~ 100–150 nm). CuS shell was designed to generate heat in response to near infrared light (808 nm) for enhancing degradation of the CaP's. Their *in vitro* and *in vivo* studies showed that intracellular degradation of the CaP nanoparticles (so called  $\text{Ca}^{2+}$  nanogenerators) inside the endosomes and release of excessive  $\text{Ca}^{2+}$  ions in cytoplasm could disrupt the mitochondrial  $\text{Ca}^{2+}$  homeostasis resulting in apoptosis of the breast cancer cells (Figure 9). This is a promising fundamental study that can possibly open up new prospects for light-triggered cancer therapy using degradable CaP nanoparticles.

### 3. Delivery of Therapeutics to Tumors Using Degradable CaP Biominerals

#### 3.1. Nucleic Acids

The ability to apply gene therapy (*e.g.*, introducing a therapeutic DNA into the nucleus of cells) holds promise for treatment of wide variety of acquired and inherited diseases, including tumors. Numerous nano-delivery systems have been developed to introduce desired genes into target cancer cells. A gene delivery vector must demonstrate biocompatibility, stability, diminished or no toxicity and sustained-released kinetics [133–137]. Among different types of nonviral vectors, calcium phosphate-based gene delivery systems have shown promising *in vitro* and *in vivo* results which makes them one of the best candidates for clinical translation [15, 138, 139]. As mentioned earlier, co-precipitation of nucleic acids (*e.g.*, DNA and siRNA) and CaP is a well-established and straightforward method for preparing a composite delivery vehicle. The reason for good adherence of nucleic acids to calcium phosphate is thought to be due to the electrostatic affinity of positively charged calcium ions in CaP to the negatively charged, phosphate groups in backbone of nucleic acids [22, 140]. Furthermore, addition of positively charged moieties such as amino groups to the surface of the CaP nanoparticles has been reported as an

effective method for higher nucleic acid loading and improving transfection [141, 142]. Also, synthesis of the nucleic acid-loaded CaP's can be easily modified to incorporate both nucleic acid and drugs molecules into CaP's for simultaneous transfection and cancer chemotherapy [143, 144].

Despite safety and efficiency of CaP/nucleic acid nanocarriers, this technique suffers from limited reproducibility and difficulty in controlling transfection parameters, which is related to the nanoparticles physical and structural properties, such as size, morphology, crystallinity and composition [34, 47, 145]. Furthermore, studies have shown that transfection efficiency not only changes with these physiochemical properties, but also depends on the ability of nano-delivery systems to effectively encapsulate and protect the nucleic acid molecules from enzymatic attacks either inside the bloodstream or cells. Different organic and inorganic additives have been incorporated into CaP's to preserve their small size and protect them from enzymatic attacks. For instance, it was shown that addition of magnesium ions to CaP mineralization solution suppresses the crystal growth by creating distortions in the crystal structure of the CaP's, which results in improving their transfection efficiency [48]. In addition studies have shown that incorporation of polymers such as block-copolymers of poly(ethylene glycol) (PEG) and poly(aspartic acid) (PAA) enhances the transfection efficiency [50]. It is known that PEG is an amphiphilic and biocompatible polymer that confers the colloidal stability of CaP nanoparticles by steric effect [117]. This polymer is also well known for its stealth property when applied *in vivo* [146, 147].

Welzel *et al.* [148, 149] reported a simple *in situ* precipitation method to prepare well-defined CaP/DNA nanoparticles with CaP core and a DNA shell. This method showed better control over size tuning, resulting in relatively higher transfection efficiency and stability of the CaP's in biological media. Despite these advantages, it was found that transfection efficiency of such core-shell system could not be further amplified, possibly due to the degradation of DNA molecules inside the lysosome compartment (before they reach the nucleus). Therefore, it is required to protect the DNA from such degradation and facilitate its transport into the nucleus. To achieve this goal, it was suggested to add an external layer of CaP (double-shell in Figure 10) to the surface of nanoparticles [130]. Since these precipitates were susceptible to agglomeration, another layer of DNA (triple-shell in Figure 10) was also added to impart the electrostatic colloidal stability. Alternative molecules such as PEI [150, 151] or its derivatives (*e.g.*, PEI-cholesterol [152]) were also used as the third layer for electrostatic stabilization of these nanoparticles, but additional considerations are required to monitor toxicity and degradation pathway of the PEI layer. The transfection efficiency of these nanocarriers has been tested in different *in vitro* studies using various cell lines, such as human cervical carcinoma (Hela), T-HUVEC and leukocyte tyrosine kinase (LTK). The *in vitro* results indicated that the transfection efficiency of triple-shell nanoparticles is significantly higher than single-shell approach in all tested cell lines, confirming that such a simple additional step can protect DNA molecules from endolysosomal enzymatic attacks.

Likewise, CaP nanoparticles can be stabilized with an outer layer of small-interfering RNA (siRNA). Similarly, triple-shell design resulted in highest gene silencing efficiency, due to the protection of siRNA molecules from intracellular degradation [153]. In addition, multi-

shell nanoparticles with a PEI layer demonstrated higher siRNA transfection efficiency in three different cancer cell lines (T24, NIH3T3 and Hela), when compared with nanoparticles without PEI [110]. Recent studies showed that other hydrophilic polymers (*e.g.*, alendronate-hyaluronan graft polymer [64]), natural cationic molecules (*e.g.*, lipids [23, 61]) and amino acids (*i.e.*, arginine molecules [154]) can also help stabilizing single core CaP nanoparticles and adsorb siRNA molecules for effective *in vitro* and *in vivo* cancer therapy, based on endosomal escape of the nanoparticles and releasing siRNA into cytoplasm after degradation. Addition of targeting molecules (*e.g.*, folic acid for breast cancer [23], galactose moieties for hepatocellular carcinoma [62], hyaluronic acid for melanoma [59], and arginylglycylaspartic acid (RGD) for bladder cancer [155]) has also been used for specific *in vitro* and *in vivo* siRNA delivery using CaP's.

CaP-based nanostructure coatings, fabricated from biomimetic solutions (*e.g.*, simulated body fluid (SBF)), can also be used for enhanced transfection. This technique was first proposed by Kokubo *et al.* more than three decades ago [156]. Although hydroxyapatite was the primary type of CaP's used for this approach, other types of CaP's with different crystallographic phases, morphologies and structures have also been prepared by adjusting the synthesis parameters such as pH, ion concentration and temperature. Results indicated that physicochemical properties of these coatings have significant influence on transfection efficacy in different cell lines. For example, Shen *et al.* demonstrated the co-precipitation of DNA with SBF solutions of different ion content and concentrations onto the cell-culture surfaces [157]. The CaP coating layers had different nanostructures and Ca:P ratios and were able to support cell attachment and proliferation. In addition, the dissolution of CaP layer provided high concentration of DNA in the vicinity of the cultured cells. The mineral stability and subsequent DNA release rate was correlated to different structures and compositions of the precipitates. Since then, many studies have been conducted to evaluate dissolution of these minerals and their effects on gene transfection efficiency in various cell types [158, 159].

Different techniques are still under progress to improve the transfection efficiency of CaP nanoparticles. For example, lipid-coated CaP nanoparticles incorporating siRNA molecules were developed using a water-in-oil microemulsion method (Figure 11) [23, 160]. In this system, the core of liposome-polycation-DNA (LPD) was replaced by acid-sensitive CaP [160]. The resulted formulation was called liposome/calcium/phosphate (LCP). The dissolution of CaP core in the acidic environment of endosomes increased the osmotic pressure. This increased pressure resulted in swelling and disruption of endosomes, followed by releasing the encapsulated siRNA into cytoplasm. Modification of these LCP nanoparticles with PEG resulted in about 70 and 50% gene silencing efficacy in cultured (*in vitro*) and xenografted (mice) H460 tumor cells, respectively.

For *in vivo* nucleic acid delivery applications, the CaP design criteria are even more demanding. For example, it is required to prepare the CaP nanoparticles that are able to efficiently incorporate adequate amount of nucleic acids and protect them in bloodstream. Zhang *et al.* [161], designed a novel hybrid micelle of siRNA and CaP's, to address these two important requirements. In this system, siRNA and PEG were conjugated *via* an environment-sensitive disulfide linkage. This complex was further modified with CaP's to

form hybrid micelles of PEG-SS-siRNA/CaP. The evaluation of physiochemical properties of these hybrid CaP's showed that they had a spherical core-shell morphology with a particle size ranging from 90 to 120 nm (Figure 12). The disulfide bond could break-down in the acidic pH of the endosomes and selectively cleaved and detached the PEG protecting layer from the nanoparticles. This cleavage resulted in destabilization and dissolution of CaP's and subsequent release of the siRNA into cytosol. The amphiphilic PEG layer also helped to improve colloidal stability and stealth property of the nanoparticles. Cellular uptake of these hybrid micelles was evaluated *in vitro* using Huh-7 cells. The results revealed significantly higher cellular uptake and subsequent gene knockdown efficiency, indicating promising characteristics of this PEGylated CaP-based nano-delivery system for *in vivo* applications.

A recent study by Huang *et al.* [62] showed efficacy of lipid/calcium/phosphate nanoparticles (LCP NPs) for preventing the tumor growth by releasing VEGF (vascular endothelial growth factor) siRNA to orthotopic hepatocellular carcinoma (HCC) tumors in murine liver. LCP NPs were loaded with siRNA and then eight different types of galactoside derivatives were conjugated to them for targeting asialoglycoprotein receptors (ASGPR) on the HCC cells. *In vitro* and *in vivo* tests demonstrated that  $\beta$ -D-galactoside derivative had the highest HCC targeting efficiency. Tail vein injected LCP NPs were accumulated in HCC tumor and degraded in acidic environment of the lysosomes, releasing VEGF siRNA to the cytosol. siRNA downregulated VEGF expression in tumor cells and prevented tumor growth by an anti-angiogenesis mechanism (Figure 13).

### 3.2. Drugs

The ability of the cancer chemotherapy drugs to induce designated therapeutic functions depends on their successful delivery to the tumor tissues, followed by their effective internalization into the target organelles such as nucleus or cytoplasm. However, most cancer drugs are fragile, impermeable and have undesirable biodistributions [162–164]. Therefore, they require a carrier system (*e.g.*, nanoparticles) that can protect them from the harsh extracellular environments and release them in a sustained manner inside targeted cancer tissue [165, 166]. CaP's have been a subject of increasing attention for cancer drug delivery due to their unique physiochemical characteristics such as controlled synthesis, pH-dependent degradability and high capacity to encapsulate different types of drugs.

Several strategies have been reported for loading cancer drugs into CaP's, depending on size and water-solubility level of the desired drug and the targeted cancers (Table 1). One of the commonly used approaches is based on loading drugs into different types of micelles that are designed to act as templates for mineralization of CaP nano-shells. For example, Wang *et al.* [60], used a multi-step procedure to synthesize CaP's for *in vivo* drug delivery to hepatocellular carcinoma. First, they linked hydrophilic polyethylene glycol (PEG) to hydrophobic phosphatidylserine (PS) to generate micelles by self-assembly of these molecules. RGD peptide (Arginyl glycyL aspartic acid) was also conjugated to these molecules (PS-PEG-RGD) for cancer targeting. As it is shown in Figure 14, Mitoxantrone (MIT, cancer drug) was trapped inside these micelles during their formation (Step 1). Then, CaP shells were formed around these micelles after addition of  $\text{Ca}^{2+}$  and phosphate ion precursors (Step 2). Finally, a solution containing Verapamil (VER, a drug-resistance



inhibitor drug) was added to MIT-loaded CaP's and the mixture was sonicated to help VER adsorption to the surface of nanoparticles (Step 3).

Mixing CaP's with solutions containing drug molecules such as Doxorubicin (DOX),  $\alpha$ -tocopheryl succinate ( $\alpha$ -TOS), and 2-deoxy-D-glucose (2DG) have also been reported as a feasible drug-loading approach in various CaP-based *in vivo* studies [54, 56, 63, 65, 72, 76, 167]. Physical adsorption of the drug molecules to the surface of CaP's *via* electrostatic or hydrogen bonding has been the major drug-loading mechanism used in these studies. However, it should be noted that these weakly-bonded drug molecules might be detached from the surface of the CaP's in blood stream and before their uptake by the tumors. Therefore, careful considerations are needed when this approach is used for loading drugs to CaP's. Mineralization of CaP's in the presence of drug molecules and entrapment of drug molecules into CaP's has also been reported as an effective drug-loading method [70]. Drug release from these nanoparticles depends on degradation of the CaP's within weakly acidic tumor microenvironment [168]. Also, controlling the amount of entrapped drug molecules and their sustained release need to be optimized for different types of drugs, considering their molecular size and therapeutic efficacy.

#### 4. CaP's for Multifunctional Cancer Therapy

CaP's have been extensively studied for multimodal cancer therapeutic applications, by combining several therapeutic strategies in a relatively more complex nanoparticle design. For example, Sokolova *et al.* [169, 170] prepared multi-shell nanoparticles with CaP core and CpG oligonucleotide/CaP shells (Figure 15). These multi-shell nanoparticles were further functionalized with CpG and poly (I:C). Furthermore, a viral peptide of the influenza A virus hemagglutinin (HA) was incorporated inside these particles. Such functionalized CaP nanoparticles were able to protect the CpG molecules from initial degradation inside the cells and stimulated the antigen-specific CD<sup>4+</sup> T-Cells strongly to produce immunostimulatory cytokines. In addition, tuning the composition of multi-shell nanoparticles enabled determining the required dose for *in vivo* targeting. The ability of these nanoparticles to encapsulate different antigens to stimulate antigen-presenting cells looks promising and paves the way for their future applications as effective cancer or antiviral vaccines.

Another common strategy has been based on adding light-responsive moieties to the CaP's for photo-induced cancer therapeutic applications such as photodynamic or photothermal therapy. These two therapeutic modalities have been used for treatment of various types of cancers, as well as curing the bacterial biofilms on the implant surfaces [171, 172]. These strategies are mostly based on ability of CaP nanoparticles to incorporate various light-responsive molecules such as photosensitive dyes. In photodynamic therapy, these non-toxic photosensitive dyes are used alongside a harmless visible light that is utilized to irradiate the tumor tissues. Interaction of these dyes with light leads to production of highly reactive oxygen species (*i.e.*, singlet oxygen) that can destroy tumor cells. The photosensitizer dyes should be delivered into the cytoplasm of the tumor cells to be effective. However, these molecules are mostly insoluble in aqueous phases and have relatively small selectivity over

malignant or healthy cells [173]. Therefore, incorporating them into nanoplateforms such as CaP's is essential for their effective delivery to tumor cells.

A study by Schwiertz *et al.* [174] demonstrated the preparation of polymer-stabilized CaP nanoparticles functionalized with methylene blue or porphyrin as model photosensitizer dyes. Their results indicated that incorporation of these dyes into CaP nanoparticles can enhance their water solubility. Therefore, CaP's could be used as carriers for their *in vitro* and *in vivo* administrations, instead of conventional administration methods such as using alcoholic solutions which impose side-effects. The phototoxicity of these nanoparticles with various concentrations of dyes was tested against different cell lines. The particles charge, incubation time, characteristics of the stabilizing polymer and type and concentration of photoactive dye altered the cell responses considerably. For instance, only moderate efficacy was observed for colon adenocarcinoma cells, whereas a relatively high phototoxicity was detected for synoviocytes.

On the other hand, photothermal therapy is based on heat generation due to interaction of the light-sensitive agents (*e.g.*, dyes and gold nanoparticles) with laser irradiation. This heat can cause cancer cell apoptosis or ablation of the tumors at higher temperatures. A recent CaP-based study reported effective treatment of subcutaneously implanted liver tumors (HepG-2) in mice by combining photothermal therapy and chemotherapy (*i.e.*, doxorubicin) [63]. In this study (Figure 16), hybrids of CaP nanoparticles and gold nanorods were loaded with doxorubicin and injected into mice *via* tail veins. A fraction of these nanoparticles were accumulated in the tumors and when irradiated with 808 nm laser, they released doxorubicin and generated heat as a result of surface plasmon effect of the gold nanorods. These synergistic therapeutic effects helped to treat the subcutaneous tumors and prolong the survival rate.

Tuning the surface chemistry is also helpful to enhance specific targeting of multifunctional CaP's. For example, Barth *et al.* [175, 176] used calcium phosphosilicate nanoparticles (CPSNPs) to target breast cancer, pancreatic cancer and leukemia. These nanoparticles were functionalized with indocyanine green (ICG) near-infrared fluoroprobe, a diagnostic and photodynamic therapy agent, and then a novel bioconjugation technique was utilized for cell targeting. *In vivo* studies on murine leukemia model showed dramatic improvements in the efficiency of photodynamic therapy by using these functionalized nanoparticles. Another study by Ganesan *et al.* [177] demonstrated the preparation of CaP nanoparticles coated by phosphate-functionalized porphyrin (p-TPPP) as a stabilizer and fluorescence agent. *In vitro* studies by NIH 3T3 fibroblast cells confirmed a considerable amount of nanoparticles uptake after a few hours. The cells showed promising viability, indicating that porphyrin-loaded CaP nanoparticles could be used as biocompatible delivery vehicles to transfer fluorescing dyes to tumors.

Iron-doped CaP's and hybrids of CaP's with iron oxide nanoparticles have also been developed for magnetothermal therapy of the tumors. For example, Pernal *et al.* [178] dispersed iron oxide nanoparticles in hydroxyapatite nanoparticles as their carriers and evaluated the magnetothermal effects of the resulted nanocomposite for apoptosis of the human brain cancer cells (E297 and U87) and mouse osteosarcomas cells (K7M2) cells.

Their *in vitro* analysis showed higher uptake and hyperthermia efficacy for hybrid hydroxyapatite/iron oxide nanoparticles, compared with iron oxide nanoparticles alone, suggesting hydroxyapatite facilitated uptake of the iron oxide nanoparticles. In a separate study, Adamiano *et al.* [179], coated superparamagnetic iron oxide nanoparticles with amorphous calcium phosphate and showed that hyperthermia effect of the resulted nanoparticles can reduce viability of the osteosarcoma and glioblastoma cancer cells in alternate magnetic fields of extremely low power. Similar results were shown by Mondal *et al.* [180] using hydroxyapatite-coated iron oxide nanoparticles for hyperthermia of the MG-63 osteosarcoma cells. Doping of the calcium phosphate nanoparticles with iron ions has also been used for magnetothermal hyperthermia [83, 84, 181, 182]. For example, Iafisco *et al.* [183] doped Doxorubicin-loaded hydroxyapatite nanorods with iron and showed more efficient Doxorubicin release from these superparamagnetic apatite nanoparticles under alternating magnetic fields. Additionally, in a recent study, Srinivasan *et al.* [182] synthesized hydroxyapatite nanoparticles in the presence of iron ions and showed that the resulted hydroxyapatite nanoparticles were partially stabilized with FeOOH and  $\alpha$ -Fe<sub>2</sub>O<sub>3</sub>. Their results showed that, under an alternating magnetic field, these nanoparticles could increase the temperature from ~23 °C to 42 °C in 4 min. Magnetic calcium phosphates have also been used for other applications such as enzyme immobilization [184] or contrast enhancement in magnetic resonance imaging (MRI) [185].

## 5. Future Direction

Development of cancer drug delivery carriers using calcium phosphate biominerals, especially in the form of nanoparticles (CaP's), has become of increasing interest. Unlike many other types of nanoparticles, CaP nanoparticles are biocompatible and biodegradable, which makes them suitable nanoplatforms with tunable body clearance pharmacokinetics. CaP degradation by-products are calcium complexes and phosphate groups which are naturally available in body. Also, they are on the same size scales as cellular ligands, receptors and channels and can be further modified or functionalized to attain specific physiochemical properties. Front-line cancer drugs are often untargeted and insoluble compounds that are easily recognized by reticuloendothelial system (RES) to get cleared from the blood by clearance organs (*i.e.*, liver, spleen and kidney) in a short time. However, their solubility and circulation time can be modified when incorporated into the CaP nanoparticles.

In order to enhance the effectiveness of the drug-loaded CaP's, it is essential to monitor their trafficking inside the target cells and other normal cells surrounding them. After endocytosis, the nanoparticles are typically dissolved in the acidic compartment of the cells that leads to increase of the intracellular calcium level. Any raise above the intracellular calcium level can cause the cell toxicity, due to imbalanced calcium exocytosis. Therefore, adjusting composition, morphology, size, surface chemistry and charge of the CaP nanoparticles play an important role in diminishing their toxicity. However, as discussed in this review, such a change in intracellular Ca<sup>2+</sup> can be used for apoptosis of the cancer cells, when designed properly.

During the last several years, a large number of studies have been focused on developing of safe and effective CaP nanocarriers that are non-immunogenic and have potential to deliver a high concentration of drugs to the target cancer tissues. Despite all the outstanding progresses, there are still major scientific and technological issues that need to be overcome. For example, delivery of these nanoparticles to the target organs and tissues has two main challenges. The first is the kinetic barrier that requires nanostructural stability and long-time circulation of nanoparticles in bloodstream in the presence of a wide variety of enzymes, proteins and immune cells. The higher the ionic strength of the nanoparticles environment, the lower the electrostatic repulsion force between particles that makes them more prone to agglomeration. In addition, protein adsorption on the surface of nanoparticles and subsequent changes in surface characteristics (*e.g.*, hydrophilicity and surface charge) is another major drawback that has to be addressed. Although, PEGylation technique is effective in extending the circulation time to some extent, it is still not able to effectively diminish the uptake of the nanocarriers by RES system. The second is the de-assembly or degradation of CaP's at the desired site of action (*i.e.*, weakly acidic tumor microenvironment or inside the endosomes and lysosomes). After successful uptake by tumors, it is required that nanoparticles undergo dissolution and release their cargo to induce the desired therapeutic effects extracellularly or intracellularly. Therefore, the ideal carrier should be able to encapsulate adequate amount of the bio-agents, transfer them stealthily *via* bloodstream and release them in a sustained manner at the targeted site.

Since calcium phosphate nanocarriers have distinctive pH-dependent physiochemical properties and are highly biocompatible, they are expected to open new prospects in the future of cancer nanomedicine. CaP's degradability helps to predict their fate and clearance mechanisms during the clinical applications in future. Unknown body clearance mechanisms and pharmacokinetics of the nanoparticles have been the major drawback of nanomedicines till now and generation of degradable nanoparticles such as CaP's can expedite the clinical translation of different nanomedicine approaches. Degradation of the CaP's in tumor microenvironment and intracellular organelles is highly dependent on their crystallographic phase. Therefore, proper phase analysis of the CaP's by using methods such as X-ray diffraction (XRD) is essential to tune their degradability. This has been a commonly neglected characteristic in most of the recent *in vivo* studies. Combination of CaP nanocarriers with the already FDA approved drugs and imaging agents are predicted to face fewer regularity problems than the other nano-delivery vehicles. Expanding integrated understanding on the fundamentals of biology, chemistry and well-designed animal studies, along with appropriate stewardship, will enable having precise control at micro- and nano-scale levels to develop more effective and successful biodegradable and clinically safe CaP-based nanovectors for improved cancer therapy.

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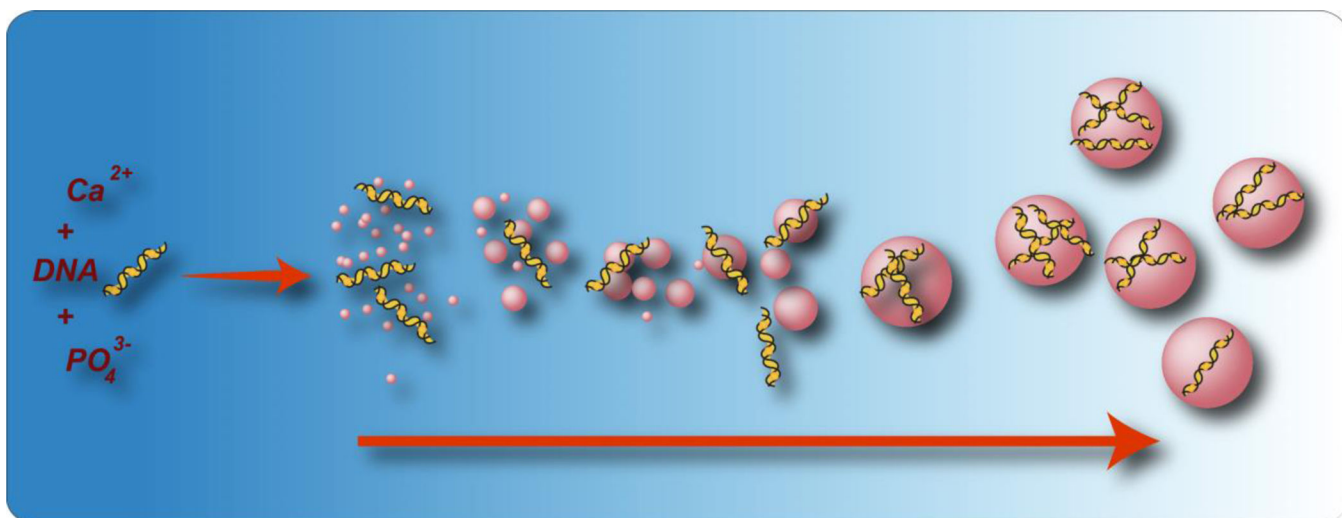
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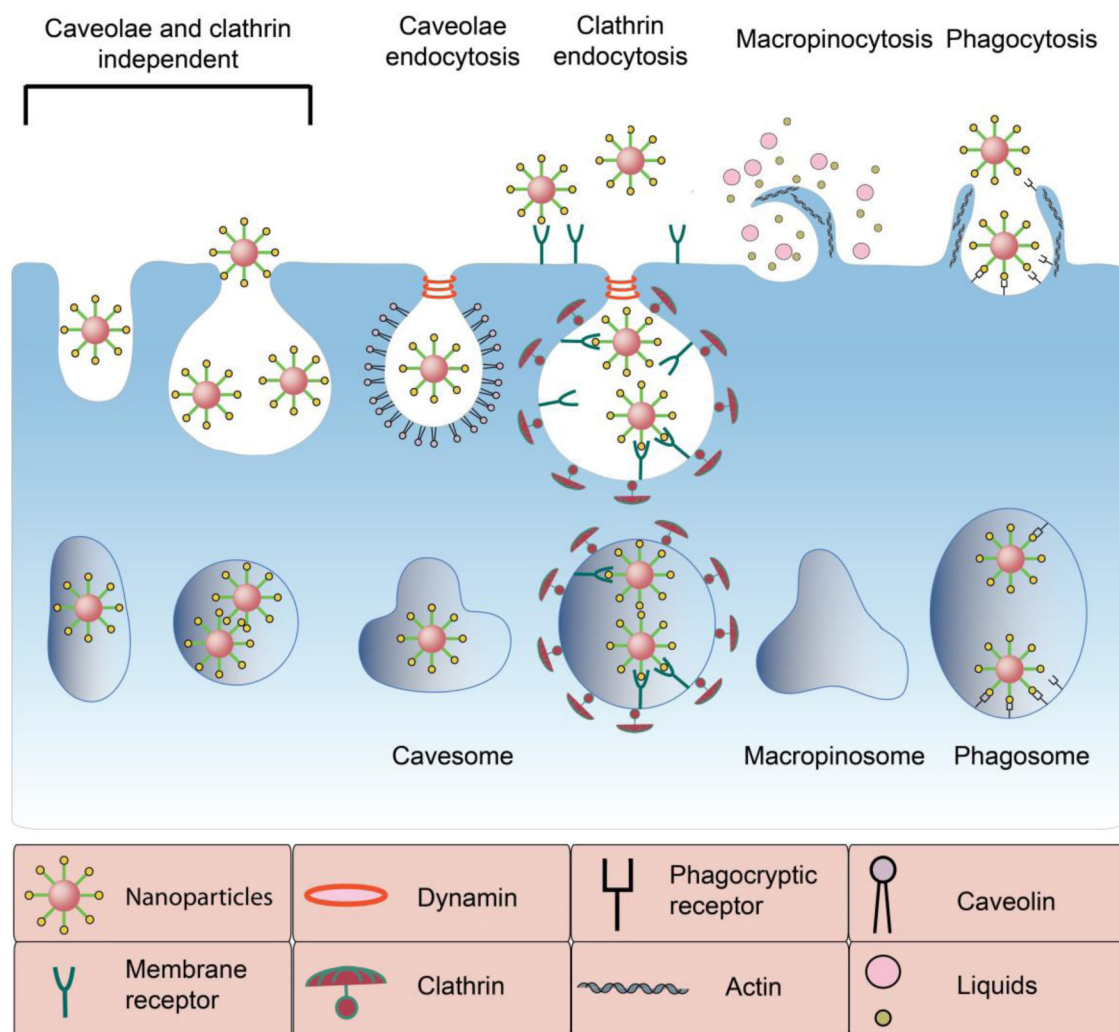
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- Degradability is a major challenge against clinical translation of nano-therapeutics.
- Calcium phosphate nanoparticles (CaPs) are degradable at low pH.
- Degradability makes CaPs suitable for clinically safer cancer therapeutic delivery.
- Degradation mechanism of CaPs in cancer tissues is discussed.
- Strategies for tuning CaPs degradation and therapeutic effects are summarized.

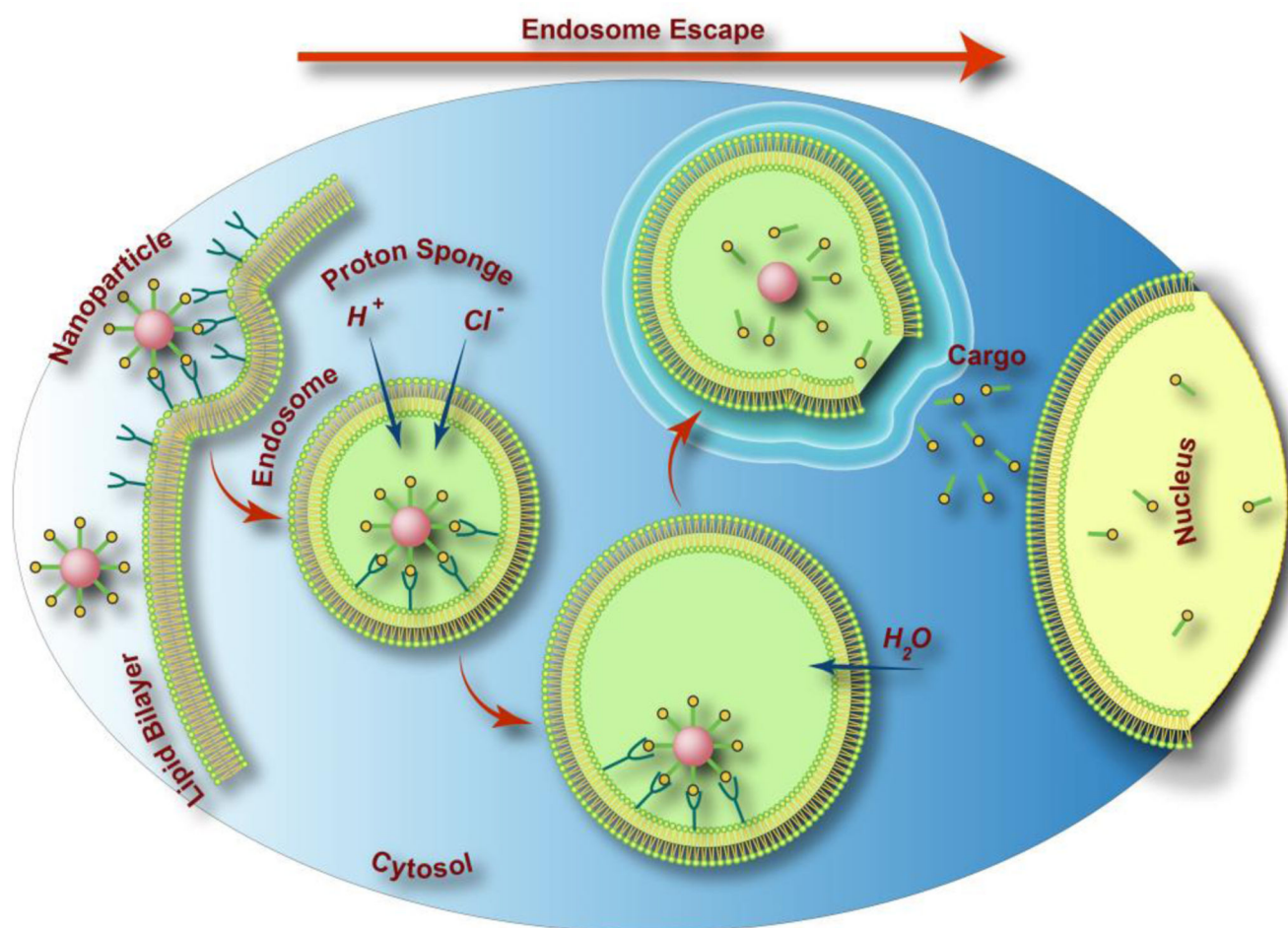


**Figure 1.**  
(a) Schematic showing co-precipitation of calcium phosphate nanoparticles in the presence of DNA molecules. (Reprinted with permission from ref. [37]. Copyright 2019 American Chemical Society)

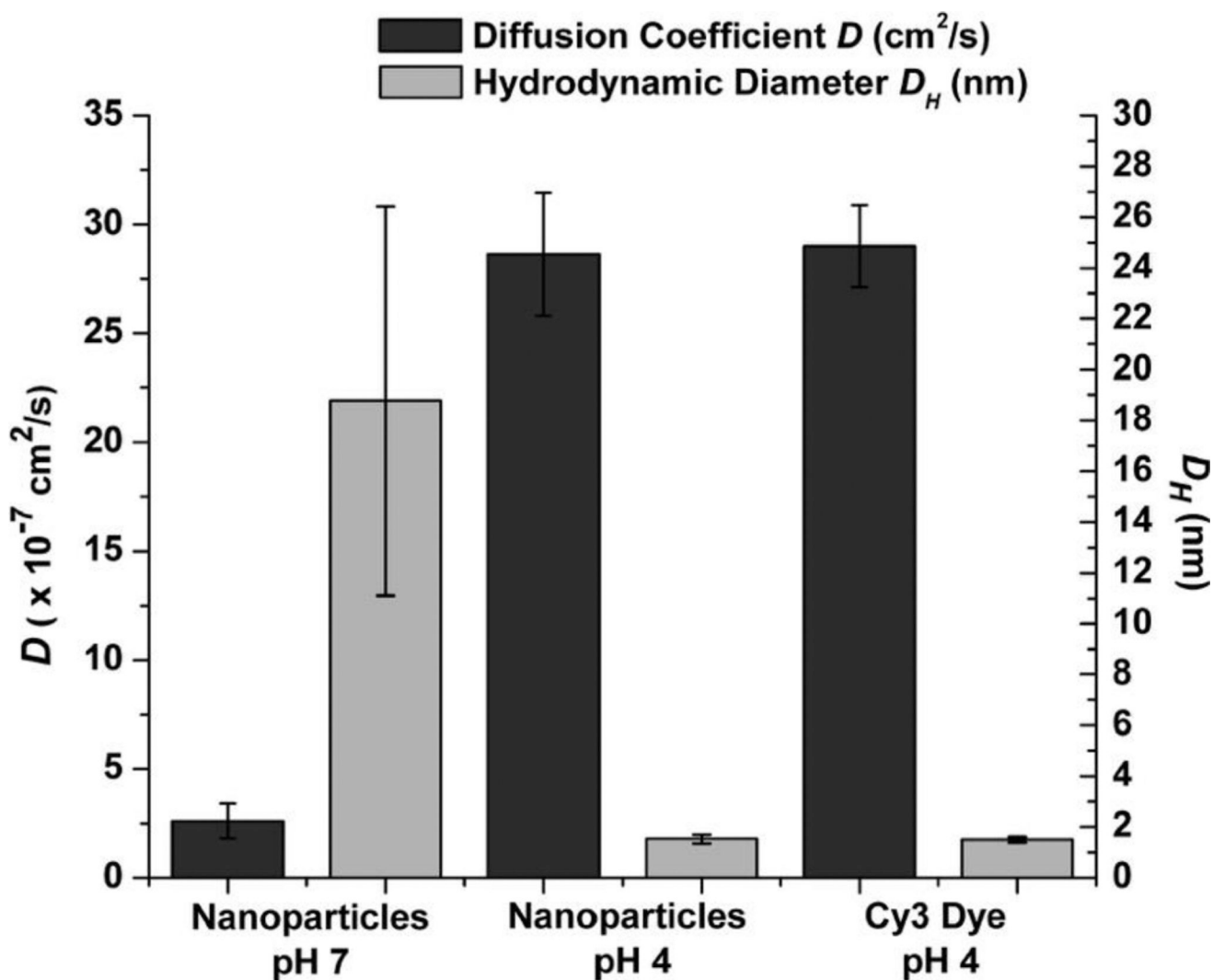




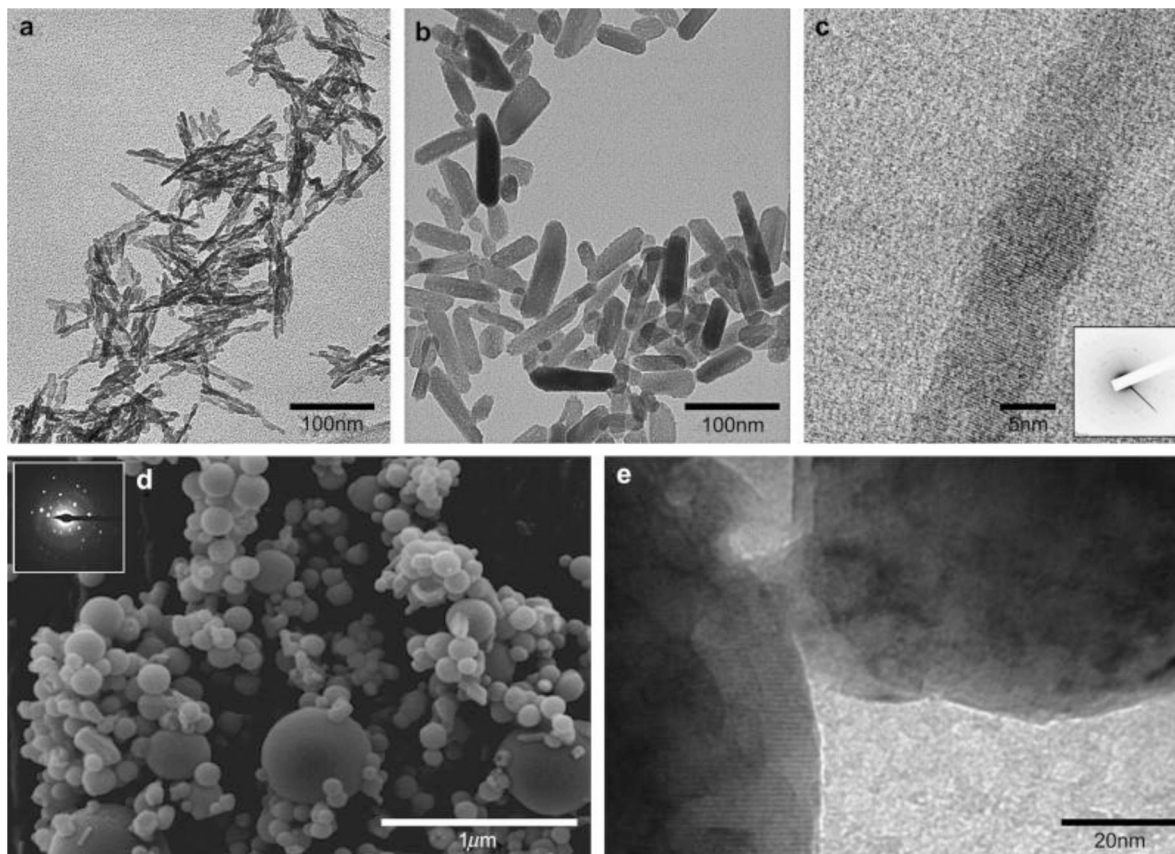
**Figure 2.** Schematic illustrating different CaP endocytic pathways. Nanoparticles are taken up into cancer cells *via* different routes including clathrin-dependent or clathrin-independent endocytosis (*e.g.*, macropinocytosis, phagocytosis and caveolae-mediated endocytosis). This process is highly complex and over 50 different proteins are participating in it. Intracellular trafficking and subsequent biological response depend on these pathways.



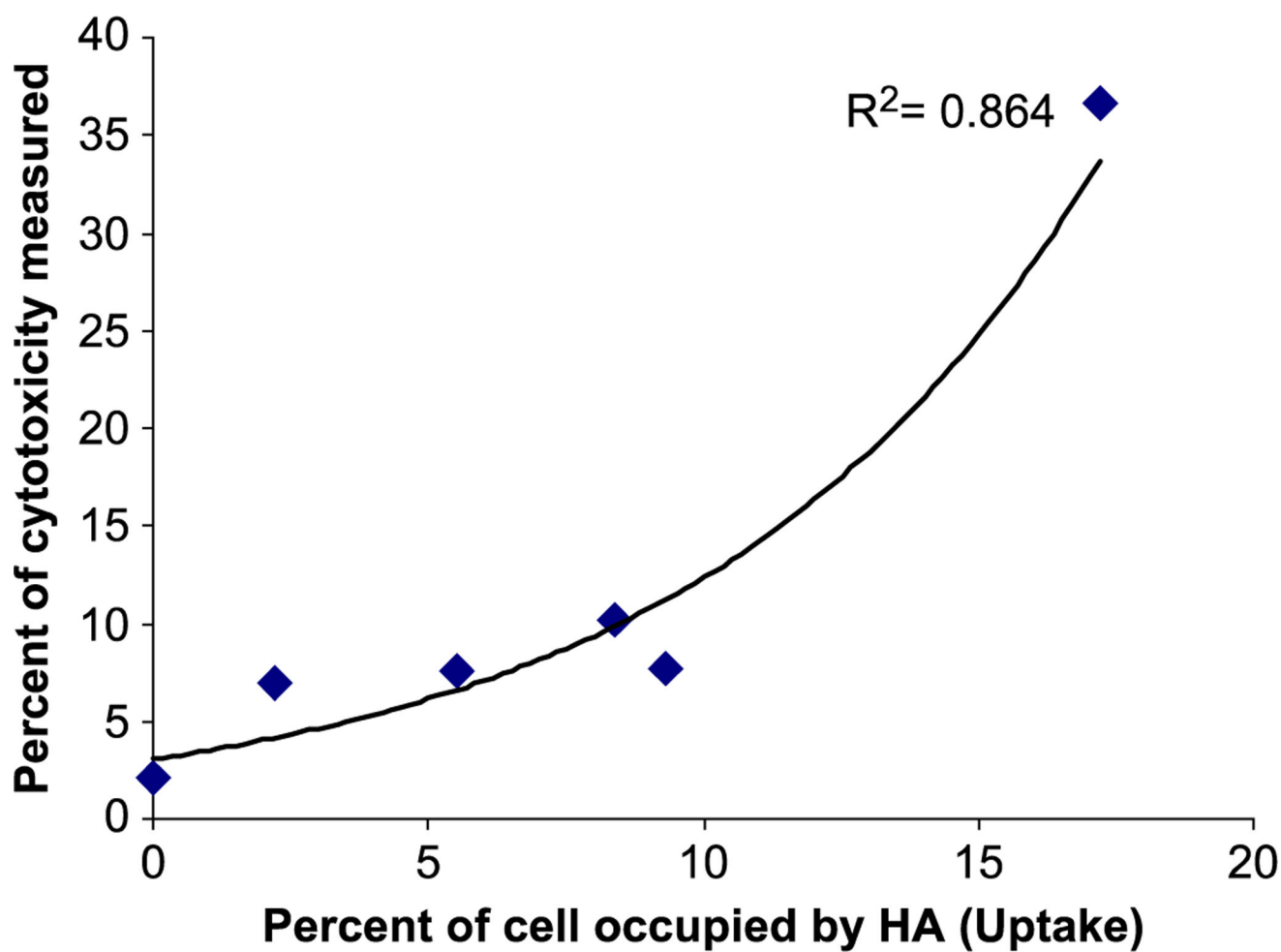
**Figure 3.** Schematic drawing of CaP's endosomal escape in cancer cells through the proton-sponge effect. The maturation of endosome to lysosome creates the acidic environment that along with various enzymes degrades the content of lysosome (*e.g.*, CaP nanoparticles).  $H^+$  ions diffuse into the endosomes by proton pumps and subsequently for maintaining the charge neutrality  $Cl^-$  ions enter to this vesicle. The influx of these ions along with influx of water causes the endosomes to swell and rupture, releasing its trapped payload inside the cytosol, a process generally known as endosomal escape.



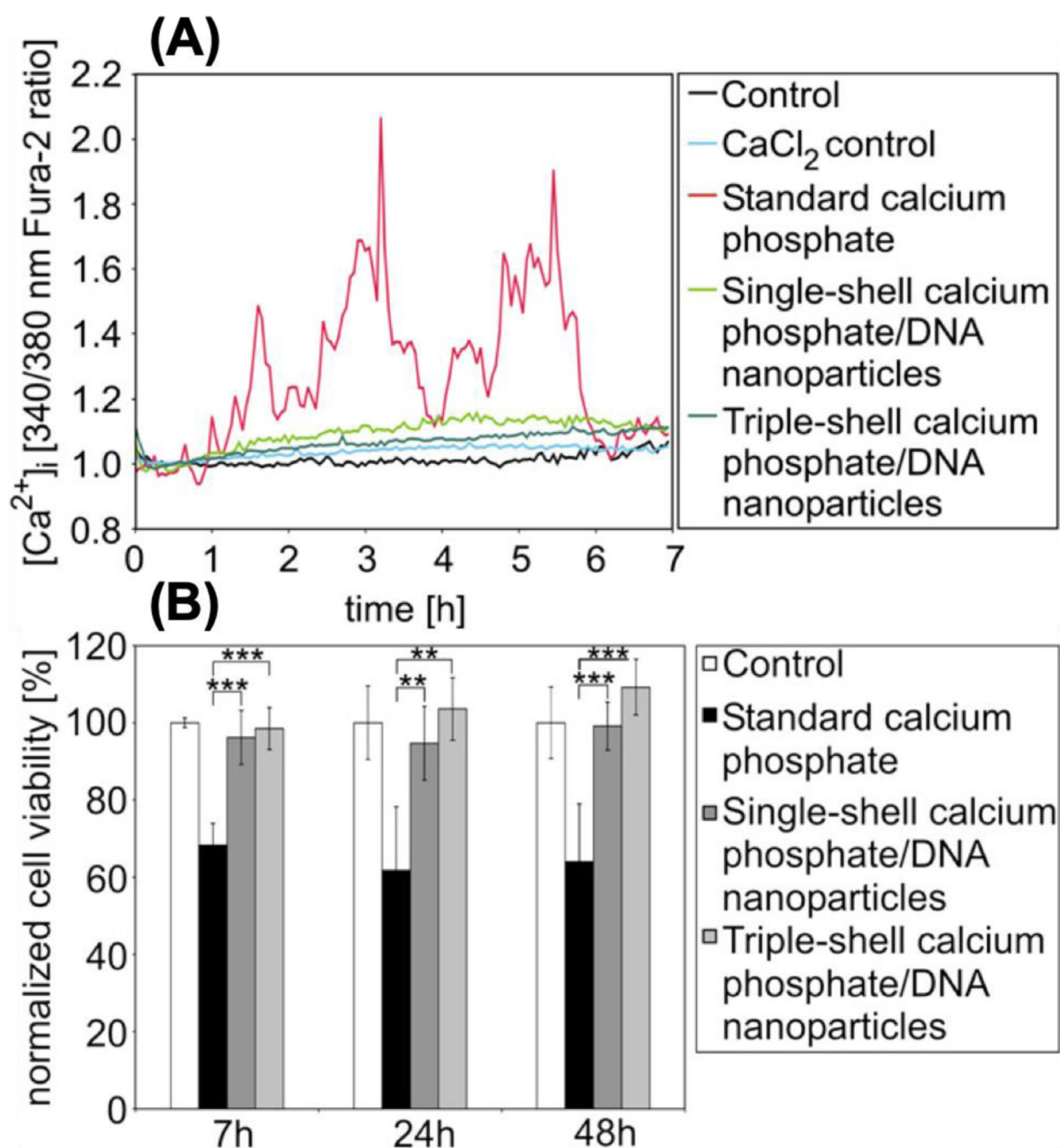
**Figure 4.** Hydrodynamic size (gray) and diffusion coefficient  $D$  (black) of nanoparticles at pH 4 and pH 7 and free Cy3 dye at pH 4. Larger hydrodynamic size at pH 7 indicates the nanoparticles specific size distribution. In addition, diffusion coefficients and hydrodynamic size of nanoparticles and free Cy3 dye at pH 4 are in the same range, suggesting dissolution of the nanoparticles at pH 4. \* $p < 0.0001$  for pH 4 vs. pH 7. (Adapted with permission from ref. [115]. Copyright 2008 American Chemical Society)



**Figure 5.** (a) TEM micrograph of as precipitated hydroxyapatite (HA) calcium phosphate-based nanoparticles; (b) The gel form of HA; (c) High resolution TEM of the gel and selected area electron diffraction (SAED) pattern of a single nanoparticle; (d) TEM micrograph and SAED diffraction pattern (inset) of commercial particles that were used for comparison; (e) High resolution TEM image of the commercial particles, presenting the smooth surface of spherical particles. (Reproduced with permission from ref. [120]. Copyright 2009 Elsevier)



**Figure 6.** Correlation between uptake of CaP-based nanoparticles (HA) and associated cytotoxicity.  $P < 0.005$ , Correlation co-efficient  $-0.929$ ,  $R^2 = 0.864$ . (Reproduced with permission from ref. [120]. Copyright 2009 Elsevier)



**Figure 7.**

Variation of intracellular calcium level (A) and cell viability results (B) obtained from transfected T24 cells at different incubation times. Cells were transfected with single-shell and triple-shell CaP nanoparticles and results were compared with standard calcium phosphate and control samples (PBS). Fura-2 (shown in A) is a sensitive indicator dye for measuring intracellular calcium. It gets excited at 340 and 380 nm and the ratio of the emissions at these wavelengths is a representative of intracellular calcium [131]. For cell viability tests, mean values of absorption were defined as 100% for control cells during the MTT assay for cell viability quantifications. Graph bars shown in (B) represent four

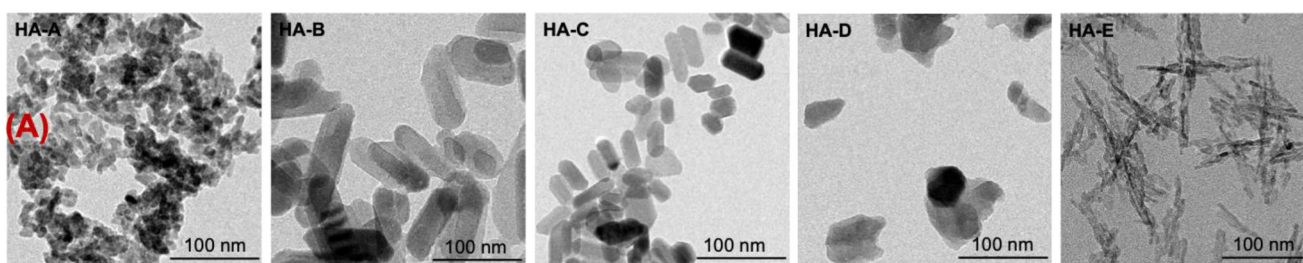
independent experiments. (\*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ). (Reprinted with permission from ref. [128]. Copyright 2009 Elsevier)

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(B)

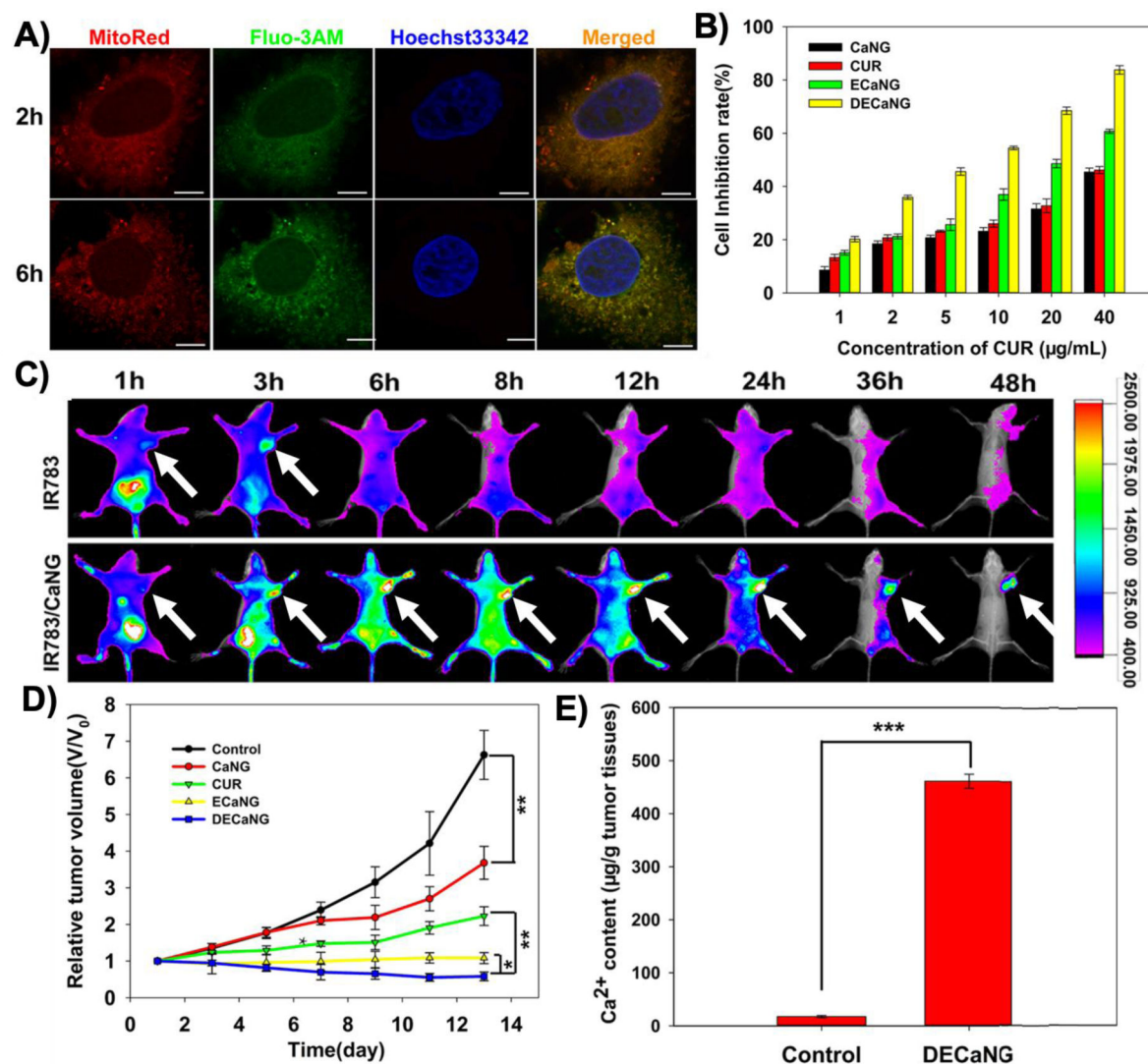
Physicochemical properties	Samples				
	HA-A	HA-B	HA-C	HA-D	HA-E
Morphology	Granular	Rod-like	Rod-like	Granular	Needle-like
Particle size (nm)	17.41±1.16	L:61.26±6.38 D:29.08±5.26	L:45.13±8.85 D:25.24±2.12	47.58±7.04	L:39.29±6.05 D:5.88±0.74
Crystallinity (%)	12.80	72.89	73.29	52.57	20.00
Zeta potential (mV)	-9.62±0.65	-10.90±0.91	-12.50±0.78	-16.60±0.60	-10.60±0.69
SSA (m <sup>2</sup> /g)	107.38±0.06	48.64±0.31	51.27±7.19	26.45±0.49	89.90±16.87
[Ca <sup>2+</sup> ] (ppm)	7.09±0.53	2.82±0.03	1.98±0.05	2.92±0.06	8.04±0.23

(C)

Cell type	Cell viability (%)				
	HA-A	HA-B	HA-C	HA-D	HA-E
A375	34.90±0.24	60.43±1.96	62.49±0.83	63.54±1.60	74.90±1.44
HSF	130.77±3.61	180.04±5.00	100.00±1.50	269.35±4.70	248.06±3.68

**Figure 8.** Effect of CaP's morphology, size, crystallinity, zeta potential, and specific surface area (SSA) on viability of melanoma (A375) and normal human epidermal fibroblasts (HSF) cells. (A) Transmission electronic microscopy images of five different types of hydroxyapatites shown as HA-A, HA-B, HA-C, HA-D and HA-E. (B) Physicochemical properties and (C) cell viability efficacy of these five batches. (Adopted with permission from ref. [69]. Copyright 2019 DovePress)





**Figure 9.**

(A) Confocal fluorescent images of the MCF-7 breast cancer cells showing distribution of Ca<sup>2+</sup> (stained with Fluo-3AM) released from degraded CaP nanoparticles (so called calcium nanogenerators or CaNG) in mitochondria (stained with MitoRed) at different incubation times (2 and 6 h). Hoechst33342 was used for staining the nucleus. (Scale bars: 7.5 µm) (B) Degradation of the CaP nanoparticles disrupted the mitochondrial Ca<sup>2+</sup> homeostasis and resulted in enhanced apoptosis (growth inhibition) of the cancer cells. (C) *In vivo* fluorescent imaging at different post-injection times indicated these nanoparticles could accumulate in subcutaneous tumors (shown by white arrows), when they were tagged with IR783 fluorescent molecules, compared with free IR783 molecules. (D) Mitochondrial disruption of the Ca<sup>2+</sup> homeostasis helped to inhibit tumor growth and enhanced the therapeutic effects, especially when combined with photothermal therapy. (E) Significantly higher amount of Ca<sup>2+</sup> in tumor tissues obtained from the mice treated with DECaNG compared with control mice only injected with saline. **CaNG**: the synthetic CaP nanoparticles; **CUR**: curcumin, an agent to enhance release of Ca<sup>2+</sup> from endosomes to

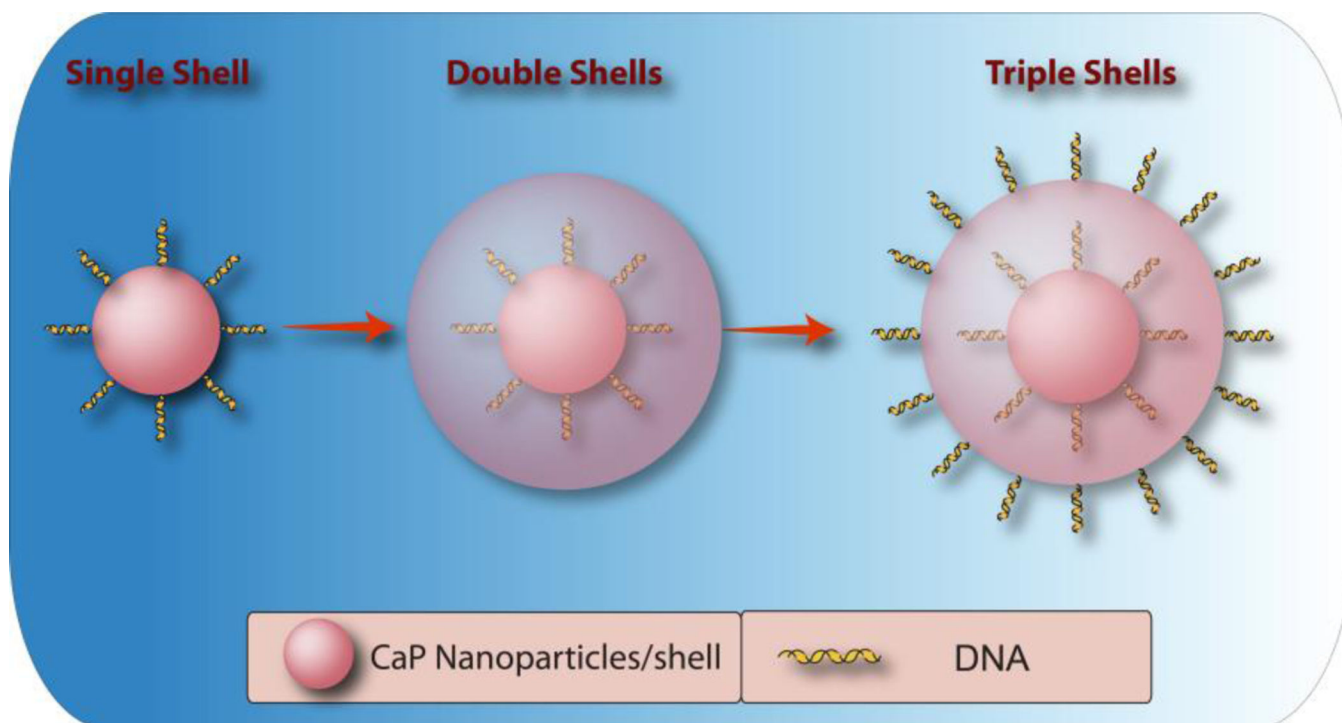
cytoplasm; **ECaNG**: enhanced  $\text{Ca}^{2+}$  nanogenerators or CUR-loaded CaNGs; **DECaNG**: dual enhanced  $\text{Ca}^{2+}$  nanogenerator, CaNG loaded with both CUR and a fluorescent molecule for simultaneous  $\text{Ca}^{2+}$  release and photothermal therapy effects. (Adopted with permission from ref. [58]. Copyright 2018 American Chemical Society)

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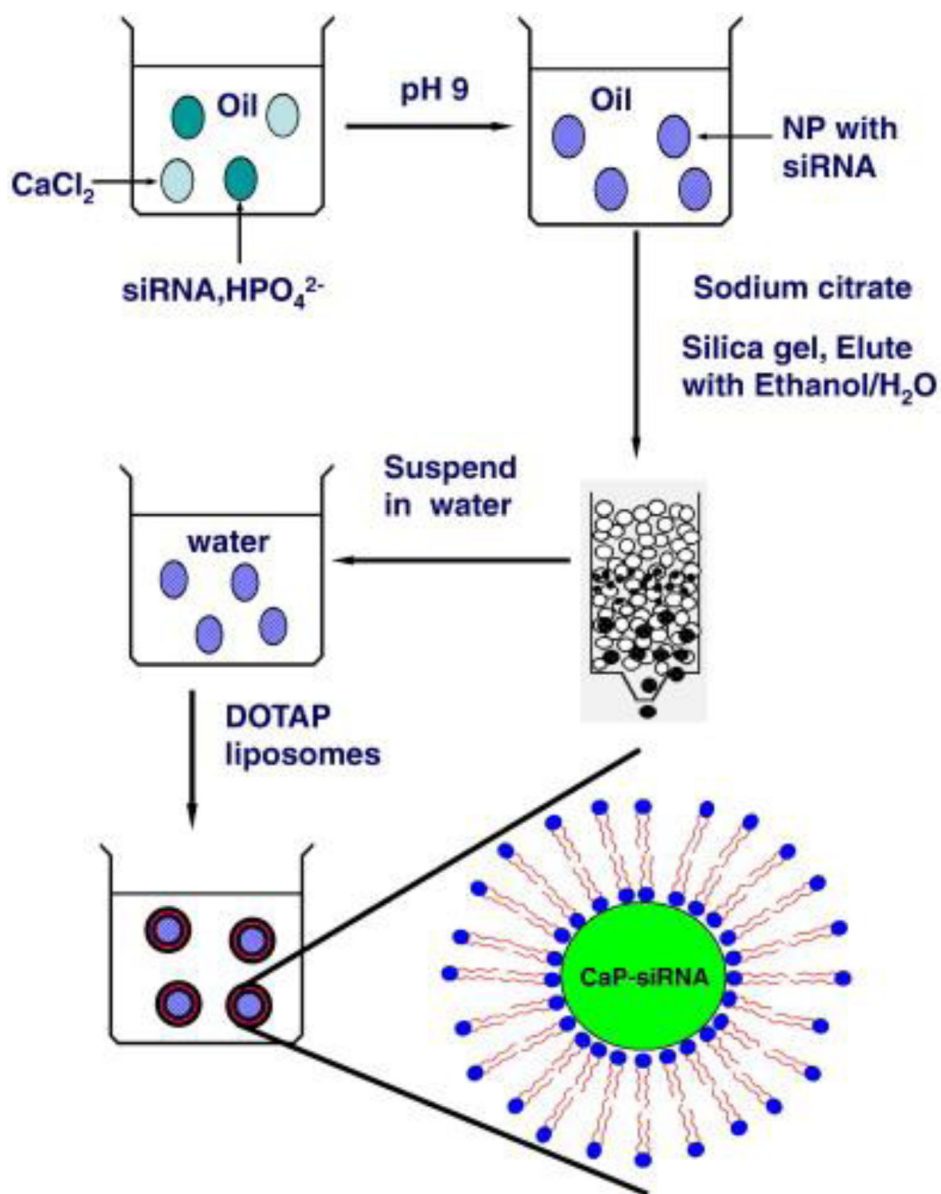
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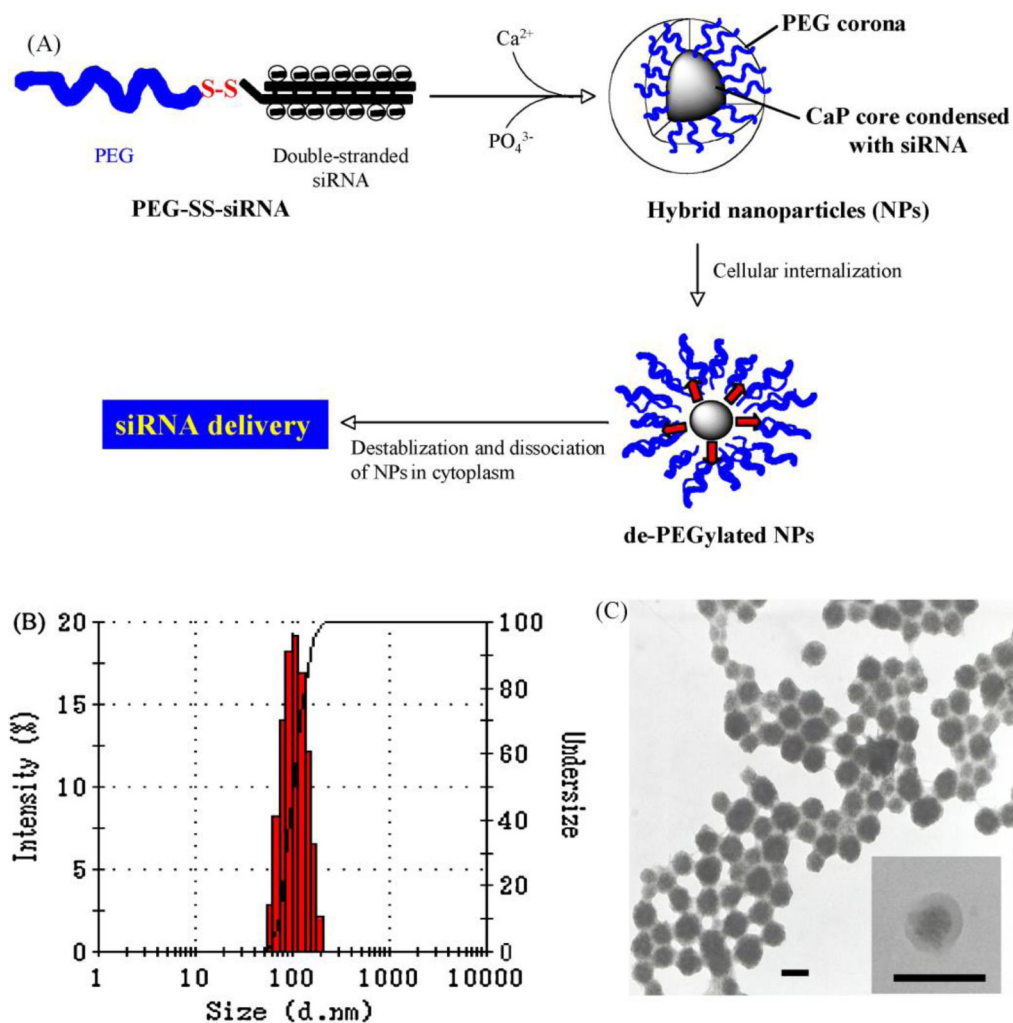
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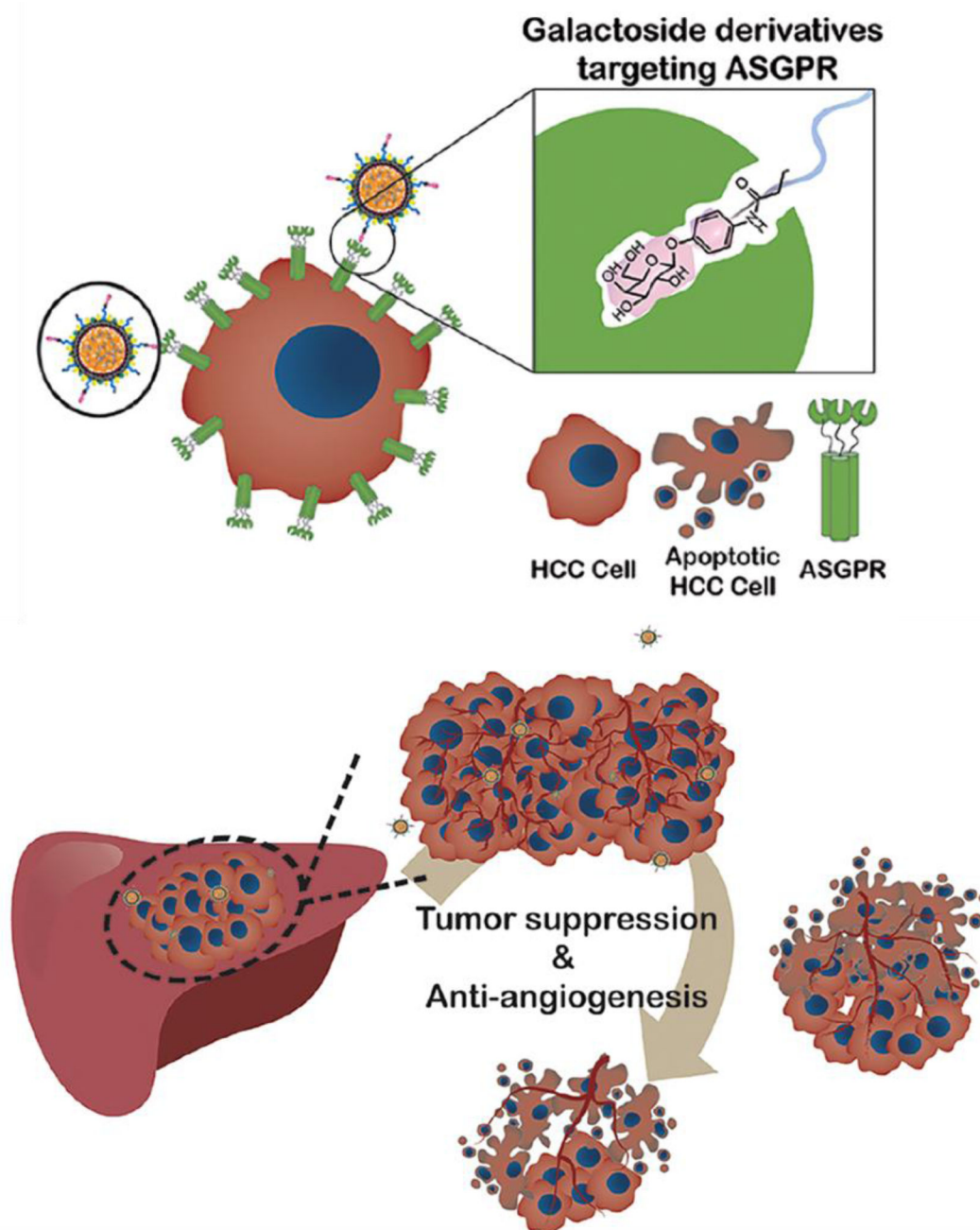
**Figure 10.** Schematic illustration of triple-shell CaP nanoparticles incorporating DNA. First DNA coated CaP nanoparticles are synthesized, followed by mineralization of an additional CaP shell. Finally the CaP coated nanoparticles are coated with another DNA layer to prevent their aggregation in biological media.



**Figure 11.** Schematic showing a typical method used for preparation of siRNA-loaded lipid/calcium/phosphate nanoparticles (LCP NPs). (Adopted with permission from ref. [160]. Copyright 2010 Elsevier)

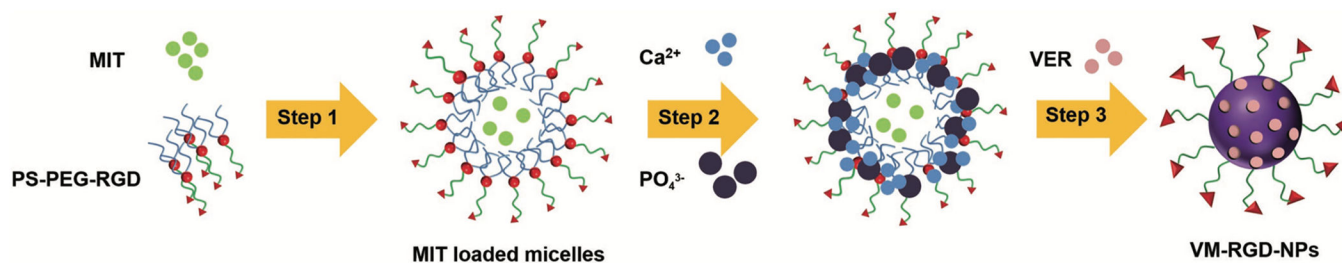


**Figure 12.** (A) Schematic showing the chemical design of the PEG-SS-siRNA/CaP nanoparticle; (B) Hydrodynamic size distribution and (C) TEM micrographs of the synthesized PEG-SS-siRNA/CaP nanoparticle; (TEM scale bar: 100 nm). (Reprinted with permission from ref. [161]. Copyright 2009 WILEY)



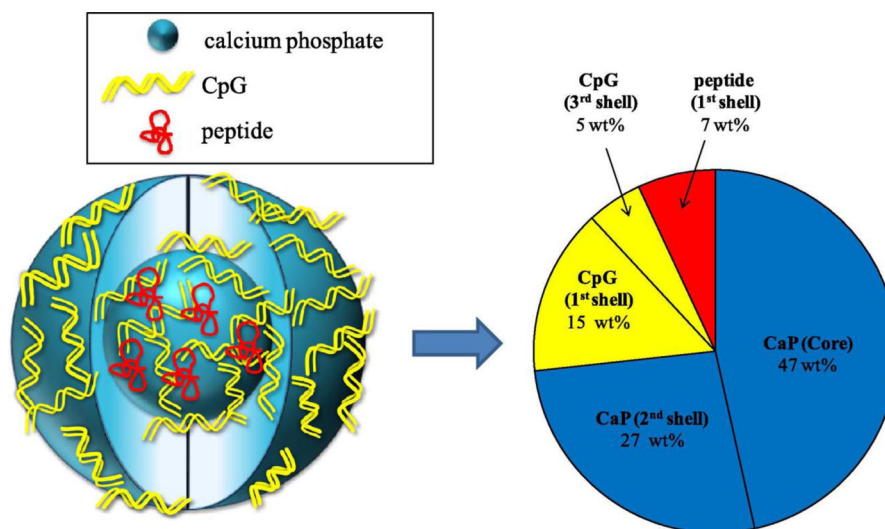
**Figure 13.**

(A) Targeted delivery of VEGF (vascular endothelial growth factor) siRNA to murine orthotopic hepatocellular carcinoma (HCC) tumors in liver, using lipid/calcium/phosphate nanoparticles (LCP NPs) as carriers.  $\beta$ -D-galactoside was conjugated to the LCP NPs for targeting the asialoglycoprotein receptors (ASGPR) on the membrane of the HCC cells. LCP NPs were degraded in low pH environment of the HCC lysosomes and released VEGF siRNA that downregulated expression of the VEGF in HCC cells and inhibited tumor growth by anti-angiogenic effect. (Adapted with permission from ref [62]. Copyright 2018 American Chemical Society)



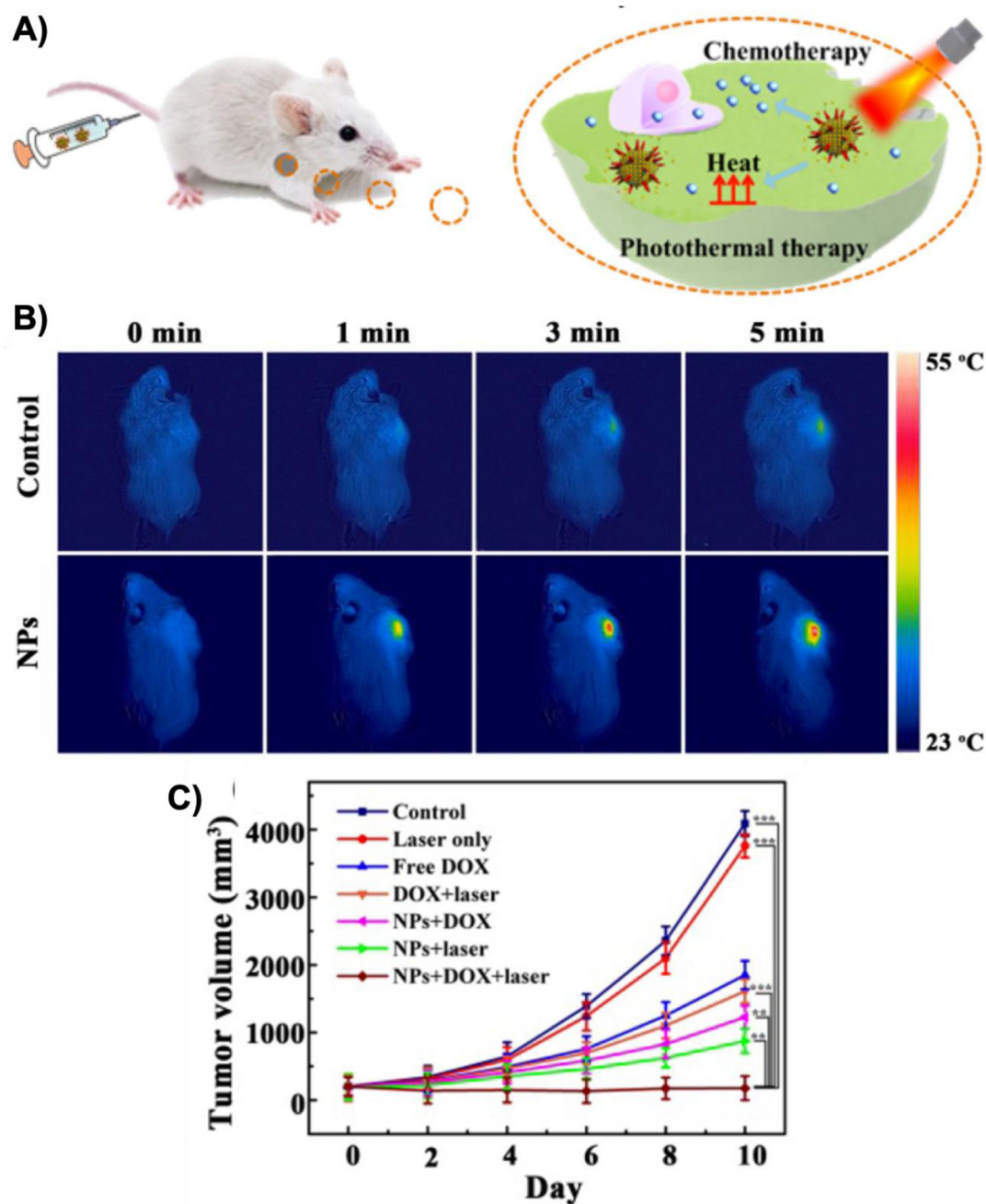
**Figure 14.**

Two common mechanisms for designing drug-loaded CaP nanoparticles. Drugs can be entrapped inside micelles that act as templates for mineralization of CaP's. Also, physical adsorption of drugs to CaP's has been commonly used for synthesizing drug-loaded CaP's. Schematic shows entrapment of Mitoxantrone (MIT) in PS-PEG-RGD micelles (Step 1). CaP shell was formed on the micelles to seal this drug (Step 2). A second drug (Verapamil or VER) was also added to the surface of CaP's by mixing and physical adsorption (Step 3). RGD is a cancer targeting peptide. (Reprinted with permission from ref. [60]. Copyright 2018 WILEY-VCH Verlag GmbH & Co.)



**Figure 15.** Composition distribution of a typical triple-shell calcium phosphate nanoparticles formulation that incorporates oligonucleotide (CpG) and a peptide. (Reprinted with permission from ref. [169]. Copyright 2011 Elsevier)





**Figure 16.**

(A) Tumor treatment using CaP nanoparticles (NPs) as platforms for simultaneous photothermal therapy and chemotherapy. Hybrids of CaP nanoparticles and gold nanorods were loaded with Doxorubicin (DOX) and injected into mice *via* tail veins. Nanoparticles accumulated in the tumors released Doxorubicin (chemotherapy agent) and generated heat in response to laser irradiation. Temperature variation in tumors were monitored using thermal imaging (B) and enhanced the therapeutic effect of the nanoparticles as shown by “NPs

+DOX+laser” in the tumor volume graph (C). (Adapted with permission from ref. [63].  
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**Table 1.**

An overview of the recent *in vivo* cancer therapeutic studies using calcium phosphate-based nanoparticles. Nanoparticles physiochemical properties (*e.g.*, crystallographic phase, hydrodynamic size, shape, surface coating and targeting molecules), chemical procedures used for their surface functionalization, cancer models and therapeutic approaches are listed for each study. Observation of endocytosis in each study is shown with YES or NO. Endocytosis in tumor cells has been reported for CaP nanoparticles with different sizes, crystallographic phases, shapes and surface coatings. (*abbreviations sorted alphabetically*: **2DG**: 2-deoxy-D-glucose;  **$\alpha$ -TOS**:  $\alpha$ -tocopheryl succinate; **ABX-EGF scFv**: EGFR- specific single chain fragment antibody; **AHA**: alendronate-hyaluronan graft polymer; **BSA**: bovine serum albumin; **CaP**: Calcium phosphate; **DOX**: Doxorubicin; **Gd**: Gadolinium MRI contrast agent; **HAp**: Hydroxyapatite; MIT: Mitoxantrone; **mPEG-SH**: methoxy-polyethylene glycol with thiol groups; **NA**: not applicable; **NPs**: Nanoparticles; NS: not characterized or specified in the reported study.; **PAA**: Poly acrylic acid; **PDT**: Photo dynamic therapy; **PEG**: Polyethylene glycol; **PEGS**: Pegylated poly(glycerol sebacate); **PTT**: Photothermal therapy; **RGD**: Arginyl glycyl aspartic acid; **TPGS**: Polyethylene glycol 1000 vitamin E succinate; **TSG-6**: (TNF)-stimulated gene 6; **uPA**: Urokinase plasminogen activator analogues peptide; **VER**: Verapamil.

CaP phase	Shape	Size (nm)	Coating	Targeting molecule	Functionalization method	Application/ Endocytosis reported?	Ref.
Amorphous CaP	Spherical	40–50	Lipid	Folic acid and/or ABX-EGF scFv	Two-step lipid coating and then covalent bonding of targeting molecules	siRNA delivery to breast tumor/ ( <b>NO</b> )	[23]
CaP (NS) (degradable)	Spherical	100–150	Copper sulfide/ Pluronic® F-68	NS	Electrostatic adsorption for F-68 capping	Mitochondrial Ca <sup>2+</sup> homeostasis and PTT of breast tumor/ ( <b>YES</b> )	[58]
CaP (NS)	Spherical	150	Hyaluronic acid	Hyaluronic acid	Electrostatic adsorption of cross-linked hyaluronic acid	siRNA delivery to mouse melanoma tumor/ ( <b>YES</b> )	[59]
CaP (NS)	Spherical	20	PEG-RGD	RGD peptide	Formation of CaP's inside drug-loaded PEG-RGD micelles	Delivery of Drug-resistance inhibitor (VER) and a chemotherapeutic agent (MIT) to hepatocellular carcinoma (liver cancer)/ ( <b>YES</b> )	[60]
CaP (NS)	Spherical	44–47	Lipid/PEG	Bispecific antibody (BsAb)	Non-covalent adsorption of BsAb to lipid-coated CaP's	Inhibit growth of the triple negative breast tumor by siRNA delivery and PTT in triple negative breast tumor/ ( <b>YES</b> )	[61]
CaP (NS)	Spherical	125	Lipid	Galactose derivative	Two-step lipid coating and non-covalent bonding of galactose derivatives	siRNA delivery to hepatocellular carcinoma (liver cancer)/ ( <b>YES</b> )	[62]
CaP (NS)	Spherical	100	PAA/ PEG/ gold nanorods	NA	Assembly of gold nanorods to the PAA-coated CaP's and then coating with mPEG-SH	DOX delivery and PTT of liver cancer/ ( <b>YES</b> )	[63]
CaP (NS)	Spherical	170	AHA	Hyaluronic acid	Electrostatic adsorption of hyaluronic acid to CaP	siRNA delivery to lung cancer/ ( <b>YES</b> )	[64]
CaP (NS)	Spherical	68	folic acid/ PEG	Folic acid	Two-step lipid coating and then covalent bonding of folic acid	Prevention of breast tumor metastasis by	[65]

CaP phase	Shape	Size (nm)	Coating	Targeting molecule	Functionalization method	Application/ Endocytosis reported?	Ref.
						vitamin E ( $\alpha$ -TOS) delivery/ ( <b>YES</b> )	
Calcium phosphonate	Spherical	42	Lipid/PEG coated	miRNA-155	Microemulsion to form miRNA loaded lipid-coated CaP's and then coating with PEG-lipid in organic phase	miRNA delivery to tumor associated macrophages in murine sarcoma cancer/ ( <b>YES</b> )	[66]
CaP (NS)	Spherical	194	Chitosan	NA	Co-precipitation of siRNA-loaded CaP's in the presence of chitosan	siRNA delivery to Hela cervical cancer/ ( <b>YES</b> )	[67]
HAp	Spherical nano-clusters	30–40	Polyglutamic acid	NA	Polyglutamic acid for self-assembly of ultrasmall HAp NPs	Synergistic DOX delivery and increased calcium influx for therapy of gastric adenocarcinoma/ ( <b>YES</b> )	[54]
CaP (NS)	CaP shell to encapsulate siRNA	113	PEG	uPA	Formation of siRNA-loaded CaP's inside PEG-uPA micells	siRNA delivery to triple negative breast cancer/ ( <b>YES</b> )	[68]
HAp	Granular and nanorods	17 and 61×29	NA	NA	No surface functionalization was used	To evaluate effects of size and shape of HAp (no drug) in melanoma/ ( <b>YES</b> )	[69]
HAp	Spherical	38	A54, TPGS and Gd	A54 peptide	CaP formation in the presence of A54-TPGS and Gd	MRI-guided DOX delivery to hepatic cancer/ ( <b>YES</b> )	[70]
CaP (NS)	Spherical	207	PEI-cholesterol	VEGF siRNA	siRNA-loaded CaP's were coated with a layer of PEI-cholesterol	siRNA delivery and inhibition of breast tumor angiogenesis/ ( <b>NO</b> )	[71]
Amorphous CaP (degradable)	CaP coated upconversion NPs	78	PAA	NA	Silica-coated upconversion NPs were coated with CaP and PAA	DOX delivery and PDT of cervical cancer/ ( <b>YES</b> )	[72]
HAp	nanorod	10×50	NA	NA	No surface functionalization was used	Mitochondria-targeting to inhibit lung cancer growth/ ( <b>YES</b> )	[53]
HAp	almost spherical and porous	217	PEG/ Folic acid	Folic acid	Folic acid was covalently conjugated to polyethylene glycol-coated HAp NPs	Drug (epirubicin) delivery to murine colorectal cancer/ ( <b>YES</b> )	[55]
HAp	mesoporous and spherical	NS	NA	NA	Electrostatic adsorption of 2DG, DOX and then radiolabeling for SPECT imaging	2DG and DOX delivery to breast tumor and SPECT imaging using radiolabeling/ ( <b>NO</b> )	[56]
CaP (NS)	Spherical	140	BSA	NA	CaP mineralization in the presence of BSA as template	TSG-6 delivery for treatment of liver fibrosis/ ( <b>NO</b> )	[73]
HAp	core/shell PEGS/ HAp micelle	20–30	PEGS	NA	HAp NPs were formed in PEGS micelles	pH responsive DOX delivery to breast tumor/ ( <b>NO</b> )	[74]
HAp	Nanorods loaded into a titanium implant	46×13	NA	NA	No surface functionalization was used	mitochondrial-dependent apoptosis and stimulating immune response in rabbit models/ ( <b>NO</b> )	[75]
HAp and oligo-HAp (degradable)	silica/HAp hybrid	99	Hyaluronan (HA) and oligo-	Hyaluronan (HA) and oligo-	HA or oHA were covalently conjugated to the surface of silica/HAp	DOX delivery to breast cancer/ ( <b>YES</b> )	[76]

CaP phase	Shape	Size (nm)	Coating	Targeting molecule	Functionalization method	Application/ Endocytosis reported?	Ref.
			hyaluronan (oHA)	hyaluronan (oHA)			
CaP (NS)	spherical	140	MMP-2 cleavable peptide and PEG-lipid	NA	Peptide and PEG-lipid coating created by microemulsion	siRNA delivery to liver cancer/ ( <b><i>YES</i></b> )	[77]

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**Table 2.**

Different crystallographic phases of calcium phosphates and their pH stability range and water solubility as representatives of biodegradability in weakly acidic microenvironment of the tumors or acidic pH of the endosomes and lysosomes. (Adapted and Reprinted with permission from refs. [32] and [31]. Copyright 2019 Royal Society of Chemistry)

Compound	Chemical formula	Ca/P ratio	Solubility at 25 °C, $-\log(K_{sp})$	Solubility at 25 °C (g L <sup>-1</sup> )	pH stability range at 25 °C
Monocalcium phosphate monohydrate (MCPM)	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O	0.5	1.14	~ 18	0.0–2.0
Monocalcium phosphate anhydrous (MCPA)	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	0.5	1.14	~ 17	<i>b</i>
Dicalcium phosphate anhydrous (DCPA, monetite)	CaHPO <sub>4</sub>	1.0	6.90	~ 0.048	<i>b</i>
Dicalcium phosphate dihydrate (DCPD, brushite)	CaHPO <sub>4</sub> ·2H <sub>2</sub> O	1.0	6.59	~ 0.088	2.0–6.0
Octacalcium phosphate (OCP)	Ca <sub>8</sub> (HPO <sub>4</sub> ) <sub>2</sub> (PO <sub>4</sub> ) <sub>4</sub> ·5H <sub>2</sub> O	1.33	96.6	~ 0.0081	5.5–7.0
Amorphous calcium phosphates (ACP)	Ca <sub>x</sub> H <sub>y</sub> (PO <sub>4</sub> ) <sub>z</sub> ·nH <sub>2</sub> O, <i>n</i> = 3–4.5; 15–20% H <sub>2</sub> O	1.2–2.2	<i>a</i>	<i>a</i>	~ 5–12
Calcium-deficient hydroxyapatite (CDHA, is also called precipitated hydroxyapatite)	Ca <sub>10-x</sub> (HPO <sub>4</sub> ) <sub>x</sub> (PO <sub>4</sub> ) <sub>6-x</sub> (OH) <sub>2-x</sub>	1.5–1.67	-85.1	~ 0.0094	6.5–9.5
Hydroxyapatite (HAP)	Ca <sub>10</sub> (PO <sub>4</sub> ) <sub>6</sub> (OH) <sub>2</sub>	1.67	116.8	~ 0.0003	9.5–12
Fluorapatite (FAP)	Ca <sub>10</sub> (PO <sub>4</sub> ) <sub>6</sub> F <sub>2</sub>	1.67	120	~ 0.0002	7–12
α-Tricalcium phosphate (α-TCP)	α-Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	1.5	25.5	~ 0.0025	<i>c</i>
β-Tricalcium phosphate (β-TCP)	β-Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	1.5	28.9	~ 0.0005	<i>c</i>
Tetracalcium phosphate (TTCP, hilgenstockite)	Ca <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> O	2.0	38–44	~ 0.0007	<i>c</i>

<sup>a</sup>Cannot be measured precisely, always metastable.

<sup>b</sup>Stable at temperatures above 100 °C.

<sup>c</sup>These compounds cannot be precipitated from aqueous solution.

**Table 3.**

Common chemical methods used for surface modification of CaP-based nanoparticles with different types of macromolecules such as nucleic acids (*e.g.*, siRNA and DNA), cancer targeting agents (*e.g.*, peptides and antibodies), and polymers (*e.g.*, PEG, PEI and hyaluronic acid), designed for *in vivo* cancer therapy studies. (**PEI**: Polyethylenimine)

Method Description	Common Macromolecules	Advantages	Disadvantages	Examples
Macromolecules used to form micelles for CaP mineralization	Liposomes, hyaluronic acid and peptides conjugated to polymers ( <i>e.g.</i> , PEG)	<ul style="list-style-type: none"> <li>- Tunable CaP shell thickness</li> <li>- Protection of CaP-coated macromolecules</li> </ul>	<ul style="list-style-type: none"> <li>- Safety of the synthetic macromolecules for clinical translation</li> <li>- Often requires additional surface modification</li> </ul>	[60, 68, 74, 77]
Macromolecules used as homogenously dispersed templates for controlled growth of mineralized CaP's (no micelles)	Nucleic acids and proteins ( <i>e.g.</i> , BSA)	<ul style="list-style-type: none"> <li>- Mostly spherical</li> <li>- Room temperature synthesis which maintains bioactivity</li> </ul>	<ul style="list-style-type: none"> <li>- Aggregation of NPs</li> <li>- Limited size and shape tenability</li> <li>- Often requires additional surface modification</li> </ul>	[67, 70]
Surface modification of mineralized CaP's by electrostatic adsorption of macromolecules	Cancer targeting molecules and nucleic acids	<ul style="list-style-type: none"> <li>- Easy procedures</li> <li>- Applicable for a large number of macromolecules</li> </ul>	<ul style="list-style-type: none"> <li>- Detachment of the physically adsorbed macromolecules due to their weak bonds</li> </ul>	[61, 62]
Covalent bonding of macromolecules to the surface of mineralized CaP's	Cancer targeting molecules, Polymers ( <i>e.g.</i> , PEG or PEI derivatives) and hyaluronic acid	<ul style="list-style-type: none"> <li>- Long-term stability in biological environment</li> <li>- Longer blood circulation time</li> <li>- Lower cost of the macromolecules</li> </ul>	<ul style="list-style-type: none"> <li>- Multiple steps and sensitive conjugation steps</li> </ul>	[23, 55, 65]