Calcium Phosphate-Based Nanosystems for Advanced Targeted Nanomedicine

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Abstract

Synthetic calcium phosphates (CaPs) are the most widely accepted bioceramics for the repair and reconstruction of bone tissue defects. The recent advancements in materials science have prompted a rapid progress in the preparation of CaPs with nanometric dimensions, tailored surface characteristics, and colloidal stability opening new perspectives in their use for applications not strictly related to bone. In particular, the employment of CaPs nanoparticles as carriers of therapeutic and imaging agents has recently raised great interest in nanomedicine. CaPs nanoparticles, as well as other kinds of nanoparticles, can be engineered to specifically target the site of the disease (cells or organs), thus minimizing their dispersion in the body and undesired organism-nanoparticles interactions. The most promising and efficient approach to improve their specificity is the “active targeting”, where nanoparticles are conjugated with a targeting moiety able to recognize and bind with high efficacy and

selectivity to receptors that are highly expressed only in the therapeutic site. The aim of this review is to give an overview on advanced targeted nanomedicine with a focus on the most recent reports on CaP nanoparticles-based systems specifically designed for the active targeting. The distinctive characteristics of CaP nanoparticles with respect to the other kinds of nanomaterials used in nanomedicine are also discussed.

Keywords: calcium phosphates, active targeting, nanoparticles, drug delivery, nanomedicine, hydroxyapatite.

1. Introduction

1.1. Nanomedicine

Modern medicine is currently undergoing a paradigm shift from the conventional disease managements to more personalized and customized treatments, exploiting specific interactions of therapeutic agents at molecular level. In this domain, encouraging results come from the emerging field of nanomedicine, which is defined as the application of nanotechnology to address healthcare problems, where the unique and novel properties displayed by nanomaterials are harnessed to achieve performances, specificity, and biological activities not exhibited by their counterparts at larger dimensional scales [1-3].

The nanometric dimensions of nanoparticles/nanosystems (that are described here by the generic abbreviation "NPs") confer several advantages when they are employed as drug nano-carriers or even as therapeutic agents. For example, water dispersed NPs can encapsulate hydrophobic drugs, increasing drug bioavailability and at the same time offering protection against *in vivo* drug early degradation [4]. The nanometric size allows NPs to escape the capture by cells of the reticuloendothelial system (RES) avoiding the rapid clearance and improving the circulation time [5]. At the same time, the small size allows their penetration into tissues and cells to reach the target site [6]. The use of NPs has been mainly studied for cancer management, and several clinically approved NPs-based formulations for the treatment of a variety of cancer types exist nowadays [3, 7-9]. Several authors have pointed out that innovative NPs-mediated formulations of conventional chemotherapeutics can enhance their efficacy [10, 11]. In addition, the conjugation of antineoplastic drugs with NPs can reduce their side effects (*i.e.* cardiotoxicity, nephrotoxicity and hepatotoxicity) by

minimizing their non-specific interactions with healthy cells and tissues [12-17]. The application of NPs to treat other diseases such as cardiovascular, neurological and musculoskeletal ones has been more recently investigated [6, 18-21].

In most of the studies, NPs act as mere carriers of biologically active molecules, and their role is to bind and deliver to the site of the disease conventional or innovative therapeutic agents [4, 22, 23]. In other cases, the composition and the intrinsic characteristics of NPs can be exploited in combination with external stimuli to exert a therapeutic effect. This is the case for example of photodynamic, hyperthermia and neutron capture therapies that have been developed employing drug-free NPs [24-31].

The use of NPs as imaging probes for cells and tissue has also been extensively explored in the last decade in virtue of several advantages provided over conventional imaging agents. Materials at the nano scale possess unique optical, magnetic, and chemical properties that allow the creation of imaging probes with increased density, amplification and quantification of the signal, as well as with improved contrast, compared to conventional imaging agents [11, 32]. In this domain NPs are efficiently used as imaging agents in magnetic resonance imaging (MRI) [33], positron emission tomography (PET) [34], near-infrared (NIR) adsorption [35], or in combinations of them as in the case of dual-imaging techniques (*i.e.* a combination of MRI and PET) [36].

NPs can also be designed to work as "theranostic" agents, designed to exert at the same time both diagnostic and therapeutic functions, thus enabling the non-invasive *in vivo* real-time monitoring of the therapy efficiency [11, 37-39]. The monitoring of NPs behavior is essential to clearly assess their bio-distribution, namely tissue penetration, organ accumulation, and excretion; these information are the key-points of important pharmacodynamics aspects of NPs such as hematic lifetime, drug delivery kinetic, toxicity, specificity and efficacy [37].

The generation of theranostic agents is at the forefront of medicine in virtue of the possibility to achieve controlled and localized therapeutic actions. In particular, the development of effective theranostic NPs is a milestone of "personalized" or "precision" medicine that aims to set up *ad hoc* patient-specific disease managements. Personalized medicine could achieve the tuning of patients treatment accordingly to the specific situation, *i.e.* tailoring of key pharmacological parameters such as drug dosage, number of treatments, drug relapse control *etc.*. Therefore, the personalized approach will enable to maximize the therapeutic action and at the same time to minimize side effects and discomfort [40, 41].

We must caution the reader that all that glitters is not gold, as long-term toxicity of several kinds of NPs has not been elucidated yet; thus, their capacity to penetrate into tissues could

become a double-edged sword as non-biodegradable NPs can accumulate also in healthy tissues inducing inflammatory and toxic effects [42, 43]. In order to overcome these drawbacks and to improve their efficiency, NPs must be engineered to be "targeted" towards the site of the disease (cells or organs) thus minimizing NPs dispersion in the organism and undesired organism-NPs interactions [44].

1.2. Targeted nanomedicine

There are several approaches to turn non-specific NPs into targeted ones. Firstly, it must be mentioned that NPs are naturally targeted for tumors through the enhanced permeation and retention (EPR) effect, consisting in their spontaneous accumulation in the leaky and over-vascularized cancerous tissues [45]. However, this form of "passive targeting" is not always efficient because the degree of tumor vascularization and porosity of tumor vessels can vary within tumor types and conditions [46, 47]. Besides, in non-cancerous diseases the EPR effect does not occur.

The most promising and efficient approach to improve the specificity of NPs is the "active targeting", where a targeting moiety is added as surface decoration to the NPs, that may provide additional or alternative delivery mechanisms to EPR.

The targeting moiety is a molecule/macromolecule capable to recognize and bind with high efficacy and selectivity to receptors expressed only by specific cells [48, 49]. Once the NPs functionalized with the targeting molecules arrive near the target cells they interact with membrane receptors via "targeting moiety-receptor" mechanisms and, in some cases, penetrate in the cells by specific receptor-mediated internalization processes. Consequently, NPs release their therapeutic payload in close proximity or inside the target cells (Figure 1) [48-50].

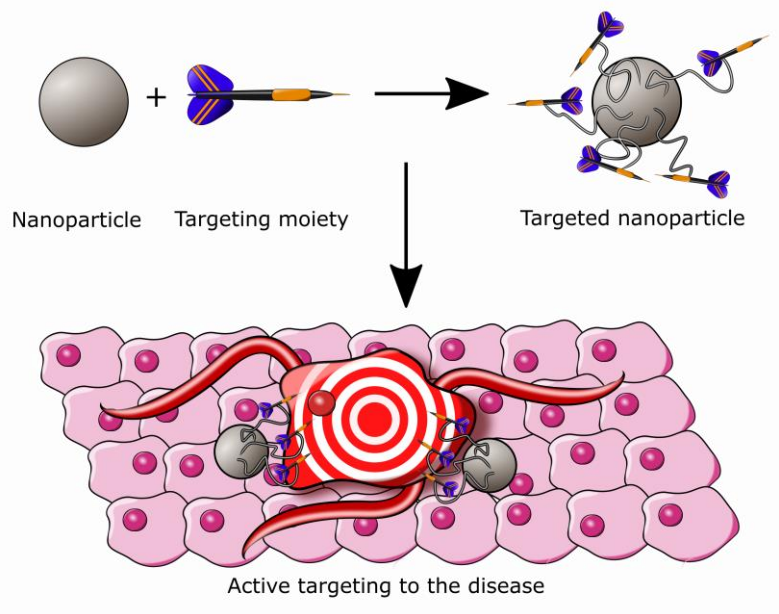

Figure 1. Schematic representation of the active targeting mechanism.

Since cancer is one of the leading causes of morbidity and mortality worldwide, the development of targeted NPs has mainly focused on tumor treatment. Specifically, death from cancer amounts to about 13% of total deaths per year (*i.e.* an average of 4.2 million men and 3.4 million women) and is expected to reach 13.1 million deaths in 2030 [51, 52]. The main issue of cancer disease is tied to the large and radical variety of tumors; therefore an ideal and efficient cancer therapy should be type-specific and selective [3, 48, 53-55]. In this regard, it is worth to mention that the research on targeted NPs for the management of cardiac, neurological and others diseases has also started but is still in its infancy [56-58].

The study of targeted NPs is at the moment a hot topic in the scientific research and the number of publications in this field has grown exponentially in the last two decades (Figure 2-A). Moreover, this topic is multidisciplinary and involves different subjects such as nanotechnology, chemistry, molecular biology, pharmacology, *etc.* (Figure 2-B).

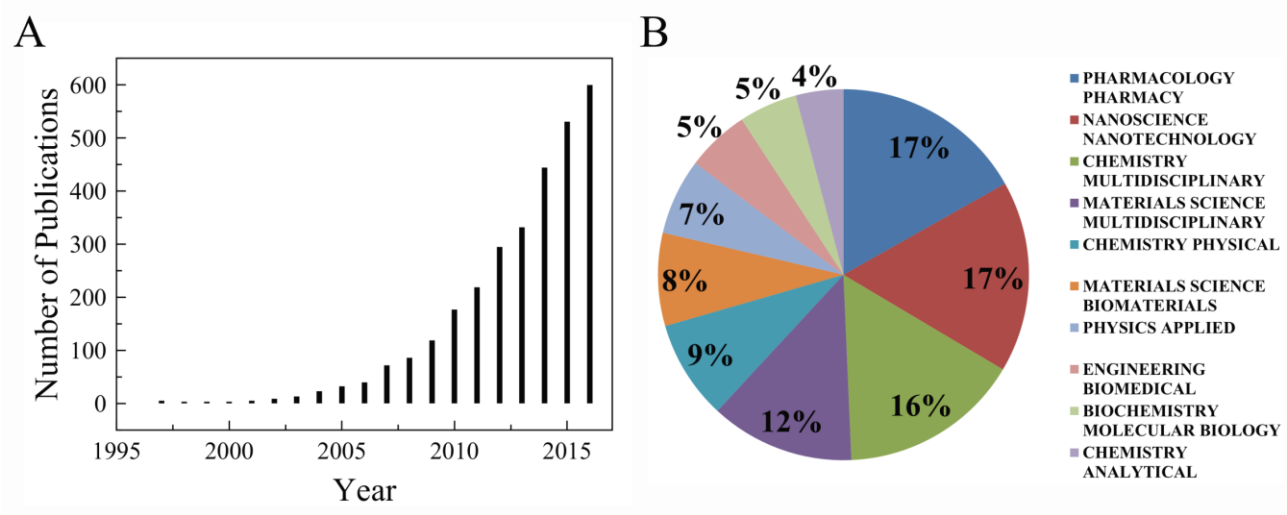

Figure 2. (A) Number of articles indexed in Thomson Reuters Web of Science reporting the terms "active targeting" and "nanoparticles" from 1995 to 2016. (B) Distribution of the papers reported in panel A by fields of study according to Thomson Reuters Web of Science.

1.3. Active targeting moieties

Several targeting moieties and functionalization procedures have been exploited to achieve active targeted NPs [50, 59-61]. The most studied and effective targeted moieties include: monoclonal antibodies [62], antibody derivatives [63], peptides [64, 65], aptamers [66], transferrin [67-69], carbohydrates [60, 70], and small organic molecules [71, 72].

Monoclonal antibodies (mAbs) are single macromolecular species that bind to their specific antigens with high affinity and selectivity (in general they can bind to only one specific target), and can be precisely designed to target a plethora of receptors [73]. However, the use of mAbs suffers of several limitations, such as (i) large molecular size, (ii) difficulties in the conjugation with NPs and (iii) highly expensive production processes [62, 74]. Moreover, mAbs often derive from animals, therefore their administration could result in an immune response. However, mAbs derived from murine proteins can be manipulated into humanized versions that can provoke low to no immune response, but on the other hand this require high production costs [62, 75].

Antibody derivatives are an interesting alternative, as they are smaller, cheaper and less immunogenic than mAbs while holding comparable affinity and selectivity [63]. These derivatives – sometimes called "nanobodies" – are naturally-derived or synthetic antigen-binding fragments (called Fab or Fab'), and usually retain the specific antigen-binding affinity of the parent mAbs while displaying improved tissue penetration [63].

Peptides and aptamers are targeting moieties consisting of small sequences of amino acids and nucleic acids, respectively. Their selection strategy is based on combinatorial peptide/aptamers libraries, consisting of random or systematic collections of oligopeptides/oligonucleotides chains. These libraries are tested against the binding target and only the molecules that possess targeting capability are selected and amplified (*i.e.* mass produced). In this regard, the reader can refer to several reviews treating in detail this strategy [76-80]. The use of peptides and aptamers allows to reduce the synthesis cost of highly specific targeting moieties with relatively low molecular size and low immunogenicity [65, 66, 79]. The targeting capacity of aptamers is mainly due to their three-dimensional conformation deriving from their nucleotide sequence [81, 82]. To maximize aptamer efficacy, whole living cells, pathogens, or even animal models are used as targets for aptamer selection and amplification [66]. Some aptamers can also promote NPs internalization enhancing the efficacy of the therapeutic agent [83-85]. However, they are prone to enzymatic degradation in the biological environment that could lead to a rapid loss of their targeting capability. To overcome this issue, aptamers can be chemically modified with small molecules or with polyethileneglycol (PEG) polymers to improve their bioavailability and pharmacokinetic properties [86, 87].

As for aptamers, a number of peptides that specifically target various tissues under normal or pathological conditions have been already identified [88]. Similarly, some proteins have the function of binding selectively to specific membrane receptors expressed by several types of cells, and the specific peptidic fragments that are involved in the binding process can be exploited as targeting peptides. A notorious example of these peptidic fragments is the arginyl-glycyl-aspartic acid (RGD) tripeptide, a sequence identified in fibronectin glycoprotein that binds to cell surface receptors known as integrins. One of the most interesting integrin is $\alpha_v\beta_3$, which is implicated in tumor angiogenesis and is the target for numerous RGD-functionalized NPs [89].

Organic molecules used as targeting moieties usually exploit the natural overexpression of their receptors in unhealthy tissues, as in the case of folate – folic acid specific – and of transferrin – transferrin specific – receptors in solid tumors [67-69, 72, 90]. Folate is the water-soluble form of vitamin B9 and is essential in humans for rapid cell division and growth, especially during the embryonic development [91]. Folate receptors are overexpressed in ovarian, brain, head, neck, renal and breast tumor cells; thus folate, which has a high binding affinity for its receptor ($K_d = 10^{-9}$ M), was widely employed as targeting moieties of imaging and therapeutic agents to tumors [92, 93]. Transferrin is a membrane

glycoprotein that binds and transports iron ions in the serum to cells via transferrin receptor (TfR) [94, 95]. As in the case of folate, when transferrin binds to its receptor it initiates the endocytosis and gets internalized into the cytoplasm [94, 95]. Due to the higher rate of proliferation of tumor cells compared to healthy ones, the transferrin receptor is 10-fold overexpressed in cancerous tissue as a consequence of the dramatic increase of iron requirement. Therefore, the increased expression of TfR makes of transferrin an attractive targeting agent for the delivery of chemotherapeutics via nano-carriers [68, 69, 96].

Some carbohydrates receptors are overexpressed too in diseased cells; for example, galactose has a high affinity for asialoglycoprotein receptors found on hepatocytes (with a density of 500000 receptors per cell) [97, 98]. Hyaluronic acid, a copolymer of N-acetyl D-glucosamine and D-glucuronic acid, can bind selectively to the cluster determinant 44 (CD44), a transmembrane protein which plays a crucial role in the activities associated with various malignant tumors [99-102].

Several of the above-mentioned targeting moieties (*e.g.* mAbs, aptamers, *etc.*) are available on the market in the conjugated forms with imaging probes to monitor their biodistribution [103, 104].

The surface decoration of NPs with targeting molecules can be achieved by several means ranging from physisorption to the formation of new chemical bonds; among them, click reactions and the use of capture nucleotide strands are two particularly effective methods [105-113]. Indeed, targeting moieties are effective only if their molecular binding region is free to recognize the receptor. Therefore, the formation of a rigid chemical bond between NPs and a non-active region of the targeting agent is the best method to have a precisely control over the orientation of the moieties. While the use of click reactions is a well-known conjugation method, the use of capture nucleotide strands is a newer approach [113, 114]. With this latter methodology, also known as nucleotide hybridization method, nucleotide single strands are attached to the NPs and the complementary strands are conjugated to the targeting moieties. The coupling between NPs and targeting agents consists in the nucleotide hybridization, that is the formation of a double strand between the nucleotides of NPs and those of the targeting agents [114-117].

A multitude of organic, inorganic and hybrid NPs have been functionalized with active targeting moieties – mainly for cancer therapy – and several review papers have been published on this topic [3, 10, 11, 48-50, 53, 54, 59, 81, 91]. Among them, calcium phosphates (CaPs) have been proved to be one of the most promising materials in nanomedicine. Several reviews on the use of CaP NPs for general nanomedical applications were already published

[39, 118-124], therefore in the next paragraphs are discussed the most recent and significant reports on CaP NPs specifically designed for the active targeting on the basis of a large literature survey. In addition, an overview about the peculiar characteristics of CaPs NPs with respect to the other kinds of nanomaterials currently studied in nanomedicine is also reported.

2. The role of calcium phosphate nanoparticles in nanomedicine

2.1 Chemical and biological properties

In biological systems, CaPs are the inorganic constituent of normal (bone, dentine, fish scales, horns of different animals) and pathological (*e.g.* dental and some urinary calculi, tendon mineralization, calcification of blood vessels) calcifications [119]. Apart from enamel, which has a high degree of crystallinity, they occur mainly in the form of ionic substituted and poorly crystalline apatites. Nanocrystalline apatites, in contrast to stoichiometric hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$] which is the most thermodynamically stable and least soluble CaP phase in physiological conditions, are nonstoichiometric (Ca/P ratio less than 1.67), nanometric in size (length 20–50 nm, width 15–30 nm, and thickness 1.5–4 nm), calcium (and OH^-)-deficient, and can incorporate substituted ions in its crystal lattice (i.e., Na^+, Mg^{2+}, K^+, F^-, CO_3^{2-} , etc.) [120, 121].

Due to their excellent biological properties, such as biocompatibility, bioactivity, osteoconductivity, osteoinductivity, and non-immunogenicity, synthetic CaPs are the most important compounds to prepare biomedical devices for hard tissues substitution and regeneration, in the form of three-dimensional dense or porous ceramics and as injectable cements [122-137].

The recent advancements in materials science and nanotechnology have prompted a rapid progress in the preparation of CaPs with tailored surface characteristics, nanometric dimensions, and colloidal stability in aqueous environment opening new interesting perspectives in different biomedical fields [138, 139].

A huge number of synthetic routes exist for CaP NPs preparation and they have been extensively reviewed by other authors [122-137]. Due to the different strategies employed up to now, the classification of the synthesis methods is very difficult and deserves dedicated review papers. Probably, the simplest way to rationally classify the CaP NPs crystallization methods is the discrimination between the processes using high or low temperature. The synthetic methods at low temperature are preferred respect to those involving higher

temperature (> 100 °C) because they offer the advantage to produce CaP NPs having features very close to the biological ones. Biomimetic CaPs are the most appealing CaP NPs for medical applications as they are better tolerated by organisms with respect to sintered ones [140]. The reason of this behavior is that they are similar in terms of chemical composition, crystal structure, and morphology to the mineral phase of bone, that makes them recognizable by organisms as a sort of endogenous materials.

The use of organic compounds as templates for the generation of CaP with nanosized dimensions and biomimetic features is another interesting strategy that has received increasing attention over the last decade. For example the role of citrate ions in controlling and stabilizing the size of CaP NPs is a biologically inspired synthetic strategy that was recently efficiently developed [141-145]. In general the synthesis of biomimetic CaPs NPs respect the principles of green chemistry, for example they are not carried out in organic or hazardous solvent and they are cheap and easy to be scaled up [119, 123, 134, 135]. These conditions are often not met in the production of other kinds of inorganic or organic NPs [146-156].

One of the most important characteristic of CaPs NPs is that they are degraded faster with respect to the most commonly used inorganic NPs (*i.e.* quantum dots, silica, magnetic NPs and carbon nanotubes) and in addition, their degradation is followed by the release of the non-toxic calcium and phosphate ions [157, 158].

From a chemical point of view, another great advantage of CaP NPs is that their chemical composition (*i.e.* Ca/P ratio, anionic or cationic substituents), hydration level, crystallinity degree, dimension, morphology, aspect ratio, polydispersity index, surface area, surface charge and colloidal stability can be tailored by changing synthesis parameters [134, 135, 159].

Moreover CaP NPs for nanomedical use, in particular of HA, have a highly flexible crystal lattice able to accommodate (doping) several substituting ions while retaining their intrinsic structure. Doping can impart peculiar functionalities (such as luminescence, magnetism, hyperthermia), or can exert specific action when in contact with the biological environment such as anticancer and antibacterial ones [30, 160-167]. For example, CaP NPs doped with fluorescent and luminescent cations, radionuclides, magnetic or antibacterial ions have been extensively reported [118].

CaP NPs are promising vectors for drug delivery since they can load a high variety of bioactive molecules on their surface or encapsulate biomolecules within the particle, thus protecting the therapeutic agent from degradation in the biological environment [145]. Furthermore,

CaP NPs possess a pH-dependent solubility as they are stable in physiological conditions and in blood plasma (pH 7.4), but are easily degraded in biological acidic environments (pH < 5) as that found in inflammatory regions or in endosomes and lysosomes after cellular intake [157]. Therefore the intrinsic stability of CaP NPs in bloodstream combined to their pH-triggered, dissolution/drug release make of them perfect nano-vectors for numerous drug delivery applications.

The main drawbacks of CaP NPs are their lower drug payload values in comparison for example to hollow organic NPs or liposomes, and their higher tendency to form aggregates in aqueous suspensions. The formation of agglomerations can hinder their penetration into cells and could lead to an immediate macrophage capture and clearance. However, several works have demonstrated that the surface decoration with ionic organic molecules (*e.g.* citrate ions, amino acids or macromolecules) can stabilize CaP NPs in their colloidal form [141, 142, 158, 168, 169]. Another common issue of CaP NPs is that their rapid surface degradation could lead to a burst release of the payload in the organism hindering their use for some applications requiring a more sustained and prolonged release [170-172]. To overcome this problem, CaP NPs can be engineered to encapsulate the drug within the crystalline matrix (the loading values may vary significantly with the chemistry of the drug) preventing the burst release effect [21, 119, 120, 158, 170, 172, 173].

2.2 Applications as nano-carrier

A huge number of therapeutic agents has been loaded on the surface of CaP NPs or even encapsulated inside them. As cancer treatment is one of the greatest challenge to modern medicine and as the use of NPs as carriers can attenuate the side effects connected with the administration of highly cytotoxic drugs, NPs are often conjugated with antitumor drugs. The most employed chemotherapeutic agents loaded onto CaP NPs are doxorubicin [157, 174-177], platinum complexes [139, 178-182] and methotrexate [183, 184]. CaP NPs were also loaded with bisphosphonates (BPs), a class of drugs based on a P–C–P backbone (where C is a carbon and P a phosphonate moiety) having high affinity for bone apatite [119, 185, 186]. BPs have been widely used for skeletal diseases (osteoporosis, osteosarcoma, etc.) since the last 40 years [119, 139, 187-192] and are conventionally dispensed by oral administration or intravenous injection. However, undesirable side effects such as fever, ulcers or osteonecrosis of the jaw are connected to these two administration routes; in addition low BPs bioavailability is commonly observed for oral administration [193]. To avoid these side effects

and to increase BPs bioavailability, the development of new strategies employing alternative administration routes mediated by CaP NPs becomes even more interesting.

Similarly to BPs, the study of the interactions between bone morphogenetic proteins (BMPs) and CaP NPs is of great biological and medical interest as BMPs have been attempted to be applied for the reconstruction of bone defects resulting from trauma, surgical resection of tumors, and congenital anomalies in orthopedic and maxillofacial surgery [122, 194-198]. BMPs are cytokines with a strong effect on bone and cartilage growth and with important roles during embryonic patterning and early skeletal formation [194, 199]. The main role of a delivery system for BMPs is to retain these growth factors at the site of injury for a prolonged time frame, possibly also providing an initial support to which cells attach and form regenerated tissue. Furthermore, the nano-carrier should protect the BMPs from degradation and maintain its bioactivity whilst releasing the protein in a time- and space-controlled way to promote the formation of new bone at the treatment site [194]. Therefore, thanks to their osteoconductivity property, CaP NPs are ideal delivery systems for BMPs. The interaction mechanism that can occur between BMPs and CaP NPs has been studied by computer simulation, proving that the atomic-level morphology of CaP NPs significantly affects the interaction between proteins and NPs, and that the orientation of BMPs influences their adsorption–desorption behavior [200, 201]. The in vitro absorption and release kinetics of BMPs from CaP NPs has been studied showing a sustained release profile of BMPs over 15 days [202]. Another interesting work by Rohanizadeh and Chung has reported three different methods to load BMPs on CaP: (i) incorporation of BMPs during hydroxyapatite precipitation, (ii) hydroxyapatite immersion in BMPs solution, and (iii) BMPs incorporation during dicalcium phosphate dihydrate conversion to hydroxyapatite. The highest BMPs uptake was achieved using the immersion method, while the BMPs loading during hydroxyapatite precipitation resulted in a more sustained and prolonged release compared to the other loading methods [173]. Collectively, these results suggest that CaP NPs has the potential to function as a carrier for BMPs delivery systems for the management of bone diseases.

CaP NPs were also extensively studied to encapsulate and deliver DNA/RNA, and to the best of our knowledge, the first use of CaP NPs for applications non-related to bone was as non-viral vectors in gene therapy [124, 145, 203-212].

CaP NPs do not possess intrinsic target specificity, apart that mediated by EPR in cancer therapy. However, the high density of positive (Ca^{2+}) and negative (PO_4^{3-} or OH^-) charges on their surface allows an efficient and stable electrostatic-driven adsorption of targeting

molecules [121, 177, 206, 213, 214]. Apart from physisorption, targeting moieties can be bound to the CaP NPs surface also by the formation of covalent bonds [113, 215, 216].

Several molecules can be integrated in the targeting agent to achieve a strong binding with the CaP surface (hereafter called ligands). Since BPs can establish a firm link with surface calcium ions of CaP, they can be employed as ligands [191, 217]. BPs are also ideal bone-targeting agents due to their affinity for apatite nanocrystals of calcified tissue (the so-called bone seeking agents) [218]. In fact, researchers are nowadays strongly focusing on the use of BPs for the targeted delivery to bones [185, 219, 220].

Small organic molecules with ionisable functional groups such as silanols, carboxylic acids, amines, thiols or nucleotide strands display high affinity for the CaP surface [221-223], and can therefore be employed as ligands. When the ligands are attached to CaP NPs, their exposed functional groups can be further conjugated with the active targeting molecules. In this regard ligands that can be connected with targeting agents through click reactions are particularly interesting due to their simplicity and efficiency [224, 225]. Amines, thiols, carboxylates, and azides are classes of molecules suitable for these reactions.

Another well-reported strategy to bind targeting agents to CaP NPs surface exploits the avidin-biotin interaction, where the macromolecule (avidin or streptavidin) and the organic molecule (biotin) form a very stable supramolecular system. Several works have reported the successful conjugation of biotinylated CaP NPs with avidin-functionalized targeting molecules [113, 226-230].

The most interesting applications of targeted CaP NPs as well as the targeting moieties and decoration methods employed so far are thoughtfully discussed in the next paragraphs.

3. Targeted calcium phosphate nanoparticles

The number of published works on targeted CaP NPs is relatively low as the application of CaP NPs in nanomedicine is in its infancy compared to other NPs such as liposomes, silica or metallic NPs. Table 1 summarizes the most important features of the targeted CaP NPs selected in this review on the base of a large literature survey, while the main compositions are schematized in Figure 3.

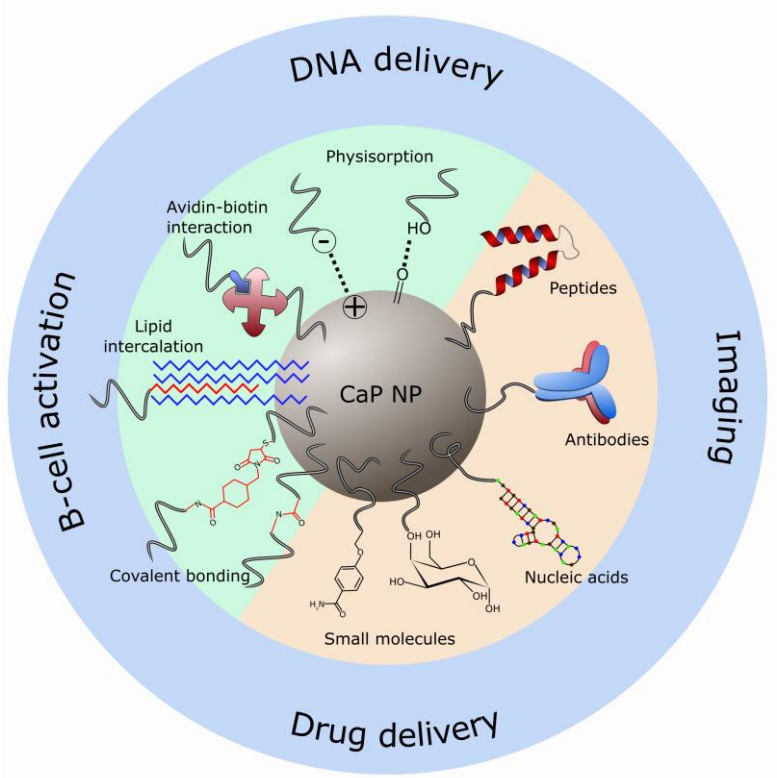

Figure 3. Schematic representation of functions (outer ring, blue), moieties (right sector, orange) and surface decoration methods (left sector, green) of targeted CaP NPs.

3.1 Applications

On the base of our survey, targeted CaP NPs were mainly used for nucleic acids delivery in the forms of siRNA [231-234], plasmids or exogenous genes [207, 235, 236] and suicide genes [237, 238]. Targeting moieties were added because they can direct the gene transfection toward the desired cells improving their therapeutic efficacy, or drive the delivery of apoptotic genes or siRNAs to malignant cells. The works on this topic have proved that the targeted transfection of therapeutic nucleotides or suicide genes by CaP NPs was efficient and selective. In particular, it was reported that cancer cells like the human colon cancer cells LoVo [238] and human gastric cancer cells SGC7901 [237] were selectively eradicated with suicide genes. SiRNAs were delivered to human lung cancer cells NCI-H-460 [231, 233], murine melanoma cells B16F10 [232] and human colon carcinoma cells HT29-luc [234] resulting in the silencing of specific genes. Important cells for the immune responses like the dendritic ones in mice, were as well successfully targeted with CaP NPs [235]. Moreover, in the works of Hu *et al.* [236] and Roy *et al.* [207] it is reported the successful *in vivo* targeted transfection of exogenous genes toward murine hepatocyte cells. It must be mentioned that

the main outcomes of these reports are only at proof of concept level and further evidences are necessary.

Targeted fluorescent CaP NPs were used as imaging agents and for photodynamic therapy. Fluorescent CaP NPs were prepared by doping with lanthanide ions such as Ce^{3+}, Eu^{3+}, Gd^{3+}, Tb^{3+} [136, 239, 240], or by the encapsulation of organic fluorescent dyes like fluorescein isothiocyanate (FITC), rhodamin B isothiocyanate (RITC) and indocyanine green (ICG) [235, 241-244]. Fluorescent CaP NPs were reported to allow the selective imaging of cancerous cells like human breast cancer cells MCF-7 [136], human breast cancer cells MDA-231 [136], human cervical epithelioid carcinoma cells HeLa [136, 235, 244], osteoblast cells MG-63 [235], human breast carcinoma cells T-47-D [239] and human nasopharyngeal carcinoma cells KB [240]. In addition some of the targeted fluorescent CaP NPs have permitted the destruction of malign cells such as murine leukemia stem cells 32D-p210-GFP [241] and human tongue–squamous epithelium carcinoma cells CAL-27 [243] through photodynamic therapy, where the cellular death is induced by a sudden release of heat after photon absorption.

The targeted delivery of conventional antineoplastic drugs like doxorubicin, platinum complexes and methotrexate by CaP NPs was investigated by different research groups [136, 177, 244-248]. It was proved that doxorubicin-loaded CaP NPs can selectively kill tumor cells like MDA-231 cells [136], MCF-7 cells [136], HeLa cells [136], human gastric carcinoma cells GTL-16 [177] and human lung cancer cells A549 [246]. CaP NPs carrying platinum complexes have shown high toxicity toward targeted cancer cells as HeLa [244] and A549 cells [248], while methotrexate-loaded CaP NPs demonstrated the capability to inhibit the growth of MCF-7 [245] and HeLa cells [245] with a similar efficacy to the free drug.

A very promising and original approach was reported by Temchura *et al.* [249] and Zilker *et al.* [250], that targeted CaP NPs stimulated the differentiation of selected B-cells into antibody secreting plasma cells; the targeting moiety was the hen egg lysozyme protein (HEL) that recognizes and activates the anti-HEL B-cells. According to the results of these works, targeted CaP NPs could be potentially used to stimulate immune response in the patients without the involvement of microbial agents.

3.2 Targeting agents

All of the most employed targeting moieties in nanomedicine as mAbs, peptides, carbohydrates, transferrin, aptamers, small organic molecules, and antigens have been conjugated to CaP NPs, with mAbs having the lion's share [177, 235, 241, 242]. For example, in the work of Iafisco *et al.* [177] CaP NPs were functionalized with mAbs specific for the

Met/Hepatocyte growth factor receptor (Met/HGFR), which is over-expressed on different types of carcinomas; therefore, the functionalized NPs were internalized only in the human gastric carcinoma cell line GTL-16 that overexpress the Met/HGFR (Figure 4). Other papers report that murine dendritic cells [235], murine leukemia stem cells 32D-p210-GFP [241] and human metastatic breast cancer cells MDA-MB-231 [242] were successfully targeted by CaP NPs functionalized with the proper mAbs.

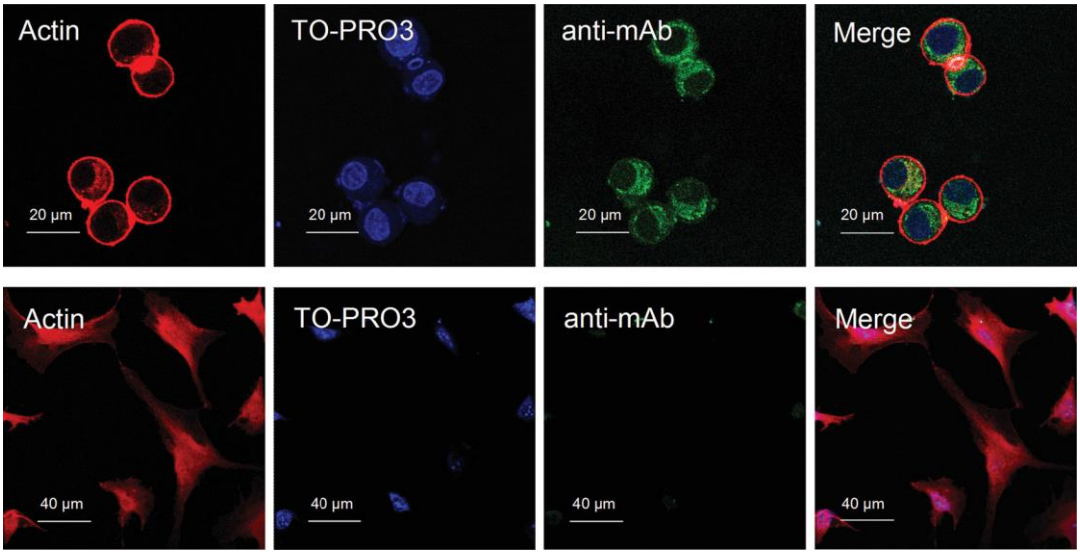

Figure 4. Confocal microscopy images of mAbs-loaded CaP NPs incubated with normal and cancer cells and their internalization. Top row: incubation with overexpressing Met/HGFR human GTL-16 cells. Bottom row: incubation with normal murine NIH-3T3 fibroblasts. CaP NPs are stained with FITC-labelled anti-mouse IgG (green), cytoskeletal actin with TRITC-labelled phalloidin (red) and nuclei with TO-PRO3 (blue). mAbs-loaded CaP NPs (green signal) can be detected only in case of GTL-16 cells, but not in case of NIH-3T3 cells. From Iafisco *et al.* 2013 with permission from Wiley-VCH.

Small organic targeting moieties have been also attached to CaP NPs; among them it is worth to mention galactose because it is a good targeting agent for a variety of hepatic diseases [207, 236]. In this respect, CaP NPs functionalized with galactose were efficiently delivered to hepatocytes [207]; moreover, galactose was found to promote CaP NPs uptake by these cells [236].

Folic acid is a targeting molecule that has been extensively studied in conjunction with CaP NPs [239, 240, 244, 251] since the folate receptor is overexpressed in several cancerous cells [72, 92, 93]. Ashokan *et al.* [240] reported that folic acid-functionalized CaP NPs (red emitting NPs in Figure 5) accumulate selectively on the surface of human nasopharyngeal carcinoma

KB cells that overexpress the folate receptor, while in the case of mouse fibroblast L929 and cancer cell line A549 having normal or very low expression level of folate receptor, even after 4 h of incubation, the red emitting folic acid-functionalized CaP NPs were randomly distributed all around the cell without any specific interaction with the membrane (Figure 5). Similar results were found by other authors that employed folic acid as targeting agent for CaP NPs and recorded targeting activity toward human breast carcinoma cells T-47-D [239] and human cervical epithelioid carcinoma cells HeLa [244]. It was also found that folic acid promotes NPs internalization into these cancerous cells [239, 244].

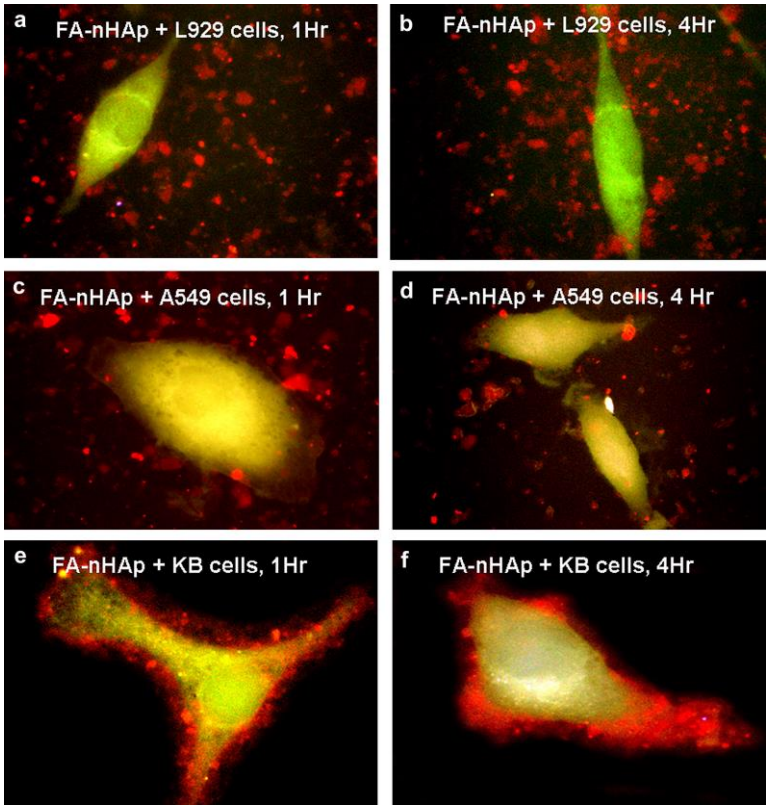

Figure 5. Fluorescence microscopy images showing interaction of folic acid-functionalized CaP NPs (possessing red fluorescence) with normal fibroblast cell line L929 after (a) 1 h, and (b) 4 h of incubation, with lung cancer cell line A549 after (c) 1 h, and (d) 4 h of incubation and with folate receptor positive human nasopharyngeal carcinoma cell line KB after (e) 1 h, and (f) 4 h of incubation. From Ashokan *et al.* 2010 with permission from Elsevier.

As mentioned above, BPs can be employed as bone seeking agents because of their high affinity for bone [245, 247]. In this respect, in a recent work of Chu *et al.* [245] alendronate has been used both as targeting molecule for bone metastases and as CaP NPs binding ligand. Specifically, alendronate was located at the head and tail of a PEG polymer chain (*i.e.*

alendronate-PEG-alendronate), then one alendronate moiety was employed to interact with CaP surface, while the other one acted as targeting moiety for the bone tissue. In the work of Wu *et al.* [247] was proved that medronate can target murine osteosarcoma cells K7M2. Moreover, medronate was found to enhance the action of a chemotherapeutic drug (JQ1, a thienotriazolodiazepine) loaded onto CaP NPs.

CaP NPs were also functionalized with anisamide [231-233], a benzamide derivative that interacts with the Sigma-1 receptor of neoplastic cells whose effectiveness as targeting molecule is a matter of debate [252]. However, some of the works in the literature affirm that anisamide efficiently acts as targeting agent toward human lung cancer cells NCI-H-460 [231, 233], murine melanoma cells B16F10 [232] and human colon carcinoma cells HT29-luc [234] allowing CaP NPs to deliver siRNAs.

Hyaluronic acid is one of the macromolecular targeting moieties used in combination with CaP NPs [234, 246, 248], with the aim to target the transmembrane glycoprotein CD44 [100, 101]. *In vitro* experiments have highlighted that hyaluronic acid is an efficient targeting agent toward cells rich of CD44 receptors such as human lung cancer cells A549 [246, 248]. In addition, these works have proved that hyaluronic acid stimulates also the CD44-mediated endocytosis of CaP NPs [246, 248].

Another common targeting macromolecule that has been also functionalized to CaP NPs is transferrin. However, Barth *et al.* [242] reported that the transferrin receptor-targeted CaP NPs were uneffective in an *in vivo* model with human metastatic breast cancer cells MDA-MB-231. To explain these results, authors supposed that the target receptors in the cells were saturated by endogenous transferrin, making the binding site unavailable for the transferrin conjugated NPs.

Peptides are an important class of molecules which can confer targeting ability to CaP NPs [242, 243]. CaP NPs were conjugated with a RGD tripeptide (variant RGDfK) which is highly selective for the integrins of the endothelium of angiogenic blood vessels [89]. The authors have proved that the RGDfK peptide promotes *in vivo* the local enrichment of CaP NPs toward human tongue–squamous epithelium carcinoma cells CAL-27 [243]. Gastrin peptide, a macromolecule involved in the digestion mechanism, together with its synthetic equivalent pentagastrin peptide, was employed successfully by Barth *et al* to CaP NPs to target *in vivo* human pancreatic cancer cells BxPC-3 [242].

Regarding the use of nucleic acids and their derivatives as targeting moieties for CaP NPs, two works have reported the use of a tailored nucleotide capable of being recognized by the carcinoembryonic antigen (CEA) gene and to promote gene delivery. The delivery was

achieved with high effectiveness toward human colon cancer cells LoVo [238] and human gastric cancer cells SGC7901 [237]. In the interesting work by Zhou *et al.* [136] an oligonucleotide aptamer was chosen as targeting molecule showing a good cell targeting and CaP NPs internalization in the human cervical epithelioid carcinoma cells HeLa.

3.3 Functionalization strategies

Several surface decoration methods have been used to conjugate targeting agents to the CaP NPs surface. Even if physisorption is the simplest strategy, several concerns are raised on the arrangement of the targeting moieties onto the NP surface. However, all the works cited in this review employing physisorption strategies [64, 136, 237, 239] have reported a successful and efficient targeting effect of CaP NPs.

An interesting physical decoration method that was used with lipid-coated CaP NPs consists in the intercalation of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-PEGylated (DSPE-PEG) into a surface lipid bilayer [231-233, 236]. This simple approach allows the production of well-oriented targeting molecules, but it is limited only to lipid-coated CaP NPs.

The targeting of CaP NPs was also achieved by exploiting the biotin-avidin non-covalent interaction [242]. However, this approach is hampered by the additional conjugation steps required to achieve biotin/avidin conjugated targeting molecules.

The most used methods to chemically bond targeting molecules to the surface of CaP NPs involve the formation of an amide or of an amine-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate-thiol bond. Both these methods are click reactions that require a "promoter" molecule. In the first case, the amide bond is formed between an amine and a carboxylic acid, and it is promoted by the use of carbodiimides [207, 234, 240-242, 244, 246]. In the second case, the conjugation between amines and thiols is promoted by a crosslinking agent, the succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) [235, 243, 249, 250]. Both methods are highly selective and allow a good reaction yield, with the disadvantages of being 2-step reactions and of using not environmentally friendly reagents [113].

Table 1. Main features of targeted CaP NPs selected in this review, reported in chronological order

CaP NPs description	Application	Targeting agent	Functionalization strategy	Reference
Polymer-	Plasmid DNA	Galactose	Formation of amide	[207]

coated CaP NPs	delivery	(asialoglycoprotein of liver cells)	bond with the surfactant polymer	
Polymer-coated CaP NPs	Suicide gene delivery	Enhanced CEA nucleotide promoter (carcinoembryonic antigen of gastric cancer)	Physisorption	[237]
Polymer-coated CaP NPs	Suicide gene delivery combined with prodrug delivery	Enhanced CEA nucleotide promoter (carcinoembryonic antigen of gastric cancer)	Physisorption	[238]
Polymer-coated CaP NPs	Cancer imaging	Folic acid (cancer folate receptor)	Formation of amide bond with the surfactant	[240]
Lipid-coated CaP NPs	siRNA delivery	Anisamide (cancer Sigma-1 receptor)	Insertion of targeted DSPE-PEG in the lipid layer	[231]
CaP/silicate NPs	Cancer imaging	Transferrin (cancer transferrin receptor), anti-CD71 mAbs (cancer transferrin receptor), gastrin peptide (cancer gastrin receptor), pentagastrin peptide (cancer gastrin receptor)	Biotin-avidin conjugation, formation of amide bond with the surfactant	[242]
CaP/silicate NPs	Leukemia imaging and photodynamic therapy	anti-CD117 antibody (leukemia receptor), anti-CD96 mAbs (leukemia receptor)	Formation of amide bond with the surfactant	[241]
Polymer- and silica-coated CaP NPs	Imaging and DNA delivery	Several monoclonal antibodies, among them anti-CD11c mAbs (dendritic cells receptor)	Formation of amine-SMCC-thiol bond with the silica shell	[235]
Lipid-coated CaP NPs	siRNA delivery	Anisamide (cancer Sigma-1 receptor)	Insertion of targeted DSPE-PEG in the lipid layer	[232]
Cobalt ferrite / CaP / polymer composite NPs	Imaging and drug delivery	Folic acid (cancer folate receptor)	Formation of amide bond with the surfactant	[244]
Lipid-coated CaP NPs	siRNA delivery	Anisamide (cancer Sigma-1 receptor)	Insertion of targeted DSPE-PEG in the lipid layer	[233]
CaP NPs	Drug delivery	DO-24 mAbs (Met/HGF receptor of cancer cells)	Physisorption	[177]
Lipid-coated	Plasmid DNA and	Galactose (asialoglycoprotein of	Insertion of targeted	[236]

CaP NPs	peptide delivery	liver cells)	DSPE-PEG in the lipid layer	
Polymer- and silica-coated CaP NPs	B-cells activation	Hen egg lysozyme antigen (HEL-specific B-cell receptor)	Formation of amine-SMCC-thiol bond with the silica shell	[249]
Phospholipid-coated CaP NPs	Cancer imaging	Folic acid (cancer folate receptor)	Physisorption	[239]
CaP coating of lanthanide fluorides NPs	Twofold imaging and drug delivery	As1411 aptamer (nucleolin cell surface protein of cancer cells)	Physisorption	[136]
CaP NPs	siRNA delivery	Hyaluronic acid (cancer CD44 receptor)	Formation of amide bond with the surfactant	[234]
Polymer- and silica-coated CaP NPs	Imaging and photodynamic therapy	RGDfK peptide (integrins of cancer endothelial cells)	Formation of amine-SMCC-thiol bond with the silica shell	[243]
Polymer-coated CaP NPs	Drug delivery	Hyaluronic acid (cancer CD44 receptor)	Formation of amide bond with the surfactant	[246]
Polymer-coated CaP NPs	Drug delivery	Alendronate (bone hydroxyapatite)	Bisphosphonate displacement of phosphate ions from the NP surface	[245]
Polymer-coated CaP NPs	Drug delivery	Hyaluronic acid (cancer CD44 receptor)	Self-assembly with surfactant	[248]
Polymer- and silica-coated CaP NPs	B-cells activation	Hen egg lysozyme antigen (HEL-specific B-cell receptor)	Formation of amine-SMCC-thiol bond with the silica shell	[250]
CaP NPs	Drug delivery	Medronate (bone hydroxyapatite)	Bisphosphonate displacement of phosphate ions from the NP surface	[247]

4. Future Approaches and Perspectives

Targeted nanomedicine is a fascinating multidisciplinary topic that combines chemistry, physics, material sciences, nanotechnology, drug delivery and pharmacology. However, the development of efficient targeted NPs is an extremely complex and expensive process. It is clear that a product of such laborious and complicate synthetic procedure may be very expensive and difficult to produce at the industrial scale [253]. Indeed the addition of new functionalities means supplementary synthetic steps and costs, more convoluted behaviors and effects when administered *in vivo*, and also onerous regulatory hurdles to be overcome [253]. Therefore the final product must bring significant and tangible benefits from the targeting approach in order to be translated from the bench to the market. However, we expect that following the costs/benefits principle a good number of successful applications for targeted nanomedical systems will be generated in the next years.

In this regard, CaP NPs for targeted nanomedicine are very versatile and open up a multitude of possible applications. Even if the development of CaP NPs in nanomedicine has begun only a decade ago, several therapeutic formulations have been already tested, spanning from gene to drug delivery, vaccines and advanced theranostic agents.

Apart from molecular targeting strategies, CaP NPs are also interesting materials for alternative targeting approaches such as magnetic driving. In this respect, several recent studies reported on the obtainment of magnetic CaP NPs by doping with iron ions [160, 165, 176, 254-258] or by the encapsulation of iron oxide phases [259-264]. Magnetic CaPs can be remotely controlled in the organism by employing external magnetic fields [28, 254, 265, 266]. The active targeting of CaP NPs by magnetic guidance offers the possibility to accumulate the nano-carriers in the diseased region, at the same time circumventing laborious and time-consuming conjugation reactions with targeting molecules. On the other hand the magnetic driving is a very complicated task due to the need of complex devices generating magnetic fields with appropriate precision and intensity for the *in vivo* driving of magnetic NPs [267].

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