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Review article

Review of potential health risks associated with nanoscopic calcium phosphate

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ABSTRACT

Calcium phosphate is applied in many products in biomedicine, but also in toothpastes and cosmetics. In some cases, it is present in nanoparticulate form, either on purpose or after degradation or mechanical abrasion. Possible concerns are related to the biological effect of such nanoparticles. A thorough literature review shows that calcium phosphate nanoparticles as such have no inherent toxicity but can lead to an increase of the intracellular calcium concentration after endosomal uptake and lysosomal degradation. However, cells are able to clear the calcium from the cytoplasm within a few hours, unless very high doses of calcium phosphate are applied. The observed cytotoxicity in some cell culture studies, mainly for unfunctionalized particles, is probably due to particle agglomeration and subsequent sedimentation onto the cell layer, leading to a very high local particle concentration, a high particle uptake, and subsequent cell death. There is no risk from an oral uptake of calcium phosphate nanoparticles due to their rapid dissolution in the stomach. The risk from dermal or mucosal uptake is very low. Calcium phosphate nanoparticles can enter the bloodstream by inhalation, but no adverse effects have been observed, except for a prolonged exposition to high particle doses. Calcium phosphate nanoparticles inside the body (e.g. after implantation or due to abrasion) do not pose a risk as they are typically resorbed and dissolved by osteoclasts and macrophages. There is no indication for a significant influence of the calcium phosphate phase or the particle shape (e.g. spherical or rod-like) on the biological response. In summary, the risk associated with an exposition to nanoparticulate calcium phosphate in doses that are usually applied in biomedicine, health care products, and cosmetics is very low and most likely not present at all.

Statement of Significance

Calcium phosphate is a well-established biomaterial. However, there are occasions when it occurs in a nanoparticulate form (e.g. as nanoparticle or as nanoparticulate bone substitution material) or after abrasion from a calcium phosphate-coated metal implant. In the light of the current discussion on the safety of nanoparticles, there have been concerns about potential adverse effects of nano-calcium phosphate, e.g. in a statement of a EU study group from 2016 about possible dangers associated with non-spherical nano-hydroxyapatite in cosmetics. In the US, there was a discussion in 2016 about the dangers of nano-calcium phosphate in babyfood.

In this review, the potential exposition routes for nano-calcium phosphate are reviewed, with special emphasis on its application as biomaterial.

Contents

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1. Introduction

Calcium phosphate is a well-established biomaterial, especially in orthopaedic and trauma surgery. It is typically applied as solid ceramic, as self-setting cement, or as implant coating. Novel developments comprise calcium phosphate nanoparticles that are used for drug and gene delivery and imaging. Nanoparticulate calcium phosphate may also result from partial degradation or mechanical abrasion from macroscopic implants.

The application of nanomaterials is controlled by regulating bodies. Although calcium phosphate is usually not in the focus of potentially hazardous nanomaterials, it is subject to the same regulatory framework as nanoscopic silica, quartz, carbon, titania, zinc oxide, silver, or gold, to give a few examples. Major concerns address the immediate and the long-term action of nanomaterials in the body, potential exposure routes (inhalation, oral uptake, skin contact, mucosal contact, implantation), and the long-term fate in the body (dissolution, excretion, accumulation). Despite its many beneficial properties in hard tissue regeneration, there is also the clear perception that pathological calcifications also consist of calcium phosphate, with atherosclerosis being the most prominent (and harmful) example.

The scientific committee on consumer safety (SCCS) of the European Commission has published an opinion paper on nano-hydroxyapatite as of 16.10.2015 (revised as of 16.03.2016) with special respect to its application in cosmetics [1]. It had received 35 notifications of cosmetic products containing nano-hydroxyapatite. The committee reviewed and assessed a large body of literature (65 publications and reports) but concluded in many cases that the studies were not performed according to OECD or EU guidelines and that important information on the nature of the particles (as outlined below) was missing. Therefore, no definite recommendation was given. The opinion report concludes that the safety of nano-hydroxyapatite in cosmetics could not be decided on the basis of the analysed literature. It furthermore states that needle-shaped hydroxyapatite "is of concern in relation to potential toxicity [and] should not be used in cosmetic products".

It is notable that other calcium phosphate phases (like tricalcium phosphate, TCP) were not taken into consideration and not mentioned in the opinion paper. This is due to the fact that the call was restricted to nano-hydroxyapatite without mentioning any other calcium phosphate. Consequently, there are no published SCCS opinion paper on other calcium phosphates.

The Food and Drug Administration (FDA) in the US has approved nano-hydroxyapatite and nano-calcium phosphate for use as bone substitution material [2]. Calcium phosphates have been generally recognized as safe (GRAS) in food by the FDA in 1975, but no special attention was paid at this time to an application in the form of nanoparticles [3]. In 2016, the discovery of needle-shaped hydroxyapatite in baby formula (about 30 nm·150 nm, looking scary to laymen in the transmission electron microscope at high magnification) has stirred a public discussion on possible side effects of such nanoparticles in the US. The content of nano-calcium phosphate was 0.4 wt% or less for the investigated baby formulas [4,5]. Given the fact that a baby formula is swallowed and that calcium phosphate will quickly dissolve in the stomach, there is clearly no risk associated with these particles. On the contrary, they enhance the beneficial dietary uptake of calcium and phosphate (in a typical baby formula, there are about 0.3–0.7 wt% calcium [6]).

It is likely that the question of the application of calcium phosphate nanoparticles in biomedicine will arise sooner or later. This review is addressing possible concerns, based on the current knowledge after decades of the application of calcium phosphate as biomaterial.

2. Chemistry of calcium phosphate

Calcium phosphate is a common mineral on earth. Calcium phosphate minerals are used to a very large extent in the fertilizer industry where the insoluble calcium phosphate mineral is treated with sulfuric acid or phosphoric acid to convert it into better soluble calcium hydrogen phosphates [7]. In general, calcium orthophosphates have a very low solubility that makes them inaccessible to plants that need phosphate for their metabolism [8].

The most common calcium phosphate mineral is hydroxyapatite: $Ca_5(PO_4)_3OH$ (often abbreviated as HAP or HA), which is actually a calcium phosphate hydroxide. Besides, a number of other calcium phosphates is known with varying degrees of protonation of the phosphate group and different composition. Traditionally, they have been assigned with abbreviations for a quick identification. The most common ones are tricalcium phosphate, $Ca_3(PO_4)_2$ (which exists in two polymorphs: α-TCP and β-TCP), octacalcium phosphate, $Ca_8(HPO_4)_2(PO_4)_4{\cdot}5\ H_2O$ (OCP), dicalcium phosphate dihydrate, $CaHPO_4{\cdot}2\ H_2O$ (DCPD), and monocalcium phosphate monohydrate, $Ca(H_2PO_4)_2{\cdot}H_2O$ (MCPM). Besides, there are also various forms of X-ray amorphous calcium phosphate (ACP) with variable composition, i.e. $Ca_x(PO_4)_{y\cdot}z\ H_2O$ [9,10]. Hydroxyapatite is the most prominent and at neutral pH the least soluble calcium phosphate [8,11,12].

All calcium phosphates are sparingly soluble or even almost insoluble in neutral water but can be dissolved in acids, typically at a pH of 4 and below [8]. In dissolved form, they are present as ions. If the pH is increased again, they will precipitate again, but not necessarily in the same phase as before [13]. A precipitation of calcium phosphate from water typically leads to DCPD, OCP, HAP or ACP, depending on the precipitation conditions (supersaturation, solution pH, precipitation rate) [14]. Of course, the ions that result from a dissolved nanoparticle do not remember their origin.

The chemistry of calcium phosphate is complex for two reasons. The first is the possibility to substitute calcium, phosphate, and

hydroxide ions on their crystallographic sites by other cations and anions. The second is the protonation equilibrium of orthophosphate that may occur as hydrogen phosphate or dihydrogen phosphate, depending on the pH during the precipitation. Especially hydroxyapatite and amorphous calcium phosphate are prominent examples for these effects [11]. For instance, they tend to incorporate other inorganic ions which are present in the mother liquor during precipitation from water, leading, e.g., to calcium-deficient hydroxyapatite, $Ca_{5-x}(HPO_4)_{2x}(PO_4)_{3-2x}OH$ (CDHA), to magnesium-substituted apatite $Ca_{5-x}Mg_x(PO_4)_3OH$, or to fluoroapatite, $Ca_5(PO_4)_3F$ (FAP) [8,12].

In pure form, most calcium phosphates give a basic reaction due to the presence of orthophosphate groups [8]. In a well-buffered and perfused biological environment, this should not cause adverse effects.

3. Calcium phosphate in the biosphere

Many living organisms use calcium phosphate as solid mineral for various purposes. Besides calcium carbonate, silica, and iron oxide, it is the most prominent biomineral on earth [15]. It occurs in bone and teeth of many higher organisms, including (but not limited to) mammals, fish and reptiles, with a total amount of about 2–3 kg per adult human. It is present as carbonate-substituted hydroxyapatite (so-called carbonated apatite, bioapatite or dahlite [16]) where carbonate typically occupies a part of the crystallographic sites of phosphate (B-type carbonated apatite) [17]. The overwhelming part in the body is present in bone where calcium phosphate forms the basis of skeletal hard tissue, together with the protein collagen in approximately equal amounts [18]. The presence of calcium phosphate in bone enhances its mechanical stability, and it also serves as calcium storage site for the body. Notably, bone mineral is nanoscopic, i.e. the calcium phosphate crystals are platelet-shaped with a width of several tens of nm and a thickness of a few nm [9,12,19]. Bone metabolism involves a constant remodelling of bone tissue, i.e. a dissolution and reformation to adapt to changing environmental conditions, e.g. to a changing mechanical load on the bone. This is accomplished by osteoclasts that dissolve calcium phosphate at low pH (around 4.5) and collagen with the help of proteases [20], in a close interplay with osteoblasts that synthesize new bone [21]. In this respect, it is especially important that biological calcium phosphate is both acid-soluble and nanocrystalline, otherwise osteoclasts would not be able to dissolve bone during remodelling [22].

In teeth, calcium phosphate is used for cutting, therefore teeth constitute the hardest tissue in the body [8]. Structurally, the interior of teeth consists of dentin, a bone-like phase containing both collagen and nano-calcium phosphate. The outer part of a tooth crown, called enamel, consists almost exclusively of calcium phosphate (Fig. 1). In enamel, calcium phosphate is present as long needles with a length of several μm and a thickness around 100 nm that form a delicately intertwined hierarchical structure, held together by a "glue" of protein [23]. Its mechanical resilience towards external shock or compression is based on this structure where the calcium phosphate crystals are deflecting cracks [24,25]. An acidic attack of foods and drinks in the mouth leads to a temporary dissolution of the outer calcium phosphate layer which is, however, recovered within a few hours by a reprecipitation of (nanoscopic) calcium phosphate from the supersaturated saliva [26]. Otherwise, our teeth would not last for decades as they usually do.

Sharks (both recent and extinct) [27–29] constitute an important exception to the use of hydroxyapatite as tooth mineral. They use the substituted variant fluoroapatite (FAP), despite the fact that a shark tooth of fluoroapatite is not harder than a human tooth of hydroxyapatite [30,31]. The reason for the peculiar choice of this unusual biomineral by sharks is unknown.

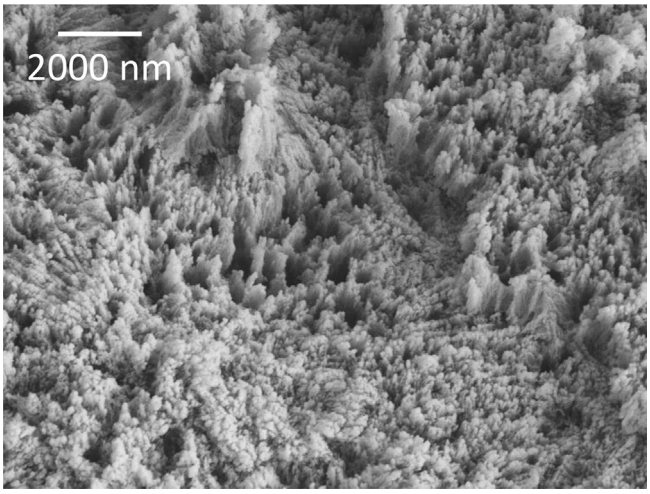

Fig. 1. The acid-etched surface of a human tooth, showing the calcium phosphate nanorods in enamel.

Finally, calcium phosphate is also present in human cartilage, typically on the way to bone formation (endochondral ossification) [8].

4. Pathological formation of calcium phosphate

Besides the beneficial effects of calcium phosphate in bone and teeth, it must be emphasized that the pathological formation of calcium phosphate in blood vessels is the major cause of death in developed countries (coronary artery disease, CAD). Atherosclerosis involves the deposition of cholesterol and lipids (in early stages) and bone mineral-like calcium phosphate (in later stages) inside arteries [32]. The exact mechanisms are still under debate, but the facts that blood serum is supersaturated with respect to calcium phosphate precipitation [33] and that nanoscopic calcium phosphate-protein aggregates have been observed in blood [34,35] indicate a physicochemical contribution to the calcium phosphate deposition. Eventually, plaque formation leads to clogging of arteries, possible rupture of arteries, heart infarcts, and strokes. The pathological deposition of calcium phosphate is not limited to atherosclerosis but a rather common phenomenon in the human body. For instance, it also occurs in cartilage (causing osteoarthritis) [36], soft tissue (ectopic calcification) [37], and on heart valve transplants [38], usually with poorly understood mechanisms.

A number of authors have demonstrated that nanoscopic calcium phosphate-protein aggregates form rapidly after contact of calcium and phosphate ions in a biological environment like blood. Although this article is directed on synthetic calcium phosphates, prepared as biomaterial or resulting from a biomaterial, this interesting aspect shall be briefly discussed. Different denotations and abbreviations have been coined, e.g. calciprotein particles (CPP) [35,39–41], biomimetic mineralo-protein nanoparticles [42], bions [43], calcium phosphate bions (CPB) [44], and calcifying nanoparticles (CNP) [45]. Such particles will be denoted as calciprotein particles in the following for the sake of simplicity, although there are slight differences in their preparation and properties. Smith et al. showed that fetuin-A forms calciprotein particles in blood and demonstrated that they can be also synthetically prepared. They found rod-like bundles of crystals with a typical size of 100 to 200 nm. These particles increased the expression of cytokines in

RAW 264.7 macrophages, interestingly more strongly than unfunctionalized hydroxyapatite particles (<200 nm) [39]. Along the same line, Peng et al. showed that such calciprotein particles can be prepared by introducing calcium and phosphate ions into blood serum. The particle size could be adjusted in the range of about 100 nm to several μm, but with some change during storage in dispersion. Their uptake by RAW 264.7 macrophages was observed, and a cytokine upregulation was found. It is remarkable that small particles (<300 nm) did not induce pro-inflammatory signals [42]. Shook et al. demonstrated that calciprotein particles also form in human amniotic fluid in fetal membranes [41]. Wu et al. showed that such protein-mineral nanoparticles are not only formed from supersaturated calcium phosphate solution but also in the presence of many other metal ions in biological fluids. The particle size (from tens of nanometers up to several microns) depended on the mineral and on the precipitation conditions. In some cases, a conversion from amorphous to crystalline mineral phases occurred in dispersion. A wide range of proteins was found to be present in such mineral-protein particles [43]. Hunter et al. isolated calciprotein nanoparticles from human serum (diameter >200 nm). Porcine aortic smooth muscle cells were incubated with such particles. The particles were well taken up by the cells and promoted vascular calcification processes (accumulation of apoptotic bodies and extracellular matrix mineralization) [45]. Kutikhin et al. showed that calciprotein particles, both isolated from atherosclerotic plaques and artificially synthesised (size about 200 nm) had some toxicity against endothelial cells (EA.hy 926 cells). However, they did not find evidence for direct calcification [44]. In contrast, Aghagolzadeh et al. demonstrated that synthetic calciprotein particles (size several 100 nm) can induce calcification in vascular smooth muscle cells at a dose of 100 mg L^{-1} calcium, corresponding to about 250 mg L^{-1} calcium phosphate (as hydroxyapatite). A remarkable observation was the fact that only "aged" calciprotein particles (7 days) induced calcification, "young" calciprotein particles (1 day) did not [40]. Powell et al. found similar particles in the intestine (see below in the chapter on intestinal exposure routes) [46].

In summary, such calcium phosphate-protein particles are readily formed in biological media if sufficient concentrations of calcium and phosphate ions are present. Thus, they are quite common in the body, especially in blood. They appear to be related to atherosclerosis but are not inherently dangerous for the body. Their biological properties strongly depend on their organic-inorganic nature (including the protein cargo), i.e. an adverse effect cannot be easily transposed to purely inorganic calcium phosphate nanoparticles as they are used in biomedicine or cosmetics.

5. Nanoscopic calcium phosphate

If calcium phosphate is precipitated from water, it typically occurs in nanoparticulate form, usually as spheres, needles or platelets of HAP, OCP, DCPD, or ACP [9,12,14,47]. A rapid precipitation at high supersaturation favours small and poorly crystalline crystals whereas a slow precipitation at low supersaturation leads to larger crystals with higher crystallinity [48,49]. Hydroxyapatite often crystallizes in the form of rod-like particles (Fig. 2). Unless the small crystals are protected by suitable capping agents, they will agglomerate to larger particles in the μm range. However, it is possible to prevent the crystal growth and the agglomeration to obtain well-defined nanoparticulate dispersions with high colloidal stability [19,50]. As stated above, calcium phosphate occurs in human hard tissue in nanocrystalline form as well, constituting the mineral of bone and teeth (dentin and enamel).

Synthetically, nanoscopic calcium phosphate is usually prepared by precipitation from the liquid phase [47,48,51–54] or by flame synthesis [55]. It may also be extracted from bovine bone

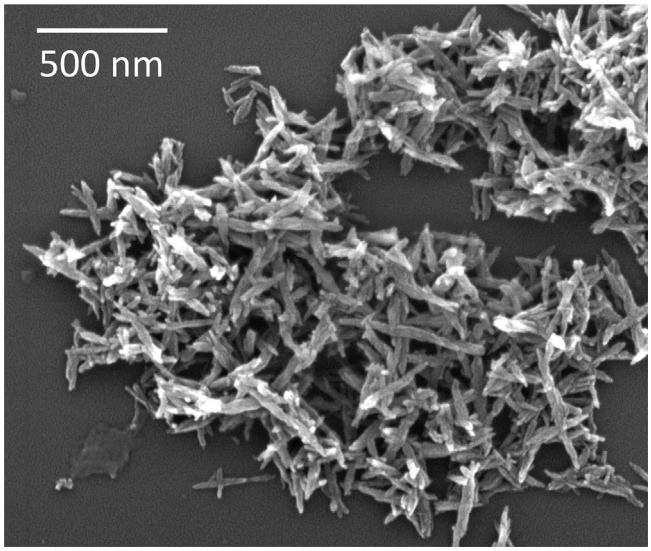

Fig. 2. Synthetically prepared calcium phosphate nanorods (from precipitation).

[56]. Usually, the calcium phosphate nanoparticles have a size between a few ten to a few hundred nm.

6. Biomedical application of nanoscopic calcium phosphate

Calcium phosphate is highly biocompatible in contact with hard tissue because the body is well accustomed to this mineral. Therefore, it has found wide application in biomedicine, especially for the treatment of bone defects [56] and the coating of metallic implants in bone contact (like total hip endoprostheses or tooth implants) [12,57]. Ceramics based on hydroxyapatite (HAP), tricalcium phosphate (TCP), and biphasic calcium phosphate (BCP; a mixture of HAP and TCP) have been clinically applied with great success for more than 50 years [12,58]. Furthermore, bone cements of various composition have been developed in the last three decades. They are leading to an in-situ precipitation of bone-like hydroxyapatite at the application site, usually with agglomerated nanoscopic calcium phosphate particles [59–65].

In general, calcium phosphate is favourable for the attachment of the bone-forming osteoblasts (a property denoted as osteoconductive) [12]. It is also resorbable by osteoclasts unless its crystallinity and particle size are not too high [66]. The fact that bone contains nanoscopic hydroxyapatite and that the remodelling process with its interplay between osteoblasts and osteoclasts is based on the higher solubility of nanoapatite in comparison to compact triggered microcrystalline apatite [22] has stimulated the development of nanocrystalline calcium phosphate formulations to fill bone defects.

Metallic implants in bone contact are often coated with calcium phosphate, either by high-temperature plasma-spraying, by precipitation from aqueous solution, or by plasma magnetron sputtering [67,68] (Fig. 3). Depending on the coating process, the calcium phosphate layer is nano- or microcrystalline [67,69]. The calcium phosphate enhances the ongrowth of bone and ensures a good mechanical interlock between implant and bone that is decisive in load-bearing implantation sites. This has been successfully applied in the clinics for more than four decades, although it has been claimed that the beneficial effect of a calcium phosphate coating may be smaller than it is often assumed [69].

Micro- and nanoscopic calcium phosphate have also been added to various toothpastes in order to enhance the tooth repair process by filling cavities and tubules in dentin [70–73].

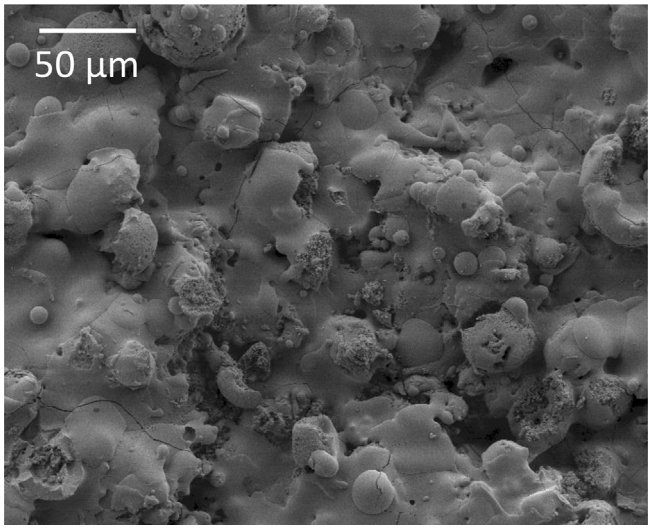

Fig. 3. A metal surface, coated with calcium phosphate by plasma-spraying.

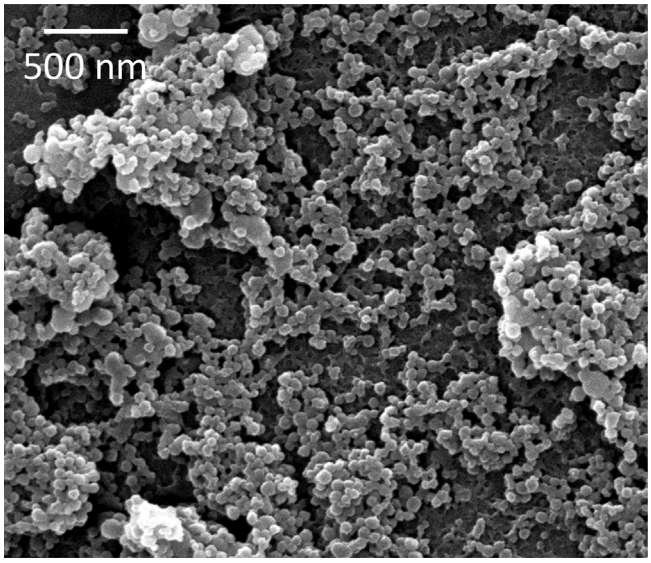

Fig. 4. Functionalized calcium phosphate nanoparticles for gene and drug delivery (from precipitation, with subsequent colloid-chemical stabilization).

If calcium phosphate nanoparticles are colloidally stabilized by suitable agents like polyelectrolytes, they can be used to deliver drugs and biomolecules into cells ("nanomedicine"; Fig. 4). Calcium phosphate nanoparticles are taken up by endocytosis and end up in an endolysosome where the calcium phosphate is dissolved. The cargo is eventually delivered into the cytoplasm (see below). Nucleic acids (for transfection and gene silencing), synthetic molecules, proteins, and antigens have been successfully delivered *in-vitro* and *in-vivo* by this strategy [12,19,50,53,54,74–77].

7. Health risks of nanoparticles

Nanoparticles are ubiquitous in nature from natural sources (like fine dust aerosols). Therefore, they are nothing new to living beings, including humans. However, an increased emission of nanoparticles by traffic and industrial processes and the application of nanoparticles in a multitude of technical products and consumer products has raised concerns among the public and among regulatory bodies about potential health risks. This also concerns the application of dedicated nanoparticles for drug delivery, immunization or cancer treatment in the area of nanomedicine.

Of course, potential health risks must be scrutinized in detail as the exposition of consumers to nanoparticles often occurs without their knowledge and explicit consent. A prominent case is the application of bactericidal silver nanoparticles in more than 1000 consumer products [78–81] which has stimulated an emotional debate [82–87]. However, most people are not aware that many popular consumer products like toothpaste (TiO_2, SiO_2) [70,88,89], sunscreen (ZnO, TiO_2) [88–90] and many foods contain considerable amounts of nanoparticles (like silica: E551 [91] or titania: E171), obviously without negative side effects [87].

The risk analysis of nanoparticles is focussed on the potential uptake routes (mainly inhalative, dermal, oral, mucosal), their action inside the body, and their eventual fate (excretion, deposition). A huge literature exists on this topic, including dedicated journals and conferences. A consensus has been reached, however, that besides the dose of a particular nanoparticle species in terms of "number of particles per body weight" or "number of particles per cell", the physicochemical properties of a nanoparticle must be taken into account. Apart from the chemical composition of a nanoparticle, these properties include, but are not limited to [86,87,92–97]

- the particle size, the particle size distribution, and the agglomeration state
- the particle surface composition (e.g. capping agents from the synthesis and the protein corona that is formed in the presence all biological media)
- the particle charge (by itself and by adsorbed molecules; typically expressed by the zeta potential)
- the particle shape (e.g. spheres, rods, needles, platelets) and
- a possible dissolution of the particle, releasing biologically active species (like in the case of silver or zinc oxide).

This makes the risk assessment of nanoparticles rather complicated, especially in the light of a variety of different exposition routes [87].

8. Reaction of eukaryotic cells to calcium phosphate nanoparticles

In general, most cell types readily take up nanoparticles [97], including calcium phosphate nanoparticles [98].

Only very small nanoparticles (a few nm in diameter) are able to penetrate the cell membrane on their own; larger particles (like almost all calcium phosphate nanoparticles reported in the literature) are taken up by pinocytosis, endocytosis or phagocytosis [97]. The critical size depends on the cell type, the nanoparticle properties and the uptake mechanism, but 150–200 nm appears to be an upper limit for non-phagocytotic uptake [99–103]. The nanoparticles end up in an endosome that transforms to an endolysosome with acidic pH [97,104,105]. In a comprehensive study with 10 different cell lines, Neuhaus et al. showed that they all take up cationic calcium phosphate nanoparticles (spherical, diameter 30–140 nm). However, the cargo (DNA for transfection) was not processed by all cell lines [98]. Sokolova et al. demonstrated that anionic and cationic calcium phosphate nanoparticles (spherical, diameter 120 nm) are taken up by endocytosis into HeLa cells [105]. Calcium phosphate nanoparticles (spherical, 75 nm, cationic) first enter the early endosome and later the lysosome where they are finally dissolved by acid within hours as shown by Kopp et al. [106]. The ions are released and pumped out of the cell within the next 48 h as shown by Neumann et al. for T24 cells [107]. This prevents a

lethal increase of the intracellular calcium concentration. Macrophages also readily take up calcium phosphate nanoparticles as shown, e.g., by Sokolova et al. [108], Motskin et al. [109], Muller et al. [110], Tay et al. [111], and Zhao et al. [112].

A large number of cell culture studies on potentially adverse effects of calcium phosphate nanoparticles has been published. Note that in many cases, the crystallographic (i.e. mineralogical) nature of the calcium phosphate is not stated, usually because it was not measured. In the following, the denotations given by the authors have been used, even if no diffraction analysis was performed. It is also possible that in some cases, the particles are not crystalline but X-ray amorphous. In some cases, the lack of information on the crystallographic nature of the particle is probably due to the fact that the particles were prepared and colloidally stabilized in dispersion. In this case, an analysis by X-ray diffraction may have been constrained by the lack of material. Furthermore, changes in the calcium phosphate phase (like a crystallization of an amorphous phase or a dehydration of a hydrated phase) upon drying cannot be excluded.

Shi et al. have analysed the effect of hydroxyapatite particles (20 and 80 nm diameter and microparticles) on osteoblast-like cells (MG-63). They found that the smallest particles induced the highest cell proliferation and the lowest cytotoxicity on the cells, but that even for the smallest particle size, the number of apoptotic cells was higher than in the control [113]. Xu et al. have analysed the protein expression in a human osteoblast cell line in response to unfunctionalized spherical (10–200 nm) and to needle-shaped (10…15 nm·80…100 nm) calcium phosphate nanoparticles. The particles had different effects on the regulation of protein synthesis, but notably, the needle-shaped particles showed an upregulation of osteocalcin and of alkaline phosphatase that should be beneficial for bone formation. One of the reasons may have been an increased intracellular calcium level after particle uptake [114]. Ha et al. have studied the gene expression of different cell types (bone marrow stromal cells, MC3T3-E1 pre-osteoblasts, and MLO-Y4 osteocytes) that were treated with calcium phosphate nanorods (about 10 nm·100 nm). They did not observe a cytotoxic effect up to and including 100 mg L^{-1} (the highest concentration studied) [115]. Xu et al. have prepared unfunctionalized hydroxyapatite nanoparticles in four different shapes (short rods, long rods, spheres, needles) with typical dimensions of 10–40 nm. The found that the nanoparticles inhibited the growth of primary rat osteoblasts in a range of 20–100 mg L^{-1}. The needle-like particles had a higher cytotoxicity [116].

Balasundaram et al. reported an increased adhesion of osteoblasts to a nanostructured hydroxyapatite surface that was ascribed to the higher specific surface area [117]. This was confirmed by Guo et al. with human osteoblasts on spark plasma-sintered micro- and nanostructured surfaces [118], by Sato et al. with osteoblasts on hydrothermally-deposited calcium phosphate nanorods on titanium [119], and by Pilloni et al. on polylysine-nano-hydroxyapatite coatings with human osteoblasts [120]. MacMillan et al. showed that nanocrystalline hydroxyapatite was resorbed much faster by osteoclasts than sintered microcrystalline hydroxyapatite [121]. This was confirmed by Detsch et al. [22]. Mestres et al. have analysed the inflammatory reaction of macrophages towards different substrates of calcium phosphate with different texture, mainly calcium phosphate cements. The differences in macrophage proliferation between the microstructured surface (faster) and the nanostructured surface (slower) were ascribed to the higher ion and protein adsorption on the nanostructured surface. However, the secretion of reactive oxygen species was higher on the microstructured surface [122].

Turkez et al. have analysed the genotoxicity of unfunctionalized hydroxyapatite nanorods (10–50 nm) on primary human blood cells. At doses larger than 100–200 mg L^{-1}, they observed adverse genotoxic and cytotoxic effects. These effects were clearly dose-related [123]. Remya et al. have studied the effect of unfunctionalized, rod-like hydroxyapatite nanoparticles (about 10 nm·50 nm) on human mesenchymal stem cells. They demonstrated that the particles were taken up by cells, and observed no adverse effects up to a dose of 800 mg L^{-1} [124]. Xu et al. have prepared nanorods of carbonated hydroxyapatite and did not find a cytotoxicity on HeLa cells at 100 mg L^{-1} [125].

Sonmez et al. have studied the toxicity of unfunctionalized hydroxyapatite nanoparticles (size about 50–150 nm) on primary rat hepatocytes. Above a concentration of 300 μg cm^{-2}, they observed an increasing cytotoxicity [126]. Jin et al. have studied the effect of unfunctionalized calcium phosphate nanorods (hydroxyapatite; 20 nm·80 nm) on MC3T3 cells. They observed an uptake by micropinocytosis and a significant cytotoxicity above 40 mg L^{-1}. They showed that the cytotoxicity was caused by oxidative stress and lysosomal rupture [127].

Tay et al. have compared the cellular reaction of epithelial cells (TR146) and macrophages (RAW 264.7) towards spherical nano-hydroxyapatite (50 nm) and nano-TiO_2 (24 nm). At the same doses, they found comparable effects of both kinds of nanoparticles: Cellular uptake, production of ROS, and inflammatory gene expression. Except for very high doses (above about 150 μM), no serious adverse effects were observed [111].

Zhao et al. have prepared hydroxyapatite nanoparticles with different size and shape: Spheres (about 50 nm), platelets (about 10 nm·200 nm), rods (about 30 nm·120 nm), and needles (about 10 nm·100 nm). They were not functionalized but analysed in the presence of the dispersant sodium hexametaphosphate (SHMP). Under these conditions, they all had a negative zetapotential (−5 to −15 mV) and a size by dynamic light scattering (DLS) between 70 and 130 nm. The cytoxicity against BEAS-2B cells (human bronchial epithelial cells) and against RAW264.7 cells (murine macrophages) was tested in the concentration range of 10–300 mg L^{-1}. Both cell lines took up all four kinds of nanoparticles. There was almost no toxicity against macrophages. However, the epithelial cells showed some necrosis and apoptosis at 100 mg L^{-1}, but no induction of reactive oxygen species (ROS). The needle-shaped nanoparticles showed the highest degree of necrosis. The authors concluded that the particle shape and the cell type are important for the cytotoxicity. However, it must be noted that in these experiments, the nanoparticles were synthesized by different routes in the presence of different additives. For instance, cetyltrimethylammonium bromide (CTAB) was only added during the synthesis of needle-shaped particles. Residues of this compound on the purified particles may well have influenced the cell reaction. The differences between the particles were also not very high so that the particle shape effect was not very pronounced. Finally, it is not clear whether the particles were dispersed in the cell culture or underwent some sedimentation after agglomeration. In any case, it is an important observation that epithelial cells were much more sensitive to all different calcium phosphate nanoparticles than macrophages [112].

Zeng et al. have studied the effect of unfunctionalized hydroxyapatite microspheres (about 1–2 μm) and microrods (about 1 μm·3 μm) on RAW264.7 cells (macrophages). The cell viability was not influenced after 24 h at concentrations up to 1000 mg L^{-1}. Some small differences in cell regulation were observed. In this case, a sedimentation of the large unfunctionalized particles on the cell layer is very likely, so that it is difficult to interprete these results in a concentration-dependent way. The uptake of the particles was not measured. However, the results clearly showed that the viability of macrophages was not impaired, even at a very high dose of calcium phosphate microparticles [128]. Lebre et al. have incubated bone-marrow derived macrophages and bone-marrow derived dendritic cells with unfunctionalized hydroxyapatite

microparticles with size fractions of 0.1 μm to 100 μm. They used very high doses (100–1000 mg L^{-1}), and it can be safely assumed that the observed adverse effects were due to a large number of sedimented particles [129].

It has been reported that an exposure of cells to large doses of unfunctionalized calcium phosphate nanoparticles was harmful due to the high intracellular calcium level. Motskin et al. have compared the response of human macrophages to unfunctionalized hydroxyapatite nanorods (20 nm·50 nm), spherical hydroxyapatite particles (170 nm) and unfunctionalized hydroxyapatite microparticles (1–2 μm). Extensive results by transmission electron microscopy were reported. All kinds of particles were taken up by phagocytosis and ended up in phagosomes/lysosomes. Depending on the dose, the cells took up very high amounts of nanoparticles which caused subsequent cell death due to an increased intracellular calcium level [130]. Subsequent high-end imaging studies on the uptake of calcium phosphate nanoparticles were reported two years later, indicating the presence of a "surface-connected compartment" in which the nanoparticles were stored [109]. This group also reported that agglomerated calcium phosphate nanoparticles were taken up better (but probably by another mechanism) than dispersed nanoparticles. Consequently, the calcium load on the cells in contact with dispersed was lower, leading to a lower cytoxicity for dispersed nanoparticles compared to agglomerated nanoparticles [110].

Ewence et al. have shown that different kinds of unfunctionalized calcium phosphate nanoparticles (agglomerated, particle size a few 100 nm) were readily taken up by vascular smooth muscle cells (VSMCs). This clearly led to an increase of the intracellular calcium concentration after lysosomal dissolution of the particles and subsequent cell death at a dose of about 150 mg L^{-1}. This indicates that a high dose of calcium phosphate will be harmful to the cell unless the calcium is pumped out of the cell soon after lysosomal degradation [131]. In a subsequent study, it was shown that the presence of proteins (albumin, fetuin-A) decreased the cytotoxicity of naked calcium phosphate nanoparticles, possibly by slowing down the dissolution inside the cell [132]. Proudfoot et al. observed that the addition of unfunctionalized calcium phosphate nanoparticles (agglomerated, primary particle size 30–60 nm; 12.5 mg L^{-1}) to human vascular smooth muscle cells strongly increased the release of interleukin-1β via activation of SYK and caspase-1, indicating the potential for inflammation inside arteries [133,134].

It is notable that the strongest effects were observed with unfunctionalized nanoparticles that necessarily must show a high degree of agglomeration. This will lead to an enhanced sedimentation onto the cells and a high local concentration. An enhanced uptake by the cells will follow, causing a high intracellular calcium concentration and subsequent cell death. Such effects have frequently been observed in nanotoxicology [87,135–138]. Possibly, the application of 3D cell culture models would give more insight into this phenomenon as it would avoid sedimentation effects [139]. It is also important to note that calcium phosphate has a high affinity to many biomolecules [140,141], i.e. it cannot be excluded that unfunctionalized nanoparticles adsorb biomolecules or ions from the cell culture medium, leading to their depletion in the medium and resulting in adverse effects on cells.

It shall be stressed here that not all authors give sufficient details on the cell culture studies with calcium phosphate nanoparticles. Parameters like particle dose, particle characteristics (like surface functionalization), agglomeration, or sedimentation phenomena are not always addressed. This problem has also led to concerns of the SCCS of the European Commission in their opinion paper in 2015/2016 [1] when it was asked to assess the risk of hydroxyapatite nanoparticles (see the Introduction). Unfortunately, in this review this was is only possible to use and assess published data, but one has to be aware that not all cell culture results were obtained with standardized protocols.

In summary, we can conclude that calcium phosphate nanoparticles are not cytotoxic on their own, but the release of calcium ions after dissolution can be harmful for a cell. As a rough estimate, calcium phosphate concentrations below 100 mg L^{-1} (100 ppm) in a cell culture appear to be harmless. Shape and size of the calcium phosphate nanoparticles play only minor roles for cytotoxicity.

9. Oral exposure to calcium phosphate nanoparticles

An oral administration of calcium phosphate may lead to a deposition on the tooth mineral (as it is exploited by some calcium phosphate-containing toothpastes) [70,73]. It should be pointed out that milk also contains calcium phosphate nanoparticles, stabilized by phosphoproteins (casein) [142]. When calcium phosphate particles are swallowed, they enter the stomach where a complete dissolution occurs at a pH of 1–2 [143]. There is no chance that dispersed calcium phosphate nanoparticles will survive these highly acidic conditions. Thus, their nanoparticulate identity is completely lost, and they are only present as calcium and hydrogen phosphate ions. After passing the stomach they enter the gut where the pH is slightly basic [144]. Under these conditions, calcium phosphate may precipitate if its solubility is exceeded and the nucleation is not inhibited by the high concentration of biomolecules [143]. In fact, this is medically exploited to reduce the phosphate concentration in blood to prevent atherosclerosis in dialysis patients [145]. Orally administered tablets of calcium carbonate are typical phosphate binders which are first dissolved in the stomach and then precipitate together with phosphate as calcium phosphate in the gut, to be finally excreted. Daily calcium carbonate doses are of the order of several grams per patient, leading to considerable amounts of precipitated calcium phosphate (probably also in nanoscopic form) in the gut [143].

In summary, adverse health effects by an oral exposition to calcium phosphate (unless tens of grams are swallowed) can be ruled out due to the dissolution in the stomach.

10. Intestinal exposure to calcium phosphate nanoparticles

Nanoparticles may cross the gastrointestinal tract epithelium and enter the bloodstream. This is of particular concern for those nanoparticles from food that can pass the stomach, but clearly not for calcium phosphate particles. As nanoparticle pathways, a transcellular transport (uptake by endocytosis or related mechanism and exocytosis on the other side) and paracellular transport (between cells) have been identified [92]. In the case of calcium phosphate, transcellular transport would involve dissolution in the acidic endolysosome, i.e. a subsequent exocytosis can be excluded. A transcellular exocytosis has been observed in osteoclasts *in-vivo* during the phagocytotic resorption of nanocrystalline bone cement (see below) [146]. However, this is not a likely mechanism in non-phagocytosing epithelial cells because they take up only smaller particles by endocytosis (see above). Paracellular transport is only possible for very small nanoparticles (a few nm in diameter), i.e. unlikely for the usually larger calcium phosphate nanoparticles. Notably, bilirubin was shown to conjugate amorphous calcium phosphate nanoparticles in the gut, making them even larger [147]. It has been stated that the healthy gut is an efficient barrier for nanoparticles [148]. Powell et al. have demonstrated that large amounts of nanoparticles, consisting of amorphous porous calcium phosphate, are formed in the small intestine. The diameter was about 100 nm. There biological function is not known, but they may adsorb and transport biomolecules to antigen-presenting cells. The authors estimated that about

$2 \cdot 10^{14}$ of these nanoparticles are formed every day. Thus, such particles are very common in the intestine, and obviously do not pose a risk [46]. They are probably related to the calciprotein particles that form in biological media as discussed above.

Calcium phosphate nanoparticles may enter the intestine orally or rectally. As discussed above, an oral administration is only possible in ionic form, leading to a reprecipitation of calcium phosphate (nano)particles in the gut. No adverse effects have been reported so far. A potential administration of acid-labile calcium phosphate nanoparticles is potentially achievable if they are enclosed into an acid-resistant shell, e.g. by a stomach acid-resistant polymer capsule that dissolves only in the gut [144]. However, this application route has not yet been reported for calcium phosphate nanoparticles. A rectal application of calcium phosphate nanoparticles within a suppository also has not been reported so far. However, it has been shown that a rectal administration of dispersed siRNA-loaded calcium phosphate nanoparticles (cationic, around 100 nm) led to a successful gene silencing in the gut [149,150]. Thus, under certain conditions it is possible for calcium phosphate nanoparticles to overcome the mucosal layer in the gut and to enter the surrounding tissue for gene silencing. This is comparable to a possible uptake of any other kind of nanoparticles in the intestine.

In summary, although calcium phosphate nanoparticles do not dissolve in the gut and have been shown to be able to transport drugs therein, the actual exposition risk through the stomach is absent and probably negligible after rectal administration.

11. Inhalative exposure to calcium phosphate nanoparticles

A prominent exposition route for nanoparticles, including fine dust, is through the respiratory tract into the lung. It has been shown that small nanoparticles (about 10–100 nm) can easily reach the alveoli inside the lung where they often cause inflammation, almost independent from their chemical composition [151]. Typically, they are taken up by macrophages which in the case of calcium phosphate would lead to dissolution in the acidic phagosome [151]. So far, no toxicological inhalation study with calcium phosphate particles has been reported, but given the unspecific pulmonary reaction to many different nanoparticles [151], an inflammatory response cannot be excluded. Nam et al. have reported a case of occupational asthma after 1.5 years exposure to calcium phosphate dust [152].

An intranasal application of a dispersion of biofunctionalized calcium phosphate nanoparticles (spherical; about 250 nm) in mice by Knuschke et al. has resulted in an efficient immunization, demonstrating that nanoparticles are taken up by the nasal mucosa [153]. Miragoli et al. have reported positive effects of peptide-loaded calcium phosphate nanoparticles (<50 nm) and demonstrated that the particles translocated from the pulmonary tree to the bloodstream and the myocardium [154].

In summary, the inhalative exposure to calcium phosphate particles may be an issue of concern at high doses, e.g. for workers exposed to calcium phosphate dust in the mineral industry or involved in mechanical machining of calcium phosphate-based materials.

12. Dermal exposure to calcium phosphate nanoparticles

Monahan et al. have treated the skin of rats with a paste of calcium phosphate nanorods (size below 50 nm; unfunctionalized) for 28 days. No statistically significant adverse effects were observed [155]. Healthy skin is an effective barrier against inorganic nanoparticles [156]. Typically, nanoparticles do not penetrate the skin deeper than a few μm as it has been demonstrated by Vogt et al. for TiO_2, silica, and silver [156]. As calcium phosphate is an inorganic mineral as well, we can safely extend the conclusion of Vogt et al. to it. Concerns have been raised, however, with respect to damaged skin where the barrier function is weakened [156]. In that case, the particles would enter the surrounding tissue and possibly the bloodstream. In tissue, the particles will be taken up by cells and dissolved. In the bloodstream, they will be rapidly covered by proteins (formation of a protein corona [157,158]) and be in the company of naturally occurring calcium phosphate nanoparticles in blood [35]. In both cases, the risk of an adverse reaction is low.

A dermal exposure is only likely if calcium phosphate nanoparticles are applied topically in high doses, e.g. as cream or cosmetics. There are only few consumer products on the market, containing different calcium phosphate phases [159]. If applied to healthy skin, the risk from a dermal exposition to calcium phosphate nanoparticles is negligible.

13. Exposure to calcium phosphate nanoparticles after implantation into the body

As described above, calcium phosphate is frequently implanted as biomaterial in contact with hard tissue (typically bone) to enhance bone regeneration. In nanoparticulate form, it may occur in the following forms:

- Formation of small particles after the setting of calcium phosphate-based bone cements, in solid and agglomerated form.
- Direct implantation as nanoparticulate bone substitution material into a bone defect, i.e. as paste or dispersion.
- Formation after mechanical, chemical or biological wear of calcium phosphate-coated metallic implants (Fig. 4) or implanted calcium phosphate ceramics [160].

In all three cases, the surrounding tissue is subjected to nanoparticulate calcium phosphate at variable concentrations. Depending on the agglomeration state, the local tissue perfusion conditions, and the particle size, the particles may migrate into the surrounding tissue. The biological response will be dominated by the cells that are exposed to the nanoparticles. Osteoclasts will dissolve the solid calcium phosphate phase (e.g. a hardened cement) by their usual acidic dissolution mechanism [161], and macrophages will take up and dissolve the calcium phosphate nanoparticles [146]. Both will excrete the corresponding ions. Notably, osteoclasts have also been shown to take up nanocrystalline calcium phosphate by phagocytosis and to excrete a part of it as solid material (transcytosis) as shown by Wenisch et al. [146]. In this particular case, we can consider this as transcytosis of solid calcium phosphate, together with other degradation products (collagen, ions etc.) of bone resorption [162,163].

Most other cells will also take up calcium phosphate nanoparticles by endocytosis or similar mechanisms and dissolve them in the lysosome [98]. Unless the intracellular calcium concentration becomes too high, adverse effects on the cells can be excluded. This is a major difference to acid-insoluble nanoparticles (e.g. polyethylene debris from artificial hip joints, carbonaceous materials, or silicates like asbestos) that are not biodegradable and can lead to a permanent inflammation due to frustrated cells, causing aseptic loosening [164,165]. Velard et al. have reviewed the inflammatory responses of cells exposed to calcium phosphate particles, including the issue of debris from calcium phosphate-coated endoprostheses. The inflammatory response depended on the particle characteristics (size, shape, charge etc.) as outlined above and was low compared to other inflammatory stimuli like LPS,

phorbolmyristate acetate or zymosan. Polymorphonuclear neutrophils (PMNs) and monocytes appear to play a major role. Velard et al. concluded that further work is necessary to understand these pathways to combat such inflammatory effects, and that in some cases, fibrosis and implant loosening may occur due to an adverse reaction to calcium phosphate particles [166].

There are no concerns regarding the application of calcium phosphate cements as a number of *in-vivo* studies has confirmed, except for an occasional slight local inflammation. This indicates that there is no negative side effect of calcium phosphate (nano)-particles migrating from the cemented site [65,167,168].

The generally favourable biological performance of solid calcium phosphate ceramics and of calcium phosphate-coated implants is beyond the scope of this article. It must be emphasized again that bone tissue contains about 50 wt% of calcium phosphate nanoparticles [18]. This means that the body is well accustomed to this material, admittedly not in dispersed form but incorporated into collagen fibres. There are studies where dispersions of calcium phosphate nanoparticles were implanted into bone defects. Typically, this involves a paste of nano-hydroxyapatite like Ostim® with a particle size of about 25 nm·150 nm and a water content of about 40% [56,169,170].

In a preclinical study by Strietzel et al., a replacement of this nano-hydroxyapatite paste in the lateral alveolar ridge by natural bone was reported. In one patient out of eight, an inflammation was observed [171]. Bone formation was also observed by Huber et al. after implantation of the same paste into a critical size defect in rabbits [172]. In a clinical study by Huber et al. with the same paste implanted into cancellous bone defects, good bone regeneration occurred and no inflammation was observed [173]. Gerlach et al. observed a good integration into bone tissue after implantation of this paste into jaw cysts in patients [174]. Spies et al. implanted this paste into the metaphyseal tibia in minipigs and observed a good osteointegration. However, the bioresorption and the bone remodelling stopped after 6 weeks implantation time for unknown reasons [175]. Busenlechner et al. implanted this paste below a titanium hemisphere in the calvariae of minipigs and found a good bone formation and no adverse effects (no inflammation) [176]. An implantation of this paste in skin chambers in hamsters by Laschke et al. showed a good vascularization, and the absence of acute inflammatory response, indicated by a lack of leukocyte activation in blood vessels located in the close vicinity of the implant [177]. Schlickewei et al. have injected a paste of spherical calcium phosphate nanoparticles (110 nm) into a critical size tibia defect of a rabbit and observed good bone healing, accompanied by material resorption after 12 weeks [178].

A migration of calcium phosphate nanoparticles away from the implantation site cannot be excluded. Depending on the perfusion and the drainage of the surrounding tissue, they might enter the bloodstream. However, as already discussed above, harmful effects of calcium phosphate nanoparticles in the blood are very unlikely (see also the reports by Ding et al. [179] and Wang et al. [180] on intravenous injection of calcium phosphate nanoparticles discussed below).

Many reports deal with the induction of inflammatory markers like cytokines after application of calcium phosphate nanoparticles to cell cultures. For instance, a moderate immunostimulatory effect of calcium phosphate nanoparticles has been reported [181]. However, it is difficult to assess these effects. First, many nanoparticles induce some kind of inflammation, regardless of their chemical composition [151,182]. Second, many nanoparticles are not "naked", i.e. they are coated with a suitable compound (like a polyelectrolyte) to keep them in dispersion. In this case, the question arises whether the calcium phosphate or the coating is inducing the cytokine response. If the calcium phosphate nanoparticles are "naked", they will usually agglomerate and precipitate on the cells, leading to a high local concentration that does not represent the actual concentration in the dispersion [137]. Therefore such results must be carefully judged with respect to the experimental conditions. Furthermore, it has been noted that the presence of impurities can lead to wrong-positive cytokine induction, e.g. if the nanoparticles are not prepared in a sterile way, they may contain endotoxins or other immunostimulatory compounds [183]. In any case, it is well conceivable that the increased calcium level in the cytoplasm after dissolution induces the production of cytokines.

Taken together, nanoparticulate calcium phosphate may cause a local inflammation at the inflammation site, but the adverse effects are minor. It must be stressed that temporary inflammation reactions near an implant [184] and also after application of all kinds of nanoparticles [96] are common observations in medicine. This must be balanced against the benefits of the implant, e.g. the bone regeneration in a defect site.

In summary, there is no sound indication for a negative side effect of calcium phosphate nanoparticles after implantation into a bone defect.

14. Calcium phosphate nanoparticles in the blood: Biodistribution

Like all other nanoparticles, calcium phosphate nanoparticles have some mobility in the body, especially in the blood stream. Parameters like size and charge play a dominant role, but it must also be emphasized that nanoparticles are quickly covered by a protein corona in contact with biological fluids [93,158]. This strongly influences the interaction with cells, e.g. the cellular uptake. Furthermore, it has been established that small nanoparticles (20–30 nm) are rapidly excreted in the kidney by renal filtration whereas larger nanoparticles are eliminated by phagocytosis [185]. Therefore, there is a size range (approximately from 30 to 200 nm) where nanoparticles have a longer circulation time in the bloodstream [186].

Ding et al. have injected dispersions of calcium phosphate nanoparticles (two types of nanorods: 10–20 nm·30–50 nm; 20–40 nm·70–90 nm) intravenously into rats at a dose of 10 mg kg^{-1}. The nanoparticles had no significant toxicity during 24 h [179]. Wang et al. injected dispersions of calcium phosphate nanorods (about 5 nm·25 nm) intravenously in rats, three times per week for four weeks, without giving the applied dose. However, no toxicity was observed, but some apoptotic cells in the liver and the kidney were observed [180]. We can conclude that the toxicity of calcium phosphate nanoparticles in the blood is negligible, unless the dose is very high.

Ignjatovic et al. have followed the biodistribution of hydroxyapatite nanoparticles (rod-shaped, about 20 nm·100 nm) after radioactive labelling with ^{125}I ($t_{1/2}$ 59 d). The particles were intravenously injected into rats and monitored for 24 h. The particles were found mainly in the liver, with smaller amounts in the lung and in the spleen. Most of the particles (or, more correctly, the radioactive ^{125}I, as it is not sure whether calcium phosphate and iodine stayed together all the time) were excreted after 2 h, and their presence was no more detectable after 24 h [187]. In the case of functionalized calcium phosphate nanoparticles, Haedicke et al. observed by *in-vivo* near infrared (NIR) imaging (mouse) that 200 nm-particles were well distributed inside the body after intravenous tail-vein injection. An accumulation was observed particularly in the lung after 24 h [188]. In a similar study by Sokolova et al., poly(I:C)-loaded calcium phosphate nanoparticles (cationic, 120 nm) were predominantly found in the lung and the liver, 1 and 3 h after the tail-vein injection into mice injection [108].

Due to the ubiquitous nature of calcium and phosphate in the body, it is difficult to trace these ions (or calcium phosphate

particles) in the body. Fluorescent labelling by NIR dyes permits *in-vivo* imaging, but only a few mm into the body. Another potent method is positron emission tomography (PET) where short-lived radioactive isotopes decay under release of a positron (β^+) that is annihilated with an electron under emission of two photons. These photons can be used to determine the location of the decaying ion. The method permits a detection deep inside the body and can also be performed *in-vivo*. The short half-life of PET isotopes restricts the observation period to a few hours.

For calcium phosphate particles, only three *in-vivo* PET studies have been reported. Jauregui-Osoro et al. have injected dispersed calcium phosphate particles (unfunctionalized, strongly agglomerated, primary particle size 100 nm) into mice. After 4 h, they found an enrichment in the lung, the liver, and the spleen. For labelling they used the isotope ^{18}F ($t_{1/2}$ 110 min) which efficiently binds to calcium phosphate [189]. Zheng et al. carried out a similar experiment with unfunctionalized calcium phosphate nanoparticles, labelled with ^{18}F, and found them mainly in lung, liver, spleen and stomach after auricular vein injection into rabbits [190]. Sandhöfer et al. have injected citrate-stabilized calcium phosphate nanoparticles (about 140 nm), labelled with ^{18}F and ^{68}Ga ($t_{1/2}$ 68 min) intraveneously into rats and followed them for 90 min. They found an enrichment in lung, liver, and spleen [191].

Of course, if the biodistribution after intravenous injection into the blood circulation is one thing, the fate of particles after implantation is another. This is especially important for an implantation into bone defects, i.e. a location with lower blood perfusion [192].

Den Hollander et al. have measured the distribution of ^{45}Ca ($t_{1/2}$ 163 d) after implantation of ^{45}Ca-labelled ceramics (sintered HAP and β-TCP) into dog femurs. After 12 months, they found a small activity of ^{45}Ca in the lymph nodes [193]. However, it is unclear whether this was an accumulation of calcium ions released from the implant or of solid material that had travelled all the way from the implantation site to the lymph node. In a preceding publication from 1989, the authors claimed that they observed crystalline material in the lymph nodes after this experiment, but no data were reported [194]. Given today's knowledge on the uptake and dissolution of calcium phosphate nanoparticles by cells, such a migration appears possible, but unlikely. Notably, Fischer-Brandies have reported a similar experiment (implantation of radioactively sintered HAP and β-TCP into rat femurs for 13 weeks) and did not find an increased level of radioactivity in the lymph nodes [195].

The question arises whether the presence of calcium phosphate nanoparticles in the blood enhances the risk of atherosclerosis. This is difficult to answer because atherosclerosis is a long-term process that cannot be reproduced by short-term experiments as in cell culture or in imaging studies. The calcium concentration in blood is highly regulated to about 100 mg L^{-1}, corresponding to 500 mg in 5 L blood for an adult human. To match this amount of calcium, one would have to disperse 1250 mg calcium phosphate nanoparticles in the blood. This is clearly much more than could reasonably be expected from any practical medical or cosmetic application. Thus, unless the applied dose is very high (which should not be the case in any conceivable application), a negative side effect is unlikely. The fact that calciprotein particles (see above) are present in the blood in high amounts indicates that the addition of a small amount of calcium phosphate nanoparticles to blood is very unlikely to have an adverse effect in the bloodstream.

In summary, it can be stated that calcium phosphate nanoparticles will migrate in the body if they enter the bloodstream. After intravenous injection, they preferentially go to lung, liver, and spleen within a few hours. In the blood, they will be in the company of naturally occurring calcium phosphate nanoparticles and eventually excreted. Negative side effects were not observed.

15. Environmental risks of calcium phosphate nanoparticles

Finally, the possible effect of a release of calcium phosphate nanoparticles into the environment will be considered. Calcium phosphate is a common mineral on earth. As such, it is not toxic. Calcium and especially phosphate are both essential ions for all living organisms, therefore the mineral itself has beneficial properties, especially if it is present in soluble and bioavailable form. If calcium phosphate nanoparticles are entering the environment, they are expected to agglomerate and, as sparingly soluble compounds, to finally enter the sediment [196]. Under acidic conditions (below a pH of about 4–5), they will be dissolved. There are no conceivable risks concerning a release of calcium phosphate nanoparticles into the environment.

16. Conclusions

Calcium phosphate is an abundant biomineral, also in the form of nanoparticles in bone and teeth. Except for a potential increase of the intracellular calcium concentration after an uptake by cells and a limited local inflammatory reaction, there are no adverse effects of calcium phosphate nanoparticles. Upon oral uptake, the particles will dissolve in the stomach, and a subsequent adverse effect in the gut can be excluded. Inhalative, mucosal, and dermal exposure may lead to a particle uptake in unfavourable cases, but a potential risk is restricted to very high particle doses. In the blood stream, calcium phosphate nanoparticles are naturally present, therefore the addition of exogeneous calcium phosphate nanoparticles in realistic doses will not cause adverse effects. Finally, there are no tangible risks of a release of calcium phosphate nanoparticles into the environment. Most published studies have looked on the effect of hydroxyapatite as the most prominent calcium phosphate phase, but not in all cases a strict crystallographic phase characterization was performed. Due to the chemical similarity of the common calcium phosphate phases, there will be no significant differences among them with respect to the biological response. Thus, the conclusions from above can be safely transferred to other common calcium phosphate phases (e.g. TCP, OCP, DCPD, ACP) as well.

Of course, the applied dose will always influence the biological response. At very high exposition levels, toxic effects will occur with almost all (nano-)materials. No material is harmless under all conditions at all concentrations in all environments. Under all reasonable conditions, calcium phosphate nanoparticles can be considered as safe for humans.

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