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RESEARCH: Review

Calcium phosphates in biomedical applications: materials for the future?

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Our populations are aging. Some experts predict that 30% of hospital beds will soon be occupied by osteoporosis patients. Statistics show that 20% of patients suffering from an osteoporotic hip fracture do not survive the first year after surgery, all this showing that there is a tremendous need for better therapies for diseased and damaged bone. Human bone consists for about 70% of calcium phosphate (CaP) mineral, therefore CaPs are the materials of choice to repair damaged bone. To do this successfully, the process of CaP biomineralization and the interaction of CaPs and biological environment in the body need to be fully understood. First commercial CaP bone graft substitutes were launched 40 years ago, and they are currently often regarded as 'old biomaterials' or even as an 'obsolete' research topic. Some even talk about 'stones'. The aim of this manuscript is to highlight the tremendous improvements achieved in CaP materials research in the past 15 years, in particular in the field of biomineralization, as carrier for gene or ion delivery, as biologically active agent, and as bone graft substitute. Besides an outstanding biological performance, CaPs are easily and inexpensively produced, are safe, and can be relatively easily certified for clinical use. As such, CaP materials have won their spurs, but they also offer a great promise for the future.

Introduction

Calcium phosphates (CaPs; Table 1) are the main constituents of bone and teeth and play as such an essential role in our daily lives. Following the logic that damaged tissue can best be repaired by something with close resemblance, biomaterials based on CaPs were already proposed for fracture treatment in 1920 [1]. CaP biomedical research soared in the 1970s and CaPs were proposed for a broad range of orthopedic and dental applications [2–6] (Table 2). These materials varied from thin coatings on metallic implants to aid implant fixation into bone [7] to sintered CaP to be used as synthetic bone graft substitutes [8]. Truly impressive clinical successes have been achieved with such materials, for example to increase the clinical survival rate of the femoral component of total hip implants [9], to reduce the risk of pin loosening for external fixators [10], or to allow earlier weight bearing after tibia plateau fractures [11]. In some cases, CaPs are even superior to autografts [12]. Nevertheless, all these achievements have become somewhat overshadowed by the advances in the field of polymers for biomedical applications that seem endlessly diverse when it comes to control of composition and related properties (e.g. co-polymers, supramolecular self-assemblies), applicable processing techniques (e.g. additive manufacturing) and functionalization possibilities (e.g. surface micro-and nanostructuring, chemical functionalization).

In the perspective of these recent developments in the field of biomaterials, which have been underlined in a large number of recent review articles (Table 3), the question arises whether CaPs are old biomaterials, functional, but not particularly elegant? Or do they stand the chance to become the materials of the future?

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TABLE 1

Main calcium orthophosphate compounds (taken from [143]). The first 6 compounds precipitate at room temperature in aqueous systems. The last 6 compounds are obtained by thermal decomposition or thermal synthesis. The 6 columns contain the name, the chemical formula, the Ca to P molar ratio, the mineral name, and the typical acronym, respectively. When $x > 0$ in the chemical composition of 'precipitated hydroxyapatite', one talks also about 'calcium-deficient hydroxyapatite' (CDHA). Generally, $x = 1$ so that CDHA has in most cases the composition $Ca_9(HPO_4)(PO_4)_5OH$.

Name	Formula	Ca/P	Mineral	Symbol
Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.50	–	MCPM
Dicalcium phosphate	$CaHPO_4$	1.00	Monetite	DCPA
Dicalcium phosphate dihydrate	$CaHPO_4{\cdot}2H_2O$	1.00	Brushite	DCPD
Octocalcium phosphate	$Ca_8H_2(PO_4)_6{\cdot}5H_2O$	1.33	–	OCP
Precipitated hydroxyapatite[a]	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$	1.33–1.67	–	PHA
Precipitated amorphous calcium phosphate	$M_u(Ca_3)(HPO_4)_{3v}(PO_4)_{3y}{\cdot}zH_2O$[b,c]	0.67–1.50	–	ACP
Monocalcium phosphate	$Ca(H_2PO_4)_2$	0.50	–	MCP
α-Tricalcium phosphate	$\alpha\text{-}Ca_3(PO_4)_2$	1.50	–	α-TCP
β-Tricalcium phosphate	$\beta\text{-}Ca_3(PO_4)_2$	1.50	–	β-TCP
Sintered hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1.67	Hydroxyapatite	SHA
Oxyapatite	$Ca_{10}(PO_4)_6O$	1.67	–	OXA
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	2.00	Hilgenstockite	TetCP

[a] x may vary between 0 and 2.
[b] u may vary between 0 and 3, v may vary between 0 and 1.5, y may vary between 0 and 0.667, and z is unclear at this point. M is typically a monovalent cation (Na^+, K^+, NH_4^+) which is only present if there is an overall negative charge on the calcium phosphate.
[c] ACP produced in basic conditions has generally $u = 0$, $v = 0$, $y = 0.667$, leading to the following composition: $Ca_3(PO4)_2{\cdot}zH_2O$ where $z = 3$–4.5. In acidic conditions, $u = 3$, $v = 1.5$, $y = 0$, leading to the following composition: $M_3(Ca_3(HPO_4)_{4.5}{\cdot}zH_2O)$ where z is unknown.

Unlike the large majority of both natural and synthetic polymers used in biomedical applications, CaPs are present in the human body and are thus relatively easy to certify. This advantage should not be underestimated at a time when the need for successful and yet affordable strategies for the treatment of diseases and the regeneration of malfunctioning organs and tissues is increasing at a high rate, as a consequence of an aging population in the Western world. CaPs meet these requirements; they can be produced in large quantities, against relatively low cost, they are stable and therefore available off-the-shelf. Nevertheless, their use is also associated with drawbacks, with poor mechanical properties being probably the most relevant one for application in orthopedics and dentistry. This, taken together, shows that additional efforts need to be placed to further advance biomedical strategies based on CaPs, but also that these materials deserve such efforts.

In the current review, we aim to highlight important recent developments in CaP research, divided into the topics biomineralization, nanoparticles for targeted delivery, and bone graft substitution. We also aim to provide an outlook toward the future of CaPs in biomedical applications.

Biomineralization

Biomineralization can be described as a phenomenon in which a mineral is integrated as a functional and often structural part of living organisms, often in direct and close contact to a matrix forming protein or carbohydrate structure. The superb properties and intriguing complexity of most mineralized structures are indeed a result of the interactions between organic molecules/matrices and the mineral itself [13]. Examples of biominerals found in nature are numerous as described in detail by Lowenstam and Weiner [14]. Most common are the calcium carbonate-based biominerals like aragonite (nacre) and calcite (mussels, exoskeletons of crayfish, *etc.*), CaPs (in vertebrate bone and teeth) and silicates (plants, sea sponges) but also much rarer natural minerals exist. A great number of studies have investigated mineral synthesis under biologically relevant conditions, with the aim to explain the mechanisms behind biological mineral. Crude simplifications of the physicochemical conditions are a necessity in these studies as the complexity of the real biological environment hampers execution of mechanistic studies. In the next chapters, we will focus on developments in the field of CaP biomineralization in both biological and synthetic systems. Important discoveries in the last decade have provided us a deeper understanding of the mechanisms of biological and abiotic CaP mineralization, especially regarding the role of amorphous precursors and charged organic molecules.

Bone mineral

The most prominent representative of CaP biomaterial is vertebrate bone, an intricate composite of collagen, non-collagenous proteins and mineral ordered in a distinct hierarchical fashion [13,15,16]. Bone mineral, which is often referred to as biological apatite or dahlite, is distinctly different from the geological apatite mineral. First of all, bone mineral consists of nanometer-sized platelets or needles [16], incorporated within collagen fibrils, and oriented with the *c*-axis in the direction of the fibril [17]. Additionally, it does not have the hexagonal crystal morphology of geological apatite and is also described as monoclinic apatite [18,19]. Furthermore, bone mineral contains a number of ionic substitutions such as CO_3^{2-} in OH^- (A-substitution) and PO_4^{3-} sites (B-substitution), or Na^+, Sr^{2+} and Mg^{2+} in Ca^{2+} sites. In fact, apatite is known for its ability to undergo ionic exchange with metal ions in aqueous solutions [20,21], hence explaining the high variability in bone mineral composition. Also, hydroxide, one of the primary constituents of *hydroxy*apatite, has been reported to be absent in bone mineral [22]. Finally, bone mineral is often described as poorly crystalline, which probably relates to the small size of the crystals as well as residual stresses in the crystal lattice. While amorphous calcium phosphate (ACP), a likely precursor for

TABLE 2

Short and non-exhaustive historical overview of important achievements in CaP research.

Year	Discovery	Reference
1920	Use of an aqueous slurry of 'Triple Calcium Phosphate'[a] to stimulate bone growth	[1]
1934	Use of tricalcium phosphate, MCP, and DCP slurries to stimulate bone growth	[327]
1936	Polyphosphates discovered in yeast	[328]
1965	Apatite precursor phase, Posner cluster	[25]
1969	Synthesis of dense HA for prosthetic applications	[329]
1970	Importance of macropores for bone regeneration	[185,186]
1971	Implantation of 'degradable' tricalcium phosphate ceramic in rats	[133]
1973	CaP-mediated transfection	[119]
1975–1979	Clinical study with β-TCP and HA	[3,330]
1975–1982	First commercial CaP products: 'Synthograft/Synthos' (β-TCP; 1975), Ceros HA (HA, 1980), Durapatite (HA, <1981), ProOsteon (HA, 1981), Calcitite (HA, 1982), Alveograf (HA, 1982), Ceros TCP (β-TCP, 1982), BioBase (α-TCP, 1982)	
1976	Description of the hydraulic properties of α-TCP	[331]
1980–1987	CaP Coatings	[332,333]
1982–1987	CaP cements (CPCs)	[137,334–336]
1985–1990	CaP used as carriers for drug delivery	[337,338]
1985	Importance of micropores for bone regeneration	[5]
1987–1999	Injectable/non-setting pastes ('Putties')	[152,153,159,339]
1990–1991	Osteoinductivity	[213,251]
1992–1999	Bone augmentation	[340,341]
1994	Production of HA Whiskers by hydrothermal synthesis	[342]
1994–1995	Clinical study with CPC, commercial launch of Norian SRS and BoneSource	[138,343]
1997	Production of CaP scaffolds by rapid prototyping	[188]
1999	Si-substituted HA	[344]
2000	Polymer-induced liquid precursor (PILP)	[345]
2001–2004	Biomimetic CaP scaffolds, macroporous CPC	[175,346–349]
2002–2008	β-TCP synthesis by precipitation in hydrothermal conditions or in organic liquids	[312,350,351]
2003	Micronization/amorphization by milling	[352]
2003	Ready-to-use CPCs, dual-paste CPCs	[144,147]
2003–2004	Custom-made CaP nanoparticle for gene delivery (transfection)	[116,353,354]
2004–2006	Hydrated layer on apatite crystals	[355,356]
2005	Flame-synthesized CaP nanoparticles	[357]
2005–2013	Re-discovery of the importance of micropores for bone formation	[202–205,358–362]
2005–2007	3D printing of CaP scaffolds	[189–191,363]
2008	Nano-particulate apatite paste as bone substitute	[151]
2008	New Ca–Mg phosphate phase diagram	[364]
2008	ACP found in evolving bone	[38]
2010–2011	Use of Ca and Phosphate ions as drugs (Bioinorganics)	[132,273]
2010	Validation of the PILP model	[58]
2012	Protein-free template mineralization	[59]
2012	Covalent functionalization of CaP nanoparticles	[109]
2013	Detailed description of ACP formation *in vitro*	[23]

[a] Most likely an apatite powder with CDHA composition.

TABLE 3

List of selected reviews published after the year 2000 in the CaP field.

Topic	Reference
Calcium phosphates (general review)	[27,365–368]
Biphasic calcium phosphates	[369]
α-TCP, DCPA, DCPD, OCP, TetCP	[370–373]
CPCs and putties	[139,143,158,374–376]
Coatings	[7,377–381]
Nanoparticles/amorphous CaP/CaP precipitation	[75,302,382–389]
Sintering, scaffold production	[375,390,391]
Osteoinductivity	[263,392,393]
Drug/gene delivery	[71,73,75,394–397]
Ionic substitution, ion exchange, bioinorganics	[21,273,281,298,324,388,398]
Particles with controlled geometry	[384,389,399,400]

the formation of bone mineral, has never been directly observed in mature bone, there is often a significant substitution of PO_4^{3-} by HPO_4^{2-} in the mature bone, which is a remainder of a transformation *via* ACP and octacalcium phosphate (OCP) precursors during the precipitation around neutral pH [23]. Taking into account all above, a complete description of bone mineral, according to the current state of knowledge, would be the following: 'a poorly crystalline, highly substituted apatite consisting of very small crystallites' [24].

Amorphous calcium phosphate

While crystalline CaP compounds are most widely used in biomedical applications, ACP appears to be involved in the formation of the majority of complex CaP structures. This hydrated, seemingly unstructured, and often very unstable material is most likely used as biological pathway for shaping and structuring bone

mineral. The first attempt to describe ACP was made by Eanes et al. [25] who studied the mineralization of a highly concentrated CaP solution with X-ray diffraction (XRD). These authors observed the presence of two broad peaks in the XRD diffraction pattern of CaP obtained at early time points. At longer maturation time, the CaP eventually transformed into an apatite whose diffraction pattern showed a remarkable resemblance to bone mineral. This observation led to the postulation of an ACP, consisting of 1.4 nm sized 'Posner's Clusters' with a composition of $Ca_9(PO_4)_6$. This model is still commonly used to describe ACP with a Ca/P ratio of ~1.5 in experiments and calculation models, and is referred to as amorphous tricalcium phosphate (TCP) [26]. Recently, ACP was described to be composed of $Ca_2(HPO_4)_3^{2-}$ clusters [23]. This observation does not stand alone, as also earlier descriptions in literature sometimes referred to a brushite-like chemistry of ACP [27,28]. The protolysis equilibria of phosphate and the ability of calcium to bind with both HPO_4^{2-} and PO_4^{3-} actually dictate that the chemistry, and perhaps also short-range structural properties of ACP change as a function of the pH, going from a PO_4^{3-} rich phase at high pH to a HPO_4^{2-}-rich phase at lower pH and physiological conditions [29].

The amorphous precursor pathway: starting from pre-nucleation species

Following the discovery of ACP, the formation of apatite in solution *via* an amorphous precursor was investigated in detail in the 1970–80s [30,31]. In all cases it reflected a cascade of events where at first an amorphous precursor precipitated from the solution, and then *via* multiple intermediate stages, often including a second ACP-stage (referred to as ACPII) and OCP-stage ($Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$)), transformed into an apatitic CaP. As these events occur rather fast, a correct analysis of this transformation is tedious, especially when sample preparation affects the properties of the analyzed material. Therefore, it is only recently that a detailed chemical, morphological and structural description of this system was given using cryo-TEM (Fig. 1) and various *in situ* and *ex situ* techniques [23]. This study described a multistep-process, starting from the aggregation of charged calcium-trihydrogenphosphate complexes ($Ca(HPO_4)_3^{4-}$) in a dendritic-like fashion in the prenucleation stage, which subsequently takes up Ca^{2+}/loses H^+ to precipitate as the earlier described spheres of acidic ACP (composed of $Ca_2(HPO_4)_3^{2-}$ post-nucleation clusters). Accordingly, ribbons of a calcium-deficient OCP ($\sim Ca_6(HPO_4)_4(PO_4)_2^{2-}$) grow out of the ACP aggregates, a phase that was originally described as ACP(II). These ribbons were observed to be only ~1.4 nm thick, thereby making them undetectable by XRD. The ribbons then transform into elongated plates of OCP, which over a long period of time generate smaller platelets of a calcium-deficient apatite. In this process, the calcium-triphosphate complex can still be found in the final apatite lattice but also in the ACP and OCP-like intermediates.

ACP precursors in biological specimens: discovery and transformation pathway

After the first description of ACP by Eanes et al. [25], and triggered by the similarity of XRD patterns between bone mineral and crystals forming from ACP in solution, it was proposed that a similar mechanism of crystal formation occurs in bone as well. However, all attempts to find ACP in (mature) bone remained unsuccessful, which dictated the general opinion for decades to follow [32]. Such evidence came only recently [33–38]. Initiated by the work of Lowenstam and Weiner [14], spectroscopic and X-ray evidence for the presence of a metastable amorphous precursor in several calcium carbonate biomineral structures was obtained [39]. Based on this work, Mahamid et al. [36,38] and Beniash et al. [34] detected the presence of an ACP in evolving zebrafish bone and in newly formed murine enamel, the apatite structure specifically found in teeth. Prerequisite for this observation was the careful extraction and analysis of the specimens, which was done by state-of-the-art techniques like X-ray absorption near edge structure spectromicroscopy (XANES), Cryo-Scanning Electron Microscopy and Synchrotron X-ray diffraction mapping. Moreover, Mahamid et al. [36] were able to visualize the transformation pathway from ACP to bone, showing cells with CaP-filled vesicles in the vicinity of the newly formed bone which excrete the ACP particles that subsequently attach to the non-mineralized bone matrix and finally fuse to the mineralized matrix. A recent study by Akiva et al. [33] indicated that the ACP particles are not necessarily produced in the direct environment of the bone growth site, but can also be supplied through blood. Studies on mouse calvaria and long-bones [37] furthermore showed that bone related cells like pre-osteoblasts, osteoblasts and osteocytes contain vesicles that are filled with 80 nm-sized CaP granules, which consist of even smaller particles, with a Ca/P ratio of 0.7. Although this Ca/P ratio seemed to be deviating from the values described in literature at that time point, and was different from the Ca/P ratio found on the mineralizing zebrafish bone (Ca/P = 1.3), it corresponded well with the one of acidic ACP, discovered afterwards [23]. Furthermore, evidence for an acidic ACP precursor in growing zebrafish bone was recently obtained in a paper by Bennet et al. [35] by use of *in situ* Raman and fluorescence imaging. Additionally, evidence was obtained for an OCP(-like) intermediate (see also [33]), corresponding to earlier work of Crane et al. [40] and the abiotic mineralization mechanism [23].

All these results seem to contradict a direct mineralization from the non-mineralized tissue by the surrounding serum. However, this possibility cannot be completely excluded since the serum is supersaturated with respect to apatite. Furthermore, the high amount of mineralization inhibitors and promotors present inside the system, which will be discussed in a following paragraph, makes it almost impossible to predict whether a collagen matrix can be mineralized in such an environment or not. Currently, the particle-excretion mechanism seems to best explain the availability of high amount of mineral that is required at the mineralization site.

Organic phosphates/polyphosphates

The observation of an amorphous orthophosphate precursor seems to be in disagreement with the enzymatic degradation of organic phosphates by alkaline phosphatase (ALP), necessary for bone cells to produce a mineralized matrix in *in vitro* culture [41]. However, if the formation of ACP occurs within a vesicle, the availability of phosphate indeed may be directed by the cleavage of organic phosphate, slowly accumulating inside these vesicles. Crystallization of the ACP could then be inhibited by control over Ca^{2+} concentration and pH. An alternative explanation for the presence of CaP inside vesicles is polyphosphate [24], a covalent

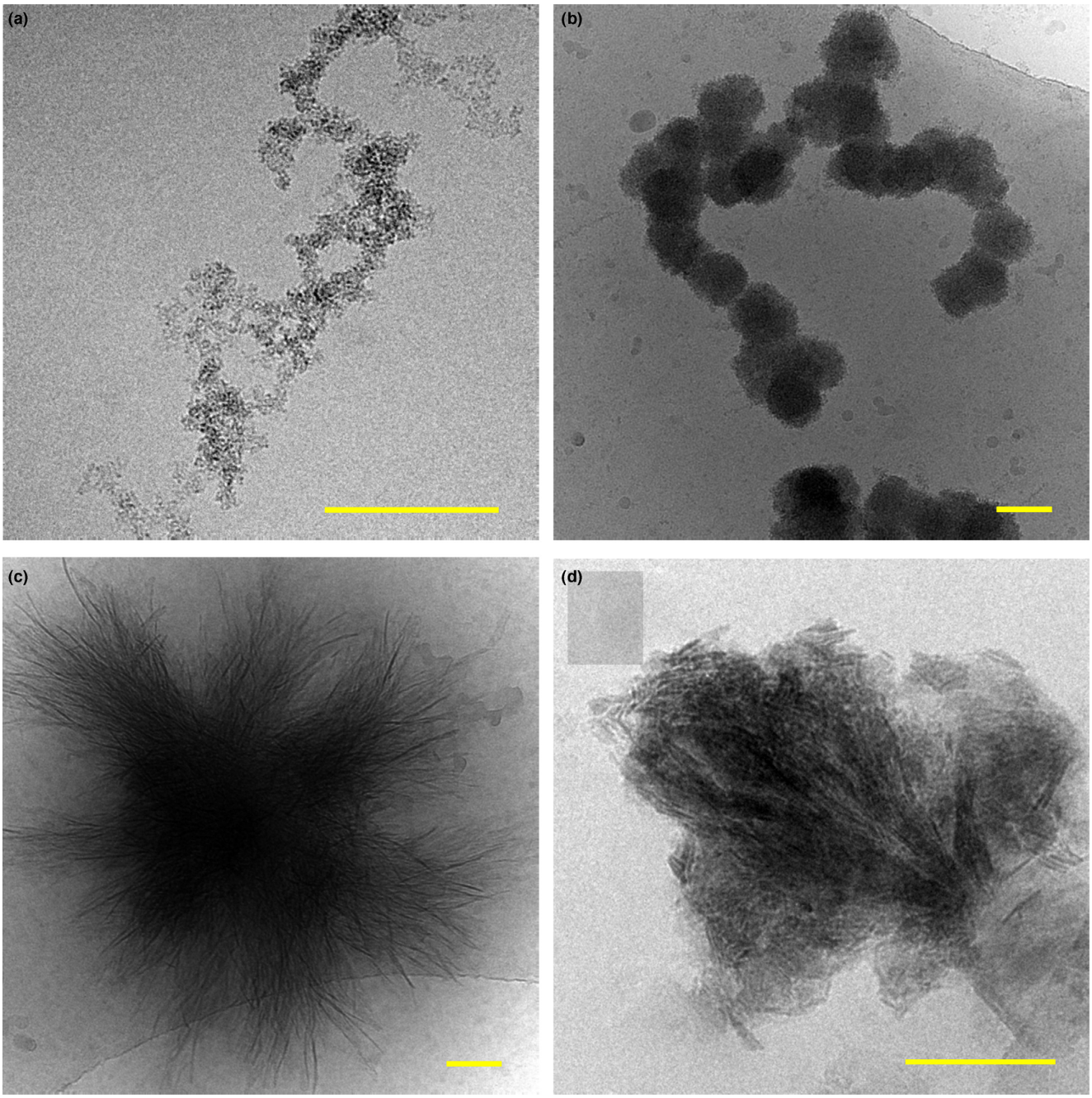

FIGURE 1

Chronological visualization of calcium phosphate solution crystallization by Cryo-TEM: (a) dendritic aggregates of pre-nucleation complexes, (b) amorphous calcium phosphate (ACP) spheres, (c) aggregate of octacalcium phosphate (OCP)-like ribbons, (d) rosette of apatite platelets, scale bars are 100 nm.

polymer of (PO_3)-repeating units, which does not crystallize into apatite unless it is cleaved by ALP into orthophosphate. The Ca/P ratio here can be similar to the 0.7 value measured for the vesicles described above (Ca/P calcium polyphosphate = 0.5–1.0), furthermore, polyphosphate can be detected using DAPI-staining (which then turns yellow [42]) and *in situ* Raman (only a very weak signal). The presence of polyphosphate-filled vesicles has been observed in marine bacteria and diatoms [43] where they act as a stable, yet easily accessible storage of phosphate, which after enzymatic degradation locally raises the levels of orthophosphate above the nucleation limit. Lately, polyphosphate was also observed to be a major source of inorganic phosphate for the slowly growing tesserae of elasmobranches (sharks and rays) [44]. Although for the growth of zebrafish bone the evidence points toward the role of an acidic ACP [33,35], polyphosphate might play a similar role in many other systems.

The polymer-induced liquid precursor phase (PILP): the role of additives

Many studies published in the last 15 years suggest that biology uses additives like proteins, foreign ions and small organic molecules to prevent or induce mineralization [45–47]. In the described particle secretion mechanism, an additive is likely involved to prevent the otherwise unstable amorphous particles from crystallizing or dissolving during their transport to the site of interest. However, increasing the viscosity of the surrounding solution, thereby delaying dissolution, might lead to the same result, as indicated by a study of Gal et al. [48]. To understand the influence of additives like proteins on CaP formation and biomineralization, numerous comparative studies have been performed. One of the most important discoveries from these studies, originally described for calcium carbonates, is the so-called polymer-induced liquid precursor process (PILP process [46,49–51,345]). Here, Gower et al. were able to stabilize a highly concentrated liquid-like mineral precursor by negatively charged carboxylic acid-containing polyelectrolytes like poly(aspartic acid) and poly(acrylic acid) [49]. The systematic presence of charged residues which can bind with Ca^{2+} or $PO_4^{3-}/HPO_4^{2-}/CO_3^{2-}$ (see also Schenk et al. [52]) on a polymeric chain, was suggested to be the main cause for the occurrence of this PILP state. Recent speculations on the formation mechanism of amorphous calcium carbonate (ACC) point toward a spinodal decomposition mechanism [53,54], that is an instant decomposition of a highly concentrated solute into an ion-rich and an ion-poor phase. The PILP could represent such a spinodal decomposition on a local scale, where the necessary increase in concentration is reached by the ion-attracting polymer chains. However, the exact properties of the PILP-phase are not clear yet [55], representing either a polymer-stabilized amorphous phase or a stage before that. In any case, however, the PILP represents an easily moldable, unstructured material, enabling a fast and convenient mineralization process of complex matrices [56]. Although originally met with skepticism as the analogy of a charged polymer and a mineralization-controlling matrix protein seemed too much of a shortcut, the highly charged amino acid sequence of all known protein-based CaP inhibitors seems to beckon that this phenomenon also occurs in biology [57]. A validation of the PILP model for the mineralization of collagen fibrils has been shown by Nudelman et al. [58]. By use of a polyaspartic acid-stabilized CaP PILP, they were able to produce oriented apatite platelets inside the collagen fibril as visualized by cryo-TEM imaging and tomography, identical to those observed in vertebrate bone. The postulated mechanism of mineralization then involves the infiltration of the PILP into the collagen by ionic interactions, where the collagen forms a template for the formation of the apatite platelets. Also without the addition of a negatively charged polymer, a bone-like mineralization of collagen fibers was observed recently [59], questioning the necessity of a PILP-like intermediate. The authors speculated that the specific attractive interactions of collagen matrix alone might be enough to initiate the formation of the typical bone mineral structure. However, also here the highly concentrated SBF solution likely led to the formation of an amorphous intermediate phase, which is stabilized by impurities like foreign ions (SO_4^{2-}, CO_3^{2-}, Mg^{2+}) [60] present in SBF. The templating action of the collagen therefore seems to be important, however, only when in the presence of a stabilized amorphous/PILP phase, as most attempts to mineralize collagen by a CaP solely led to peripheral deposition of mineral. In a recent paper of Pompe et al. [61], the templating action of the collagen is described to be a result of the coherent evolution of tropocollagen and OCP. This process is enabled by the interaction of units of post-nucleation complexes inside the OCP and its precursors [23] and amino-acid triplets within the tropocollagen, which show a perfect structural fit. It indicates that the structure of collagen can be interpreted as being the result of a 'survival of the fittest' in the formation of CaP-based biocomposites in living organisms.

The efficiency of additives to stabilize an amorphous or PILP stage is largely dependent on their nature. For example, in contrast to most foreign ions, crystallization of the ACP is significantly delayed by adding only a small amount of (poly)-glutamate or polyphosphate [60]. In fact, many examples in biology indicate that ACP-stabilizing agents are highly phosphorylated [45,62–64]. A classical example, although beyond the scope of biomineralization, is casein micelles, phosphoprotein–CaP complexes [62,65] that are found in milk. Thanks to the formation of these micelles, the level of calcium present in milk is raised without causing precipitation. Additionally, it is suggested that this complexation improves the intestinal resorption of Ca^{2+}, and evidence is given for the remineralization of enamel caries by human and animal studies [62]. The CaP inside these micelles is X-ray amorphous, and especially the phosphorylated seryl-cluster motif inside the casein is responsible for the interaction with the CaP [62], although the overall charge of the protein also has an effect. This system is therefore clearly in accordance with the PILP-model, where only the complex between the organic and the inorganic material is able to execute its biological function. Another example is fetuin-A, a liver-derived blood protein, which is an important inhibitor of ectopic calcification as shown in a study in knock-out mice [47]. In biological samples, fetuin-A/CaP complexes are observed as elongated 200 nm-long crystalline colloidal structures [64]. Although phosphorylated residues are also present here, a comparative study has shown that the binding behavior between the fetuin-A and CaP is lost upon blocking the carboxylic acid residues, and the inhibitory effect is mostly regulated by the cystatin-like Domain D1 [66]. In contract to the casein, phosphorylation might aid mineralization here, but is not necessary to control it. Another system which has been studied in great detail is the formation of dental enamel and the role of amelogenin [34,45,67,68]. Here, Cryo-TEM analysis and structural analysis of the protein have demonstrated a templating interaction between the forming mineral and amelogenin [67,68]. As expected, phosphorylation of the amelogenin seems to be beneficial for the stabilization of ACP, although it is not a prerequisite for the formation of enamel-like structures in mineralization reactions [45]. The ternary structure of the amelogenin seems to be important in the templating interaction, and is influenced by the presence of phosphorylation groups [45]. A similar templating interaction where the protein undergoes structural deformations upon binding with the CaP was also observed with the dentin matrix-protein-1 [69], a phosphorylated protein involved in dentin formation, and with osteopontin [63], a highly phosphorylated glycoprotein which is expressed in many mineralized and soft tissues. Finally, a well-known example of a phosphorylated molecule that stabilizes ACP is DNA, forming

nanometer-sized DNA–CaP complexes [70], which are used for cell transfection (see below), although little is known about the actual stabilization mechanism. Overall, phosphorylation seems to be important for the stabilization of ACP to achieve a possible templating interaction between the protein and the CaP and to influence the ternary structure of the protein. It is plausible that most of these functions can also be achieved by carboxylic acid residues, although chemical similarity between the phosphorylated residue and the CaP could be an important advantage.

Calcium phosphate nanoparticles and gene delivery

While understanding the fundamentals behind biomineralization will undoubtedly lead to new ideas for design of CaPs in the biomedical field, impressive results have already been achieved in a variety of applications. One such application is targeted drug delivery using CaP nanoparticles. CaP nanoparticles can be synthetically prepared and used for the delivery of (bio)molecules in the body and also into cells [71–75]. A clear advantage compared to other nanoparticles is their high similarity to bone mineral as stated above, making them biocompatible, and also their biodegradability under moderately acidic conditions (like during the resorption by osteoclasts or inside a cell in a lysosome) [76]. In addition, CaP is well known as excellent adsorbent for many biomolecules, a property that has led to its application in the chromatographic separation of biomolecules like nucleic acids [77]. As other nanoparticles, CaP nanoparticles are taken up by cells by endocytosis and related mechanisms [78–83]. They end up in endosomes which subsequently fuse with lysosomes with an acidic pH (around 4). Under these conditions, CaP dissolves [84]. The corresponding ions can be metabolized or excreted by the cell. Whereas phosphate is harmless, an increased level of calcium ions in the cytosol can be harmful and may lead to subsequent cell death [85,86]. Unless the cell is able to pump out the calcium ions within a few hours, it will eventually die. This happens if cells are subjected to large quantities of CaP nanoparticles, an effect which is also discussed in the context of atherosclerosis [87].

However, if moderate amounts of CaP nanoparticles are applied, they are well tolerated [88], and the nanoparticles can serve as carriers to transport all kinds of molecules across the cell membrane [89,90]. It is important to note that many molecules cannot cross the cell membrane alone due to their charge or their size, and that many receptors for drug molecules are located inside the cell [91]. In the following, we will discuss current applications of functionalized CaP nanoparticles.

A prerequisite for a successful application of nanoparticles is their colloidal stability in the dispersion medium. This medium is often water after the synthesis, but in the cell culture and in the body it consists of an aqueous solution of salts, carbohydrates, lipids, and proteins [92–95]. While an increasing salt concentration often leads to the destruction of an electrostatic colloidal stabilization and subsequent agglomeration, the adsorption of proteins can enhance the dispersibility due to an additional steric stabilization. If the nanoparticles are not sufficiently colloidally stabilized, they will agglomerate to microparticulate aggregates which will not be able to penetrate the cell wall due to their size, and which will be rapidly degraded by phagocytosis [96].

Prominent biomolecules which alone cannot penetrate the cell wall due to their negative charge are nucleic acids, that is DNA, RNA and other oligonucleotides. With the help of nanoparticles, they are able to enter cells and influence the protein synthesis inside a cell [97–99]. The non-viral introduction of DNA into a living cell is called transfection. If the DNA travels into the cell nucleus, it will cause the production of the protein whose sequence is encoded in the DNA. This is a way to specifically upregulate the protein synthesis inside a cell. The other option is the introduction of small-interfering RNA (siRNA) or micro-RNA (μRNA) into a cell which can suppress the production of a specific protein ('gene silencing') already by their presence in the cytosol. Together, these two techniques can be used to genetically manipulate cells and tissues and to enhance or suppress specific proteins, constituting the exciting field of 'gene therapy' [97–100].

Cells take up nanoparticles as long as they have a suitable size (up to about 200 nm) and charge (a positive charge helps), regardless of their chemical composition [78,79,81]. Obviously, this creates problems when a specific tissue is to be addressed by nanoparticles. To address tumors, the so-called 'enhanced permeation and retention effect' (EPR effect) is often proposed [96,101]. In this process, the facts that the blood vessels leading to a tumor are leaky (i.e. possess larger pores than normal blood vessels) and that a tumor does not possess a lymphatic system lead to an enrichment of nanoparticles inside a tumor. Broadly speaking, the access of nanoparticles to a tumor is enhanced and their removal is restricted, causing an enrichment of nanoparticles inside the tumor. A second option is the surface-functionalization of nanoparticles with suitable targeting moieties like antibodies, peptides or aptamers to direct them to a specific cell or tissue type [102–106]. This can be easily achieved for metallic nanoparticles (e.g. gold, silver) by thiol coupling chemistry [107,108], but does not work straightforward for ionic CaP where a direct covalent functionalization is impossible. However, after the addition of a coating layer of silica, CaP can be easily functionalized by well-established siloxane chemistry [109].

Gene therapy in biomaterials science typically involves the upregulation of genes which enhance bone or vessel growth. This leads to the production of proteins like bone morphogenetic proteins (BMPs) or vascular endothelial growth factor (VEGF) [110,111]. With DNA being cheaper and easier to prepare and purify than proteins, this is a promising alternative to conventional delivery of the proteins, for example into an implantation site or a bone defect. Conceptually, the protein level is also more constant due to the permanent production by the surrounding cells. As the transfection with nanoparticles is only temporary (a couple of days or weeks), there is no risk of a permanent genetic manipulation of the host.

Besides other types of nanoparticles and polymers, custom-made CaP nanoparticles have attracted attention as biocompatible, inorganic gene delivery systems [75,112–116]. Different ways to achieve a local transfection have been proposed, ranging from an electrophoretic deposition of DNA-loaded nanoparticles on metal surfaces [66] to their incorporation into polymer films in a layer-by-layer process [117] to a water-based paste of CaP nanoparticles for direct introduction into a bone defect (Fig. 2) [118]. It should be noted that *in situ* precipitated CaP nanocrystals have been used as transfection agent in cell biology since 1973 [119], albeit with limited reproducibility and efficiency [120,121].

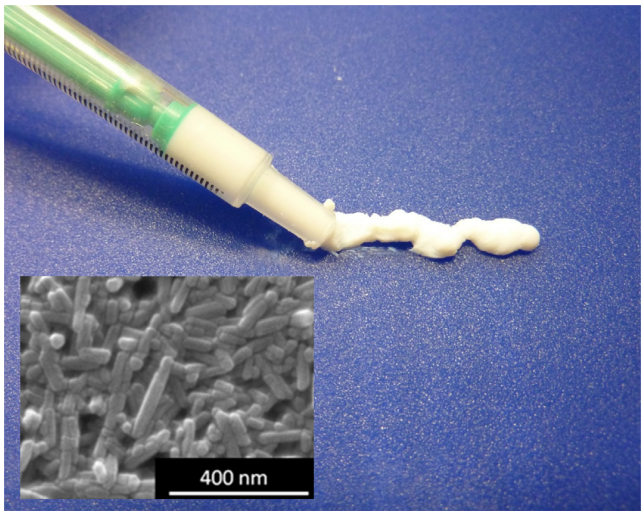

FIGURE 2

Calcium phosphate nanorods, loaded with DNA encoding for BMP-7 and VEGF-A, as genetically active paste for bone defect augmentation [118].

CaP nanoparticles can be visualized in cell cultures and *in vivo* by adding suitable fluorescent moieties. One way is the incorporation of fluorescent lanthanide ions like Eu^{3+} into the CaP lattice [122–124]. The other way is the functionalization (either covalent or by adsorption) with fluorescent dyes [84]. This permits an easy detection of nanoparticles inside cells and also *in vivo* when near-infrared dyes (NIR) are used [125].

In principle, CaP nanoparticles can be loaded with almost all molecules and drugs, except for hydrophilic small molecules which will be washed out by the surrounding aqueous medium. If they are prepared in a multi-shell way, they can carry more than one drug or fluorophore (Fig. 3). Their applicability in immunology has also been demonstrated, together with other kinds of nanoparticles [126]. They were loaded with TLR ligands to stimulate an immune response and with specific antigens against viral infections. It was shown both *in vitro* and *in vivo* that such CaP nanoparticles can upregulate the immune response and lead to both prophylactic and therapeutic vaccination [127], also against retroviruses [128]. Remarkably, it has been shown that CaP nanoparticles alone can enhance the immune response, even without biomolecules [129,130].

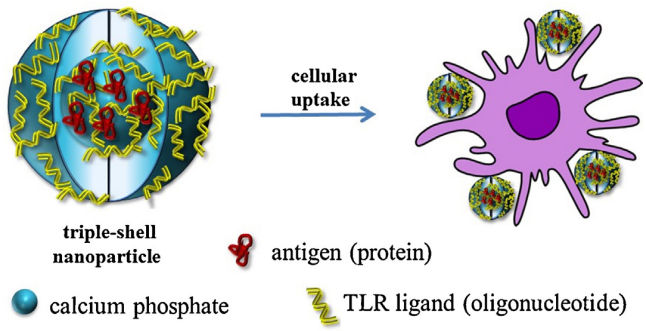

FIGURE 3

A multi-shell calcium phosphate nanoparticle, carrying an antigen and a TLR ligand as adjuvant for stimulation of the immune system [401].

In summary, CaP nanoparticles represent a promising alternative to other nanoparticles (e.g. gold, silica, polymers) because the body is already accustomed to them, because they dissolve after cellular uptake, and because they can be loaded with many different (bio-)molecules. Their synthesis is comparatively easy, straightforward and inexpensive.

Calcium phosphate bone graft substitutes

CaPs are among the best bone graft substitutes because they promote rapid bone formation on their surface, and may assure bone healing within a year. Several reasons can be invoked to explain these excellent properties: (i) the main constituent of our bone is biological apatite (as demonstrated above), therefore calcium and phosphate ions are present in large quantities in human body; (ii) various CaPs are resorbed by a cell-mediated process [5,131], ensuring not only a concomitant material resorption and bone formation process, but also an absence of biocompatibility issues due to the uncontrolled release of large amounts of degradation products; (iii) calcium and phosphate ions have a direct potent effects on bone cells, with in particular phosphate ions being thought to trigger an osteoinductive response [132].

CaPs were first proposed as bone graft substitute in the 1970s [2,133], but it was only in the late 1990s that their use spread out as a consequence of the appearance of diseases such as AIDS [134], and BSE [135], as well as stricter regulation for nature-derived products (xeno- and allografts). Even though the basics of the use of CaP materials for bone substitution were laid early on, important improvements have been achieved in the last decade: design of ready-to-use injectable pastes, much better understanding of the factors triggering the osteoinductivity of CaP bone graft substitutes, or development of additive manufacturing approaches allowing the production of custom-made implants in the operating room.

Handling of CaP bone graft substitutes

Originally, CaP bone substitutes were only available as sintered blocks and granules [4,133]. Porous blocks (pre-forms) excel in filling of defects with a predictable shape and size, such as burr holes, osteotomy gaps, and cavities in inter-body fusion cages, but tight filling of irregular defects with good implant-to-bone contact is almost impossible. Contrarily, granules can fill any defect form but their handling is poor. Indeed, filling a narrow defect with granules is very difficult, and if granules fall outside the borders of the defect, they need to be removed, which may be cumbersome. Cleaner application is facilitated by matching the granule size to the defect size. As a result, granules are smaller in the dental field (<1 mm), where bone defects are relatively small, than in the orthopedic field (1–6 mm) [136]. Also, large efforts have been made over the past 4 decades to improve the handling of CaP bone graft substitutes with the aim to provide injectable, moldable, and/or ready-to-use pastes.

Injectable CaP cements

In 1983, Brown and Chow [137] presented a CaP hydraulic cement consisting of two CaP powders (tetracalcium phosphate and dicalcium phosphate) and an aqueous solution. Hardening occurred within less than an hour by a combination of raw material dissolution and apatite precipitation (i.e. crystallization). This discovery opened up the possibility to provide injectable pastes to clinicians. In 1995, a report on one of the first clinical uses of CaP cements

suggested that CaP cements could not only heal bone, but even stabilize bone fractures, thus rendering the use of metallic internal fixators, the so-called osteosynthesis plates and screws, obsolete [138]. However, it soon appeared that internal fixators were still needed because of limited mechanical properties of CaP cements, and that the mixing system of the cement was difficult to use and prone to errors. Also, not all of the paste could be extruded with the injection system due to the so-called filter-pressing phenomenon: the liquid migrating between the solid CaP particles while injecting the paste, eventually leading to a wet densified powder plug at the plunger side, unamendable to injection [139]. This problem was solved in more recent CaP cement formulations by increasing the viscosity of the mixing liquid [140–143]. Nevertheless, this approach is costly and CaP cement mixing still requires a large number of manual operations before an injection can be performed. Also, once the CaP cement is mixed, there is only a limited period of time during which the cement can be injected.

Ready-to-use CaP cements and pastes

The very limited handling and injection window of CaP cements has triggered the development of ready-to-use CaP cements consisting of one or two pastes. One approach consists in keeping the cement components in a non-aqueous hydrophilic or hydrophobic liquid [144–146]. This approach works with any formulation, and hardening only occurs once the paste is injected into the bone defect. On the negative side, the use of an additional component makes production and certification more difficult, and the release of large amounts of a foreign liquid during injection may result in adverse biological reactions. Also, hardening is slow and volume-dependent because it relies on the exchange or replacement of water with the hydrophilic or hydrophobic liquid [146]. A second approach to formulate ready-to-use pastes consists in dispersing the reactive CaP powders in aqueous solutions. Unfortunately, there are only very few reactive CaP powders that can be stabilized in aqueous conditions. Lemaitre et al. [147] proposed to combine an acidic (reactive) monocalcium phosphate monohydrate (MCPM) paste with a basic (fairly inert) β-tricalcium phosphate (β-TCP) paste to obtain brushite after reaction. Recently, Bohner et al. [148] showed that small amounts of divalent cations, such as Mg^{2+} can stabilize α-TCP aqueous pastes for at least one year at room temperature, and that the latter paste can then be reactivated by adding a small volume of highly concentrated Ca^{2+} solution. To minimize the drawbacks of the non-aqueous and aqueous approaches, Chow and Takagi [149] placed the reactive CaP components in a non-aqueous paste and triggered the reaction by mixing the non-aqueous pastes with an aqueous paste. Currently, there are only very few commercial formulations due to issues such as shelf-life stability [150], limited reactivity [148], sterilization, price, and phase separation during storage, but there is a clear trend and clinical desire toward ready-to-use CaP cements.

The difficulties to design ready-to-use CaP cements have led to the development of non-hardening, ready-to-use pastes made of a mixture of CaP particles and an aqueous solution [151–154]. Such formulations are not only very easy to use ('open the package and inject'), but cheaper and easier to produce than CaP cements. Nevertheless, there are concerns about the biological response of dispersed CaP particles. Indeed, Malard et al. [152] described inflammatory reactions after implanting 10–20 μm biphasic calcium phosphate (BCP, consisting of HA and TCP) particles. Similar results were reported with sintered CaP particles [155] and CaP cements [156]. Wang et al. also reported a loss of osteoinductive potential with particles smaller than 45 μm [157]. However, what is true for microparticles might not be true for nanoparticles, because nanoparticular pastes have a much better cohesion than microparticular pastes [158] and because nanoparticles – contrary to microparticles – are much smaller than cells.

Moldable CaP pastes

Another trend of the new millennium is to combine the good handling properties of a paste with the good biological properties of a granular bone substitute. For that purpose, CaP granules are embedded in a viscous polymer matrix which can be rapidly cleared from the body by enzymatic cleavage or transport once implanted [154,159]. A wide range of polymers has been considered, including fibrin [160], hyaluronan [161,162], collagen [163,164], alginate [159], various kinds of cellulose [153,165,166], and poly(trimethylene carbonate) [167]. Generally, these pastes contain particles larger than 50–100 μm, and may require to be mixed in the operating room. In the latter case, it is sometimes possible to produce or combine the pastes with blood, bone marrow, or platelet-rich plasma. In contrast to the previously mentioned problem of granules spreading around the defect site, the polymer provides cohesion and keeps the particles in place until the wound is closed and clotting occurs.

To conclude this section on the handling properties of CaP bone graft substitutes, it is likely that ready-to-use pastes will soon replace granules as the most frequently used form of CaP bone graft substitute.

Calcium phosphates and bone healing

Historically, first efforts to synthesize and design CaP bone graft substitutes were focused on sintered CaPs, namely sintered HA [3], β-TCP [2,133], or their mixtures (BCP) [154,168]. The aim was to obtain a material with sufficient mechanical strength for load-bearing applications, in particular for internal fixation ('osteosynthesis'), but still resorbable enough to allow a full conversion into bone during bone regeneration. A special interest was paid to sintered HA due to its crystallographic and compositional similarity to bone mineral, its high strength, and its comparatively easy production. However, whereas bone mineral can be remodeled by the action of osteoclasts, sintered HA is biologically practically inert and not biodegradable [169]. Furthermore, dense CaPs are too brittle to be used in load-bearing applications, and polymer–CaP composites have failed due to insufficient mechanical properties [170] or inadequate resorption behavior [171]. Therefore, a paradigm shift occurred at the turn of the millennium: instead of designing load-bearing bone graft substitutes, researchers aimed for CaP bone graft substitutes providing a fast healing response, that is a fast turnover from a bone defect to mature (= mechanically competent) bone. Strategies have included a change in composition, and an improvement of the material architecture at both the micro and macro level, as discussed in the following sections.

Change of composition to achieve faster bone defect healing

Besides sintered HA and β-TCP, two other Ca orthophosphate phases can be obtained by sintering: tetracalcium phosphate

(TetCP) and α-TCP. Due to its basicity, TetCP has only been proposed as antimicrobial agent [172]. Therefore, early efforts to find a CaP material with fast resorption were focused on α-TCP, a metastable polymorphic form of β-TCP that has a higher solubility than β-TCP. CaP cements made of α-TCP powder are considered to have excellent biocompatibility [138,173], not just after hydration to CDHA, but also immediately after injection when the ceramic is still mostly constituted of α-TCP. Also, the few studies reporting the *in vivo* performance of implanted α-TCP granules are generally positive [174]. Nevertheless, α-TCP is thought to be resorbed too fast and has hardly been investigated as raw material for larger granules and shaped blocks. So, until the discovery of CaP cements, research remained focused on HA, β-TCP and their mixtures (BCP).

The discovery of CaP cements widened the research scope by allowing the synthesis of solids consisting of phases only stable at or close to room temperature. Of particular interest are not only the two typical end-products of CaP cement reactions, namely calcium-deficient HA ('CDHA') [175,176], and dicalcium phosphate dihydrate (DCPD; 'brushite'), but also OCP and dicalcium phosphate anhydrate (DCPA, 'monetite') [177–180], which can be obtained by hydrolysis or dehydration of DCPD. Apart from having often a higher solubility than that of β-TCP, CaP materials produced at or close to room temperature have two interesting features compared to sintered CaPs. First, they have specific surface areas (SSA) that are often close to the values of bone mineral (≈80 m^2/g) and as such up to two orders of magnitude higher than the values exhibited by sintered CaPs (typically below 1 m^2/g). High SSA values are believed to stimulate protein adsorption, which is a very important event in the healing cascade. Second, DCPD and OCP are precursor phases for apatite formation. Since bone mineral is also formed from precursor phases, as is described in the section on biomineralization above, several authors have speculated that the *in vivo* conversion of DCPD and OCP into apatite enhances bone formation [181,182]. Indeed, DCPD [173,183] and OCP [181] are rapidly converted to apatite after implantation. Also, DCPA, DCPD, OCP, and CDHA have been suggested to have superior biological properties compared to sintered CaP ceramics [184]. Nevertheless, the number of studies comparing sintered and non-sintered CaP bone graft substitutes is very limited and their interpretation is constrained by the difficulty to control all physicochemical properties of CaP materials affecting their biological response. To date, it is possible to produce almost any type of CaP in almost any shape, but studies proving the superior biological behavior of CaP phases obtained at or close to room temperature are still missing.

Architecture

n 1970, at the start of bone substitution research, Hulbert et al. [185,186] already described the importance of pore and pore interconnection size for the biological response to porous ceramic bone substitute. He recommended to incorporate pores larger than 100 μm (=macropores) into bone graft substitutes. In 1984, Klein et al. [5] underlined the fact that not only macropores but also micropores (typically close to 0.1–10 μm) were essential to provide a fast resorption. However, it was only after the establishment of tissue engineering by Langer and Vacanti in 1993 [187] that a boom occurred in the research devoted to the control of the architecture of CaP bone graft substitutes and to the understanding of the link between implant architecture and biological response.

The first efforts to perfectly control the architecture of CaP bone graft substitutes relied on stereolithography: polymer molds were first printed and subsequently used for HA slip casting and sintering [188]. In 2005, Seitz et al. [189] started to directly print 3D scaffolds using an ink-jet printer, a water-soluble polymer ink, and HA powder. In 2007, Gbureck et al. [179,190,191] removed the need to sinter the printed pieces by jetting phosphoric acid onto an α-TCP powder to directly form a ready-to-use 3D printed brushite scaffold without organic additives. Since several inks can be used simultaneously, chemically complex scaffolds can be produced, for example printed pieces with spatially localized drugs [190]. Unfortunately, 3D printing is manually demanding and printing hollow structures is strongly limited by the need to remove the powder from unprinted volumes (the so-called 'depowdering'; Fig. 4a) [192]. Also, it is not easy to perfectly control the composition of the printed pieces, and post-treatments in acids are generally required.

Another additive manufacturing technique called 'robocasting' is based on the extrusion of a thick (solid-rich) CaP slurry through a thin nozzle [193] (Fig. 4b). Compared to 3D printing, robocasting is not as versatile in terms of materials and geometries (not possible to print overhanging parts) but provides a higher printing accuracy (typically below 100 μm [193] compared to ≈500 μm with 3D printing [194,195]), and is not subject to the strong limitations of the depowdering step encountered in 3D printing [192]. Initially, robocast scaffolds relied on a two-step process consisting of (i) printing, and (ii) sintering [193], but in the meantime, various companies have developed CaP pastes that can be directly used to print a scaffold in the operating room. Several companies have started commercializing products based on additive manufacturing, for example scaffolds with an oriented architecture to promote bone ingrowth in a specific direction [196], or innovative craniofacial implants combining a 3D-printed titanium mesh and DCPA ceramic tiles (Fig. 4c).

Considering that 60–70% of all bone graft substitutes are still sold as granular materials, various research groups started working on granules with controlled geometry, the goal being to control the pore size created between the granules [197,198]. A particularly interesting approach was proposed by Choi et al. [199] who used injection molding to produce 'tetrapods'. Nevertheless, despite all efforts that have been made to produce CaP bone graft substitutes with controlled architecture, the ideal architecture providing an optimal biological response has been defined only in very general terms [200] and is still a matter of debate [201–205]. Indeed, most researchers will agree that micropores (around 1–10 μm) and macropores (>100 μm) have a favorable effect on bone formation, but precise recommendations regarding the pore volume fraction or size are still missing.

Osteoinductive calcium phosphate ceramics and the use of bioinorganics

One of the important achievements in the field of CaPs in the past few decades was the development of materials with intrinsic osteoinductivity. The importance of the phenomenon of osteoinduction, initially defined by Friedenstein as the process of the

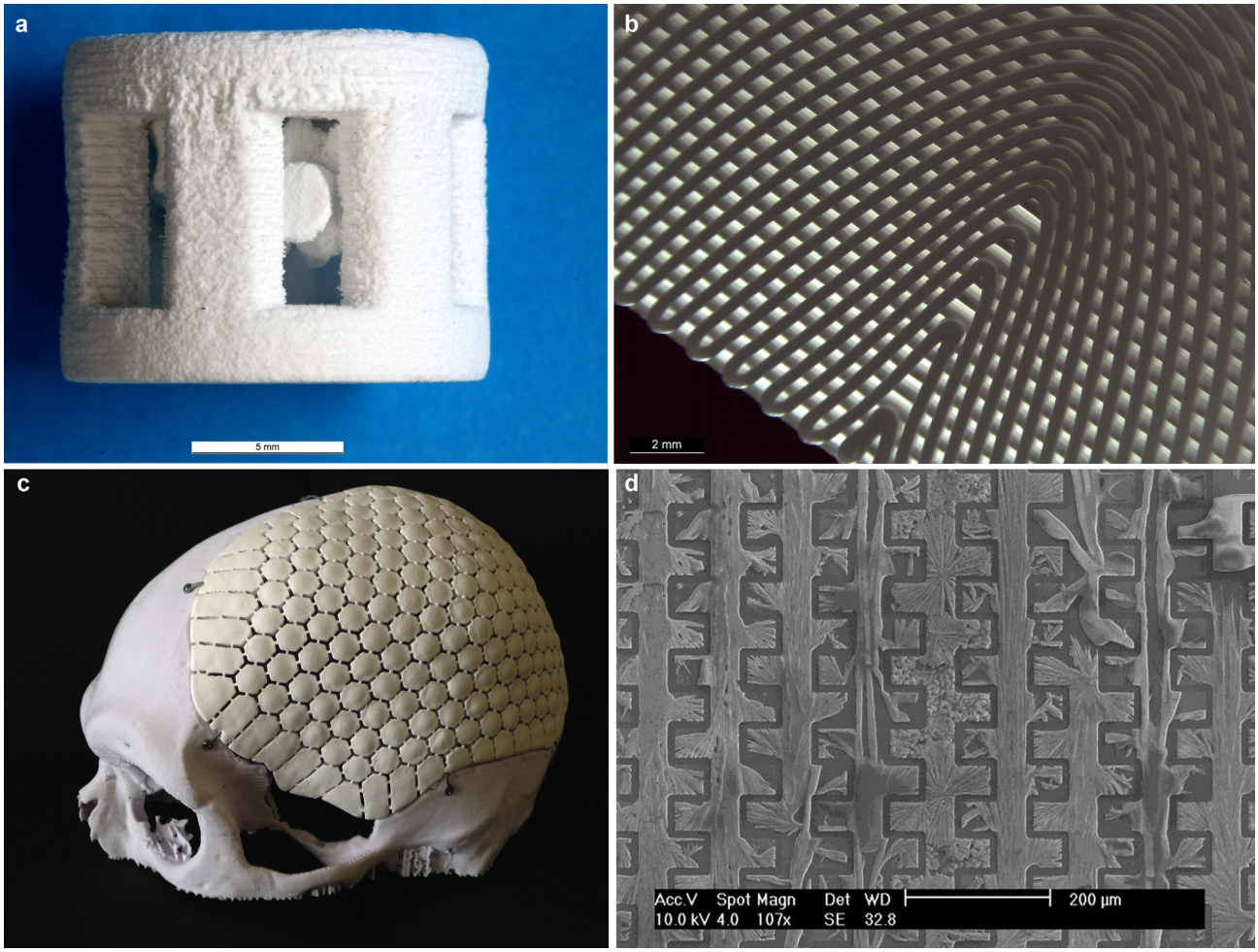

FIGURE 4

Examples of structures obtained by additive manufacturing techniques. (a) 3D printed scaffolds made of DCPA/monetite (scale bar: 5 mm) [192], (b) solid obtained by robocasting (scale bar: 2 mm; courtesy of S. Heinemann, InnoTERE GmbH, Germany), (c) 'Craniomosaic': a DCPA based implant for treatment of cranial bone defects. The device uses a 3D-printed titanium mesh covered with DCPA ceramic tiles (courtesy of J. Aberg, OssDsign, Sweden), (d) pattern of CaP created on a silicon substrate using soft lithography (scale bar: 200 μm).

'induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells' [206] was recognized following the seminal work by Urist, who showed bone formation by hydrochloric acid-decalcified diaphyseal bone in muscles of rabbits, rats, mice and guinea pigs [207]. Further work, directed toward understanding of the mechanism of this heterotopic bone formation, led to identification of Bone Morphogenetic Proteins (BMPs) as inducer of the cascade of chemotaxis, mitosis, differentiation, callus formation and finally bone formation [208]. In general, a successful clinical application of BMPs, with emphasis on commercially available BMP-2 and BMP-7 (OP-1) in spinal fusion and defect caused by trauma [209–211], logically strengthened the perception of osteoinduction as being a highly important property of a bone graft substitute. First reports on heterotopic bone formation triggered by a synthetic biomaterial that did not contain BMPs or any other biological factors caused both disbelief and excitement, considering important advantages of synthetics *versus* biologics, such as generally lower cost of production and better stability.

One of the first reports on *de novo* bone formation induced heterotopically by a synthetic biomaterial was published in 1969, where Winter and Simpson described an observation of bone induction by a sponge made of polyhydroxyethylmethacrylate (poly-HEMA) in the soft tissue of pigs [212]. It was, however, not until 1990s that researchers actively started searching for synthetic biomaterials with intrinsic osteoinductive potential. Until now, a range of CaP-based biomaterials and a handful of other materials, all with the ability to mineralize *in vitro* and *in vivo*, have been shown to induce heterotopic bone formation to various extents. Osteoinduction has been demonstrated for various CaP phases, including HA [213–228], TCP [223,225,229–238], various blends of the two in the form of BCP [218,219,221–225,236,238–247], DCPD [179], DCPA [179], carbonated apatite (CA) [239,240,248] and OCP [249,250], and in various forms such as sintered ceramics [213,214,216,225,227,251,252], cements

[179,224,247], coatings [249,250], as well as coral-derived ceramics [213,214,227,228,253,254], in a number of animal models.

While a growing number of studies confirmed that osteoinductivity can be an intrinsic property of some CaP ceramics, the clinically relevant question remained whether such ceramics would also result in an improved regeneration of challenging, critical-sized bone defects. In other words, can we consider osteoinductive CaP as a true alternative to natural bone grafts? In a number of studies, a direct comparison was made between osteoinductive and non-osteoinductive ceramics in regenerating bone defects, including critical-sized ones, in general concluding that a more pronounced bone formation occurred with ceramics with an osteoinductive capacity [179,239,241,248,255,256]. Although in some of these studies an attempt was made, either by implant design [179] or by analysis method [241], to prove that osteoinduction, independent of osteoconduction, occurs in large bone defects as well, no conclusive evidence has been presented yet. Probably the best proof for clinical relevance of CaP ceramics with intrinsic osteoinductivity was given in the study by Yuan et al. in which an osteoinductive TCP ceramic was compared to an autograft and to a rhBMP-2 construct [238]. The osteoinductive ceramic was shown to be at least as successful in bridging an ovine critical-sized iliac wing defect by newly formed bone as either the autograft or the rhBMP-2 construct.

Despite these clinically relevant achievements, the mechanisms behind the CaP-induced bone formation are still incompletely understood. The facts that ceramic-induced bone formation is always intramembranous, in contrast to BMP-2-induced formation that mainly occurs *via* the endochondral pathway [257], and that material-induced ectopic bone formation is relatively slow as compared to the BMP-2-driven case [218,235,258,259], suggests different mechanisms, but the reason for this is still unknown. An issue that strongly hampers the study of the biological mechanism behind osteoinduction is the fact that a ceramics-induced heterotopic bone formation preferentially occurs in large animals, such as baboons, dogs, sheep and goats and less so in rodents such as mice and rats, as was shown in a number of studies comparing osteoinductive potential of ceramics in different animal species [219,245]. In a recent study by Barradas et al. in which 10 different mouse strains were compared, only one was found to be suitable for studying osteoinduction by biomaterials, suggesting the importance of genetic make-up [231]. Finally, also the exact implantation location (subcutaneous *versus* intramuscular) and size of the implant were shown to affect the extent of new bone formation [240].

Within the limitations described here, various attempts have been made, on the one hand, to understand which material properties are essential for rendering a ceramic osteoinductive, and on the other hand, to describe biological mechanisms behind the phenomenon of osteoinduction. Chemical composition, macrostructure and surface micro- and nanostructural properties have all been looked into for their relevance in the process of osteoinduction. Regarding the effect of chemical composition, as was mentioned earlier, CaPs with different phase composition have shown the ability to induce bone formation. A few studies comparing HA and TCP, or HA and BCP, have demonstrated that the presence of a more soluble phase is beneficial for the amount of induced bone [218,219,222], while in other studies, it was shown that the addition of TCP negatively affected bone induction [236] and that a relatively stable surface was needed for *de novo* bone formation to occur [239]. Based on the current knowledge, it is suggested that an increase in *in vivo* degradability of CaPs in general is beneficial for osteoinduction, however, a relatively stable surface is required to facilitate *de novo* bone formation. In other words, a compromise needs to be reached between the level of dissolution/reprecipitation events occurring on the material surface and the rate of surface disintegration due to *in vivo* degradation [236,240]. In general, the effect of chemical composition can be dual; on one hand, increased calcium and/or inorganic phosphate ion concentrations as a consequence of release from the ceramic upon implantation can have a direct effect on the osteogenic differentiation of stem cells, as was recently demonstrated *in vitro* [260,261] and *in vivo* [132]. On the other hand, the proposed origin of bioactivity of all CaPs, that is the precipitation of biological apatite on the material surface *in vivo*, presumably incorporating endogenous proteins is also considered as possible indirect effect of materials chemistry [262].

The overall geometry of the implant, that is the presence of pores or predefined geometries, such as concavities and channels, or their formation post-implantation, has also been shown to be important to facilitate osteoinduction [179,215,224,240]. It has been suggested that these 'protected' spaces were needed to reach optimal conditions (e.g. calcium and phosphate ion concentrations) to trigger the process of osteoinduction [263]. Recently, Davison et al. demonstrated that osteoinduction indeed occurs on a planar surface of ceramics as well, that is in the absence of protective areas such as pores [264], although in lower amounts than with the macroporous counterparts [238]. This suggests that macrostructure is important but not essential property in the process.

Finally, surface structural properties including microporosity, grain size and therewith-related specific surface area have been shown to play a determinant role in the process of osteoinduction by biomaterials (Fig. 5). Concerning CaPs, important information about the effect of surface structural properties stems from studies in which sintering temperature was used as a tool to control grain size and microporosity [234,238,239,241]. Based on these studies, it was concluded that the submicrometer scale of grains and pores on the surface is the property which renders a ceramic osteoinductive [234]. This was specifically demonstrated for sintered ceramics, and although this finding cannot be directly extrapolated to all CaPs, the importance of surface-structural features is evident.

The identification of properties which influence a ceramic's ability to induce heterotopic bone formation (a more comprehensive overview can be found elsewhere [263]), has resulted in improvement of the existing and development of new biomaterials with intrinsic osteoinductivity, yet, no recipe exists for the optimal osteoinductive materials. One of the important reasons is that CaP materials are highly complex structures with intertwined properties, making it difficult to change one without affecting the others [265]. This explains why researchers have not succeeded yet at pinpointing a property or combination of properties that is essential for osteoinduction to occur. This is also true for the biological cascade leading to *de novo* bone formation. A few mechanisms have been proposed that are involved in this process, but no conclusive evidence exists so far. For example, it is not

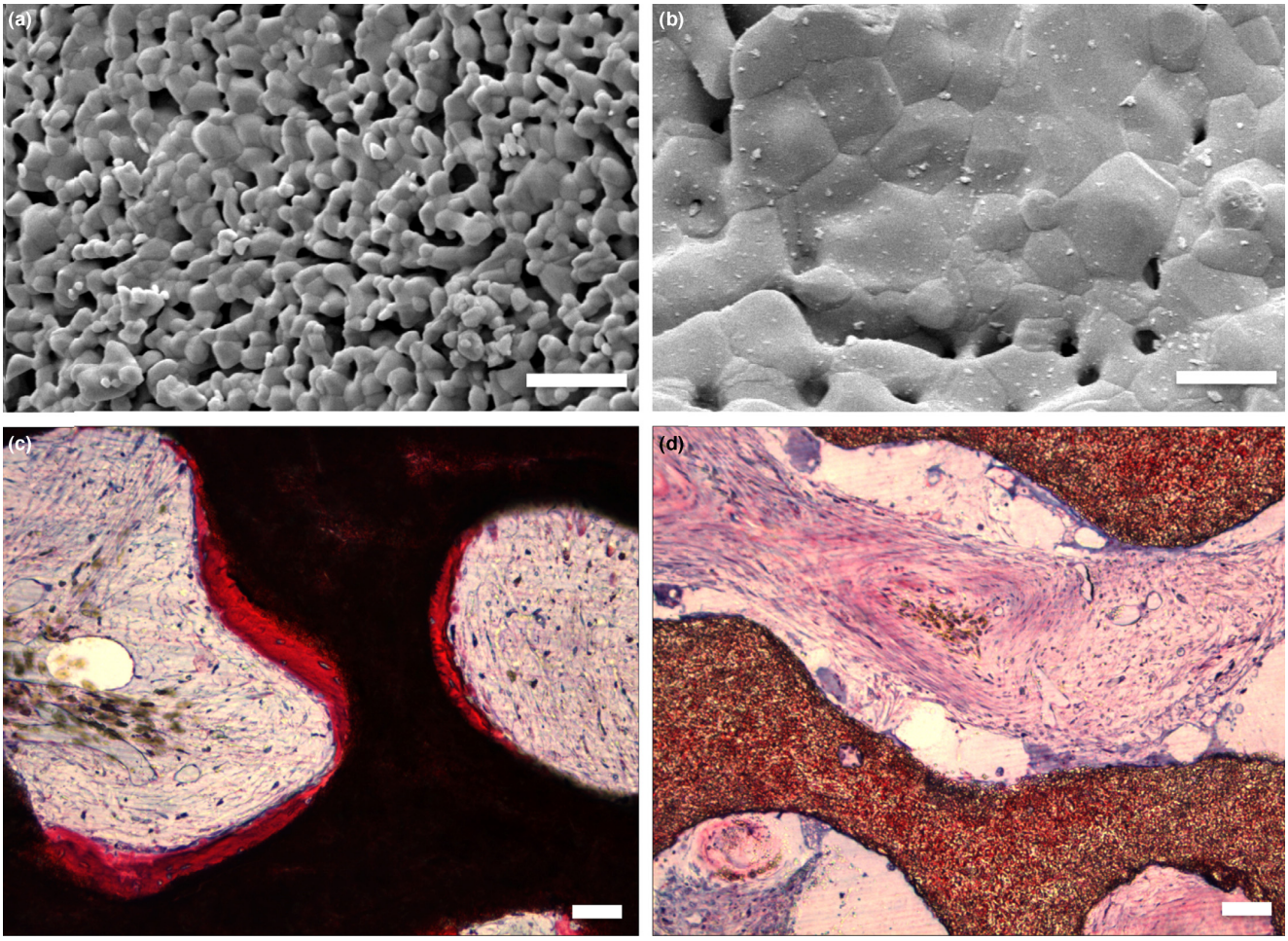

FIGURE 5

Physicochemical properties of calcium phosphate ceramics influence their intrinsic osteoinductivity. Osteoinductive biphasic calcium phosphate ceramic with small grains and micropores (a) induces bone formation upon 12-week intramuscular implantation in a goat (c), in contrast to its non-osteoinductive counterpart with larger grains and fewer micropores (b) that is only infiltrated by fibrous tissue (d). Scale bar: in (a) and (b) 5 μm; in (c) and (d) 100 μm.

known which cells in the heterotopic environment of muscle or subcutis are the ones being triggered to differentiate into the osteogenic lineage. Different theories exist in this regard, including cells associated with microvessels, such as pericytes and myoendothelial cells [218,235,245,246,266]. Using a canine model, Song et al. demonstrated that bone marrow stromal cells migrate from bone marrow through blood circulation to the heterotopic implantation site, possibly contributing to ceramic-induced *de novo* bone formation [267]. Different theories have also been proposed to explain the mechanism leading to bone formation. These include (i) direct trigger of osteogenic differentiation through physicochemical properties described above [222], (ii) trigger of osteogenic differentiation through accumulation or local production of endogenous osteoinductive proteins such as BMP-2 [215,253,268], (iii) osteogenic differentiation that is indirectly triggered as a consequence of the inflammatory response [269], and/or (iv) the process of osteoclastogenesis [233,234].

The mechanism governing osteoinduction by biomaterials cannot be discussed without shedding light upon a frequently observed clinical problem that closely resembles osteoinduction: heterotopic ossification. Heterotopic ossification, sometimes also called pathological ossification, is often simply defined as the presence of bone in soft tissue where bone normally does not exist. Heterotopic ossification can roughly be divided into two forms. The first, acquired form is often associated with trauma (fracture, total hip arthroplasty, muscular trauma) or has a neurogenic cause (spinal cord or central nervous system injuries) and is most common. In addition, there is the rare hereditary form, including diseases such as *fibrodisplasia ossificans progressiva*, progressive osseous heteroplasia and Albright hereditary osteodistrophy [270]. In both forms of heterotopic ossification, trauma or injury to the skeleton or soft tissue are the inducing events [271,272]. While clearly during heterotopic implantation of a material the surrounding tissue experiences injury, a material-induced *de novo* bone formation cannot be defined as simply a case of heterotopic ossification. As it was already mentioned, only some synthetic biomaterials, with specific properties, possess osteoinductive potential. Besides, a material-induced heterotopic

bone formation is always strongly associated to the implanted material, and is never found in the soft tissue distant from the material's surface. This, however, does not mean that similar biological mechanisms could not be involved in both phenomena.

It is probably a matter of time for the mechanisms behind osteoinduction to be fully unraveled, and for instructions as of how to produce a material with most pronounced or fastest occurring osteoinductivity, to be written. To achieve this, it is important to put efforts into separating individual properties of functional materials from one another and investigating their independent effects on biological response. Advantages for the clinical setting that synthetic biomaterials with superior biological performance, including intrinsic osteoinductivity and fast and successful healing of challenging bone defects, offer over biologics, justify these efforts.

One of the attractive approaches to enhance and accelerate osteoinductivity and bone defect healing capacity, while retaining their synthetic character, is the use of the, so-called, bioinorganics. These are relatively simple compounds, often present in the human body in trace amounts (e.g. in bone tissue) and yet known to play an essential role in normal functioning of organs and tissues. As extensively described in a recent review [273], strontium ranelate [274–276], which is an anti-osteoporotic agent, and fluoride [277,278], being an anti-cariogenic agent, are two well-known examples of a clinical application of bioinorganics in orthopedics and dentistry. While strontium ranelate is generally administered systemically, a growing need exists for local delivery methods, for example at the location of bone defects in the case of bone graft substitutes. Initially, bioinorganics were used in conjunction with bone graft substitutes and other orthopedic, craniomaxillofacial and dental implants with the aim to accelerate bone formation and improve bone bonding. For example, a large amount of work on silicon-substituted CaPs has been published since the 1990s [279–283]. But also strontium- [284–289], magnesium- [285–287], fluoride- [290,291] and zinc- [288,292–294] incorporation into CaPs, among other elements, as well as incorporation of combinations of bioinorganics [288,292,295–297] have been extensively investigated in this context. As mentioned before, CaPs are known to be capable of a large variety of anionic and cationic substitutions, making them a relatively easy-to-produce delivery vehicle for bioinorganics. Indeed, the incorporation of elements of interest can be achieved by the addition of a precursor during CaP powder precipitation, sol–gel process or in a solid-state reaction, and a great number of ion-substituted synthetic bone grafts exist, varying from bulk ceramics, cements and coatings on metallic and polymeric substrates [298]. While it is relatively easy to prepare bioinorganics-containing CaPs, the question remains whether these structures are optimal carriers and delivery vehicles for bioinorganics. If it is difficult to describe which property or properties are essential for a CaP ceramic to be osteoinductive as was described earlier, the addition of another ion into the CaP lattice will complicate this understanding even further. An important reason for this is that ionic substitution and, to a lesser extent, even physical entrapment of the compound of interest inside the ceramic material, will affect its physicochemical and biological properties. As an example, Ca can be fully substituted by Sr in the HA crystals. However, this results in a change of the surface morphology, with smaller and less sharp crystals formed, due to a difference in the ionic diameter between Ca^{2+} and Sr^{2+} [299,300]. Furthermore, strontium-substituted HA is more soluble than the phase-pure ceramic [301]. Finally, the release of Sr^{2+} is in principle dependent on the degradation of the structural ceramic phase, meaning that Sr^{2+} release is always accompanied by calcium and phosphate release. In other words, even when we observe differences between the biological responses to a phase-pure and a substituted ceramic, it is very difficult to conclude whether the difference is a consequence of the bioinorganic release, or of the change to the structural ceramic introduced by the addition of the compound. Also here, these individual effects will have to be separated to understand the mechanisms behind their action, which in turn can be used as input for the development of improved or completely new bone graft substitutes. This may require alternative approaches toward biomaterial development, where different materials and technologies are combined, instead of relying on the processing parameters that often limit the freedom of design.

Outlook

So far, we have highlighted important recent developments in CaP research, divided into topics of biomineralization, nanoparticles for targeted delivery and bone graft substitution. In this last section, we will touch upon a few topics, which we believe are worth investing in to secure the place for CaPs in future biomedical applications.

Identifying the relevant mineralization pathway

The results presented on biomineralization starting from the 2000s have brought valuable insights, of which some are already implemented in (preliminary) biomedical research [302]. In particular, the fact that in most biomineralization systems an amorphous phase is used as a precursor, indicates that this material could be the material of choice for bone implants, especially in the case of tissue-engineered constructs. ACP can be expected to be easily resorbed and restructured *in vivo*, although additives like charged molecules/proteins may be necessary to stabilize the material and perhaps manipulate its crystallization pathway. The most challenging task in the future, however, will be to identify the mineralization processes relevant for human bone remodeling. As most biomineralization studies are filled with short-cuts and artifacts that hamper the correct interpretation of the data, *in situ* investigation of bone formation and remodeling, such as recently performed with zebrafish larvae [33,35], is hereby the most promising pathway to follow.

Multifunctional nanoscopic CaP materials

CaP with nanoscale dimensions is well suited to interact with (bio-)molecules of all kinds due to the polar surface. This, together with the inherent non-toxicity and biodegradability inside cells and the body has the potential of a cell- and tissue-specific application, for example to fill bone defects (with agglomerated nanoparticles) or to combat diseases or infections in the body (with dispersed nanoparticles, for example in the bloodstream). Multifunctional CaP nanoparticles which combine drug delivery, imaging and targeting capabilities still have

to be developed, but in general, they are more versatile than metallic nanoparticles (with a solid core and an unknown degree of biodegradability), polymeric nanoparticles which typically consist of non-biological compounds, and liposomal or micellar constructs that are 'soft' by nature without a solid core.

Load-bearing bone graft substitutes

Currently, bone graft substitutes have three important weaknesses: their handling properties, their insufficient osteoinductivity/bone regenerative potential, and their inability to provide both cortical bone-like mechanical properties and excellent biological properties. The first two topics were discussed in length in this review. The last topic is an 'old new' topic. Indeed, researchers already tried in the 1970s and 1980s to provide bone graft substitutes with mechanical properties of cortical bone, but their efforts failed due to the intrinsic brittleness of ceramics, the biocompatibility issues caused by the use of large volumes of degradable polymers or metals [303], and the difficulties to combine polymers with ceramics without compromising the mechanical properties [170,171]. Recent knowledge from the field of biomineralization has clearly indicated that the architecture of natural composites such as bone and nacre is responsible for their high tensile and toughness properties [304,305]. Also, various authors have proposed innovative methods to produce architecturally complex structures with high level of control and have shown that outstanding properties can be achieved [306–310]. Finally, uniform CaP particles displaying high aspect ratios, and sub-micrometric thicknesses can be

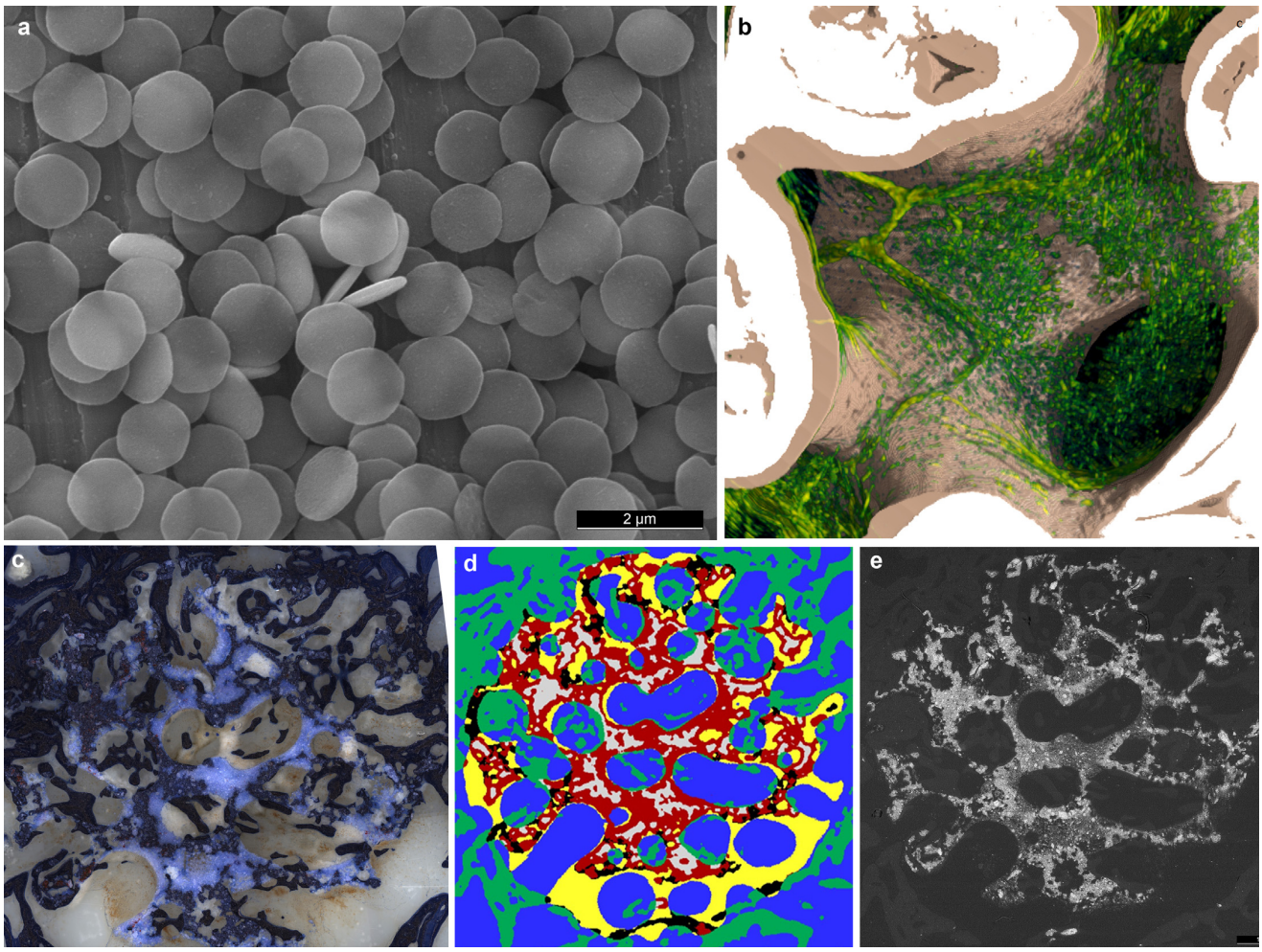

FIGURE 6

(a) Almost uniform β-TCP platelets with high aspect ratio and small thicknesses. Scale bar: 2 µm. (b) 3D pseudo-holotomographic images of a tissue-engineering construct after 24 weeks of implantation showing vessel growth (green) and newly-formed bone (brown/pink) in one single pore of the implanted scaffold (white); Image width: roughly 2 mm (Courtesy of V. Komlev and R. Cancedda) [319]; (c–e) direct comparison between histology and micro-computed tomography (µCT) image. Scale bar: 1 mm. (a) Histological section of a β-TCP scaffold after 6 weeks of implantation [402]. The section is stained in toluidine blue. Color code: gray/white: soft tissue, light blue: ceramic, dark blue: 'bone'. (d) µCT image of the histological section; the image is obtained by comparing the µCT image of the β-TCP implant before and after implantation and extracting the histological section from the 3D image. Color code: blue: soft tissue; red = ceramic; gray = zones within the ceramic with a higher radio-density than the ceramic in red; green = bone; yellow = zone where the ceramic was resorbed during implantation; black: zones where the ceramic was resorbed during implantation and replaced by new bone (Courtesy of Courtesy of A. Sweedy and G. Baroud, Université de Sherbrooke, Canada). (e) SEM image in back-scattered electron mode of the histological section shown in (c). Color code: white = high radio-density; black = low radio-density.

produced [311–315] (Fig. 6a). This, taken together, should lead to development of resorbable bone graft substitutes combining high bioactivity and cortical bone-like tensile properties in the near future.

Understanding and controlling the in vivo *behavior of bone graft substitutes*

As underlined in the present manuscript, many aspects of the *in vivo* behavior of bone graft substitutes are still poorly understood: What is the exact mechanism behind osteoinductivity? What is the optimal chemistry and optimal architecture of a bone graft substitute? Answering these questions will lead to major advancements in properties and performance of the existing bone graft substitutes. To this end, both technological developments allowing a more precise design and production of biomaterials with well-defined properties and improved assays to study interactions of biomaterials with a biological system will play an important role. For example, it is envisioned that conventional techniques to produce bone graft substitute will be replaced by those allowing design of scaffolds with precise architectures to obtain new insights into the cell–material interactions, such as the importance of surface curvature [316,317]. Another example includes the application of patterning techniques of chemical or structural cues to obtain spatial and/or temporal control over a biological response (Fig. 4d). Advancements in biological assays will, on the other hand, bring deeper understanding of cell–material and tissue–material interactions. For example, the availability of genomics data describing these interactions is growing [318]. Also advanced and/or multi-imaging approaches provide unprecedented details about the healing process of soft and hard tissues, not only post – but also during implantation [319–322] (Fig. 6b–e). A particularly interesting novelty is the possibility to get spatio-temporal information: how much resorption and bone formation occurs in one specific pore over time? Is there a correlation between these two phenomena [265,323]? It is envisioned that, eventually, we will have information on correlation between a specific property of a biomaterial and a specific biological response. A next step will then be development of smart strategies to combine right properties to obtain desired response. This approach will also require the use of heavy and complex data processing tools, but for some research groups, this is already reality.

CaPs as bioinorganics reservoirs

Increasing amount of evidence gathered over the past 15 years demonstrates that the delivery of specific ions can trigger biological responses. The next step in the bioinorganics research is to step away from the idea of these ions being a structural component of CaPs as substitute in CaP crystal lattice, like in natural bone, but to consider them as drugs. This will require different strategies to control and monitor their release and consequent biological response [324], like in the case of classical drugs or growth factors. CaP could then be reservoirs of calcium or phosphate ions, or other bioinorganics, but polymers can also be used to locally deliver calcium or phosphate [325]. *In vitro* screening methods to identify biological response to a (combination of) bioinorganics is the first step in this direction [326], but eventually, *in situ* ion release should be achieved and coupled to an analysis of the *in vivo* response.

Conclusion

Here we have given an overview of some truly impressive advances achieved in the past 15 years in the field of CaPs. Not only did the knowledge of fundamental processes governing biomineralization of CaPs grow tremendously, but their application as targeted delivery vehicles and as synthetic bone graft substitutes has demonstrated very important successes. Maybe because they have been out there and clinically used for over 40 years now, these successes have been somewhat downplayed among other developments in the field of biomaterials. This, however, does not reflect the enormous diversity CaPs have to offer both in terms of products and their applications. And these have not yet been explored to their maximum extent. Recent technological developments will bring the CaP research and development another step further, which fits well in the search for largely available and affordable strategies for damaged and diseased bone in our aging population.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mattod.2015.10.008.

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