Deconvoluting the Bioactivity of Calcium Phosphate-Based Bone Graft Substitutes

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Deconvoluting the Bioactivity of Calcium Phosphate-Based Bone Graft Substitutes: Strategies to Understand the Role of Individual Material Properties

*Víctor Pablo Galván-Chacón and Pamela Habibovic**

Calcium phosphate (CaP)-based ceramics are the most widely applied synthetic biomaterials for repair and regeneration of damaged and diseased bone. CaP bioactivity is regulated by a set of largely intertwined physico-chemical and structural properties, such as the surface microstructure, surface energy, porosity, chemical composition, crystallinity and stiffness. Unravelling the role of each individual property in the interaction between the biomaterial and the biological system is a prerequisite for evolving from a trial-and-error approach to a design-driven approach in the development of new functional biomaterials. This progress report critically reviews various strategies developed to decouple the roles of the individual material properties in the biological performance of CaP ceramics. It furthermore emphasizes on the importance of a comprehensive and adequate material characterization that is needed to enhance our knowledge of the property-function relationship of biomaterials used in bone regeneration, and in regenerative medicine in general.

1. Introduction

Calcium phosphate (CaP)-based ceramics are currently the most widely applied synthetic biomaterials for repair and regeneration of damaged and diseased bone, and their use is expected to further increase. Currently a few million bone graft procedures are performed worldwide each year,[1] as a treatment of bone defects caused by trauma or tumor removal, and spinal fusion. An increasing need exists for effective and affordable bone graft substitutes, and CaPs have the potential to play a pivotal role in a socially responsible tissue engineering.[2] The production and storage of CaPs are inexpensive as compared to growth factors- or cells-based strategies, and their chemical composition, mainly calcium and phosphate, resembles that of natural bone mineral, simplifying the regulatory path towards clinical application.

Some distinct clinical successes have been achieved with CaPs, and in a few studies, the bone regenerative potential in vivo has been shown comparable to that of autologous bone.[3,4] Indeed, CaPs are considered bioactive materials, meaning that they are able to elicit or modulate the biological activity.[5] In a bony environment, the bioactivity of CaPs encompasses their bone-bonding capacity, osteoconductivity, and in some cases, osteoinductivity. While it may seem straightforward that their chemical composition is the key factor for their bioactivity, it should be emphasized that CaPs are complex functional biomaterials, with a set of largely intertwined physico-chemical and structural properties, many of which have been reported to affect the biological response to these materials. For example, surface microstructure,[6–8] particle size-[9–13] and morphology,[10,11,14] grain size,[15–17] surface energy and wettability,[18–20] porosity,[17,21] pore size and shape,[21] phase composition,[14,22] crystallinity[23,24] and stiffness[25–27] have all been shown to influence the bone regenerative capacity of CaPs.

While different properties of CaPs are known to affect their bioactivity, it should be mentioned that already the very first contact of the material with the biological environment is largely dependent on the material properties. Upon implantation in a bone defect, the initial interaction of the biomaterial surface with the biological environment occurs via contact with blood, leading to the formation of a blood clot that supports the healing process,[28] and that is known to contain platelets, erythrocytes and leukocytes.[28,29] The process of blood clot formation is influenced by the physicochemical properties of the material. For example, surface roughness is determinant for the process of protein adsorption on the surface, which in turn facilitates platelet adhesion and activation, and eventually controls the bone healing process.[30,31]

Furthermore, the bioactivity of CaPs in vivo is closely related to their degradation, by means of physico-chemical dissolution as well as resorption via cellular mechanisms.[32,33] The activated platelets release growth factors, such as platelet factor 4 (PF-4), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β),[28,34] which stimulate the chemotaxis of inflammatory cells like neutrophils and monocytes (with the ability to differentiate into macrophages and osteoclasts), favoring their migration towards the site of implantation.[35] In addition, proliferation of osteoblasts, as well as the migration and proliferation of other bone marrow-derived

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adhm.201601478.

DOI: 10.1002/adhm.201601478

cells occurs.[30,36,37] The resorption processes is often preceded by the process of chemical dissolution or mechanical degradation, whereby material particles are released from the implant, interacting with the inflammatory cells, which then become activated and release inflammatory mediators.[32,33] Depending on the size of the particles, they will be processed by phagocytosis and intracellular digestion by the macrophages (size below 10 micrometers), giant cells (size between 10 and 100 micrometers), or through extracellular degradation by macrophages or foreign body giant cells (FBGCs) if the particle size is larger than 100 micrometers.[32,38] Furthermore, direct resorption by osteoclasts can occur on the surface of some CaPs, a process that is, among others, dependent on the chemical phase of the ceramic and the presence of dopants.[32,39]

Elucidating the role of each individual material property on the biological response to the material is of utmost importance for designing and developing new, more efficient bone graft substitutes. Indeed, understanding the role of the individual properties and designing new materials that encompass a combination of the desired properties would change the way synthetic bone graft substitutes are currently developed, which is largely based on trial-and-error experiments. Nevertheless, three main obstacles can be defined that hamper the understanding of the role of individual properties in affecting the biological response to a material. First, a thorough and adequate characterization of the material properties is crucial to make a comparison among different materials and different studies. Nevertheless, most studies focus on characterizing only a limited set of properties, thus neglecting the influence of the rest of them.

Second, there is a large variety of methods to assess the biological performance of a given biomaterial, making the comparison of the results difficult. In vitro studies with stem cells or (pre)osteoblast (-like) cells and the measurement of the expression of osteogenic markers at the mRNA level, such as alkaline phosphatase (ALP), osteopontin (OP) or osteocalcin (OC) is a common procedure for the evaluation of cell differentiation,[40] although the use of different cell types prevents a reliable comparison of the results from different studies. Other researchers focus more on protein adsorption[19,24,41,42] or osteoclast activity.[7,43–46] In vivo studies add additional challenges, as animal models suffer from interspecies[47] and intraspecies[48] variations, while the model itself is also largely determinant for the outcome.

Third, in contrast to polymers, the chemistry or topography of which can be, to a certain extent, tuned individually without affecting the other parameters, the properties of ceramic materials are difficult to control independently during the synthesis process.[49,50] For example, it is very difficult to change the chemical composition of CaPs without affecting their crystal structure or morphology,[51] or the micropore size without changing the total porosity.[17] Therefore, any experiment designed to correlate the effect of an individual property with a certain biological response is largely affected by this entanglement of the properties (**Figure 1**).

Nevertheless, recently, a number of strategies have been developed as an attempt to decouple the role of individual material properties in bone regeneration. Yet, the use of different strategies may lead to different conclusions. For example, in our recent study,[52] the decoupling of the effect of topography and chemistry led to the conclusion that the effect of chemistry on the osteogenic differentiation of human mesenchymal stromal cells (hMSCs) was more important than that of the topography, while in a study using a different strategy and osteoblast-like cells,[53] topography seemed to be the main factor influencing the expression of osteogenic markers. Similarly, while the results of some studies support the hypothesis that surface chemistry is the most important factor,[52,54,55] others stand for a predominance of the effects of surface structure.[55,56] Decoupling the properties also implies the loss of the possible synergistic effects among them, which have been suggested to exist.[57,58] For instance, the incorporation of only calcium or only phosphate ions instead of CaP into a polymer to form a composite material allowed to evaluate the individual effects of the ions on the proliferation and osteogenic differentiation of hMSCs, but the potential synergistic effects of the chemistry and topography were lost.[59]

This progress report reviews the strategies developed to understand the roles of chemical (CaP phase, presence of additives, individual ions, release kinetics), physical/structural (crystal structure, crystallinity, surface energy, particle size and shape, grain size, surface roughness, porosity, pore size and shape) and mechanical properties, along with the results obtained for their influence on the biological response to the material. We discuss the advantages and the possible downsides

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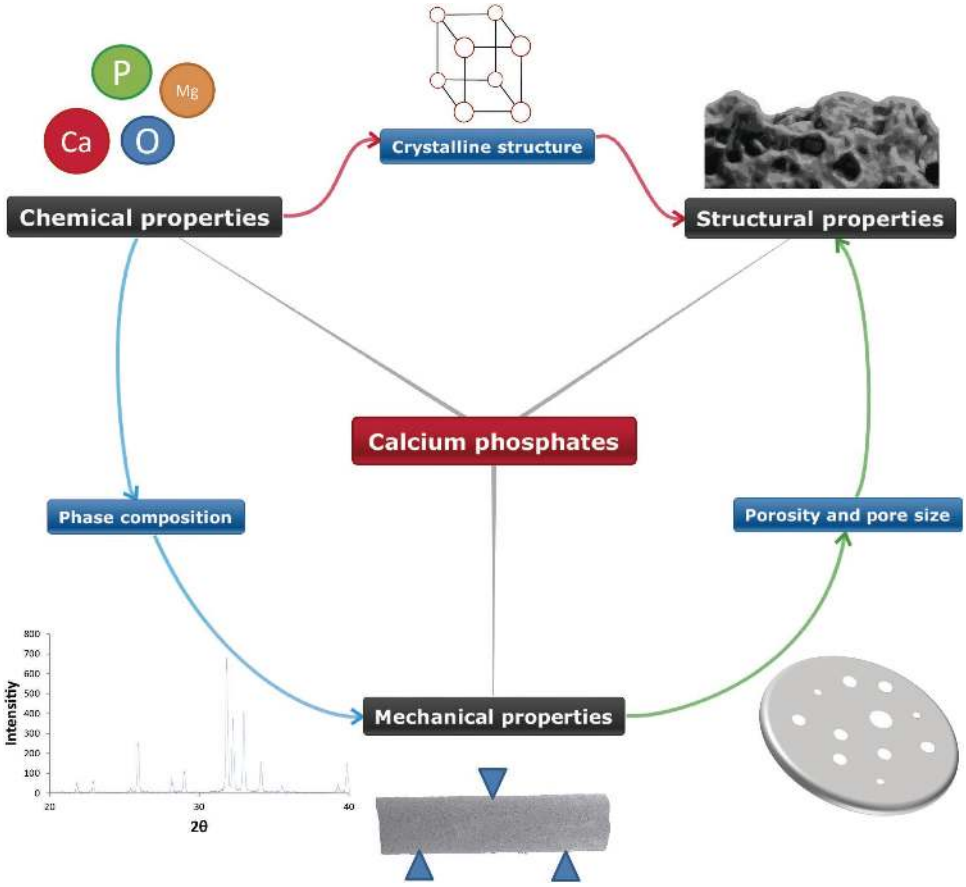

Figure 1. The material properties of calcium phosphates are highly intertwined. A change in the chemical composition, e.g. by substitution of an ion in the crystal lattice, results in a modification in the crystalline structure that affects the surface topography, and at the same time the mechanical properties. Similarly, an attempt to modify the mechanical properties by controlling the sintering parameters results in a modification of the porous structure, surface topography as well as phase composition.

of the selected strategies. The aim of this report is to provide a better insight in the effects of individual material properties on the bone regenerative potential of synthetic bone graft substitutes, which may be useful for developing new materials.

2. Role of Physical Properties

2.1. Structural Properties

Understanding the influence of the structural properties of CaP-based biomaterials for bone repair and regeneration on their performance is still a challenge, given that it is difficult, if not impossible, to change the surface topography without affecting the surface chemistry.[60] To provide a definitive answer regarding the individual contribution of these properties on the bioactivity of the biomaterial, we have recently employed a range of techniques, including soft lithography and various coating methods,[52] to decouple and subsequently recombine the chemical and surface topography effects of biomimetic crystalline CaPs.

There are several surface topographical parameters that have been suggested to affect the in vitro and in vivo performance of CaP bone graft. Surface roughness is usually characterized through R_a, which is the mean deviation of the height on the surface measured along a line profile, and S_a, i.e. the mean deviation of the height on an area, that is considered a more representative and descriptive parameter than R_a.[60,61] Surface roughness has been reported to affect both in vitro[7,8,54,62–64] and in vivo[56,65] performance of the bone graft substitutes. The suggested mechanisms behind this effect seem to be similar in vitro and in vivo. In vitro, it has been reported that rougher surfaces promote protein adsorption[66,67] and facilitate cell attachment and proliferation,[8] while in vivo the roughness has been shown to additionally affect the inflammatory response to the material.[68]

The porous nature of the structure has also been widely reported to have an effect on processes related to bone regeneration. Porous structure is usually characterized by two parameters, the pore size and the porosity. It has been shown that in vivo the proteins accumulate inside the micropores and ion-exchange through dissolution and (re)precipitation are facilitated.[69–71] Macropores are the key factor for the migration and proliferation of (pre)osteoblasts and the facilitation of the angiogenesis, as a network of interconnected pores, in general larger than 50 µm[58,69,72–74] is required to allow the formation of blood vessels.[69,75,76]

An increase in crystallinity has been reported to reduce the reactivity of CaP,[23,77,78] including dissolution, thereby affecting their biological performance. As reported by ter Brugge et al.,[79] the effect of CaP crystallinity and associated solubility both in vitro and in vivo is still controversial. In vitro, some studies found that amorphous materials promote proliferation and differentiation of mouse fibroblasts and rat calvarial cells,[80,81] while others stated that the higher dissolution rate of amorphous materials can result in cytotoxicity due to the elevated ion concentration.[23,79,82] In vivo, highly crystalline CaP have been reported to enhance new bone formation or to hamper it.[83–85] The crystallinity of CaP can be changed by modifying the synthesis and/or sintering procedure, and, again, it is likely that by doing so other parameters will change as well, which may lead to the different conclusions about its role in the biological performance of CaP that are described above. Although the parameters grain size and particle size are frequently interchangeably used, they refer to different properties of crystalline materials. Whereas a grain is defined as a region, mono- or polycrystalline, where the crystal or crystals have the same lattice orientation, a particle is an agglomerate and can contain multiple grains. Both grain- and particle size have been shown to affect cell behavior, although their effects are very difficult to decouple from the effect of surface roughness, as an increase in grain size generally leads to an increase in surface roughness.

The presence of artificial, often machined, micro- and nanostructured patterns on the biomaterial surfaces seems also to affect the biological response,[86] and even at the nanoscale, patterns have been shown to have the potential to control the osteogenic differentiation of MSCs in vivo.[87]

2.1.1. Coating Methods to Isolate the Effect of Surface (Micro) Structure

This strategy employs "masking" of the surface chemistry of CaPs by depositing a thin layer of another material on the ceramic surface with the aim to isolate or at least highlight the role of the surface structural features (**Figure 2**.1). Obviously, this method eliminates the influence of the CaP surface

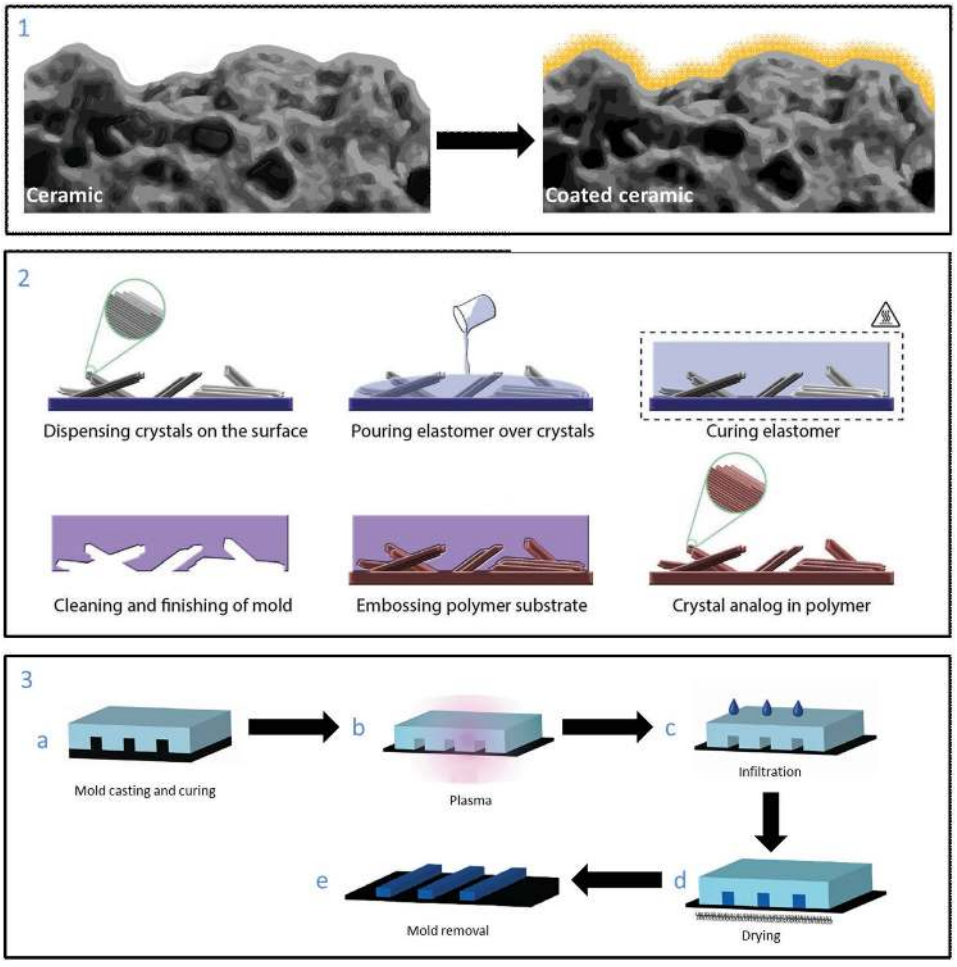

Figure 2. Strategies to decouple the role of topography from other material properties. 1) Coating of a CaP ceramic with a thin layer of other material, e.g. gold, to retain the surface structural properties of the ceramic while changing the surface chemistry. 2) Fabrication of structural analogs of crystalline CaPs in a polymer using soft embossing. Adapted with permission.[52] 3) A schematic representation of the process of surface micropatterning with CaP ceramics by micromoulding in capillaries, including a) casting of polydimethylxyloxane (PDMS) and curing to develop a mould, b) air plasma treatment, c) infiltration of a CaP solution into the channels, d) drying and e) mold removal. Adapted with permission.[105] 2015, the Royal Society of Chemistry.

chemistry but it introduces the chemistry of the coating material. Therefore, biocompatible but inert materials, such as gold or palladium, are generally preferred for this application, despite a few reports on the negative effects of these inert materials on cell proliferation and differentiation, as compared to titanium coatings.[55,88] It should be mentioned that the use of line-of-sight coating methods, i.e. methods in which only surfaces that are exposed to the source of the coating material, such as plasma spraying or most physical vapor deposition techniques,[89,90] have the important disadvantage of leaving some areas uncoated, in particular in the case of geometrically complex or porous structures.[89–91] In these cases, the effect of the underlying chemistry cannot be fully eliminated. Therefore, non line-of-sight techniques, such as chemical vapor deposition (CVD), electrodeposition or solution-based biomimetic methods, are preferred within the context of isolating the effect of surface structure.[92–94] In the work by Dos Santos et al.,[55] a gold coating with a thickness of 20 nm was applied to the surfaces of hydroxyapatite (HA) and tricalcium phosphate (TCP), two widely used CaP bone graft substitutes, having a high density in order to avoid the additional effects of surface microporosity. Although the effect of calcium and/or phosphate ions that diffused through the, not fully homogenous, gold layer could not be completely excluded, the authors were able to demonstrate that the effects of nanoroughness on the adhesion of human osteoblastic cells SaOs2 were negligible, while nanoroughness significantly affected cell proliferation and differentiation. Cairns et al. deposited thin CaP films on top of glass substrates having different topographies produced by magnetron sputtering deposition of polycrystalline titanium, and then applied a second layer of fibronectin, an extracellular matrix protein known to bind to implant surfaces in vivo.[95] Upon culture of MG-63 osteosarcoma cells on such coated materials, effects on cell attachment, proliferation and differentiation were observed not only as a result of the presence of the fibronectin layer, but were also shown dependent on the nanoscale topographical features underlying the CaP layer. Pegg et al. applied a thin sputter-coated gold layer over the surface of titanium alloy substrates coated with an HA coating, by using the commercially available BoneMaster biomimetic technique.[96] While the surface topography between the uncoated and HA-coated titanium alloy substrates was significantly different, the gold coating did not affect surface roughness. Furthermore, the analysis of the surface chemistry using x-ray photoelectron spectroscopy (XPS) confirmed that no calcium or phosphate ions were present on the gold-coated samples. Cell proliferation, as well as ALP and OC expression levels were higher on HA-coated than on the uncoated metal, while no significant differences were found between the gold-sputtered HA-coated titanium alloy and HA-coated titanium alloy without the gold coating. Based on this, the authors concluded that the topography is the main contributing factor in the osteogenic differentiation of cells on these surfaces. Nevertheless, it was impossible to completely exclude the possible leaching of calcium and phosphate ions from the gold-coated samples, which could also have affected the growth and differentiation of the cells. In a subsequent clinical trial, 55 patients received an uncemented femoral stem coated with a layer of HA deposited either using the BoneMaster technique or by plasma spraying. After five years, there was no clinically relevant difference between the two groups of patients.[97] The coating method for decoupling the effects of surface chemistry from that of the surface structure was evaluated in vivo in a study by Hacking et al.[56] Titanium implants with a plasma-sprayed HA coatings were coated with an ultrathin titanium layer using physical vapor deposition, and implanted in a canine femoral intramedullary implant model. The analysis of the surface chemistry of the implants, made by XPS, did not show any remnants of HA on the surface, that is, the only influence on the surface chemistry was due to the titanium coating. The comparison between the two groups of implants, titanium coated with HA and titanium coated with HA and subsequently with titanium led to the conclusion that 80% of the maximum bone formation was a direct effect of the surface topography.

2.1.2. Replication of the Calcium Phosphates Structure in Other Materials

Another possibility to decouple the role of the surface structure from that of the surface chemistry is to fabricate a replica of a given biomaterial in another material with different surface chemistry. The majority of studies employing this method were performed on titanium, which is a widely used material for medical devices, such as total hip implants.[98,99] Wieland et al. fabricated replicas in epoxy of titanium surfaces varying in roughness.[98] A combination of non-contact laser profilometry (LPM) and stereo-SEM was used to prove that there were no statistically significant differences in surface roughness between the Ti surfaces and the epoxy replicas in the wavelength range from 100 nm to 1 mm. The authors were able to demonstrate that the roughness, independently of the chemistry, affected the morphology and thickness of cultured human fibroblasts.[98] In another study, they coated the titanium replicas in epoxy with a thin titanium layer by applying physical vapor deposition, thereby faithfully reproducing the surface topography and chemistry of the original titanium substrates.[99] Rat calvarial osteoblast, cultured for 24 h on smooth and rough coated replicas, showed no differences in cell number, footprint area or cell morphology.

In recent studies,[52,100] replicas in cyclic oleofin copolymer (COC) of crystalline biomimetic CaPs and of microstructured CaP/polylactic acid (PLA) composites were produced using a soft embossing technique applying an intermediate polydimethylsiloxane (PDMS) mold (Figure 2.2). The structures were reliably reproduced in polymer down to the submicrometer level, thus obtaining a material with same surface topography but different surface chemistry. In order to recover the effect of the surface chemistry, some of the replicas in COC were subsequently coated with a thin amorphous CaP layer or, to introduce an alternative chemistry, with a thin titanium layer. The results of the hMSC culture on uncoated and coated replicas indicated that the chemical effect, i.e. the effect of the presence of CaP, was more pronounced that the microstructural effect, in terms of the differentiation of hMSCs into the osteogenic lineage.

A criticism to this strategy may be that the replicas in different materials not only have different surface chemistry, but consequently also different mechanical properties and

wettability, which has also been shown to have an effect on the cell behavior. An attempt to decouple the effect of the chemical composition from that of the mechanical properties is addressed in a later section of this report.

2.1.3. Surface Structure Modification

The modification of the surface structure after the synthesis and/or sintering process is a possible way to study the effect of micro- and nanostructure of CaPs. There are different surface modification methods, such as polishing, grinding, sandblasting and etching. These methods of surface modification, however, may simultaneously affect more than one parameter; for example the polishing process may not only reduce the surface roughness, but also affect the porosity of the surface or the grain size. Therefore, a meticulous characterization is needed to attribute the change in cell- or tissue response to a single property.

Deligianni et al. fabricated HA pellets and polished them after sintering to achieve three different surface roughness levels.[8] The analysis of the phase composition by x-ray diffraction (XRD) showed that the three different samples were all composed of pure HA. However, no analysis of the change in surface porosity or grain size was performed. There were no differences in cell number, morphology or ALP expression levels of cultured bone marrow-derived hMSCs among the three groups of materials. In contrast, cell attachment and the force required for the detachment increased with the increase in surface roughness.

In another study, Li et al. produced biphasic CaP (BCP) discs, consisting of HA and β-TCP, and polished them to achieve different surface roughness.[101] The discs were then used to study the behavior of three different cell types: C2C12, a myoblast cell line, primary bone marrow-derived hMSCs and MC3T3-E1, an osteoblast cell line. The discs used for the study not only differed in surface roughness, but also in crystal size and microporosity, making a reliable comparison challenging. Nevertheless, in contrast to the study by Deligianni et al.,[8] cell attachment, although dependent on the cell type, was not significantly affected by the surface roughness. As a conclusion, surface roughness was not considered an important factor in cell behavior.

A similar procedure was followed by Mazón et al., who polished α-TCP ceramic discs to vary surface roughness without affecting the chemical composition.[102] The material characterization procedure included XRD, x-ray fluorescence (XRF), surface profilometry and scanning electron microscopy (SEM). Adult hMSCs were cultured on the discs, and the results showed that cell proliferation and differentiation were significantly affected by the difference in surface roughness.

2.1.4. Patterning

Rather than relying on methods such as polishing or etching that result in an average value for e.g. surface roughness, derived from structural features over a larger area, patterning is an elegant way to obtain biomaterial surfaces with finely controlled features in terms of properties and spatial distribution. Although this technique has so far not been extensively used for decoupling the roles of chemistry and topography of CaPs, it is worth mentioning it here for its great potential in designing parametric studies. Structural patterning has already been widely used for polymeric biomaterials,[103] since polymers are more amenable to various patterning techniques as compared to brittle and hard ceramic materials. Nevertheless, several techniques, such as micromachining or laser ablation, have been used to obtain patterned surfaces in (bioinert) ceramics, although their accuracy is below 100 μm.[104,105] The use of microfabrication technologies, such as soft lithography, allows for ceramic patterning with higher accuracy. Pelaez-Vargas et al.[106] used a combination of soft lithography and sol-gel processing to produce anisotropic silica microtextures with varying spatial density, followed by culture of human osteoblast-like cells. Cell alignment was remarkably influenced by the effect of the surface topography. Cell metabolic activity and osteogenic differentiation measured by ALP activity were also affected by the density of the patterns, with the lower-density topographies showing the significantly higher values that the flat controls. A photolithography-based method was used by He et al. to produce patterns of HA in the shape of micro-islands on a polyethylene glycol (PEG)-passivated glass substrates.[107] The HA patterns were obtained by precipitation from simulated body fluid (SBF), and had, according to XRD data, a relatively low crystallinity. The culture of MC3T3-E1 cell line on the patterned substrates showed that the cell adhesion took place exclusively on the HA micro-islands. The authors proposed micropatterning as a method to decouple chemical from geometrical cues in CaP. A recent paper by Barata et al.[105] reported patterning of CaP (dicalcium phosphate anhydrous (DCPA) and β-TCP) on silicon substrates using a soft lithography approach (Figure 2.3). The linear ceramic patterns consisted of elongated crystals with radially branching morphology, occupying the volume of the channels used for the infiltration of the mineral solution. While the formation of the patterns was successful, further optimization is required to improve the homogeneity of the patterns and to better control the properties of the ceramic phase. Nevertheless, the alignment, morphology, and the retention of the morphology in time of MG-63 osteosarcoma cells were affected by the dimensions of the ceramic patterns.

2.1.5. Control Over the Porosity

In conventional sintered ceramic materials, the final porosity depends on the porosity of the green body and the sintering temperature. Therefore, two different methods (and their combinations) have been developed to achieve control over the porosity: the modification of the green body by creating artificial pores or by introducing porogens prior to the sintering process (**Figure 3**),[108] and the modification of the sintering temperature. Ideally, the porogens should be substances that are removed during the sintering process, leaving pores behind. The modification of the sintering route generally also affects several other material properties, such as the phase composition or the grain size.

Yuan et al. produced two different porous bioceramics, HA and BCP, by introducing different porogens such as

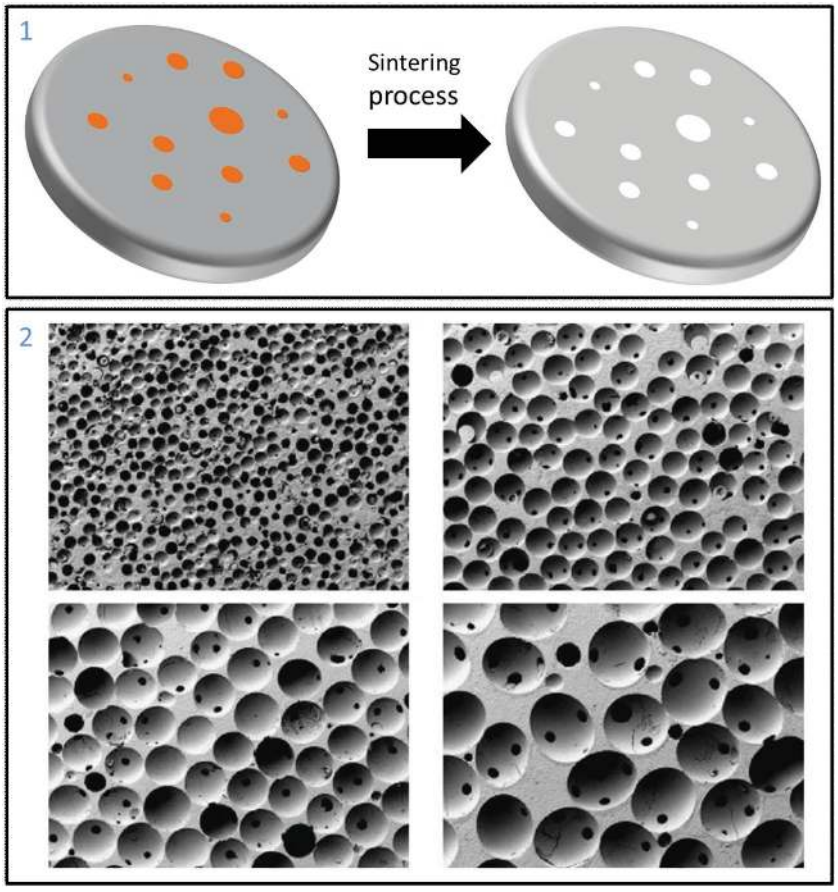

Figure 3. Use of porogens to elucidate the role of properties of porous structure in CaP bioactivity. 1) Porogens are incorporated to the green body, and during the sintering process they are removed, resulting in a porous structure. The use of porogens of different sizes and in different amounts allows to tune the size distribution and porosity of the structures. 2) SEM images of β-TCP ceramics with different pore sizes produced by the use of PMMA porogen particles with different diameters. Reproduced with permission.[108] 2008, Elsevier.

naphthalene and wax particles in order to achieve controlled porosity and pore size.[109] Both HA and BCP had macroporous structures, but the surface of the macropores in BCP contained abundant micropores, while the surface of HA macropores was dense and showed no microporosity. The ceramics were shaped as cubes and implanted intramuscularly in a goat model. The results showed that all the BCP implants were able to induce new bone formation, while the macropores of HA were filled with fibrous tissue, without bone. As the authors acknowledged, this study compared two ceramics, the material properties of which differed in more than one parameter, namely the chemical phase and the surface microstructure, making a reliable comparison difficult. In two other studies,[4,110] BCP ceramics were sintered at different temperatures, 1150 and 1300 °C; the increase in sintering temperature led to materials with lower microporosity but also different volume distribution of the pores and larger average grain size. Additionally, differences in sintering temperature are also likely to modify the phase composition of the surface and the bulk of the material.[111,112] Upon implantation in a posterolateral spinal fusion model in dog[4] and in a paraspinal muscle goat model,[110] both studies found that bone formation was induced in the material with higher microporosity, i.e., those which were sintered at 1150 °C. These results suggest that the difference in bone formation was caused by the change in the structural properties, however, the question to which of the structural properties this effect can be attributed, and to which extent, remains unanswered. In a similar study, two different types of ceramics, HA and BCP, were fabricated with different microporosity and crystal size through variations in the sintering temperature.[113] The phase composition in both HA and BCP did not change with the increments in sintering temperature, but the microporosity diminished and the crystal size increased, thus the specific surface area was lower in the ceramics sintered at higher temperatures. The results of an in vivo experiment testing ectopic bone formation potential suggested that an optimal specific surface area exists at which the amount of newly formed bone is maximal.[113]

Klenke et al. produced BCP particles with different macropore sizes by using naphthalene particles of different dimensions as porogens and hydrogen peroxide for creating micropores, while keeping the overall porosity constant.[76] The ceramic bodies were implanted in critical-sized cranial defects in mice, showing that angiogenesis, followed by bone formation, was mainly stimulated in samples having the pores with a size over 140 μm. Another example of the use of porogens to control the porosity was provided by Kasten et al.,[21] who analyzed the effect of the porosity of β-TCP scaffolds on the in vitro behavior of hMSCs, as well as on in vivo bone formation of the same scaffolds loaded with hMSCs in a subcutaneous mouse model. The results showed that in vitro, an increase in porosity increased the protein production, but it did not influence ALP expression levels, i.e., osteogenic differentiation. In vivo, while no effect of the porosity was observed on the ALP expression, scaffolds with a higher porosity exhibited a more pronounced mineralization by the cells.

In order to decouple the effects of microporosity from those of grain size, in an elegant study Lapczyna et al. developed four different TCP ceramics, with independent variation of these properties.[17] Two different porogens were used: PEG particles for the generation of macropores, and stearic acid and wheat flour with a bimodal size distribution to vary micropore sizes. Different sintering routes were used to obtain different grain sizes and maximize the purity of the phase composition. Although the aim was to obtain samples that are phase-pure β-TCP, some of the samples contained up to 16 wt.% α-TCP. Furthermore, the micropore size distribution differed among the samples, showing that it is very difficult, if not impossible, to vary a single parameter in CaP ceramics, by employing

conventional production techniques. Nevertheless, the material characterization in this study was highly comprehensive, including XRD, SEM, micro computed tomography (micro-CT), mercury porosimetry, and measurement of the density and specific surface areas, which is of utmost importance in correlating the properties of a material to a biological response. The materials were implanted in proximal and distal humerus and femur defects is sheep,[114] and the amount of bone formed after the implantation was evaluated by means of micro-CT image analysis. The results showed that there were no significant differences between the different implants, despite their different structural parameters. These results are in contrast with other studies in which the effects of microporosity and grain size of CaPs were investigated. Besides several studies previously mentioned in this section, Chan et al. implanted silicon-substituted CaP ceramics with different microporosities in an ectopic sheep model for 24 weeks, finding that the level of bone formation was significantly higher in the ceramics with higher porosity.[73] In a study by Hong et al.,[115] BCP and HA with different grain sizes were implanted into thigh bones of dogs, showing that in BCP, an increase in the grain size (from 86 nm to 768 nm) had a positive effect to the amount of newly formed bone, whereas in HA, a grain size increase from 115 nm to 731 nm did not strongly affect the amount of new bone formation, suggesting that the effect of grain size is dependent on the chemistry of the ceramic.

The differences in the results obtained from different studies may be attributed to the fact that the final biological response is more dependent on biological factors than on material properties.[17] Taking a more deterministic perspective, we should reflect on the effects of the animal model, the sometimes incomplete material characterization that might mask changes in multiple properties beside those being the object of the study, or the experimental design, including the strategy chosen to decouple the role of the different properties. Therefore, it seems necessary to improve the decoupling strategies reviewed in this section. This could be achieved by developing and employing alternative fabrication techniques able to provide a more precise control over the ceramic synthesis and sintering process, such as the microwave sintering reported by Hong et al.,[115] the slip-casting shaping method developed by Feng et al.,[116] or by using other techniques able to create pores in the ceramics without modifying other properties, such as additive manufacturing techniques allowing to customize and control the pore structure of bone graft substitutes.[117,118] For example, Tarafder et al. 3D-printed TCP scaffolds with controlled interconnected porosity and different pore sizes (500, 750 and 1000 micrometers), sintered them using two different methods (conventional sintering furnace at 1150 °C and microwave sintering at 1250 °C, both resulting in a decrease of pore size) and tested them in vitro using human osteoblast cells and in vivo by implantation in femoral defects of Sprague-Dawley rats.[119] In vitro, the ceramics with smallest interconnecting pore size showed the most pronounced cell proliferation, while in vivo all implants were found to promote new bone formation. So far, additive manufacturing techniques can create controllable macro- and micropores, however, their resolution is in general not high enough to control nanotopographical features, including nanopores.[120]

2.2. Mechanical Properties

Various studies have demonstrated that the mechanical properties of the substrate the cells are in contact with, affect cell fate. For example, substrate stiffness has been shown to affect MSCs attachment, proliferation and osteogenic differentiation.[121] Furthermore, it has been demonstrated that stiffness-mediated focal adhesion kinase (FAK) is essential for the onset of osteogenesis.[122,123] Although these studies have demonstrated that substrate stiffness may affect cell behavior, they were performed on polymeric materials, the stiffness of which is generally several orders of magnitude lower than that of CaPs. In ceramic substrates, it is not possible to modify the mechanical properties without changing other properties, including chemical composition and porosity, which are both known to also play also an important role in regulating cell fate, and consequently, the cell and tissue response to the material. Therefore, decoupling mechanical from other properties is challenging. In a recent elegant study, Mattei et al. suggested a strategy to decouple the effect of the mechanical properties from other material-related cues in HA.[25] Composite HA/gelatin hydrogel constructs were produced with varying weight ratios of the two components, and in parallel HA-free scaffolds were produced with different amounts of glutaraldehyde (GTA), a crosslinking agent (**Figure 4**). The variations in the stiffness of the HA/gelatin constructs due to differences in the HA content were matched in the HA-free constructs by varying the GTA content, thus achieving HA-free mechanical equivalents of the HA/gelatin constructs with similar surface roughness. Human periosteal derived progenitor cells (PDPCs) were cultured on the different materials, and the BMP2, Runx2 and BGLAP mRNA levels were measured after one week to investigate the differentiation of the cells towards the osteogenic phenotype. The results indicated that the extent of osteogenic differentiation was dependent on the HA content in HA/gelatin constructs, and on the stiffness of the HA-free scaffolds, suggesting that the instructive signals for these markers may have their origins in the substrate stiffness.

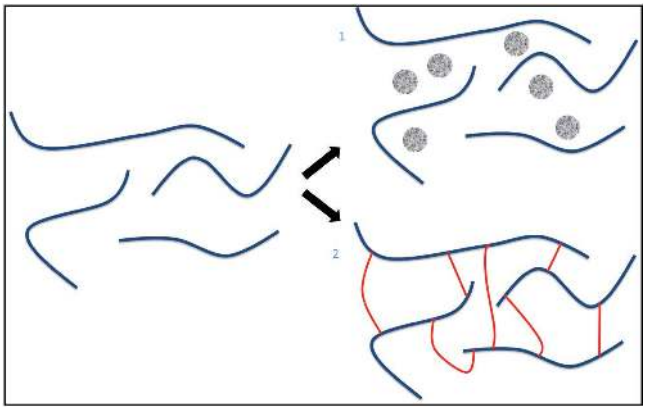

Figure 4. Strategy to elucidate the role of stiffness in CaP bioactivity suggested by Mattei et al.[25] Gelatin scaffolds were loaded with hydroxyapatite (1), which increased their stiffness, while a range of mechanical equivalents in HA-free gelatin (2) were produced by changing the extent of crosslinking.

To the best of our knowledge, there are no other studies that specifically focused on decoupling the mechanical from other properties in CaPs. Nevertheless, the work by Mattei et al. may be a basis for combining this strategy with others, such as the one developed by us,[52] achieving further control over the individual material properties.

2.3. Surface Energy, Wettability and Polarization

A number of studies have reported changes in cell behavior due to variations in surface energy or wettability. Although both physical parameters are related, with higher surface energy causing an increase in the contact angle, the former is an intrinsic property of the material, while the latter is also affected by the surface-structural properties, including roughness.[124] The polarization of a material surface leads to an increase in wettability, and both surface energy and wettability are important for the adsorption of molecules, including proteins, on the material surface,[19,125] which is the first event occurring upon contact between the material and a biological environment. This initial adsorption of the proteins is largely determinant for cell attachment on the material.[126] In general, it has been reported that surfaces with high surface energy promote cell adhesion.[18,124] Several techniques exist that allow to change the surface energy or the wettability of the materials: deposition of self-assembled monolayers, application of electromagnetic fields, electrochemical methods, changes in the environmental conditions, changes in temperature or pH, application of surface pressure or low-electron energy irradiation,[127] although they have not been extensively applied to CaP biomaterials. The use of any of these techniques implies a change in the surface chemistry of the material, as both surface energy and surface chemistry are intertwined properties.

3. Role of Chemistry

The success of CaPs as bone graft substitutes has been traditionally attributed to the similarity of their chemical composition with that of bone mineral.[128] During the degradation process it has been shown that at the surface of CaPs, a dynamic process occurs whereby calcium and inorganic phosphate ions are released, and, upon reaching supersaturation, reprecipitated in the form of a carbonated apatite layer, along with coprecipitation of organic compounds, such as proteins, from the surrounding body fluids.[129] The reality of the degradation of CaPs in vivo, is however, more complex. Three main routes of degradation of CaPs are identified; physico-chemical dissolution as a result of hydrolysis of the surface, cellular degradation by mineral-resorbing cells and mechanical degradation. Each of these processes is dependent on the properties of the ceramic, as well as on the (micro)environmental conditions, and each process affects (the extent of) the other two.[129]

Different phase composition is associated with different surface reactivity and dissolution kinetics. Differences in the biological performance in accordance with the different phase compositions of the materials have been reported,[22] and, for example, BCP is considered to have a higher osteoinductive potential than HA.[130] Besides the differences in reactivity and dissolution kinetics, the differences in crystal structures may lead to a different surface energy,[124] a factor that can also change the biological performance of CaPs.

Previous studies have demonstrated that both calcium and inorganic phosphate ions have an effect on skeletal cells.[50,131] The presence of calcium in the cell culture medium has been shown to have a stimulatory effect on osteoblast proliferation and differentiation and to induce a number of intracellular signaling pathways through the activation of calcium receptors related to the regulation of osteoblasts and osteoclasts.[131,132] High levels of inorganic orthophosphate were shown to induce osteoblasts apoptosis in vitro,[50,133] while low levels have been related to an increase in both osteoclastic resorptive activity and osteoblast differentiation.[134]

CaPs admit the substitution of (a portion of) calcium and phosphate ions in the crystal lattice by other ions with therapeutic potential, usually called bioinorganics.[50] For example, magnesium can completely replace calcium, thus producing magnesium phosphate, or ceramics having different ratios of calcium and magnesium.[135] Other bioinorganics that can be incorporated into CaPs or combined with them are, for example, strontium,[136,137] silicate,[138] cobalt,[139] fluoride, carbonate, copper or zinc.[137,140] It should however be noted that by substituting a calcium, phosphate, or, in the case of hydroxyapatite a hydroxide ion by a guest ion, one not only changes the chemical composition of the ceramic, but it also modifies many other physico-chemical properties, including its crystallinity, mechanical or degradation properties.[50,137,141] Consequently, when investigating the behavior of cells or tissues on such a ceramic, it is difficult to attribute an effect solely to the change in chemistry, i.e. addition of a new ion.

Another difficulty in identifying the cause of a biological response arises from the degradation of the ceramic in a biological environment, whereby not only the guest ion, but also the host ceramic ions are released, in different amounts.[142]

Due to the issues discussed above, strategies are needed to isolate the role of the different ions that may be present in CaPs, and to evaluate how important the chemistry is in relation with other factors, such as the topography or the mechanical properties.

3.1. Incorporation of Calcium and Phosphate Ions into Polymeric Delivery Systems

The individual influence of the calcium and phosphate ions on cell and tissue behavior is difficult to elucidate in CaPs, because their release and uptake generally occur simultaneously. Furthermore, the effects of other ions present in the lattice, and other physico-chemical factors, cannot be excluded. To overcome this issue, the incorporation of salts containing only calcium or only phosphate into polymeric carriers has been explored as a way to exclude the effects of other parameters. While such a system provides advantages of having a functional material, that allows cell culture on the surface and study of a single ion, obviously, the system is not ideal, as it introduces a new material, i.e. the polymer, with its own chemistry, mechanical properties, etc. The attention should therefore be

paid to the choice of the polymer in terms of properties that could affect the experiment.

An example of study in which the above described strategy was applied is based on the findings suggesting that inorganic pyrophosphate (PPi) prevents the mineralization of type I collagen by binding to the CaP crystals.[143] ALP-mediated hydrolysis of PPi increases the local concentration of inorganic orthophosphate (Pi), promoting collagen mineralization by overcoming the inhibitory effect of PPi.[144,145] In an attempt to mimic this process by employing synthetic materials, we have developed constructs consisting of polycaprolactone (PCL), into which mildly acidic and alkaline sodium phosphate powders, and the pH-neutral mixture of the two were added.[146] 15-day ectopic implantation of the constructs in the thoracic muscles of CD1 mice resulted in ectopic mineral formation following release of sodium phosphate, indeed suggesting that an increase in local saturation of soft tissue with inorganic orthophosphate induced collagen mineralization, even in absence of additional calcium.

Danoux et al. incorporated, in separated experiments, calcium ions (as calcium carbonate salt) or phosphate ions (as sodium phosphate salt) into a polylactic acid (PLA) matrix and tested the individual effects of the ions on growth and differentiation of bone marrow-derived hMSCs.[59] The results of this study indicated that calcium ions stimulated cell proliferation and differentiation into the osteogenic lineage in a dose-dependent manner, which was in accordance with a previous study, in which the cells were cultured in media with increased calcium concentrations.[147] Similarly, the effect of the phosphate concentration on cell proliferation was dose-dependent: cell proliferation was stimulated between 2 and 4 mM, but inhibited beyond 5 mM, a fact that was attributed to the induction of cell apoptosis.[133] The osteogenic differentiation, in particular the expression of BMP2 and OP, was significantly increased in cells cultured on materials containing inorganic phosphate, which was also in accordance with previous work.[148] Interestingly, the measurements of the calcium ion concentrations of the cell culture medium containing PLA-calcium composite were equal to or lower than of medium containing control PLA samples or PLA-phosphate samples, which may suggest the formation of a new CaP layer on the surface of the composites, and/or the mineralization of the extracellular matrix produced by the hMSCs. This again emphasizes the importance of the biological environment in which the material is tested (i.e. cell culture medium contains calcium and phosphate ions itself as well as serum proteins), which interacts with the material, even in the absence of cells. It would therefore be important to invest in new analytical tools that allow for spatiotemporal chemical and other analyses within a (in vitro) biological system. As an example, recently, a study demonstrated the use of time-of-flight secondary ion mass spectroscopy to measure uptake of strontium ions released from CaP cement by osteoclasts.[149] This and similar techniques, based on mass spectrometry imaging, for example, are highly promising to further unravel the physico-chemical processes occurring at the interface of biomaterials and cells or tissues.

Within the context of delivery and study of individual ions, one could also consider delivery systems as used for biologics, such as growth factors. For example, biodegradable PLA-based microspheres have the advantage of providing a sustainable release of drugs encapsulated inside them,[150] while the carrier is degraded into lactic acid, which is finally transformed into carbon dioxide and water in biological systems.[142,151] Recently, we took advantage of these properties to design a calcium delivery system by incorporating a calcium chloride salt into PLA microspheres without affecting their surface morphology, and to release calcium ions upon immersion of the microspheres in cell culture media.[142] While in this proof-of-concept study, no significant effect of calcium-containing microspheres was observed on proliferation and osteogenic differentiation of hMSCs in a transwell in vitro set-up (**Figure 5**.1), the system may be valuable for screening the effects of individual ions in various biological conditions, before designing new functional biomaterials, for example for bone regeneration.

While this strategy provides an excellent way for assessing the individual role of the different ions present in CaPs, a criticism can be raised that all possible synergies between the chemistry and other physical properties are completely lost, as well as the possible synergies between different ions. A bottom-up approach could be a possible solution, starting by assessing the role of the individual ions, then the role of the combinations of the selected ions embedded in a polymer construct, and finally shaping the polymer such that it resembles the surface topography, or even approximates the mechanical properties of a CaP.

3.2. Coating Techniques

The role of surface chemistry can also be elucidated by depositing a thin layer of CaP on the surface of another material (Figure 5.2), using for example electrodeposition[152] or biomimetic techniques.[43,153] This method is also often used to improve the bioactivity of inert materials, such as titanium, in a bony environment. Alternatively, depositing a thin layer of another material on the surface of CaP may potentially be a suitable method for retaining the surface structure of the ceramic, while changing the surface chemistry.

For example, in our in vivo studies,[154,155] it was shown that a thin (20–60 micrometers) octacalcium phosphate coating produced using a biomimetic technique, was able to render the otherwise bioinert porous titanium alloy (Ti6Al4V), osteoinductive.[156] Although it is plausible that this change of surface chemistry is responsible for the observed effect, it is worth mentioning that the highly crystalline OCP coating also modified the surface roughness of the metal.

In a paper that was discussed in the topography section, Danoux et al. coated a structural polymer replica of a CaP with a thin amorphous layer of CaP, using a biomimetic precipitation method to reintroduce the chemistry of the ceramic.[52] The results of this study confirmed that the effect of the surface chemistry, i.e. presence of CaP, on the osteogenic differentiation of hMSCs was more pronounced than the effect of the (surface) geometrical features.

Burke et al. used photolithography to produce a silicon substrate with well-defined surface microstructure, followed by a chemical etching process and deposition of a thin CaP coating by radio frequency magnetron deposition.[157] In order

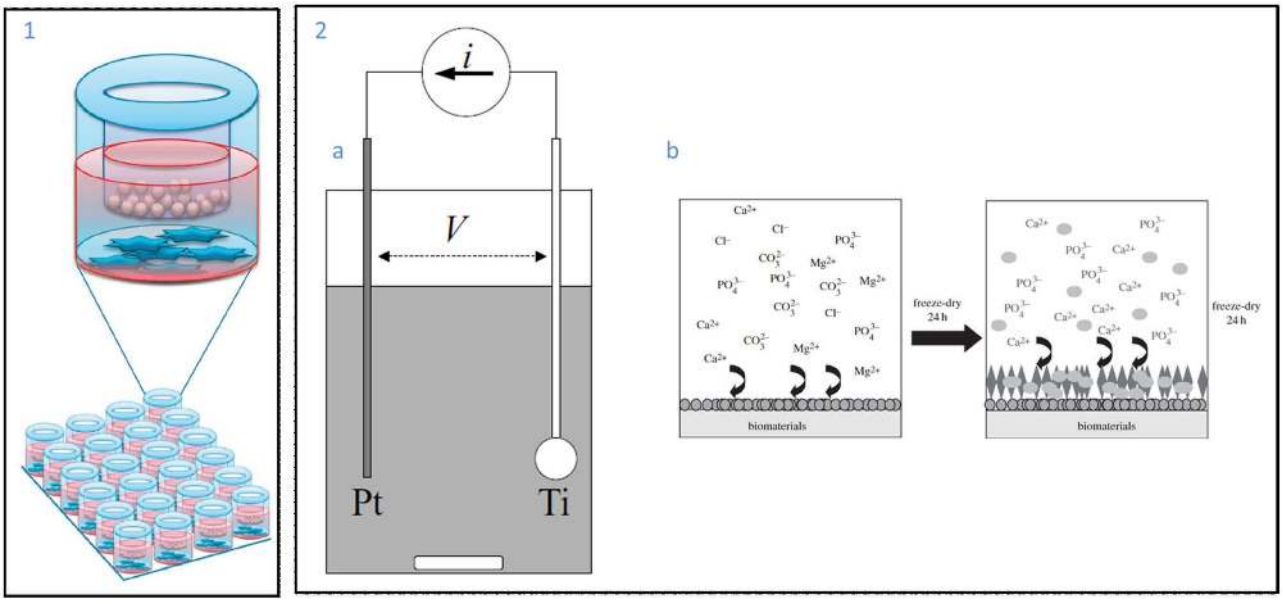

Figure 5. Strategies to elucidate the role of the chemistry in CaP bioactivity. 1) Incorporation of calcium ions into polymeric microspheres to study the effect of the ions on cell behavior using a transwell cell culture system. Reproduced with permission.[142] Copyright 2014, Russian Academy of Sciences. 2) Different methods for producing thin CaP coatings: a) electrodeposition setup for CaP deposition on titanium discs, consisting of a two-electrode (Pt and Ti) cell configuration immersed in a supersaturated solution containing calcium and phosphate ions. Adapted with permission.[152] 2006, Springer. B) Biomimetic deposition of CaP from a supersaturated simulated body fluid. Adapted with permission.[153] 2006, The Royal Society.

to increase the crystallinity of this thin CaP layer, a post-deposition thermal annealing process was used. The extensive characterization confirmed the deposition of a phase-pure HA coating, that was relatively stable in cell culture medium over a period of 10 days. The culture of SaOS-2 osteoblasts on the coated surface showed an increase of the markers of osteogenic differentiation, ALP, BMP4 and collagen type I as compared to uncoated surfaces.

Similarly, Hu et al. used a hydrothermal process to coat sand-blasted and acid etched titanium disks with nano-HA.[158] XRD and FTIR were used to confirm that the coating was made of crystalline HA. MC3T3-E1 cells were cultured on the materials, and it was shown that the HA coating significantly improved the cell attachment after 4 h and increased the ALP after prolonged culture time.

In a recent study, van Oirschot et al. evaluated the osteophilicity of a range of titanium substrates with different surface modifications, namely machined, grit-blasted and grit-blasted/acid-etched, and, in the case of grit-blasted titanium surfaces, coated with CaP using different techniques, namely pulsed laser deposition (PLD), plasma-spraying, electrospray deposition (ESD), biomimetic coating and magnetron sputtering.[159] A thorough physico-chemical and structural characterization showed that differences existed both in chemistry and surface roughness among the different materials. Bone conduction-cassettes containing the materials were attached on the goat transverse processes for a comparative in vivo analysis of the bone deposition on the surfaces of the materials over a period of twelve weeks. The results of the histomorphometrical analyses showed that predominantly plasma-sprayed coatings had a positive effect on the osteophilicity, plausibly due to a higher surface roughness as compared to other materials, or due to the existence of amorphous CaP regions in the coating, that may release calcium ions, subsequently stimulating the process of bone formation. While this study exemplifies an elegant attempt to investigate the role of CaPs versus titanium surface, and the role of surface structure, providing conclusive evidence regarding the effect of individual properties remains challenging, considering the differences in the chemical composition among the coatings.

The coating strategy constitutes an excellent method to decouple the role of surface chemistry from the rest of the properties, which remain almost intact. The thin layer of CaP may affect the surface roughness of the material, but depending on the coating method this effect can be almost negligible.

3.3. Reducing Surface-Structural Features

A more basic approach to reduce the influence of the topography when studying that of surface chemistry is to actually reduce the size of the topographical features present on the surface of the material. This approach can be combined with the coating procedures described in the previous section.

To test the hypothesis that the microstructure of CaP ceramics is important for the osteoinductive capacity of CaP ceramics, Davison et al. generated BCP discs having different surface microstructural feature dimensions.[44] Upon sintering at 1150 °C, the discs were coated with a 50 nm-thick film of titanium using magnetron sputtering following a previously established procedure,[160] to "cover" CaP on the surface, thus decoupling the chemical from the topographical effect. After intramuscular implantation in dogs for 12 weeks, both the BCP disc with small microstructural features and the respective

Ti-coated BCP disc were able to promote the formation of osteoclast-like cells and to induce de novo bone formation, but the size of the osteoclast formed on the Ti-coated BCP discs was between two and four times smaller than the size of osteoclasts formed on non-coated BCP discs. It should be noted that the titanium coating was not fully homogenous to completely "seal" the surface, still allowing the ions exchange between the underlying ceramic and the biological environment. Nevertheless, the results of this paper suggested that the size of the surface topography features plays an important role in osteoinduction.

In a recent study, Park et al. combined two of the previously described strategies, namely the coating techniques and the reduction of the structural features.[161] By coating flat β-TCP discs with HA, Mg and both HA and Mg, they studied the effect of the surface chemistry on osteoconductive properties of β-TCP ceramic. The HA coating was deposited using radio frequency magnetron sputtering, the Mg coating by DC sputtering, and the HA/Mg by the combination of the two sputtering techniques. SEM imaging and EDX analyses confirmed that the microstructure of the HA-coated β-TCP was very similar to that of β-TCP, while the surfaces of Mg-coated β-TCP and MgHA-coated β-TCP were similar to one other, but different from that of the uncoated β-TCP and HA-coated β-TCP. Osteoblastic MC3T3-E1 cell line culture indicated that cell morphology was shown to change from round or spindle-like on uncoated β-TCP to more polygonal shaped on the coated discs. While cell proliferation, evaluated using the MTT assay, was similar for all materials, the ALP activity, a marker for osteogenic differentiation, was higher on the HA-coated β-TCP and Mg-coated β-TCP, and significantly higher on the MgHA-coated β-TCP, showing the potential benefit of this method to modify the biological performance of the ceramic.

The method of reducing or eliminating microstructural features can be useful for evaluating the independent role of the chemistry. Nevertheless, it remains questionable whether with the currently available techniques, the topographical effect can indeed be fully eliminated, in particular considering that several studies have shown that cells may be responsive to structural features at the nanoscale,[162–164] even as small as a few nanometers in size.[164,165] Moreover, the use of this strategy to test CaP coatings with different ion substitutions is still challenging, as the incorporation of the ions can change the surface morphology of the coatings.[166]

4. Conclusions and Outlook

This progress report focused on discussing strategies to elucidate how individual properties of CaPs affect cell and tissue response to the material. In our view, this knowledge is essential for the development of new bone graft substitutes with improved bone regenerative potential. Indeed, in order to develop synthetic bone graft substitutes that can actually replace natural grafts in all, or at least the majority of clinical application, a design-driven approach is needed, in contrast to the processing-driven approaches, which are commonly used nowadays. The overview of different strategies described here has shown that a number of, often contradictory, results exist regarding the importance of a certain property in the biological response. Three main reasons can be defined for these contradictions: (1) an inadequate or incomplete material characterization, (2) a wide variety of methods used to assess the biological performance, along with the unavoidable inter- and intra-species variations, in particular in in vivo studies and (3) the fact that the material properties of CaPs are largely intertwined and cannot be independently tuned.

The importance of a comprehensive material characterization, including all those parameters that have been reported in the literature, or suspected to affect cell behavior, is crucial for achieving a full understanding of the mechanisms of action of CaPs. Only by fully understanding the materials and their properties, it will be possible to describe why and how these properties affect the biological performance. An adequate and complete characterization is also critical to evaluate the efficiency of the strategies developed to decouple individual properties, and to avoid claims that are based on selective observations.

The development of strategies to decouple the role of individual properties of CaPs in the biological performance of the materials has been put forward as a necessity for the field of biomaterials where the demand for efficient and affordable treatments for damaged and diseased organs and tissues is growing, and where the conventional methods of developing new biomaterials may fall short. However, the use of these strategies can also be heavily criticized, as they are not flawless yet, and furthermore, possible synergies among different material properties may be lost. Therefore, decoupling strategies should be seen as a first step toward the design of new materials for healthcare applications. The obvious next step will be to combine the desired properties in an intelligent manner, an effort that is not trivial and may require the development of new technologies. On a positive note, there is much room for creativity in the years to come in a field that has already made a big difference to the quality of patients' lives.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This research has been made possible with the support of the Dutch Province of Limburg.

Keywords

biomaterials-biological systems interactions, biomaterials design, bone graft substitutes, calcium phosphates, property-function relationships

Received: December 30, 2016
Revised: March 24, 2017
Published online: May 23, 2017

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