Manipulating Calcium Phosphate Materials
with **Surface Topography**
for **Bone Regeneration**

Rongquan Duan

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The research described in this thesis was fully supported by

The printing of this thesis was generously sponsored by

MANIPULATING CALCIUM PHOSPHATE MATERIALS WITH SURFACE TOPOGRAPHY FOR BONE REGENERATION

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus
Prof.Dr. T.T.M. Palstra,
on account of the decision of the graduation committee,
to be publicly defended
on Thursday, May 23rd, 2019, at 12:45

By

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ISBN: 978-90-365-4773-4

DOI: 10.3990/1.9789036547734

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献给勤劳善良的爷，娘

To my diligent and kind-hearted father and mother

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CHAPTER 1

General introduction

1. BONE BIOLOGY

1.1. Function of bone

Bone is a dynamic and highly vascularized tissue with some 'smart' capacities such as healing itself without leaving a fibrous scar formation, adapting its mass and morphology and rapid mobilizing mineral stores upon metabolic demand [1]. Bone tissue mainly provides structural support for the body by withstanding major biomechanical loads, facilitates movement via muscular contraction, and protects internal organs. Furthermore, bone acts as a mineral storehouse for calcium and phosphate and provides an environment for fat and bone marrow for hematopoiesis in adults [1].

1.2. Bone composition

Bone is formed by a series of complex events rigorously orchestrated by different types of bone cells interacting with each other, as well as the extracellular matrix. Its cellular makeup includes four main cell types: osteoblasts, bone lining cells, osteocytes and osteoclasts with relevant functions respectively [2,3].

Osteoblasts are cuboidal cells derived from mesenchymal stem cells (MSCs) and are responsible for bone matrix synthesis and subsequent extracellular mineralization. MSCs proliferate and are differentiated into osteoblasts during both intramembranous and endochondral bone formations [4]. Some cytokines are involved in these processes such as bone morphogenetic proteins (BMPs), runt-related transcription factors 2 (RUNX2), and parathyroid hormone (PTH). Runx2 is a master gene of osteoblast differentiation and has demonstrated to up-regulate osteoblast associated genes such as alkaline phosphatase (ALP) [5]. Osteoblasts synthesize and secrete type 1 collagen and other non-collagenous proteins such as osteopontin, osteocalcin and bone sialoprotein, which are involved in the formation of bone by mineralization [6]. During the bone matrix formation, some osteoblasts are entrapped with the newly formed matrix and continue their lives as osteocytes.

Osteocytes contribute to over 90% of bone cells, are the most abundant and long-lived cells [7]. Osteocytes located in bone lacuna keep contact with neighboring osteocytes in osteoid, as well as osteoblasts and bone lining cells on the bone surfaces, via an extensive network of cell processes. These connecting processes are thought to be essential in intercellular transport and nutrition [8]. Osteocytes are thought to act as mechanosensors to detect changes in physical forces upon bone, and as orchestrators of bone remodeling by adjusting bone resorption or formation [9].

Bone lining cells with flattened shape cover the bone surface, where neither bone resorption nor bone formation happens [10]. Bone lining cells contain only a few cell organelles, indicating their low metabolic activity. Bone lining cell functions are not completely understood. By covering the bone surface, they may prevent bone against any resorptive activity by osteoclast [11].

Osteoclasts are much large multinucleated (3-20 nuclei) cells and are originated from fusion of monocyte-macrophage lineage [12]. Osteoclastogenesis critically depends on several cytokines such as receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [13, 14]. Mature osteoclasts have calcitonin receptors, and vitronectin receptors, express the tartrate resistant acid phosphatase (TRAP) activity and vacuolar proton ATPase activity [15]. Osteoclasts are generally attached on the surface of bone to resorb mineralized tissue. This function is characterized with a ruffled edge, where active digestion occurs with the secretion of bone resorbing enzymes [15].

Extracellular bone matrix: In addition to bone cells, the remaining part in bone is bone matrix, which comprise approximately 65% inorganic matter and 35% organic component on a weight basis [16]. The *inorganic matrix* consists mainly of calcium phosphate (CaP) mineral in crystalline fraction (70%) and amorphous phase (30%), as well as magnesium, carbonate, fluorite, zinc, barium et al. as trace elements. Calcium and phosphate ions nucleate to form the hydroxyapatite (HA) crystals, which

are represented by the chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. The Inorganic component provides the major portion of the tensile yield and plays essential physiological function related to the storage of ions [17]. The *organic matrix* composes of 90% type I collagen, which is responsible for the bone tensile properties, and 10% non-collagen proteins (e.g. BMP, proteoglycan, osteopontin, osteonectin, osteocalcin, bone sialoprotein) and growth factors, which are important for bone metabolism [18]. Inorganic and organic matrixes make up a multilayered and organized framework which provides mechanical support and plays crucial role in bone homeostasis. With age, the percentage of inorganic matrix increases accompanying with the decrease of organic matter [19].

1.3. Bone remodeling

The bone remodeling process involves sequential osteoclast-mediated bone resorption and osteoblast-mediated bone formation, as well as is also associated with blood vessels and the peripheral innervation at the same location, and it is performed by the so-called basic multicellular unit (BMU) [20]. BMU is covered by a canopy of cells (e.g. bone lining cells) that form the bone remodeling compartment (BRC), connecting to bone lining cells on bone surface and communicating with osteocytes enclosed within the bone matrix [21]. The remodeling process consists of five distinct stages: activation, resorption, reversal, formation and quiescence [22]. Bone remodeling plays an important role in maintaining mature and healthy bone tissue. Bone remodeling can promote the replacement of apoptotic osteocytes in bone, the remodeling of the fracture callus into normal lamellar bone during the secondary stage of fracture healing and the restoring of microdamage in the mineralized matrix [23].

1.4. Bone fracture and healing

Bone fracture causes the loss of anatomic continuity and mechanical stability, as well as excruciating pain, motion deficits, functional disorders and lameness [24]. It is estimated that, by the year 2020, there are over 200 million people world-wide at risk for bone fracture because of osteoporosis or low bone mass. Bone fracture not only decreases quality of life (especially for elder people) and delays return to active life (e.g.

going back to work, to sport activity), but also brings out social and economic problems (increasing public expense on healthcare) [25].

As noted above, bone is a highly vascularized connective tissue with a remarkable self-healing. This process can be split into two categories: primary and second bone healing [26]. Primary healing apparently occurs when the fracture site is rigidly fixated, and the fracture gap is smaller than 0.01 mm. Under these situations, cutting cone consisting of osteoclast crossing the fracture line forms and generates longitudinal cavity, which is later occupied with bone tissue produced by osteoblast, fracture gap is finally filled directly by continuous ossification and subsequent Haversian remodeling [26].

Figure 1. Illustration of a typical fracture healing process, biological events, and cellular activities at different phases. The primary metabolic phases (blue bars) of fracture healing overlap with biological phases (brown bars). The time scale of healing is equivalent to a mouse closed femur fracture fixed with an intramedullary rod (Adapted from *Nat Rev Rheumatol*, 2015, 11:45-54).

Secondary bone healing is the more common form of fracture healing and takes place when the fracture edge is smaller than twice the diameter of the damaged bone [27]. Generally, a cascade of biological events such as haematoma formation, innate immune response, migration and differentiation of MSCs, cartilaginous callus formation and replacement of cartilage by bone and bone remodeling is involved in the secondary bone fracture repair. An anabolism is triggered initially in the form of increasing formed cartilage volume and continues in prolonged phase. Subsequently, this anabolic phase is replaced by catabolic activities as shown by reducing callus tissue volume. When the vascular bed and blood flow rate recover to pre-injury level, the catabolic phase reaches the final state. Finally, the stage of coupled bone remodeling happens. During this period the marrow space and hematopoietic tissue are reconstructed and recovery of the original structural and functional features of the injured bone tissue are achieved [28]. The cascade of biological events including the relevant activities and cells involved in bone fracture healing is illustrated as shown in Figure 1.

Local vascularity at the fracture site has been recognized as one of the most significant parameters affecting bone healing. The greatest amount of new bone formation often happens in the most vascularized areas, and inadequate vascularization at bone defect sites is associated with decreased bone healing. The combination of angiogenesis and osteogenesis is a decisive factor in the bone healing environment. In the bone fracture healing process, vascular endothelial growth factor (VEGF) is initially released from hematoma and facilitates the development of endothelial cells to form vascular network at the hypoxia environment. The formed blood vessel supplies nutrients and oxygen [23]. Although VEGF has been demonstrated as an essential player in induction of vascularization for bone healing, prolonged expression and overexpression can result in deleterious consequences via activation of osteoclasts or increased vascular permeability.

When the bone defect is too large to repair spontaneously by the two methods described above, it becomes a critical-sized bone defect.

Critical sized bone defects are defined as the smallest osseous defect in a particular bone and species of animal that does not heal spontaneously during the lifetime or shows less than 10% bony regeneration during the lifetime [29]. The critical sized bone defect is an extreme condition in bone healing, which can be resulted from inflammation, diseases, trauma, tumor resection and revision surgery. The vast bone loss in the critical-sized bone defect has been shown to directly affect revascularization and tissue regeneration, and eventually leads to non-union without intervention [30]. Non-union can highly affect the quality of patients' lives due to the prolonged and postoperative treatment costs and also pose a major surgical, socio-economical and research challenges [31]. In these cases, bone grafts are urgently needed to help repair the critical-sized bone defects.

2. BONE GRAFTS AND SUBSTITUTES

Bone grafts have been predominantly used to aid in the promotion or regeneration of bone, such as in fracture or spinal fusion, for decades [32]. There are more than two million bone grafting surgeries to be operated annually worldwide, which is indeed the second most frequent tissue transplantation right after blood transfusion [33].

Up to now, there is a wide spectrum of materials used for the purpose of bone grafting, which can be categorized into different types such as natural bone grafts (autograft, allograft, xenograft), ceramic-based (e.g. CaP ceramics and cements, calcium sulfate), metal-based, and polymer-based bone graft substitutes either with or without various growth factors (i.e. MSCs BMP, VEGF) and cells, as well as polymer-CaP composites [34]. The ideal bone substitute should be biocompatible and possess necessary properties required for bone regeneration such as osteoconduction, osteoinduction and osteogenesis [35].

Osteoconduction refers to the provision of an appropriate framework for bony growth with a surface whose biocompatibility supports and facilitates host progenitor cell migration, osteoblast attachment and eventual deposition of new bone. This ability depends on direct contact with host bone. Osteoinduction describes the graft to allow the

recruitment and osteogenic differentiation of host MSCs. This complex process involves the mediation of multiple signaling factors, such as those of the transforming growth factor β (TGF- β) superfamily, which includes BMPs. Osteogenesis means the synthesis of new bone from osteoblasts or progenitor cells within the graft that can survive in a host environment [35].

2.1. Natural bone grafts

2.1.1. Autologous bone grafts (autograft)

Autologous bone grafting is defined as the transplantation of an osseous graft harvested from a non-essential site (e.g. iliac crest) to the required site within the same individual. Autografts not only obviate graft-host reactions mediated by histocompatibility mismatches, but also can offer all necessary properties required for bone regeneration, it is thus still the “gold standard” in treating bone defects and the benchmark in evaluating bone substitutes [36]. With the possession of osteoconductivity, osteoinductivity and osteogenicity as well as osteogenic cells and growth factors, an autologous bone graft can integrate into the host bone rapidly and be replaced by newly formed bone completely without associated immune and infective risks [36]. However, as extensively reported [34-37], associating to the harvesting procedures, autologous bone grafting causes post-operative pain and other complications such as fracture, hematoma formation, infection and nerve palsy. In addition, the volume of harvested autologous bone is limited. The disadvantages restrict the clinical application of autogenic bone grafting [37].

2.1.2. Allogenic bone grafts (allograft)

Allogenic bone graft refers to bone tissue harvested from one individual and transplanted to a different individual of the same species, which is the second most common bone grafting technique as an alternative to autograft [38]. Allogeneic bone may be customized machined and is available in various forms, including demineralized bone matrix (DBM), cortical and cancellous chips, as well as osteochondral and whole bone

segments. Advantages of allografts in the treatment of critical sized defects such as in musculoskeletal malignancies could be seen [39]. However, because of irradiation or freeze-drying processing, allografts have been devitalized as shown by less osteoinductivity and no cellular component. In addition, allografts are related to risks of immunoreactions and transmission of infections. Compared with autografts, allografts result in a higher failure rate [40].

2.1.3. Xenogeneic bone grafts (xenograft)

Xenograft is harvested from a species other than human being such as bovine or porcine which can be freeze dried or demineralized and deproteinized. The first documented xenografting was operated in 1668 in the Netherlands by Jacob van Meekeren who employed a piece of dog bone to repair a skull defect of an injured soldier [41]. Xenograft is usually considered as a calcified matrix. Various types of corals are harvested and processed into coralline xenografts [42]. In addition, recently, different species of wood have been pyrolyzed in an inert atmosphere to get a carbonaceous residue, which is saturated with calcium salts and finally reheated to achieve a highly porous crystallized material. The wood-based material has better penetration during bone growth and more flexion than metal or ceramic grafts [43]. Commercially available coral derived products are Interpore and Pro-osteon as well as bovine-based products such as Bio-Oss and Endobon. Though xenograft has some advantages such as the availability, osteoconductivity and low costs, the results of clinical practice are contradictory with the favorable data by some authors and non-favorable results by others. Additionally, xenografts have potential risks of zoonose diseases transmitted from animals to humans, like bovine spongiform encephalopathy (BSE) or porcine endogenous retroviruses (PERV) [44].

In order to overcome the drawbacks of natural bone grafts such as donor site morbidity, volume availability, potential disease transmission and immunogenicity, a variety of synthetic bone graft substitutes has been triggered to blossom in clinics.

2.2. Ceramic-based bone grafts

Due to the similarity to inorganic matrix of bone, biocompatibility and osteoconductivity, ceramic-based bone grafts generally composed of calcium or/and phosphate are the largest class of synthetic bone graft substitutes and have been widely used in clinics. CaP ceramics, CaP cements, calcium sulfate or combinations thereof are most commonly synthetic bone substitutes available at the present [45].

2.2.1. Calcium phosphate ceramics

Based on composition, CaP ceramics presently used as bone substitutes are classified as of HA, beta-tricalcium phosphate (β -TCP) and biphasic calcium phosphate (BCP) with both HA and TCP as the mixture. They are generally produced in a highly thermal process known as "sintering" where they are heated between 700 °C and 1300 °C to form their crystalline structure [46].

HA is the primary mineral component of bone and teeth. Because of its excellent osteoconductivity and similar initial mechanical properties to the cancellous bone, HA ceramic has been popularly used in orthopedic, craniofacial and orthognathic surgery since the 1970's [46]. For example, Werber et al. (2000) used HA bone substitute to repair bone defects after distal radius fractures, magnetic resonance imaging (MRI) and biopsy indicated that HA is a well-tolerated bone graft substitute with the evidence of osseous integration and incorporation [47]. However, the relatively high Ca/P ratio and crystallinity delay the resorption process *in vivo*, the residual HA grafts may not compromise the intrinsic intensity of the bone due to the decreasing of mechanical strength [48]. Therefore, HA alone is frequently used in non-loaded sites, or as a coating on implants and external fixator pins [49].

Using β -TCP with the chemical formula of $\text{Ca}_3(\text{PO}_4)_2$ for bone repair was firstly reported by Albee in 1920 [50]. With the Ca/P molecular ratio of 1.5 lower than that of HA, TCP possesses higher degradation and resorption rate, it could eventually be replaced by new bone tissue. This advantage gives the TCP bone substitute to repair effectively bone defects caused by trauma and benign tumors [51]. Recently, it has been shown that β -TCP enhanced angiogenesis in augment of bone defects,

for instance, Chen et al. (2015) found that higher content of TCP phase significantly upregulated proliferation of human umbilical vein endothelial cells (HUVECs) and subsequently angiogenesis *in vitro*. Meanwhile, CaP ceramics with higher content of TCP also induced higher density of microvessels in a mice intramuscular implantation model [52]. Though various hypotheses are proposed to explain this phenomenon, such as the porous structure, ions transfer and strain changing et al., the exact mechanism is still unknown [53]. There are several potentially usable agents in the TCP category including AttraX (NuVasive, Inc.), ChronOS (DePuy/Synthes, Paoli, PA), Vitoss (Stryker/Orthovita, Malvern, PA) and Osferion (Olympus, Tokyo, Japan).

BCP bone substitutes relate to another widely used group of commercial ceramics obtained by mixing HA and β -TCP in different ratios for the purpose of combining their advantages [54]. The combination of HA and TCP enables a faster and higher bone ingrowth than using HA alone while offering better mechanical properties than β -TCP alone. By adjusting the formulation, the dissolution rate and mechanical properties can be controlled within ranges and subsequently applied in bulk or as implant coatings. Given that degradability of BCP bone substitutes is mainly dependent on the ratio HA/ β -TCP, optimal ratios were addressed. Yamada et al. (1997) reported that resorption activity of osteoclasts was observed on β -TCP and BCP 25/75 (25% HA/75% β -TCP) and not on BCP 75/25 (75% HA/25% β -TCP) and HA. Interestingly, they found that solubility affected the pattern of osteoclastic resorption in terms of distribution and shape of resorption lacunae [55]. Some of commercially available BCP bone substitutes are MBCP (Biomatante, France) and MagnetOs (Kuros Biosciences, the Netherlands).

2.2.2. Calcium phosphate cements

For the purpose of extending the adaptability and moldability of CaP bone substitutes, CaP cements were invented by Brown and Chow in 1983 and were approved by FDA for the treatment of non-load bearing bone defects in 1996. CaP cements can be conveniently injected to fill defects with irregular shapes and subsequently solidified by mixing with

an aqueous phase through setting reaction. The hardening reaction forms nanocrystalline which makes CaP cement microporous, biocompatible and mechanically supportive with low bending strength [56]. Compared to polymethyl methacrylate (PMMA) cement, hardening of CaP cement does not change the temperature and physiologic pH, resulting in no harm to surrounding tissue [57]. However, compared to CaP ceramics with macroporosity, CaP cements degrade layer by layer *in vivo*, theoretically hampering ingrowth of neovascular and bone tissue [58]. Based on its flow feature before setting reaction, CaP cements mainly favor bone replacement, in percutaneous vertebroplasty [59], but not as bone substitutes in general.

2.2.3. Calcium sulfate

Calcium sulfate (CS), also known as gypsum or plaster of Paris, is a kind of osteoconductive and biodegradable synthetic composed of CaSO_4 . It was firstly used in humans as a void filler of tuberculous osteomyelitis at 1892 [60]. CS is a heated gypsum and made into powder form, with eventual crystalline structure described as alpha hemihydrate [61]. With its rapid resorption rate but weak internal strength, CS can only be used to fill small bone defects with rigid internal fixation [62]. Due to its faster degradation in early stage of bone regeneration, CS did not give optimal fusion rate in spinal arthrodesis [63]. CS could be combined with antibiotics (i.e. vancomycin) to treat the chronic osteomyelitis in achieving rapid control of infection, however the replacement of the composite by new bone was not uniformly reached [63]. As such, CS application in clinics is limited.

2.3. Metal-based bone graft substitutes

Metals are used for bone repair because of their mechanical properties, while metals are recognized as non-bioactive materials in general. However, through the specific treatments, metals such as titanium can be made bioactive. For example, Santander et al. (2012) found that refining surface roughness endowed titanium enhanced osteogenic differentiation of hBMSCs *in vitro* [64]. Given the fact that implant surface interacts with biological environment, controlling surface topography

could be a crucial parameter for determining the surface ion interchange, protein adhesion, and cell interaction [65]. Numerous methods have been employed for titanium to obtain favorable micro-scaled roughness such as acid etching, sandblasting and spark anodization [66]. In addition to mechanical surface refinement, a chemical surface treatment is also generally applied. Surface ion impregnation allows the materials to act chemically for the promotion of new bone formation or bone loss reduction because ions are essential factors for enzymes [67]. For example, phosphate ion has been associated with proteins in bone formation and thus was employed to modified titanium surface [68].

2.4. Polymer-based bone graft substitutes

Having distinctly different physicochemical and mechanical properties from other bone substitutes. polymers can be divided into natural polymers and synthetic polymers. As one of the most abundant protein found in bone, is collagen has been widely used in orthopedic applications due to its excellent biocompatibility and availability, but its mechanical properties remain in question [69]. The synthetic polymers can be classified as nondegradable (e.g. PMMA) and fully biodegradable (e.g. polylactic acid, PLA) [33]. Due to its high mechanical property and feasibility for handling, PMMA bone cement has been widely used in total joint replacement and the percutaneous vertebroplasty for the fixation of components. However, the exothermicity during polymerization, mechanical mismatch and aseptic loosening were reported inevitably resulting in the failure of arthroplasties with PMMA [70].

The biodegradable PLA allows a total bone replacement in time without remaining foreign implants, and thus have been used as standalone devices and as extenders of autografts and allografts [33]. For example, Chou et al. (2016) used a PLA cage as a carrier for bone chips to regenerate bone in a rabbit model [71]. Due to its biocompatibility, biodegradability and hydrophilicity, poly ethylene glycol (PEG) is another synthetic compound used in orthopedic surgery. It could be used in functionalizing other bone substitutes and linking growth factors to improve bone formation [72]. Polymer-based bone substitutes are

suitable to be used as carriers for growth factors or bioactive molecules, potentially conferring osteogenic properties. Allowing their fabrication with macropores/micropores and in the shapes as desired (e.g. PLA or PEG), polymer-based bone substitutes are mainly scrutinized for their wide potentials in tissue engineering as scaffolding materials [73].

2.5. Polymer-ceramic composites

Bone tissue is a composite material consisting of minerals (e.g. HA) and polymers (e.g. collagen), polymer-CaP composite materials, which use the advantages of both materials, represent attractive candidates for bone graft substitution. CaP fillers improve the shapeability and mechanical strength of polymers (e.g. compressive strength and modulus) and enhance, osteoconductive property. Composite materials have demonstrated their superior outcomes in bone regeneration in comparison to the use of the individual materials on their own [74]. For example, collagen type I and hydroxyapatite enhanced osteoblast differentiation, when combined accelerated osteogenesis [75]. Ma et al. (2001) reported that porous poly (L-lactic acid) (PLLA)/HA composite scaffolds had a higher osteoblast survival rate, better growth and over express of bone specific gene *in vitro*, but also improved bone formation *in vivo* compared with pure polymer scaffold [76]. In addition, the addition of HA to natural polymer scaffolds has been shown to potentially reduce adverse effects associated with the degradation of certain synthetic polymers [77]. Overall, composites of HA and various polymers such as PLA, collagen, chitosan and gelatin, have been successfully assembled and have demonstrated to improve bone tissue formation *in vivo*. Polymer-ceramic composites are considered to be biomimetic, to stimulate the deposition of CaP from simulated body fluid (SBF), leading to enhanced bone matrix interface strength [78].

3. OSTEOINDUCTIVE BIOMATERIALS

Next to material optimization with respect to biocompatibility, bioactivity, handling property, and mechanical property, growth factors and cells are suggested to combine with scaffold materials to enhance bone regeneration. For instance, BMPs, especially BMP-2 (e.g.

recombinant human BMP-2, rhBMP-2) is a member of the TGF- β superfamily with superior osteoinductive property and is possibly the most extensively investigated growth factor in treating bone defects [26]. However, the swelling, inflammation and excessive bone formation of BMPs, the high cost and burst release of the growth agents, as well as the difficulty in storage and transportation of the living cells hindered their applications in orthopedic surgery [79].

One of the reasons why that autografts are considered as gold standard of bone graft is their inherent initial osteoinductivity, which is the ability to induce osteogenic differentiation of MSCs to give rise to *de novo* bone formation without the presence of additional osteogenic factors. The osteoinductive property of a material is usually evaluated by bone formation after implantation in non-osseous sites (e.g. in muscle or under skin). Osteoinduction was firstly reported by Urist in 1965 who found that demineralized bone matrix (DBM) induced bone formation in muscles of different animals [80]. CaP biomaterials are generally known to be osteoconductive but not osteoinductive until the early 1990's when an array of CaP materials was demonstrated to possess innate osteoinductive property [81-83]. Since then, plenty of CaP-based biomaterials were demonstrated osteoinductive in different animal models, such as porous synthetic HA, coralline HA, porous β -TCP, porous BCP, CaP cements, dicalcium phosphate anhydrous (DCPA), dicalcium phosphate dihydrate (DCPD) et al [84-88]. It has been shown that the osteoinduction is material dependent and is an isolated phenomenon heavily influenced by their physicochemical properties [89].

Studies have demonstrated that osteoinductive biomaterials not only induced ectopic bone formation in non-osseous sites but also enhanced bone regeneration in osseous sites, in comparison with non-osteoinductive counterparts [90,91]. Furthermore, an osteoinductive TCP material showed an equivalent performance to autograft and collagen loaded with BMP-2 in repairing critical-sized bone defects [92]. These findings arouse increasing interest in engineering biomaterials with higher osteoinductivity in order to repair bone defects as well as possible.

4. THE INFLUENCE OF PHYSICOCHEMICAL PROPERTIES ON OSTEOINDUCTION

Osteoinduction was often observed in specific materials but not in others, indicating the possible roles of chemical composition, macrostructure, microstructure, surface topography, porosity of the CaP in material-driven osteoinduction [89].

4.1. The influence of chemical composition on osteoinduction

The majority of biomaterials demonstrated as osteoinductive to date comprises calcium and phosphate groups, indicating that the presence of calcium and phosphate is a prerequisite for inducing ectopic bone formation. Meanwhile, it was shown that calcium and phosphate ions in the microenvironment enhanced the osteogenic differentiation of stem cells *in vitro* [93]. Calcium and phosphate ions released from CaP materials were assumed to trigger osteogenic differentiation of stem cells ultimately leading to bone formation [91]. As CaP materials with different compositions have different solubility resulting in various calcium phosphate ion releases, it appeared that a higher solubility of CaP ceramics could result in higher osteoinductivity. For example, having similar macropores and micropores, BCP with higher content of TCP induced more ectopic bone formation compared to HA, which is obviously associated with the higher dissolution rate of BCP than that of HA [92,93]. However, things do not always happen in this way. Kurashina et al. (2002) reported that an increase in the amount of TCP had a negative effect on osteoinductive potential [95]. Chemical composition and associated dissolution behavior may not be appointed determinant for osteoinductive potential. As a support, inductive bone formation could be triggered by poly-hydroxyethyl methacrylate (Poly-HEMA) [96] and titanium implants [97] in which no calcium or phosphate are present or released.

4.2. The influence of macrostructure on osteoinduction

Inductive bone formation was always detected in the pores of materials with interconnected pores, or in dense microparticles in between which

individual microparticles provide porosity, indicating the necessity of macroporous structure in inducing bone formation. The basic function of macropores is generally considered to accommodate blood vessel ingrowth, which bring along adequate exchange of nutrients, oxygen, cells and metabolic wastes. It has been suggested that 100-200 μm pores are enough to support cell migration, 300-500 μm pores appear to be recommended to allow the formation of capillaries [98]. For example, In the study by Fujibayashi et al. (2004) who found titanium blocks with predefined porous structure could induce bone formation in dogs, in contrast to titanium fiber meshes. In addition, the pore dimension plays important roles in osteoinduction caused by materials [97]. Bodde et al. (2007) found that the CaP cement cylinders with small pores (150 μm) failed to induce bone formation as compared to those with bigger pores after 90 or 180 days of implantation [99]. Besides the presence of pores with suitable dimensions, geometry of porous structure has been shown important in osteoinduction. For example, Ripamonti et al. (2004) observed that bone formation always started in the concave and never on the convex spaces of HA ceramic implanted in the muscle of baboons, indicating that specific geometries could be a prerequisite for ectopic bone formation [100].

4.3. The influence of microstructure on osteoinduction

In addition to chemical composition and macrostructural properties, micropores (pore size < 10 μm) play a crucial role in promoting osteogenesis. The micropores on the walls of macropores are not only beneficial to penetrating body fluids, but also produce rough surfaces on the walls, which are favorable for cell attachment and the expression of osteogenic phenotype. It has been demonstrated that biomaterials with same chemistry, but different microstructural properties could give different performances when implanted heterotopically. For instance, macroporous HA scaffolds with micropores could trigger bone formation in non-osseous sites, while the one without such micropores failed [101]. It has been suggested that the micropores on the walls of macropores play a positive role in favoring the adsorption of proteins with low

molecular weights (e.g. TGF- β) [102]. Furthermore, it has been proven that the microporosity positively affected osteoinductive potential of CaP-based materials [103]. In fact, another important parameter to be considered is the dimension of the micropores. When TCP ceramics having similar strut porosities were intramuscularly implanted, the one with submicron sized pores was more favorable for inductive bone formation compared to the one having micron scaled pores [104,105]. A similar phenomenon was observed in BCP ceramics [106]. It is likely that there is an optimal dimension of micropores necessary for the occurrence of inductive bone formation in CaP materials.

4.4. The influence of surface topography on osteoinduction

The surface topography of materials has been shown to affect cell adhesion and differentiation, it seems particularly important to material-driven bone formation. In the study of Dalby et al. (2007), who showed that disordered nanoscale topography significantly increased osteogenic differentiation of MSCs, while the symmetric and random nanoscale arrays did not [107]. Chen et al. (2008) reported that micropore structure on HA surface upregulated the early osteoblastic differentiation, but the macropore structured one favored cell proliferation [108]. Zhang et al. (2014) observed that having the same chemistry, macroporosity and microporosity, submicron scaled surface topography facilitated osteogenic differentiation of MSCs *in vitro* and induced ectopic bone formation *in vivo* [104]. These studies revealed the possibility of surface topography on cell behavior including osteogenic differentiation. In CaP materials, the surface topography might also be reflected in surface microstructure variation, resulting from different crystal sizes by different sintering temperatures.

In summary, osteoinduction by biomaterials can be influenced by chemical composition, macro- and micro-pore structure and surface topographical feature. However, the detailed links between these material properties and inductive bone formation are still unclear.

5. THE MATERIAL MECHANISM UNDERLYING OSTEOINDUCTION

To disclose the relationship between physicochemical properties and osteoinduction, some hypotheses have been proposed in the literature, in speculating their function, such as protein adsorption, surface biomineralization and ion release (Figure 2).

Figure 2. Schematic proposed mechanisms underlying osteoinduction by biomaterials. Physicochemical properties of osteoinductive biomaterials may trigger ectopic bone formation directly or indirectly. Micro and nano structural properties can favor the interaction with BMPs and other essential endogenous proteins that in turn trigger stem cell differentiation into osteoblasts and hence bone formation. Surface apatite layer and inorganic ion release may also be a direct trigger of the process of osteogenic differentiation and bone formation (Adapted from Eur Cell Mater, 2011, 21:407-429).

5.1. Protein adsorption

Urist et al. (1965) firstly observed that osteoinduction by DBM and identified BMP in DBM responsible for DBM-induced bone formation [80]. Because growth factors such as BMPs are present in the body and thanks to their high affinity to CaP materials, biomolecules adsorption

was suggested as a potential process favoring bone induction [109,110]. It is generally thought that the porous structure of CaP materials acts as solid substrate offering adsorption sites for the local accumulation of proteins. It is thus assumed that a concentration threshold of growth factors could be reached to trigger inductive bone formation. As such, De Groot et al. (1998) proposed the rational design and development of BMP concentrators that can concentrate and immobilize the endogenous BMP complex facilitating bone regeneration [111]. The protein adsorption theory explained well the osteoinduction in microporous HA ceramics, in materials with high strut porosity and in submicron surface structured TCP ceramics. However, it is difficult to explain why not all materials, or at least all CaP materials with their high affinity to bind BMPs are osteoinductive.

5.2. Calcium and phosphate ion release

Due to the fact that the osteoinductive phenomenon is mainly observed in CaP based biomaterials and osteoinductive potential varied with CaP chemical composition and solubility, calcium and phosphate ions are suggested to be of great importance for osteoinduction caused by CaP materials [89]. Additionally, when bone remodeling, calcium and phosphate at supersaturated level have significant impact on the proliferation and differentiation of osteoblasts and on the subsequent bone formation [112]. Furthermore, it was reported that calcium and phosphate ions in the microenvironment enhance the osteogenic differentiation of stem cells and osteogenic cells *in vitro*. It is reasonable to speculate that the possible roles of the release of calcium and phosphate ion in material-driven osteoinduction [93,94]. It is likely that release of calcium and phosphate from more soluble β -TCP phase in BCP caused a local increase in ion concentration, resulted in more calcium and phosphate precipitation, hence promoted protein adsorption and bone formation in comparison to stable HA [106]. In support of this, the most powerful osteoinductive synthetics ever reported were TCP ceramics, which dissolve faster, providing more calcium and phosphate sources as building blocks of bone [91,114]. However, it should be kept in mind the

cases of inductive bone formation in poly-HEMA and titanium implants with the absence of calcium and phosphate ions [96,97], which question the essential role of calcium and phosphate in material-driven osteoinduction.

5.3. Surface biomineralization

It is suggested that the biological apatite layer forming on the surface of biomaterials plays important roles in osteoinduction, especially in the cases of osteoinductive materials free from calcium and phosphate, such as titanium and poly-HEMA. Prior to ectopic bone formation, those materials underwent biomineralization *in vivo* [97]. The apatite layer consisting of calcium hydroxyl carbonate apatite is equivalent in composition and structure to the inorganic matrix of bone, the formation of such biological apatite layer has become a standard for evaluating bioactivity of a material. The formation of an apatite layer on biomaterials includes the nucleation, growth and crystallization and mainly depends on the concentration of calcium and phosphate ions, pH value, temperature and surface feature of the solid onto which the apatite will be deposited [106,114]. Material's total surface area and chemical composition affect the dissolution and reprecipitation events at their surface finally leading to the formation of a biological apatite layer [89]. Proteins eventually entrapped in the biological apatite layer might induce osteogenesis in such osteoinductive material. However, it's worth noting that many CaP-based biomaterials which can induced biological apatite layer formation on their surface do not possess osteoinductivity.

5.4. Mechanotransduction

In the absence of chemical signals, surface topography of biomaterials has been shown to directly affect the behavior of adherent cells through mechanotransduction, i.e. gene regulation through physical and interactions between surface topography and cells [115]. For instance, Abagnale et al. (2015) reported that ridges with 15 μm increased differentiation of MSCs to adipogenic lineages, while smaller ridges enhanced osteogenic differentiation [116]. Zhang et al. (2014) found that submicron scaled surface topography of TCP enhanced ALP activity and

expression of osteocalcin and osteopontin *in vitro* and induced ectopic bone formation following implantation, while micron scaled one failed [104]. Furthermore, Abagnale et al. (2015) reported that randomly arranged surface pits induced more MSCs differentiation than pits arranged in an orderly grid, without osteogenic medium supplements [116].

In addition to the surface structure scale, the surface structure morphology has been shown to affect differentiation of MSCs. For instance, Kilian et al. (2010) found that the star-like surface structure promoted differentiation of MSCs towards osteoblasts while the flower-like one favored adipogenesis from MSCs [117]. This finding provides another interesting method to further improve osteoinductive potential of CaP materials.

The mechanical forces from actin rearrangement and cytoskeletal tension are transferred to the cell nucleus through various signaling pathways where gene transcription is changed [118]. Following this principle, it is likely that osteoinductive surface topography adjust the adherent MSCs to adapt their shapes and subsequently trigger a series of biological responses through the mechanotransduction, such as recruiting TGF receptor on primary cilia and undergo osteogenic differentiation and finally inducing ectopic bone formation upon implantation [119].

6. BIOLOGICAL MECHANISM UNDERLYING OSTEOINDUCTION

Osteogenic differentiation of MSCs is a key step in inductive bone formation in materials following ectopic implantation and it has been shown that osteogenic differentiation of MSCs is affected by physicochemical properties of materials [105,106]. While MSCs may not be the only cells respond to physicochemical properties of materials. For instance, macrophages have shown to be regulated by specific surface topographies. Fellah et al. (2010) observed that the number of macrophages varied according to the grain size of the CaP materials [120]. Furthermore, Davison et al. (2014) showed that CaP ceramics with submicron sized surface architecture could induce inflammatory response of macrophages and their subsequent osteoclastogenesis and

osteogenesis [105]. When macrophages were selectively depleted in mouse model by liposome-encapsulated clodronate, ectopic bone formation in osteoinductive materials was significantly blocked [121]. De Bruijn et al. (2008) proposed that the processes leading to material-directed heterotopic bone formation start with the injury at the implantation, followed by inflammation and invasion of macrophages into the material, which are stimulated to release cytokines causing chemotaxis of MSCs, subsequent osteogenic differentiation to eventually form bone [122].

6.1. Biological responses after implantation

When a biomaterial is implanted into the living body, a cascade of biological events takes place in the surrounding tissue, which not only predicts the fate of the implant but also modulates immune responses to overcome translational challenges in regenerative medicine. The biological events start with the formation of a provisional matrix and end with the production of foreign body giant cells [123] (Figure 3).

6.1.1. Blood protein adsorption and provisional matrix formation

In the early stages of implant insertion, plasma components extravasated from injured blood vessels adhere to the surface of the implant and develop into a provisional matrix, which consists of fibrin rich clots, platelet granule components, growth factors and cytokines. The provisional matrix not only serves as a depot for inflammatory cells, cytokines and growth factors, but it is also capable of mediating macrophage activity and proliferation as well as activation of other cells involved in the inflammatory and wound healing responses [124,125].

6.1.2. Acute inflammation

Immediately following protein deposition and provisional matrix formation, an acute inflammatory response occurs, which is characterized by the presence of blood-derived polymorphonuclear leukocytes (PMNs), predominantly neutrophils [126]. The PMNs migrate from blood vessels to the injury site and act as the first line of defense against invading foreign body that is deemed to be 'dangerous'.

Figure 3. Immune response toward biomaterials. **A:** Adsorption of blood proteins and activation of the coagulation cascade, complement and platelets result in

the priming and activation of PMNs, monocytes and resident macrophages. B: Danger signals (alarmins) released from damaged tissue additionally prime the immune cells for enhanced function via PRR engagement. C: The acute inflammatory response is dominated by the action of PMNs. PMNs secrete proteolytic enzymes and ROS, corroding the biomaterial surface. D: Macrophages are the driving force of chronic inflammation. Constant release of inflammatory mediators like TNF- α , IL-6, and MCP-1 results in permanent activation of macrophages. E) Macrophage-derived cytokines and PRR engagement activate DCs on the biomaterial surface (Adapted from Biomaterials, 2011, 32:6692-6709).

The PMNs meet with the provisional matrix at the injury site, where engagement of molecules and their corresponding receptor on granulocyte surface. Activated PMNs secrete proteolytic enzymes and reactive oxygen species (ROS), which may erode the biomaterial surface depending on the properties of the biomaterial [127].

6.1.3. Chronic inflammation

At the end of the acute inflammation, if the foreign body is not removed, a chronic inflammation arises, which is characterized by the presence of monocytes, macrophages and lymphocytes, with the proliferation of blood vessel and connective tissue. Especially, the macrophages secrete chemokines to induce invasion of other inflammatory cells [125].

6.1.4. Macrophage phenotypes and their functions

Macrophages are mainly derived from circulating monocytes and migrate to local implant site, where they can secrete pro-inflammatory cytokines to recruit more macrophages and produce ROS and degradative enzymes in an attempt to phagocytize the implant [128]. The activation of inflammatory macrophages triggers wound healing process by recruiting fibroblast and endothelial cells, which proliferate and form granulation tissue. The endothelial cells organize into capillary tubes to form new small blood vessels, while fibroblasts synthesize proteoglycans and later collagen [125]. To fulfill their functional diversity, macrophages exhibit a spectrum of transient polarization states: originally referred to as M1 (classically activated) and M2 (alternatively activated) macrophages [129, 130].

Classical activation macrophages (M1)

The activation of M1 macrophages are typically depended on interferon- γ (IFN- γ), tumor necrosis factor α (TNF- α) or bacterial lipopolysaccharide (LPS). They exhibit a pro-inflammatory expression profile during the early stage of the normal tissue repair process [131]. M1 macrophages can secrete pro-inflammatory cytokines and perform microbicidal activity mediated by increased synthesis of ROS and nitrogen radicals [130]. However, a prolonged M1 presence leads to a severe foreign body reaction (FBR) and fibrous encapsulation resulting in failure of biomaterial integration. This is especially detrimental for regenerative biomaterials where the goal is to replace lost tissue and avoid scar tissue formation [132].

Alternative activation of macrophages (M2)

The M2 macrophages are activated by signals such as interleukin-4 (IL-4) and IL-13 during adaptive immune responses [130]. IL-4 programs macrophages to down-regulate pro-inflammatory mediators and to promote wound healing processes by contributing to the production of extracellular matrix (ECM) and by activation of fibroblasts [130]. Furthermore, the presence of such anti-inflammatory cytokines and the tissue remodeling response can facilitate the vascularization of regenerative biomaterials by inhibiting fibrous tissue formation, and enhance the integration of the biomaterial and enable it to fulfill its intended function [133]. In conclusion, M2 macrophages are associated with a more regulatory and recovery character and can promote tissue repair. If for any reason the host immune system fails to enhance M2 macrophage levels for switching to the healing stage, it could lead to the inability of macrophages to resolve the inflammation, resulting in a state named 'frustrated phagocytosis' [134].

Although historically, the terms "pro-inflammatory" and "pro-healing" have been associated with the M1 and M2 phenotypes respectively, the current state of knowledge in the field does not support the oversimplified notion of M1 macrophages as being detrimental for healing and M2 macrophages as being positive for healing. M1 and M2

macrophages are both needed at different time points in the healing process. Promotion of a rational and timely control of the M1/M2 balance throughout the healing process seems to be key in tissue engineering and regenerative medicine [135].

6.2. The influence of physicochemical properties of implant on immune response

It is well established that microenvironmental cues presented by implant play an important role in immune response of host tissue [136]. The physical and chemical surface properties of the implant are thus largely responsible for the foreign body reaction propagated by infiltrating immune cells. Physicochemical properties have been shown to affect the behavior of adherent cells, and great progress has been achieved in understanding these effects on both stem cells and somatic cells. However, the influence of such cues on immune cells specifically macrophages is less well known [137].

6.2.1. The influence of physical properties on immune response

The physical properties of the biomaterial, such as surface topography has been shown crucial in immune response. From a biological perspective, the physical properties of biomaterial, such surface topography can directly control the shape and elasticity of adherent immune cells and affect cellular responses [138].

Surface topography, especially at micron/nano scale, has been shown to control events that are instrumental in the immune response to biomaterial. For example, Cao et al. (2010) found that scaffolds with an aligned fiber topography had a significantly decreased capsule formation and enhanced cell infiltration compared with those with randomly aligned fibers [139]. Chen (2010) reported the effect of nano/microtopography in macrophage behavior in the FBR using gratings (500 nm to 2 mm parallel) imprinted on selected polymer surface. They found that different grating topographies induced changes in macrophage behavior independently of surface chemistry compared to planar controls [140]. In addition to the dimension of surface topography, several studies have indicated the role

of the shape of surface topography in controlling immune response. For example, Matlaga et al. (1976) noted that rods with circular cross-sections triggered the least-extensive FBR, compared to pentagonal and triangular cross-sections [141].

6.2.2. *The influence of chemical component on immune response*

In addition to the physical surface properties of implants, the surface chemistry has been shown to be a straightforward way to regulate protein adsorption and subsequently cellular behavior. It is generally believed to alter protein adhesion and therefore direct downstream cell recruitment resulting in producing "repellent" surface [142]. The typical characteristics of protein resistant functional groups include hydrophilic nature, hydrogen bond acceptors and neutral charge et al [143]. Generally, hydrophilic functionality provides low interfacial free energy resulting in decreased protein adsorption, cell adhesion, and blood compatibility [144]. Thus, increasing surface hydrophilicity has been shown to improve the biocompatibility of the implant at least *in vitro*. Despite the fact that there are very few studies which have looked at the effects of surface functional groups *in vivo*, *in vitro* results have supported the view that surface functionality may alter biomaterial-mediated acute inflammatory responses *in vivo* [145,146].

Taken together, surface architecture and chemistry of implants can be manipulated to alter immune response, which may have a role in osteoinduction. For example, Le Nihouannen et al. (2005,2006) showed that *de novo* bone formation may be stimulated by inflammatory microparticles which activated macrophage phagocytosis and secretion of osteogenic cytokines [147,148]. However, without the counterpart with other surface structures to compare immune response to *de novo* bone formation, the relationship between immune response and osteoinduction is mainly speculative.

7. AIM OF THIS THESIS

The progress made in the last two decades has made it is possible to alter physicochemical properties of CaP materials to endow them with

osteoinductivity [90,99]. Among physicochemical properties, the dimension of surface topography has been demonstrated to be crucial in inducing ectopic bone formation in multiple studies [93,105,106]. Next to the dimension of surface topography, the surface morphology is another characteristic of surface topography which has been reported to affect osteogenic differentiation of MSCs *in vitro* [118]. It may be possible to tune surface morphology to further accelerate osteoinductive potential of CaP materials.

Although the key physicochemical properties relevant to CaP-instructed osteoinduction have been deeply outlined [89,90,99], the mechanism underlying material-driven osteoinduction is still unknown. Some theories such as protein adsorption, calcium and phosphate ion release and biomineralization have been proposed, but can't explain all phenomena observed so far in material-instructed bone formation.

How the physicochemical parameters of materials initiate the biological response involved in material-instructed bone formation is unknown as yet, while the biological mechanism underling material-driven bone formation is even less addressed. Osteogenic differentiation of MSCs through chemistry or special surface topographies of materials would be a key step in CaP-instructed bone formation [95,108,149]. However, CaP-instructed bone formation in non-osseous sites does not occur immediately but weeks after implantation. Other biological responses in wound healing process may therefore take place prior to osteogenic differentiation of MSCs [93,105,106]. Firstly, in order to ensure steady transport of MSCs from bone marrow to implant site, angiogenesis in and around the implant would be necessary [150,151]. Next, the injury elicited upon implantation would attract and stimulate immune cells (e.g. macrophages) to secrete cytokines that could favor osteogenic differentiation of MSCs and drive ectopic bone formation [120,128]. In this way, the biological processes underlying ectopic bone formation need to be explored.

Osteoinductive CaP bone grafts do not only trigger ectopic bone formation via inducing osteogenic differentiation of MSCs, but they can

also enhance orthotopic bone formation. The significance of osteoinductive materials has indeed been demonstrated in critical-sized preclinical models [92,93]. However, in order to translate the fundamental findings from bench to bedside, it would necessary to evaluate osteoinductive materials in clinically relevant models.

The flowing questions are addressed in this thesis:

- What are the key material factors evoking inductive bone formation and how do they initiate biological responses?
- Are the key material factors relevant to osteoinduction also favorable for bone regeneration in orthotopic sites?
- Can the shapeability and handleability of osteoinductive CaP materials be improved for clinical applications?
- Is an immune response involved in material-driven osteoinduction? If so, how does it work?
- Is it possible to improve the osteoinductive potential of CaP materials by finetuning physicochemical properties (e.g. by optimizing the morphology of surface architecture)?

Step by step, the questions are addressed in the following chapters.

Chapter 2. Clarify crucial material parameters for material-instructed bone formation and address possible mechanisms underlying osteoinduction, by comparing different materials with respect to physicochemical properties (chemistry and microstructure), evaluating their response to (simulated) body fluid (protein adsorption, ion release and biomineralization) and evaluating ectopic bone formation.

Chapter 3. Evaluate the bone regenerative potential of osteoinductive and non-osteoinductive CaP materials in a spine environment.

Chapter 4. Explore the influence of surface topography scale on bone formation and material resorption in a critical-sized bone defect model.

Chapter 5. Assess the performance of osteoinductive CaP materials in either a granules or putty formulation in posterolateral lumbar fusion of sheep.

Chapter 6. Investigate the relationship between surface topography dimension, macrophage phenotype, angiogenesis and osteoinduction.

Chapter 7. Improve osteoinductive potential of CaP materials by optimizing surface topography with hydrothermal treatment.

Finally, a general discussion of the thesis and future directions described in **Chapter 8**.

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CHAPTER 1

CHAPTER 2

Variation of bone forming ability with the physicochemical properties of calcium phosphate bone substitutes

2

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Published in Biomaterials Science 2017, 6:136- 145

A B S T R A C T

Because of their bioactive property and chemical similarity to the inorganic component of bone, calcium phosphate (CaP) materials are widely used for bone regeneration. Six commercially available CaP bone substitutes (Bio-Oss, Actifuse, Bi-Ostetic, MBCP, Vitoss and chronOs) as well as two tricalcium phosphate (TCP) ceramics with either micron-scale (TCP-B) or submicron-scale (TCP-S) surface structure are characterized and their bone forming potential is evaluated in a canine ectopic implantation model. After 12 weeks of implantation in the paraspinal muscle of four beagles, sporadic bone ($0.1 \pm 0.1\%$) is observed in two Actifuse implants (2/4), limited bone ($2.1 \pm 1.4\%$) in four MBCP implants (4/4) and abundant bone ($21.6 \pm 4.5\%$) is formed in all TCP-S implants (4/4). Bone is not observed in any of the Bio-Oss, Bi-Ostetic, Vitoss, chronOs and TCP-B implants (0/4). When correlating the bone forming potential with the physicochemical properties of each material, we observe that the physical characteristics (e.g. grain size and micropore size at the submicron scale) might be the dominant trigger of material directed bone formation via specific mechanotransduction, instead of protein adsorption, surface mineralization and calcium ion release.

1. INTRODUCTION

Calcium phosphate (CaP) ceramics such as beta-tricalcium phosphate (β -TCP), hydroxyapatite (HA) and biphasic calcium phosphate (BCP), have been developed to aid the healing of bone defects [1,2]. Next to their biocompatibility, bioactivity and osteoconductivity, CaP ceramics with specific physicochemical properties have also been shown to initiate bone formation on their surface after ectopic implantation (e.g. intramuscular, and subcutaneous). As bone cells are not present at these locations, this phenomenon is described as material directed bone formation (i.e. osteoinduction) [3-5].

With the ability to induce bone formation, osteoinductive CaP bone substitutes do not only trigger bone formation faster than those lacking osteoinductivity [6], but also allow the repair of critical-sized bone defects [7,8]. Moreover, an osteoinductive CaP bone substitute showed equal bone regeneration capacity to autograft and collagen load with recombinant human bone morphogenetic protein 2 (rhBMP-2) in critical bone defects [9]. The osteoinductive potential of CaP materials is largely dependent on their physicochemical properties [10-18]. The chemical composition plays a role in inductive bone formation. For instance, BCP (i.e. biphasic ceramic of HA and TCP) has a higher osteoinductive potential than HA [6,10], indicating a role of the faster resorbing TCP. However, a negative influence of TCP content was reported in a study where an increase of TCP in CaPs adversely affected the osteoinductive ability and no bone formation was detected in pure TCP ceramic [11]. Next to the chemistry, the presence of micropores in the material has proven to be crucial for osteoinduction [12]. For example, Ariizumi et al. found that TCP with interconnected macropores and micropores gave rise to inductive bone formation while in absence of micropores it did not [13]. Furthermore, it was shown that the osteoinductive potential increased with microporosity [14]. Moreover, the size of micropores has also been reported to greatly affect inductive bone formation by CaP ceramics. For instance, a study where two BCP ceramics were compared

in muscle of goats showed that only the BCP with smaller pore size and crystal size induced bone formation [15].

Given the fact that osteoinduction of CaP materials is material-dependent, it is likely that the bone forming potential of commercially available CaP bone substitutes varies due to differences in their physicochemical properties. In this study, six commercially available CaP bone substitutes (Bio-Oss, Actifuse, Bi-Ostetic, MBCP, Vitoss and chronOs) as well as two β -TCP ceramics with either micron-scale (TCP-B) or submicron-scale (TCP-S) surface architecture were characterized and their intramuscular bone forming potential was evaluated using a well-established canine osteoinduction study model [8,16].

2. MATERIALS AND METHODS

2.1. Calcium phosphate materials and characterization

2.1.1. Calcium phosphate materials

Six CaP bone substitutes (Bio-Oss, Actifuse, Bi-Ostetic, MBCP, Vitoss and chronOs) (Table 1) were purchased and used as received. Two β -TCP ceramics with micron (TCP-B) or submicron (TCP-S) granules were prepared as previously described [8] and were sterilized with gamma irradiation (dose 25-40 kGy, Isotron Netherland BV, Ede, the Netherlands) prior to use.

2.1.2. Physicochemical characterization

The chemistry and crystal structure of the eight CaP materials were evaluated with X-ray diffraction (XRD, Miniflex II, Rigaku, Tokyo, Japan). The surface structure of the materials was observed with scanning electron microscope (SEM, JEOL JSM-5600, JEOL Ltd, Tokyo, Japan). Grain size (the vertical lengths crossing the center of each grain) was measured by randomly selecting 100 grains from 10 SEM images ($\times 5000$). The microporosity (i.e. the volume percentage of pores smaller than 10 μm in the material), micropore size distribution, and surface area were obtained by mercury intrusion ($n = 1$ per material, Micromeritics Instrument Incorporation, Norcross, USA).

2.1.3. Protein adsorption

The materials (0.1 cc ml⁻¹, triplicates) were immersed in cell culture medium [minimal essential medium-alpha (a-MEM, Gibco, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS, Lonza, Germany) and 1% penicillin/streptomycin (Gibco, Invitrogen, UK)], and incubated at 37 °C in a humid atmosphere with 5% CO₂ for 4 days. After washing three times with phosphate buffer solution (PBS, Invitrogen, Darmstadt, Germany), 0.5 ml of 1% Triton solution was added to each sample. Thereafter, the amount of protein detached from the samples into the Triton solution was measured with the QuantiPro™ BCA Assay Kit (Pierce Biotechnology Inc., Rockford, USA) following the manufacturer's instructions and absorbance was measured with a spectrophotometer (Anthos Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria) at 620 nm. Protein adsorption was converted to the same amount of material used for the *in vivo* implants (1.0 cc) and expressed as mean ± SD.

2.1.4. Calcium and phosphate ions release

With stirring at 70 rpm and at 37 °C, 0.5 cc of each material (in triplicates) was incubated in 100 ml simulated physiological saline solution (SPS, 0.8% NaCl, 50 mM HEPES, 0.4 mM NaN₃, 37 °C, pH = 7.3) for 1, 4 and 7 days. Calcium and phosphate concentrations in the SPS solution were measured with a QuantiChrom™ Calcium assay kit (BioAssay Systems, Hayward, USA) and PhosphoWorks™ Colorimetric Phosphate assay kit (AAT Bioquest®, Inc., Sunnyvale, USA) respectively following the manufacturer's guidelines. Thereafter, absorbance was measured with spectrophotometer (Anthos Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria) at 620 nm. The calcium and phosphate concentrations were quantified respectively with a standard calibration curve and expressed as mean ± SD.

2.1.5. Surface mineralization

The bioactivity of the materials was evaluated *in vitro* with simulated body fluid (SBF) [17]. SBF was prepared by dissolving reagent grade chemicals

in distilled water (1000 ml) strictly in the order of NaCl (8.035 g), NaHCO₃ (0.355 g), KCl (0.225 g), K₂HPO₄·3H₂O (0.231 g), MgCl₂·6H₂O (0.311 g), CaCl₂ (25 ml) (calcium ion standard solution, 0.1 M, Metrohm, Herisau, Switzerland) and Na₂SO₄ (0.072 g). The solution was then buffered to pH=7.4 at 36.5 °C with Tris (CH₂OH)₃CNH₃ (6.118 g) and 1M HCl (25 ml). Fifty granules of each material as well as aluminum oxide ceramic granules (an internal negative control¹⁸ and prepared as described elsewhere¹⁹) were soaked in 200 mL of SBF at 37 °C for up to 14 days. A minimum of 5 granules were taken out at each time point of 1, 4, 7, and 14 days. The granules collected were carefully rinsed with distilled water, dried, gold-sputtered and observed with SEM in secondary electron modality.

2.2. Ectopic bone formation

2.2.1. Animal experiments

Following the permission of the local animal care committee (i.e. the management committee of experimental animals of Sichuan province, China), all animal experiments were performed in compliance with the laws and institutional guidelines of the animal care committee. The eight CaP materials (n=4, 1.0 cc per material) were intramuscularly implanted in the para-spinal muscles of 4 canines (beagles, male, 12-month old, 8 samples per animal). The surgical operation was conducted under general anesthesia by intravenous injection of sodium pentobarbital (30 mg kg⁻¹ body weight) and sterile conditions. Following the surgeries, buprenorphine (0.1 mg per animal) was intramuscular injected to the dogs for 2 days to relieve pain, while penicillin (40 mg kg⁻¹) was intramuscular injected for three consecutive days to prevent infection. After operation, the animals received normal diet. Fluorochromes were intravenously injected at 3 weeks (calcein, Sigma, Louis, USA, 10 mg kg⁻¹), 6 weeks (xylenol orange, Sigma, 50 mg kg⁻¹) and 9 weeks (tetracycline, Sigma, 10 mg kg⁻¹) after surgery to monitor the onset time of bone formation. Twelve weeks after operation, animals were sacrificed by a celiac injection of an excessive amount of pentobarbital sodium, and implants were harvested with the surrounding tissues.

2.2.2. Histological and histomorphometric analysis

The explants were fixed in 4% buffered formaldehyde solution (pH = 7.4) at 4 °C for one week, then dehydrated with a series of gradient ethanol solutions and finally embedded in methyl methacrylate (MMA, K-plast, LTI Nederland, Bilthoven, the Netherlands). Serial non-decalcified sections with an average thickness of 10-20 µm were obtained using a microtome (SP-1600, Leica, Wetzlar, Germany) equipped with diamond saw blade. Sections for light microscopy observation were stained with 1% methylene blue (Sigma-Aldrich, Louis, USA) and 0.3% basic function (Sigma-Aldrich) after etching with acidic ethanol (Merck, Darmstadt, Germany). Non-stained sections were used for fluorescent microscopical observation with the FITC Texas Red filter (bandpass mirror wavelengths of 510-555 nm and 585-665 nm; Nikon, Tokyo, Japan). Overview images were made from the stained sections with a slide scanner (Dimage Scan Elite 5400II, Konica Minolta Photo Imaging Inc, Tokyo, Japan) for histomorphometric analysis, which was performed using Adobe Photoshop Elements 4.0 software (CS5, v12, Adobe Systems Benelux BV, Amsterdam-Zuidoost, the Netherlands). The area with CaP materials was selected as a region of interest (ROI) and the corresponding number of pixels was read as ROI. Then the bone tissue area and CaP materials were pseudo-colored and their respective pixels were counted as B and M respectively. The percentage of bone in the available space was determined as $B\% = B \cdot 100 / (ROI - M)$ and presented as mean $\pm$ SD.

2.3. Statistics

Statistical analysis was carried out using one-way analysis of variance (ANOVA) with Tukey's test. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Calcium phosphate materials and characterization

3.1.1. Physicochemical properties of the CaP materials

The physicochemical properties of the materials are summarized in Table 1. The XRD patterns (Figure 1) show that the CaP materials are chemically

Table 1. Physicochemical properties of calcium phosphate materials

Material	Supplier	Chemistry ^{a)}	Micropore size ^{b)}	Grain size [μm] ^{c)}	Surface structure dimension	Microporosity [%] ^{b)}	Specific surface area by weight [m ² /g] ^{b)}	Specific surface area by volume [m ² /ml] ^{b)}
Bio-Oss	Geistlich Pharma AG	HA	Nano	Nano-scale	Nano	43.01	28.46	18.01
Actifuse	Apatech Baxter	Si-HA	Micron	1.02 ± 0.43	Micron	22.05	0.33	0.32
MB CP	Biomatante	55H A/ 45TCP	Submicron	0.71 ± 0.20	Submicron	40.53	2.93	2.09
Bi-Ostetic	Berkeley Advanced Biomaterials	45H A/ 55TCP	Micron	0.91 ± 0.24	Micron	53.74	1.42	1.10
Vitoss	Orthovita Stryker	TCP	Micron	1.93 ± 0.70	Micron	42.74	0.36	0.30
chronOs	Synthes	TCP	Micron	5.03 ± 1.90	Micron	31.52	0.51	0.59
TC P-B	Home made	TCP	Micron	2.52 ± 0.34	Micron	46.32	0.83	0.73
TC P-S	Home made	TCP	Submicron	0.81 ± 0.21	Submicron	45.14	1.85	1.59

^{a)} as evaluated by X-ray diffractometry; ^{b)} obtained from mercury intrusion; ^{c)} as confirmed by quantitative measurements on scanning microscopic images (5000×).

different and consist of either HA (Bio-Oss and Actifuse) or TCP (Vitoss, chronOs, TCP-B and TCP-S) or BCP (Bi-Ostetic and MBCP). The wider peaks in the XRD pattern of Bio-Oss indicate its lesser crystalline nature compared to the other materials. SEM images show that the eight CaP materials have different surface structure regarding grain size, dimension and amount of micropores (Figure 2 and Table 1). Mercury intrusion data indicate variations in the micropore size, micropore distribution, microporosity and specific surface area among these CaP materials (Table 1 and Figure 2A). The nano-scaled crystal grains and nano-scaled micropores indicate that Bio-Oss has a nano-scale surface architecture. Having both the crystal grains and the micropores smaller than 1 μm , MBCP and TCP-S belong to submicron-scale surface structured CaP materials. With either crystal grains or micropores larger than 1 μm , the surface structure of Actifuse, Bi-Ostetic, Vitoss, chronOs and TCP-B is at the micron scale.

Figure 1. XRD pattern showing the chemistry of the eight CaP materials as compared with standard HA and β -TCP.

3.1.2. Calcium and phosphate ions release

The release of calcium and phosphate ions from the CaP materials into SPS solution varied with the content of TCP component. In general, TCP materials (Vitoss, TCP-S, TCP-B and chronOs) released the most calcium and phosphate ions, while HA materials (Bio-Oss and Actifuse) released the least, with the BCP materials (Bi-Osetic and MBCP) in between (Figure 3B, C). In addition, having same chemistry, Vitoss released more calcium and phosphate ions, followed by TCP-S TCP-B and chronOs.

Figure 2. A: distribution plots of the micropore with size; **B:** SEM images showing the surface structure of seven CaP materials.

3.1.3. Protein adsorption

The amount of protein adsorbed by the eight CaP materials is shown in Figure 3A, and it is related to the specific surface area of the materials. Having the largest surface area ($18.01 \text{ m}^2 \text{ ml}^{-1}$), Bio-Oss has the highest protein adsorption capacity, followed by MBCP, TCP-S, Bi-Osetic, TCP-B and chronOs. Because of their smaller surface area, Vitoss ($0.30 \text{ m}^2 \text{ ml}^{-1}$) and Actifuse ($0.32 \text{ m}^2 \text{ ml}^{-1}$) adsorbed the least.

3.1.4. Surface mineralization

No apatite formed on the surface of alumina ceramic after 14-day soaking in SBF (data not shown), which validated the *in vitro* bioactivity evaluation system [17,18]. Layers of CaP apatite formed on the surface of all the tested materials after 1-day soaking in SBF at 37°C (Figure 3D), but the degree of mineralization varied among the CaP materials. Scattered mineralization was detected on the surface of Vitoss, chronOs and TCP-B, while the rest other materials were completely covered by a layer of mineralized apatite. With the soaking time, the apatite layers gradually deposited eventually covering the surface of all CaPs after 14 days soaking in SBF.

Figure 3. A: amount of proteins adsorbed into implants (1 ml) from culture medium after 4-day incubation; B, C: calcium and phosphate ions release from

the materials into SPS at day 1, day 4 and day 7 (*: $p < 0.05$); D: SEM images showing the apatite formation on the seven CaP materials after soaking in SBF for 1 day.

3.2. Tissue response after intramuscular implantation of the CaP materials

Newly formed bone was observed in TCP-S (4 out of 4), MBCP (4 out of 4) and Actifuse implants (2 out of 4), while no bone formation was seen in any of Bio-Oss, Bi-Ostetic, Vitoss, chronOs and TCP-B (Figure 4).

Normal bony structures with osteocytes embedded in the lacunae and osteoblasts laid on *de novo* bone were often observed in TCP-S and MBCP implants, while sporadically in Actifuse implants (Figure 5). In addition, macrophage-like cells engulfing fine ceramic particles were mainly observed in TCP-S and MBCP samples (Figure 5).

Figure 4. The histological overviews showing the tissue formation in CaP materials intramuscularly implanted in beagles for 12 weeks (un-decalcified sections stained with methylene blue and basic fuchsin, bone was stained fresh-red).

Figure 5. Histological images showing tissue responses to CaP materials intramuscularly implanted in beagles for 12 weeks (B: bone; M: material; O: osteoid; Black arrow: Osteoblast, Red arrow: foreign body giant cells).

Under fluorescent microscopy, three colors (green, calcein 3 weeks; red, xylenol orange, 6 weeks; and yellow, tetracycline, 9 weeks) were seen in TCP-S implants, while only green color was observed in MBCP, Actifuse, Bio-Oss and Bi-Ostetic implants, and no color was detected in Vitoss, chronOs and TCP-B implants (Figure 6). Since the green color was seen on the CaP materials, it suggests mineralization *in vivo*. The red and yellow colors stained the bone tissues in TCP-S implants, indicating that bone formation started between 3 and 6 weeks after implantation, and bone was still actively formed at week 9. Red and yellow colors were not seen in MBCP and Actifuse implants, suggesting that the bone formation process in these two materials started 9 weeks after implantation.

Figure 6. The fluorescent images showing the mineralization of materials and onset time of bone formation within CaP materials intramuscularly implanted in

beagles for 12 weeks (Green, calcein, 3 weeks; Red, xylenol orange, 6 weeks; Yellow, tetracycline, 9 weeks; M: materials; B: bone).

Quantitatively TCP-S gave rise to significantly more bone formation in the available space ($21.6 \pm 4.5\%$) than MBCP ($2.1 \pm 1.4\%$). The bone formed in Actifuse ($0.1 \pm 0.1\%$) was significantly less than in TCP-S and MBCP (Figure 7).

Figure 7. The area percentage of bone in the available spaces of the CaP material implants (*: $p < 0.05$).

4. DISCUSSION

4.1. Which material factors are triggers for ectopic bone formation?

In the current study, Actifuse, MBCP and TCP-S implants triggered bone formation after 12 weeks, with different incidence, onset time and amount of bone. No bone formation was detected in Bio-Oss, Bi-Ostetic, Vitoss, chronOs and TCP-B. The ectopic bone forming potential among the eight CaP materials is related to their physicochemical properties.

4.1.1. Chemical composition

When the chemical composition was evaluated as potential material factor for inductive bone formation, the influence of HA/TCP ratio was observed in individual studies [6,10,11]. In this study, bone formation was induced in Actifuse (Si-HA), MBCP (BCP) and TCP-S (TCP), indicating that

osteoiduction was not constrained to only one chemistry. It has been reported that the presence of TCP could release more calcium and phosphate thus enhance inductive bone formation [6,10]. However, despite their identical chemistry, we observed that Vitoss released most calcium and phosphate ions *in vitro* but did not induce any bone formation while TCP-S did. Similarly, Bio-Ostetic contained more TCP and released more calcium and phosphate ions than MBCP, but it was MBCP rather than Bio-Ostetic to induce bone formation. Furthermore, other studies demonstrated that some materials free of CaP induced ectopic bone formation [19,20]. Therefore, the chemical composition may not be appointed as determinant trigger in CaP material-driven osteoiduction.

4.1.2. Macroporous structure

Bone formation has never been observed in dense structure of CaPs and was always detected in the pores of osteoinductive CaPs, indicating the necessity of macroporous structure in inductive bone formation [3-6]. All eight CaP materials evaluated in this study have macroporous structure, but only three of them induced ectopic bone formation, suggesting that the macrostructural property is a prerequisite but is not a sufficient factor to trigger osteoiduction.

4.1.3. Microporous structure

Micropores (i.e. smaller than 10 μm) and microporosity have been shown essential for CaPs material-driven osteoiduction [12-15]. In the current study, when TCP-S was compared to chronOs, the role of the microporosity on inductive bone formation was shown. The higher microporosity in TCP-S led to inductive bone formation, while the less microporous chronOs did not. However, given the same chemistry and similar microporosity, TCP-S induced bone while TCP-B and Vitoss did not. This indicates that the high microporosity does not guarantee inductive bone formation in CaP materials. At the same time, the variation in micropore size and crystal grain size among TCP-S, TCP-B and Vitoss suggests the importance of the dimension of surface structure in material-driven osteoiduction, which was further confirmed in BCP materials. In

fact, despite having higher microporosity than MBCP, Bi-Ostetic did not induce bone formation but MBCP with finer pore and grain size did. It should be stressed again that MBCP and TCP-S have different chemistry but similar submicron scale structure, and both induced bone formation. Further, having either nano-scale (Bio-Oss) or micron-scale (Vitoss, Bi-Ostetic, chronOs and TCP-B) surface structure, the materials did not induce any bone formation. These results suggest the essential role of surface structure when at the submicron scale rather than at the nano- or micron-scale as trigger in CaP materials-driven bone formation. Despite its micron-scale surface architecture, Actifuse triggered sporadic bone formation which may be attributed to silicon incorporation [21].

We have previously said that the chemistry may not be a crucial trigger of material-driven bone formation. However, once the bone formation process is triggered, TCP-S having more TCP content induced more bone as compared with MBCP. Therefore, the influence of the chemistry on inductive bone formation is still important for the quantity of new bone tissue formation.

4.2. How do material factors trigger bone formation?

As described above, the surface structure at the submicron-scale may be the essential trigger for osteoinduction by CaP materials, but how it triggers CaP-induced bone formation is not clear as yet. It is generally thought that bone induction of CaP materials is the result of protein adsorption [3,5,9,13,22,23], surface mineralization [10,24,25] and calcium and phosphate ions release [26-28].

4.2.1 Protein adsorption

When CaP materials are implanted in the body, they adsorb proteins from the body fluids (including growth factors). Inductive bone formation of CaP materials was often addressed as the secondary response of protein adsorption [22,23]. Indeed, the protein adsorption theory explains well the role of the presence of micropores (including the quantity of micropores) and the size of the crystal grains and micropores in CaP-induced bone formation [3,5,9,13]. With the presence of micropores and

the decrease in size of micropores and crystal grains, the specific surface area of CaP materials increases and more protein can be concentrated favoring bone induction. However, the protein adsorption theory is not fully demonstrated because the possible proteins involved in material-induced bone formation were not identified. The data obtained in the current study does not support the protein adsorption theory either. When the same volume of CaP materials was soaked in simulated body fluids (cell culture medium containing foetal bovine serum), a large amount of proteins was adsorbed onto Bio-Oss, which has the highest surface area but no bone was formed in any of Bio-Oss implants.

4.2.2. Surface mineralization

Next to the protein adsorption, the ability to favor mineral deposition from body fluids appears to be a characteristic of osteoinductive materials as it could facilitate osteogenesis and explained bone induction in surface-active titanium implants [29]. The surface area and chemical composition of CaP materials affect the dissolution and reprecipitation events eventually leading to apatite layer formation on their surface [10,24]. However, as seen in the current study, the green signal of calcein was present in Bio-Oss, Actifuse, Bi-ostetic, MBCP and TCP-S implants but not in Vitoss, chronOs and TCP-B (Figure 6). As fluorochromes form chelate complexes with apatite via their iminodiacetic acid groups, which is a phenomenon depending on the availability of the binding sites that could originate from ongoing bone formation or surface mineralization processes [30,31]. Thus, the green color observed in implants indicates *in vivo* biomineralization of materials within 3 weeks after implantation. Cerruti et al [32] also showed that higher surface area led to faster compositional changes of the surrounding fluids hence facilitating early apatite formation. BioOss, with the highest surface area and more amorphous structure, had the strongest calcein signal. Because of their lower surface area, Vitoss, chronOs and TCP-B did not mineralize *in vivo* and thus no calcein was detected in the implants. The *in vivo* mineralization is well correlated with the 1-day *in vitro* data, where scattered mineralization was observed on the surface of Vitoss, chronOs

and TCP-B, while all the other materials were completely covered by a layer of mineralized apatite. Actifuse has as similar specific surface area as Vitoss, but the former underwent mineralization *in vivo* and was completely covered by an apatite layer *in vitro*, which might be attributed to the silica incorporation [21]. Nevertheless, mineralization occurred in all materials *in vitro*, and in 5 out of 7 materials *in vivo*, but only 3 of them induced bone formation. This suggests that surface mineralization of CaP materials may be a prerequisite but is not sufficient to trigger ectopic bone formation.

4.2.3. Calcium and phosphate ions release

CaP materials could release calcium and phosphate ions with degradation or resorption. Such ions have shown their positive influence on osteogenic differentiation of mesenchymal stem cells (MSCs) and osteogenesis of osteogenic cells [26-28]. For instance, Shih et al., recently reported that phosphate ions released from CaP materials could induce osteogenic differentiation of human MSCs through phosphate-ATP-adenosine signaling [28]. It was thus assumed that bone induction by CaP materials could be the result of calcium and phosphate ions release. Indeed, these ions release may have enhanced the bone formation in CaP materials having a high content of TCP [6,10]. However, calcium and phosphate ions release may not be considered as the triggers of bone induction. As shown in Figure 3B, C, Vitoss released the most calcium and phosphate ions but it did not induce bone formation at all. Furthermore, the ion release theory is not supported by literature as other studies showed ectopic bone induction of non-CaP materials such as alumina [19] and polymeric implants [20].

4.2.4. Surface topography

From the discussion above, the overall results in this study could not assign any of the biochemical theories of protein adsorption, surface mineralization and ion release to CaP-induced bone formation. Indeed, without the presence of chemical signals, special surface topographies, more especially with a scale range from nano- to micron-meters, have shown to directly modulate the cellular behavior and guide specific

biological responses [33-45]. For example, Giulio et al. found that ridges with 15 μm increased the differentiation of mesenchymal stem cells (MSCs) into adipogenic lineages, while smaller ridges enhanced osteogenic differentiation [45]. Similar phenomenon has been described by Hatano et al. who observed that rough surface (0.81 μm) enhanced the proliferation, alkaline phosphatase (ALP) activity and osteocalcin expression of rat osteoblasts in comparison with smoother one [46]. Presumably the submicron surface topography directly induce osteogenesis through mechanotransduction, i.e. the translation of mechanical stimuli at the cell-substrate interface into electrochemical signals which elicit specific cellular responses, through YAP and TAZ regulators [47-49]. It is also likely that through the mechanotransduction, mesenchymal stem cells adapt their shape to surface structure, recruit TGF receptor on primary cilia and undergo osteogenic differentiation [50].

In addition to modulating stem cells and osteoblasts, giant cells (e.g. macrophage) are sensitive to surface topography. For instance, Fella and colleagues observed that the number of macrophages and giant cells varied according to the surface grain size of CaP ceramic implants [51]. Furthermore Davison et al. showed that CaP ceramics with submicron sized surface architecture could induce an inflammatory response of macrophages, and their subsequent secretion of cytokines instructed stem cell differentiation [6,52]. In line with the findings by Kondo et al, who observed plenty of active multinucleated giant cells prior to osteoinductive bone formation in CaP implants [53], we observed in this study macrophage-like cells on the submicron structured surface of osteoinductive TCP-S and MBCP. It is possible that the crosstalk between macrophages and mesenchymal stem cells on the submicron scaled surface topography initiated the CaP-directed osteoinduction through mechanotransduction.

Osteoinductive CaP materials are useful for bone regeneration [6-9], and identifying the crucial material factors would be helpful for further optimization of CaP materials with respect to bone forming ability. The

submicron dimension of the surface structure of CaP materials was clearly shown to be the stronger trigger of ectopic bone formation. However, more studies are necessary to further understand the material-driven osteoinduction from a biological perspective.

5. CONCLUSION

We compared the bone forming potential of eight synthetic CaP bone substitutes and found that the bone forming ability varied with materials. It was observed that the specific topography engineered by submicropores and submicron crystal grains appear to be the necessary trigger for osteoinduction, and that ceramics releasing ions could enhance the amount of inductive bone formation. The submicron-scale surface topography, via specific mechanotransduction, might be the direct trigger of osteoinduction in CaP bone substitutes rather than the theories already in the literature such as proteins adsorption, surface mineralization and calcium ions release.

A C K N O W L E D G E M E N T S

The authors would like to thank the Netherlands Institute for Regenerative Medicine (NIRM) and Rapid Prototyping of Custom-Made Bone-Forming Tissue Engineering Constructs (RAPIDOS Project, Ref. NMP-2013-EU-China604517), the Seventh Research Framework Program of the European Union (REBORNE under Grant agreement no. 241879) and the European Union's Horizon 2020 research and innovation program for the financial support to this research project (CHARME, grant agreement no. 674282) for their financial support in this study.

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CHAPTER 3

Submicron-surface structured tricalcium phosphate ceramic enhances the bone regeneration in canine spine environment

3

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Published in Journal of Orthopaedic Research 2016, 34:1865-1873

A B S T R A C T

Calcium phosphate ceramics with submicron-scaled surface structure can trigger bone formation in non-osseous sites and are expected to enhance bone formation in spine environment. In this study, two tricalcium phosphate ceramics having either a submicron-scaled surface structure (TCP-S) or a micron-scaled one (TCP-B) were prepared and characterized regarding their physicochemical properties. Granules (size 1–2 mm) of both materials were implanted on either left or right side of spinous process, between the two lumbar vertebrae (L3-L4), and in paraspinal muscle of eight beagles. After 12 weeks of implantation, ectopic bone was observed in muscle in TCP-S explants ($7.7 \pm 3.7\%$), confirming their ability to inductively form bone in non-osseous sites. In contrast, TCP-B implants did not lead to bone formation in muscle. Abundant bone ($34.1 \pm 6.6\%$) was formed within TCP-S implants beside the two spinous processes, while limited bone ($5.1 \pm 4.5\%$) was seen in TCP-B. Furthermore, the material resorption of TCP-S was more pronounced than that of TCP-B in both the muscle and spine environments. The results herein indicate that the submicron-scaled surface structured tricalcium phosphate ceramic could enhance bone regeneration as compared to the micron-scaled one in spine environment.

1. INTRODUCTION

Spinal impairments, e.g. scoliosis and trauma, represent an important part of musculoskeletal disorders worldwide [1, 2]. They are nowadays treated with various techniques such as mini-invasive spinal surgery [3] and posterolateral or interbody spinal fusion via grafting materials [4]. When spinal fusion is chosen, autologous bone harvested from the patient's iliac crest is currently the golden standard grafting material for an adequate fusion, but it can lead to significant donor site morbidity, pain and has limited availability [5, 6]. Allograft and demineralized bone matrix (DBM) are also used but, next to disease transmission risk, they are weakly osteoinductive and can hardly promote new bone formation [5, 6]. The use of human recombinant bone morphogenetic proteins (rhBMPs) has been proposed as well [7, 8]. However, the clinical need of high doses of rhBMPs not only generates elevated costs but also raises safety concerns [9]. Being chemically similar to the main inorganic component of bone, and thanks to their biocompatibility, osteoconductivity and adjustable resorbability [10, 11], calcium phosphate (CaP) ceramics have been reported to support bone formation in spine [12-14]. However, fusion of larger segments is not currently expected from these ceramics because the conductive bone forming process is limited to regions close to the host bone bed [2, 13].

Studies performed at the beginning of the 1990s demonstrated that certain CaP ceramics are osteoinductive as indicated by the bone formation following their implantation in muscle or under the skin [15-17]. Later it was shown that osteoinduction of such CaP ceramics is material-dependent [15-26]. In particular, the chemistry [18], geometry [19] and surface microstructure [20-21] were suggested as relevant material properties for osteoinduction to occur. Among them, the presence of micropores (i.e. pores smaller than 10 μm) in the ceramics has been proven to be crucial. This was demonstrated when microporous hydroxyapatite (HA) triggered subcutaneous [17] and intramuscular [20] bone formation in dogs, while the same ceramic without micropores did not. Furthermore, the osteoinductive potential of CaP ceramics was

shown to increase with their microporosity [22]. When the surface of such CaP ceramic is comprised of grains and micropores, the dimension of the surface structures was shown to be the dominant factor governing osteoinduction [23]. In particular, given the same chemistry and similar microporosity, a tricalcium phosphate ceramic (TCP) with a submicron-scaled surface structure enhanced the osteogenic differentiation of bone marrow stromal cells *in vitro* and led to inductive bone formation *in vivo* [24, 25]. Conversely, a similar TCP ceramic with a micron-scaled surface structure had significantly lower performances *in vitro* and *in vivo* [24, 25].

The significance of osteoinductive CaP ceramics in repairing osseous voids has been shown when implanted in non-critical and critical-sized defects. For instance, when a CaP ceramic having high osteoinductive potential was implanted in a femoral non-critical sized bone defect model, it could repair the defect faster than the material with lower osteoinductivity [26]. Furthermore, an osteoinductive CaP ceramic repaired critical-sized defects in goat iliac crest model while a non-osteoinductive one failed [27]. Moreover, a resorbable osteoinductive CaP ceramic was reported to have equal potential to autologous bone and rhBMP-2 in repairing ovine iliac crest critical-sized defects [28].

To evaluate the bone regeneration capacity of an osteoinductive CaP ceramic in spine surgery, in this study we compared two TCP ceramics having either an osteoinductive submicron or a non-osteoinductive micron surface structure in canine spine environment. The two materials were also implanted intramuscularly to determine their osteoinductivity. We expected that, due to its osteoinductive potential, the ceramic having submicron surface structure would form more bone in the spine environment than the non-osteoinductive one.

2. MATERIALS AND METHODS

2.1. Preparation of the two TCP ceramics

Two types of TCP powders (TCP-S and TCP-B) were synthesized by adding a phosphoric acid solution (H_3PO_4 , Fluka) to a calcium hydroxide suspension [$\text{Ca}(\text{OH})_2$, Fluka] at different rates, while keeping the Ca/P

ratio constant at 1.5. To obtain TCP-B, the calculated volume of phosphoric acid solution was directly poured into the calcium hydroxide suspension. Conversely, to prepare TCP-S, the addition was dropwise. After aging at room temperature for six weeks, the slurries were filtered, dried and grinded. The obtained TCP powders were then foamed by adding diluted H_2O_2 (1% in distilled water, Merck) and a porogen (wax particles, 600-1000 μm , Merck) at 60°C. TCP-S and TCP-B ceramics were then obtained by sintering the green bodies for 8 hours at 1050 °C and 1100 °C respectively. Irregularly shaped granules of both ceramics, with size range of 1–2 mm, were prepared by crushing and sieving the sintered bulk materials. Meanwhile ceramic discs ($\varnothing 9 \times 6$ mm) were machined from the sintered bulks for mechanical test. All the materials were then ultrasonically cleaned with the series of acetone (Fluka), 70% ethanol (Fluka) and demineralized water, dried at 80 °C and sterilized by gamma irradiation (dose 25-40 kGy, Isotron Nederland BV) before use.

2.2. Physicochemical characterization of the ceramics

The surface structure of the ceramics was evaluated using a scanning electron microscope (SEM; XL30, ESEM-FEG, Philips). The grain size was determined from SEM images taken at 5,000x magnification and the chemical composition of the CaP ceramics was identified by X-ray diffraction (XRD; Miniflex II, Rigaku). Total porosity (i.e. the volume percentage of pores in the materials), macroporosity (i.e. the volume percentage of pores greater than, or equal, to 10 μm in the materials), microporosity (i.e. the volume percentage of pores smaller than 10 μm in the materials), pore size distribution in the ceramic strut and specific surface area of the ceramics were obtained with mercury intrusion ($n=1$ per material, Micromeritics Instrument Corporation).

2.3. Mechanical performance of the ceramics

The mechanical strength at rupture of the two ceramics was analyzed with uniaxial compression tests on ceramic discs ($n=3$ per ceramic). The analyses were performed using an electromechanical testing machine (model 5565, Instron) at a constant crosshead compression speed of 0.5

mm/min. The elastic modulus and the compressive strength were measured.

2.4. Dissolution behavior of the ceramics

Calcium ion release was determined by placing 0.5 cc of the ceramic granules in 100 ml of simulated physiological saline (SPS; 0.8% NaCl, 50 mM HEPES, pH = 7.3) and monitoring the variations of calcium ion concentration in SPS using a calcium electrode (Metrohm, Switzerland) during a time frame of 200 minutes at 37°C.

2.5. Protein adsorption potential of the ceramics

Protein adsorption was performed using two types of protein solutions. A volume of 0.2 cc ceramic granules was soaked into 2 ml of bovine serum albumin (BSA) solution (concentration = 200 µg/ml) or 2 ml of 1% fetal bovine serum (FBS) solution respectively for 24 hours at 37°C (n=6 per material). The protein concentration in the solutions before and after the adsorption was measured using a protein assay kit (Micro BCA Protein Assay Kit, Thermo Scientific, USA) and then the percentage of proteins adsorbed onto the ceramics was calculated.

2.6. Animal studies: intramuscular and spinal surgeries

A total of 8 adult male beagles (1-2 years old, 10-15 kg) were used for this study. Surgery was performed with the permission of the local animal ethic committee (i.e. the management committee of experimental animals of Sichuan province, China). General anesthesia conditions were achieved after intraperitoneal injection of pentobarbital sodium (30 mg per kg body weight). After shaving and sterilizing the skin with iodine, the spinous processes L3-L4 were exposed with a skin incision on the back. Then the spinous processes and the lamina between L3 and L4 were exposed in both sides. With blunt separation, tissues at both sides of the spine were separated from the intra-spinous and supraspinous ligaments without damaging the ligaments, and two implantation beds next to the L3 and L4 spinous processes were created. After superficial decortication on either side of the exposed spinous processes and lamina using a scraper without damaging the facet joint, 5 cc granules of either TCP-S

or TCP-B was placed on one side to bridge the two spinous processes. The implantation scheme ensured that each animal received both materials, one at each side of the spinous processes. The muscles of both sides were then tightly closed with silk suture.

Meanwhile two paraspinal muscle pouches were created with blunt separation at least 3 cm away from the spinal bone. Ceramic granules (1 cc per material) were implanted separately in the muscular pouches and sealed with silk suture. Finally the wound was closed layer by layer with silk suture. After surgery, the animals were given buprenorphine intramuscularly (0.1 mg per animal) for two days to relieve pain, and penicillin (40 mg/kg) was injected intramuscularly for three consecutive days to prevent infection. Animals were allowed to undertake full activity in large cages and received normal diet after surgery. Fluorochromes were injected intravenously at 3 weeks (calcein, Sigma, 2 mg/kg body weight), 6 weeks (xylenol orange, Sigma, 50 mg/kg body weight) and 9 weeks (tetracycline, Sigma, 10 mg/kg body weight) after surgery to monitor the onset of bone formation. After 12 weeks, the animals were sacrificed with overdose intraperitoneal injection of pentobarbital sodium and the samples were harvested with the surrounding tissues.

2.7. Animal studies: histology and histomorphometry

The explants were trimmed and fixed in 10% buffered formalin for at least one week, and subsequently they were dehydrated with a series of ethanol solutions. The intramuscular implants were dehydrated as they were, while spine samples were cut parallel to their sagittal plane across the middle of the spinous processes using a circular saw prior to dehydration. Thereafter, all samples were embedded in methyl methacrylate (MMA). Non-decalcified sections (10-20 μm thick) were made using a diamond saw (SP-1600, Leica, Germany) and stained with 1% methylene blue (Sigma-Aldrich) and 0.3% basic fuchsin (Sigma-Aldrich) for light microscopy observation. Non-stained slides were also prepared for observation at the fluorescent microscopy equipped with the FITC Texas Red filter (bandpass mirror wavelengths of 510-555 nm and 585-665 nm; Nikon). In particular, coronal and cross sections were

made for the spine and intramuscular samples respectively. All stained sections were scanned (Dimage Scan Elite 5400 II scanner, AF5400-2, Konica Minolta) to collect overview images to quantify *de novo* bone formation and material resorption. Quantitative histomorphometry was performed using Adobe Photoshop software (CS5, v12, Adobe Systems Benelux BV). For spine samples, the area including all granules excluding the spinous processes was selected as the region of interest and the number of pixels was subsequently read as ROI. For the intramuscular implants, the ROI included the pixels of the entire sample. Thereafter, in both cases, the material and the mineralized bone were pseudo-colored and their pixels were read as Me and B respectively. The area percentage of ceramic in the explants (Me%) was then calculated as $\text{Me} \times 100 / \text{ROI}$ and the area percentage of bone in the available space (B%) was calculated as $\text{B} \times 100 / (\text{ROI} - \text{Me})$. The resorption rate of ceramics was calculated as $(\text{Mi}\% - \text{Me}\%) \times 100 / \text{Mi}\%$, where the initial area percentage of the ceramic material (Mi%) in the implants was determined after making 10-20 μm thick sections of MMA-embedded ceramic granules ($n=8$).

2.8. Statistical analysis

Two tail paired t-test was performed using the software OriginPro SR1 (v8.0773 B773, OriginLab Corporation) to evaluate any statistical difference, which was considered significant when $p < 0.05$. The power of both spine and muscular experiments was computed with a post-hoc power analysis (two-tail t-test for difference in mean) using the freeware G*Power software [29] to see whether the studies had sufficient statistical power.

3. RESULTS

3.1. Physicochemical characterization of the ceramics

XRD indicated that the two ceramics were chemically identical (Figure 1A), in particular they were β -TCP as confirmed by comparison with the international standard chart JCPDS 9-0169. SEM imaging showed that both ceramics had a microporous surface with grain-like texture (Figure

1B), where TCP-S presented significantly smaller grains than TCP-B ($p<0.01$, Table 1).

Table 1. Physicochemical properties of the calcium phosphate ceramic implants.

		TCP-S	TCP-B
Chemistry ^a		β-TCP	β-TCP
Grain size (μm) ^b		0.81±0.24*	2.50±0.19
porosity ^c	Total porosity	72%	73%
	Macroporosity (≥10μm)	50%	50%
	Microporosity (<10μm)	22%	23%
Micropore size range (μm) ^c		0.3-1.3	0.5-2.0
Specific surface area (m ² /g) ^c		1.4	0.7
Ca ²⁺ ion released (ppm, after 200 min)		16.5±2.0*	11.4±1.7
Proteins adsorbed after 24 hours (%)	BSA	61.1±3.0*	49.5±2.9
	FBS	27.6±2.8*	14.4±1.7
Material area percentage (Mi%) ^d		47.5±2.5	46.5±3.2
Elastic modulus (GPa)		155.8±9.1*	107.6±0.0
Compressive strength (MPa)		5.5±0.7*	2.9±0.2

^a as determined via X-ray diffractometry; ^b as determined via quantitative measurements on scanning microscopic images (5000×); ^c as determined via mercury intrusion; ^d as determined via histomorphometry; * significantly different ($p<0.01$), comparison done between TCP-S and TCP-B.

Mercury intrusion testing indicated that the total porosity, macroporosity and microporosity were similar between the two materials (Table 1). However, TCP-S possessed mainly submicron pores while TCP-B had mainly micron-scaled pores (Table 1). This was clearly shown by the distribution curves (Figure 1C), where the majority of pores in TCP-S had dimension of 500 nm, while in TCP-B most pores had size of 1.5 μm.

Furthermore, TCP-S had approximately a two-fold higher specific surface area than TCP-B (Table 1).

Figure 1. Physicochemical properties of calcium phosphate ceramics. **A:** XRD pattern showing the chemistry of the two CaP ceramics; **B:** SEM images showing the surface structure of both the ceramics; **C:** distribution plots of the submicro- and micro-pore dimension; **D:** calcium ion release from the ceramics into SPS during 200 minutes; **E:** percentage of proteins adsorbed from 2 ml of 200 μ g BSA/ml and 2 ml 1% FBS solution onto 0.22cc ceramic granules after 24 hours (stars indicate a significant difference, $p < 0.05$).

3.2. Mechanical strength

The two ceramics have high stiffness as shown by their elevated elastic modulus, where TCP-S is significantly stiffer and has higher compressive strength than TCP-B (Table 1).

3.3. Dissolution behavior of the ceramics

The curves (Figure 1D) indicate a continuous release of calcium ions from the two studied ceramics into the medium. It can be seen that the two trends have a parallel behavior where TCP-S released more ions in 200 minutes into the SPS 7.3 solution than TCP-B (Figure 1D, Table 1).

3.4. Protein adsorption potential of the ceramics

After one day of soaking, TCP-S could adsorb significantly more proteins from both BSA and FBS solutions than TCP-B (Figure 1E, Table 1) ($p < 0.01$ in both cases).

3.5. Intramuscular bone formation

After 12 weeks of implantation, all intramuscular explants were encapsulated by a thin layer of connective tissue (Figures 2A and 2B), while soft tissues infiltrated into both types of materials. Newly formed bone was observed in all TCP-S explants but was not completely homogenous (Figures 2A and 2C). Calcified bone tissue with entrapped osteocytes was observed (Figure 2E), and osteoid with a seam of osteoblasts lying on its outer surface was seen as well (Figure 2E). Multinucleated giant cells phagocytizing small fragments were found on the surface of TCP-S granules (Figure 2E). Conversely, no bone formation was observed in any of TCP-B explants (Figure 2B and 2D) and multinucleated giant cells were hardly noted (Figure 2F). As indicated by fluorescent microscopy, bone formation initiated between week 3 and week 6 in TCP-S and was still actively occurring at week 9 (Figure 2G).

Quantitatively, $7.7 \pm 3.7\%$ of the available space in TCP-S explants was occupied by bone at week 12 and the area percentage of material remaining in the explants was $28.6 \pm 5.5\%$. The material area percentage in TCP-B explants was $44.9 \pm 10.1\%$ at week 12. When compared to the area percentage of the materials prior to implantation, significant material decrease occurred in TCP-S (40%, $p < 0.01$), while the decrease seen in TCP-B (3%) was not significant (Table 2 and Figure 6).

Figure 2. Tissue formation in CaP ceramics intramuscularly implanted in dogs for 12 weeks (un-decalcified sections, either stained with methylene blue and basic fuchsin for histological observation or not stained for fluorescent microscopy). **A:**

histological overview of TCP-S; **B**: histological overview of TCP-B; **C**: higher magnification of the selected area in A; **D**: higher magnification of a selected region in B; **E**: selected area in C at higher magnification showing the ongoing process of TCP-S resorption; **F**: higher magnification of the selected region in D showing the absence of giant cells on TCP-B surface; **G**: fluorescent image of TCP-S, indicating bone formation was triggered within 6 weeks (red) and continued at least up to 9 weeks (yellow) after implantation (bone: bone; M: ceramic; O: osteoid; *: material fragments detached from TCP-S; black arrow: giant cell phagocytizing material fragments).

Table 2. A summary of the explants from muscle and spine of dogs after 12 weeks.

		TCP-S	TCP-B	Power (effect size) ^{§§}
Intramuscular samples	Number of explants	8	8	--
	Bone incidence	8/8	0/8	--
	Bone in available space (B%)	7.7±3.7**	0	0.99 (3.01)
	Material percentage (Me%)	28.6±5.5**	44.9±10.1	0.97 (2.13)
	Resorption rate	40%**	3%	--
Spine samples	Number of explants	8	8	--
	Bone in available space (B%)	34.1±6.6**	5.1±4.5	1.00 (5.11)
	Material percentage (Me%)	22.7±7.9*	39.5±5.6	0.99 (2.44)
	Resorption rate	52%*	15%	--

** significantly different ($p<0.01$), comparison done between TCP-S and TCP-B;

* significantly different ($p<0.05$), comparison done between TCP-S and TCP-B;

^{§§} the statistical power was calculated for each experiment with post-hoc statistical power analysis for t-test of means between two independent groups (significance level $\alpha=0.05$); the results show that a number of 8 animals carrying both materials is sufficient in this study for both the muscular and spine experiments.

3.6. Bone formation in spine environment

Eight spine samples per ceramic were available for histological and histomorphometrical evaluations (Table 2). Histology showed abundant bone formation between the two spinous processes in the TCP-S group (Figure 3A). However, the spatial distribution of this *de novo* bone

Figure 3. Tissue formation in TCP-S implanted in canine spine (un-decalcified sections stained with methylene blue and basic fuchsin). **A:** histological overview; **B:** higher magnification of the region B in A showing bone formation in TCP-S implant; **C:** higher magnification of the area C in A showing cartilage formation in TCP-S implant; **D:** selected region in B at higher magnification showing the process of TCP-S resorption *in vivo*; **E:** selected rectangle in C at higher magnification showing the remodeling of cartilage towards bone (M: TCP-S; O: osteoid; bone: bone; cartilage: cartilage; *: material fragments detached from TCP-S; black arrow: giant cell engulfing material fragments).

formation was not homogenous and solid spinal fusion was not achieved (Figure 3A). Osteoid, mineralized bone and bone marrow were mainly

observed in the regions close to the spinous processes (Figures 3A and 3B), while less bone was seen in the central region where fibrous cartilage and osteochondral ossification were more regularly presented (Figures 3A, 3C and 3E). Phagocytes with fine ceramic particles engulfed in their cytoplasm were mainly observed in TCP-S samples (Figure 3D).

In contrast to TCP-S, little bone was seen nearby the bone bed hosting TCP-B implants (Figure 4A) and fibrous tissue occupied most of the available space (Figures 4B and 4C). Phagocytes were less presented in TCP-B (Figure 4C) as compared to TCP-S.

Figure 4. Tissue formation in TCP-B implanted in canine spine (un-decalcified sections stained with methylene blue and basic fuchsin). **A:** histological overview; **B:** higher magnification of the selected area in A showing bone formation in TCP-B nearby the host bone bed; **C:** selected region in B at higher magnification showing tissue formation and material resorption in TCP-B (HB: host bone; NB: new bone; M: TCP-B; *: material fragments detached from TCP-B; black arrow: giant cell phagocytizing material fragments).

Fluorescent microscopy of the spinal implants showed that, nearby the host bone, new bone formation started in TCP-S implants as early as 3 weeks after implantation (Figure 5A, green color). In those regions far away from the host, bone formation started between 3 and 6 weeks

(Figure 5B, red color). In TCP-B implants bone formation generally occurred at a later time point (6 to 9 weeks) and was less active (data not shown).

Quantitatively, more bone was formed in TCP-S ($34.1 \pm 6.6\%$) as compared to TCP-B ($5.1 \pm 4.5\%$) (Table 2, Figure 6A, $p < 0.01$). In addition, significantly more material resorption was observed for TCP-S (52%) as compared to TCP-B (15%) ($p < 0.05$, Table 2).

Figure 5. Sequence of bone formation in TCP-S in spine. **A:** fluorescent image nearby the host bone bed; **B:** fluorescent image far away from the host bone bed (3 weeks, calcein, green; 6 weeks, xylenol orange, red and 9 weeks, tetracycline, yellow; HB: host bone; NB: new bone; M: ceramic).

Figure 6. Histomorphometric data of CaP ceramics implanted in muscle and spine of dogs for 12 weeks. **A:** area percentage of bone in the available space in the region of interest; **B:** area percentage of ceramic material in implants and explants (Stars indicate a significant difference, $p < 0.05$).

3.7. Notes on bone formation between intramuscular and spinal experiments

When comparing intramuscular and spinal implants, the amount of bone formed in TCP-S was significantly more in spine implants ($34.1 \pm 6.6\%$) than in intramuscular implants ($7.7 \pm 3.7\%$) (Table 2 and Figure 6A, $p < 0.05$). With regard to TCP-B, limited amount of bone was formed in the spinal implants ($5.1 \pm 4.5\%$), while no bone was observed intramuscularly (Figure 6A).

3.8. Statistical analysis

Power analysis results are reported in Table 2 showing that the animal data had sufficient statistical power.

4. DISCUSSION

Heterotopic bone formation was found in TCP-S but not in TCP-B after 12 weeks implantation in muscle of dogs. In spine environment, more bone formed in TCP-S than in TCP-B and it was also observed that, in the case of TCP-S, more bone was formed in spine than in muscle. Furthermore, TCP-S resorbed more than TCP-B in both muscle and spine environments. Statistical analyses showed that the aforementioned differences were significant.

TCP-S has similar chemistry and porosity but different dimensional scale of the surface structure as compared to TCP-B (Figures 1A, Table 1). Therefore, the higher bone formation in TCP-S observed in both ectopic and spinal sites may be attributed to its submicron scaled surface structure. Thanks to its smaller grains and micropores (Figures 1B and 1C, Table 1), TCP-S had a larger surface area which allows it to concentrate higher amounts of proteins (Figure 1E, Table 1) and to release more calcium ions (Figure 1D, Table 1) as compared to TCP-B. It has been generally proposed that, via concentrating growth factors like proteins (e.g. BMPs) locally, CaP ceramics with enhanced surface area may not only induce the osteogenic differentiation of mesenchymal stem cells but also prompt osteogenic cells to form bone [16, 17, 20, 28, 30, 31]. Next to protein adsorption, the presence of calcium ions in the microenvironment enhances the osteogenic differentiation of stem cells and osteogenic cells [32, 33]. Thus the faster release of calcium ions from

TCP-S may have further enhanced the osteogenic differentiation of cells leading them to form bone. Moreover, it has been recently shown that submicron surface topography itself is sufficient to induce osteogenic differentiation of stem cells and ultimately lead to bone induction [24]. Nevertheless, how the surface structure enhances bone formation is not fully understood as yet and needs to be further studied.

TCP ceramics are generally considered as resorbable materials [10, 11], and both the ceramics studied herein underwent resorption in spine environment (Figure 6B, Table 2). In particular, after 12 weeks of implantation, larger amounts of TCP-S were resorbed than TCP-B (Table 2 and Figure 6B). The resorption in muscle was generally slower than in the spine environment (Table 2 and Figure 6B). Chemical dissolution and cell-mediated resorption have been suggested as the main resorption mechanisms for CaP ceramics [34, 35]. Both TCP ceramics are dissolvable as indicated by the calcium ion release *in vitro*, but only TCP-S had abundant phagocytes on its surfaces *in vivo* (Figures 2E and 6B) and was significantly resorbed in the muscle. On the contrary, TCP-B lacked resorbing cells on its surface and was not resorbed in the muscle (Figures 2F and 6B). Our observations, therefore, indicate a predominant role of cell-mediated resorption rather than chemical dissolution of CaP ceramics *in vivo*. The role of cell-mediated resorption of CaP ceramics was also shown in previous studies, where TCP-S enhanced the formation of osteoclasts from mononuclear cells and thus the resorption process [36]. Conversely, it could be that phagocytes did not colonize the intramuscular TCP-B implants, while their activity could have been inhibited in spinal samples of TCP-B.

The dimension of surface structure scale affected not only the bone formation and resorption of TCP ceramics but also their mechanical strength. Thanks to its higher compactness given by the submicron-structure, TCP-S had higher compressive strength and elastic modulus than TCP-B. The initial compressive strength (5.5 ± 0.7 MPa) of TCP-S may decrease with the resorption, while such decrease may be supplemented by the formation of bone.

Although the submicron-surface structured TCP-S was shown to enhance bone formation in spine environment, solid bone bridge was not achieved. The short implantation duration used in this study (i.e. 12 weeks) could have been an influencing factor. Spine fusion rates increased with time and higher fusion rates could be achieved with an implantation time longer than 12 weeks [37-39]. Non-instrumentation might be one factor as well relevant to the absence of bone bridging. Non-instrumented spine surgery resulted in high non-fusion rate and cartilage islands in spine implants [40]. Formation of cartilage tissue between the spinous processes was observed also in this study (Figures 3A, 3C and 3E). Since osteoinduction of CaP ceramics starts directly as bone (i.e. intramembranous ossification) [41, 42], the formation of cartilage in TCP-S may not be because of the osteoinductive property of the material. More likely, it could be the result of micro-motions occurring between the spinal segments as they were not stabilized by instrumented fixation [43]. In addition to the implantation time and the instrumentation, the spinous process model could be another factor resulting in no solid fusion in this study. In a posterior segmental canine spinal fusion model, which was similar to the model used in the current study, solid fusion was seen at low rate with either autologous bone or rhBMP-2 implants when instrumented fixation was applied [44]; while in the cases of an internal fixation of spinous processes with only steel wire, spine fusion was largely dependent on the excision of facet joint and lamina [45]. At week 12, no solid fusion could be achieved with autograft if the excision was not aggressive enough [45]. Without instrumented fixation and the excision of facet joint and lamina, solid fusion might not be expected in the current study.

The data obtained in the current study could not fully demonstrate as yet the functionality of submicron surface structured osteoinductive TCP ceramic in spine fusion, since the model was conducted between spinous processes rather than posterolateral lumbar. However, the current data demonstrated that a submicron surface structure renders TCP ceramic osteoinductive and leads to superior bone regeneration as compared to non-osteoinductive TCP ceramics in spine environment. It is thus worthy

to evaluate the functionality of submicron surface structured osteoinductive tricalcium phosphate ceramic for spine fusion using the well-validated spine fusion models.

5. CONCLUSION

By comparing the bone forming capacity of two TCP ceramics, with either a submicron (TCP-S) or a micron (TCP-B) surface structure in muscle and spine environment, we demonstrated that submicron surface structured tricalcium phosphate ceramic could be osteoinductive and perform superiorly than the micro surface structured one in spine environment.

A C K N O W L E D G E M E N T S

The authors would like to thank the Netherlands Institute for Regenerative Medicine (NIRM) and Rapid Prototyping of Custom-Made Bone-Forming Tissue Engineering Constructs (RAPIDOS Project, Ref. NMP-2013-EU-China604517) for their financial support in this study.

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CHAPTER 4

Modulating bone regeneration in rabbit condyle defects with three surface-structured tricalcium phosphate ceramics

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A B S T R A C T

Tricalcium phosphate (TCP) ceramics are used as bone void fillers because of their bioactivity and resorbability, while their performance in bone regeneration and material resorption vary with their physical properties, e.g. the dimension of the crystal grain. Herein, three TCP ceramic bone substitutes (TCP-S, TCP-M, and TCP-L) with gradient crystal grain size ($0.77 \pm 0.21 \mu\text{m}$ for TCP-S, $1.21 \pm 0.35 \mu\text{m}$ for TCP-M and $4.87 \pm 1.90 \mu\text{m}$ for TCP-L), were evaluated in a well-established rabbit lateral condylar defect model (validated with sham) with respect to bone formation and material resorption up to 26 weeks. Surface structure-dependent bone regeneration was clearly shown after 4 weeks implantation with TCP-S having most mineralized bone ($20.2 \pm 3.4\%$), followed by TCP-M ($14.0 \pm 3.5\%$), sham ($8.1 \pm 4.2\%$) and TCP-L ($6.6 \pm 2.6\%$). Afterwards, the amount of mineralized bone was similar in all the three groups, but bone marrow and material resorption varied. After 26 weeks, TCP-S induced most bone tissue formation (mineralized bone + bone marrow) ($61.6 \pm 7.8\%$) and underwent most material resorption ($80.1 \pm 9.0\%$), followed by TCP-M ($42.9 \pm 5.2\%$ and $61.4 \pm 8.0\%$ respectively), TCP-L ($28.3 \pm 5.5\%$ and $45.6 \pm 9.7\%$ respectively) and sham ($25.7 \pm 4.2\%$). Given the fact that the three ceramics are chemically identical, the results indicate that the surface structure (especially, the crystal grain size) of TCP ceramics can greatly tune their bone regeneration potential and the material resorption in rabbit condyle defect model, with the submicron surface structured TCP ceramic performing the best.

1. INTRODUCTION

In orthopaedic surgery, autograft is still the golden standard to treat critical-sized bone defects. However, its drawbacks such as complications and limited availability drove the development of synthetic bone substitutes [1-3]. Thanks to their chemical similarity to the inorganic component of bone and teeth, biocompatibility, bioactivity and osteoconductivity, calcium phosphate (CaP) ceramics including beta-tricalcium phosphate (β -TCP), hydroxyapatite (HA) and biphasic calcium phosphate (BCP) are often considered as bone grafting materials, either alone or as autograft extenders [4-7].

Despite osteoconductive CaP ceramics could conduct bone formation, the conductive bone is restricted to areas nearby the host bone bed, limiting their clinical application in the repair of critical-sized bone defects [8,9]. However, a subgroup of CaP ceramics with special physicochemical properties was found to trigger bone formation in heterotopic sites (e.g. intramuscular or subcutaneous), i.e. osteoinduction [10-12].

When tested in the ectopic implantation models, the bone forming potential of CaP ceramics varied with their physicochemical properties [13-19]. For instance, bone formation in CaP ceramics was affected by the HA/TCP ratio in intramuscular implantation models [13,14]. Next to the chemistry, CaP ceramic-directed bone formation in ectopic sites depended largely on physical factors (e.g. macroporosity [10-12], microporosity [15,16] and the dimension of surface crystal grain [17-19]). In particular, without any osteogenic additives, the reduction of the dimension of the surface crystal grain could render CaP ceramics osteoinductive [17-19]. For example, a BCP ceramic with small crystal size and large amount of micropores induced bone formation in muscle of goats, while the one having larger crystal size and fewer micropores did not [13]. The importance of surface structure in material-driven osteoinduction was clearly shown in studies, where TCP ceramic having submicron surface crystal grains triggered bone formation in muscles while micron surface structured TCP failed [17-19].

Although material-driven osteoinduction is animal-dependent [20], CaP ceramics with submicron surface crystals led to ectopic bone formation in goats [13], canines [17-19] and FVB mice [21]. Meanwhile these submicron scaled surface structured CaP ceramics enhanced bone regeneration in iliac crest of goats [22] and spine of beagles [23]. Furthermore, such osteoinductive CaP ceramics were as good as autograft, and outperformed a non-osteoinductive CaP ceramic in ovine critical-sized iliac wing defects [24].

In addition to serve as temporary scaffold to facilitate bone regeneration, an ideal bone substitute should gradually degrade during the bone remodeling process and ultimately be replaced by the newly formed bone [25,26]. It is well known that resorption rate of CaP ceramics could be chemically controlled by the means of HA/TCP ratio. β -TCP is of major interest and is often used in clinics because of its higher resorption rate [27,28]. Next to its chemistry, the resorption rate of TCP ceramic is affected by the physical properties (e.g. the dimension of the surface architecture). For instance, functional osteoclast-like cells could form on the TCP surfaces with submicron crystal grains but not on those with micron scale crystal grains. As a result, TCP ceramics with submicron crystal grains were more resorbable as compared to their counterparts with micron scale grains following an intramuscular implantation [17-20].

Given the fact that the crystal grain size largely impacted the bone forming potential and resorption rate of TCP ceramic in ectopic sites, it is assumed that the performance of such TCP ceramics varies also in orthotopic sites. In this study, we selected three TCP ceramics with gradient crystal grain size and compared in a well-established rabbit lateral condylar defect model with respect to bone regeneration and material resorption [29-30].

2. MATERIALS AND METHODS

2.1. Preparation of TCP ceramics

TCP-M (Osferion, Olympus, 1.0 - 3.0 mm) and TCP-L (chronOS, Synthes, 1.4 - 2.8 mm) were purchased and used as received. TCP-S was prepared

as previously described elsewhere [24]. In brief, phosphoric acid solution (H_3PO_4 , Fluka, Steinheim, Germany) was added dropwise to a calcium hydroxide suspension ($\text{Ca}(\text{OH})_2$, Fluka) at a Ca/P ratio of 1.5. After aging at room temperature for 6 weeks, the slurries were filtered and TCP-S powder was eventually obtained after drying and grinding. This powder was then foamed with diluted H_2O_2 (1% in distilled water, Merck, Darmstadt, Germany) and a porogen (wax particles, 600-1000 μm , Merck) at 60 °C to get green bodies. TCP-S ceramic was finally obtained by sintering the green bodies at 1050 °C for 8 hours. Irregularly shaped granules (1.0 - 2.0 mm) were prepared by crushing the sintered bodies and sieving. The granules were then ultrasonically cleaned with acetone (Fluka), 70% ethanol (Fluka) and demineralized water, dried at 80 °C and sterilized with gamma irradiation (dose 25 - 40 kGy, Isotron Netherlands B.V., Ede, the Netherlands) prior to use.

2.2. Physicochemical characterization

The chemistry of the TCP ceramics was characterized by X-ray diffraction (XRD; Miniflex II, Rigaku, Tokyo, Japan). The surface structure was evaluated with scanning electron microscope (SEM; XL30, ESEM-FEG, Philips, Eindhoven, the Netherlands) where 100 crystal grains were randomly selected from 10 SEM images ($\times 5000$) and were measured with AxioVision LE (Carl Zeiss Microimaging, Inc., Breda, the Netherlands). The pore size distribution in the ceramic strut and the surface area of the ceramics were obtained with mercury intrusion ($n = 1$ per material, Micromeritics Instrument Incorporation, Norcross, GA, USA).

2.3. Protein adsorption *in vitro*

Ceramic granules (0.2 cc, $n = 6$) were soaked into 2 ml of medium composed of minimal essential medium-alpha (a-MEM; Gibco, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS, Lonza, Germany) and 1% penicillin/streptomycin (Gibco, Invitrogen, UK), and incubated at 37 °C in a humid atmosphere with 5% CO_2 for 4 days. After washing three times with phosphate buffer solution (PBS, Invitrogen, Darmstadt, Germany), 0.5 ml of 1% Triton solution was added to each sample. Thereafter the amount of protein detached from the samples into the

Triton solution was measured with the QuantiPro™ BCA Assay Kit (Pierce Biotechnology Inc., Rockford, USA) following the manufacturer's instructions, and absorbance was measured with a spectrophotometer (Anthos Zenyth 3100, Anthos Labtec Instruments GmbH, Salzburg, Austria) at 620 nm. A calibration curve was prepared using standard FBS solutions. The amount of proteins adsorbed onto the ceramics was then converted to the same amount of the material (1.0 cc) and expressed as mean $\pm$ SD.

2.4. Calcium and phosphate ion release

Calcium and phosphate ion release from the ceramics was determined by placing 0.5 cc of ceramic granules ($n = 3$) in 100 ml of simulated physiological saline (SPS; 0.8% NaCl, 50 mM HEPES, 0.4 mM NaN_3 , 37 °C, pH = 7.3) for 1, 4 and 7 days. The calcium and phosphate ion concentrations in the SPS solution were measured with a QuantiChrom™ Calcium assay kit (BioAssay Systems, Hayward, USA) and a PhosphoWorks™ Colorimetric Phosphate assay kit (AAT Bioquest®, Inc., Sunnyvale, USA) respectively following the manufacturer's guidelines. Absorbances were measured with the spectrophotometer at 620 nm. The calcium and phosphate ion concentrations were quantified respectively with the respective standard calibration curves and expressed as mean $\pm$ SD.

2.5. Animal experiment

A total of 36 New Zealand white rabbits (female, 6 months, 3.5 - 4.5 kg) were used for this study. Rabbits were housed separately in stainless steel cages in laboratory animal at 20 - 25 °C with 50 - 60% humidity, and a light cycle coinciding with daylight hours. They were acclimatized to their new housing facilities for 2 weeks before surgery. Operations were performed with the permission of the local animal ethic committee (The management committee of experimental animals of Sichuan province, China) under general sterile conditions. All surgeries were conducted under general anesthesia by intravenous injection of sodium pentobarbital (30 mg Kg^{-1} body weight; Merck) in the marginal ear vein of rabbits. One defect ($\varnothing 6 \times 8$ mm) was created on each lateral condyle.

In brief, a longitudinal skin incision of 3 cm was made on the lateral femoral condyle, which was exposed following the entire removal of the overlying periosteum. At this point, a 2.0 mm pilot hole was generated with a drill ($\varnothing = 2$ mm) perpendicular to the long axis of the femoral shaft, and then the pilot hole was gradually widened with a larger drill ($\varnothing = 6$ mm) eventually creating a critical sized defect ($\varnothing 6 \times 8$ mm). Both lateral femoral condyles of each rabbit were operated, and each defect was either randomly treated with one of the three CaP ceramic granules till the defect was completely filled ($n = 6$ per group), or was left untreated (sham, $n = 6$). Afterwards, the muscle and skin were closed layer by layer with sutures in a routine surgical manner.

After surgery, the animals were intramuscularly given buprenorphine (0.1 mg per animal, Merck) for two days to relieve pain, and penicillin (40 mg kg^{-1} ; Merck) for three consecutive days to prevent infection. Animals were fed *ad libitum* and were allowed to move inside their cages without restriction. At each post-operative time interval (i.e. 4, 12 and 26 weeks after implantation) 12 rabbits were sacrificed with a marginal ear vein injection of excessive amount of sodium pentobarbital, and samples were harvested for the radiographic analysis and histological evaluation.

2.6. Radiographic examination

After stripping away the soft tissues, the femoral condyles were fixed in formaldehyde solution for 3 days, and then the bone defects were evaluated with X-ray radiography in the sagittal plane by three people in a blinded fashion. The conclusions were drawn based on the gray level differences between ceramic and bone tissue, and the consequent change of gray level distribution in the defects with implantation time.

2.7. Histological evaluation

After radiographic examination, all explants were trimmed as much as possible without harming the host bone surrounding the implants and embedded in methyl methacrylate (MMA) after dehydration with a series of ethanol solutions. The embedded samples were trimmed and mounted on a diamond saw microtome (Leica SP-1600, Wetzlar,

Germany). Three non-decalcified sections (10 - 20 μm) were made crossing the middle of each explants (on the axial plane) and stained with 1% methylene blue (Sigma-Aldrich, St, Louis, MO) and 0.3% basic fuchsin (Sigma-Aldrich) for light microscope observation. All the stained sections were digitalized with a scanner (Dimage Scan Elite5400 II, Konica Minolta, Tokyo, Japan) to obtain overview images for the quantification of the *de novo* bone formation and material resorption. The analysis was performed by pseudocoloring pixels representing mineralized bone (MB), bone marrow (BM), soft tissue (ST) and the remaining material (Me) in the region of interest (ROI, a 5 $\times$ 5 mm square overlapping the center of the defect) using Adobe Photoshop software (CS5, v12, Adobe Systems Benelux BV, Amsterdam-Zuidoost, the Netherlands). The Me%, MB%, BM%, Bone tissue% and ST% were calculated as follows:

$$\text{Me\%} = \text{Me} \times 100/\text{ROI}$$

$$\text{MB\%} = \text{MB} \times 100/\text{ROI}$$

$$\text{BM\%} = \text{BM} \times 100/\text{ROI}$$

$$\text{Bone tissue\%} = \text{MB\%} + \text{BM\%}$$

$\text{ST\%} = (\text{ROI} - \text{Me} - \text{Bone tissue}) \times 100/\text{ROI}$, the ST includes fibrous tissues as well as blood vessels.

The resorption rate of the ceramics was calculated as $(\text{Mi\%} - \text{Me\%}) \times 100/\text{Mi\%}$, where the initial area percentage of the ceramic material (Mi%) in the implants was determined after making 10 - 20 μm thick sections (ROI, a 5 $\times$ 5 mm) of MMA-embedded non-implanted ceramic granules (0.2 - 0.3 mL, n = 6).

2.8. Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) with Tukey's test ($p < 0.05$ for statistically significant differences), while normality of the data was assessed using the Shapiro-Wilk test ($p > 0.05$ for normally distributed data). The power of all experiments was determined with a post-hoc statistical power analysis (one-way omnibus ANOVA test with fixed effects).

3. RESULTS

3.1. Physicochemical properties of the TCP ceramics

The XRD patterns of the CaP ceramics are given in Figure 1A, which shows that the three CaP ceramics are chemically equivalent and belong to β -TCP. SEM images showed that the three CaP ceramics had gradient crystal grain size as expected (Figure 1B), with crystal grain size of $0.77 \pm 0.21 \mu\text{m}$ for TCP-S, $1.21 \pm 0.35 \mu\text{m}$ for TCP-M and $4.87 \pm 1.90 \mu\text{m}$ for TCP-L (Table 1). The microporosity was affected by both the number and the size of micropores and varied with materials (Figure 1B, Table 1). TCP-M had slightly higher strut porosity (51.90%) compared with TCP-S (45.14%), while TCP-L had the lowest strut porosity (31.52%). As also shown in Figure 1C, the micropores were mainly 0.3 - 1.0 μm in size for TCP-S, 0.5 - 1.5 μm for TCP-M and 0.5 - 7.0 μm for TCP-L. TCP-S also had the highest specific surface area by volume ($1.62 \text{ m}^2 \text{ mL}^{-1}$) followed by TCP-M ($1.28 \text{ m}^2 \text{ mL}^{-1}$) and TCP-L ($0.59 \text{ m}^2 \text{ mL}^{-1}$) (Table 1).

Figure 1. **A:** XRD pattern showing that the chemistry of the three TCP ceramics is β -TCP; **B:** SEM images show that the three TCP ceramics have different surface crystal grain size; **C:** Distribution plots of the surface pore dimension.

Table 1. *Physicochemical properties of CaP ceramics*

Mate rials	Supp liers	Che mistr y ^a	Parti cles size [mm]	Strut pore size ^b	Surfac e grain size ^c [μm]	Strut poro sity ^b [%]	Specific surface area [by weight] ^b [m ² g ⁻¹]	Specific surface area [by volume] ^b [m ² mL ⁻¹]
TCP- S	hom e mad e	β- TCP	1.0- 2.0	subm icron	0.77±0 .21	45.1 4	1.87	1.62
TCP- M (Osfe rion)	Olym pus	β- TCP	1.0- 3.0	subm icron	1.21±0 .35	51.9 0	1.47	1.28
TCP- L (chro nOS)	Synt hes	β- TCP	1.4- 2.8	micr on	4.87±1 .90	31.5 2	0.51	0.59

^a as evaluated by X-ray diffractometry;

^b obtained from mercury intrusion;

^c as confirmed by quantitative measurements on scanning microscopic images (5000×).

3.2. Protein adsorption *in vitro*

After soaking in culture medium containing FBS for 4 days, the amount of protein adsorption by CaP ceramics was correlated to their specific surface area

(Figure 2A and Table 1). TCP-S with the largest specific surface area adsorbed significantly more protein ($183.9 \pm 21.7 \mu\text{g ml}^{-1}$), followed by TCP-M ($137.3 \pm 15.7 \mu\text{g ml}^{-1}$) and TCP-L ($67.1 \pm 10.4 \mu\text{g ml}^{-1}$).

3.3. Calcium and phosphate ion release

The amount of calcium ions released by the three CaP ceramics is shown in Figure 2B. TCP-S released the most calcium ions into SPS 7.3 solution ($155.1 \pm 9.2 \mu\text{M}$, $163.3 \pm 12.2 \mu\text{M}$ and $166.6 \pm 10.3 \mu\text{M}$ at 1, 4 and 7 days respectively), followed by TCP-M ($130.1 \pm 5.7 \mu\text{M}$, $135.8 \pm 8.8 \mu\text{M}$ and $135.7 \pm 6.8 \mu\text{M}$ at 1, 4 and 7 days respectively) and TCP-L ($79.6 \pm 7.4 \mu\text{M}$,

Figure 2. **A:** Amount of protein adsorbed into implants (1 ml) from basic medium after 4-day incubation; **B, C:** Calcium and phosphate ions release from the materials into SPS at day 1, day 4 and day 7 (stars indicate a statistically significant difference, $p < 0.05$). For all the three experiments, the Shapiro-Wilk normality test gave $p > 0.05$. The power (and effect size) were 1.00 (2.95), 1.00 (3.74) and 0.99 (1.74) for protein adsorption, calcium and phosphate ion release experiments respectively.

$91.5 \pm 8.3 \mu\text{M}$ and $88.9 \pm 5.9 \mu\text{M}$ at 1, 4 and 7 days respectively). Similarly, as shown in Figure 2C, TCP-S also released the most phosphate ions into SPS 7.3 solution, followed by TCP-M and TCP-L. Differences among materials were statistically significant at all endpoints, however ion release did not increase with soaking time.

3.4. Radiographic examination

As shown in the X-ray images (Figure 3), 4 weeks after implantation the three materials were observed in the defects because of their higher radiologic density compared to the adjacent cancellous bone. In particular, the margins between the adjacent bone and the defects filled

with TCP-S and TCP-M were already indistinct, indicating that the implanted materials were well incorporated with the surrounding bone.

Figure 3. X-ray photos of the rabbit condylar defects with TCP-S, TCP-M TCP-L and sham ranging from 4 to 26 weeks post-operatively.

On the contrary, the margin of the sham and defects treated with TCP-L were still clearly visible, indicating no closure. At 12 weeks, the contour of all the implanted materials became vaguer and most likely was continuously replaced by *de novo* bone, meanwhile the defects treated with sham were still not fully closed. At 26 weeks, the TCP-S implants almost disappeared, and the defects had similar gray levels to the cancellous bone, followed by TCP-M. Indeed, the resorption and material replacement with bone in TCP-L was pronouncedly delayed as the material was still clearly recognized in the defects. A partial closure of sham defects was visible.

3.5. Histological evaluation in summary

As shown in the histological overviews after 4 weeks (Figure 4), a considerable creeping MB had been penetrated well into the whole defects treated with TCP-S, and relatively less MB was observed at the defect margins in TCP-M implants. In defects filled with TCP-L, limited MB was restricted to peripheral regions close to the host bone bed and no bone tissue could be observed in the center of the defects. In addition, the ceramic material within the defects was still largely intact in all the three materials.

Figure 4. Histological overviews showing the bone regeneration and material resorption within condylar defects of rabbits ranging from 4 to 26 weeks post-operatively (un-decalcified sections stained with methylene blue and basic fuchsin, where bone was stained fresh-red).

After 12 weeks, the bone tissue (consisting of MB and BM) increased continuously within the defects treated with all three ceramics. The MB increased within defects implanted with TCP-M and TCP-L, but was significantly decreased in those treated with TCP-S (where much MB was remodeled into BM). Meanwhile, the amount of the residual materials decreased as compared to the earlier endpoint. In addition, the condyle

defects treated with TCP-S were closed by the newly formed bone (6/6), while those treated with TCP-M, TCP-L and sham were partially repaired and no fully repair was achieved in any of the defects.

By 26-week post-operatively, *de novo* bone formation, bone remodeling and material resorption were continuously proceeding within the defects containing ceramics. An increase of MB was noted in condyle defects with TCP-L. Bone remodeling primarily dominated with the evidence of decreased MB and increased BM in those defects treated with TCP-S and TCP-M. Limited amount of resident ceramic was observed in the center of the defects filled with TCP-S, while more was seen in the TCP-M group. The TCP-L material was still predominantly present in the defects. In addition, defects treated with TCP-M were now completely closed with new bone (6/6), but defects treated with TCP-L and sham were still open (6/6).

3.6. Histological evaluation in detail

Examination with light microscopy revealed the presence of normal bony structures with osteocytes embedded in the lacunae and osteoblasts lining *de novo* bone after 4 weeks treatment all three groups. However, BM was detected only in TCP-S, while the space in defects treated with TCP-M and TCP-L was generally occupied by loose connective tissue. Additionally, numerous phagocytic cells colonized the surface of TCP-S and phagocytized ceramic debris. Limited phagocytes adhered on TCP-M surfaces and rarely noted on TCP-L surfaces, the sham consisted of much soft tissue and limited MB (Figure 5).

After 12 weeks implantation, the bone regeneration and remodeling continuously proceeded with the evidence of BM increase in TCP-S and TCP-M, and MB increase in TCP-L (Figure 5). Meanwhile, higher phagocytic activity in TCP-S than in TCP-M and TCP-L was seen as phagocytes engulfed particles and there was scalloped structure formation on the ceramic surfaces.

Figure 5. Light microscope images taken in the central of the defects showing bone formation, bone remodeling and degradation of CaP ceramics implanted in condylar defects of rabbits ranging from 4 to 26 weeks post-operatively (undecalcified sections stained with methylene blue and basic fuchsin, B: bone; O: osteoid; M: material; BM: bone marrow; ST: soft tissue; red arrows: osteoblasts; white arrows: osteocytes; red asterisks: phagocytic cells, white asterisks: scalloped structures of ceramics).

After 26 weeks implantation, MB was remodeling into cancellous bone and BM was seen in all the three implants as well as in sham, and osteoblast-like cells were rarely seen in the center. When such cells were seen, they were much flatter than those at 12 weeks. In addition, the number of phagocytes attached on TCP-S and TCP-M surface decreased compared to those seen at the 12-week endpoint, while the phagocytes were still rarely seen on TCP-L.

3.7. Quantitative histology

At the 4-week post-operative time interval, most MB were noted in defects treated with TCP-S ($20.2 \pm 3.4\%$) and followed by TCP-M ($14.0 \pm 3.5\%$), sham ($8.1 \pm 4.2\%$) and TCP-L ($6.6 \pm 2.6\%$). By 26-week post-surgery, TCP-S gave rise to most bone tissue formation ($61.6 \pm 7.8\%$), followed by TCP-M ($42.9 \pm 5.2\%$) and TCP-L ($28.3 \pm 5.5\%$) and sham ($25.7 \pm 4.2\%$). Quantification of the absolute percentage of ceramic residuals

within the defect showed that the most resorption occurred in TCP-S ($80.1 \pm 9.0\%$), followed by TCP-M ($61.4 \pm 8.0\%$) and TCP-L ($45.6 \pm 9.7\%$) (Figure 6). Differences in total bone tissue between materials were always statically significant at all endpoints.

Figure 6. Quantitative histomorphometry data on (A) the area percentage of mineralized bone, bone marrow, other soft tissue and residual material in ROI, and (B) the material resorption rate ranging from 4 to 26 weeks post-operatively (stars indicate a statistically significant difference, $p < 0.05$). For all the three experiments, the Shapiro-Wilk normality test gave $p > 0.05$. The power (and effect size) were 1.00 (3.90) and 1.00 (3.62) for bone tissue and resorption experiments respectively.

4. DISCUSSION

This study aimed at evaluating the bone regeneration and material resorption as function of crystal grain size of TCP ceramics in a well-established rabbit lateral condylar defect model. The main finding was that the three TCP ceramics had different bone regeneration potential and underwent resorption at different rates according to the crystal grain size.

4.1. Bone regeneration

Although with different rates, the newly formed bone tissue increased with the implantation time for all the three TCP ceramics. As shown in Figure 4, the most *de novo* MB occurred throughout TCP-S implants at 4-week post-operative time interval, followed by TCP-M and TCP-L where limited MB was restricted in peripheral areas adjacent the host

bone. Afterwards, the amount of MB was comparable in the three groups, but the BM, which indicates ongoing bone remodeling, varied among the three implanted materials (TCP-S > TCP-M > TCP-L), resulting into a direct relationship between bone tissue (BM+MB) and the crystal grain size. Bone regeneration in osseous sites could be formed both by inducible mesenchymal stem cells via osteoinduction and by osteogenic cells via osteoconduction. In our previous study, following intramuscular implantation in beagles, TCP-S could induce significantly more ectopic bone formation (21%) than TCP-M (8%), while TCP-L with micron scaled surface did not trigger any bone formation [19]. Therefore, the enhanced bone regeneration and remodeling in TCP-S implants should be the result of both the enhanced conductive bone formation in regions close to the host bone and the inductive bone formation in the central area of the implants [17-19,23].

Indeed, it has been shown that a specific surface topography, resulted from the combination of different dimensions of crystal grains and micropores with the microporosity, could tune adherent cell fate through mechanotransduction [19,21,31-34]. More specifically, the surface structure with a range from nano- to micro-scale could profoundly influence the cellular orientation, attachment, proliferation and differentiation irrespectively of the chemistry, even without osteogenic additives [33-41]. In the current study, TCP-S with crystal grains and micropores at the submicron scale constructed a specific surface topography, which directly affected the osteogenic differentiation of inducible mesenchymal stem cells (osteoinduction) and the osteogenesis of bone forming cells (osteoconduction) in orthopedic sites. As a result, bone regeneration in condyle defects treated with TCP-S was enhanced. This conclusion is also supported by our previous work, where TCP-S was osteoinductive and in ectopic site [19,23] and enhanced bone regeneration in spine environment [23].

In addition to the surface topography, the increase of microporosity and the concomitant decrease of crystal grain and micropore dimensions, augmented the surface area of CaP ceramics. The enlarged surface area

of TCP-S could have entrapped and concentrated more proteins (e.g. BMP) than TCP-M and TCP-L, which could have later induced the differentiation of undifferentiated cells to achieve osteogenesis favoring bone formation [41-44]. At the same time, calcium and phosphate ion release was related to the physical properties of the ceramics (e.g. surface area and crystallinity). Thus, the enlarged surface area of TCP-S could have provided a richer calcium ion microenvironment, which is not only a source for the building blocks of bone but could also enhance the proliferation, osteogenic differentiation and mineralization of bone forming cells to finally synthesize bone matrix [45,46]. Conversely, TCP-L with the largest crystal grains and lowest microporosity had the least surface area and formed the least bone. It appeared that the influence of crystal grain size on the bone forming potential of CaP ceramics could be attributed to the surface area that tunes protein adsorption and ion release.

Among the factors affecting bone regeneration with CaP ceramics, the size and interconnectivity of macropores (pores > 50 μm) are important ones [10-12]. The macropores inside the individual particles were interconnected, as evidenced by the presence of infiltrated tissues in the pores (Figure 5). By virtue of the fact that the three materials studied here had similar particle size range (1.0 - 3.0 mm, Table 1), they could have similar macroporosity and fully connected microporous structures after being packed in the defects. Therefore, the influence of pores larger than 50 μm on bone formation between the three groups could be minimized in this study.

4.2. Material resorption

Similarly with ectopic implantation model, the resorption of TCP ceramics varied with the crystal grain size in osseous site. The finer the crystal grains, and the faster resorption was seen. TCP-S had the highest rate of resorption, followed by TCP-M and TCP-L (Figure 6B).

The resorption of CaP ceramics was suggested to involve two types of process: solution-mediated disintegration (i.e. passive dissolution) and cell-mediated resorption [47,48]. It has been suggested that increasing

the surface area could enhance the contact between the ceramic and the surrounding body fluids rendering the material more prone to passive dissolution [23]. As shown in Figure 2B, TCP-S released the most calcium ions indicating that it dissolved faster than TCP-M and TCP-L due to its larger surface area (Table 1). In addition, cell-mediated resorption has been proven to play important roles in the resorption of CaP ceramics *in vivo*, where typical osteoclastic resorption pits on CaP ceramic surface were observed [49]. As shown in detailed histological images at the 12-week time period, a number of multinucleated osteoclast-like cells colonized the surface of TCP-S phagocytizing material fragments and scalloping much surface (Figures 5), which indicates an active cell-mediated material resorption. A limited amount such phagocytes and scalloped structure surface were detected in TCP-M. Conversely, such resorbing cells were missing in TCP-L implants (Figure 6B). Indeed, it has been shown that the surface structure dimension greatly affected the cell-mediated resorption of TCP ceramics, via the differential formation of multinucleated osteoclast-like cells and resorption capacity of osteoclasts. For example, TCP with submicron scale surface structure not only enhanced the formation of osteoclasts from mononuclear cells, but it was also resorbed by the osteoclasts formed on the surface, as compared to its micron surface structured counterpart [50]. In this study, having a larger surface area facilitating chemical dissolution and the right surface structure, suitable for the formation and function of osteoclasts, the submicron surface structure may have enhanced the resorption of TCP-S ceramic.

4.3. Bone remodeling and material resorption

When comparing the bone regeneration and material resorption, the resorption of the three TCP ceramics in osseous sites appeared to be coupled with bone remodeling. Bone tissue consisting of MB plus BM increased with time, ranging from 4 to 26 weeks poste-operatively, in all the three groups (Figure 6A). The mineralized bone in TCP-S reached its peak at week 4 and decreased thereafter with no significant difference to TCP-M and TCP-L at week 12 and week 26. However, at week 12 and

26, significantly more BM was formed in TCP-S than in TCP-M and TCP-L. This result is in line with that previously demonstrated [13,14,17,24], as TCP-S with the specific surface architecture enhanced the formation of osteoclasts. This fact not only enhanced the material resorption, but it also facilitated the remodeling of the bone formed at the early endpoints. Thus, more bone marrow could be detected in TCP-S, while TCP-L with micron-scale surface structure did not favor osteoclast generation and underwent the less material resorption and bone remodeling. The group of TCP-M has a performance between TCP-S and TCP-L.

The results obtained in this study demonstrate that, despite the same chemical composition, the bone regeneration capacity and resorption of CaP ceramics were different. Physical properties, especially the crystal grain size, are important in determining the functionality of CaP ceramics in bone regeneration and remodeling, and in material resorption in rabbit condyle defect model.

These finding may provide scientists clues for further optimization of CaP ceramic bone substitutes, and would be helpful for clinicians in selecting the right CaP ceramic bone substitutes for bone regeneration. However, studies with larger critical-size defects (e.g. iliac crest defect $\varnothing = 17$ mm) and functional studies (e.g. posterolateral lumbar fusion) would be necessary to further confirm the function of submicron scale surface structured calcium phosphate ceramics.

5. CONCLUSION

When three TCP ceramics with various dimensions of crystal grains were compared in rabbit femoral condyle defects for up to 26 weeks, the influence of surface structure scale on bone regeneration and material resorption was shown. Submicron-structured TCP-S gave rise to most bone formation and underwent most resorption as compared to TCP ceramics with larger crystal grains. The data could be useful for further optimizing CaP ceramic bone substitutes and selecting the most suitable CaP ceramic bone substitutes for clinical application.

A C K N O W L E D G E S

The authors would like to thank the Seventh Framework Programme of the European Union (REBORNE under grant agreement no. 241879), the Netherlands Institute for Regenerative Medicine (NIRM) and Rapid Prototyping of Custom-Made Bone-Forming Tissue Engineering Constructs (RAPIDOS Project, Ref. NMP-2013-EU-China604517), and the Horizon 2020 Framework Programme (CHARME, grant agreement no. 674282) for their financial support in this study.

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CHAPTER 4

CHAPTER 5

Efficacy of submicron surface structured calcium phosphate ceramics for posterolateral spinal arthrodesis

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Submitted for publication

A B S T R A C T

Iliac crest autograft is the gold standard for posterolateral lumbar fusion (PLF), while its drawbacks propel the development of synthetic alternatives. In this study, we evaluated the efficacy of a submicron surface structured calcium phosphate (CaP) ceramic, both in granular and shapeable putty formulations (Granules and Putty), in an instrumented ovine PLF model using iliac autograft as control. Harvested samples were subjected to manual palpation, radiography (X-rays), micro-computed tomography (μ CT), biomechanical tests and histological analysis to assess fusion rate, rigidity of the fusion mass, *de novo* bone formation and material resorption at the endpoints of 4, 8 and 12 weeks. Fusion rates increased with time for all groups as shown by manual palpation, X-ray, μ CT and mechanical test, with slightly better fusion outcomes for Granule and Putty than autograft at week 12. Next to slightly higher fusion rate, the fusion masses formed in Granules and Putty at week 12 were significantly mechanically stronger than that in autograft. Furthermore, larger fusion masses and more mineralized bone tissue were noted in Granules and Putty groups than in autograft at week 12. Spine fusion efficacy was equivalent for Granules and Putty formulations, with slightly more bone and slightly higher resorption in Putty. The overall information presented herein suggest that the submicron surface structured CaP ceramic could be at least as efficient as autograft in PLF arthrodesis in either Granules or Putty formulation, with an improved handling property and slightly improved performance in the latter.

1. INTRODUCTION

Having all the characteristics of an ideal bone graft substitute such as osteoconductivity, resorbability, osteoinductivity and non-immunogenicity, iliac crest autograft is still the gold standard for posterolateral spinal fusion (PLF) [1-4], while it bears the drawbacks such as donor site pain, morbidity and limited availability. With respect to allografts, the high risks of disease transmission and immunological rejection restrict their application [5,6]. Bone morphogenetic protein (BMP)-containing materials are thought to be promising alternatives to autologous bone, but their clinical application faces the need of high dosage, protein instability, high cost and side effects [7,8]. The shortcomings of the available bone grafting materials make it necessary to search for synthetic bone grafting materials that are as efficient as autologous bone for PLF.

Due to their chemical similarity to the native bone minerals, bioactivity, osteoconduction and controllable resorbability, synthetic calcium phosphate (CaP) ceramics are widely employed as autograft expanders, or as bone grafting alternatives, in spine fusion procedures [9-11]. Among them, beta-tricalcium phosphate (β -TCP) ceramics raised more attention due to their natural resorbability [12-14]. However, because of the lack of osteoinductive and osteogenic properties, the spine fusion rate of CaP materials is often inferior to that of autograft [15,16].

It has been demonstrated that the physicochemical properties such as chemistry [17-18], macrostructure [19-20] and surface microstructure [21-30] affect the functionality of CaP ceramics in bone regeneration. Especially, surface microstructure was shown as a crucial factor for the bone forming ability and resorbability of CaP ceramics. For instance, a comparison between two β -TCP ceramics with the same macrostructure but different size of the surface microstructure, demonstrated that only the submicron sized one induced ectopic bone formation in canines and rodents [25-27]. Meanwhile the submicron surface structured TCP supported the differential formation of osteoclast-like cells, facilitating

the material resorption as compared to the micron surface structured one [28].

More importantly, the specific submicron surface properties of osteoinductive CaP ceramics could lead to abundant bone formation in spine environment [29] and repair critical defects as good as the autologous bone and rhBMP-2 in goat iliac crest model [30]. These data indicate that osteoinductive submicron scale surface structured CaP ceramic could be a promising synthetic alternative to autologous bone in spine fusion.

Most of the available CaP ceramics are supplied in the granular form. Next to their unlimited on-the-shelf availability, it would be more desirable if CaP ceramics can be moldable to enhance surgical handling and favor the spanning of the granules between distant bony surfaces such as two spinal segments [31,32]. A way to produce shapeable CaP ceramic granules is to combine them with polymeric carriers [33,34]. Since the special surface structure of osteoinductive CaP ceramics is pivotal for their bone forming ability, the polymer binder should preserve the surface physicochemical characteristics of the granules during their shelf-life and it should be fast cleared from the site upon implantation to expose the osteoinductive surface structure to the surrounding biological environment [35,36].

In this study, the efficacy of a submicron surface structured CaP ceramic (in both granular and shapeable putty forms) was compared against autograft in a well-established instrumented ovine PLF spine fusion model [37-39]. The samples harvested at the time points of 4, 8 and 12 weeks were subjected to manual palpation, radiography (X-rays), micro-computed tomography (μ CT), biomechanical tests and histological analysis to assess fusion rate, rigidity of the fusion mass, bone formation and material resorption.

2. MATERIALS AND METHODS

2.1. Characterization of CaP bone substitutes

The Granules and Putty formulations of the submicron scaled surface structured CaP ceramic (Attrax® Granules and Attrax® Putty) were purchased from NuVasive Inc (san Diego, USA).

The chemical composition of the Granules was identified via X-ray diffraction (XRD; Miniflex II, Rigaku, Tokyo, Japan). The surface structure of the Granules was evaluated using a scanning electron microscope (SEM; XL30, ESEM-FEG, Philips, Eindhoven, the Netherlands), where 100 crystal grains were randomly selected from 10 SEM images ($\times 5000$) and measured with AxioVision LE (Carl Zeiss MicroImaging, Inc., Breda, the Netherlands). The mercury intrusion porosimetry ($n=1$, Micromeritics Instrument Incorporation, Norcross, GA, USA) was employed to determine the total porosity (volume percentage of pores in the materials), macroporosity (volume percentage of pores greater than, or equal to $10\text{ }\mu\text{m}$ in the materials), microporosity (the volume percentage of pores smaller than $10\text{ }\mu\text{m}$ present in the strut of the ceramic granules), pore size distribution in the ceramic strut and specific surface area of the ceramic.

The Putty was subjected to chloroform treatment to isolate the polymer binder for chemical characterization. Proton nuclear magnetic resonance spectroscopy (^1H NMR; Bruker Ascend 400/Avance III, Bruker, Leiderdorp, the Netherlands) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR; Spectrum Two, Perkin Elmer, Groningen, the Netherlands; ZnSe crystal) were employed to study the polymer binder identity. XRD, SEM and mercury intrusion were performed as described above for physicochemical analyses of the CaP ceramic granules isolated from the Putty.

2.2. Dissolution test *in vitro*

Dissolution test was performed by soaking 0.5 cc of Granules and Putty ($n = 5$) respectively in 100 mL of simulated physiological saline (SPS; 0.8% NaCl, 50 mM HEPES, 0.4 mM NaN_3 , pH = 7.3, at 37°C , 50 rpm). The cumulative calcium ion concentration in SPS was monitored by a calcium electrode (Metrohm, Herisau, Switzerland) for 400 minutes with refreshment of PBS solution after 200 minutes. Meanwhile, pictures of the

Putty were taken to evaluate its dissolution state up to 400 minutes. Thereafter the ceramic granules were rinsed with distilled water for 3 times and dried at 60°C for physicochemical characterization.

2.3. Surgical procedure

This study was performed with the permission from Spring Valley Laboratories (SVL) Institutional Animal Care and Use Committee (IACUC). In compliance with the laws and institutional guidelines of the local animal care committee, 21 skeletally mature sheep (n=6 for 4 weeks, n=6 for 8 weeks and n=9 for 12 weeks) underwent instrumented surgical procedures at two operative lumbar motion segments (L2-L3 and L4-L5) with random implantation of one of the three grafting materials (Granules, Putty and Autograft) as previously described [37,40,41].

In brief, Acepromazine (0.05mg/kg, IM) and Buprenorphine (0.1 mg/kg, IM) were administered approximately 15 minutes prior to induction by Diazepam (0.2 mg/kg, IV), Ketamine HCL (5 mg/kg, IV) and Cefazolin sodium (antibiotic) (1 g IV) via jugular vein. After sedation, animals were intubated and maintained on general inhalation anesthesia with isoflurane (2%) with continuous intravenous fluids (3 to 6 mL/lb/hr) administered for the duration of surgery through an ear vein catheter. Then the posterior lumbar region and iliac crest were aseptically prepared. 10 cc of cortico-cancellous bone grafting was harvested from each iliac crest for autograft before the spine surgery. An initial skin incision was made in the dorsal mid-line over the L2-S1 levels. Blunt dissection was done to expose the L2-L3 and L4-L5 facets and transverse processes. The posterior cortices of the bilateral operative level transverse processes were decorticated to be punctate bleeding, and two operative levels (L2-L3 and L4-L5) were instrumented bilaterally with polyaxial pedicle screws (5.5 mm diameter × 25 mm length) and solid titanium rods (5.5 mm dia.). Following thorough irrigation, 20 cc of each material were equally distributed to the bilaterally decorticated arthrodesis site at the appropriate lumbar level (10 cc per side) (Figure 1). Then the paraspinal muscles, dorsolumbar fascia and subcutaneous

layers were approximated with 1-0 and 2-0 Vicryl sutures and skin closed with staples.

Figure 1. A: Granules (1), Putty (2) and Autograft (3) harvested from iliac crests; **B:** Granules, Putty and Autograft were implanted (20 cc/level) as stand-alone at two spine levels (L2-3 and L4-5) with instrumentation.

After surgery, the sheep were kept in the post-operative care pen and received observations of general condition. Flunixin Meglumine (1 mg/kg, IM) and Cefazolin (1 g/animal, IM) were administered to reduce pain and prevent infection. A single administration of Dexamethasone (20 mg/animal, IM) was administered for anti-inflammation and the surgical sites were dressed with Biozide Gel.

At each time point, animals were humanely sacrificed using an overdose (150 mg/kg) of concentrated pentobarbital solution (390 mg/mL). The spinal column of the animal was carefully removed and immediately placed in double wrapped plastic specimen bags and frozen at -20°C for subsequent radiographic, biomechanical and histologic evaluation of the operative motion segments.

2.4. Assessments of the spine implants

2.4.1. Radiographic analysis (X-radiography, X-Ray)

Plain films (X-ray) were taken on the post-sacrifice and the radiographic status of the all posterolateral spinal arthrodeses was evaluated utilizing the Lenke four-point grading [42] (Table 1).

Table 1. *The fusion grading scales for X-ray, μ CT and histological evaluations*

The Lenke grading scales for X-ray and μ CT evaluations		
Grade	Score	
A	4	Solid, big trabeculated fusion bilaterally (definitely solid)
B	3	Solid, big fusion mass unilaterally with a small fusion mass on the contralateral aspect (possibly solid)
C	2	Small, thin fusion masses bilaterally with apparent crack (probably not solid)
D	1	Graft resorption bilateral or fusion mass with an obvious bilateral pseudarthrosis (definitely not solid)
The fusion grading scales for histological evaluation		
A	4	Definitely solid with robust bridging bone;
B	3	Probably solid with thin bridging bone;
C	2	Probably not solid with some bone formation but without bridging;
D	1	Definitely not solid with obvious pseudarthrosis or bone graft dissolution.

2.4.2. Radiographic analysis (micro-computed tomography, μ CT)

μ CT was performed for each lumbar spine specimen post-sacrifice. Images were obtained at 1 μ m thick slice intervals (American Radiology Inc., White Marsh, Maryland, USA) and underwent three-dimensional reconstruction (axial, coronal and sagittal) at 0.5 mm intervals. The entire stack of images in three planes was evaluated for fusion assessment using the Lenke grading scale (Table 1).

μ CT images were also used to quantitatively assess the fusion mass volume (cm^3) within the posterolateral treatment levels. Using a similar method to Wheeler et al. [43], the calculation was carried out on each fusion mass for the right and left sides at both operative levels, from the cranial to caudal transverse process edges spanning the fusion site but

excluding the transverse processes. This calculation represents the total volume of the fusion mass, including both new bone and residual implant.

2.4.3. Manual palpation

Non-destructive manual palpation was performed after removing instrumentation in flexion extension and lateral bending to assess the range of motion of the operative functional spinal units. Sites was categorized as fusion and non-union.

2.4.4. Biomechanical testing (range of motion)

Quantitative multidirectional flexibility testing was performed to assess the range of motion of the spinal units after removing the spinal instrumentation. To determine the multidirectional flexibility, six pure, unconstrained moments, i.e. flexion and extension (± 4 Nm X-axis), left and right lateral bending (± 4 Nm Z-axis) and left and right axial rotation (± 4 Nm Y-axis), were applied to the superior end of the vertically oriented specimen while the caudal portion of the specimen remained fixed to the testing platform. A maximum applied moment of ± 4 Nm was used for each loading mode and applied at a ramp rate of 0.25 degrees/second using a six degree of freedom spine simulator (6DOF-SS). A total of three load/unload cycles were performed for each motion, with data analysis based on the final cycle. For the six main motions - corresponding to the moments applied - the operative level vertebral rotations (degrees) were quantified in terms of peak range of motion (ROM). To prevent desiccation during assessment, specimens were moistened with 0.9% NaCl sterile irrigation solution.

2.4.5. Histological evaluation

The harvested lumbar segments were para-sagittally sectioned along the right and left posterolateral fusion sites. Histologic preparation of samples included dehydration in series of ethanol, undecalcified solution processing and embedding in polymethyl-methacrylate (PMMA). Using the EXAKT Microgrinding Device, the embedded specimens were cut at approximately 300 μ m in thickness and subsequently ground and polished to 120 μ m. A total of two slides from each right and left site were

obtained and stained using the techniques of Harris' hematoxylin and eosin. High-resolution images were taken and used for fusion assessment by two reviewers blinded to the treatment groups. The success of fusion and extent of implant resorption were graded using the scales as shown in Table 1.

2.4.6. Quantification of bone and materials

High-resolution microradiographic images were made from the mounted histological sections with Faxitron Radiography. Using the Image J image analysis system, one microradiographic image from each side (left and right) of the operative segments was used to quantify trabecular bone area (mm^2) and residual implant area (i.e. CaP Granule) (mm^2) within the posterolateral region, excluding the transverse processes.

2.5. Statistical analysis

All data were shown as mean plus/minus one standard deviation and statistically compared using student's t-test and ANOVA were used to assess significance of differences ($p < 0.05$).

3. RESULTS

3.1. Physicochemical characterization of materials

Granules resulted to be biphasic calcium phosphate (BCP: 5%wt. HA, 95%wt. TCP) via XRD (Figure 2A). SEM showed that Granules had a submicron scaled surface structure with grain-like topography (Figure 2B), and mercury intrusion showed that Granules had a pore size distribution within the submicron scale, while microporosity, macroporosity and specific surface area are summarized in Table 2.

As shown in Figure 3, the polymer binder belonged to the alkylene oxide copolymer (AOC) family, which can either be a copolymer, or a mixture, of poly (ethylene glycol) (PEG) and poly (propylene glycol) (PPG). The CaP ceramic granules extracted from Putty had similar physicochemical properties as Granules (Figure 1, Table 2).

Table 2. Physicochemical properties of the CaP ceramics form Granules and Putty

Parameters	Granules	Putty
Chemistry ^a	5%HA/95%TCP	5%HA/95%TCP
Grain size ^b (μm)	0.71 ± 0.21	0.73 ± 0.18
Total porosity ^c	72	70
Macroporosity ^c ($\geq \mu\text{m}$)	50	47
Microporosity ^c ($< \mu\text{m}$)	22	23
Micropore size range ^c (μm)	0.3 - 1.1	0.3 - 1.2
Specific surface area ^c (m^2/g)	1.85	1.79
Calcium ion release (ppm, at 400mins)	14.54 ± 1.62	15.06 ± 0.77

^a as evaluated by X-ray diffractometry;

^b obtained from mercury intrusion;

^c as confirmed by quantitative measurements on scanning microscopic images (5000 $\times$);

Figure 2. Physicochemical properties of Granules and CaP ceramic extracted from Putty: **A:** XRD pattern showing the chemistry of Granules and Putty; **B:**

Distribution plots of the submicron pore dimension of Granules and CaP ceramic extracted Putty; C and D: SEM image showing the surface structure of Granules and CaP ceramic extracted from the Putty.

Figure 3. **A:** ^1H NMR revealed that the binder from the Putty is composed of poly (ethylene glycol) (PEG: e) and poly (propylene glycol) (PPG: g₁, g₂, g₃, g₄, g₅), the symbol s indicates the shift for water; **B:** FTIR spectra confirmed the NMR results: the binder is composed of PEG (h₂, h₄) and PPG (h₁, h₃, h₅).

3.2. Dissolution test *in vitro*

As shown in Figure 4A, after 60 minutes some CaP ceramic granules dissociated from the Putty that began to disrupt indicating that the binder was dissolving. After 120 minutes most of the CaP ceramics granules were liberated, indicating that the Putty was almost completely disrupted. After 200 minutes all CaP ceramic granules were dispersed.

Figure 4. **A:** Shape change of Putty during dissolution in SPS 7.3 solution within 200 mins. **B:** Calcium ion release profile of Granules and Putty in SPS 7.3 solution during 400 mins (* $p < 0.05$).

The presence of AOC binder restrained the calcium ion release as compared with Granules alone during the first 200 minutes (Figure 4B).

With the dissolution of AOC, the release kinetics of Putty reached that of Granules after 200 minutes. The cumulative calcium ion release averages for Putty after 290 mins (11.52 ± 2.92 ppm) and 400 mins (14.54 ± 1.62 ppm) were statistically the same as the average calcium ion release observed for Granules at the same endpoints (12.90 ± 0.60 ppm and 15.06 ± 0.77 ppm).

3.3. Assessments of the spine samples

3.3.1. X-ray

Representative radiographic images over time among the groups are shown in Figure 5, where the size of fusion mass of autograft visibly

Figure 5. Representative anteroposterior X-ray images of Granules, Putty and Autograft ranging from 4 to 12 weeks poster-operatively showing the resorption of implanted materials as well as bone formation. Please note that the instrumentation at 4 and 8 weeks was removed before taking X-ray images.

decreased with the implantation time. Slight decreases of fusion mass volume were seen for Granules and Putty groups. At week 12, the fusion mass for Granules and Putty was bigger than with the mass in autograft. The fusion rates were estimated with plain film radiography according to the fusion grading scale of Lenke (Table 3). Solid fusion was not observed in any sample at 4 and 8 weeks post-implantation. However, by 12 weeks post-operation significant increases in the overall fusion success were observed in all groups ($p < 0.05$). Both the Granules and Putty led to three specimens graded as B and three as A (3.5 ± 0.5), while autograft led to two C, three B and one A (2.8 ± 0.8).

3.3.2. μ CT

3.3.2.1. μ CT assessment of fusion

Similarly to the radiographic observations (Figure 5), the 3D reconstruction of the μ CT images showed a clear change in the fusion

Figure 6. A: Fuse mass volume of Granules, Putty and Autograft are shown by 3D reconstructed posterior views based on μ CT images; **B:** Representative 3D reconstructed views comparing the 3 axes rendering of the operative lumbar spine and fusion sites based on the imported CT images; **C:** 3D model containing only the segmented fusion mass following reconstruction using the 3D Slicer program; **D:** Volume change of the implants with time from time zero to week 12.

mass of autograft group during time (Figure 6A). At week 12, the fusion mass was larger in Granules and Putty groups than in the autograft group. Grading the fusion rates with μ CT images according to Lenke's scale resulted in no significant degree of fusion at either week 4 or 8 in any of the groups. At 12 weeks, a significant increase in overall fusion success was observed in the three groups ($p < 0.05$): both Granules and Putty had two specimens graded as B and four as A (3.7 ± 0.5), while the autograft indicated two C, two B and two A (3.0 ± 0.9) (Table 3).

3.3.2.2. Quantitative μ CT assessment of posterolateral fusion mass volume

The total fusion mass volume (cm^3) within the posterolateral treatment was quantified with μ CT images (Figure 6D). At time zero, the implant volume was $12.71 \pm 0.74 \text{ cm}^3$, $13.24 \pm 0.86 \text{ cm}^3$ and $7.42 \pm 1.66 \text{ cm}^3$ for Granules, Putty and autograft respectively. Not much mass change was observed in Granule group and Putty groups by week 4, while a decrease was noted in the autograft group, with a mass volume of $14.02 \pm 0.86 \text{ cm}^3$ for the Granules, $12.19 \pm 1.00 \text{ cm}^3$ for the Putty and $3.87 \pm 0.32 \text{ cm}^3$ for autograft. The decrease of mass volume from 4 to 8 weeks was significant for all groups. At week 8, the fusion mass volumes were $10.34 \pm 1.61 \text{ cm}^3$ for Granules, $8.78 \pm 1.55 \text{ cm}^3$ for Putty and $2.47 \pm 1.80 \text{ cm}^3$ for Autograft. Thereafter the volume mass remained stable, with $9.89 \pm 2.14 \text{ cm}^3$ for Granules, $8.31 \pm 1.47 \text{ cm}^3$ for putty and $2.66 \pm 1.23 \text{ cm}^3$ for autograft at week 12. Less mass volume was seen for autograft at all the timepoints.

3.3.3. Manual palpation

CHAPTER 5

No fusion could be detected with manual palpation in any sample at either 4 or 8 weeks. At week 12, both the CaP-based groups (Granules and Putty) achieved 100% fusion and had higher rates than autograft (4/6=67%) (Table 3).

Table 3. A summary of fusion assessment per evaluation method.

Methods		Granules			Putty			Autograft		
		4 W*	8 W	12 W	4 W	8 W	12 W	4 W	8 W	12 W
Manual palpation		0/4	0/4	6/6	0/4	1/4	6/6	0/4	0/4	4/6
X-ray	Grade	4/4 D	4/4D	3/6A 3/6B	4/4 D	2/4C 2/4D	3/6A 3/6B	4/4 D	4/4 D	1/6A 3/6B 2/6C
	Score	1	1	3.5± 0.5	1	1.5± 0.6	3.5± 0.5	1	1	2.8± 0.8
μCT assessment	Grade	4/4 D	1/4C 3/4D	4/6A 2/6B	4/4 D	1/4B 1/4C 2/4D	4/6A 2/6B	4/4 D	4/4 D	2/6A 2/6B 2/6C
	Score	1	1.3± 0.5	3.7± 0.5	1	1.8± 1.0	3.7± 0.5	1	1	3.0± 0.9
Histology	Grade	8/8 D	8/8D	4/12 A 3/12 B 5/12 C	8/8 D	3/8C 5/8D	6/12 A 3/12 B 3/12 C	8/8 D	8/8 D	2/12 A 2/12 B 8/12 C
	Total Score	1	1	2.9± 0.9	1	1.3± 0.5	3.3± 0.9	1	1	2.5± 0.8

*: weeks

3.3.4. Range of motion mechanical analysis

Range of motion (ROM) decreased with time from week 4 to week 12 for all the tested groups (Figure 7). Please note that the flexion/extension and axial rotation of autograft at week 8 had large variations (Figure 7B and C). When attentions were paid to the 12-week samples, significantly

higher degree of lateral bending was found for autograft than for Granules and Putty (Figure 7A).

Figure 7. Multi-direction flexibility, **A:** lateral bending; **B:** flexion-extension; **C:** axial rotation of the operative motion segments ranging from 4 to 12 weeks post-operatively.

3.3.5. Histological evaluation

At week 4, new bone tissue was formed nearby the host transverse process bone bed in Granules and Putty implants (Figure 8), but bone formation was not observed in their central region resulting in no fusion (Table 3). As regards the autograft at week 4, formation of bone was not limited to the host bone bed but also in the central region, however cortico-cancellous bone fragments presented within the intertransverse space, with a connective tissue interface primarily consisting of fibrocartilage and no bridging of the individual bone chips (Figure 8). The fusion masses were histologically graded as D for all samples, with no histological fusion at week 4 (Table 3).

At week 8, bone formation progressed from the host bone bed to the central region of the Granules and Putty implants, while continuous bone between the two transverse processes was not found in any specimen of Granules (graded to D), while 3 out of 8 segment sides were graded to C (Figure 8 and Table 3). The trabecular bone was observed at week 8 in the autograft group with a combination of fragmented iliac crest remnants and immature woven bone extending from the transverse processes towards the center of the intended arthrodesis site, but a continuous bone bridge between the transverse processes was not achieved yet (Figure 8). All the 8 segment sides with autograft were graded to D (Table 3).

By the 12-week post-operative time endpoint, the Granules treatment demonstrated a dense network of woven trabecular bone in direct apposition onto the CaP granules extending across the arthrodesis sites, with occasional pockets of fibrous tissues leading to non-unions in few sites (Figure 8). Out of 12 segment sides, 4 were scaled to A, 3 to B and 5 to C (with the score of 2.9 ± 0.9 , Table 3). The histological appearance of the Putty group was essentially the same as Granules (Figure 8). In 12 segment sides, 6 were graded to A, 3 to B and 3 to C (with the score of 3.3 ± 0.9 , Table 3). In both the Granule and Putty groups, reduction of CaP granules within the posterolateral regions was seen as compared to the 4 and 8-week time intervals. Trabecular bone was noted between the transverse processes with smaller size in autograft group (Figure 8). Complete intertransverse fusion bridges were less often in autograft group at week 12, which had 2 grade A, 2 grade B and 8 grade C out of the 12 segment sides (with the score of 2.5 ± 0.8 , Table 3).

Figure 8. The histological overviews of Granules, Putty and Autograft at week 4, 8 and 12 (un-decalcified section, H&E staining).

3.3.6. Quantification of bone formed and residual materials

The mineralized trabecular bone and CaP granules could be clearly distinguished in the microradiographic images obtained from the histological sections using Faxitron Radiography (Figure 9A), allowing the quantification of mineralized bone and CaP materials in the specimens at the different time intervals.

By area, an increase of bone with time was seen in all groups (Figure 9B). A higher increase of bone tissue with time was seen in the Granules and Putty group than in autograft group, with the most bone (at week 4) as well as the least bone (at week 12) in autograft group. At each time point, the bone was slightly more in the Putty group than in the Granules (Figure 9C). Decrease of materials with time was noted in both the Granules and Putty groups (Figure 9D). In Granules group, the CaP ceramic decreased from week 4 to 12, mainly happened during the period from week 8 to 12, the granules in the Putty had as comparable resorption rate (38.1% for Putty vs 27.6% for Granules from week 4 to week 12) as the Granules alone.

Figure 9. A representative microradiographic image (A) that was used for histomorphometric calculations for trabecular bone area in the implants (B) trabecular bone area varied with time (C) and residual implant area (D) using the ImageJ image analysis in the explants during implantation time.

4. DISCUSSION

Submicron structured surface CaP ceramics triggered bone formation following soft tissue implantation [25-28] and could repair critical-sized defects [30] as well as could enhance bone formation in spine environment [29]. Therefore, it is expected that osteoinductive submicron surface structured CaP ceramics could be promising grafting materials for spine fusion in clinics. Using the well-established pre-clinical model of instrumented sheep PLF [37-39], we evaluated the efficacy of a submicron surface structured CaP ceramic in both granular and putty forms. Meanwhile, the possible influence of a polymeric binder (AOC) on the performance of the submicron surface structure CaP ceramic was addressed.

Physicochemical characterization of the CaP ceramic granules used in this study showed a submicron surface structured material (Figure 1, Table 2) with a trace HA in β -TCP (Figure 1, Table 2), indicating the CaP ceramic was a resorbable osteoinductive CaP material [12-14].

A shapeable putty revealed to be a combination of a polymeric binder and CaP ceramic granules. Chemical assays (NMR and FTIR) showed that the binder was composed of poly (ethylene glycol) (PEG) and poly (propylene glycol) (PPG), both of which are biocompatible and often used for clinical applications [44-46]. Quick dissolution of the binder was shown (Figure 4A) and was further confirmed with the calcium release (Figure 4B). Further analyses of the granules extracted from the Putty after dissolution confirmed that the presence of the binder did not alter their surface structure and dissolution kinetics (Figure 2 and Table 2). The overall physicochemical data of the Putty indicated that the used binder may not negatively affect the bone forming ability of the ceramic granules as previously demonstrated [47,48].

Following a standard surgical procedure, the three grating materials were implanted in an instrumented sheep PLF (Figure 1). The fusion assessed by manual palpation at week 4 and 8 was not achieved, except for one fusion out of 4 Putty samples at week 8, indicating that not much difference in fusion rate was seen among the three groups at the earlier time points. Fusion rates increased at week 12, reaching 100% fusion in Granules and Putty groups and 67% for autograft (4 out of 6 samples). The fusion rate of 67% achieved with autograft in this study is in line with those reported in literature for autograft at the same endpoints [49]. The results thus suggest that both Granules and Putty performed slightly better than autograft in instrumented sheep PLF.

The radiographic assessment (X-ray) supported the fusion scores observed with manual palpation. The Lenke scores increased during time for all groups, with significantly higher fusion scores in Granule and Putty groups than in autograft group at week 12 (Table 3). The fusion scores obtained from radiographic films may not be accurate because of the difficulties in distinguishing bone and CaP ceramic in Granule and Putty groups, while the smaller fusion mass volume in the autograft group at week 12 (Figure 5) indirectly supports a better performance in Granule and Putty groups.

Increase of fusion rates with time and higher fusion scores were seen in Granule and Putty groups than in autograft group with μ CT assessment, while this assessment method may not be accurate either as μ CT detects both mineralized bone and CaP ceramics. However, similarly to radiographic evaluation, larger fusion mass volume was observed in Granule and Putty groups at week 12 (Figure 6A), supporting their better performance at week 12. Larger volume of fusion mass was not only qualitatively seen (Figure 6A), but also quantitatively shown (Figure 6D). The less fusion mass of autograft in volume than those of Granules and Putty before implantation (Figure 6D) does not mean that less autograft was used in the implantation since the volume detected by μ CT includes the mineralized components of bone in autograft and CaP ceramic in Granules and Putty. Given the fact that mineral density accounts for about

40% of the autologous bone [50,51], the total volume of cancellous bone could be calculated back to 18.5cc (close to the volume measured with syringe in Figure 1A). The larger volume detected in the Granule and Putty groups with μ CT is the result of a higher mineral densities in the Granules and Putty. With time, the volume of fusion mass decreased for all groups. The less volume of fusion mass in autograft group at all the considered time points (Figure 6D) suggested a better performance of the Granules and Putty than autograft.

However, the fusion mass volume alone may not be used to judge the fusion. For instance, fusion mass with Granules and Putty groups was large at week 4 and week 8 (Figure 5 and Figure 6), but no fusion could be achieved (Table 3). While, with the same mass volume between 8 weeks and 12 weeks for all groups, fusion was achieved only at week 12. Fusion could be achieved only when the integration between new bone tissue and grafting materials was formed (Figure 8, 12 weeks and Figure 9A). Both the bone formation and resorption (including resorption of the CaP ceramic in case CaP ceramic was used) are important to reach osteointegration between the newly formed bone tissue and grafting materials. In the case of autograft, it appeared that the formation of bone was less than the resorbed bone, resulting in a volume decrease in 8 weeks (Figure 6D). It is impossible to judge bone formation, bone resorption and material resorption for the Granule and Putty groups via μ CT, while histological observations showed a progressive bone formation and material resorption during time (Figure 8), and this progress was confirmed with the quantification of mineralized bone area in the samples (Figure 9). Bone area increased with time for all groups, while the largest increase was seen for Granules and Putty (Figure 9B). More bone was present in autograft group at week 4 (likely because of the bone chips implanted), while new bone formation progressed during time, eventually resulting in more bone in Granules and Putty than in autograft group at week 12 (Figure 9C). The more bone in Granules and Putty at week 12 implied again their better performance in PLF of sheep.

The better performance of Granules and Putty in PLF could be partially supported by manual palpation, radiographic assessment, μ CT assessment, histological evaluation and morphometrical analysis of mineralized bone. The mechanical data led to the same conclusion. As indicated by the lateral bending (Figure 7A), fusion mass became more rigid with time for all groups while significantly smaller range of motion was detected at 12 weeks in the Granule and Putty groups than in autograft.

Bearing the osteoconductive, osteogenic and osteoinductive properties, autograft is considered the golden standard graft for bone substitute, while 100% success may not be expected in posterolateral spine fusion with autograft [1-4] because of its limited bone forming ability or faster resorption rate or both [5,6]. Having submicron structured surface, and thus being osteoinductive [26-29], CaP ceramics may not only enhance conductive bone formation from the host bone bed but also trigger bone formation in the soft tissue region far from the host bone bed, eventually resulting in spine fusion. The hypothesis of successful spine fusion with osteoinductive CaP ceramics was demonstrated in this study. At week 12, 100% fusion could be achieved with osteoinductive submicron surface structured CaP ceramic in both granular and putty forms in a well-established preclinical sheep model.

The outcome at week 12 was the same regarding fusion rate for both Granule and Putty formulations, while the Putty had advantages. The first advantage is its shapeability, which improves the surgical handling of the grafting material in practice. Further, the shapeability may have allowed for better accommodation of CaP ceramic granules onto the host bone bed, so that the conductive bone formation from the host bone bed was improved (Figure 9C) resulting in possible earlier spine fusion. As seen in Table 3, better performance was seen with Putty at week 8.

Being mainly β -TCP phase, the submicron surface structured CaP ceramic was resorbed in the spinal region, ceramic in both granular and putty forms had comparable resorption rate during the period from week 8 to 12 (Figure 9D).

5. CONCLUSION

The head to head comparison among autograft, AttraX granule and AttraX putty was performed with a well-established sheep instrumented PLF model. Manual palpation, radiographic assessment, uCT evaluation, mechanical test, histological observation and morphometric assay showed a better performance of Granules and Putty at the 12-week endpoint. The results presented herein indicated that submicron surface structured CaP ceramics in both granular and shapeable putty formulations could be promising alternatives to autograft for spinal fusion applications.

ACKNOWLEDGEMENTS

The authors would like to thank the Seventh Framework Programme of the European Union (REBORNE under grant agreement no. 241879), the Netherlands Institute for Regenerative Medicine (NIRM) and Rapid Prototyping of Custom-Made Bone-Forming Tissue Engineering Constructs (RAPIDOS Project, Ref. NMP-2013-EU-China604517), and the Horizon 2020 Framework Programme (CHARME, grant agreement no. 674282) for their financial support in this study.

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CHAPTER 6

Nexus between macrophage phenotype, angiogenesis and bone formation in calcium phosphates

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S u b m i t t e d f o r p u b l i c a t i o n

A B S T R A C T

The ability of calcium phosphate (CaP) materials to initiate bone formation varies with their physicochemical properties, among them the dimension of the surface topography is one of the most crucial triggers. Given the fact that, following surgical implantation, CaP materials initiate the natural wound healing processes of inflammation, angiogenesis, tissue formation and remodeling, we herein investigated the biological cascades occurring upon ectopic implantation of a tricalcium phosphate with submicron surface topography (TCP-S, osteoinductive) compared against a tricalcium phosphate with micron-scale topography (TCP-B, non-osteoinductive). *In vitro*, TCP-S facilitated the M2 polarization of macrophages derived from a human leukemic cell line (THP-1) as shown by the enhanced secretion of TGF- β 1 and CCL18. Subsequently the media with cytokines secreted by M2 macrophages polarized on TCP-S enhanced tube formation from human umbilical vein endothelial cells (HUVECs). Following implantation in a canine intramuscular defect, TCP-S samples led to production of more of the typical M2 macrophage markers (e.g. IL-10) at week 1, had enhanced blood vessel formation after week 3 compared to TCP-B samples. Bone formation was observed by histology in TCP-S 6 weeks after implantation, while such bone formation was reduced when an angiogenesis inhibitor (KRN633) was added to TCP-S. No bone formed in TCP-B implants. The data presented herein demonstrates strong links between surface topography, macrophage polarization, angiogenesis and bone formation in CaP materials implanted in non-osseous sites. It is likely that the submicron surface topography instructs the differentiation of macrophages of the M2 phenotype, which secrete cytokines to enhance angiogenesis, facilitating bone formation.

1. INTRODUCTION

Calcium phosphate (CaP)-based materials are often considered as bone substitutes [1-2], and their capacity to trigger bone formation varies with their physicochemical properties [3-5]. By altering their size of surface topographical features, CaP materials can be endowed with osteoinductivity and have been shown to perform equally well to the gold standard autograft bone in repairing critical-sized bone defects [6]. In addition, the osteoinductive potential could be further accelerated by optimizing the surface morphology of CaP materials [7]. Although the key physicochemical properties relevant to CaP-instructed bone formation have been outlined in the last two decades [3-6], the biological mechanism underlying the material-driven osteoinduction is still largely unknown [8].

It is well known that any surgical implantation evokes a series of biological responses including inflammation, angiogenesis, tissue formation and remodeling [9]. The inflammation starts with a rapid infiltration of monocytes and differentiation of macrophages, which drive innate and adaptive immune responses. Such immune response not only play an essential role in innate immunity for host defense but are also involved in cellular processes and tissue repair [10,11]. To fulfil various functions, macrophages exhibit a wide range of polarization states. Classically activated macrophages (M1) are characterized by the production of pro-inflammatory cytokines such as TNF- α , IL-1 β and high levels of reactive oxygen [12]. These cytokines have been shown to lead to granuloma and fibrous encapsulation resulting in chronic inflammatory and failure of the implants interaction [13]. Alternatively, activated macrophages (M2) secrete anti-inflammatory cytokines like IL-10, CCL18 and TGF- β et al. [12], such cytokines favor the integration biomaterials by inhibiting fibrous tissue formation [18]. Meanwhile, macrophages are suggested to be involved in bone formation [14], where a switch of M1 into M2 macrophages was observed during the natural bone healing process [15]. Further, macrophages have been shown to have a potential role in ectopic bone formation. When macrophages were selectively depleted

in a mouse model of heterotopic ossification, osteogenesis was significantly blocked [16]. By inhibiting macrophage formation with liposome-encapsulated clodronate CaP-instructed bone formation following subcutaneous implantation in FVB mouse was blocked [17].

The natural inflammatory response is a major regulator of vascularization through the secretion of cytokines from different types of macrophages and the cytokines they secrete [18]. More precisely, macrophages polarized towards an M2 phenotype have been shown to elicit a higher angiogenic potential compared to other subsets *in vivo* [19]. Following an acute inflammatory response, the formation of new blood vessels and microvasculature from pre-existing blood vessels occurs. Angiogenesis has been shown to be crucial for the successful tissue repair and regeneration [20,21]. For instance, bone occurring in nature always starts after from capillaries formation [22], indicating angiogenesis a prerequisite process for bone formation and successful osteo-integration during bone healing. Studies have demonstrated that angiogenesis predominantly occurs before the onset of osteogenesis, and it was suggested that the newly formed blood vessels not only transport oxygen, nutrients and waste, but also steadily supply osteoblast precursors for bone formation [23,24]

It has been shown that the physicochemical properties of materials significantly influence macrophage responses [25], although the molecular mechanisms are not completely understood [11]. For example, Chen et al. reported that topography, independent of surface chemistry, affected macrophage morphology and cytokine secretion *in vitro* and *in vivo* [26]. Fellah et al. found that the number of macrophages changed with the surface grain size of CaP materials *in vivo* [27]. Furthermore, Davison et al. reported that submicron scaled surface topography of CaP material could induce an inflammatory response of macrophages, which subsequently secreted cytokines that instructed osteogenic differentiation of mesenchymal stem cells (MSCs) [28].

The osteogenic differentiation of MSCs is considered to be a key step in CaP-instructed bone formation following implantation in non-osseous

sites [29-31]. However, *in vivo*, other biological processes must take place prior to osteogenic differentiation of MSCs, because CaP-instructed bone formation in non-osseous sites does not occur immediately but weeks after [3-7].

The available knowledge indicated links between physicochemical properties, macrophage phenotypes, angiogenesis and bone formation, and it is reasonable to speculate that CaP-instructed bone formation follows a cascade of 1) the shifting of macrophage phenotype from M0 to the anti-inflammatory M2 phenotype; 2) enhancing of angiogenesis by the angiogenic cytokines secreted by M2 macrophages; 3) ensuring steady transport of MSCs from bone marrow to implantation sites with the enhanced angiogenesis; 4) finally MSCs respond to specific surface topography of CaPs to undergo osteogenic differentiation to form bone. We hereby conducted studies to demonstrate the possible links between submicron surface structure, macrophage phenotype, angiogenesis and bone formation. To test the hypothesis, an osteoinductive tricalcium phosphate with submicron surface topography (TCP-S, positive control) and a non-osteoinductive tricalcium phosphate material with micron-scale surface topography (TCP-B, negative control) were employed to investigate the effect of their surface microstructure on macrophage polarization, angiogenesis and bone forming ability. An angiogenesis inhibitor (KRN633) was applied to further evaluate the possible roles of angiogenesis in material-driven osteoinduction.

2. MATERIALS AND METHODS

2.1. Preparation and characterization of CaP materials

Two materials (osteoinductive TCP-S and non-osteoinductive TCP-B) were prepared in porous disc forms ($\varnothing 9 \times 2$ mm for *in vitro* and $\varnothing 9 \times 6$ mm for *in vivo*) and characterized as described elsewhere [5,6] and sterilized with gamma irradiation (dose 25–40 kGy, Isotron Netherlands B.V., Ede, The Netherlands) prior to use. The crystal chemistry of the materials was determined by X-ray diffraction (XRD; Rigaku Miniflex II Japan) scanning the range $2\theta = 25 - 45^\circ$ (step size = 0.01° , rate = 1° min^{-1}). The surface structure was analyzed using scanning electron

microscopy (SEM, JEOL JSM-5600, JEOL Ltd, Tokyo, Japan), and the grain size ($n = 50$) was subsequently measured with AxioVision LE (Carl Zeiss MicroImaging, Inc., Breda, The Netherlands) from SEM images taken at the magnification of 5000 $\times$. The microporosity (i.e. the volume percentage of pores smaller than 10 μm in the material) and total pore area of the materials were determined by mercury intrusion testing ($n = 1$, micromeritics Instrument Incorporation, USA).

2.2. Evaluation *in vitro*

2.2.1. Expansion of monocytes and differentiation into M0 macrophage

Monocytes, of the human monocytic cell line (THP-1, ATCC, Manassas, USA), were cultured in RPMI-1640 media (Grand Island, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, USA) and 100 U/mL penicillin-streptomycin (P/S, Gibco, Grand Island, USA) at 37 °C, 5% CO₂ in a 95% humidified atmosphere for expansion with refreshment media twice a week. Then, 1×10^6 THP-1 cells were plated in 6-well plates in 3 mL RPMI media containing 25 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) and cultured for 48 hours to get macrophages (M0) according to established protocols [32].

2.2.2. Macrophage culture on material surface

To assess the influence of surface microstructure on macrophage polarization, 2.5×10^5 M0 macrophages suspended in 0.5 mL RPMI-1640 media were seeded onto TCP-S/TCP-B discs ($\phi 9 \times 2$ mm) in the 48-well suspension culture plates. In addition, 2.5×10^5 M0 macrophages were seeded on normal 48-well plates in 0.5 ml media either with an addition of 20 ng/ml interferon gamma (IFN- γ , PeproTech, London, UK) and 100 ng/ml lipopolysaccharide (LPS, PeproTech) to achieve M1 macrophages or with 20 ng/ml interleukin 4 (IL-4, PeproTech) and 20 ng/ml interleukin 13 (IL-13, PeproTech) to polarize M2 macrophages as previously described [33]. All plates were incubated at 37 °C, 5% CO₂ in a 95% humidified atmosphere for 4 day with refreshing media every 2 days. The sample size was 5 ($n=5$) per condition.

To assess the morphology of cells, samples after a 4-day culture were rinsed twice with PBS and subsequently fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffered solution for 15 minutes, then washed twice with cacodylate buffered solution and dehydrated in a graded ethanol series, finally dried with tetramethylsilane and sputter coated with gold for SEM observations. Macrophage size was quantified with SEM micrographs (magnification, 100 $\times$) by calculating the mean surface area of cells at 3 random locations per CaP disc using automated threshold, edge detection, and particle analysis functions in ImageJ software, as previously described [34].

To identify the phenotype of the cells, the supernatant of cultures was evaluated at day 2 and day 4 with respect to the production of TNF- α , IL-1 β , TGF- β and CCL18, using corresponding ELISA assay kits following the instructions of the manufacturer with a multi-mode spectrophotometer (Biotek, USA). The total production of the cytokines was expressed as average $\pm$ SD. Meanwhile, DNA of cells at Day 4 was quantified with the QuantiFluor dsDNA system kit (Life Technologies, the Netherlands) after the samples were rinsed with PBS and underwent freeze and thaw cycles in 1 mL MilliQ water. The production of cytokines at day 4 was normalized to DNA and presented as average $\pm$ SD.

2.2.3. Angiogenesis evaluation in vitro

To investigate the possible influence of cytokines secreted by cells cultured on materials on angiogenesis, supernatant harvested from TCP-S/TCP-B at day 4 was mixed and used to culture human umbilical vein endothelial cells (HUVECs) using available protocols [35]. Briefly, HUVECs (passage 3, Mixed donors, Lonza) were expanded in T175 flasks coated with 0.2% gelatin and maintained in endothelial growth media (EGMTM-2, Clonetics, Lonza, Basel, Switzerland) for 1 week. Growth Factor Reduced (GFR) Basement Membrane Matrix (Matrigel, Corning, New York, USA) was thawed overnight at 4 °C to coat 15-well μ -Slide angiogenesis chambers (Ibidi, Planegg, Germany) (10 μ l/well) and incubated at 37 °C and 5% CO₂ for 1 hour. HUVECs were then seeded in μ -Slide angiogenesis chambers at 6,000 cells/well in either EGM-2

medium (positive control) or endothelial basal media (EBM, Lonza) (negative control), as well as TCP-S/TCP-B conditioned media. All μ -Slides were incubated in humidified atmosphere of 5% CO₂ at 37 °C for 8, 12 and 16 hours. At each interval, photomicrographs were made with a light microscope (Nikon Eclipse E200, Japan) under bright field at 4 × magnification to show cells, nascent and developed tubules. Angiogenesis parameters (e.g. number of meshes, junctions and segments, total segments length) were evaluated with images captured using ImageJ software [36]. Sample size was 4 per condition (n=4).

2.3 Evaluation *in vivo*

2.3.1. Intramuscular implantation

Under the permission of local animal care committee (Animal Center, Sichuan University, Chengdu, China), 16 adult male beagles (10 - 15 kg, n = 4 for per timepoint) were employed in the study for 1, 3, 6 and 12 weeks respectively. TCP-S and TCP-B were used as received, in order to evaluate the effect of angiogenesis on bone formation, an angiogenesis inhibitor (KRN633) was employed. Under aseptic conditions, 150 μ g KRN633 dissolved in 250 μ l DMSO was loaded on each TCP-S, followed by vacuum evaporation of DMSO. The three tested samples were implanted in the paraspinal muscles. Surgeries were conducted under sterile conditions and general anesthesia by intravenous injection of sodium pentobarbital (30 mg k⁻¹). Following the surgeries, buprenorphine (0.1 mg per animal) was given to the animals intramuscularly as pain relief for 2 days, while penicillin (40 mg kg⁻¹) was injected intramuscularly for 3 consecutive days to prevent infection. After operation, the animals were allowed to bear their full weight and received a normal diet.

2.3.2. Sample harvesting

At the end point, animals were anesthetized with intravenous injection of sodium pentobarbital (30 mg kg⁻¹) and following an intravenous irrigation of Chinese ink to monitor blood vessels formation in the implants until the skin of abdomen became dark. Then the animals were sacrificed with overdose of pentobarbital sodium and the implants were harvested with

the surrounding tissues. The samples for 1 and 3 weeks were cut into two parts, with one part fixed in 10% formalin for histology and one part stored in liquid nitrogen for the biochemical assays of TNF- α and IL-10. The samples for week 6 and week 12 were fixed in 10% formalin for histological evaluation.

2.3.3. Bioassays

After trimming the surrounding tissues, the samples for bioassays were ground to powder in the presence of liquid nitrogen, collected to 10 mL centrifugal tubes and weighed. Then RIPA buffer containing proteinase inhibitors was added at a ratio of 2.5 mL buffer/ 200 mg sample, mixed with vortex and subjected to 3 freeze/thaw cycles at -20 °C with vortex in between. Finally, lysates were obtained after centrifugation at 4000 rpm, 4 °C for 10 minutes. TNF- α (Elisa assay kit, R&D system) and IL-10 (Elisa assay kit, R&D system) were measured according to the instructions for the ELISA assay kit. The data were normalized to the mass of the samples (n=4 per sample per time point).

2.3.4. Histology and histomorphometry

The samples for histological evaluation were fixed in 10% formalin for one week, and then embedded in methyl methacrylate (MMA, K-plast, LTI Netherland, Bilthoven, the Netherlands) after dehydration in a series of gradient ethanol solutions. Serial non-decalcified sections with an average thickness of 15 μ m were obtained using a microtome (SP-1600, Leica, Germany) equipped with diamond saw blade, and stained with 1% methylene blue (Sigma-Aldrich) and 0.3% basic fuchsin (Sigma-Aldrich) for light microscopy. Non-stained slides were prepared to view and quantify blood vessels. Meanwhile, sections were prepared from MMA-embedded material blocks (not implanted) to be used as controls.

The histological sections were scanned using a slide scanner (Dimage Scan Elite 5400II, Konica Minolta, Tokyo, Japan) for gross evaluation and histomorphometric analysis. Histomorphometry of *in vivo* bone, blood vessel formation and CaP resorption was performed by pseudo-coloring pixels representing bone (B), blood vessel (BV) and remaining material

(Me) in the region of interest (ROI) using Adobe Photoshop Elements 4.0 software. The area percentage of bone (B%) and blood vessel in the available space were calculated as $B\% = B \times 100 / (\text{ROI-Me})$ and $V\% = V \times 100 / (\text{ROI-Me})$ respectively. The area percent of material resorbed (M%) was calculated as $M\% = (M_0 - \text{Me}) \times 100 / M_0$, where M_0 was the area of CaP materials that were not implanted.

2.4. Statistical analysis

Statistical comparisons were performed using One-way ANOVA and Tukey's post hoc tests; p values of <0.05 were considered to indicate a statistically significant difference.

3. RESULTS

3.1 Physicochemical properties of the materials

The X-ray diffractometry patterns of the two materials are given in figure 1A, which shows the two materials had the same β -TCP chemistry. SEM images revealed that the TCP-S surface topography consisted of smaller grains and smaller micropores compared to TCP-B (Figure 1B). Quantitatively, TCP-S had a grain size of $0.84 \pm 0.29 \mu\text{m}$, while TCP-B

Table 1. *Physicochemical properties of the calcium phosphate material implants.*

Materials		TCP-S	TCP-B
Chemistry ^a		β -TCP	β -TCP
Grain size (μm) ^b		0.84 ± 0.29	2.3 ± 0.71
porosity ^c	Total porosity	75%	72%
	Microporosity (<10 μm)	24%	22%
	Macroporosity ($\geq 10\mu\text{m}$)	51%	50%
Micropore size range (μm) ^c		0.2 - 0.7	0.6 - 2.8
Specific surface area (m^2/g) ^c		1.6	0.6

^a as determined via X-ray diffractometry;

^b as determined via quantitative measurements on scanning microscopic images (5000 $\times$);

^c as determined via mercury intrusion;

displayed larger grains with $2.30 \pm 0.71 \mu\text{m}$ (Figure 1C). Mercury intrusion data revealed that the total porosity, strut porosity and microporosity were similar between the two materials (Table 1). The differences in micropore size observed via SEM were confirmed by the distribution curves (Figure 1D), which indicated a micropore range of $0.2 - 0.7 \mu\text{m}$ with the peak at $0.45 \mu\text{m}$ for TCP-S, while the micropores in TCP-B ranged from $0.5 - 2.8 \mu\text{m}$, with the peak at $2.1 \mu\text{m}$. In addition, TCP-S had higher specific surface area than TCP-B (Table 1).

Figure 1. *A:* XRD pattern of TCP-S and TCP-B showing that the two materials have same chemistry; *B:* SEM images showing TCP-S and TCP-B had either

submicron or micron scaled surface structure; *C*: Mercury intrusion indicating distribution plots of the submicron- and micro-pore dimension of both materials; *D*: TCP-S and TCP-B had either submicron or micron scaled grains (* $p < 0.05$).

Figure 2. SEM micrographs of the morphology of macrophages cultured on (A-C) TCP-S and (D-F) TCP-B after 4 days culture. At low magnification, macrophages formed on (A) TCP-S are larger than those formed on (D) TCP-B.

Prominent cellular filopodia connected adjacent cells and attached to the surface on (B) TCP-S versus (E) TCP-B. At high magnification, (C) macrophages appeared tightly bound to TCP-S, with spread cell membranes decorated with active fusion; (F) macrophage on TCP-B appeared rounded with less frequent fusion. (G) Osteoclast size after 4 days culture calculated using ImageJ software ($p < 0.05$).*

3.2 Evaluation *in vitro*

3.2.1 Cell morphology on material surface

After a 4-day culture, cells seeded on TCP-S were prominently larger, with more connections in a highly networked cell population (Figure 2A). In contrast, cells were smaller in size on TCP-B and fused but to a far less extent (Figure 2B). Cell membranes were tightly integrated with TCP-S surface as seen at higher magnifications, and prominently fused each other (Figure 2C). Conversely, cells on TCP-B were far less connected by less frequent filopodia and appeared rounder with less integration to the material surface (Figure 2F). Quantitatively, the cells on TCP-S were twice as big as those on TCP-B (Figure 2G), with the size of $> 230 \mu\text{m}^2$ in some cases.

3.2.2 Cytokines secreted by the cells

As shown in figure 3, IFN- γ and LPS enhanced production of TNF- α and IL-1 β , while IL-4 and IL-13 promoted TGF- β and CCL18 production at both day 2 and day 4. Cells cultured on TCP-S produced significantly more TGF- β and CCL18 at both day 2 and day 4, while cells on TCP-B secreted significantly more TNF- α and IL-1 β at both time points (Figure 3). Not much difference was found with cell numbers between the groups at day 4 as shown by the DNA contents (Figure 4A), resulting in a similar trend for the cytokines were normalized by DNA contents (Figure 4B, C) to the total cytokines among the groups at day 4.

Figure 3. Quantification of anti-inflammatory cytokines TGF-β (A) and CCL18 (B), as well as pro-inflammatory cytokines TNF-α (C) and IL-1β (D) in supernatant harvested from THP-1 derived macrophages cultured on TCP-S/B for 2 and 4 days (*: $p < 0.05$).

Figure 4. Quantification of DNA (A) and pro-inflammatory cytokines $TNF-\alpha$ and $IL-1\beta$ (B), as wells as anti-pro-inflammatory cytokines $TGF-\beta$ and $CCL18$ (C) in supernatant harvested from THP-1 derived macrophages cultured on TCP-S/B for 4 days (*: $p<0.05$).

3.2.3 Effects of macrophage-conditioned media on angiogenesis in vitro

As shown in Figure 5, 8 hours after culture, no mesh was formed in any groups tested; 12 hours after culture, a few tubular networks were formed in positive control and TCP-S group; 16 hours after culture, the tubular network increased to form meshes in positive control and TCP-S group, tubular network was not often detected in negative control or TCP-B group.

Figure 5. Representative phase-contrast images of tubular network on Matrigel formed by HUVECs with conditioned medium at 8, 12 and 16 hours after seeding (magnification: 40 $\times$).

Quantitative assays of angiogenesis parameters, the number and length of segments increased with time, and significantly more and longer segments were presented in the positive control and TCP-S as compared to the negative control and TCP-B at all timepoints (Figure 6A, B), resulting significantly more segment junctions and finally mesh formation in positive control and TCP-S with no mesh in either negative control or TCP-B group (Figure 6C,D).

Figure 6. Quantitative analysis of specific parameters of capillary tube formation with Angiogenesis Analyzer (ImageJ) on harvested images at three intervals: (A) number of segments; (B) total segments length; (C) number of junctions; (D) number of meshes ($p < 0.05$). (Image Analysis with the Angiogenesis Analyzer Module of ImageJ (* $p < 0.05$)).

3.3 Evaluation *in vivo*

3.3.1 $TNF-\alpha$ and $IL-10$ at week 1 and week 3

Both $TNF-\alpha$ and $IL-10$ were detected in all implants at both week 1 and

Figure 7. Biochemical assays of IL-10 (A), TNF- α (B) *in vivo*. Significant differences were shown only between the materials at each endpoint with * ($p < 0.05$).

week 3 (Figure 7). Significantly higher levels of IL-10 were seen in TCP-S implants as compared to TCP-B implants at both timepoints, while no difference in TNF- α was detected between TCP-S and TCP-B at both week 1 and week 3.

3.3.2. Angiogenesis and bone formation in TCP-S and TCP-B

One week after implantation, the macropores of the implants were filled with loose fibrous tissue and clotted blood cells. Blood vessels, bone

Figure 8. Histological overview and detailed images of TCP-S (a, b, c), TCP-S+KRN633 (d, e, f) and TCP-B (g, h, i) after 3 weeks (undecalcified sections without staining) (M: material; ST: soft tissue; red arrow: blood vessel).

tissue and material resorption were not observed at this time (figures were not shown). Three weeks after implantation, all explants were

encapsulated by a thin layer of connective tissue, whilst the implants performed differently with respect to vascularization, with the most vessels in TCP-S implants and the least in TCP-B (Figure 8). Abundant blood vessels were detected in all TCP-S implants, as shown by Chinese ink in black in the non-stained sections (Figure 8). Quantitatively, by area percentage, TCP-S had $3.85 \pm 0.34\%$ vessels in available space, TCP-S+KRN633 had $0.97 \pm 0.18\%$ and TCP-B had $0.50 \pm 0.14\%$. At week 3, no bone was formed in any of the implants (Figure 11A).

Figure 9. Histological overview and detailed images of TCP-S (a, b, c), TCP-S+KRN633 (d, e, f) and TCP-B (g, h, i) after 6 weeks (undecalcified sections stained with methylene blue and basic fuchsin) (M: material; B: mineralized bone; O: osteoid; red arrow: blood vessel; black arrow: osteoblast; yellow arrow: osteocyte; ST: soft tissue).

Six weeks after implantation, the proportion of blood vessels increased in TCP-S/KRN633 compared to week 3 (from $0.97 \pm 0.18\%$ to $1.67 \pm$

0.30%), but the proportion of blood vessels in both TCP-S and TCP-B did not increase. While TCP-S had the most proportion of blood vessels, followed by TCP-S/KRN633 and TCP-B (Figure 11A). Bone formation was found in all TCP-S implants. Bone was identified with the presence of newly formed bone matrix, immature osteocytes and osteoblasts (Figure 9). By area percentage, $8.43 \pm 1.45\%$ available space was occupied by newly formed bone. In contrast, no bone but soft tissue was formed in the TCP-S+KRN633 and TCP-B implants (Figure 9).

Figure 10. Histological overview and detailed images of TCP-S (a, b, c), TCP-S+KRN633 (d, e, f) and TCP-B (g, h, i) after 12 weeks (undecalcified sections stained with methylene blue and basic fuchsin) (M: material; B: mineralized bone; O: osteoid; red arrow: blood vessel; black arrow: osteoblast; yellow arrow: osteocyte; yellow star: giant cell phagocytizing material fragments).

After twelve weeks, the proportion of blood vessels in the implants was maintained in all groups. TCP-S still had the most blood vessels and TCP-

B presented the least blood vessels (Figure 11A). The majority of the macropores in TCP-S was occupied with mature bone with osteocytes embedded in mineralized bone matrix (Figure 10) and the amount of bone increased to $25.06 \pm 5.21\%$ in the available space. Bone formation had initiated in TCP-S/KRN implants at this time, with both immature bone matrix and mineralized bone in the macropores (Figure 10). However, the amount of bone in TCP-S/KRN was significantly less as compared to TCP-S at this moment. No bone was formed in any TCP-B implants (Figure 10).

The presence of foreign body giant cells and CaP debris in histological sections (Figure 10) indicated the materials were undergoing cell-mediated resorption. The resorption rates significantly varied between the groups and with the implantation time. TCP-S underwent the most material resorption, followed by TCP-S/KRN633 and TCP-B at each time point (Figure 11).

Figure 11. Quantitative histomorphometry of blood vessel (A), mineralized bone formation (B) in available space and resorption rate of the materials (C) during implantation time (*: $p < 0.05$).

4. DISCUSSION

In a classical intramuscular model for evaluating osteoinduction, a TCP material composed of submicron scaled grains and pores was found to potently induced blood vessel formation, along with subsequently ectopic bone formation likely by tuning macrophage phenotype. Whereas an equivalent TCP material with micron scaled topographical features stimulated limited blood vessels and failed to trigger ectopic bone formation. These arouse a potential link between submicron-scaled surface topography, macrophage phenotype, angiogenesis and inductive bone formation.

The determination of angiogenesis observed in TCP-S and TCP-B *in vivo* sheds lighter on how submicron scaled surface topography can induce ectopic bone formation. *De novo* bone formation started in TCP-S at week 6 (Figure 9), whereas abundant blood vessels were already detected in TCP-S at week 3 (Figure 8), indicating that angiogenesis precedes bone formation in TCP-S. In contrast, it's counterpart TCP-B induced sparse blood vessels at week 3 and no bone formation could be seen until week 12 (Figure 8, Figure 11A). These results suggest the important role of angiogenesis in material-driven inductive bone formation. This finding was further confirmed when using the anti-angiogenic reagent KRN633 in TCP-S discs. As a selective inhibitor of VEGF receptor-2, KRN633 has been shown to suppress tumor angiogenesis and growth [37,38]. Similarly, KRN633 treatment impeded blood vessel formation and subsequently *de novo* bone formation compared with TCP-S (Figure 8, 9, 10, 11).

The quantification of macrophage markers *in vivo* shows a possible link between surface topography and angiogenesis in TCP-S/TCP-B implants. At 1 week after implantation, macrophages have been recruited to the implants as indicated by the presence of TNF- α and IL-10 cytokines (Figure 7). TNF- α is generally seen as a marker typical for M1

macrophages, but certain M2 subtypes are also known to secrete TNF- α in conjunction with IL-10 [25]. IL-10 is a specific cytokine secreted by M2 macrophages [12]. The significantly higher production of IL-10 in TCP-S implants, likely indicating a major presence of macrophages recruited in TCP-S are M2 phenotype macrophages. The earlier presence of M2 macrophage (week 1) in TCP-S implants than the occurrence of blood vessel (week 3), implies the important role of M2 macrophage, i.e. M2 macrophages likely play an important role in angiogenesis in submicron surface structured TCP-S implants. In contrast, TCP-B recruited less M2 macrophages and got less blood vessels formation.

The coupling between macrophage phenotype and angiogenesis observed *in vivo* was further confirmed *in vitro* using additional M1/M2 stimulators. With the presence of PMA, TPH-1 monocytes were differentiated into M0 macrophages, which responded to the specific submicron surface topography of TCP-S to polarize into M2 macrophages *in vitro* and produced significantly more TGF- β and CCL18 (Figure 3A, B; Figure 4B). Subsequently the condition media harvested from M0 macrophages cultured on TCP-S enhanced angiogenesis *in vitro* as the angiogenic agents did (Figure 5). Indeed, studies have indicated that an increasing ratio of M2/M1 macrophages is associated with improved biomaterial vascularization [39,40]. TGF- β and CCL18, which are produced at high levels by M2 macrophages, have previously been shown to induce endothelial sprouting *in vitro* and promote neovascularization *in vivo* [41,42]. It has been well established that TGF- β facilitates angiogenesis *in vitro* and *in vivo* through an autocrine or paracrine activation of VEGFR2 by VEGF [43,44]. CCL18 has been shown to enhance HUVECs migration and proliferation *in vitro* and promoted angiogenesis *in vitro* and *in vivo* as well [41].

In addition, the cytokines secreted by M2 macrophages have been shown to direct osteogenic differentiation of MSCs to form bone [45]. However, in this study, bone formation occurred several weeks (week 6) after M2 macrophage formation (week 1). This suggests that M2 macrophage-related cytokines play roles in material-driven inductive bone formation

may not be through directing osteogenic differentiation of MSCs, but more likely via angiogenesis in advance.

Generally, the implantation of CaP materials triggered the inflammation with the infiltration of monocyte into the implants and the formation of macrophages (e.g. M0). The recruited macrophages may anchor to the surface structure of the implant and responded to the submicron surface topography to differentiate into M2 macrophages. The cytokines secreted by M2 macrophages then enhanced angiogenesis and led to formation of a better developed blood vessel system, which not only provides the nutrition [20,21] but also transports cytokines and cells to the implants, including BMSCs derived from bone marrow for ectopic bone formation [46]. The BMSCs infiltrated into implants via blood vessel system may respond to submicron scaled surface topography to undergo osteogenic differentiation [47] and form eventually bone.

Still, how exactly surface topography may stimulate macrophage differentiation can currently only be speculated. Other groups have demonstrated that surface features physically tailor macrophage morphology and cytokine secretion profiles accordingly [48,49]. For example, macrophages that polarized toward different phenotypes *in vitro* exhibited dramatic changes in cell shape [50]. The similar phenomenon was reported by Davison et al. who observed that osteoclasts formed on TCP-S were larger than those formed on TCP-B [28]. It appeared that macrophage polarization by changing cell shape, associated with various surface topographies, through morphological adaption. For example, Dupont et al. suggested that alterations of cell shape may directly affect the transcription of M1 or M2 gene programs through mechanical signals on transcription factor activity [51]. Meanwhile, studies showed that cellular enlargement affected nuclear organization, chromatin condensation, and histone modification [52,53], which could have an influence on the genetic programs associated with macrophage polarization. In this study, macrophages seeded on TCP-S were larger and connected, while those on TCP-B appeared rounder with less frequent connection (Figure 2). It is possible that the submicron

scaled surface topography of TCP-S may have enlarged the seeded macrophages and induced them to polarize into M2 phenotype, while macrophages on TCP-B appeared isolated with round morphology and differentiated into M1 macrophages.

Taken together, the *in vivo* and *in vitro* findings presented here show the possible links between surface topography, macrophage polarization, angiogenesis and material-driven inductive bone formation. It is likely that the submicron scaled surface topography tuned the immune response shifting from M0 to anti-inflammation phenotypes (M2), which subsequently secreted the specific cytokines facilitating angiogenesis, the formed blood vessels ensured steady transport of MSCs for osteogenic differentiation towards ectopic bone formation.

The submicron scaled surface topography not only induced M2 macrophage differentiation which enhanced angiogenesis and ultimately inductive bone formation, but also facilitated macrophages fusion into foreign body giant cells (FBGCs) [54], which could secrete cytokines having osteogenic ability [55]. More relevant, it was reported that prior to material-driven inductive bone formation, osteoclast like FBGCs had been present on the surface of implants following non-osseous implantation [56]. Moreover, animal dependence of material-driven osteoinduction was linked to the FBGCs formation *in vivo*. For example, when a porous calcium-deficient hydroxyapatite induced bone formation in dogs numerous TRAP-positive multinucleated cells were detected, in contrast, only a small number of TRAP-positive cells were detected in the same material in rats and no bone formation was observed [57].

Additionally, the formed FBGCs not only affect inductive bone formation, but are also involved in cell-mediated material resorption through phagocytosis [53]. In this study, FBGCs were observed predominantly on the surface of TCP-S implants, while less FBGCs were detected on surface of TCP-S+KRN633, and hardly seen on TCP-B. Consequently (figure 10), TCP-S underwent the most material resorption, followed by TCP-S+KRN633 and TCP-B (Figure 11).

The data presented in this study give a potential coupling between surface topography, macrophage phenotype, angiogenesis and ectopic bone formation, however, the exact processes between surface topography and macrophage state is unknown and needs to be explored.

5. CONCLUSION

A CaP material with submicron scaled surface topography was found to promote differentiation of M2 macrophages which secreted cytokines favoring angiogenesis *in vitro* and to enhance blood vessels formation *in vivo*. Meanwhile, the importance of angiogenesis in ectopic bone formation was seen. The couplings between surface topography, macrophage phenotype, angiogenesis and ectopic bone formation in material implants suggest a biological pathway of material-instructed bone formation.

A C K N O W L E D G E M E N T S

The authors would like to thank the Rapid Prototyping of Custom-Made Bone-Forming Tissue Engineering Constructs (RAPIDOS Project, Ref. NMP-2013-EU-China 604517), the Seventh Framework Programme of the European Union (REBORNE under grant agreement no. 241879) and the Horizon 2020 Framework Programme (CHARME, grant agreement no. 674282) for their financial support to this research project.

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CHAPTER 6

CHAPTER 7

Accelerated bone formation by biphasic calcium phosphate with a novel submicron surface topography

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A B S T R A C T

Osteoinductive calcium phosphate (CaP) bone grafts have been shown to have equivalent performance to autograft in repairing critical-sized bone defects. The osteoinductive potential of CaP has been linked to the size of surface topographical features. We performed a study in which two novel biphasic calcium phosphate (BCP) bone grafts were synthesized with either submicron ($\text{BCP}_{<\mu\text{m}}$) or micron-scale ($\text{BCP}_{\mu\text{m}}$) needle-shaped surface topography and compared to dimensionally similar tricalcium phosphate with grain-shaped surface structures ($\text{TCP}_{<\mu\text{m}}$ and $\text{TCP}_{\mu\text{m}}$). To clarify the possible function of surface morphology (needle-like vs. grain-like) in initiating bone formation, the four CaP test materials were physicochemically characterized and implanted in the dorsal muscle of beagles for 12 weeks. The submicron needle-shaped topography of $\text{BCP}_{<\mu\text{m}}$ triggered earlier bone formation (3 - 6 weeks) compared to the grain-shaped surface topography of $\text{TCP}_{<\mu\text{m}}$ which formed bone at 6 - 9 weeks. After 12 weeks, the amount of induced bone formation in both materials was equivalent based on histomorphometry. The micron-sized needle-shaped surface topography of $\text{BCP}_{\mu\text{m}}$ led to limited formation of new bone tissue, whereas its counterpart, $\text{TCP}_{\mu\text{m}}$ with grain-shaped surface topography, failed to trigger *de novo* bone formation. We found that the relative strength of effect on CaP-driven bone induction is as follows: surface feature size > surface feature morphology > substrate chemistry. BCP materials with needle-shaped submicron surface topography gave rise to accelerated bone formation and a slower rate of resorption than a comparable TCP in this intramuscular implantation model. These characteristics may translate to improved bone healing in orthotopic defects.

1. INTRODUCTION

Owing to their similarity to the inorganic component of bone, their bioactivity, biocompatibility and osteoconductivity, calcium phosphate (CaP) materials are used clinically as bone void fillers [1,2]. However, because they have historically lacked osteoinductivity, their efficacy has been perceived as being lower than that of the gold standard bone graft, autologous bone, in repairing critical-sized bone defects [3,4].

Unless osteogenic agents were added to the graft material pre-implantation, osteoinduction of CaP bone grafts on their own was not reported until the beginning of the 1990s. Since then, initiation of bone formation by various CaP bone grafts with specific physicochemical properties has been observed in studies of non-skeletal defect sites (e.g. subcutis and muscle) [5-8].

Studies comparing osteoinductive versus non-osteoinductive CaP in orthotopic skeletal sites have demonstrated the benefits of osteoinductivity in bone regeneration [9]. Osteoinductive CaP bone grafts not only trigger ectopic bone formation, but also enhance orthotopic bone formation via inducing osteogenic differentiation of mesenchymal stem cells (MSCs)¹⁰. One such osteoinductive CaP bone graft substitute has been shown to have equivalent performance to autograft, considered the gold standard bone graft, in repairing critical-sized bone defects [11]. Having now established a link between osteoinductivity and bone-forming potential in orthotopic sites, researchers are focused on developing osteoinductive CaP bone grafts that can trigger high quality bone formation as early as possible after implantation.

It has been proven that osteoinduction by CaP bone grafts is material-dependent, and the osteoinductive potential varies with their physicochemical properties [12,13], such as chemistry (i.e. the ratio HA/TCP) [14], microporosity (i.e. the volume percentage of pores < 10 μm in the material) [15,16], surface architecture and geometry [10,17,18]. Among them, the presence of a microporous structure has been shown as essential. For example, one study demonstrated that microporous

hydroxyapatite (HA) induced ectopic bone formation, while a non-microporous HA did not [19]. Furthermore, it has been shown that reducing the size of features on a microporous surface is critical to endowing CaP materials with osteoinductive properties. For instance, submicron scale surface structured TCP could induce ectopic bone formation, while an equivalent but micron scale surface structured alternative failed [10,20]. The influence of surface feature size was also seen in biphasic calcium phosphates (BCP), where materials with smaller grain crystals initiated ectopic bone formation, while materials with larger structures did not [18,21]. This research confirms that the size of surface features plays an important role in initiating bone formation by CaP materials in ectopic defect sites.

Nowadays, there is still limited knowledge regarding the molecular mechanism underlying surface topography induced differentiation of MSCs. It has been reported that geometrical features can enhance the actomyosin contractility and facilitate osteogenesis via enhancement of c-Jun N-terminal kinase and with the activation of the extracellular related kinase in conjunction with elevated wingless-type (Wnt) signaling [22]. Furthermore, Zhang et al. reported that the surface topography of CaPs can tune MSCs with respect to morphology, primary cilia length and TGF β R recruitment to the cilium, all of which have been associated with osteogenic differentiation *in vitro* and bone formation *in vivo* [23].

Next to the size of features on the surface structure, the morphology is another characterization of surface topography which has been reported to evoke specific cellular responses *in vitro* [24-28]. For example, Kolhar et al. found that rod-shaped nanoparticles had higher affinity with endothelial cells in *in vitro* and *in vivo* experiments compared with their spherical counterparts[24]. Similarly, Agarwal et al. reported that elongated nanoparticles exhibited higher efficiency for the adhesion of cells and facilitated the multivalent interaction between cells and surface, compared to spherical nanoparticles [25]. Dasgupta and co-workers observed that the elongated particles with higher aspect ratio had extensive cellular uptake compared to spherical particles with the

average diameter [26]. Furthermore, *in vitro* studies have indicated that surface morphology changes modulate adhesion, cytokine release, and gene expression of osteogenic cells [27,28]. Based on the data above, we hypothesize that, besides the surface feature size, the morphology of the surface structure may play a crucial role in triggering bone formation in CaP materials.

We investigated the relative influence of surface feature size, surface morphology and substrate chemistry on osteoinductivity of CaP bone grafts by employing a hydrothermal treatment to obtain two needle-shaped surface structures on BCP at submicron and micron scale, and by comparing their *in vivo* bone-forming potential to submicron and micron grain-shaped TCP counterparts.

2. MATERIALS AND METHODS

2.1. Engineering the surface morphology

Submicron grain-like-surface-structured osteoinductive CaP (TCP_{<μm}, positive control) and micron grain-like-surface-structured non-osteoinductive CaP (TCP_{μm}, negative control) were prepared in granular format (1 - 2 mm) as previously described [9,10].

The two novel CaP materials were manufactured by Kuros Biosciences B.V. (the Netherlands). Briefly, BCP powder was synthesized using a wet precipitation of apatite powder, followed by foaming with H₂O₂ (1 % in distilled water, Merck, Darmstadt, Germany), and sintering at either 1125 °C or 1200 °C for 6 h respectively to obtain BCP plaques. After crushing the plaques and sieving, BCP granules (1 - 2 mm) were then prepared and autoclaved at 135 °C for 99 min to form novel CaP materials with either submicron (BCP_{<μm}) or micron scale (BCP_{μm}) surface topography. All materials were sterilized with gamma irradiation (dose 25 - 40 kGy, Isotron Nederland B.V., Ede, the Netherlands) prior to use.

2.2. Physicochemical characterization of CaP materials

The chemical composition of the CaP materials (n=1 per material) was identified by X-ray diffraction (XRD; Miniflex II, Rigaku, Tokyo, Japan) using a scanning range of 25 - 45 deg., step size of 0.01 deg., and a

scanning rate of 1 deg. per min. The amount of HA phase was determined by using an internal calibration system.

The surface structure of the CaP materials was analyzed with scanning electron microscope (SEM; JEOL JSM-5600, JEOL Ltd, Tokyo, Japan). The surface grain size (i.e. the vertical length crossing the center of each grain) and the shortest axis of the needles were measured with AxioVision LE (Carl Zeiss MicroImaging, Inc., Breda, the Netherlands) for at least 100 random grains or needles visualized from 10 SEM images (magnification: 5000×).

The microporosity and the specific surface area were determined with mercury intrusion porosimetry ($n=1$ per material; Micromeritics Autopore 9600 Mercury Porosimeter, Norcross, USA) with a mercury temperature of 18.36 °C, and Hg contact angle of (I) 130.00°, (E) 130.00°.

2.3. *In vitro* assay: dissolution rate

Calcium ion release was evaluated by soaking 0.5 mL of CaP granules ($n=5$ per material) in 100 mL of simulated physiological saline (SPS; 0.8 % NaCl, 50 mM HEPES, 0.4 mM NaN_3 ; 37 °C; pH = 7.3) solution for 200 min. While carefully stirring at 150 rpm to avoid the contact between the stirring bar and granules, the calcium ion concentration in SPS was recorded every minute using a calcium electrode (Metrohm, Herisau, Switzerland).

2.4. *In vitro* assay: protein adsorption

Protein adsorption was measured for two types of protein solutions. Sterile CaP granules (0.2 mL, $n = 5$ per material) were added to 2 mL of 1 % vol. fetal bovine serum (FBS) solution or 2 mL of 400 µg/mL bovine serum albumin (BSA) solution at 37 °C and 5 % CO_2 for 1 week. Protein adsorption was measured after 12 h, 1, 4 and 7 d using a micro BCA assay kit (Micro BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) and a spectrophotometer with an absorbance filter of 595 nm. The amount of proteins adsorbed by the samples (expressed in mg/mL for FBS, and in µg/mL for BSA) was estimated via internal calibration protein curves and

was then reported per 1 mL which corresponds to the volume of material implanted *in vivo*.

2.5. *In vivo* assay: intramuscular implantation

All surgeries were performed following ethical approval by the local animal care committee (i.e. the management committee of experimental animals of Sichuan province, China) and in accordance with the local laws and institutional guidelines. To evaluate their osteoinductive potential, the four CaP materials ($n = 8$ for each group) were implanted in the dorsal muscles of healthy beagles ($n = 8$, 2 - 4 years, 10 - 15 kg), for 12 weeks as described previously [9,10]. Briefly, under general anesthesia by intravenous injection of sodium pentobarbital (30 mg/kg body weight) and in sterile conditions, 1 mL of each test group was implanted into paraspinal muscle pockets created by blunt dissection. The pockets were kept isolated from each other to prevent contact between the samples. Subsequently, the muscle wound was closed with non-resorbable sutures and the skin incisions were closed layer by layer. Following the surgeries, buprenorphine (0.1 mg per animal) was injected intramuscularly for 2 d to relieve pain, while penicillin (40 mg/kg) was injected intramuscularly for 3 consecutive days to prevent infection. Animals were allowed to undertake full activity and received a normal diet immediately after surgery. To determine the time of onset for bone formation, we utilized three fluorescent histological labels to indicate osteogenesis at: 1 - 3 weeks (calcein; green staining); 3 - 6 weeks (xylenol orange; red staining), and 6 - 9 weeks (tetracycline; yellow staining). Fluorochromes were intravenously injected at 3 weeks (calcein, Sigma, Louis, USA, 5 mg/mL 2% NaHCO_3 solution, pH = 7.41, injection volume: 2 mL/kg body mass), 6 weeks (xylenol orange, Sigma, 50 mg/mL 1% NaHCO_3 solution, pH = 7.38, injection volume: 2 mL/kg body mass) and 9 weeks (tetracycline, Sigma, 10 mg/mL 0.9% NaCl solution, pH = 7.41, injection volume: 2 mL/kg body mass) after implantation.

2.6. *In vivo* assay: implants harvest and histological processing

Twelve weeks after implantation, animals were sacrificed by intravenous injection of an excessive dose of sodium pentobarbital and the implants

were harvested with their surrounding soft tissue, trimmed and then fixed in 4 % buffered formaldehyde solution (pH = 7.4) at 4 °C for at least 1 week. Following fixation, the samples were dehydrated using a series of gradient ethanol solutions and embedded in methyl methacrylate (MMA, K-plast, LTI, Bilthoven, the Netherlands). Consecutive histological sections (10 - 20 μ m) of non-decalcified samples were obtained by sectioning the whole samples using a diamond saw blade microtome (SP-1600, Leica, Wetzlar, Germany). The sections were alternately not stained and stained with 1 % methylene blue (Sigma-Aldrich, Louis, USA) and 0.3 % basic fuchsin (Sigma-Aldrich) after etching with acidic ethanol (Merck, Darmstadt, Germany). The stained sections were analyzed with light microscopy (Nikon Eclipse E200, Tokyo, Japan) and histomorphometry. Non-stained sections were used for fluorescence microscopy using a FITC Texas Red filter (Nikon, Tokyo, Japan) at bandpass mirror wavelengths of 510 - 555 nm and 585 - 665 nm.

2.7. *In vivo* assay: histology and histomorphometry

Stained histological sections were scanned with a slide scanner (Dimage Scan Elite 5400II, Konica Minolta Photo Imaging Inc, Tokyo, Japan) to obtain overview images for histomorphometric analysis (i.e. the area percentage of *de novo* bone formation and residual material). The amount of multinucleated osteoclast-like cells was counted from 40 randomly selected histological images (magnification 20 $\times$) and expressed as number of cells per mm². Histomorphometric analysis was performed using Adobe Photoshop Elements 4.0 software (CS5, v12, Adobe Systems Benelux BV, Amsterdam-Zuidoost, the Netherlands) as follows. First, the area encompassing the whole sample was selected as a region of interest and the corresponding number of pixels was read as ROI. Then the bone tissue and residual material areas were pseudo-colored, and their respective pixels were counted as B and Me respectively. The percentage of bone in the available space was determined as $B \% = B \cdot 100 / (ROI - Me)$, while the percentage of residual material was calculated as $Me \% = Me \cdot 100 / ROI$. The percent area of material resorbed (M %) was calculated as $M \% = (M0 - Me) \cdot 100 / M0$,

where M0 was the mean pixel area of similarly embedded, sectioned, and pseudo-colored materials (1 mL, n = 8) that had not been implanted.

Unstained histological sections were observed using fluorescence microscopy (Nikon Eclipse E600, Japan; camera Nikon FDX-35) to determine the time of onset of bone formation and material biomineralization. The incidence (for total animals operated) of three fluorescent labels in the explants was recorded, while the number of xylene orange staining spots (6 weeks, red color) in the samples was counted. A quantitative index (X_{fluor}), which indicates early bone formation (< 6 weeks), was calculated as the ratio between the total spots counted in all animals and the incidence of fluorescent labels.

2.8. Statistical analysis

Normality of the data was assessed using the Shapiro-Wilk test ($p > 0.05$ for normally distributed data), and statistical comparisons were carried out by one-way ANOVA and Tukey's post hoc tests, where a p-value smaller than 0.05 was considered statistically significant.

3. RESULTS

3.1. Physicochemical characterization of materials

X-ray diffraction analysis confirmed that the two novel CaP materials were BCP (i.e. BCP_{<μm}: 25 % HA/ 75 % TCP, BCP_{μm}: 22 % HA/ 78 % TCP), while both the positive and the negative controls were pure β-TCP (Figure 1a and Table 1).

SEM imaging revealed that the two novel CaP materials had either submicron (median, 0.60 μm) or micron-scale needles (median, 1.52 μm) on their surface, comprising an epitaxial polygon surface structure. Both TCP_{<μm} and TCP_{μm} possessed grain-shaped surface features, at the submicron scale for TCP_{<μm} (median, 0.70 μm) and micron-scale for TCP_{μm} (median, 1.96 μm) (Fig. 1b, 1c and Table 1).

Mercury intrusion porosimetry indicated that BCP_{<μm} and BCP_{μm} had lower microporosity (9.0 % and 5.1 % respectively), compared to TCP_{<μm} and TCP_{μm} (22.0% and 23.0 % respectively). By volume, TCP_{<μm} had a

slightly larger surface area ($2.02 \text{ m}^2/\text{mL}$) than $\text{BCP}_{<\mu\text{m}}$ ($1.78 \text{ m}^2/\text{mL}$), followed by $\text{TCP}_{\mu\text{m}}$ ($0.72 \text{ m}^2/\text{mL}$) and $\text{BCP}_{\mu\text{m}}$ ($0.65 \text{ m}^2/\text{mL}$) (Table 1).

Table 1. Physicochemical properties of the calcium phosphate ceramic implants.

Materials		$\text{BCP}_{<\mu\text{m}}$	$\text{TCP}_{<\mu\text{m}}$	$\text{BCP}_{\mu\text{m}}$	$\text{TCP}_{\mu\text{m}}$
Chemistry ^a		25 % HA/ 75 % TCP	0 % HA/ 100 % TCP	22 % HA/ 78 % TCP	0% HA/ 100% TCP
Grain/needle size (μm) ^b		0.33 - 0.90 Median 0.60	0.10 - 1.20 Median 0.70	1.01 - 2.28 Median 1.52	1.30 - 2.45 Median 1.96
Microporosity ($<10 \mu\text{m}$) _c		9.0%	22.0%	5.1%	23.0%
Specific surface area	by weight (m^2/g) ^c	2.77	1.71	1.21	0.79
	by volume (m^2/mL) ^c	1.78	2.02	0.65	0.72
Ca^{2+} ion released (ppm, after 200 min)		9.5 ± 0.8	18.0 ± 1.8	5.2 ± 0.9	11.1 ± 1.7
Proteins adsorbed after 7 d	BSA ($\mu\text{g}/\text{mL}$)	$1722.9 \pm$ 104.5	$1796.5 \pm$ 172.7	$786.3 \pm$ 48.1	$920.5 \pm$ 62.5
	FBS (mg/mL)	$114.9 \pm$ 3.7	$127.6 \pm$ 7.2	49.8 ± 4.1	60.6 ± 3.9

^a as determined via X-ray diffractometry;

^b as determined via quantitative measurements on scanning microscopic images (5000 $\times$);

^c as determined via mercury intrusion.

Figure 1. Physicochemical properties of CaP materials. **a:** XRD pattern showing the chemistry of the CaP materials; **b:** distribution plots of the dimension of shortest axis of the needle of BCP_{<μm} and BCP_{μm}; **c:** SEM images showing the surface structure of the CaP materials.

3.2. *In vitro* assays: dissolution rate

After soaking in SPS solution for 200 minutes, the amount of calcium ion released from the CaP materials into SPS solution varied between test groups. TCP_{<μm} released most calcium ions, followed by TCP_{μm}, BCP_{<μm} and BCP_{μm} (Fig. 2a and Table 1).

3.3. *In vitro* assays: protein adsorption

As shown in Figure 2b and 2c, BCP_{<μm} and TCP_{<μm} adsorbed similar amount of proteins from BSA but TCP_{<μm} absorbed more protein from FBS. Both TCP_{μm} and BCP_{μm} absorbed significantly less protein from BSA and FBS than their submicron counterpart test materials (Table 1).

Figure 2. *In vitro* evaluation of ion release and protein adsorption. **a:** calcium ion release from the materials into SPS over 200 min; **b, c:** percentage of proteins adsorbed from 2 mL 1 % FBS and 2 mL of 200 μg BSA ml⁻¹ solution onto 0.2 cc CaP granules for up to 7 d (**p* < 0.05).

3.4. Histology and histomorphometry

After 12 weeks intramuscular implantation, all explants were encapsulated by a thin layer of connective tissue which was also infiltrated into the CaP materials. *De novo* bone formation was observed in 8 out of 8 BCP_{<μm} and TCP_{<μm} (positive control) explants, 7 out of 8 BCP_{μm} explants

and 0 out of 8 TCP_{μm} (negative control) explants (Figure 3a, Table 2). As

Figure 3. Histological images of BCP_{μm} and TCP_{μm} after 12 weeks implantation. **a:** histological overviews showing the bone regeneration and material resorption of CaP materials; **b, c:** detailed histological images showing bone formation and remodeling as well as degradation of CaP substrates (non-decalcified sections stained with methylene blue and basic fuchsin, B: bone; M: material; black arrow: osteoblast; yellow arrow: osteocytes; red arrow: phagocytic cells).

Figure 4. Histological images of $BCP_{\mu m}$ and $TCP_{\mu m}$ after 12 weeks implantation. **a:** histological overviews showing the bone regeneration and material resorption of CaP materials; **b, c:** detailed histological images showing bone formation and remodeling as well as degradation of CaP substrates (non-decalcified sections stained with methylene blue and basic fuchsin, B: bone; M: material; black arrow: osteoblast; yellow arrow: osteocysts; red arrow: phagocytic cells).

shown in the detailed histological images (Figure 3b, 3c), osteoid with a seam of cuboidal osteoblasts lying on its outer surface as well as osteocytes entrapped in lacunae of lamellar and woven bone were clearly observed in $BCP_{<\mu m}$, $TCP_{<\mu m}$ and $BCP_{\mu m}$ explants. In addition,

multinucleated osteoclast-like cells phagocytizing small fragments of CaP were localized on the surface of the test CaP material for BCP_{<μm} (22 ± 7 cells/mm²) and TCP_{<μm} (14 ± 6 cells/mm²) groups. In contrast, the multinucleated osteoclast-like cells were sporadically present in BCP_{μm} (4 ± 3 cells/mm²), and scarcely present in any of the non-osteoinductive TCP_{μm} (0 cells/mm²) (Figure 4). More condensed collagen fibrils, bone marrow and blood vessels were observed in BCP_{<μm} and TCP_{<μm} implants, which were less compact in BCP_{μm}. Looser connective tissue, with very limited presence of blood vessels, was detected in TCP_{μm}.

Table 2. A summary of the explants from muscle of beagles after 12 weeks.

Explants		BCP _{<μm}	TCP _{<μm}	BCP _{μm}	TCP _{μm}
3 weeks (green color)	Calcein incidence	8/8	8/8	8/8	0/8
6 weeks (red color)	Xylenol orange incidence	5/8	2/8	0/8	0/8
	Σ(spots)	21	2	0	0
	X _{fluor}	4.2	1.0	0	0
9 weeks (yellow color)	Tetracycline incidence	8/8	8/8	0/8	0/8
12 weeks	Bone incidence	8/8	8/8	7/8	0/8
	Bone area in available space (B %)	24.5 ± 4.3	23.9 ± 6.3	2.1 ± 1.7	0
	Material resorption (%)	28.1 ± 4.1	53.8 ± 5.7	9.3 ± 2.4	4.7 ± 2.1

Under the fluorescent microscopy (Figure 5, Table 2), xylenol orange was detected in 5 out of 8 BCP_{<μm} and in 2 out of 8 of TCP_{<μm} explants. Calcein and tetracycline were observed in all the BCP_{<μm} and TCP_{<μm} explants. Only calcein was found in BCP_{μm} explants, and no fluorescent signal was seen in any of the TCP_{μm} explants. Xylenol orange was detected in the bone inside the BCP_{<μm} implants, indicating that the marked bone formed between 3- and 6-weeks post-implantation. Whereas, tetracycline was the predominant fluorophore observed in the bone formed in TCP_{<μm}, revealing that it formed between 6 and 9 weeks. No xylenol orange or tetracycline fluorophores were detected in BCP_{μm}, indicating that any

bone found in these defects was formed after 9 weeks post-implantation. These qualitative observations were corroborated by the quantitative data. In fact, the X_{fluor} index and the xylenol orange incidence (Table 2) indicate that much more bone was formed between week 3 and week 6 in $\text{BCP}_{<\mu\text{m}}$ as compared to the positive control ($\text{TCP}_{<\mu\text{m}}$). In sharp contrast, $\text{BCP}_{\mu\text{m}}$ and $\text{TCP}_{\mu\text{m}}$ showed no xylenol orange incidence (Table 2).

Figure 5. Fluorescent images showing the mineralization of materials and onset time of bone formation within CaP materials intramuscularly implanted in dogs for 12 weeks (Green, calcein, 3 weeks; Red, xylenol orange, 6 weeks; Yellow, tetracycline, 9 weeks; M: materials; B: bone; red arrow: bone tissue stained with xylenol orange; yellow arrow: bone tissue stained with tetracycline).

$\text{BCP}_{<\mu\text{m}}$ led to faster bone formation, but by the time of sacrifice at 12 weeks $\text{BCP}_{<\mu\text{m}}$ and the positive control, $\text{TCP}_{<\mu\text{m}}$, led to similar amounts of bone tissue, as measured by histomorphometry: $24.5 \pm 4.3 \%$ and $23.9 \pm 6.3 \%$ respectively, followed by $\text{BCP}_{\mu\text{m}}$ ($2.1 \pm 1.7 \%$). No bone formed in $\text{TCP}_{\mu\text{m}}$ (Figure 6a). In addition, the percent area of resorbed material (by histomorphometry) profoundly varied among the four materials:

TCP_{<μm} underwent the most material resorption (53.8 ± 5.7 %), followed by BCP_{<μm} (28.1 ± 4.1 %), BCP_{μm} (9.3 ± 2.4 %) and TCP_{μm} (4.7 ± 2.1 %) (Figure 6b).

Figure 6. Quantitative analyses of bone formation and material resorption. Quantitative histomorphometry data on (a) the area percentage of bone tissue in the available spaces, and (b) the material resorption at 12 weeks post-operatively (* $p < 0.05$). The Shapiro-Wilk normality test gave $p > 0.05$ indicating normal distribution with a negative skewness of all data sets.

4. DISCUSSION

In the present study, a hydrothermal treatment was employed to promote the growth of a layer of epitaxial crystals on the surface of BCP materials, leading to a novel needle-shaped surface topography. By adjusting the sintering temperature (i.e. 1125 °C or 1200 °C) of the BCP materials, needle-like crystals at either the submicron or micron scale were achieved (Figure 1). *In vivo*, the novel CaP with submicron needle-shaped topography (BCP_{<μm}) triggered earlier bone formation (3 - 6 weeks) compared to TCP with a submicron grain-shaped surface topography (TCP_{<μm}) which mainly formed bone at 6-9 weeks (Figure 5). By 12 weeks post-implantation, the amount of bone formed by the positive control, TCP_{<μm}, was equivalent to the amount formed by the novel needle-like surface topography of BCP_{<μm} (Figure 6a). Similarly, BCP_{μm} with a micron scale needle-shaped surface topography underwent *in vivo* biomineralization and gave rise to ectopic bone formation, while its counterpart TCP_{μm} with micron-scaled grain-like crystals did not (Figure 5, 6a).

In agreement with previous reports, the feature size of the surface structure greatly affected the osteoinductive potential of CaP materials [9-11,18,20,29]. As expected, inductive bone formation occurred in greater amounts for the submicron surface structured BCP_{<μm} and TCP_{<μm} grafts but very limited bone formed in BCP_{μm} and no bone formed in TCP_{μm} with a micron-scale surface topography (Figure 3, 4, 5 and 6). The strength of the observed effect suggests that the scale of topography influences osteoinductivity independently from chemistry and surface morphology. Based on the available data for grain size, surface area and protein adsorption for BCP_{<μm} and TCP_{<μm} versus BCP_{μm} and TCP_{μm}, this could either be due to a direct or an indirect effect of the smaller surface feature size on bone formation.

Let us first consider whether the specific surface topography could exert a direct effect on bone formation by affecting attached cells. In the absence of chemical factors, surface topographies ranging from nano- to micron scale have been reported to evoke specific cell attachment, orientation, proliferation and guide their differentiation towards various lineages [30-35]. At the micron scale, surface structure has been shown to enhance adipogenic differentiation of MSCs, while submicron scaled surface structures favored osteogenic differentiation [9,10,34]. More recently, research has demonstrated that submicron surface topographies can preferentially direct early wound healing toward the bone-forming pathway by influencing macrophage polarization [29,36-38]. The role of macrophages as either mediators of a pro-inflammatory response or mediating a wound-healing and angiogenic response is well-understood [36]. The key to successful bone healing, that is to promote osteogenesis and avoid the formation of scar tissue, is to tip the balance of wound healing toward the M2, or deactivated, macrophage response following an initial phase of inflammation [37,38]. CaP materials with a submicron surface topography have been shown to direct the transition of macrophages to the M2 phenotype, as shown by the higher production of TGF-β and CCL18 from cells cultured on CaP with submicron surface structures [29]. At the same time, CaP materials with micron surface topography displayed more M1 macrophages formation

as indicated by an enhanced TNF- α and IL-1 β secretion [29]. The conditioned media harvested from THP-1 cells cultured on CaP with submicron surface topography enhanced tube formation by human umbilical vein endothelial cells (HUVECs) *in vitro*. Furthermore, submicron surface topography enhanced angiogenesis and triggered bone formation in ectopic defect sites [29].

Let us next consider whether the specific surface topography could exert an indirect effect on bone formation. Protein adsorption is often suggested as crucial in inductive bone formation [39-41], where osteoinduction of CaP materials is thought to be a secondary response following the adsorption of growth factors/ cytokines from body fluids *in vivo* [42-44]. Since the function of growth factors/cytokines is dose-dependent [45,46], theoretically the more proteins concentrated in the implants should lead to higher osteoinductive potential. In this study, TCP_{<μm} adsorbed significantly more proteins from FBS per implant than BCP_{<μm} (Figure 2b, 2c and Table 1), while the latter triggered earlier bone formation than the former. Similarly, BCP_{μm} adsorbed similar or little more proteins than TCP_{μm}, but the former triggered bone formation, the latter did not (Figure 2b, 2c and Table 1). Therefore, we conclude that the initiation and enhancement of inductive bone formation in BCP_{<μm} and TCP_{<μm} is less likely to have been due to a higher surface area (and associated increased protein adsorption) but, more likely, instructed by the possible physical cues created by the smaller features in the surface topography.

It is important to consider which of the other effects of chemistry and surface feature morphology is most dominant in driving earlier bone formation in ectopic defects, as observed for BCP_{<μm} (3 - 6 weeks), compared to TCP_{<μm} (6 - 9 weeks). Firstly, BCP_{<μm} consists of 25 % HA/ 75 % β -TCP, while TCP_{<μm} is phase-pure β -TCP. Although the role of chemical composition appears, according to some literature, not to be an essential material factor to trigger inductive bone formation in CaP materials [20], it is generally thought that a higher content of TCP can affect the dissolution of CaP and thus the ion release (e.g. calcium and

phosphate) from its surface. This increased ion release may, in turn, enhance inductive bone formation [12,47-51]. However, in this study, BCP_{<μm} released less calcium ions *in vitro* than TCP_{<μm} (Figure 2a) and resorbed at a slower rate than TCP_{<μm} *in vivo* (Figure 6b). Therefore, the earlier trigger or enhancement of bone formation observed in our study for BCP_{<μm} seems not to be attributed to the rate of calcium ion release.

Both BCP test materials had resorption rates between those observed for TCP_{<μm} and TCP_{μm} *in vivo* (Figure 6b), contrary to the general view that a higher TCP content would lead to faster resorption. Since BCP_{μm} had the lowest dissolution *in vitro* (Figure 2a) but a faster rate of resorption compared to TCP_{μm} *in vivo*, it indicates that cell-mediated resorption played the major role in the *in vivo* resorption for the BCP test articles. This is further corroborated by the histological analysis (Figure 3b, 3c), in which multinucleated osteoclast-like cells were observed on the surface of the materials. Could chemistry dictate the mechanism of resorption and indirectly drive earlier bone formation for BCP_{<μm}? We suggest this is not a dominant factor, since abundant resorption by multi-nucleated giant cells was also observed using histological analysis for TCP_{<μm} (Figure 3c). The slower resorption we observed for BCP_{μm} compared to BCP_{<μm} corroborates the findings of Davison et al. that CaP ceramic with submicron scaled surface structures favored the formation of osteoclasts from mononuclear cells and facilitated material resorption as compared to its micron scale structured counterpart [52]. Therefore, if the presence of multinucleated osteoclast-like cells is a driver for osteoinductive potential, according to our research, the size of feature in the surface topography is more likely to be the dominant factor than the chemistry.

It is worth noting that the BCP and TCP families contained a different level of microporosity (Table 1). Microporosity is often suggested to determine the osteoinductive potential of CaP materials because it creates an ideal microenvironment for protein adsorption and/or cell attachment, differentiation and proliferation [9-11,15,16,20], where higher microporosity has been reported to lead to higher osteoinductive potential [16]. Since lower microporosity was detected in the BCP family

than in the TCP family, the trigger of earlier inductive bone formation observed in BCP_{<μm} is not likely to be linked to microporosity.

Based on our data and the supporting literature, we conclude that the most dominant secondary factor (after surface feature size) for CaP driven osteoinductivity is the surface feature morphology of the substrate. In the case of this study, we found that needle-shaped surface features gave rise to earlier bone formation than grain-shaped surfaces. As chemistry is still likely to have a certain level of influence in osteoinductivity, we propose the strength of effect to be as follows: surface feature size > surface feature morphology > substrate chemistry. The improved bone formation outcomes for BCP_{<μm} and BCP_{μm} in this intramuscular model indicates that they have higher osteoinductive potential than TCP_{<μm} and TCP_{μm} respectively. Previous studies have shown that osteoinductive materials facilitated bone regeneration in orthotopic skeletal sites compared to non-osteoinductive materials [9,11], and that a higher osteoinductive potential correlates with faster bone repair in orthotopic defects [47]. Therefore, of all the articles tested in this study, BCP_{<μm} may be expected to have good bone regeneration potential in clinical applications.

Using the current settings, the superiority of BCP_{<μm} to other 3 materials in the study was seen. However, besides the histological and histomorphometrical data, more analyses such as quantitative μCT, biochemical assays of bone forming signals and immunohistostaining of bone markers would make the conclusion stronger. Fluorescent labeling was used to monitor the onset of bone formation at different timepoints, without the need to include more animals in the study. Another approach could have been to use animal cohorts at each time point, which would allow for a more precise analysis and include bone quantity assessment at each time point. Finally, a clinically relevant animal study (i.e. orthotopic implantation) would be required to more accurately predict the potential clinical efficacy of these materials.

5. CONCLUSION

Evaluation of ectopic bone formation by two novel CaP materials with a needle-like surface topography showed that they can give rise to earlier and accelerated bone formation as compared to their counterparts with grain-like surface structures. Our data suggests that the relative strength of effect on osteoinductive potential is as follows: surface feature size > surface feature morphology > substrate chemistry. Of all materials tested in this study, BCP_{<μm} with a needle-shaped surface topography is expected to have the best bone regeneration potential in clinical applications, although the performance of this material still needs to be evaluated in an orthotopic implantation site.

A C K N O W L E D G E M E N T S

The authors would like to thank the Netherlands Institute for Regenerative Medicine (NIRM), the Rapid Prototyping of Custom-Made Bone-Forming Tissue Engineering Constructs (RAPIDOS Project, Ref. NMP-2013-EU-China, no. 604517), the Seventh Framework Programme of the European Union (no. 241879) and the Horizon 2020 Framework Programme (no. 674282) for their financial support in this study. The authors also acknowledge financial support of Kuros Biosciences BV (the Netherlands) and Sichuan University (China) for the animal study and histological analysis.

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CHAPTER 8

General summary, discussion and further perspectives

1. SUMMARY

In the first experimental chapter (**Chapter 2**), six commercially available calcium phosphate (CaP) bone substitutes and a submicron or micron-scaled CaP were characterized and evaluated in an intramuscular beagle model. The results pointed out that, as compared to the chemical composition, surface topography was crucial to trigger osteoinduction, where materials with a submicron surface topography were most osteoinductive. Moreover, it was shown that surface topography may bring about biological processes via physical cues rather than the theories described in the literature such as protein adsorption, ion release and biomineralization.

In order to evaluate the influence of surface topography on bone formation and material resorption in orthotopic sites, submicron/micron scaled tricalcium phosphate (TCP) materials were implanted in the dorsal muscle and in a spine environment respectively in **Chapter 3**. The results showed that the submicron scaled surface topography does not only induce ectopic bone formation in dorsal muscles of beagles, but also improve orthotopic bone formation as compared to a chemically equivalent counterpart with a micron scaled surface topography. The resorption rate of the materials varied with the dimension of surface topography in both ectopic and orthotopic (spine) sites, with a higher resorption rate for the osteoinductive material with a submicron surface topography. Meanwhile, higher resorption rates of materials were seen in the spinal environment than in the muscle.

The effect of surface topography dimension on bone regeneration and material resorption in a critical-sized bone defect was further investigated in **Chapter 4**, where three TCP-based bone substitutes with various scales of surface topography were implanted in a rabbit condylar defect for three timepoints. The results showed that submicron scaled surface topography of TCP give rise to more bone formation and undergo more material resorption in comparison to the other two chemically equivalent materials with a larger scale of surface topography. These results

indicated that the dimension of surface topography could affect bone regeneration and material resorption in osseous sites.

In **Chapter 5**, commercially available CaP bone graft substitutes with a submicron scaled surface topography in Granules and Putty formulations were implanted in an instrumented posterolateral lumbar fusion (PLF) of sheep for 4, 8 and 12 weeks, with the gold standard autograft as a positive control. The results showed that both submicron surface structured CaP ceramics were at least as efficient as autograft in spinal fusion, with an improved handling property and slightly improved performance of the Putty formulation.

In **Chapter 6**, CaP materials with either a submicron or micron scaled surface topography were employed *in vitro* regarding to macrophage differentiation and *in vivo* with respect to angiogenesis and ectopic bone formation. The results indicated a strong link between surface topography, macrophage polarization, angiogenesis and osteoinduction. It is likely that the submicron surface topography instructs the shifting of macrophages from uncommitted macrophages (M0) to alternatively activated macrophages (M2), which can secrete cytokines favoring angiogenesis *in vitro* and *in vivo*, and finally facilitating ectopic bone formation.

In order to clarify the role of surface morphology in inducing ectopic bone formation, two novel CaP materials with either submicron or micron scale needle-like surface structure were engineered with hydrothermal treatment and compared to the grain-shaped counterparts with corresponding scaled surface topography in terms of bone formation in **Chapter 7**. The data indicated that the surface morphology of CaP materials played a role in the onset of ectopic bone formation, with the needle-like surface structure being superior to the grain-shaped one. The relative strength of effect on CaP-driven bone induction could be as follows: surface feature size > surface feature morphology > substrate chemistry.

2. GENERAL DISCUSSION

In order to design optimal CaP bone graft substitutes with improved bone formation potential that meet clinical requirements, we investigated what material parameters necessary for material-driven bone formation and which biological processes occurring in osteoinduction. A coupling between surface topography, macrophage phenotype, angiogenesis and ectopic bone formation was observed and, as a result, a novel CaP material with accelerated osteoinductive potential was discovered. Furthermore, clinically relevant animal studies were performed to further support the transition of osteoinductive CaP materials from bench to bedside.

Throughout the experimental chapters, one of overarching findings is that submicron scaled surface topography of CaP-based materials is a dominant material parameter determining their bone forming ability in both ectopic and orthotopic sites. Submicron structured CaP materials not only enhanced bone formation in a spine environment and in condylar defect, but also performed equivalently to autograft in a preclinical sheep PLF model. Another finding is the coupling of immune response, angiogenesis and material driven bone formation. The submicron surface topography of CaP-based materials was shown to modulate the macrophage phenotype to M2 leading to the secretion of specific cytokines upon implantation, followed by enhanced angiogenesis and bone formation. Moreover, next to the dimension of surface topography, the morphology of surface topography was found to be another physical parameter playing a role in material-instructed bone formation. The osteoinductive potential of CaP-based materials could therefore be further improved with a needle-like surface feature as compared with grain-shaped surface topography.

While the findings of chapters 2-7 stand on their own, they also come together to address the broader questions proposed in chapter 1, which will be further discussed below.

2.1. Material's parameters for osteoinduction

Without adding growth factors and osteogenic cells, CaP materials with specific physicochemical properties can induce ectopic bone formation.

This indicated that CaP-driven osteoinduction must be related to the intrinsic material parameters, such as physical structure and chemical composition [1.2].

Ectopic bone formation has not been reported on CaP materials without a microstructure, indicating that microstructure is a primary factor necessary for osteoinduction. The role of microstructure in material-driven bone formation has been highlighted by several research groups. Yamasaki et al. (1992) firstly demonstrated that microstructured HA triggered *de novo* bone formation when implanted subcutaneously in the abdomen of dogs, but non-microstructured HA failed [3]. The indispensability of microstructure was further confirmed by Yuan and Habibovic who found that CaP materials including HA, BCP and TCP with microstructure were osteoinductive while those without were not [4-8]. Next to the presence of microstructure, the importance of dimension of surface microstructure (i.e. the size of grain and pore) in material-driven bone formation was defined by Yuan et al. (2007) who demonstrated both surface grains and pores should less than 1.5 μm to induce ectopic bone formation upon intramuscular implantation [9].

The first experimental chapter (Chapter2) validated that the dimension of surface microstructure was essential in material-inducing ectopic bone formation. TCP-S and MBCP having different chemical compositions but similar dimension of surface microstructure triggered *de novo* bone formation in dorsal muscle of beagles. Conversely, materials with either smaller (e.g. nano) or larger scales (e.g. micron), failed to give rise to ectopic bone formation.

In Chapter 7, the importance of surface microstructure dimension in inductive bone formation was further confirmed using CaP with either submicron or micron scaled structure in dorsal muscles of beagles. Irrespective of surface chemistry, both BCP_{<μm} and TCP_{<μm} with submicron scaled surface structures induced ectopic bone formation, while their micron scaled counterparts triggered sporadic bone formation or did not give rise to bone formation at all. Additionally, the needle-like topography of BCP_{<μm} stimulated earlier bone formation (3 - 6 weeks)

compared to the grain-shaped surface topography of TCP_{<μm} which formed bone at 6 - 9 weeks. These results indicated that, next to size, the surface microstructural morphology was another material parameter affecting the onset of ectopic bone formation.

In addition to surface microstructure, the importance of chemical composition in the extent of osteoinduction has been highlighted in the literature. Because CaP materials with different ratio of HA/β-TCP lead to various calcium and phosphate ions release. It appears that a higher solubility of CaP ceramics can result in higher osteoinductivity. For example, having similar microstructure, BCP with more soluble β-TCP induced more ectopic bone formation as compared to HA. Similar phenomenon was observed when TCP was compared to BCP [6,10,11].

Similarly, in Chapter 2, both TCP-S and MBCP with similar surface microstructures stimulated *de novo* bone formation, while the amount of bone formed in MBCP was less than in TCP-S. Since TCP-S is more soluble than MBCP, it is possible that chemical composition enhances *de novo* bone formation by through calcium and phosphate ion release.

However the role of ion-release in material-driven bone formation may not be that necessary. In Chapter 7, less soluble BCP_{<μm} and more soluble TCP_{<μm} with similar scaled surface structure induced a similar amount of *de novo* formation after 12 weeks of implantation. While less soluble BCP_{<μm} stimulated earlier bone formation (3-6 weeks) as compared to TCP_{<μm} which formed bone at 6-9 weeks.

2.2. Physicochemical mechanisms on osteoinduction

Several theories have been presented to explain how CaP physicochemical properties serve to induce ectopic bone formation. Among them protein adsorption, calcium and phosphate ions release, biomineralization were often addressed in the literature [1].

Because CaPs have high affinity to growth factors such as BMPs, which have been suggested as possible triggers of osteoinduction by CaP materials [12, 13]. Ripamonti et al. (1991) firstly proposed that the porous structure of CaP materials act as a collector for local accumulation of

endogenous BMPs [14]. It was thus assumed that a concentration threshold of growth factors could be reached to trigger inductive bone formation. The protein adsorption theory explained well the osteoinduction in CaP materials with high porosity. However, it is difficult to explain the results of Chapter 2, in which nano-structured Bio-Oss adsorbed the most amount of protein *in vitro* but did not induce any bone formation *in vivo*.

Prior to ectopic bone formation, Poly-HEMA underwent biomineralization *in vivo* [15], surface mineralization has been then proposed necessary for osteoinduction. CaP material's total surface area and its chemical composition affect the dissolution and reprecipitation events at its surface, finally leading to the formation of a biological apatite layer [1,16]. It could well be that the surface mineralization affects the bone forming ability of CaP materials with various surface area and different chemical compositions. However, as seen in Chapter 2, eight CaP materials underwent biomineralization *in vitro*, but only 3 CaP materials triggered ectopic bone formation, indicating that biomineralization may be a prerequisite but is not sufficient to trigger ectopic bone formation in CaP materials.

Calcium and phosphate ions have shown a positive influence on osteogenic differentiation of MSCs as well as osteogenesis by (pre)osteoblasts [17-19]. It is therefore reasonable to speculate an influence of released calcium and phosphate ions on material-driven bone formation. It is likely that calcium and phosphate ions released from more soluble β -TCP phase in BCP hence promoted bone formation in contrast to stable HA [10,11]. Similarly, in Chapter 2 TCP-S released more calcium and phosphate ions and induced more ectopic bone formation compared to less soluble MBCP. However, it's worth noting that Vitoss TCP released the most calcium and phosphate ions but failed to induce ectopic bone formation. The ion-release theory is not supported either by the ectopic bone formation reported in non-CaP materials such as titanium [20] and poly-PMMA [21]. As such, ion release may be involved in material-driven ectopic bone formation but is not the driving factor.

It has been reported that surface topography can initiate biological responses through mechanotransduction – the conversion of mechanical signals at the cell-substrate interface to gene transcription and lineage commitment in the nucleus. For example, it has been reported that changing the micropore size of polycarbonate surfaces could modulate the differentiation of pre-osteoblasts [22]. Hatano et al. (1999) who observed that a surface average roughness up to 0.8 micron enhanced the proliferation, alkaline phosphatase (ALP) activity and osteocalcin expression of osteoblasts in comparison with a smoother surface [23]. The molecular mechanism underlying adherent cell response to mechanical/topographical stimuli and the subsequent conversion to transcriptional regulation of cell fate is still unclear. Presumably the surface microstructure uniquely changes the focal adhesions and cytoskeletal tension of adherent cells, which in turn upregulates transcription of osteoblast genes through YAP and TAZ regulators [24-26]. It is likely that cells respond to osteoinductive CaP materials in such a way to undergo specific cell differentiation [27].

2.3. Biological processes underlying osteoinduction

Osteogenic differentiation of MSCs is the key step in inductive bone formation by materials following ectopic implantation and has shown to be affected by physicochemical properties of materials [28,29]. In addition to MSCs, immune cells (e.g. macrophages) can also be modulated by the surface microstructure. For instance, Fellah et al. (2010) reported that the number of macrophages and giant cells varied according to the surface grain size of CaP ceramics [30]. Davison et al. (2014) observed that submicron scale surfaced CaP materials induced an inflammatory response of macrophages, and their subsequent secretion of cytokines resulted in stem cell differentiation [29]. Interestingly, when macrophages were selectively depleted in this mouse model by liposome-encapsulated clodronate, ectopic bone formation was blocked [31].

The results in Chapter 6 showed a link between surface topography, macrophage phenotype, angiogenesis and ectopic bone formation.

Submicron scaled surface structure shifted the macrophage from uncommitted phenotype (M0) to alternatively activated state (M2), which secrete cytokines favoring angiogenesis *in vitro*. It was also shown in Chapter 6 that angiogenesis greatly affected inductive bone formation.

2.4. Ectopic and orthotopic models of osteoinductive materials

Ectopic models to test osteoinductive materials, such as in the muscle or under skin are useful to analyze *de novo* bone formation in soft tissues where no bone is present. In the past decades, osteoinduction has been reliably demonstrated in dogs, sheep, goats, baboons, pigs and FVB mice [1,31,32]. In order to prompt fundamental findings from bench to bedside, it is necessary to evaluate their functions in clinically relevant animal models, which serve to more directly explain how osteoinductive materials could be applied in the repair of critical sized bone defects in the clinic. Yuan et al. (2010) and Habibovic et al. (2006) demonstrated that osteoinductive CaP materials could give rise to more *de novo* bone formation as compared to non-osteoinductive materials in critical sized ilium defects. Osteoinductive CaP materials could perform equally well to autologous bone or collagen loaded with BMPs [7,11].

Chapter 3, 4 and 5 addressed further the bone regeneration capacities of osteoinductive CaP materials in orthotopic defects. In both a spine environment (Chapter 3) and in critical sized bone defects (Chapter 4), osteoinductive materials performed better than non-osteoinductive materials. The Sheep PLF model has been commonly considered a more clinically relevant critical-sized bone defect model because osteoinductive stimulus is needed to form bone between the transverse processes of the vertebrae and make a bony bridge [33, 34]. As seen in Chapter 5, osteoinductive CaP materials, as either a granule or putty formulation, achieved equivalent spinal fusion to autograft in a sheep PLF model. The results demonstrated that the bone regenerative ability of bone substitutes in orthotopic sites was closely related to their osteoinductive potential and the material parameters affecting the bone forming ability of materials. For example, submicron scaled surface topography of CaP materials with higher osteoinductive potential

performed much better in bone regeneration in osseous sites, in comparison with micron scaled materials with lower osteoinductive potential.

3. CONCLUSION

Throughout this thesis, the overarching findings were 1) submicron scaled surface topography was a crucial material factor to initiate material-instructed bone formation; 2) rather than protein adsorption, ion release and biomineralization, surface topography played its crucial roles in material-instructed bone formation by its physical cues; 3) the physical cues provided by surface topography may first affect the macrophage phenotype which are coupled with angiogenesis and bone formation; 4) either alone or in a moldable putty form, the materials with specific surface topography enhanced bone regeneration in orthotopic sites and could performed equivalently to autograft in clinically relevant bone regeneration model; 5) surface morphology was another physical parameter playing roles in material-instructed bone formation and osteoinductive potential could be further improved with the needle-shaped surface feature.

The overall data presented in this thesis gave more insights on material mechanism and biological mechanism of material-instructive bone formation and paved a way for further improvement of bone substitutes and the clinical applications of such bone substitutes with improved bone forming ability.

4. PERSPECTIVES AND FUTURE DIRECTIONS

4.1. How to further improve osteoinductivity of CaPs?

In view of the findings of this thesis, it is possible to maximize the osteoinductive potential of CaPs by optimizing their surface architecture. In line with previous findings [4-11,31,32], the results of Chapter 2, 3 and 7 demonstrated that surface microstructure at a submicron scale is essential to trigger ectopic bone formation. Additionally, Chapter 7 demonstrated the possibility to further improve the osteoinductive potential of CaP-based materials through tuning the surface morphology.

It is therefore possible to further improve the osteoinductive potential of materials through a more accurate control of physicochemical parameters including chemistry and surface topography.

4.2. How does the submicron scaled surface structure affect macrophage phenotype and function?

In Chapter 6, we show that surface topography can affect macrophage polarization, i.e. submicron scaled surface topography facilitated macrophage shifting from uncommitted macrophages (M0) to alternatively activated macrophages (M2). Though hypotheses have been proposed, the precise mechanism underlying this phenomenon is still unknown. Some researchers have suggested that surface topography may alter the morphology of adherent cells and subsequently affect their biological behavior by mechanotransduction. This refers to the processes through which cells sense and respond to mechanical stimuli by converting them to biochemical signals that elicit specific cellular responses [22-26]. In chapter 6, the influence of surface topography on mechanotransduction was seen with cell morphological changes. Macrophages cultured on submicron scaled topographical TCP material appeared larger in size with a flat morphology, while macrophages seeded on micron scaled surfaces presented a small and round morphology. How cells adapt their morphology to surface topography and how this affects osteoinduction need further studies.

4.3. How does angiogenesis favor ectopic bone formation?

Numerous studies have demonstrated that angiogenesis predominantly occurs before the onset of osteogenesis, and it has been suggested that these newly formed blood vessels not only transport oxygen, nutrients, and wastes [35-37] but are also a source of inducible (stem) cells. As seen in Chapter 6, prior to *de novo* bone formation, angiogenesis was observed in the osteoinductive materials. On the contrary, limited blood vessels were observed in non-osteoinductive materials. Moreover, inhibition of angiogenesis with KRN633 hampered bone formation in osteoinductive materials. Although a relation between angiogenesis and material-driven ectopic bone formation was clearly shown, the exact link

between angiogenesis and ectopic bone formation is not yet known. It is possible that the invading blood vessels ensured a steady transport of blood vessel derived MSCs (i.e. pericytes) to the implant surface [28,38] but, without the direct biological markers of the osteoblast precursors, any link between angiogenesis and osteoinduction is mainly speculative.

4.4. Improve osteoinductivity of CaPs through tuning immunomodulation?

From the literature and our data, it could be deduced that osteoinduction is orchestrated by macrophages through an effective immune response. Would it therefore be possible to design biomaterials with higher osteoinductivity by introducing M2 macrophages into implants?

Immune cells are beneficial in bone formation through induction of certain biological events, such as angiogenesis [35,36]. Several pro-angiogenic factors such as TNF- α and IL-1 are produced in high concentrations by macrophages [39]. Using macrophages as a proangiogenic reservoir is therefore a possible way of addressing one of the fundamental problems in bone tissue engineering. For example, by treating osteoblast/endothelial cell co-cultures with macrophages derived from THP-1 cells, Dohle et al. (2014) induced the formation of microvessel-like structures with an increase in the expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) [40]. Because methods for isolating specific blood-derived monocytes (such as CD14+ monocytes) and their differentiation to desired cell types such as macrophages of a given phenotype are readily available, it is possible to develop such therapies with autologous cells. Incorporation of macrophages to improve tissue formation could be a viable method eliciting a positive effect on bone regeneration.

Different strategies could be the choices to design tissue-instructive biomaterials, by either tuning physicochemical properties of materials or immunomodulation [41]. Functional repair of damaged bone may not be a dream in the future.

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Ph.D. PROFILE

Biomaterials Science & Technology (BST)

Twente University

Promoter

Prof. Dr. J.D. de Bruijn

Prof. Dr. D.W. Grijpma

Co-promoter

Dr. H. Yuan

Ph.D. candidate:

Rongquan Duan

1. Discipline

Name	Organizer	Date	EC
Dutch Polymer Days -15 (2015)	The National Dutch Graduate School of Polymer Science & Technology	16/03/2015 - 17/03/2015	1.5
The 27th European conference on biomaterials (ESB) (2015)	Polish Society for Biomaterials	29/08/2015 - 04/09/2015	2.5
The annual conference of Netherlands society for biomaterials and tissue engineering (NBTE) (2015)	The Netherlands society for biomaterials	03/12/2015 - 04/12/2015	1.5
Advanced Functional Polymers for Medicine (AFPM) (2016)	University of Twente	15/06/2016 - 17/06/2016	1.5
The annual conference of Netherlands society for biomaterials and tissue engineering (NBTE) (2016)	The Netherlands society for biomaterials and tissue engineering	01/12/2016 - 02/12/2016	1.5
The 6th China-Europe Symposium on Biomaterials in Regenerative Medicine (CESB) (2017)	European Society for Biomaterials, Chinese Society	21/05/2017 - 24/05/2017	2.5
The 28th European conference on biomaterials (ESB) (2017)	Hellenic Society for Biomaterials	04/09/2017 - 08/09/2017	2.5

The annual meeting of orthopaedic research society (ORS) (2018)	American Orthopaedic Research Society	09/03/2018 - 13/03/2018	2.5
Total			16.0

2. Generic

Name	Organizer	Date	EC
English level 2 course (2014)	Babel	10/09/2014 - 30/10/2014	2.5
TGS-2 days introductory PhD workshop (2014)	University of Twente	03/12/2014 - 04/12/2014	1.0
Intensive individual English language training course (2015)	Regina Coeli	02/11/2015 - 11/11/2015	2.5
Animal studies/ histology training (2014)	Xpand Biotechnology BV	01/07/2014 - 26/08/2014	2.5
XRD testing (R&D samples) (2014-2016)	Xpand Biotechnology BV	03/07/2014 - 17/05/2016	1.0
SEM testing (R&D samples) (2014-2016)	Xpand Biotechnology BV	29/07/2014 - 29/03/2016	1.0
Cell culturing (2014-2015)	Xpand Biotechnology BV	26/08/2014 - 02/06/2015	1.0
FT-IR testing (R&D samples) (2014-2015)	Xpand Biotechnology BV	11/11/2014 - 01/01/2015	1.0
Calcium release/ pH-Ion meter (R&D samples) (2015-2016)	Xpand Biotechnology BV	04/01/2015 - 18/01/2016	0.5
Colour inspection (R&D samples) (2015)	Xpand Biotechnology BV	04/01/2015 - 16/02/2015	0.5
Determination of Calcium Phosphate or Polymer identity by FT-IR	Kuros Biosciences BV	17/04/2017- 20/04/2017	1.0
H-NMR analysis of polymers	Kuros Biosciences BV	08/05/2017 - 11/05/2017	1.0
Inherent viscosity of polymers with Ubbelohde viscometer	Kuros Biosciences BV	16/10/2017- 20/10/2017	1.5
Thermal properties of polymers by DSC	Kuros Biosciences BV	27/11/2017- 01/12/2017	1.0
Total			18.0

PUBLICATIONS

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ACCEPTED CONFERENCE ABSTRACTS

❖ **ORS 2018:** 64th Annual Meeting of the Orthopaedic Research Society. New Orleans USA.

- Oral presentation "Relation between macrophage differentiation, angiogenesis and topology-directed osteoinduction of calcium phosphate ceramics".

- Poster "Efficacy of a Novel Surface Structured Bone Graft Material in Rabbit Posterolateral Lumbar Spine Fusion".

❖ **ESB 2017:** 28th Annual Meeting of the European Society for Biomaterials. Athens, Greece.

- Oral presentations "Performance of an anhydrous copolymer for moldable composites with calcium phosphate ceramic".

- Oral presentations "Relation between angiogenesis and osteoinduction of calcium phosphate ceramics".

- Poster "Efficacy of a moldable calcium phosphate ceramic putty for posterolateral spinal arthrodesis".

❖ **CESB 2017:** 6th China-Europe Symposium on Biomaterials in Regenerative Medicine. Porto, Portugal.

- Oral presentations "Possible role of angiogenesis in osteoinduction of calcium phosphate ceramics".

- Poster "A Study Investigating the Use and Efficacy of the AttraX Putty and Osferion Materials for Posterolateral Spinal Arthrodesis".

❖ **NBTE 2016:** 25th Annual Meeting of the Netherlands society for biomaterials and Tissue Engineering. Lunteren, the Netherlands.

- Poster "Tuning the resorption and bone regeneration potential of β -tricalcium phosphate ceramics via the dimension of their surface microstructure".

❖ **AFPM 2016:** Advanced Functional Polymers for Medicine 2016. Enschede, the Netherlands.

- Poster "Sterilizable anhydrous copolymer as calcium phosphate ceramic binder for bone tissue engineering".

❖ **NBTE 2015:** 24th Annual Meeting of the Netherlands society for biomaterials and Tissue Engineering. Lunteren, the Netherlands.

- Oral presentation "Bone Regeneration in Rabbit Condyle Defects with Different Surface Structured Calcium Phosphate Ceramics".

❖ **ESB 2015:** 26th Annual Meeting of the European Society for Biomaterials. Cracow, Poland.

- Oral presentation "Ectopic bone formation by commercial calcium phosphate bone grafts substitutes".

- Poster "Improving Bone Forming Ability of Calcium Phosphate Ceramics with Surface Structure Modification".

❖ **Dutch Polymer days (DPD) 2015:** Lunteren, the Netherlands.

- Poster "Strontium addition in composite implants increased the local bone volume in rabbits undergoing osteoporosis".

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Zhengchao & Liangjia Karin Shaun
Jiaping & Kang

EΥΧΑΡΙΣΤΩ TÄNAN HVALA GRACIAS DZIĘKUJĘ
GRAZIE ありがとう MERCI TACK
THANK YOU DIAKUIU
PALDIES
ACIU TACK DANKE DANK U WEL ДЗЯКУЮ
СПАСИБО 谢谢 OBRIGADO diolch KIITOS
TESEKKUR EDERIM

Davide & Zhen Myrtille Wouter
Luuk Henry Ingeborg Dirk Wengjie & Xuhong
Ruiqing Winlan Viola Rens Furren
my dear parents Ruud
& all my family, friends
Zhang Yang my beloved Yi
Duan Ke Marijn Frank-Jan Jinlong & Bing

ABOUT THE AUTHOR

Rongquan Duan was born in a small village in Qufu, China, where he spent his carefree childhood there. His father, an unlettered but open-minded man, believed that knowledge is important and forced him to go to school.

After stubborn resistance in adolescent period, he was getting fascinated with life sciences and earned his bachelor's degree in clinical medicine.

To study abroad is the bravest decision that he ever made, the road is full of hardships, but he never regrets. Before the University of Twente, Rongquan had started his study in the Master-Doctor combine program focusing on biomedical engineering in Southwest Jiaotong University.

Cover design: Zhaojie Jiang

ISBN: 978-90-365-4773-4

DOI: 10.3990/1.9789036547734