DIVIDE, COMBINE AND CONQUER

on the Effect of Individual Properties
of Biomaterials for Bone Regeneration

Charlène Danoux

**Divide, Combine and Conquer:
on the Effect of Individual Properties of Biomaterials
for Bone Regeneration**

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On the cover: SEM micrograph of an OCP crystal.

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**DIVIDE, COMBINE AND CONQUER:
ON THE EFFECT OF INDIVIDUAL PROPERTIES OF
BIOMATERIALS FOR BONE REGENERATION**

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
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Summary

The increase of life expectancy in the developed countries has resulted in a growing demand for replacement of damaged and degraded bone. To overcome the limitations of autologous bone grafts, research efforts have focused for decades on developing synthetic substitutes that can be produced in large quantities, are readily available and relatively inexpensive. Among various synthetic bone graft substitutes, calcium phosphate (CaP)-based ones have been extensively used in bone repair and regeneration because of their close resemblance with natural bone mineral. CaP bone graft substitutes are biocompatible, osteogenic and osteoconductive, and some have shown osteoinductive properties, which are believed to be a prerequisite for reaching the same bone regenerative potential as natural bone grafts. Nevertheless, none of the CaP-based bone graft substitutes developed has yet reached the status of a full alternative to natural bone grafts.

Bone graft substitutes are traditionally developed with the aim to find a material with the highest bone regenerative potential, using a somewhat trial-and-error approach. In an iterative process of, often small, modifications to a single material property and, often simplified, biological evaluations, large numbers of biomaterials are developed with similar performance. However, in most functional biomaterials properties are interlinked, making it very difficult to control and modify a single material parameter without affecting the others, and to describe the exact relationship between this material property and a biological response.

The strategy developed in this thesis was to step away from the conventional, processing-driven biomaterials development to a design-driven one, whereby

combining and converging of materials and technologies is a logical step. The overall aim was twofold: (1) to explore strategies to isolate individual properties of complex biomaterials in order to investigate their independent effects on a biological response and based on this knowledge (2) to recombine individual properties into new, improved functional biomaterials. Using this approach, the chemical and structural properties of CaP materials were investigated and exploited throughout this thesis by designing innovative implants in CaP-based composites (Chapter 2), investigating the independent effect of calcium and phosphate ions (Chapter 3), studying the effect of CaP crystal topography and decoupling this effect from that of the CaP chemistry (Chapter 4), and finally by recombining the desired chemical and structural properties into improved functional materials (Chapters 4 and 5).

This exploratory journey aspired to identify tools and techniques that can be used to change the way bone graft substitute materials are designed and developed. The final aim is thereby to deliver fundamental answers regarding the effect of specific material properties on the biological system, while developing innovative and improved materials.

Samenvatting

Als gevolg van bevolkingsvergrijzing in de ontwikkelde landen neemt de vraag naar behandeling en vervanging van beschadigd bot toe. Gebruik van patiënt-eigen bot in deze toepassing gaat gepaard met een aantal nadelen. Daarom is er in de afgelopen tien jaar veel onderzoek gedaan naar synthetische botvervangers, die relatief goedkoop en eenvoudig te produceren zijn in grote hoeveelheden. Botvervangers op basis van calciumfosfaat keramiek worden het meest gebruikt voor reparatie en regeneratie van beschadigd botweefsel. Dit omdat de chemische samenstelling van deze materialen sterk overeenkomt met de samenstelling van natuurlijk botmineraal. Calciumfosfaat botvervangers zijn biocompatibel, osteogeen, osteoconductief, en in een aantal gevallen zelfs osteoinductief. Osteoinductiviteit wordt vaak gezien als een van de belangrijkste eigenschappen voor succesvolle regeneratie van bot. Ondanks deze eigenschappen, worden synthetische botvervangers nog steeds niet beschouwd als een volwaardig alternatief voor patiënt-eigen bot. Voor een deel is dit te wijten aan de wijze waarop de meeste synthetische botvervangers voor regeneratie van grote botdefecten worden ontwikkeld. In een iteratief proces worden vaak kleine veranderingen aangebracht aan één van de eigenschappen van het materiaal, gevolgd door een vaak simplistische biologische evaluatie. Dit resulteert in een groot aantal materialen met kleine verschillen in klinische prestatie. Daarnaast zijn individuele eigenschappen van functionele materialen meestal sterk met elkaar verbonden, en is het moeilijk, zo niet onmogelijk, om één eigenschap te wijzigen zonder dat dit een effect heeft op andere eigenschappen. Hierdoor is het moeilijk de relatie te beschrijven tussen een eigenschap van een materiaal, en de prestatie ervan in een

klinische setting.

De strategie die in dit proefschrift is ontwikkeld is erop gericht de conventionele manier van het produceren van biomaterialen, die gedreven wordt door de mogelijkheden van beschikbare productietechnieken, te vervangen door een ontwerp-gedreven methode. Daarbij is het combineren van verschillende soorten materialen en technologieën een logische stap. Het globale doel van dit werk was tweeledig: (1) de strategieën te verkennen waarmee individuele eigenschappen van complexe materialen geïsoleerd kunnen worden om zo hun individuele effect op biologische processen te onderzoeken, en op basis van deze kennis (2) de gewenste eigenschappen opnieuw te combineren om zo tot een verbeterde botvervanger te komen. Deze benadering is gebruikt om chemische en structurele eigenschappen als ook de relatie ervan tot het gedrag van het materiaal in een biologische omgeving te onderzoeken. In Hoofdstuk 1 is de relevantie van macrostructuur onderzocht door middel van een innovatief implantaat ontwerp, gemaakt van een polymeer/calciumfosfaat composiet. In Hoofdstuk 3 zijn de individuele effecten van calcium en fosfaat ionen nader onderzocht. Hoofdstuk 4 is gericht op het loskoppelen van kristalstructuur en chemische samenstelling van calciumfosfaten. In hoofdstukken 4 en 5 zijn de mogelijkheden onderzocht om de gewenste chemische en structurele eigenschappen te combineren tot een verbeterd materiaal.

Deze ontdekkingsreis had als doel gereedschappen en technieken te identificeren die gebruikt kunnen worden om de conventionele manier van het ontwikkelen van botvervangers te veranderen. Daarbij is geprobeerd fundamentele antwoorden te geven betreffende het effect van individuele eigenschappen op het biologisch systeem, in parallel met het ontwikkelen van nieuwe, verbeterde materialen.

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General Introduction

1.1 Bone tissue

1.1.1 Definition and roles

Bone is a highly specialized form of connective tissue with an important role in the internal support system of the majority of higher vertebrates. Bone is a complex composite material that is built for approximately 60 weight percent of a mineral phase, for 30 weight percent of an organic matrix and for 10 weight percent of water [1]. Two morphological categories of osseous tissue can be distinguished: cortical (compact) bone and cancellous (trabecular) bone. Cortical bone that is built of cylindrical lamellae is dense, and mechanically stronger and metabolically less active than cancellous bone. In contrast, cancellous bone has a spongy, highly porous structure, with a porosity of above 75%, which orients in response to the direction of the external load. Most bones comprise an outer shell of cortical bone that is strong and stable, protecting an inner core of cancellous bone. Together, bones provide an internal frame that anchors the musculature, tendons and ligaments to enable support and locomotion. Cranial and thoracic bones also ensure the protection of vital organs. The cancellous part of bone, which is highly vascularized, contains bone marrow, where blood cellular components are produced (hematopoiesis). Furthermore, bone, being highly vascularized and possessing a large specific surface area, plays an important metabolic role as a reservoir of calcium and phosphate, which can be drawn upon when needed in the

homeostatic regulation of these ions in blood and other body fluids.

1.1.2 A composite structure

At a microscopic level, bone can be seen as a highly organized composite material with multiple hierarchical levels. The organic matrix of bone is made up for 90% of collagen type I while the remaining 10% is a mix of non-collagenous proteins such as osteocalcin (OC), osteopontin (OP), osteonectin, bone sialoprotein (BSP), decorin and biglycan [2]. A collagen molecule is composed of three strands of amino acids arranged in a triple helix with a length of approximately 300 nm. Such collagen molecules assemble and cross-link in a repetitive structure presenting a constant thickness of five collagen molecules. This assembly is defined as a collagen fibril. Fibrils can assemble as well to form collagen fibers of up to 10 μm in diameter. The mineral phase of bone is composed of calcium phosphate (CaP) ceramic in the form of hydroxyapatite (HA) crystals, which present some substitutions with carbonate groups in the lattice (carbonated apatite), and some calcium vacancies (calcium-deficient apatite). As a result of these substitutions with relatively instable and highly reactive groups, bone mineral is more soluble than phase-pure HA, which is an important property in the process of the formation and remodeling of bone tissue. In bone tissue, the CaP crystals are observed between the collagen fibrils. Aligned collagen fibers are deposited in concentric sheets called osteons. This composite assembly provides exceptional mechanical properties to bone tissue by combining the hardness and compressive strength of its mineral phase with the elasticity and energy dissipating properties of its organic phase.

1.1.3 A living material

During bone formation, osteoblasts deposit the organic matrix of bone by secreting collagen type I that will later mineralize (ossification). Mature osteoblasts border the surface of newly formed bone and evolve as flat-bone lining cells before eventually being integrated into bone tissue as osteocytes. Besides collagen type I, osteoblasts produce molecules that control the organic matrix mineralization such as alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OP) and bone sialoprotein (BSP). ALP is known to influence phosphate homeostasis as this enzyme can convert extracellular pyrophosphate, a mineralization inhibitor, into inorganic phosphate that can form CaP deposits [3]. OC regulates the growth and size of HA crystals while OP is thought to inhibit HA formation and growth during bone mineralization [4, 5]. BSP plays a significant role in nucleation of HA crystals and mineralization by binding to calcium and inorganic phosphate ions from body fluids [6]. Osteoblasts originate from mesenchymal stem cells and the osteoblast differentiation process is regulated by a large number of growth factors [7]. The family of bone morphogenetic proteins (BMPs) is known to initiate osteoblast differentiation from uncommitted precursors. Among this family,

BMP-2 has a well-known role in the process of osteogenic differentiation as well as in the maintenance of bone homeostasis and bone regeneration [8]. In this thesis, ALP, OC, OP, BSP and BMP-2 are considered as valuable markers of osteogenic differentiation.

1.2 Bone repair

As a living material, bone tissue possesses a fascinating self-healing capacity. However, the magnitude of a defect can sometimes exceed this capacity of regeneration. Nonunion fractures as a result of trauma, tumor removal and infections can for instance lead to critical size defects. Bone grafting is then the preferred treatment. This treatment is also used to increase the bone volume during surgical procedures such as maxillary augmentation, cleft palate repair and spinal fusion. An estimated 2.2 millions bone grafting procedures occur every year in the world [9].

1.2.1 Natural bone grafts

Natural bone graft materials can originate from three different sources: the patient (autologous), other humans (allogeneic) and animals (xenogeneic). Autologous bone is considered the gold standard of bone graft materials because it possesses the following properties:

- Osteogenicity, which refers to the capacity to form new bone. It is directly related to the presence of osteoblasts and osteoprogenitor cells [10].
- Osteoconductivity, defined as spreading of bone over the surface preceded by ordered migration of differentiating osteogenic cells [11].
- Osteoinductivity, being the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteogenic cells [12]. Osteoinduction is usually demonstrated by de novo bone formation in the area where bone does not naturally occur, i.e. at heterotopic sites.

Autologous bone also presents the advantages of being resorbable, possessing satisfactory mechanical properties and not causing an immunogenic response. However, the use of autologous bone as graft material also implies longer operations, as healthy bone tissue needs to be harvested, and potential risk to the patient with surgical complications such as chronic pain and infection. And most importantly, the availability of autologous bone is limited, which is a serious concern considering an increasing demand for bone regenerative strategies as a consequence of population ageing. While availability is not an issue with

allogeneic and xenogeneic grafts, their clinical outcome is variable as they have to undergo physico-chemical treatments to reduce the risk of disease transmission and immunogenicity.

1.2.2 Synthetic bone graft substitutes

To overcome the limitations of autologous bone grafts, research effort have focused for decades on development of synthetic substitutes. An important advantage of synthetic materials is that they can be produced in large quantities and stored to be readily available without issues related to stability. Furthermore, when using synthetic bone graft substitutes, a single surgical procedure is sufficient, and there is much lower risk of disease transmission or immunogenic response. The properties of synthetic bone graft substitutes are defined by their design and should ideally include biocompatibility in a given application, osteoconductivity, osteoinductivity and in vivo degradability. In the case of load-bearing applications, synthetic substitutes should also provide the proper mechanical support. Synthetic bone graft substitutes have been clinically available for decades but they are only used in 10% of the procedures requiring bone grafts and they still have to be improved to match the results obtained with autologous grafts [13]. While all material classes, i.e. ceramics, metals, polymers, and combinations thereof in the form of composites and mixtures have been used for bone regenerative strategies, CaP ceramics are the best example of successful bone graft substitutes as they have already been extensively used in dentistry and orthopedics for the past four decades [14, 15]. The rationale behind the use of CaP's as bone graft substitutes is their close resemblance with natural bone mineral. Besides, the ability of a material to mineralize in vivo or upon immersion into a simulated body fluid is considered an important indicator of its bioactivity in bone regeneration, as was demonstrated for osteoinductive metal [16] and polymer [17]. In vivo precipitation of a biological apatite layer on an implant surface changes both the chemical and topographical properties and is believed to play a paramount role in the material bioactivity.

1.3 Design of synthetic bone graft substitutes

The ideal synthetic bone graft substitute should provide optimal environment and right signals for the process of regeneration to be initiated and successfully completed. Therefore, modern design of synthetic bone graft substitutes should rely on the comprehension of how individual material parameters can initiate and stimulate bone formation and on the control and combination of these parameters to optimize their effects on bone growth. The cues to trigger a biological response can in general be divided into chemical and physical cues.

1.3.1 Chemical cues

The chemical composition of a synthetic material is of prime importance for its applicability, as it will largely determine whether the material will be well accepted by its environment upon implantation, i.e. whether the material is biocompatible at a given implantation site. For instance, the composition of CaP ceramics is very similar to that of bone inorganic phase, which ensures the biocompatibility of this class of materials. Chemical composition also determines the degradation properties of the material, which can occur passively via physico-chemical dissolution and/or actively by the action of osteoclasts and macrophages [18]. Ideally, the degradation rate of a synthetic bone graft substitute, intended for regeneration of a bone defect, matches the rate of formation of new bone tissue to ensure continuous support and provide space for tissue growth. The release of degradation products is a simultaneous consequence of degradation. For all synthetic materials it is important that degradation products do not trigger a strong inflammatory response. In the case of CaP ceramics, degradation products consist of free calcium (Ca^{2+}) and inorganic phosphate (Pi) ions, the building blocks of bone inorganic phase. Apart from being building blocks of bone mineral, these two ions play a more complex role in processes related to bone formation and remodeling. Ca^{2+} , for instance, has been shown to induce a signaling cascade in human mesenchymal stromal cells that led to their osteogenic differentiation [19]. Pi has also been shown to influence osteoprogenitor cell fate, promoting osteogenic differentiation at specific dosage [20]. The degradation and release properties of bone substitute materials can be further exploited by the addition of compounds with therapeutic potential such as growth factors and bioinorganics. BMPs, for example, have been shown to enhance the osteoinductive potential of CaP ceramics, as BMPs can bind to CaP while remaining active [21]. However, the addition of BMPs to a graft substitute increases its price and generates a risk of uncontrolled bone growth. Therefore, the interest in bioinorganics as a synthetic and potentially safer alternative to biological growth factors is increasing as a method to improve the biological performance of bone graft substitutes, while retaining their synthetic character. For example, recently, the positive effect on bone formation of the addition of zinc and of zinc and strontium to β -tricalcium phosphate cement was qualitatively observed in a porcine tarsal bone model in a pilot study [22]. The chemical composition of bone graft substitutes is not the only parameter that can affect cells on a chemical level as implant design, overall geometrical properties such as porosity and surface structural properties including roughness and topography also play an important role in the events occurring at the interface of the material and the biological system. These parameters determine the specific surface area, which affects degradation and related ion release properties as well as the adsorption of endogenous proteins. Design and porosity also create a sheltered environment that modifies the diffusion and concentration profiles of compounds present or released in body fluids, which in

turn can affect the local biological response. It was, for example, demonstrated that ceramic material-induced heterotopic bone formation is predominantly observed inside pores, channels and concavities and rarely on the implant periphery [23, 24].

1.3.2 Physical cues

More recently it has been shown that the structural parameters of materials also provide mechanical signals that have the potential to directly control cell fate, a phenomenon called mechanotransduction. For instance, the mechanical properties of a material (e.g. stiffness), a bulk property of a material that depends on its chemical composition, has been shown to affect lineage commitment [25]. Surface properties such as microporosity, specific surface area and topography also have an impact on the cell response to a material, independently of the chemical composition or indirectly related to it. Indeed, an increase in microporosity and specific surface area of a ceramic has been shown to lead to a higher osteoinductive potential of calcium phosphate ceramics with identical chemical composition [26, 27]. In an attempt to understand and control the mechanisms behind mechanotransduction, a few elegant approaches to design surface topographies are currently being explored [28, 29].

1.3.3 Rational design versus processing-driven materials development

Listing the different material parameters and their effects on a biological process also unravels the complex links that bond these parameters to each other. Some material properties are largely interdependent in their nature. For example, an increase in surface microporosity of a CaP ceramic does not only mean a change of a physical property of the material, but it also means a change in the specific surface area, and therewith degradation properties and consequently the release of Ca^{2+} and Pi ions. Other parameters are simultaneously affected by changes in the material synthesis process. For instance, changes in sintering conditions of a CaP ceramic do not only affect the surface micro- and nano-structure, but also the mechanical properties. Another example is the incorporation of a bioinorganic such as fluoride into a CaP ceramic, that not only means a change in chemistry, but also a modification in crystal lattice, and thus surface structural and mechanical properties [30]. It is therefore very difficult to describe the exact relationship between a single material property and a biological response. Furthermore, the conventional approach towards biomaterials development, that is largely material synthesis/processing-driven strongly hampers significant advancements in the biomaterials field. Therefore, in the current thesis, a modern biomaterial design approach is explored, where the material properties are first decoupled to better understand and control their role. A combinatorial approach is then followed to create a new generation of optimized functional biomaterials, whereby different material types and different techniques are employed to this end.

1.4 Outline of the thesis

The general aim of this research is twofold: (1) to explore strategies to isolate individual properties of complex biomaterials in order to investigate their independent effects on a biological response and based on this knowledge (2) to recombine individual properties into new, improved functional biomaterials. For the majority of the experiments, composite materials combining a polymeric and a ceramic phase were used and biological response was studied either *in vivo*, or *in vitro* using clinically relevant bone marrow-derived hMSCs.

In Chapter 2, an overview is given of strategies employed to design and develop biomaterials for application in regenerative medicine. A changing clinical need is discussed as a consequence of demographical changes, as well as the consequences of this change on the way biomaterials are designed, developed and evaluated.

Chapter 3 presents an *in vivo* study in which osteoinductive potential of a polymer/ceramic composite is investigated, with emphasis on the importance of porosity, or “sheltered” areas in the process of biomaterial-triggered osteoinduction. To this end, extrusion of the composite was used, to overcome issues related to solvent-based processing procedure, and, since extrusion does not allow direct formation of porous implants, an innovative implant design was developed to create artificial “sheltered” space.

Chapter 4 focuses on the independent chemical effects of calcium and inorganic phosphate ions in the process of bone regeneration. Instead of using classical composite materials consisting of a polymer and a CaP ceramic, the ceramic filler was replaced by only a calcium- or only a phosphate salt. Attachment, growth and osteogenic differentiation of clinically relevant hMSCs was studied. In parallel, the experiments were performed on tissue culture plates in Ca^{2+} - or Pi - conditioned medium, to isolate the chemical effect from the effect of the polymeric carrier.

In Chapter 5 a practical challenge is undertaken to decouple and later recombine chemical from geometrical properties of biomimetically grown CaP crystals. As tools, crystal precipitation process, nanoimprinting and various coating techniques were applied, and hMSCs were investigated for their response on different material properties.

In Chapter 6, the biomimetic approach towards control over geometrical properties was replaced by a rational topography design. The effect of the selected surface topographies imprinted in a polymer/ceramic composite material was studied on the behavior of hMSCs, as well as the effect of the molecular weight of the polymer, that in turn affects the release of Ca^{2+} - and Pi ions from the composite material.

Finally, Chapter 7 contains a general Discussion of the results obtained from different studies in the thesis, as well as recommendations for future work.

References

1. Marks SC, Hermey DC. The structure and development of bone. In: Bilezikian JP, Raisz LG, Rodan GA, editors. *Principles of bone biology*. San Diego, USA: Academic Press; 1996. p. 3-14.
2. Derkx P, Nigg AL, Bosman FT, Birkenhager-Frenkel DH, Houstmuller AB, Pols HA, et al. Immunolocalization and quantification of noncollagenous bone matrix proteins in methylmethacrylate-embedded adult human bone in combination with. *Bone*. 1998 Apr;22(4):367-73.
3. O'Neill WC. Pyrophosphate, alkaline phosphatase, and vascular calcification. *Circ Res*. 2006 Jul 21;99(2):e2.
4. Roach HI. Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralisation and resorption. *Cell Biol Int*. 1994 Jun;18(6):617-28.
5. Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. *Crit Rev Oral Biol Med*. 2004 Jan;15(3):126-36.
6. Tye CE. Delineation of the Hydroxyapatite-nucleating Domains of Bone Sialoprotein. *J Biol Chem*. 2002 Dec 18;278(10):7949-55.
7. Karperien M, Roelen B, Poelmann R, Groot AG, Hierck B, DeRuiter M, et al. *Tissue Engineering*. null. Elsevier; 2008.
8. Rosen V. BMP2 signaling in bone development and repair. *Cytokine Growth Factor Rev*. 2009 Jan;20(5-6):475-80.
9. Giannoudis P V, Dinopoulos H, Tsiridis E. Bone substitutes: an update. *Injury*. Elsevier; 2005 Nov 11;36 Suppl 3(3):S20-7.
10. Williams DF. *The Williams dictionary of biomaterials*. 1999.
11. Davies JE, Hosseini MM. Histodynamic of endosseous wound healing. In: Davies JE, editor. *Bone Engineering*. Toronto, Canada: Em squared Inc.; 2000. p. 1-14.
12. Friedenstein AY. Induction of bone tissue by transitional epithelium. *Clin Orthop Relat Res*. 1968;59:21-37.
13. Bucholz RW. Nonallograft osteoconductive bone graft substitutes. *Clin Orthop Relat Res*. 2002 Feb;(395):44-52.
14. Hollinger JO, Brekke J, Gruskin E, Lee D. Role of bone substitutes. *Clin Orthop Relat Res*. 1996 Mar;(324):55-65.
15. Habraken W, Habibovic P, Epple M, Bohner M. Calcium phosphates in biomedical applications: materials for the future? *Mater Today*. 2015 Oct; in press.
16. Fujibayashi S, Neo M, Kim H-M, Kokubo T, Nakamura T. Osteoinduction of porous bioactive titanium metal. *Biomaterials*. 2004 Feb;25(3):443-50.
17. Winter GD, Simpson BJ. Heterotopic Bone formed in a Synthetic Sponge in the Skin of Young Pigs. *Nature*. 1969 Jul 5;223(5201):88-90.
18. LeGeros RZ. Biodegradation and bioresorption of calcium phosphate ceramics. *Clin Mater*. 1993 Jan;14(1):65-88.
19. Barradas AMC, Fernandes HAM, Groen N, Chai YC, Schrooten J, van de Peppel J, et al. A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells. *Biomaterials*. 2012 Apr;33(11):3205-15.
20. Chai YC, Roberts SJ, Schrooten J, Luyten FP. Probing the osteoinductive effect of calcium phosphate by using an in vitro biomimetic model. *Tissue Eng Part A*. Mary Ann Liebert, Inc. 140 Huguenot Street,

3rd Floor New Rochelle, NY 10801 USA; 2011 Apr 22;17(7-8):1083-97.

21. Oda S, Kinoshita A, Higuchi T, Shizuya T, Ishikawa I. Ectopic bone formation by biphasic calcium phosphate (BCP) combined with recombinant human bone morphogenetic protein-2 (rhBMP-2). *J Med Dent Sci.* 1997 Sep;44(3):53-62.
22. Pina S, Vieira SI, Rego P, Torres PMC, da Cruz e Silva OAB, da Cruz e Silva EF, et al. Biological responses of brushite-forming Zn- and ZnSr- substituted beta-tricalcium phosphate bone cements. *Eur Cell Mater.* 2010 Jan;20:162-77.
23. Ripamonti U, Crooks J, Kirkbride A. Sintered porous hydroxyapatites with intrinsic osteoinductive activity: Geometric induction of bone formation. *S Afr J Sci. Bureau Scientific Publications;* 1999;95:335-43.
24. Habibovic P, Gbureck U, Doillon CJ, Bassett DC, van Blitterswijk CA, Barralet JE. Osteoconduction and osteoinduction of low-temperature 3D printed bioceramic implants. *Biomaterials.* 2008 Mar;29(7):944-53.
25. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* 2006 Aug 25;126(4):677-89.
26. Habibovic P, Yuan H, van Der Valk CM, Meijer G, van Blitterswijk CA, de Groot K. 3D microenvironment as essential element for osteoinduction by biomaterials. *Biomaterials.* 2005 Jun;26(17):3565-75.
27. Yuan H, Fernandes H, Habibovic P, de Boer J, Barradas AMC, de Ruiter A, et al. Osteoinductive ceramics as a synthetic alternative to autologous bone grafting. *Proc Natl Acad Sci USA.* 2010 Aug 3;107(31):13614-9.
28. Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, et al. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat Mater. Nature Publishing Group;* 2007;6(12):997-1003.
29. Unadkat H V, Hulsman M, Cornelissen K, Papenburg BJ, TruckenmÄijller RK, Carpenter AE, et al. An algorithm-based topographical biomaterials library to instruct cell fate. *Proc Natl Acad Sci USA.* 2011 Oct 4;108(40):16565-70.
30. Yang L. Calcium phosphate coatings for bone regeneration. University of Twente; 2010.

Development of materials for regenerative medicine: from clinical need to clinical application

Abstract

Given the demographic challenges of an ageing population combined with rising patient expectation and the growing emphasis placed on cost containment by healthcare providers, economic regenerative medicine approaches for regeneration of damaged and diseased organs and tissues are a major clinical and socio-economic need. The scope of this chapter is to use skeletal regeneration as the exemplar to discuss classical and high-throughput screening approaches to biomaterials development for regenerative medicine, including choice and design of materials based on clinical need, biological assessment and regulatory issues.

2.1 Basic principles: development of materials for regenerative medicine

The increase in an ageing population in developed countries is accompanied with a growing need for replacement and repair of damaged organs and tissues. Transplantation of the patient's own tissue is still considered the gold standard in many applications, but limited availability and complications associated with harvesting of the so called autograft, are becoming an important drawback. Tissues and organs from human or animal donors present issues of disease transmission and functional failure. Alternative strategies, based on biological growth factors, cell therapy and tissue engineered constructs are explored as alternatives to the patient's own tissue, but their use is hampered by biological instability and high costs. These issues demonstrate the need for strategies based on biomaterials, which are often synthetic, and thus less prone to instability problems. In addition, the fact that (synthetic) biomaterials can often be produced in large quantities, and thus be off-the-shelf available is an important advantage when coping with an increasing need for regenerative approaches.

Following a consensus by experts in the field, a biomaterial is defined as a "nonviable material used in a medical device to interact with biological system" [1]. A number of other, non-medical applications also include an interaction between a material and a biological system; for example materials used to produce diagnostic gene arrays or equipment to process biomolecules in various biotechnological applications and can therefore be considered biomaterials. However, the focus of this chapter is to describe the process from synthesis of a material to clinical application and to discuss how high-throughput screening of materials can make such a process more efficient.

2.1.1 The changing role of materials in medicine

Historically, the primary application of materials in medicine was in implants, the role of which was to replace and take over the function of a diseased/damaged organ or tissue. From this perspective, the choice of materials for and design of implants are such that the natural organ/tissue that needs replacement is mimicked as closely as possible. The implant needs to be accepted by the *in vivo* surrounding and perform its function, without actively affecting the functioning of other organs or tissues. Heart valve prostheses, for example, which can be made from various materials including stainless steel and carbon, instantly recover cardiac function upon implantation. The use of intraocular lenses, used to replace a natural lens when it becomes cloudy due to cataract formation are made of poly(methyl methacrylate), silicone rubber or a hydrogel and their insertion results in immediate restoration of vision (see also classical experiments). Dental implants, usually made of titanium, provide an artificial tooth anchor upon which

a crown is placed. Hip joint prosthesis is a good example of an implant where different materials need to be combined in order to restore the function of the natural joint: a metallic stem is required in order for the implant to withstand high levels of mechanical stress, a polymeric liner allows for smooth movement of the femoral head inside the acetabular cup, and PMMA cement is needed to fix the stem inside the femur upon placement [2]. This is to demonstrate that the complexity of the organ or tissue that needs to be replaced determines the complexity of the implant both from raw material and implant design perspective.

Classical experiment

Development of intraocular lens

Sir Harold Ridley, M.D. (1906–2001) was a pioneer of intraocular lens (IOL) development. IOL are meant to replace the crystalline lens of the eye in patients suffering from cataract, which is a clouding of the lens leading to partial or complete blindness. Surgery procedures for this condition were developed as early as in the ancient times, but the sole removal of the crystalline lens resulted in a blurry and unfocused vision. In the 1930s, Harold Ridley, an ophthalmic surgeon, recognized the need for a synthetic lens. After World War II, Ridley treated injured pilots from the Royal Air Force, and observed the interesting consequences of fragments of Hurricane cockpit canopies lodged in the eyes of the pilots. He noted that no reaction had been triggered by the fragments and that the material seemed to be well tolerated by the human eye. Ridley's observations would today relate to the concept of biocompatibility. He researched the canopy material, which was poly(methyl methacrylate) (ICI Perspex, also known under the trademark name of Plexiglas), and used this material to design the first IOL. In 1949, Ridley performed his first implantation of IOL in a cataract patient at Saint Thomas Hospital in London. Although the outcome of the surgery was very encouraging, he faced a strong opposition from the medical community that fiercely disapproved implantation of a foreign material in the eye. Between 1949 and 1966, approximately a thousand of IOL were implanted by Ridley in Europe and in the United States in order to prove the robustness of the technique. The success rate of these surgeries was evaluated to be around 70%. However, it was not until the 1980s that this technique was accepted as a general treatment of cataract. Nowadays, tens of millions of IOL are implanted each year, with a successful restoration of sight in cataract patients. Ridley's talent and merit were first acknowledged in 1986, with his election to the Fellowship of the Royal Society, and in 2000, Harold Ridley received the honour of being knighted by Queen Elizabeth II [3, 4].

Figure 2.1: Examples of prosthetic heart valves: a) Caged-ball (Starr-Edwards) valve (1960). This artificial valve proved to have an excellent durability; however, it also triggered a very high thrombogenicity.⁽⁵⁾ b) Trileaflet valve scaffold, made of a polyglycolic-acid / poly-4-hydroxybutyrate composite, seeded with myofibroblasts and endothelial cells. The construct was implanted in an ovine model for 20 weeks and showed promising results: the scaffold is able to provide support and guidance for tissue growth and to resorb to leave place to a regenerated valve [6].

Current strategies in treatment of damaged organs and tissues require a more active role from implanted materials. Instead of, relatively passively, replacing and thus taking over the role of an organ or tissue, the goal is now to repair the natural tissue therewith restoring its function. This regenerative medicine approach, poses different requirements to the biomaterials to be implanted. They have to actively facilitate new tissue formation and, in some cases, to even instruct the *in vivo* environment to exert a certain function. Such an active role is often associated with changes in the material itself after implantation, which needs to be predicted before the materials can be used in the clinic. The changing role of materials in regenerative medicine is illustrated in Figure 2.1, in which two examples of prosthetic heart valves are shown.

2.1.2 Classical approach to materials development

Independent of whether a material will be used in a “passive” device, or as a tool to regenerate an organ or tissue, it is evident that a number of choices need to be made before and during development. These choices are in the first place determined by the role the material needs to play in the body. In addition, the process of implantation itself, that needs to be efficient, safe and minimally

invasive for the patient will also determine certain properties of the materials. In bone regeneration, for example, the biomaterial should be able to initiate and/or facilitate new bone formation and to (temporarily) provide mechanical support to defected bone [7]. Constructs for treatment of peripheral nerve injuries should possess an oriented morphology, in order to guide regeneration of a damaged nerve [8]. Materials used in peripheral stent grafts should provide a mechanical barrier to prevent intravascular pressure from being transmitted to the weakened wall of the aneurysm [9]. From this it is obvious that development of a biomaterial is a highly multidisciplinary process, where knowledge of materials science, engineering, biology and anatomy needs to be combined, while taking into account requirements from the clinic.

By taking development of bone graft substitutes as an example, we will now discuss the classical process of biomaterials development, based on rational design, including the material choice and design, pre-clinical *in vitro* and *in vivo* tests of safety and functionality, and regulations that are important in order to bring a material to clinical application.

Material choice and implant design

As mentioned before, what determines the properties of a material intended for implantation are the characteristics of the tissue/organ that needs to be regenerated and the function the material needs to fulfil. The role of synthetic bone graft substitutes is to restore a bone defect that is caused by e.g. trauma, tumour removal, extensive resorption or congenital diseases.

Bone is a highly specialized form of connective tissue that is nature's provision for an internal support system in higher vertebrates. Bone provides for the attachment of muscles and tendons essential for locomotion, protects the vital organs of the cranial and thoracic cavities, and it encloses the blood forming elements of the bone marrow. In addition to these mechanical functions, bone plays an important metabolic role as a store of calcium and phosphate, which can be drawn upon when needed in the homeostatic regulation of calcium and phosphate in blood and other fluids of the body [10]. By weight, bone contains approximately 60% mineral (mainly type AB carbonated calcium-phosphate apatite), 10% water and about 30% organic matrix (predominantly type I collagen, as well as proteoglycans and non collageneous proteins, such as osteocalcin, osteopontin, osteonectin, bone sialoprotein, decorin and biglycan) [11, 12]. Morphologically, there are two forms of bone: cortical (compact) bone and cancellous (trabecular) bone. Compact bone, which is rigid and dense, is found mainly in the middle shaft of long bones or shells of other bones. Found predominantly in epiphysis, ribs and spine, cancellous bone has a highly porous structure (>75%), with numerous small bone trusses or trabeculae interconnected with each other, and tends to orient along the principal directions in adaptation to the external loading environment. In the formation of the skeleton, mesenchymal cells aggregate

and form condensates of loose mesenchymal tissue, prefiguring the skeletal elements. Within these aggregates, cells may differentiate into osteoblasts, when in association with adequate vascularisation, thereby directly initiating ossification, which eventually results in either compact or cancellous bone (intramembranous bone formation). Alternatively, condensates of mesenchymal cells can differentiate into chondrocytes in an avascular environment, producing cartilage which is eventually replaced by bone (endochondral bone formation) [13].

The role of a bone graft substitute is to aid repair of a bone defect or to restore bone volume. Therefore, a bone graft substitute should be osteoconductive, meaning that it should allow migration of potentially osteogenic cells at the site of (orthotopic) implantation [14], which eventually results in deposition of bone on the surface of a bone graft substitute. Ideally, a bone graft substitute should also be osteoinductive, a process defined as the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells [15], meaning that the material should induce bone formation. In order to restore the function of defected bone as efficiently as possible, the process of bone formation and the resulting structural properties of the newly formed bone should be equal, or at least similar to what is occurring in the body. For example, when a bone graft substitute is used to treat a calvarial defect, bone formation should follow the direct pathway, whereas for the repair of a long-bone, for example femoral defect, the pathway of bone formation should be endochondral. Similarly, bone formed in a femoral defect should be cortical, in contrast to the bone found in the interior of vertebrae, which is predominantly cancellous, which will also determine its mechanical properties.

In classical, candidate approaches towards biomaterials development, a logical starting point is to mimic the composition of natural tissue. As mentioned before, the mineral component of bone is a calcium phosphate ceramic, and it is therefore not surprising that the most widely applied bone graft substitutes are based on calcium phosphates [7]. The exact composition (for example calcium to phosphorus ratio, presence of other additives such as fluorine, magnesium, etc.) varies, which determines properties of these materials such as degradation rate and mechanical strength, but the rationale in all cases is to mimic bone mineral or its precursors. That such a biomimetic approach is logical, is witnessed by the fact that the majority of calcium phosphate ceramics are biocompatible and bioactive in terms of osteoconductivity, and in some cases even osteoinductivity [16].

Besides chemical composition, other physical properties, such as macro- and microstructure are often mimicked too. In the majority of calcium phosphate ceramics, one aims at introducing an interconnected porous structure into the material, which allows infiltration by cells, blood vessels, and therewith nutrients and oxygen as well as in growth of *de novo* tissue. A great number of studies are therefore focused on determining optimal pore size, importance of interconnectivity, and methods to obtain these [17]. Finally, surface properties are often used as

a tool to control interactions of the material with its *in vivo* environment. For example, roughness of the surface, its size, presence of micro- and nanofeatures are all of importance in determining initial contact interaction with body fluids, including immune response, protein adsorption, as well as subsequent cell proliferation, differentiation, and matrix formation [18, 19, 20].

Apart from these material properties, which are designed in such a way that upon implantation they lead to bone formation that is similar to the natural process, there are also needs or wishes from the clinicians that should be satisfied. For example, in many cases, an orthopaedic or maxillo-facial surgeon would prefer a bone graft substitute that is mouldable or injectable. This is why calcium phosphate cements have been developed [21], as well as why there is much research performed on mixing granules of calcium phosphate ceramics, with polymeric gels, etc. to allow injectability [22, 23]. The restrictions that are associated with such modifications are that it is much more difficult to introduce pores into cement, and besides, the size of ceramic granules needs to be modified to allow injectability. Thus, a compromise is to be reached. Furthermore, while ceramic materials based on calcium-phosphates perform very well *in vivo*, they are intrinsically brittle, which means that they cannot be used in loaded applications, which of course is an important disadvantage, considering the load bearing functions of bone. In order to improve mechanical properties, other materials, for example polymers, or ceramic/polymer mixtures are considered, but this usually is a guarantee for a decrease in bioactivity.

All this is to demonstrate how difficult it is to reproduce the complexity of natural tissue and that often many choices and compromises need to be made along the way of material development. This also shows why, despite over 60 years of intensive work on synthetic bone graft substitutes, there is still not yet a truly comprehensive alternative to patient's own bone.

Biological assessment of biomaterials

Once a material is designed and developed, a lengthy process of tests needs to be followed in order to bring the potential bone graft substitute to the clinic. In general, this process starts with preclinical *in vitro* assays using a cell - or an organ culture system. It should be emphasized that classical *in vitro* assays have initially been developed to study the influence of growth factors and hormones on attachment, proliferation, differentiation and mineralization of cells for example. These assays have been implemented in the field of biomaterials research without significant modifications. What is, however, often ignored is that the *in vitro* setting may significantly be changed by the presence of a material due to e.g. material-cell culture medium interaction and that this change is highly dependent on the type of material tested. As a consequence, it is difficult to distinguish between the true responses of cells to a material from the unwanted response of cells to the changes in the cell culture environment [24]. Needless to say, *in vitro* assays are attractive

because of their simplicity, but, at the same time, this simplicity is an important limitation, in particular because presence of a material adds to the complexity of the cell culture system.

In vitro cell- and organ culture assays are in the first place used to investigate the “safety” of the material in terms of cytotoxicity and biocompatibility. Three main cell culture assays are used for evaluating biocompatibility: direct contact, agar diffusion and elution, and the L-929 mouse fibroblast cell line has been most extensively used for testing biomaterials. In addition to the tests that focus on safety aspects of biomaterials and are independent of the intended application of the material, *in vitro* bone formation assays are used to predict the performance of the material *in vivo* in its role of a bone graft substitute, meaning that one aims at getting an idea about osteoconductive and osteoinductive potential of the material. For this, again, a rational choice for a cell type and cell culture conditions is made in an attempt to mimic the *in vivo* environment. In the case of bone graft substitutes, this often means that one uses osteoblasts (either as cell lines such as MC3T3-E1 or MG63, or primary osteoblasts) and mesenchymal stromal cells, known to possess the ability to differentiate into the osteogenic lineage and form bone, as a model of choice. With components of cell culture medium, specific stimuli, acting on osteogenic differentiation or mineral production are added in order to simulate various phases of bone formation. Read-out of such assays is usually cell proliferation, expression of markers of osteogenesis at gene and protein level, production of extracellular matrix and mineral formation. Besides these assays which are directed to study potential bone forming capacity, other assays relevant to bone formation and remodelling, such as angiogenesis (new blood vessel formation) and osteoclastogenesis (degradation by bone-resorbing cells) are performed too, with relevant cells types (endothelial cells and osteoclasts, respectively) [24]. Similar to orthopaedic research, functional *in vitro* assays with either primary cells or cell lines are used to test applicability of materials in other applications. For example, the ability of primary chondrocytes to retain their chondrogenic phenotype and the ability to produce type II collagen in culture is an important assay in cartilage research [25]. In cardiac research, the ability of cells to differentiate into functional and beating cardiomyocytes is of great relevance for treatment of infarcted myocardium [26]. An example of a relevant assay in nervous system regeneration research is the ability of Schwann cells to provide a growth substrate to central nervous system axons [8].

While *in vitro* assays present a relatively easy way to test biological performance of potential bone graft substitutes, the truth is that they are too simple and therefore often fail to predict the *in vivo* behaviour of the materials. This is why the next step in testing these materials is pre-clinical *in vivo* studies involving animals. As reviewed by Jansen [27], the first test following *in vitro* assays is *in vivo* compatibility of materials for short and prolonged periods of time. Soft tissue implantation is an attractive model to study safety of the materials in terms of e.g.

toxicity and carcinogenicity, as it is rather inexpensive, readily available and yet relevant, as many materials used as bone graft substitutes come in contact with subcutaneous tissue, muscles, fasciae and tendons. The two most frequently used soft tissue models are subcutaneous and intramuscular implantation. It has been shown that the biocompatibility response of implant materials can differ between the two test sites, due to differences in vascularisation, regenerative capacity and intrinsic stress. The selection of a suitable animal for biocompatibility testing is another complex issue. Mice, rats and rabbits are most often used for soft tissue implantations. The advantage of these, relatively small animals is their availability and low cost. However, their metabolic and wound healing properties differ from those of large animals and humans. In addition to testing safety of biomaterials, soft tissue models are needed to study osteoinductive potential materials, as a true proof of osteoinductivity of bone formation at heterotopic sites. An and Friedman gave an overview of the frequently used soft tissue models (e.g. subcutaneous, intramuscular, intraperitoneal and mesentery) to assay osteogenicity prior to orthotopic implantation [28].

Finally, functional *in vivo* tests are performed to test the capacity of a bone graft substitute in its true role, thus the ability to close a bone defect, help regenerating bone and restoring its function. Four types of defect are typically used: calvarial-, long bone (or mandible) segmental-, partial cortical- (e.g. cortical window, wedge defect, or transcortical drill hole) and cancellous bone defects. Various animals are used for these functional assays, including rat, sheep, dog, goat and non-human primates, and ideally, bone metabolism and mechanical loading should be highly representative for those found in humans. An elegant overview of different animal models of bone defect repair are reviewed by An and Friedman [29]. While bone metabolism and mechanical loading of the skeleton determine which animals are most suitable for testing materials in orthopaedic applications, in biomaterials intended for regeneration of other organs and tissues, other characteristics of an animal are more relevant. In cardiac research, for example, cardiovascular physiology, ease of vascular access and low body fat have made canines the model of choice for cardiac and peripheral vascular studies for many years, although more recently, swine and sheep have been used too [30]. To study repair of articular cartilage defects, the thickness of articular cartilage in, for example femoral condyle, the thickness of the defect as well as joint size and loading conditions determine the suitability of an animal model. Regarding the thickness of cartilage and the volume and nature of the defect, the horse shows the best resemblance with humans. Regarding the loading, goats and sheep seem a more suitable model [31].

From R&D results to clinical application

Biomaterials are subjected to a regulatory process, similar to that of therapeutic drugs, before they are allowed to be marketed. This process is conducted by the

manufacturer, who has to provide sufficient evidence regarding the biomaterial safety and efficacy in the intended application, to the regulatory authority. For the U.S. market, the Food and Drug Administration (FDA) is responsible for the evaluation of the applications. To help the manufacturers building their report - also called 510(k) submission, the FDA provides guidelines on tests that need to be performed to provide the required data (e.g. FDA documents for bone void and dental bone grafting devices). There are no specific or quantified requirements to meet. However, it is advised to establish a detailed comparison between the new biomaterial and a material already marketed or approved.

Depending on what is already known about the biomaterial entering the regulatory process, the manufacturer has to choose between a traditional, an abbreviated or a specific 510(k) submission (see The New 510(k) Paradigm, Figure 2.2. For a traditional submission, requirements are described in 21 CFR 807, subpart E. An abbreviated submission has to include a description of the device and its intended use, a description of the device design and performances, an identification of the risk analysis method, and a discussion of the device characteristics. For specific submissions, precise guidelines are provided; “Class II Special Controls Guidance document: Resorbable Calcium Salt Bone Void Filler Device”, for example, gives a list of physical properties (porosity, size, shape, surface area, mass to volume ratio) and chemical analysis (X-ray diffraction and Fourier transform infrared spectroscopy) to be provided. It also integrates animal testing to evaluate performances such as bone formation, biomaterial resorption and mechanical properties. To facilitate the evaluation of 510(k) submissions, FDA advises the use of standards, such as ISO-10993 “Biological Evaluation of Medical Devices”, or ASTM – American Society for Testing and Materials standards, which give an insight on how to perform the different tests.

2.2 Relation to materiomics: High-throughput screening of libraries of polymers produced using parallel chemistry to large animal studies and beyond

The lengthy and expensive process from material design to clinical trial involves a number of assumptions and choices along the way, some of which may be incorrect, meaning that there is a fair chance that potentially excellent bone graft substitutes will be missed. In the absence of iterative and high throughput robust analysis the current portfolio of bone graft substitutes has remained remarkably unchanged over a number of years. Current research strategies therefore aim at replacing the traditional “one-sample for one-measurement” approach to biomaterial evaluation by high-throughput (HT) and combinatorial chemistry strategies. In conjunction with polymer microarray platforms, these have streamlined the process of material discovery, synthesis and screening for

Figure 2.2: The new 510(k) paradigm showing the regulatory process for obtaining FDA approval for a medical device.

skeletal tissue regeneration applications including mesenchymal stem cell (MSC) isolation, growth and differentiation. In general, HT screening of materials is expected to improve the efficiency of materials development, simply by providing the possibility to include more variations in the material properties and to combine these variations in order to build the optimal material for intended application. In addition, the application of a HT strategy for evaluation of materials is particularly promising when it comes to developing *in vitro* assays with a higher predictive value for the *in vivo* or even clinical functioning of a bone graft substitute. HT screening of materials for bone graft substitutes is still at a relatively early stage, and in the following sections, we will elaborate on our experience with regard to HT screening of materials intended for orthopaedic applications.

We have examined the efficacy of a large number of polymer blends, generated by mixing different combinations and ratios of well characterised polymers including binary and tertiary polymer blend formulations, to support the attachment, growth and osteogenic differentiation of human skeletal stem cells for skeletal repair. We used a combination of a high-throughput approach for material formulation along with a microarray platform to analyse over 135 binary polymer blends and an array of tertiary polymer blends for their ability to function as biocompatible matrices for a variety of human skeletal cell populations. The binary mixture of poly (L-lactic acid)/ PLLA and poly (ϵ -caprolactone)/ PCL (blended in a ratio of 20%:80%) exhibited a high binding affinity for STRO-1+ skeletal stem cells, as identified by microarray screening, and also by scanning electron microscopy (SEM) demonstrated a remarkable bone-like 3D architecture. To examine their bone regenerative potential, 3D scaffolds of polymer blends permissive for skeletal cell attachment were seeded with human bone marrow STRO-1+ skeletal stem cells. In the murine femoral segmental defects, STRO-1+ skeletal stem cells seeded on the PLLA + PCL polymer blend scaffolds demonstrated significant bone regeneration. This approach demonstrates multidisciplinary approaches that integrate high-throughput polymer blend and microarray analysis for skeletal tissue regeneration for de novo tissue formation using skeletal progenitor and stem cell populations has clear potential to improve the quality of life for many, and emphasizes the need for interdisciplinary strategies to achieve with significant therapeutic outcome.

2.2.1 Application of HT combinatorial polyurethane libraries for isolation of MSCs

Conventional approaches such as fluorescently-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) for isolation of desired cell types are often time-intensive, require expensive instrumentation and trained personnel. In an alternative approach, a library of 120 comprehensively characterised polyurethanes (PUs) were contact printed in a microarray format on an agarose-coated standard microscope slide, and screened for the identification of PUs capable of bone

marrow-derived MSC attachment and immobilization [32]. This approach allows the effective and rapid generation of detailed structure-function relationships since the high multiplexing ability of the microarray enables functional screening of all 120 PUs for MSC attachment and immobilisation under identical conditions in a single experiment.

The approach adopted a simple yet effective protocol involving overnight incubation of the MSC-enriched STRO-1+ population of human bone marrow on the PU microarrays and identified STRO-1+ cell binding to 31 PUs from the library of 120 PUs. These 31 PUs could be categorised as showing low, moderate and high-affinity on the basis of 2-7, 15-25 and 80-120 STRO-1+ cells bound to individual PU spots of the microarray respectively. From the complete library of 120 PUs, only 4 PUs, namely PU-16, PU-17, PU-61 and PU-71, were identified as high-affinity PUs and determined to be highly selective for the STRO-1+ population of adult human bone marrow (incapable of binding to the STRO-1+ fraction of human foetal skeletal cells and STRO-1 immunoreactive early osteoblast-like human MG63 cells). Furthermore, the 4 high-affinity PUs were able to selectively immobilise STRO-1 immunolabelled cells from a heterogeneous population of freshly isolated bone marrow-derived mononuclear cells, thereby demonstrating the significant methodological implications of applying these PU substrates in the development of facile strategies for the isolation of MSCs from human bone marrow.

2.2.2 Application of HT polymer blend libraries for augmentation of MSC growth and differentiation

Polymer blending refers to the judicious mixing of two or more polymers to yield new composite materials (e.g. binary blends, ternary blends etc.) with unique properties and functionalities that are often not accessible from the individual polymeric compounds themselves. In an attempt to develop distinctive polymeric biomaterials for skeletal tissue engineering applications, a HT approach was employed to generate 135 binary polymer blends by mixing different combinations and ratios of 7 commercially available, inexpensive and comprehensively characterised polymers, namely polyethylenimine (PEI), chitosan (CS), poly(L-lactic acid) (PLLA), poly(caprolactone) (PCL), poly(ethylene oxide) (PEO), poly(vinyl acetate) (PVAc) and poly(2-hydroxyethyl methacrylate) (PHEMA) [33].

The 135 pre-mixed binary blends along with the 7 individual polymers were contact printed onto agarose-coated standard microscope slides in a microarray format for rapid identification of composite materials capable of supporting attachment, growth, viability and differentiation of a variety of human skeletal cell populations including primary MSCs. Following overnight incubation with human bone marrow-derived STRO-1+ MSCs on the microarray slides, total number of cells immobilised onto each polymer spot was determined using a high content

imager (PathfinderTM software) and the data was analysed to identify STRO-1+ MSC-compatible polymer blends. The analysis demonstrated immobilisation and attachment of STRO-1+ cells on microarray spots of PLLA/PCL, PEI/PEO, PEI/PHEMA, PEI/PVAc and CS/PEI binary polymer blends, from which the PLLA/PCL binary blend was selected as the “hit” composite material for further studies since it exhibited distinctive characteristics conducive for MSC attachment, growth and skeletal tissue engineering.

Classical experiment

Development of Bioglass for bone regeneration

In 1967, Prof. Larry Hench made the seminal discovery of Bioglass, the first synthetic material able to bond to bone. He was then assistant professor at the University of Florida, working on semi-conducting glass ceramics resistant to high energy radiation for satellite electrical systems. On his way to present his findings at the U.S. Army Materials Research Conference in Sagamore, he made the acquaintance with colonel Klinker, who had just returned from Vietnam. After having given a brief description of his work to the colonel, Hench was surprised to see himself challenged by Klinker: “If you can make a material that will survive exposure to high energy radiation, can you make a material that will survive exposure to the human body?”. The colonel depicted to Hench the great number of limb amputations performed on the war casualties, and how the injured bodies systematically rejected the metallic and plastic implants - only materials available at that time - used by the surgeons to repair the limb. In 1968, Hench submitted a proposal to the U.S. Army Medical Research and Development Command, with the following hypothesis: “The human body rejects metallic and synthetic polymeric materials by forming scar tissue because living tissues are not composed of such materials. Bone contains a hydrated calcium phosphate component, hydroxyapatite [HA] and therefore if a material is able to form a HA layer in vivo it may not be rejected by the body.”. The project was funded; Hench started to develop a glass containing calcium and phosphate oxide. He provided Dr. Ted Greenlee with rectangular samples of his first glass for in vivo testing in a rat femoral model. After six weeks, Greenlee called - “Larry, what are those samples you gave me? They will not come out of the bone. I have pulled on them, I have pushed on them, I have cracked the bone and they are still bonded.” The 45S5 composition of the first Bioglass has since been extensively studied to understand the bioactive properties of the material. The bone-bonding ability of the Bioglass has been linked to the dissolution /reprecipitation phenomenon occurring at the surface of the material in the presence of body fluids. To date, bioactive glasses are still evolving and are used in clinical procedures such as maxillofacial reconstruction [34, 2].

The polymer blend integrates properties of two polymers, PLLA and PCL, which have FDA-approval for clinical application. Although PLLA and PCL individually displayed negligible STRO-1+ MSC attachment, the cells were able to bind to most microarray spots composed of PLLA and PCL blended in different proportions/ ratios (i.e. 10:90, 20:80, 30:70, 50:50, 60:40). In particular, a significantly high number of cells were bound to microarray spots of PLLA and PCL blended in a ratio of 20:80. Moreover, scanning electron micrographs of the PLLA/PCL (20/80) blend revealed a highly porous and inter-connected 3-D architecture that was comparable to natural trabecular bone porosity. Therefore, 3-D scaffolds of the PLLA/PCL (20/80) binary blend were fabricated to assess whether the composite material supported robust MSC growth and osteogenic differentiation *in vitro* and *in vivo* and, hence was suitable for use in bone regeneration strategies.

Examination of long-term *in vitro* cultures of STRO-1+ MSCs on 3-D PLLA/PCL (20/80) polymer blend scaffolds indicated that the binary blend functioned as an excellent biomimetic template that was able to promote MSC attachment, viability and expression of alkaline phosphatase, an early marker of osteoblast differentiation. Furthermore, PLLA/PCL (20/80) scaffolds supported the generation of mature osteoblasts from the multipotent STRO-1+ MSC population following a 28-day culture period in presence of osteogenic growth factors such as BMP-2 and dexamethasone. *In vivo*, the PLLA/PCL (20/80) scaffolds were able to support the generation of new bone/osteoid to restore the lost bone tissue in critical-size defects created in femora of immunodeficient mice. Although bone regeneration was observed in defects containing PLLA/PCL (20/80) scaffolds alone and scaffolds seeded with MSCs, indices of bone histomorphometry, namely bone volume, bone volume/total volume, trabecular number and trabecular spacing, routinely applied to assess new bone generation were significantly improved in defects containing MSC-seeded scaffolds compared to scaffolds without cells. The *in vitro* and *in vivo* studies therefore demonstrate the suitability of the PLLA/PCL (20/80) scaffold as a temporal substrate supporting the activities of MSCs including viability, migration, proliferation and comprehensive osteogenic differentiation that are fundamental for bioengineering robust skeletal tissues.

2.2.3 Identification, fabrication and testing of candidate polymers for large animal studies

The single most promising binary blend polymer (PLLA/PCL 20/80), as defined by appropriate physical as well as biological properties was selected for large scale fabrication and evaluation within a large animal study. Polymers were fabricated into 18 × 35 mm cylinders by chloroform dissolution and blending, followed by freeze-drying. In order to maximise cell penetration, a central 8mm diameter medulla was drilled and the surface layer and polymer film removed. The overall size was chosen to replicate the approximate requirements in human clinical

Figure 2.3: a) and b) SEM demonstrates pores of between 0.3 and 1.5 mm within the PLLA/PCL scaffold. c) Polymer blend scaffold cylinder prepared for implantation.

practice. Initial large-scale cell permeability tests were performed using negative pressure stain penetration analysis and electron microscopy, to ensure adequate porosity and pore interconnectivity (Figure 2.3).

2.2.4 Identification of the appropriate large animal model

Practical obstacles to *in vivo* scale-up must be addressed to reliably assess new strategies for skeletal tissue engineering before pre-clinical studies can take place. In addition to the requirement to manufacture larger biomaterials and the need to culture cells on a much greater scale than is routine in most research laboratories, further challenges include the establishment of appropriate large animal models for the clinical scenarios to be modelled [35]. Although the bone composition of the dog, sheep, goat and pig is similar to those of humans, the age and rate at which osteonal remodelling occurs varies considerably. Additional key factors include cost, local availability, temperament and husbandry expertise [35, 36]. For skeletal regeneration models, sheep and goats are particularly relevant as their body weight is comparable to humans and the dimensions of their long bones allow for the use of human implants and fixation techniques [36–38]. In the current study, we refined a segmental critical-sized tibia defect model in adult Northern Mule sheep, using an external fixator for stabilisation. External fixation is a common clinical technique for human fractures in long bones and variants are also used in distraction osteosynthesis and bone transport techniques for bone regeneration [39], thus this model was felt to be most appropriate to the human scenario.

2.2.5 Experimental and surgical technique using the candidate polymer

Following cadaveric pilot studies to refine techniques and confirm an adequate projected sample size, 5ml iliac crest bone marrow was harvested from each of 12 sheep under general anaesthetic and with full aseptic precautions and cultured

Figure 2.4: An empty tibia defect with external fixator in situ b) tibia defect with PLLA/PCL scaffold seeded with cells in situ, c) lateral radiograph made immediately post-operatively, demonstrating a 3.5cm tibia diaphyseal defect and stable construct.

in osteogenic conditions (basal culture medium supplemented with ascorbate and dexamethasone). Sheep were divided into 3 groups, each containing 4 sheep: group 1 - empty defect (negative control); group 2 - unseeded scaffold alone (positive control), and group 3 - scaffold seeded with autogenous bone marrow cells (treatment group). Scaffolds were incubated *in vitro* in the absence or presence of 5×10^5 cells/ml in osteogenic medium for 7 days prior to transfer into the *in vivo* ovine segmental defect. After pre-medication, antibiotic prophylaxis, induction and general anaesthetic, a unilateral 3.5 cm tibia defect was created in each sheep, and a scaffold inserted for groups 2 and 3 only (Figure 2.4).

A custom-made external fixator (Orthofix, Maidenhead, UK) was applied using 3 bi-cortical Schanz pins on each side of the defect. The sheep were allowed to fully weight bear with no additional restrictions and radiographs were made immediately post-operatively and at two, six and twelve weeks, at which point the animals were killed and each entire tibia was disarticulated for subsequent CT, histological and mechanical analysis (Figures 2.5 and 2.6)

Despite encouraging results in preliminary *in vitro* and in small animal *in vivo*

Figure 2.5: Representative CT reconstructions of the defect site after 12 weeks *in vivo* ovine incubation with a) empty defect b) polymer scaffold alone c) polymer scaffold and cells. In b) and c) there is evidence of bone formation within the pores of the scaffold and new bone appears to track through the central canal of the scaffold from both proximal and distal bone ends.

Figure 2.6: Quantification of new bone volume at the tibia defect site shows a trend towards increasing bone healing with the scaffold and with cell-seeded scaffold blend.

studies, the use of the PPLA/PCL polymer blend failed to significantly enhance new bone formation in the large animal model as demonstrated by radiological, histological and mechanical analysis. Potential reasons for the failure of this construct in the large scale tests are myriad, including biological, mechanical, surgical, chemical and analytical factors, all of which are subject to significant variation when up-scaling from small animal work and often lead to a disparity between initial encouraging results in laboratory testing and the poorer outcomes of trials in larger species. However, this does highlight the caution that should be exercised before attributing and extrapolating encouraging findings in laboratory tests to the clinical scenario, particularly when implanting heterogenic cells from one species to another and the importance of predictive assays (see chapter of assay development). By necessity, many independent factors vary simultaneously when up-scaling to a large animal study and these must be individually defined and addressed before successful clinical translation can occur. Rather, we present here our approach as an example of a continuous facile process through which candidate biomaterials can be identified, fabricated, screened and tested for a variety of clinically useful attributes prior to potential clinical application. The stage is now set to use a similar approach after careful attention to the vascularisation of this construct and cell seeding and for the analysis of many alternative biomaterial options for tissue regeneration strategies. The pivotal importance of such processes in the development of new biomaterials is clear: putative scaffolds can progress to pre-clinical evaluation only after confirmation of safety and replicable biological and structural efficacy in a large animal model.

2.3 Future perspectives

So far, we have attempted to give an overview of classical approaches towards the development of materials for regenerative medicine and to describe the effects that high-throughput screening of materials may have on this process. It is obvious that screening large numbers of materials in the beginning of the process significantly increases the chances of finding positive hits, but also of excluding poor candidates at the early stages of research. Currently, libraries of materials are usually made by varying a single property of the material (e.g. polymer chemistry as described in section 2). However, as described above, not a single, but different properties of a material, including its chemistry, physical and structural properties at different length scales, are responsible for its final performance in the human body. Furthermore, clinicians' requirements regarding handling properties should be taken into account at all times. Therefore, from the clinical perspective, the future of high-throughput screening of materials lies in systems in which different materials properties are simultaneously varied in a systematic way. It is envisioned that arrays of materials are produced that combine physico-chemical and structural properties, to create materials that closely resemble the final implant, but in a

less random way than is the case by taking classical approach towards materials development. Undoubtedly, such systems will require a much more complex library design, assay development and data analysis, but when functional, they will further improve the efficiency of the development of materials for regenerative medicine.

Another envisioned development in the field of high-throughput screening of materials, that is expected to make an enormous impact from the clinical perspective, is coupling of patient diagnostics to materials screens. This offers an approach towards customized health care. It is plausible that ideal treatment of a bone defect caused by trauma in a young, active patient may be different from the ideal treatment of a defect created by tumour dissection in an older patient suffering from osteoporosis. If this information on the health condition of a specific patient can be used as an input parameter for the material screen, the success rate of the treatments based on these materials are expected to increase tremendously. Again, coupling patient information to materials screen is far from trivial, and the question remains whether such coupling is possible at all without compromising the reliability of the screens or making them too complex, but materiomics certainly does offer larger chances of developing such systems in the future, than classical implant development approaches do.

2.4 Snapshot summary

- An increasing ageing population and increased expectation from patients to retain a high quality of life drive a growing need for regenerative medicine approaches to regenerate damaged and diseased organs and tissues.
- Synthetic biomaterials present an interesting alternative to a patient's own tissue in regenerative medicine owing to their off-the-shelf availability in large quantities, relative ease of production and low costs.
- Classical, candidate approach towards development of materials for regenerative medicine include rational design that often originates in mimicking of the physico-chemical, (surface) structural and mechanical properties of natural tissue.
- Upon design and development, a biomaterial needs to be exposed to a number of preclinical *in vitro* and *in vivo* tests aimed to demonstrate the safety and functionality of the material.
- In addition to the process of preclinical tests related to the research and development phase, specific regulatory requirements need to be met in order to bring a medical device based on a synthetic material to the clinic.

- High throughput evaluation of biomaterials for regenerative medicine in the early stages of development is expected to increase the efficiency as well as the success rate of this process.
- In order to make high-throughput evaluation of materials the standard approach for biomaterial research and development, it will be critical to develop reliable assays that are predictive of clinical performance.
- In the future, material libraries will become more complex, and will allow systematic evaluation of various properties of the materials in a single screen.
- From the clinical perspective, future developments will need to focus on combining material screens with patient diagnostics in order to develop customized regenerative approaches.

Further Reading

Ratner BD, Bryant SJ. Biomaterials: where we have been and where we are going. *Annu Rev Biomed Eng.* 2004;6:41-75.

Huebsch N, Mooney DJ. Inspiration and application in the evolution of biomaterial. *Nature.* 2009 Nov;462(7272):426-32.

Hook AL, Anderson DG, Langer R, Williams P, Davies MC, Alexander MR. High throughput methods applied in biomaterials development and discovery. *Biomaterials.* 2010 Jan;31(2):187-98.

Khan F, Tare RS, Kanczler JM, Oreffo ROC, Bradley M. Strategies for cell manipulation and skeletal tissue engineering using high-throughput polymer blend formulation and microarray techniques. *Biomaterials.* 2010 Mar;31(8):2216-28.

References

1. Williams DF. The Williams dictionary of biomaterials. 1999
2. Ratner BD. A history of biomaterials. In: Ratner B, Hoffman AS, Schoen FJ, Lemons JE, editors. *Biomaterials science: an introduction to materials in medicine.* San Diego, USA: Elsevier Academic Press; 2004. p. 10-9.
3. Apple DJ. Sir Harold Ridley and his fight for sight: he changed the world so that we may better see it. Thorofare, USA: SLACK Incorporated; 2006.
4. Davison JA, Kleinmann G, Apple DJ. Intraocular Lenses. In: Tasman W, Jaeger EA, editors. *Duane's Clinical Ophthalmology.* Lippincott Williams & Wilkins; 2006.
5. Vongpatanasin W, Hillis LD, Lange RA. Prosthetic heart valves. *New England Journal of Medicine.* 1996;335(6):407-16.
6. Hoerstrup SP, Sodian R, Daebritz S, Wang J, Bacha EA, Martin DP, Moran AM, Guleserian KJ, Sperling JS, Kaushal S, Vacanti JP, Schoen FJ and Mayer JE. Functional Living Trileaflet Heart Valves Grown In Vitro. *Circulation.* 2000 Nov;102(90003):III-44-49.

7. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. *Journal of applied biomaterials*. 1991 Jan;2(3):187-208
8. Dalton P, Harvey A, Oudega M, Plant G. Tissue engineering of the nervous system. In: van Blitterswijk CA, editor. *Tissue Engineering*. San Diego, USA: Academic Press; 2008. p. 611-647.
9. Padera Jr RF, Schoen FJ. Cardiovascular medical devices. In: Ratner B, Hoffman AS, Schoen FJ, Lemons JE, editors. *Biomaterials science: an introduction to materials in medicine*. San Diego, USA: Elsevier Academic Press; 2004. p. 470-494.
10. Fawcett DW. Bone. In: Bloom W, Fawcett DW, editors. *A textbook of histology*. Philadelphia, USA: W.B. Saunders Company; 1986. p. 199-238.
11. Marks SC, Hermey DC. The structure and development of bone. In: Bilezikian JP, Raisz LG, Rodan GA, editors. *Principles of bone biology*. San Diego, USA: Academic Press; 1996. p. 3-14.
12. Derkx P, Nigg AL, Bosman FT, Birkenhager-Frenkel DH, Houstmuller AB, Pols HA, van Leeuwen JP. Immunolocalization and quantification of noncollagenous bone matrix proteins in methylmethacrylate-embedded adult human bone in combination with. *Bone*. 1998 Apr;22(4):367-73.
13. van Gaalen S, Kruijt M, Meijer G, Mistry A, Mikos A, van den Beucken J, Jansen J, de Groot K, Cancedda R, Olivo C, Yaszemski M, Dhert W. Tissue engineering of bone. In: van Blitterswijk CA, editor. *Tissue Engineering*. San Diego, USA: Academic Press; 2008. p. 560-610.
14. Davies JE, Hosseini MM. Histodynamic of endosseous wound healing. In: Davies JE, editor. *Bone Engineering*. Toronto, Canada: Em squared Inc.; 2000. p. 1-14.
15. Friedenstein AY. Induction of bone tissue by transitional epithelium. *Clinical orthopaedics and related research*. 1968;59:21-37.
16. Barradas AM., Yuan H, van Blitterswijk CA, Habibovic P. Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms. *European cells & materials*. 2011 Jan;21:407-29.
17. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials*. 2005 Sep;26(27):5474-91.
18. Habibovic P, Yuan H, van Der Valk CM, Meijer G, van Blitterswijk CA, Groot K. 3D microenvironment as essential element for osteoinduction by biomaterials. *Biomaterials*. 2005 Jun; 26(17):3565-75.
19. Habibovic P, de Groot K. Osteoinductive biomaterials – properties and relevance in bone repair. *Journal of tissue engineering and regenerative medicine*. 2007 Jan;1(1):25-32.
20. Covani U, Giacomelli L, Krajewski A, Ravaglioli A, Spotorno L, Loria P, Das S, Nicolini C. Biomaterials for orthopedics: a roughness analysis by atomic force microscopy. *Journal of biomedical materials research*. Part A. 2007 Sep;82(3):723-30.
21. Schmitz JP, Hollinger JO, Milam SB. Reconstruction of bone using calcium phosphate bone cements: A critical review. *Journal of Oral and Maxillofacial Surgery*. 1999 Sep;57(9):1122-6.
22. Weiss P, Gauthier O, Bouler J-M, Grimandi G, Daculsi G. Injectable bone substitute using a hydrophilic polymer. *Bone*. 1999 Aug;25(2):67S-70S.
23. Hou Q, De Bank PA, Shakesheff KM. Injectable scaffolds for tissue regeneration. *Journal of Materials Chemistry* . 2004 May;14(13):1915-23.
24. Habibovic P, Woodfield T, de Groot K, van Blitterswijk CA. Predictive Value of In Vitro and In Vivo Assays in Bone and Cartilage Repair -What do They Really Tell Us about the Clinical Performance? *Advances in Experimental Medicine and Biology*. 2007;585:327-60.
25. Brittberg M, Lindahl A. (2008). Tissue engineering of cartilage. In: van Blitterswijk CA, editor. *Tissue Engineering*. San Diego, USA: Academic Press; 2008. p. 534-557.

26. Karam JP, Muscari C, Montero-Menei CN. Combining adult stem cells and polymeric devices for tissue engineering in infarcted myocardium. *Biomaterials*. 2012 in press.
27. Jansen JA. Animal models for studying soft tissue biocompatibility of biomaterials. In: An YH, Friedman RJ, editors. *Animal models in orthopaedic research*. Boca Raton, USA: CRC Press; 1999. p. 393-405.
28. An YH, Friedman RJ. Animal models of bone defect repair. In: An YH, Friedman RJ, editors. *Animal models in orthopaedic research*. Boca Raton, USA: CRC Press; 1999. p. 241-60.
29. An YH, Friedman RJ, editors. *Animal models in orthopedic research*. Boca Raton, USA: CRC Press; 1999.
30. Bianco RW, Grehan JF, Grubbs BC, Mrachek JP, Schroeder EL, Schumacher CW, Svendsen CA, Lahti M. Large animal models in cardiac and vascular biomaterials research and testing. In: Ratner B, Hoffman AS, Schoen FJ, Lemons JE, editors. *Biomaterials science: an introduction to materials in medicine*. San Diego, USA: Elsevier Academic Press; 2004. p. 379-396.
31. de Vries R, Buma P, Leenaars M, Titskes-Hoitinga M, Gordijn B. Reducing the number of laboratory animals used in tissue engineering research by restricting the variety of animal models. *Articular cartilage tissue engineering as a case study. Tissue Engineering Part B Reviews*; 2012 in press.
32. Tare RS, Khan F, Tourniaire G, Morgan SM, Bradley M, Oreffo ROC. A microarray approach to the identification of polyurethanes for the isolation of human skeletal progenitor cells and augmentation of skeletal cell growth. *Biomaterials*. 2009 Feb;30(6):1045-55.
33. Khan F, Tare RS, Kanczler JM, Oreffo ROC, Bradley M. Strategies for cell manipulation and skeletal tissue engineering using high-throughput polymer blend formulation and microarray techniques. *Biomaterials*. 2010 Mar;31(8):2216-28.
34. Hench LL. The story of Bioglass. *Journal of materials science. Materials in medicine*. 2006 Nov;17(11):967-78.
35. Reichert JC, Saifzadeh S, Wulschleger ME, Epari DR, SchÄijtz MA, Duda GN, Schell H, van Griensven M, Redl H, Hutmacher DW. The challenge of establishing preclinical models for segmental bone defect research. *Biomaterials*. 2009 Apr;30(12):2149-63.
36. Muschler GF, Raut VP, Patterson TE, Wenke JC, Hollinger JO. The design and use of animal models for translational research in bone tissue engineering and regenerative medicine. *Tissue engineering. Part B, Reviews*. 2010 Feb 9;16(1):123-45.
37. Newman E, Turner AS, Wark JD. The potential of sheep for the study of osteopenia: Current status and comparison with other animal models. *Bone*. 1995 Apr;16(4):S277-S284.
38. Reichert JC, Epari DR, Wulschleger ME, Saifzadeh S, Steck R, Lienau J, Sommerville S, Dickinson IC, SchÄijtz MA, Duda GN, Hutmacher DW. Establishment of a preclinical ovine model for tibial segmental bone defect repair by applying bone tissue engineering strategies. *Tissue engineering. Part B, Reviews*. 2010 Feb;16(1):93-104.
39. Smith JO, Aarvold A, Tayton ER, Dunlop DG, Oreffo ROC. Skeletal tissue regeneration: current approaches, challenges, and novel reconstructive strategies for an aging population. *Tissue engineering. Part B, Reviews*. 2011 Oct;17(5):307-20.

In vitro and *in vivo* Bioactivity Assessment of a Polylactic Acid / Hydroxyapatite Composite for Bone Regeneration

Abstract

Synthetic bone graft substitutes based on composites consisting of a polymer and a calcium-phosphate (CaP) ceramic are developed with the aim to satisfy both mechanical and bioactivity requirements for successful bone regeneration. In the present study, we have employed extrusion to produce a composite consisting of 50 wt.% poly(D,L-lactic acid) (PLA) and 50 wt.% nano-sized hydroxyapatite (HA) powder, achieving homogeneous distribution of the ceramic within the polymeric phase. *In vitro*, in both a simulated physiological saline (SPS) and a simulated body fluid (SBF), a greater weight loss was observed for PLA/HA than for PLA particles upon 12-week immersion. Furthermore, in SPS, a continuous release of calcium and phosphate from the composite was measured, whereas in SBF, decrease of the amount of the two ions in the solution was observed both for PLA and PLA/HA accompanied with the formation of a CaP layer on the surface. *In vitro* characterization of the composite bioactivity was performed by culturing human mesenchymal stromal cells (hMSCs) and assessing proliferation and osteogenic differentiation, with PLA as a control. Both PLA/HA composite and PLA control were shown to support hMSCs proliferation over a period of two weeks. In addition, the composite significantly enhanced alkaline phosphatase (ALP) activity of hMSCs in osteogenic medium as compared to the polymer control. A novel implant design was employed to develop implants from dense, extruded materials,

suitable for testing osteoinductivity *in vivo*. In a preliminary study in dogs, PLA/HA composite implants induced heterotopic bone formation upon 12-week intramuscular implantation in all animals, in contrast to PLA control, which was not osteoinductive. Unlike *in vitro*, a more pronounced degradation of PLA was observed *in vivo* as compared to PLA/HA composite.

3.1 Introduction

Synthetic biomaterials have been developed and improved over more than five decades as an alternative to natural bone grafts. Among other benefits, synthetic bone graft substitutes could overcome the limited availability of autologous bone grafts, an issue that is becoming increasingly important with continuous population ageing. To be accepted as a comprehensive alternative to natural bone, synthetic bone graft substitutes need to meet a number of requirements, including provision of mechanical support, and bioactivity in terms of osteoconductivity and osteoinductivity. While the existing synthetic bone graft substitutes are generally accepted as being osteoconductive, i.e. able to promote “the recruitment and migration of osteogenic cells into the wound site” [1], recent research efforts have strongly focused on osteoinductivity of materials, defined by Friedenstein as “the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells” [2], because osteoinductivity is considered to be essential for successful healing of large, critically-sized bone defects. The exact mechanism of osteoinduction by synthetic biomaterials has not yet been fully deciphered; however, a growing number of materials has shown osteoinductive potential, demonstrated by the induction of bone formation upon implantation in heterotopic sites such as muscle or subcutis in various animal models. While osteoinduction has occasionally been shown for polymers [3] and porous metals [4], biomaterials with osteoinductive potential are mainly calcium-phosphate (CaP) ceramics. With chemical compositions close to that of bone mineral, CaP ceramics have logically raised considerable interest in bone regeneration. Previous studies have recorded heterotopic bone formation in, among others, hydroxyapatite (HA) [5], β -tricalcium phosphate (β -TCP) [6], biphasic calcium phosphate (BCP) (a mixture of HA and β -TCP) [7], brushite [8] and octacalcium phosphate [9] in various animal models.

While CaP ceramics have shown the strongest osteoinductive potential among synthetic materials, intrinsic brittleness limits their use as bone graft substitutes to non-load bearing sites. This could possibly be overcome by the development of composite materials. Combinations of polymers and ceramics offer numerous opportunities for improving the mechanical properties of ceramics while retaining their bioactivity [10]. For example, composites based on polylactic acid (PLA), a biocompatible and biodegradable aliphatic polyester and HA have been investigated as potential bone graft substitutes in a number of studies. Addition of

HA microparticles to PLA was shown to increase the compressive and bending strengths in a dose-dependent manner [11]. Similarly, diametral tensile strength of PLA improved upon incorporation of HA [12]. In the study by Nejati *et al.* it was furthermore shown that the size of ceramic particles can be used to tailor mechanical properties of the composites: HA nanoparticles increased the compressive strength of the composite in comparison to HA microparticles, making it comparable to cancellous bone [13]. *In vitro*, support of attachment and growth of MSCs and (pre)osteoblasts has been described in a number of studies [13–16]. *In vivo*, porous composites made of PLA and HA microparticles were shown osteoconductive when implanted in a 6 mm defect in a rabbit femoral intercondylar notch model [17]. Similarly, porous PLA/HA composite materials produced using gas foaming showed successful healing of a critical-sized cranial defect in rats [18]. Osteoinductivity of a PLA/HA porous composite produced using a solvent-based method was demonstrated upon intramuscular implantation in dogs [19, 20].

Besides chemistry, structural properties of biomaterials have also been suggested to affect level of osteoinductivity [21]. Microporosity, for instance, contributes to this phenomenon: an increase in microporosity and specific surface area of a ceramic, has been shown to lead to a higher osteoinductive potential [22, 23]. Geometrical features of the materials at the macroscopic level have also been shown to strongly affect material-induced bone formation. Indeed, heterotopic bone formation has been shown to preferentially form inside pores, channels and concavities and rarely on the implant periphery [8, 24], suggesting that “protected” environment inside the material is required for osteoinduction to occur and/or be maintained.

In the current study, a PLA/HA composite was developed using extrusion, a manufacturing technique that does not require the use of solvents. *In vitro*, human mesenchymal stromal cells (hMSCs) were cultured on the composite material and proliferation and osteogenic differentiation were assessed, with PLA as a control. Furthermore, the osteoinductive potential of the composite was investigated in a preliminary *in vivo* study in a canine intramuscular model. Since extruded materials are dense, not containing “natural” protected areas like pores or channels, we designed the implants such that an “artificial pore” is created between plates of extruded materials in order to test the ability of these extruded materials to induce heterotopic bone formation (Figure 3.1).

3.2 Results

3.2.1 Materials characterization

The HA powder was phase-pure as determined by XRD analysis (Figure 3.2.A) and had a particle size of 69.9 ± 12.8 nm in width and 308.7 ± 61.2 nm in length (Figure 3.2.B), as determined by imaging using SEM. FTIR spectra of PLA and PLA/HA composite powders are shown in Figure 3.2.C. In contrast to PLA control, PLA/HA

Figure 3.1: Implant design. Digital photograph (A) and schematic representation with dimensions (B) of implants used for intramuscular implantation in a canine model to assess osteoinductivity.

Figure 3.2: Characterization of PLA, PLA/HA and HA. XRD spectrum of nano-HA powder (A), SEM micrograph of nano-HA powder (B), and FTIR spectra of nano-HA powder, PLA and PLA/HA composite (C).

composite spectrum contained phosphate (604 and 562 cm^{-1}) and hydroxyl (632 cm^{-1}) bands, characteristic of HA, confirming incorporation of the ceramic in the polymer phase [25]. A modification of the main PLA peak (symmetric and asymmetric deformational vibrations of C-H in CH_3 groups), was observed in the composite spectrum around 1000 cm^{-1} due to the HA phosphate band situated at the same wavelength. The pronounced intensities of HA-specific bands in the composite spectrum are in line with the quantity of HA incorporated.

EDAX analysis of a gold-sputtered PLA/HA pellet, after polishing and sterilization, confirmed presence of HA by the pronounced calcium and phosphorus peaks in the EDAX spectrum (Figures 3.3.A and 3.3.B), while no calcium or phosphorus were observed in the spectrum of the PLA control (data not shown). Phosphorous and calcium mapping of PLA/HA composite showed homogeneous distribution of both elements (Figures 3.3.C and 3.3.D).

Figure 3.3: Composite homogeneity. SEM micrograph (A), EDAX spectrum (B) and elemental maps of calcium (C) and phosphorous (D) atoms of PLA/HA pellet after polishing and sterilization. (scale bars: 20 μm).

3.2.2 Calcium and phosphate release and degradation study

Degradation and ion release tests were carried out on material particles with a size of 0.5-1 mm. The behavior in an aqueous environment of PLA/HA composite and PLA control materials was tested over three months in SPS, a buffered solution with an ionic strength similar to that of blood plasma, and in SBF, which mimics the mineral composition of blood plasma and is saturated towards dicalcium phosphate dihydrate (Figure 3.4). Measurements of the wet weight of the immersed particles showed that the PLA water uptake approximately doubled the initial dry weight of the particles after 12 weeks of immersion in both solutions. The swelling of PLA/HA particles appeared to be less pronounced with a wet weight increase of approximately 1.5 times the initial weight (Figure 3.4.A). After drying, particles were weighed again to assess the mass loss. While no measurable decrease could be detected in the case of PLA particles in either solution, the dry weight of PLA/HA particles decreased with about 4% in SPS and with about 7% in SBF over the three months of the experiment. The pH of SPS and SBF solutions was stable over time

in the case of PLA particles, whereas for PLA/HA particles a decrease from 7.32 to 6.92 was observed in SPS and from 8.53 to 7.16 in SBF between 12 hours and 12 weeks of immersion (Figure 3.4.B).

Release of calcium and phosphate from the PLA/HA composite was measured in SPS and SBF solutions over the time of the degradation study (Figures 3.4.C–3.4.F). In SPS, calcium signal was first detected after two weeks of immersion, whereas phosphate was detected as early as one week after immersion in SPS. A continuous release of both calcium and phosphate from the composite was observed during the time frame tested, without reaching a plateau. No calcium or phosphate were observed in SPS upon immersion of PLA particles over the 12-week period. In the case of samples immersed in SBF, precipitation occurred shortly after mixing of the salts, leading to a milky solution after 12 hours. Simultaneously, the concentrations of calcium and phosphate dropped over the first week of immersion for both PLA and PLA/HA, before reaching a plateau. For PLA/HA samples, an increase in concentration of calcium and phosphate can be observed at 8 and 10 weeks, respectively.

The SEM micrographs of cross-sections of PLA and PLA/HA particles, immersed for 12 weeks in SPS, showed a dense surface of both PLA and PLA/HA. Underneath the surface, the material was porous in the case of PLA particles, whereas in the PLA/HA particles a dense “core” could still be observed (Figures 3.8.A and 3.8.B). In the case of samples immersed in SBF, the SEM micrographs of both PLA and PLA/HA particles surfaces, showed the formation of globular precipitates, which were identified as CaP by EDAX analysis (data not shown). The deposition of this layer was observed as early as 12 hours upon immersion, and at all later time points. After 12 weeks in SBF, PLA particle surface exhibited holes suggesting degradation, and deposits of CaP. Similar CaP deposits were observed on PLA/HA particles with porous bulk material underneath the surface (Figures 3.8.C and 3.8.D).

3.2.3 hMSCs proliferation and osteogenic differentiation

For *in vitro* cell culture experiments, PLA and PLA/HA pellets with a diameter of 10 mm and a thickness of 2 mm were seeded with hMSCs. DNA was quantified after 7 and 14 days of culture in basic and osteogenic medium as an indirect measure of cell proliferation (Figure 3.5.A). Both PLA/HA composite and PLA control sustained hMSCs proliferation over the 2-week culture period. A significant increase in DNA amounts between day 7 and day 14 was observed for cells cultured in basic medium on both PLA and PLA/HA. An increase in time was also observed for cells cultured in osteogenic medium on both material types, however, the differences were not statistically significant. No differences between PLA and PLA/HA were observed at either time point, independent of the medium used. Similarly, no effect of the medium type on cell proliferation was observed on either material type at days 7 and 14.

Figure 3.4: *In vitro* degradation and calcium phosphate release. Wet and dry weights of the PLA and PLA/HA particles upon immersion in SPS and SBF over a period of 12 weeks (A) pH of the solutions (B). Calcium and phosphate release from PLA/HA particles in SPS (C and D respectively) and of PLA and PLA/HA in SBF (E and F respectively). Statistical analysis was performed using one way ANOVA with Tukey's multiple comparison post-hoc test ($p < 0.05$ and $n = 3$).

Figure 3.5: HMSCs proliferation and osteogenic differentiation. DNA content (A) and ALP activity corrected for DNA content (B) of hMSCs cultured on PLA and PLA/HA pellets in basic and osteogenic medium for 7 and 14 days. Statistical analysis was performed using one way ANOVA with Tukey's multiple comparison post-hoc test ($p < 0.05$ and $n = 3$).

ALP activity of hMSCs was quantified and corrected for DNA content after 7 and 14 days of culture in basic and osteogenic medium (Figure 3.5.B). In osteogenic medium, ALP level increased between 7 and 14 days on both PLA/HA composite and PLA control, although a statistically significant increase was only observed for PLA/HA. No temporal increase was observed when cells were cultured in basic medium. Regarding the effect of the material type, the trend observed was that, independent of the medium used, ALP level of hMSCs cultured on PLA/HA composite was higher than that of cells cultured on PLA control at both 7 and 14 days. The difference in ALP expression between cells cultured on PLA/HA and PLA was, however, only statistically significant after 14 days in osteogenic medium.

3.2.4 Osteoinductive potential

For the *in vivo* study, implants were used that consisted of two paired identical plates of PLA or PLA/HA composite with a size of $24 \times 14 \times 5 \text{ mm}^3$ with two 0.5 mm thick PEOT/PBT spacers between them (Figure 3.1). There were no surgical complications and all animals experienced an uneventful recovery from the surgery. At implant retrieval, no clinical signs of inflammation or infection (i.e. swelling or redness) were observed upon visual inspection of the implantation area. Only two out of five implanted PLA samples were found and explanted. In the areas where the remaining three PLA samples were implanted, no material remnants or signs of tissue other than muscle were observed. In contrast, all 12 implanted PLA/HA samples were retrieved, and while 2 were lost during processing (dissolved in

MMA monomer), 10 could be histologically analyzed. Macroscopic and microscopic observation showed that a fibrous capsule with a thickness varying between 50 and 200 μm had formed around all samples of both PLA and PLA/HA (Figure 3.6.A–3.6.D). The fibrous capsule seemed somewhat thicker around the PLA than around the PLA/HA implants, but as only two PLA samples were retrieved, a good comparison was difficult to make. The PLA samples were largely degraded (Figures 3.6.A and 3.6.C) which may be an explanation why the remaining three samples were impossible to trace at explantation. Degradation of composite samples was observed to be less pronounced than that of PLA control (Figure 3.6.B). Analysis of the samples showed that in all cases, the PEOT/PBT spacers used to create the gap between the two biomaterial plates were completely degraded. As a result, the two plates were in close contact with each other, limiting ingrowth of tissue inside the implant. The unexpected degradation of spacers, and thus loss of the gap were attributed to the improper choice of PEOT/PBT copolymer composition. Microscopic analysis of tissue formed around the implants showed no signs of bone formation or mineralization in PLA controls (Figure 3.6.C). Fibrous tissue surrounding PLA/HA implants contained no mineral or bone either, however, in all 10 analyzed PLA/HA samples, heterotopic bone formation was observed inside the implants, particularly in the areas where the original entrance of the gap between the two material plates was located (Figures 3.7.A and 3.7.B). Based on qualitative observations, the amount of bone formation was relatively limited. Nevertheless, the maximum depth of ingrowth of bone into the implant was observed to be about 4 mm. Bone formed was normal in appearance, showing mineralized matrix aligned with a layer of osteoblast (Figures 3.7.C and 3.7.D). Another qualitative observation suggested that heterotopic bone formation was most pronounced in the highly degraded areas of implant (Figure 3.7.E).

3.3 Discussion

Synthesis of ceramic/polymer composite materials by extrusion does not require use of organic solvents, which is an important advantage when aiming for implantable materials, as remnants of solvents are difficult to completely remove from the material [26]. In the present study, we have confirmed that up to 50 wt% nano-sized HA particles can homogeneously be incorporated into PLA by using extrusion. Previous studies performed on PLA and HA blends have shown that presence of ceramic in the polymeric matrix has a dose dependent effect on various properties of the polymer, such as mechanical behavior, degradation rate, and bioactivity [11, 20, 27, 28]. Verheyen *et al.* have demonstrated that a 50 wt% content of HA mixed with PLA improved the osteoconductivity as compared to the polymeric control in a transcortical implantation model in goats [27]. Hasegawa and colleagues provided first evidence for osteoinduction by porous scaffolds made of PDLLA/HA composite with a weight ratio 70/30, upon intramuscular implantation

Figure 3.6: *In vivo* degradation of implants and formation of fibrous capsule. Images of methylene blue and basic fuchsin stained histological sections of implants after 12-week intramuscular implantation in dogs. Digital photographs of a PLA (A) and a PLA/HA (B) implant section (scale bars: 5 mm), showing a more pronounced degradation of the polymer as compared to the composite implant. Low magnification micrographs of a PLA (C) and a PLA/HA (D) implant section, showing fibrous capsule (FC) that had formed around both implant types and the surrounding muscle tissue (M) (scale bars: 100 μm).

Figure 3.7: *In vivo* bone formation in composite implants. Low magnification micrographs (A and B) and enlargements of black square areas in A and B, respectively (C and D) of representative methylene blue and basic fuchsin stained sections of PLA/HA composite implants from two dogs. Images a and b show the entrance of the protective gap between the two composite plates surrounded with fibrous tissue (FT). Bone formation can be observed inside the gap (Bone). The newly formed bone was normal in appearance, with osteoid containing osteocytes and aligned with a layer of osteoblasts (C and D). Bone formation was also observed surrounding composite particles formed upon degradation (indicated by arrows, scale bars: A and B 500 μm , C and D 50 μm , e 200 μm).

Figure 3.8: SEM micrographs of cross-sections of PLA and PLA/HA particles immersed in SPS for 12 weeks (A and B respectively, scale bars: 200 μm) and of surface of PLA and PLA/HA immersed in SBF for 12 weeks (C and D respectively, scale bars: 50 μm).

in dogs [19]. In contrast, no heterotopic bone formation was observed in PDLLA scaffolds without HA. In a study by Barbieri *et al.*, in which porous PDLLA/nano apatite composites with a ceramic content of 10 wt%, 20 wt% and 40 wt% produced using a solvent-based method were tested, only the composite with 40 wt% apatite led to heterotopic bone formation upon intramuscular implantation in dogs for 12 weeks [20]. In a more recent study by Barbieri *et al.*, a similar PDLLA/nano apatite blend with a ceramic content of 50 wt% produced by extrusion was shown to trigger osteoinduction in a sheep intramuscular model [29]. Regarding the extrusion process, it could be argued that a temperature of 150°C may affect the molecular weight of the polymer, as PLA is known to be temperature sensitive [30]. Analysis of polymer before and after extrusion using Ubbelohde viscosimeter, however showed a minor effect of the extrusion process on molecular weight of the polymer (data not shown). It has previously been suggested that ceramic fillers limit the thermal degradation of the polymer during extrusion [30], while the amount of

ceramic incorporated correlates with frictional degradation [28].

Degradation is a critical parameter of biomaterials and in the case of bone graft substitutes; degradation rate of the material should be comparable to the rate of new bone formation, in order for the material to provide sufficient support while leaving space for tissue growth. Degradation must therefore be controlled. PLA is known to undergo autocatalytic hydrolysis, producing lactic acid, and its degradation rate is strongly influenced by the molecular weight: the lower the molecular weight is, the faster PLA degrades [29, 31]. Indeed, strong degradation of PLA occurred upon implantation of the polymer in paraspinal muscles of dogs, to the extent that a number of implants could not be retrieved from the animals 12 weeks after implantation. This level of degradation is not surprising, considering the low molecular weight of the polymer used. Although degradation of PLA/HA implants was also observed, its extent was significantly lower than that of PLA, which could be explained by the buffering effect of the ceramic phase of the composite that hampered the autocatalytic degradation of the polymer. The observed decrease in degradation of PLA/HA composite as compared to the PLA control was in accordance with earlier studies [32, 33]. Degradation of the polymeric phase of the composite is also crucial for the release of calcium and phosphate ions from the ceramic phase, which is suggested to be the origin of bioactivity of CaPs [34, 35]. For example, *in vitro* effects of free calcium ions have been extensively studied on various cell types, including hMSCs [36], osteoblasts [37], macrophages [38], human periosteum-derived cells (hPDCs) [39], etc. Calcium ions have shown to influence the proliferation, morphology and osteogenic differentiation of hMSCs [36]. Similarly, *in vitro* studies on the effect of inorganic phosphate ions have shown a concentration-dependent effect on osteogenic differentiation of hPDCs [39], whereas osteoblast-like cells were shown to undergo apoptosis when treated with high levels of inorganic phosphate [40]. On the other hand, hypophosphatemia has been shown to inhibit osteoclast formation and osteoblast differentiation to mature osteocytes [41]. Despite this body of evidence for the effect of free calcium and phosphate ions on cell proliferation and osteogenic differentiation, as we recently reviewed, it is difficult to pinpoint a single parameter that is responsible for biological response to a CaP material *in vitro* or *in vivo* [21]. Release of calcium from a CaP ceramic is accompanied by other events, such as phosphate release, reprecipitation of a biological apatite layer, possibly containing endogenous proteins and other factors, and change of surface topography, all of which can affect bioactivity of the material as well. In our study, we have demonstrated a continuous release of calcium and phosphate ions from the composite over 12 weeks in SPS *in vitro*. In addition, a small but more pronounced weight loss was observed in for PLA/HA composite particles as compared to PLA control particles along with a stronger decrease of pH of the solution. SPS is a solution with ionic strength similar to the blood plasma, but it is, unlike blood plasma not saturated towards dicalcium phosphate dihydrate. Upon immersion in SBF, which simulates the

mineral composition of blood plasma, weight loss of PLA/HA particles was again more pronounced than that of PLA particles, and so was the pH decrease. However, a decrease of calcium and phosphate ions measured was probably a consequence of the formation of a CaP layer on the surface of both PLA and PLA/HA. In the case of PLA/HA, an increase in calcium and phosphate concentrations in SBF at the latest time points may be attributed to the drop in pH observed. Although SBF is theoretically supposed to better mimic the mineral composition of body fluids [42], *in vivo* conditions of fluid refreshment regimes, local pH, temperature and presence of endogenous factors are difficult, if not impossible to mimic *in vitro*, which is why release data as described here can at best be considered an indication of whether or not ions release from the composite material takes place, and to which extent this release is dependent on the degradation of the polymer phase. Nevertheless, considering clear signs of PLA/HA degradation *in vivo*, and differences in the extent of degradation between the polymer and the composite, it is plausible that release of calcium and phosphate from the ceramic phase occurred in parallel.

Both PLA/HA composite and PLA control sustained proliferation of hMSCs over a period of 14 days. In general PLA is considered a biocompatible material, and indeed in our study no detrimental effect on cell proliferation was observed within the time frame cultured, which was in accordance with a study in which MC3T3-E1 osteoblast were cultured on electrospun PLA and PLA/HA fibres [14]. While no effect of material or medium type was observed, cell numbers on both PLA/HA and PLA increased between days 7 and 14, independent of the cell culture medium used. This increase was, however, less pronounced for PLA/HA in osteogenic medium than for the other conditions, which may be explained by an increase in osteogenic differentiation, as was demonstrated by a significant increase in ALP levels of cells cultured on PLA/HA in osteogenic medium, between days 7 and 14. A trend of ALP levels of cells cultured on PLA/HA being higher than those on PLA control was observed at both time points and in both basic and osteogenic medium, although the difference was only significant at 14 days in osteogenic medium. These data suggest that PLA/HA composite improved osteogenic differentiation of hMSCs as compared to PLA without the ceramic and are in accordance with earlier studies using fetal bone cells [43].

In vivo, although some cases of inflammatory response were reported upon PLA implantation, this polymer is considered to have an overall satisfactory biocompatibility [44]. In our *in vivo* study, the thin dense fibrous capsule observed around both PLA and PLA/HA implants after 12 weeks of intramuscular implantation in dogs, suggested a mild tissue response [45]. Presence of bone in all PLA/HA samples, implanted intramuscularly was the proof of osteoinductivity of the composite. Previous studies already reported osteoconductivity [27] and osteoinductivity [19, 20, 29] of PLA/HA blends. While use of solvents may be a disadvantage, solvent-based methods typically result in porous materials, in contrast to extruded material particles, which are dense. As stated earlier, protective areas in an implant, in the

form of concavities, channels or pores are considered essential for osteoinduction to occur [21]. For this reason, implants for the *in vivo* testing in this study were designed such that a protective area was artificially created between two dense composite plates. With improvement of the choice of spacer material, such as Teflon, the implant design with the protective gap is expected to retain its shape to allow formation of bone even deeper inside the implant. The observation that bone formation was primarily observed in the areas close to the original openings of the gap and in the areas of pronounced degradation of the composite were in accordance with results from an earlier study in which calcium phosphate cement blocks with open and closed channels were tested for osteoinductivity [8]. This observation could be related to a better oxygen and nutrient supply in the areas close to gap openings as well as to better availability of calcium phosphate ceramic in the areas of strong degradation of the composite. Indeed, considering that no bone was found in PLA implants, despite extensive degradation, it is suggested that bone formation observed in PLA/HA implants was related to increased availability of the HA ceramic and ionic dissolution/reprecipitation processes occurring in the vicinity of HA particles. Despite the unexpectedly fast degradation of the material used for spacer in the 3D implants, we have shown that osteoinduction by the PLA/HA composite produced by extrusion is possible, when dense materials are shaped into an implant that allows bone induction. In general, such dense materials may be interesting in studies aiming to describe the effect of individual material parameters in phenomena such as osteoinduction, for example, by allowing the change of a single parameter, such as ceramic phase without variability in macro- or microporosity.

3.4 Materials and methods

3.4.1 Materials production and characterization

The composite used in this study consisted of 50 wt.% amorphous poly(D,L-lactic acid) (PLA), (ANaBior or Purac), and 50 wt.% in-house made nano-sized HA powder. HA was produced using a wet precipitation method as described earlier [20]. In short, aqueous solutions of $(\text{NH}_4)_2\text{HPO}_4$ and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ were mixed at a pH above 10, and the resulting powder was allowed to age, washed and finally resuspended in acetone. A low molecular weight of 55,000 - 59,000 Da was chosen for PLA in order to build a rapidly degradable composite. HA powder was characterized using X-ray diffraction (XRD, Rigaku Miniflex) and an environmental scanning electron microscope (SEM; XL30, ESEM-FEG, Philips) in the secondary electron mode with an acceleration voltage of 20 kV (Figures 3.2.A and 3.2.B). Composite was produced by extrusion using a twin screw extruder with conical non-converging screws (Artecs BV, Enschede, the Netherlands). The extruder was set at 150°C and the screw rotation speed at 100 rpm, which allowed the composite to flow out of the extruder after a mixing time of

5 minutes. Controls made of PLA were prepared using the same process. Following extrusion, PLA/HA composite and PLA control were shaped into particles, pellets and complex three-dimensional implants to be used for the degradation study, *in vitro* evaluation and *in vivo* implantation respectively.

Chemical characterization was performed using transmission mode Fourier Transform Infrared spectroscopy with KBr pellets (FTIR; Spectrum100, Perkin Elmer Analytical Instruments). Surface morphology and homogeneity of the ceramic distribution in the polymeric phase were assessed for sterilized PLA/HA and PLA pellets using SEM with an acceleration voltage of 5 kV, coupled to energy dispersive X-ray analyzer (EDAX; Apollo X, Ametek).

3.4.2 *In vitro* degradation and ion release dynamics

For the degradation study, materials particles with a size of 0.5-1 mm were produced by grinding of extruded material and subsequent sieving. 100 mg of particles were immersed in 5 ml of either simulated physiological saline (SPS) or simulated body fluid (SBF) in triplicate. SPS contained 137 mM Na⁺, 177 mM Cl⁻ and 50 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES). The pH of SPS was adjusted to 7.3 using 1 M NaOH. SBF was prepared using the method proposed by Böhner and Lemaître [42] and contained 142 mM Na⁺, 109.90 mM Cl⁻, 34.88 mM HCO₃⁻, 2.31 mM Ca²⁺ and 1.39 mM HPO₄²⁻. The pH of SBF was adjusted to 7.4 using 1 M HCl. Samples were placed in a shaking waterbath at 37°C for a maximum of three months. Calcium and phosphate concentrations and the pH of the solutions were analyzed after 12 hours, 1, 2, 4, 6, 8, 10 and 12 weeks. Calcium and phosphate concentrations were determined using a quantitative colorimetric method (QuantiChrom™ Calcium assay kit (DICA-500) and QuantiChrom™ Phosphate assay kit (DIPI-500), respectively). Optical density of solutions after adding reagents from the kits was read with a microplate spectrophotometer (Thermo Scientific Multiskan GO) at 612 nm and 620 nm for calcium and phosphate release, respectively. The degradation experiment was performed on independent triplicates for the time points 1, 4, 8 and 12 weeks. The intermediate measures of calcium and phosphate concentrations after 12 hours, 2, 6, and 10 weeks were carried out by sampling 300 µl of solution, which were not refreshed, from the independent triplicates aforementioned. This procedure was selected to avoid the error induced by solution refreshment. The material particles were collected, rinsed with MilliQ water and air-dried for two days before being characterized by SEM. The wet and the dry weights of the particles were recorded after 12 hours and 12 weeks of immersion in SPS or SBF.

3.4.3 *In vitro* cell culture

Bone marrow aspirates were obtained after written informed consent, and hMSCs were isolated and proliferated as described previously [46, 47]. Briefly, aspirates

were resuspended by using 20-gauge needles, plated at a density of 5×10^5 cells/cm² and cultured in hMSC proliferation medium containing α -minimal essential medium (Gibco), 10% fetal bovine serum (Lonza), 0.2 mM ascorbic acid (Sigma Aldrich), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), 10 μ g/ml streptomycin (Gibco), and 1 ng/ml basic fibroblast growth factor (FGF) (Fisher Scientific). Cells were grown at 37°C in a humid atmosphere with 5% CO₂. Medium was refreshed twice a week, and cells were used for further sub-culturing or cryopreservation. Cells were trypsinised prior to seeding on materials.

For *in vitro* cell culture experiments, the extruded rods of PLA and PLA/HA composite were moulded into pellets with a diameter of 10 mm and a thickness of 2 mm using a Teflon mould and a heating press at 120°C and 20 kN for 5 minutes. Pellets were then polished using sandpaper (grades 500 and 2400) to ensure a smooth surface. After sterilization with 70% isopropanol for two times 3 minutes and complete evaporation at room temperature, pellets were placed in ultra-low attachment 24 well-plates (Corning) to minimize the adhesion of cells to the plate. Prior to cell seeding, pellets were conditioned overnight in proliferation medium. 4000 hMSCs of passage 3 were seeded on each pellet in proliferation medium. After 24 hours, proliferation medium was replaced by either basic (proliferation medium without FGF) or differentiation medium (basic medium supplemented with 10 nM dexamethasone). Cells were cultured for 7 and 14 days at 37°C in a humid atmosphere with 5% CO₂, with refreshment of medium every 2 to 3 days. hMSCs proliferation was evaluated by quantifying DNA content (CyQUANT® Cell Proliferation assay) and ALP production was assessed at the enzymatic level (CDP-Star® Reagent) as an early marker for osteogenic differentiation. Expression of ALP by hMSCs cultured on tissue culture plastic under osteogenic conditions has been shown to occur at around day 11 [48], whereas on different biomaterials, including polymer/ceramic composites, hMSCs have been shown to express ALP even earlier [49], which is why we selected 7 and 14 days of culture as endpoints for our *in vitro* experiment. All analyses were performed in triplicate.

3.4.4 *In vivo* study

Implant preparation and implantation procedure Implants were produced by moulding the extruded rods of PLA and PLA/HA composite into 24×14×5 mm³ plates by using a poly(dimethylsiloxane) mould with smooth surface, two microscope glass slides and two foldback clips, by applying pressure. The setup was left at 120°C for 5 minutes. Upon removal from the mould, the plates were paired and glued together with smooth surfaces facing one another using a 0.5 mm thick poly(ethylene oxide terephthalate) / poly(butylene terephthalate) - (PEOT/PBT) (PolyVation BV) spacers and super glue (Pattex) (Figure 3.1). Following an aPEOTbPBTc nomenclature, the composition used in this study was 300PEOT55PBT45 where, (a) is the molecular weight in g/mol of the starting poly(ethylene glycol) (PEG) blocks used in the copolymerisation, while (b) and (c)

are the weight ratios of the PEOT and PBT blocks. Prior to implantation, samples were sterilized by ethylene oxide (Isotron Nederland BV).

Twelve PLA-HA composite and 5 PLA control implants were inserted in paraspinal muscles of 8 skeletally mature male mongrel dogs (1-4 years old, weighing 10-15 kg) upon approval by the local animal care committee (Animal Center, Sichuan University, Chengdu, China; protocol P11029). The surgical procedure was performed under general anesthesia of the animals (pentobarbital sodium, Merck; 30 mg/kg body weight). After shaving the lumbar area and disinfection of the skin with iodine, the paraspinal muscles were exposed. Using blunt dissection, intramuscular pockets were created bilaterally, and each pocket was filled with one implant. Distance among pockets was sufficient to avoid effect of individual implants on each other's behavior. Upon implant placement, the wound was closed in layers using silk sutures. Following the surgeries, animals were given buprenorphine (0.1 mg per animal) intramuscularly for 2 days as analgesics and penicillin (40 mg/kg) by intramuscular injection for 3 consecutive days to prevent infection. Twelve weeks after implantation, the animals were sacrificed with overdose of pentobarbital sodium to harvest the samples.

3.4.5 Histological analysis

After explantation, samples, with surrounding muscle tissue were fixed in 4% buffered paraformaldehyde (pH 7.4) and kept at 4°C for one week. Fixed samples were rinsed with phosphate buffer solution (PBS), dehydrated by ethanol series (70%-100%) and infiltrated with a methylmethacrylate (MMA, LTI Nederland) solution that polymerized at 30°C within 1 week. Longitudinal non-decalcified sections with a thickness of 10-15 μm were cut using a diamond saw microtome (Leica Leitz 1600) and stained with 1% methylene blue and 0.3% basic fuchsin after etching with an HCl/ethanol mixture.

Tissue formation was qualitatively analyzed using a light microscope (Nikon Eclipse E600, DS Cooled Camera Head DS-Fi1c).

3.4.6 Statistical analysis

One way ANOVA with Tukey's multiple comparison post-hoc test was performed to analyze weight measurements of the materials upon degradation study and DNA and ALP values following cell culture. The level of significance was set at $p < 0.05$. All data presented are expressed as mean $\pm$ standard deviation.

3.5 Conclusion

In the present study, extrusion was used as a solvent-free method to synthesize dense composite materials consisting of 50 wt% (poly)D,L lactic acid and 50 wt%

nano-sized hydroxyapatite, for bone regeneration. While proliferation of hMSCs was comparable between the composite and the polymer control not containing ceramic, ALP expression was higher on the composite materials. Furthermore, intramuscular implantation in dogs of composite implants produced using an innovative design, resulted in heterotopic bone formation, in contrast to polymer-based implants, that were not osteoinductive.

References

1. Davies JE, Hosseini MM. Histodynamic of endosseous wound healing. In: Davies JE, editor. Toronto, Canada: Em squared Inc.; 2000. p. 1-14.
2. Friedenstein AY. Induction of bone tissue by transitional epithelium. *Clin. Orthop. Relat. Res.* 1968;59:21-37.
3. Winter GD, Simpson BJ. Heterotopic Bone formed in a Synthetic Sponge in the Skin of Young Pigs. *Nature* 1969;223:88-90.
4. Fujibayashi S, Neo M, Kim HM, Kokubo T, Nakamura T. Osteoinduction of porous bioactive titanium metal. *Biomaterials* 2004;25:443-50.
5. Ripamonti U. Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. *Biomaterials* 1996;17:31-5.
6. Yuan H, de Bruijn JD, Liu Y, Feng J, Yang Z, de Groot K, et al. Bone formation induced by calcium phosphate ceramics in soft tissue of dogs: a comparative study between porous alpha-TCP and beta-TCP. *J. Mater. Sci. - Mater. Med.* 2001;12:7-13.
7. Yang Z, Yuan H, Tong W, Zou P, Chen W, Zhang X. Osteogenesis in extraskeletally implanted porous calcium phosphate ceramics: variability among different kinds of animals. *Biomaterials* 1996;17:2131-7.
8. Habibovic P, Gbureck U, Doillon CJ, Bassett DC, van Blitterswijk CA, Barralet JE. Osteoconduction and osteoinduction of low-temperature 3D printed bioceramic implants. *Biomaterials* 2008;29:944-53.
9. Barrère F, van Der Valk CM, Dalmeijer RAJ, Meijer G, van Blitterswijk CA, de Groot K, et al. Osteogenicity of octacalcium phosphate coatings applied on porous metal implants. *J. Biomed. Mater. Res. Part A.* 2003;66:779-88.
10. Tanner KE. Bioactive ceramic-reinforced composites for bone augmentation. *J. R. Soc. Interface.* 2010;7 Suppl 5:S541-57.
11. Lin PL, Fang HW, Tseng T, Lee WH. Effects of hydroxyapatite dosage on mechanical and biological behaviors of polylactic acid composite materials. *Mater. Lett.* 2007;61:3009-13.
12. Wang T, Chow LC, Frukhtbeyn SA, Ting AH, Dong Q, Yang M, et al. Improve the Strength of PLA/HA Composite Through the Use of Surface Initiated Polymerization and Phosphonic Acid Coupling Agent. *J. Res. Natl. Inst. Stan.* 2011;116:785-96.
13. Nejati E, Firouzdor V, Eslaminejad MB, Bagheri F. Needle-like nano hydroxyapatite/poly(l-lactide acid) composite scaffold for bone tissue engineering application. *Mater. Sci. Eng. C.* 2009;29:942-9.
14. Jeong SI, Ko EK, Yum J, Jung CH, Lee YM, Shin H. Nanofibrous poly(lactic acid)/hydroxyapatite composite scaffolds for guided tissue regeneration. *Macromol. Biosci.* 2008;8:328-38.
15. Wang XH, Shi S, Guo G, Fu SZ, Fan M, Luo F, et al. Preparation and characterization of a porous scaffold based on poly(D,L-lactide) and N-hydroxyapatite by phase separation. *J. Biomat. Sci. - Polym. E.* 2011;22:1917-29.
16. Rizzi SC, Heath DJ, Coombes AG, Bock N, Textor M, Downes S. Biodegradable poly-

mer/hydroxyapatite composites: surface analysis and initial attachment of human osteoblasts. *J. Biomed. Mater. Res.* 2001;55:475-86.

17. Hasegawa S, Tamura J, Neo M, Goto K, Shikinami Y, Saito M, et al. In vivo evaluation of a porous hydroxyapatite/poly-DL-lactide composite for use as a bone substitute. *J. Biomed. Mater. Res.* A. 2005;75:567-79.

18. Montjovent M-O, Mathieu L, Schmoekel H, Mark S, Bourban P-E, Zambelli P-Y, et al. Repair of critical size defects in the rat cranium using ceramic-reinforced PLA scaffolds obtained by supercritical gas foaming. *J. Biomed. Mater. Res. A.* 2007;83:41-51.

19. Hasegawa S, Neo M, Tamura J, Fujibayashi S, Takemoto M, Shikinami Y, et al. In vivo evaluation of a porous hydroxyapatite/poly-DL-lactide composite for bone tissue engineering. *J. Biomed. Mater. Res. A.* 2007;81:930-8.

20. Barbieri D, Renard AJS, de Bruijn JD, Yuan H. Heterotopic bone formation by nano-apatite containing poly(D,L-lactide) composites. *Eur. Cells Mater.* 2010;19:252-61.

21. Barradas AMC, Yuan H, van Blitterswijk CA, Habibovic P. Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms. *Eur. Cells Mater.* 2011;21:407-29.

22. Habibovic P, Yuan H, van Der Valk CM, Meijer G, van Blitterswijk CA, de Groot K. 3D microenvironment as essential element for osteoinduction by biomaterials. *Biomaterials* 2005;26:3565-75.

23. Yuan H, Fernandes H, Habibovic P, de Boer J, Barradas AMC, de Ruiter A, et al. Osteoinductive ceramics as a synthetic alternative to autologous bone grafting. *PNAS.* 2010;107:13614-9.

24. Ripamonti U, Crooks J, Kirkbride AN. Sintered porous hydroxyapatites with intrinsic osteoinductive activity: Geometric induction of bone formation. *S. Afr. J. Sci.* 1999;95:335-43.

25. Nejati E, Mirzadeh H, Zandi M. Synthesis and characterization of nano-hydroxyapatite rods/poly(l-lactide acid) composite scaffolds for bone tissue engineering. *Composites Part A.* 2008;39:1589-96.

26. Freier T, Kunze C, Schmitz KP. Solvent removal from solution-cast films of biodegradable polymers. *J. Mater. Sci. Lett.* 2001;20:1929-31.

27. Verheyen CCPM, de Wijn JR, van Blitterswijk CA, de Groot K, Rozing PM. Hydroxylapatite/poly(L-lactide) composites : an animal study on push-out strengths and interface histology. *J. Biomed. Mater. Res.* 1993;27:433-44.

28. Barbieri D, de Bruijn JD, Luo X, Far S, Grijpma DW, Yuan H. Controlling dynamic mechanical properties and degradation of composites for bone regeneration by means of filler content. *J. Mech. Behav. Biomed.* 2013;20:162-72.

29. Barbieri D, Yuan H, Luo X, Far S, Grijpma DW, de Bruijn JD. Influence of polymer molecular weight in osteoinductive composites for bone tissue regeneration. *Acta Biomater.* 2013; In press.

30. Mathieu L, Bourban P, Manson J. Processing of homogeneous ceramic/polymer blends for bioresorbable composites. *Compos. Sci. Technol.* 2006;66:1606-14.

31. Henton DE, Gruber P, Lunt J, Randall J. Polylactic acid technology. In: Mohanty AK, Misra M, Drzal LT, editors. Boca Raton, USA: Taylor & Francis; 2005. p. 527-77.

32. Sui G, Yang X, Mei F, Hu X, Chen G, Deng X, et al. Poly-L-lactic acid/hydroxyapatite hybrid membrane for bone tissue regeneration. *J. Biomed. Mater. Res. A.* 2007;82:445-54.

33. Deng XL, Sui G, Zhao ML, Chen GQ, Yang XP. Poly(L-lactic acid)/hydroxyapatite hybrid nanofibrous scaffolds prepared by electrospinning. *J. Biomat. Sci. - Polym. E.* 2007;18:117-30.

34. Geesink RG, de Groot K, Klein CP. Bonding of bone to apatite-coated implants. *J. Bone Joint Surg. Br.* 1988;70:17-22.

35. Hanawa T, Kamiura Y, Yamamoto S, Kohgo T, Amemiya A, Ukai H, et al. Early bone formation around calcium-ion-implanted titanium inserted into rat tibia. *J. Biomed. Mater. Res.* 1997;36:131-6.
36. Barradas AMC, Fernandes HAM, Groen N, Chai YC, Schrooten J, van de Peppel J, et al. A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells. *Biomaterials* 2012;33:3205-15.
37. Nakamura S, Matsumoto T, Sasaki JI, Egusa H, Lee KY, Nakano T, et al. Effect of calcium ion concentrations on osteogenic differentiation and hematopoietic stem cell niche-related protein expression in osteoblasts. *Tissue Eng. Part A.* 2010;16:2467-73.
38. Honda Y, Anada T, Kamakura S, Nakamura M, Sugawara S, Suzuki O. Elevated extracellular calcium stimulates secretion of bone morphogenetic protein 2 by a macrophage cell line. *Biochem. Biophys. Res. Commun.* 2006;345:1155-60.
39. Chai YC, Roberts SJ, Schrooten J, Luyten FP. Probing the osteoinductive effect of calcium phosphate by using an in vitro biomimetic model. *Tissue Eng. Part A.* 2011;17:1083-97.
40. Meleti Z, Shapiro IM, Adams CS. Inorganic phosphate induces apoptosis of osteoblast-like cells in culture. *Bone* 2000;27:359-66.
41. Zhang R, Lu Y, Ye L, Yuan B, Yu S, Qin C, et al. Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J. Bone Miner. Res.* 2011;26:1047-56.
42. Bohner M, Lemaire J. Can bioactivity be tested in vitro with SBF solution? *Biomaterials* 2009;30:2175-9.
43. Montjovent M-O, Mathieu L, Hinz B, Applegate LL, Bourban P-E, Zambelli P-Y, et al. Biocompatibility of bioresorbable poly(L-lactic acid) composite scaffolds obtained by supercritical gas foaming with human fetal bone cells. *Tissue Eng.* 2005;11:1640-9.
44. Athanasiou K, Niederauer GG, Agrawal CM. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/ polyglycolic acid copolymers. *Biomaterials* 1996;17:93-102.
45. Gogolewski S, Jovanovic M, Perren SM, Dillon JG, Hughes MK. Tissue response and in vivo degradation of selected polyhydroxyacids: polylactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/VA). *J. Biomed. Mater. Res.* 1993;27:1135-48.
46. Fernandes H, Mentink A, Bank R, Stoop R, van Blitterswijk CA, de Boer J. Endogenous collagen influences differentiation of human multipotent mesenchymal stromal cells. *Tissue Eng. Part A.* 2010;16:1693-702.
47. Both SK, van der Muijsenberg AJC, van Blitterswijk CA, de Boer J, de Bruijn JD. A rapid and efficient method for expansion of human mesenchymal stem cells. *Tissue Eng.* 2007;13:3-9.
48. Karperien M, Roelen B, Poelmann M, Gittenberger-de Groot A, Hierck B, DeRuiter M, Meijer D, Gibbs S. Morphogenesis, generation of tissue in the embryo. In: *Tissue Engineering*, van Blitterswijk CA et al. editor. Elsevier: 2008. p.28-72
49. Barbieri D, de Bruijn JD, Yuan H. Surface structure of nanocomposites and its properties: a practical example. In: *Frontiers in Nanobiomedical Research Series. Tissue Regeneration: Where Nano Structure Meets Biology*, Liu Q and Wang H editors. World Scientific Publishing and Co.

Elucidating the individual effects of calcium and phosphate ions on hMSCs by using composite materials

Abstract

The biological performance of bone graft substitutes based on calcium phosphate bioceramics is dependent on a number of properties including chemical composition, porosity and surface micro- and nanoscale structure. However, in contemporary bioceramics these properties are interlinked, therefore making it difficult to investigate the individual effects of each property on cell behavior. In this study we have attempted to investigate the effects of calcium and inorganic phosphate ions independent from one another by preparing composite materials with polylactic acid (PLA) as a polymeric matrix and calcium carbonate or sodium phosphate salts as fillers. Clinically relevant bone marrow derived human mesenchymal stromal cells (hMSCs) were cultured on these composites and proliferation, osteogenic differentiation and ECM mineralization were investigated with time and were compared to plain PLA control particles. In parallel, cells were also cultured on conventional cell culture plates in media supplemented with calcium or inorganic phosphate to study the effect of these ions independent of the 3D environment created by the particles. Calcium was shown to increase proliferation of cells, whereas both calcium and phosphate positively affected alkaline phosphatase enzyme production. QPCR analysis revealed positive effects of calcium and of inorganic phosphate on the expression of osteogenic markers, in particular BMP2 and osteopontin. Higher levels of mineralization were also observed upon exposure

to either ion. Effects were similar for cells cultured on composite materials and those cultured in supplemented media, although ion concentrations in the composite cultures were lower. The approach presented here may be a valuable tool for studying the individual effects of a variety of soluble compounds, including bioinorganics, without interference from other material properties.

4.1 Introduction

Calcium phosphate-based compounds have a long history of use in bone replacement and regeneration, mainly owing to their chemical likeness to bone mineral [1]. As discussed in a recent review, inorganic bone graft substitutes are generally considered to play a beneficial role in bone repair only in the solid state [2]. In other words, their main function is “structural”, meaning that they act as a barrier to soft tissue infiltration, offer temporary or lasting mechanical support to damaged hard tissues and facilitate the onset and growth of new bone tissue on their surface.

It is also important to consider that all calcium phosphate-based compounds are, to varying extents, degradable in the physiological environment, either by physico-chemical dissolution or through cellular activity [3]. During the process of degradation, structural function of the substitute is diminished, but this process is accompanied by release of calcium and phosphate ions. Indeed, it is believed that in this process of calcium and phosphate release, followed by the re-precipitation of a carbonated apatite layer, along with co-precipitation of endogenous proteins, lies the origin of bioactivity of calcium-phosphates [4]. A majority of the literature regarding calcium phosphate bioactivity focuses on the formation of this biological apatite layer, and the *in vivo* effects of free calcium (Ca^{2+}) and phosphate (Pi) ions both individually and in combination are largely ignored [5]. Ca^{2+} and Pi are considered to be inorganic ions with therapeutic potential, also called bioinorganics. The interest for bioinorganics as a synthetic and potentially safer alternative to biological growth factors is increasing as a method to improve the biological performance of bone graft substitutes, while retaining their synthetic character [2].

Several examples of *in vitro* studies investigating the effects of Ca^{2+} and Pi on growth and differentiation of cells relevant for bone repair and regeneration can be found. For example, increase of calcium ion concentration in cell culture medium has been shown to enhance the proliferation and osteogenic differentiation of both osteoblasts [6] and human periosteum derived stem cells (hPDCs) [7] in a dose-dependent manner. Elevated Ca^{2+} concentration [7.8 mM] in hMSCs culture resulted in upregulation of a number of osteogenic markers, including bone morphogenetic protein-2 (BMP-2), osteocalcin (OC), osteopontin (OP) and bone sialoprotein (BSP) [8]. It has recently been shown that stimulation of osteogenic differentiation of osteoprogenitor cells by calcium occurs by signaling through L-type calcium channels rather than through calcium sensing receptors [8, 9].

The inorganic phosphate ion concentration in medium has also been shown to

influence osteoprogenitor cell fate in a dose dependent manner. For example, a 96-hour exposure to 7 mM Pi, led to apoptosis of primary human osteoblast-like cells, induced by a mitochondrial membrane permeability transition caused by the anion [10]. However, a Pi concentration between 2 and 10 mM were also shown to support hPDCs proliferation and osteogenic differentiation in a dose-dependent manner [7] and acts as a specific signal for the induction of OP gene expression in osteoblasts [11]. In a recent study by Shih *et al.* it was demonstrated that extracellular phosphate uptake through SLC20a1, a sodium phosphate symporter, supports osteogenic differentiation of hMSCs via adenosine, an ATP metabolite, which acts as an autocrine/paracrine signaling molecule through the A2b adenosine receptor [12].

The use of conventional calcium phosphate ceramic bone graft substitutes does not allow for in depth studies of the effects of individual physicochemical parameters on the bioactivity of the material. During degradation, release of calcium ions is naturally also accompanied by a release of phosphate ions. Furthermore, surface structural properties, such as macro- and micro-porosity, specific surface area, roughness [13–16] and overall geometry [17–19], may affect the biological performance in terms of osteoconductivity and osteoinductivity, either independently or partially dependently to the degradation properties. In order to isolate chemical effects from physical and structural effects, one of our earlier studies involved loading highly soluble inorganic phosphate salts into an inert polymeric delivery vehicle that was implanted intramuscularly in mice. This study showed that local supersaturation of soft tissue surrounding the implant with inorganic phosphate resulted in extensive collagen mineralization proximal to the implant [20].

To further explore the potential of polymeric carriers as delivery vehicles for bioinorganics, and to study the effects of calcium or phosphate released from such carriers independently, composite materials, consisting of PLA, a biocompatible and biodegradable aliphatic polyester, and calcium or phosphate salts were developed in this study. These composites were shaped into particles, offering a three-dimensional substrate for the culture of hMSCs and simultaneously acting as calcium- or phosphate-delivery systems over 14 days of culture. In parallel, hMSCs were cultured in media supplemented by Ca^{2+} and Pi. We assessed the independent effects of Ca^{2+} and Pi on hMSCs proliferation, differentiation towards osteogenic lineage and extracellular matrix (ECM) mineralization.

4.2 Materials and Methods

4.2.1 Synthesis of composite materials

Calcium carbonate was selected as a source of calcium ions (Fisher Biotech, USA). A sedimentation cut-off was performed in ethanol in order to select salt particles with

a maximum diameter of 35 μm . A combination of sodium phosphate monobasic (25 wt%) and dibasic (75 wt%) salts (Fisher Biotech, USA) was prepared to obtain a pH neutral source of inorganic phosphate ions. This mixture was ground in a pestle and mortar and sieved through a 38 μm sieve. The polymeric phase of the composites used in this study consisted of amorphous poly(D,L-lactic acid) (PLA) (Purac, Gorinchem, the Netherlands) with a molecular weight of 59,000 Da.

The three composites, i.e. composite containing 5 wt% calcium carbonate salt (PLA-Ca5%), composite containing 50 wt% calcium carbonate salt (PLA-Ca50%) and composite containing 5 wt% sodium phosphate salt mixture (PLA-Pi5%) (Table 4.1), and PLA controls were prepared as follow: salts were dispersed and PLA was dissolved in chloroform. The mixture was then added drop by drop using a separating funnel to isopropanol under constant stirring. The precipitate was collected and rinsed overnight in isopropanol, drained and dried. The material was placed in a silanized glass dish and dried further at 150°C and at a pressure of 10 inHg for 5 minutes in order to remove solvent traces. Finally, the material was ground and sieved to collect particles between 0.5 and 1 mm in diameter. It should be noted that we were able to develop a composite with a higher (20 wt%) sodium phosphate salt content; however, a burst release of Pi was observed. This burst release was overcome by the addition of PLA with a higher molecular weight, but since this change in polymeric carrier would not allow a proper comparison with other conditions, we decided not to include it in the study.

Materials	PLA 59,000 Da	Calcium salt	Phosphate salt
PLA-Ca5%	95 wt%	5 wt%	
PLA-Ca50%	50 wt%	50 wt%	
PLA-Pi5%	95 wt%		5 wt%
PLA	100 wt%		

Table 4.1: Composition of the composites and controls.

4.2.2 Material characterization

Salt grains and composite particles were sputter coated with Au/Pd and observed by scanning electron microscopy (FE-SEM, Hitachi S-4700; ESEM-FEG, Philips XL30) under secondary electron mode with an acceleration voltage of 10 kV. Salt incorporation and homogeneity of distribution in the polymeric phase were assessed using SEM and Energy Dispersive X-ray Spectroscopy (EDAX; Apollo X, Ametek).

4.2.3 Ion release and particle degradation

Ion release from the composite particles was assessed over 2 weeks in a buffer of trishydroxymethyl aminomethane (0.1 M TRIS (CH_2OH)₃CNH₂), 0.15 M NaCl, adjusted to a pH of 7.3 with 1 M HCl). 100 mg of particles were immersed in 15 ml of TRIS buffer and maintained at 37°C in a still waterbath. Upon immersion in the solution, particles of all material types settled at the bottom of the vial. The calcium and phosphate content of the buffer was analyzed by ion chromatography (DX2500, Dionex, Sunnyvale, CA). The degradation and release experiment was performed in independent triplicate samples to prevent any error introduced by solution refreshment. To assess the surface modification of the composite particles after the 2-week immersion, particles were dried, sputter coated with Au/Pd and observed using a SEM (FE-SEM, Hitachi S-4700) with an acceleration voltage of 2 kV.

4.2.4 Cell culture on composites

Human bone marrow aspirates were obtained after written informed consent, and hMSCs were isolated and proliferated as described previously [21, 22]. Briefly, aspirates were resuspended by using 20-gauge needles, plated at a density of 5×10^5 cells cm^{-2} and cultured in hMSC proliferation medium containing α -minimal essential medium (Gibco), 10% fetal bovine serum (Lonza), 0.2 mM ascorbic acid (Sigma Aldrich), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), 10 $\mu\text{g}/\text{ml}$ streptomycin (Gibco), and 1 ng/l basic fibroblast growth factor (FGF) (Fisher Scientific). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. Medium was refreshed twice a week, and cells were used for further sub-culturing or cryopreservation. Cells were trypsinised prior to seeding on materials.

Particles were sterilized by immersion in 100% isopropanol followed by complete evaporation. The materials were then rinsed with sterile PBS and conditioned overnight in proliferation medium. Replicates consisting of 100 μl of particles were placed in ultra-low attachment 24 well-plates (Corning) to minimize the adhesion of cells to the plate. hMSCs of passage 3 were seeded on the particles at the density of 400,000 cells per replicate in 200 μl of proliferation medium. Regular cell culture 12-well plates were used as control material and cells were seeded at a density of 25,000 cells per well in 1.5 ml of proliferation medium. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. After 24 hours the proliferation medium was collected and replaced with 1.5 ml of basic medium (proliferation medium without FGF). Medium was collected and refreshed after 3, 6, 8, 10, 12 and 14 days. For attachment, proliferation, ALP activity and mineralization, cells from one donor were used, while independent cultures of cells from two donors were used for qPCR analysis.

Cell attachment was observed 24 hours after seeding by fixing cells in formalin overnight at 4°C and staining the samples with methylene blue for 3 minutes. The

samples were then rinsed with PBS and observed using an optical stereomicroscope.

Calcium and phosphate concentrations in the culture medium were determined using a quantitative colorimetric method (QuantiChrom™, Calcium assay kit (DICA-500) and QuantiChrom™ Phosphate assay kit (DIPI-500), respectively). Optical density of the medium after adding reagents from the kits was measured using a microplate spectrophotometer (Thermo Scientific Multiskan GO) at 612 nm and 620 nm for calcium and phosphate release, respectively.

The proliferation of hMSCs was evaluated at 7 and 14 days of culture by quantifying DNA content (CyQUANT® Cell Proliferation assay) and ALP activity was assessed at the enzymatic level (CDP-Star® Reagent) as a marker for osteogenic differentiation.

A combination of the TRIzol (Invitrogen) method with the NucleoSpin RNA II isolation kit (Macherey-Nagel) was used to perform RNA isolation from the cultures, with the exception of hMSCs grown on PLA-Ca50%. 1 ml of TRIzol reagent was added to each well. Following one freeze/thaw cycle, 200 µl chloroform was added per sample. After mixing and centrifugation, the aqueous phase of each sample was collected and mixed with 350 µl 70% ethanol before loading onto the RNA binding column of the NucleoSpin RNA II isolation kit. Subsequent steps were in accordance with the manufacturer's protocol. In the case of PLA-Ca50%, the manufacturer's protocol was followed from the first step on, where mercaptoethanol was used instead of TRIzol. First strand cDNA was synthesized using iScript (Bio-Rad). Quantitative real time PCR was performed and CT values were normalized to the GAPDH housekeeping gene and fold induction was calculated using the comparative Δ CT method. Primer sequences of the selected markers are given in Table 4.2.

Mineralization of the ECM produced by hMSCs was analyzed after 14 days of culture by fixing cells in formalin overnight at 4°C and staining the samples with alizarin red for 3 minutes. The samples were then rinsed with PBS and observed under an optical stereomicroscope.

4.2.5 Cell culture in supplemented media

Four supplemented media were prepared from basic medium by enriching them in calcium or phosphate to 4 mM (Ca4, Pi4) or 8 mM (Ca8, Pi8) as final concentrations. Medium conditioning was achieved by adding appropriate volumes of 100 Å pH buffer solutions consisting of demineralized water, 25 mM Hepes (Invitrogen) and 140 mM NaCl (Sigma-Aldrich) and respectively 220 mM of calcium, 620 mM of calcium (CaCl₂, Sigma-Aldrich), 280 mM of phosphate or 680 mM of phosphate (pH neutral mixture of NaH₂PO₄ and Na₂HPO₄, Sigma-Aldrich) to the basic medium. Basic medium and osteogenic medium (basic medium supplemented with 10 nM dexamethasone) were used as controls.

hMSCs from the same two donors as used for cultures on composites were

Name	Primer Sequence
ALP	5'-GACCCTTGACCCCCACAAT-3' 5'-GCTCGTACTGCATGTCCCCT-3'
BMP-2	Purchased from SA Biosciences
Collagen type 1	5'-GAGGGCCAAGACGAAGACATC-3' 5'-CAGATCACGTCATCGCACAAAC-3'
GAPDH	5'-CGCTCTCTGCTCCTCCTGTT-3' 5'-CCATGGTGTCTGAGCGATGT-3'
Osteocalcin	5'-GGCAGCGAGGTAGTGAAGAG-3' 5'-GATGTGGTCAGCCAACTCGT-3'
Osteopontin	5'-CCAAGTAAGTCCAACGAAAG-3' 5'-GGTGATGTCCTCGTCTGTA-3'

Table 4.2: Primers sequences for human genes.

seeded in conventional cell culture 12-well plates at a density of 25,000 cells per well in 1.5 ml of basic medium. Cells were left in basic medium overnight to attach. The following day, medium was refreshed using the supplemented media. The culture was kept at 37°C in a humidified atmosphere with 5% CO₂, and medium was refreshed every other day. All analyses were performed on cultures from two independent donors after 4, 7 and 10 days of conditioning. These time points were chosen so as to gather information at an early stage (4 days) and to observe the evolution of the proliferation, ALP activity and gene expression until cell confluence was reached in the wells (10 days).

HMSCs proliferation was evaluated at 4, 7 and 10 days of culture by quantifying DNA content (CyQUANT® Cell Proliferation assay) and ALP activity was assessed at the enzymatic level (CDP-Star® Reagent).

Total RNA was isolated from the cultures of two donors after 4, 7 and 10 days by using the combination of the TRIzol (Invitrogen) method with the NucleoSpin RNA II isolation kit (Macherey-Nagel) as previously described for the cultures on composite materials. Primer sequences of the selected markers are given in Table 4.2.

The mineralization of the ECM produced by hMSCs was observed after 10 days of culture by fixing cells in formalin for 20 minutes and staining the samples with alizarin red for 3 minutes. The samples were rinsed with PBS and digital photographs were subsequently taken.

4.2.6 Statistical analysis

One-way ANOVA with Tukey's multiple comparison post-hoc test was performed to analyze DNA, ALP and qPCR data. All experiments were carried out in triplicate. All data presented are expressed as mean $\pm$ standard deviation. For clarity, only the significant differences among different conditions analyzed at the same time point are indicated by asterisks on the graphs. Patterned lines are used to distinguish different comparisons. The level of significance was set at $p < 0.05$.

4.3 Results

4.3.1 Material characterization

Analysis of salt particles using SEM showed grains ranging in size from approximately 1 to 35 μm for CaCO_3 (Figure 4.1.A), and from 5 to 38 μm for Na_2HPO_4 and NaH_2PO_4 (Figures 4.1.B and 4.1.C, respectively). Upon salt incorporation and independent to the quantity or type of salt added, holes on the surface of the composite particles were observed, which were probably formed during the solvent evaporation phase (Figure 4.2, upper row). The surface of PLA-Ca5% appeared smoother than that of PLA-Ca50% indicating that the quantity of incorporated salt grains influenced the texture of the material. EDX spectra of the particles (Figure 4.2, third row) confirmed the presence of calcium in PLA-Ca5% and PLA-Ca50%, and of phosphorus and sodium in PLA-Pi5%. EDX analyses of PLA control particles detected only carbon and oxygen. EDX mapping of the composite particles (Figure 4.2, fourth row) allowed the visualization of salt distribution throughout the polymeric matrix. Calcium carbonate particles appeared well distributed within PLA, with a much denser signal for PLA-Ca50% than for PLA-Ca5%. In the phosphate composite, salt grains appeared larger and more aggregated compared to those of the calcium composites; however, their distribution within PLA also seemed homogeneous.

4.3.2 Ion release and particle degradation

PLA-Ca5% and PLA-Ca50% samples released calcium continuously over the 14-day degradation study and no burst release was observed (Figure 4.3.A and 4.3.B, respectively). The calcium release rate from PLA-Ca50% samples was approximately 10 times higher than PLA-Ca5% samples. After 14 days, 9% of the theoretical maximum release value was reached for PLA-Ca5% (Figure 4.3.A), and 10% for PLA-Ca50% (Figure 4.3.B). For PLA-Pi5%, phosphate release was also continuous (Figure 4.3.C) and no burst release was observed. After 14 days, approximately 10% of the theoretical maximum release value was reached for PLA-Pi5%.

Figure 4.1: Calcium and phosphate salts as composite fillers. SEM micrographs of composite fillers: calcium carbonate (A), sodium phosphate dibasic (B), and sodium phosphate monobasic (C) (scale bars: 50 μm). The size of CaCO_3 particles varied between 1 and 35 μm , whereas NaH_2PO_4 and Na_2HPO_4 had a comparable size of between 5 and 38 μm .

Observation of the composite particles following the degradation study revealed that holes had formed on the surface of all samples as a result of salt grain dissolution; small cracks in the polymer matrix were also visible, probably due to water uptake and polymer swelling (Figure 4.4). Despite these microscale changes, all of the samples remained structurally intact.

4.3.3 *In vitro* cell culture on composites

Cell attachment, proliferation and osteogenic differentiation of hMSCs cultured on the different composite particles were evaluated. Methylene blue staining of the hMSCs after 24 hours in culture showed good attachment of cells on all materials tested. Cells were seeded at the same density for each sample and showed similar attachment density with plenty of remaining naked surface available to allow for cell proliferation (Figure 4.5).

Chemical analysis of culture medium compositions showed that the calcium-containing composites did not enrich the medium with Ca^{2+} , as the Ca^{2+} concentrations of PLA-Ca5% and PLA-Ca50% media were comparable or at some time points lower than that of plain PLA (Figure 4.6.A). However, the phosphate-containing composite induced a sustained Ca^{2+} depletion of the medium as compared to plain PLA and calcium composites, which was especially noticeable on days 1, 3 and 8 (Figure 4.6.A). The phosphate-containing composite enriched the cell culture medium in Pi , with the highest release at early time points, which slowly decreased with time (Figure 4.6.B). In PLA-Ca50% samples, Pi depletion in the culture medium was observed, while the effect of PLA-Ca5% on Pi concentration in the medium was less apparent and comparable to that of plain PLA samples (Figure 4.6.B).

After 7 and 14 days of culture in basic medium, total DNA from the attached hMSCs was quantified as an indirect measure of cell proliferation (Figure 4.7.A). All materials sustained cell proliferation over 14 days of culture. The amounts of DNA

Figure 4.2: Characterization of composite particles. First row: SEM micrographs of composite particles (scale bar: 500 μm). Second row: SEM micrographs of particle surfaces (scale bar: 50 μm). Third row: EDX spectra of composite materials. Fourth row: EDAX elemental map of composite particle surfaces. All composite particles presented holes in the surface, probably formed during solvent evaporation. Furthermore, the surface of PLACa50% appeared rougher than that of other materials, as a consequence of a higher salt content. The EDX analysis confirmed incorporation of both calcium and phosphate salt, with CaCO_3 being homogeneously distributed and with phosphate salt particles showing some aggregation.

Figure 4.3: Ion release from composite materials. Ionic concentrations in TRIS buffer at 37°C over 14 days: calcium from PLA-Ca5% composite (A), calcium from PLA-Ca50% composite (B) and phosphate from PLA-Pi5% composite (C). PLA-Ca5% and PLA-Ca50% showed a continuous release of Ca^{2+} over the 14-day period, up to respectively 9% and 10% of their theoretical maximum release. PLA-Pi5% also released Pi in a continuous manner up to 10% of its theoretical maximum release.

measured in the cultures increased slightly between 7 and 14 days, but this increase was only significant for cultures on tissue culture plates. After 7 days, the DNA level of cells grown on PLA-Ca5% was significantly higher than that of cultures on tissue culture plates. Cells grown on PLA-Ca50% particles showed the highest proliferation rate with DNA levels significantly higher than those observed for all other materials at both time points, with the exception of PLA-Ca5% after 7 days of culture.

ALP activity of hMSCs was quantified and normalized for DNA content after 7 and 14 days of culture (Figure 4.7.B). A significant increase in ALP expression between 7 and 14 days was observed for PLA-Ca5% and PLA-Pi5% composites only. ALP levels of hMSC cultures on composite samples were higher than those measured in PLA and plate cultures, independent of the time point. The difference

Figure 4.4: Surfaces of composite particles after a 14-day immersion. SEM micrographs of PLA-Ca5% (A), PLA-Ca50% (B) and PLA-Pi5% (C) particles after 14 days in TRIS buffer at 37°C (scale bars: 20 μm). While for all composites, the formation of holes was observed on the surface upon immersion in the buffer, the particles retained their overall structural integrity.

in ALP levels measured in cultures on composites versus controls was statistically significant for PLA-Ca50% and PLA-Pi5% at 7 days and for all three composites at 14 days. In terms of differences among composites, it was observed that hMSCs cultured on PLA-Ca50% and PLA-Pi5% particles expressed significantly higher amounts of ALP after 7 days than hMSCs cultured on PLA-Ca5%. After 14 days, ALP production was significantly enhanced in cultures with PLA-Pi5% as compared to cells cultured on other composites.

The expression profile of osteogenic markers of hMSCs from two independent donors cultured on the composite materials was determined using qPCR at 7 and 14 days (Figure 4.8). ALP gene expression was downregulated in cells cultured on particles at both time points compared to cells cultured on tissue culture plastic, both in basic and osteogenic medium. In the case of donor 1, this downregulation was statistically significant, with the exception of cells cultured for 7 days on PLA-

Figure 4.5: hMSC attachment. Light micrographs of hMSCs stained with methylene blue after 24 hours of culture on the indicated polymer and composite particles. All materials allowed the attachment of hMSCs, without apparent differences among the materials.

Figure 4.6: Analysis of culture medium. Calcium (A) and phosphate (B) ion concentrations in medium collected from the culture of hMSCs on composite particles for 14 days in basic medium. While PLA-Ca5% and PLA-Ca50% did not affect the Ca²⁺ content of the medium, PLA-Pi5% depleted the medium of Ca²⁺. Conversely, PLA-Ca50% depleted the medium of Pi and PLA-Pi5% enriched it.

Figure 4.7: HMSCs proliferation and osteogenic differentiation on composite particles. DNA content (A) and ALP activity normalized for DNA content (B) of hMSCs cultured on PLA-Ca5%, PLA-Ca50%, PLA-Pi5%, PLA particles, and tissue culture plate in basic medium for 7 and 14 days (*= $p < 0.05$). The three composites and the polymeric control sustained cell proliferation over 14-day period, although the increase in DNA content between day 7 and day 14 was limited for all materials. Cells grown on calcium composites showed the highest proliferation rate. ALP activity was higher when cells were grown on composite materials as compared to PLA or tissue culture plastic. While at 7 days, cells grown on PLA-Ca5% showed a lower ALP activity than those cultured on PLA-Ca50% or PLA-Pi5%, at 14 days, the phosphate composite showed a higher ALP activity than the calcium composites.

Ca50%, which exhibited ALP mRNA expression level comparable to that of cells cultured on tissue culture plastic in basic medium. For donor 2, ALP expression in cells cultured on tissue culture plastic in osteogenic medium was significantly higher as compared to ALP mRNA levels of cells grown on all four particle samples.

BMP2 gene expression was upregulated in cells cultured on particles for 7 days as compared to cells cultured on tissue culture plastic in basic and osteogenic media for the same duration, independent of the donor. Donor 1 cells cultured on PLA-Pi5% expressed significantly higher levels of BMP2 mRNA than controls. It should be noted that cells cultured on PLA-Ca50% expressed BMP2 mRNA levels significantly higher than those of all other cultures at both time points. Cells from donor 2 grown on PLA-Ca50% and PLA-Pi5% showed a significantly higher BMP2 expression than cells cultured on all other materials, with PLA-Pi5% cultures also expressing higher levels of BMP2 mRNA than PLA-Ca50% cultures.

Collagen type I expression was generally downregulated in cells cultured on particles at both time points as compared to controls in basic medium. However,

donor 1 hMSCs expressed a significantly higher level of collagen type I mRNA when cultured on PLA-Ca50% particles than cells cultured on all other materials, independent of the time point.

Analysis of osteocalcin expression showed similarities between donor 1 and donor 2 profiles. The highest signal was obtained for cells cultured for 14 days on PLA-Ca50% particles, which was significantly higher than the values obtained

Figure 4.8: Osteogenic profile of cultures on composite particles. hMSCs gene expression after 7 and 14 days of culture on composite particles. For clarity, the PLA part of the particle notation has been removed, BM stands for basic medium and OM for osteogenic medium (*= $p < 0.05$). ALP gene expression was downregulated when cells were cultured on composite or polymeric particles as compared to tissue culture plastic. Cells from both donors cultured on PLA-Ca50% or PLA-Pi5% showed an upregulation of BMP2 gene expression after 7 days. A significant upregulation was also observed for donor 2 cells cultured on PLA-Ca5%. Furthermore, cells cultured on PLA-Ca50% showed an upregulation of OC and OP gene expression for both donors, and of collagen type I expression for donor 1. Donor 2 cells cultured on PLA-Pi5% also expressed significantly higher levels of OC and OP mRNA.

from all other cultures. For donor 2, the osteocalcin expression of cells cultured on PLA-Ca50% particles for 7 days was significantly higher than those of cells cultured on the other materials, and the expression of cells cultured on PLA-Pi5% was significantly higher than that of cells grown in osteogenic medium on plastic plates after 7 days, and than those of cells grown on all other materials (except

Figure 4.9: Osteogenic profile of cultures on composite particles. hMSCs gene expression after 7 and 14 days of culture on composite particles. For clarity, the PLA part of the particle notation has been removed, BM stands for basic medium and OM for osteogenic medium ($\ast=p<0.05$). ALP gene expression was downregulated when cells were cultured on composite or polymeric particles as compared to tissue culture plastic. Cells from both donors cultured on PLA-Ca50% or PLA-Pi5% showed an upregulation of BMP2 gene expression after 7 days. A significant upregulation was also observed for donor 2 cells cultured on PLA-Ca5%. Furthermore, cells cultured on PLA-Ca50% showed an upregulation of OC and OP gene expression for both donors, and of collagen type I expression for donor 1. Donor 2 cells cultured on PLA-Pi5% also expressed significantly higher levels of OC and OP mRNA.

PLA-Ca50%) after 14 days.

Osteopontin expression by donor 1 cells was significantly upregulated only for hMSCs cultured for 7 days on PLA-Ca50% particles. This result was confirmed in the profile of donor 2, where the upregulation was still significant after 14 days of culture. However, osteopontin expression by cells from donor 2 was also significantly upregulated for cells cultured on PLA-Pi5% for 7 days.

Alizarin red staining of the particle cultures after 14 days provided a qualitative indication of ECM mineralization (Figure 4.9). Controls consisting of particles immersed for 14 days in basic medium in the absence of cells did stain when exposed to alizarin red, but the stain seemed less homogeneously distributed than on the particles stained after cell culture. In contrast to the “spot-wise” staining of the particles in absence of cells (supplementary data), samples with cells were homogeneously stained over the entire surface and between the particles, plausibly as a consequence of mineralization of the layer of cells and ECM covering the particles. Although all cultures displayed a red stain after treatment, qualitative differences were observed among the various composite types: the lowest level of stain was observed on PLA, followed by PLA-Ca5% and PLA-Pi5%. Cultures on PLA-Ca50% clearly displayed the most intensive stain.

4.3.4 *In vitro* cell culture in ion-supplemented media

To investigate the effect of calcium and phosphate ions on the behavior hMSCs from the two donors, independently from the PLA carrier, an additional cell culture was performed on tissue culture plates in basic media that were supplemented with Ca^{2+} or Pi, followed by cell attachment, proliferation and osteogenic differentiation analysis.

After 4, 7 and 10 days of culture, hMSCs DNA was quantified to assess cell proliferation in supplemented media (Figure 4.10.A and 4.10.B). All media sustained cell proliferation over 10 days of culture. A significant increase in total DNA was observed between days 4 and 7 in all the conditions tested, independent of the donor. Between day 7 and 10, the increase in DNA amount was significant for Ca4, Ca8 and Pi8 media for donor 1 and Ca8 medium for donor 2. For cells from donor 1, Ca8 medium showed the only significant effect on the cultures after 4 days of exposure with a significantly higher level of DNA than those of Pi4 and basic medium culture. After 7 days, the DNA amount in Ca8 condition was still significantly higher compared to Pi4, Pi8 and basic medium, and Ca4 was also shown to significantly increase cell proliferation in comparison to Pi8 and basic medium. After 10 days, the same trend was noticeable, with Ca8 culture giving a significantly higher signal than those observed for all other cultures. Pi8 culture showed a significantly lower level of DNA in comparison to all other cultures. The profile of donor 2 showed similarities with that of donor 1, especially regarding Ca8 effect on the culture. At day 4, the amount of DNA measured in Ca8 culture was already significantly higher than those measured in Pi4 and basic medium cultures. At this time point it was also noted that Ca4 and Pi8 significantly increased the proliferation rate of the cultures compared to basic medium. After 7 days Ca8 showed a significantly higher level of DNA compared to all other conditions. After 10 days the effect of Ca8 was still noticeable, although Ca4, Pi4, Pi8 also significantly increased the culture proliferation rate as compared to basic medium.

ALP activity of hMSCs was quantified and corrected for DNA content after 4, 7 and 10 days of culture (Figure 4.10.C and 4.10.D). At day 4, ALP levels detected were negligible, independent of the donor. At day 7, ALP levels were quantified but no significant differences were found between the experimental conditions. At 14 days, Ca4 and Ca8 cultures of both donors expressed a significantly higher level of ALP compared to Pi4, Pi8 and basic medium cultures. However, cells of donor 1 also showed a significant effect of Pi4 medium, with ALP levels higher than those of Pi8 and basic medium cultures; and cells of donor 2 grown in Ca8 expressed significantly higher amounts of ALP than cells cultured in Ca4 medium.

The expression profiles of osteogenic markers of hMSCs from the two donors cultured in supplemented media are displayed in Figure 4.11. No significant differences were observed among the ALP mRNA levels detected in supplemented basic media cultures, with the exception of donor 2 cells cultured for 10 days in

Figure 4.10: HMSCs proliferation and osteogenic differentiation in supplemented media. DNA content (A and B, donor 1 and 2 respectively) after 4, 7, and 10 days, and ALP activity normalized for DNA content (C and D, donor 1 and 2 respectively) after 7 and 10 days of culture of hMSCs in tissue culture plates with supplemented media (*= $p < 0.05$). All media sustained cell proliferation over 10 days of culture, independent of the donor. Ca8 medium showed an early and consistent effect on the cultures with the highest proliferation rate. A positive effect of Ca4 medium on hMSCs proliferation was also observed for both donors. At 14 days, Ca4 and Ca8 cultures of both donors expressed a significantly higher level of ALP compared to Pi4, Pi8 and basic medium cultures. ALP level increased when cells were cultured in Pi4 medium and was significantly higher as compared to the cultures in Pi8 or basic medium for donor 1.

Ca4 medium, which showed significantly higher levels of ALP mRNA than cells cultured in Ca8, Pi4, Pi8 and basic medium. The strongest effect was observed in the osteogenic medium cultures, independent of the donor. For donor 1, significantly higher levels of ALP were detected in osteogenic medium cultures at day 4 compared to levels obtained in Pi4, Pi8 and basic medium cultures, and by day 10, compared to levels measured in Ca8, Pi4, Pi8 and basic medium cultures. Donor 2 cells cultured in osteogenic medium expressed significantly higher levels

Figure 4.11: Osteogenic profile of cell cultured in supplemented media. hMSCs gene expression, after 4, 7 and 10 days in supplemented media. BM stands for basic medium and OM for osteogenic medium (*= $p < 0.05$). Only the osteogenic cell culture medium had a significant positive effect on the ALP gene expression. A temporal increase in BMP2 mRNA levels was observed for cells grown in Ca8 and in Pi8 supplemented media, with, at 10 days, significantly higher BMP2 levels than those of all other cultures. Cells cultured in calcium supplemented media showed an upregulation of collagen type I, whereas no statistically significant differences could be detected among other media, independent of the time point or donor. Furthermore, the Ca8 medium showed a significant upregulation of OC and OP mRNA levels occurring in time for both donors. Donor 2 cells cultured in Pi8 medium also expressed significantly higher levels of OC and OP mRNA.

of ALP mRNA than all other cultures at day 7 (with the exception of cells in Ca4) and at day 10.

BMP2 expression profiles of cells from the two donors presented similarities with an upregulation of BMP2 mRNA for cells grown in Ca8 and in Pi8 sup-

plemented media. A temporal increase in BMP2 levels was observed for these cultures, which was statistically significant from the first time point for Pi8 medium and from the second time point for Ca8 medium compared to all other conditions, independent of the donor. Considering donor 1, hMSCs cultured for 7 days in Pi8 medium expressed a significantly higher level of BMP2 as compared to Ca4, Pi4, basic and osteogenic media, while cells grown in Ca8 only showed a significantly higher level of mRNA in comparison to osteogenic medium cultures. After 10 days, BMP2 expression was significantly increased in Ca8 and Pi8 cultures, as compared to cultures in all other media. It could be noticed that cells grown in Pi8 also expressed significantly higher levels of BMP2 than cells grown in Ca8. Data on donor 2 cultures presented significant BMP2 upregulation at day 7 in cells grown in Pi8 medium and at day 10 in hMSCs cultured in Ca8 and Pi8 as compared to all other conditions.

Analysis of collagen type I expression showed that only cells cultured in calcium supplemented media showed an upregulation of this gene, as no statistically significant differences could be detected among other media, independent of the time point and donor. For donor 1, cells cultured in Ca8 medium expressed a significantly higher level of collagen type I mRNA, at 7 and 10 days, as compared to all other cultures. HMSCs from the second donor cultured in Ca8 medium presented a significantly higher expression of collagen type I at 7 days compared to cells cultured in basic medium, and at 10 days compared to cells grown in all other media. The effect of calcium on these cells was also clear after 10 days of exposure to Ca4 medium, where cells expressed a significantly higher level of mRNA than cells cultured in basic medium.

Osteocalcin gene expression was also enhanced by Ca8 medium. Donor 1 hMSCs levels of osteocalcin mRNA were significantly increased in Ca8 cultures at 7 and 10 days in comparison to all other cultures. In hMCs from the second donor, the effect of Ca8 medium was noticeable as early as day 4 with an upregulation of gene expression compared to basic medium culture. After 7 days of exposure to Ca8, cells expressed significantly higher levels of osteocalcin than cells grown in all other conditions. From this second time point, upregulation of osteocalcin also became significantly higher for cultures in Pi8 medium compared to basic medium cultures. After 10 days of culture, cells cultured in both Ca8 and Pi8 media presented a statistically significant upregulation of osteocalcin expression compared to all other media.

The only significant increase in osteopontin expression of cells from donor 1 was detected for cells cultured for 10 days in Ca8 medium. Cells from donor 2 showed a similar effect of Ca8 medium, however upregulation of osteopontin occurred at day 7 and this pattern was also observed for cells cultured in Pi8 medium. However, the osteopontin levels measured in Ca8 cultures were significantly higher than that recorded for Pi8 cultures at 10 days.

hMSCs cultured in supplemented media were stained with alizarin red after

Figure 4.12: Mineralization of cultures grown in supplemented media. Alizarin red staining of hMSCs cultured for 10 days in supplemented media, and in basic and osteogenic media (scale bars: 1 mm). Small clusters of red stain were observed in the Pi4 cultures, whereas abundant and homogeneous staining was observed in Ca8 and Pi8 cultures. Cells cultured in Pi8 medium exhibited the most intensive stain.

10 days to qualitatively assess ECM mineralization (Figure 4.12). To exclude the possibility that supplemented media could precipitate on the ECM and be stained by the alizarin red solution, collagen coated petri dishes were filled with the supplemented media and placed in culture conditions for 24 hours. After this treatment, collagen coated petri dishes were rinsed and stained in the same way as the cultures. None of the dishes were stained (results not shown), which confirmed that supplemented media did not precipitate on the collagen films. Only Ca8, Pi4 and Pi8 cultures were stained and the result was similar for both donors. Microscopic red clusters were observed in the Pi4 cultures, whereas staining was abundant in Ca8 and Pi8 cultures and could be seen easily with the naked eye. Cells grown in Pi8 medium exhibited the strongest stain with nearly the entire surface of the wells strongly coloured red.

4.4 Discussion

Calcium phosphate based bioceramics are the largest class of synthetic bone graft substitutes. A wide range of calcium phosphate based biomaterials have been developed since the 1970's and several have reached clinical application for orthopaedic and maxillofacial surgery, due largely to their physicochemical

similarity to natural bone mineral and osteoconductive properties [23]. In an attempt to find an optimal calcium phosphate ceramic for application in bone repair and regeneration, a contemporary literature search reveals a somewhat general conclusion that a favorable combination of complex features such as chemical composition, degradation rate, grain size, porosity and specific surface area is required. These different properties are, however, interlinked, making it very difficult to change one without affecting another; hence design of calcium phosphate bone graft substitutes remains a highly complex issue. Consequently it is also difficult to identify which property, or combinations of properties, of a bone graft substitute is responsible for a specific biological effect.

In order to obtain a greater understanding regarding the role of individual material properties on the biological processes related to bone formation and remodeling, one needs to separate the individual properties and test them independently to one another. Here, we have attempted to investigate the individual effects of calcium and phosphate ions on the growth, differentiation and mineralization of clinically relevant hMSCs. To this end, we designed composites with calcium or phosphate salts as fillers, instead of a calcium phosphate ceramic. By doing so, we ensured that these composites would serve two purposes: (1) independent release of either Ca^{2+} or Pi from the polymeric carrier and (2) control over surface properties (like porosity or roughness) since the polymeric phase and the production process remained identical. Furthermore, to test the effect of the two ions in the absence of the 3D environment created by the composite particles, hMSCs were also cultured in conventional tissue culture well plates in media conditioned with the two ions of interest.

Polymeric particles have been extensively studied as carriers of drugs and other biologics, but they can also be used as release vehicles for bioinorganics. PLA is a good candidate for this application, since the degradation rate of this biocompatible polymer, and hence the release profile of the incorporated compound, can be controlled by selecting a specific molecular weight [24]. Different combinations of calcium carbonate and PLA have already been achieved, such as composites [25] and hollow spheres [26], and it has been shown that such materials support cell attachment. In the present study, we were able to confirm a homogeneous incorporation of calcium carbonate and sodium phosphate salts into the PLA matrix, with a sustained release of both Ca^{2+} and Pi over 14 days in a simulated physiological solution. The ion release rate decreased with time, although the duration of the experiment did not allow the system to reach equilibrium. Much of the inorganic salt grains were trapped inside the polymer matrix and unable to dissolve and therefore ions were not released. Changing the particle size or introducing macroporosity (e.g. foaming) to modify surface area would make more of the entrapped salt accessible and would increase the ions release rate. Another advantage of polymeric carriers is their ability to retain structural integrity while releasing compounds in a biological environment, making them suitable for *in vitro*

[26] and *in vivo* [19, 24] studies. The composite particles developed in the current study developed only minor microscale damage (surface holes and cracks) after 14-day immersion in physiologically relevant environment that did not prevent cell attachment and proliferation. Changes in the concentrations of Ca^{2+} and Pi in our *in vitro* composite-cells-medium systems were evaluated. Even though an increase in Ca^{2+} concentrations would be expected in experiments containing calcium composites, we observed Ca^{2+} concentrations comparable, or even lower, than those measured in the medium containing PLA control samples. In cases of lower than expected Ca^{2+} , there was also a depletion of Pi in the medium, which was especially noticeable in PLA-Ca50% samples. While PLA-Pi5% did enrich the culture medium in phosphate ions, there was also a reduction in Ca^{2+} . This decrease in Ca^{2+} and Pi concentration indicates either spontaneous precipitation of a calcium phosphate layer on the surface of the materials as previously observed for composites consisting of PLA and calcium-phosphate apatite [24] or of the mineralization of the ECM produced by the cells [20], or both.

Although the Ca^{2+} and Pi concentrations measured in the medium during cell culture on the different composites were lower than those initially present in the supplemented media, similarities could be observed in the effects of both ions on hMSCs growth and differentiation. Cells cultured on calcium composites exhibited a higher proliferation rate than those cultured on the phosphate composite and PLA control. This positive effect was especially significant in the PLA-Ca50% culture, which correlates with the results obtained from cultures in supplemented media where calcium was shown to stimulate hMSCs proliferation in a dose-dependent manner. This observation confirms previous results obtained with hMSCs exposed to a calcium concentration of 7.8 mM [8]. While PLA-Pi5% did not seem to affect the proliferation rate of hMSCs, the effect of phosphate on cell proliferation in supplemented media depended on the donor. The cells of donor 1 showed a decrease in proliferation when exposed to the highest phosphate concentration for 10 days. However, an increase in proliferation was observed in donor 2 cells exposed for 10 days to 4 and 8 mM of phosphate. A stimulating effect of phosphate on cell proliferation has previously been described for human periosteum derived cells (hPDC) [7] and osteoblasts [27], when these cells were exposed to Pi concentrations between 2 and 4 mM. However, the exposure to high concentrations of Pi (>5mM) also led to osteoblast apoptosis [10]. This implies that the control of Pi release from bone substitute materials is highly important.

The favourable effect of a combination of calcium and phosphate ions on ALP production by hMSCs has already been demonstrated in an earlier study where nanosized hydroxyapatite powder was combined with PLA to produce a composite [19]. ALP is considered an early marker of osteogenesis [28] and is known to influence phosphate homeostasis, as this enzyme can convert extracellular pyrophosphate, a mineralization inhibitor, into inorganic phosphate [29]. In the present study, it was demonstrated that Ca^{2+} and Pi also independently increase

production of ALP in hMSCs, both from composites and supplemented media cultures. Our results indicate that Ca^{2+} has a dose-dependent effect on ALP production, as high concentration of Ca^{2+} resulted in higher ALP activity in both composite cultures and in supplemented media. Interestingly, higher levels of ALP expression were observed in composite cultures, though Ca^{2+} concentration in the medium was in general lower than that in supplemented media. Pi strongly enhanced ALP expression in the composite culture, whereas in the conditioned media, only a low concentration of 4mM and only at the later time point of 10 days showed a positive effect as compared to the control. Considering that also here, the Pi concentration in the medium of composite culture was lower than in the supplemented media, it is suggested that there is an upper limit to Pi concentration above which the ALP expression is inhibited.

Consistent effects of calcium and phosphate ions in composites and supplemented media cultures could also be observed in the gene expression of hMSCs. For instance, an upregulation of BMP2 gene expression of hMSCs cultured in presence of elevated levels of calcium or phosphate both on composites and in supplemented media was observed, particularly at early time points. BMP2 levels of cells cultured in Ca8 and Pi8 media for 10 days were significantly increased as compared to expression levels measured in all other cultures. Also cells cultured on PLA-Ca50% and PLA-Pi5% for 7 days expressed significantly higher BMP2 mRNA levels than cells cultured on controls. These results are in accordance with the literature as hMSCs were shown to express significantly higher levels of BMP2 mRNA when exposed for 6 days to 7.8mM of calcium ions [8]. The effect of phosphate on BMP2 expression is documented for hPDCs, which were shown to express higher levels of BMP2 mRNA when exposed to 8mM of phosphate ions for more than 7 days [7]. BMP2 has a well-known role in the process of osteogenic differentiation as well as in the maintenance of bone homeostasis and bone regeneration [30].

This study indicates that the expression of OC and OP in response to phosphate may be dependent on the cell source. Here we tested hMSCs from 2 different donors and while cells from donor 1 expressed significantly higher levels of both genes when exposed to Ca8 or cultured on PLA-Ca50%, cells from donor 2 expressed higher levels of both genes also when exposed to Pi8 medium and when cultured on PLA-Pi5%. The positive effects of calcium and phosphate on the expression of OC and OP mRNA were also drawn in previous studies [7, 8]. OC is known to regulate the growth and size of hydroxyapatite crystals [31] and OP is thought to inhibit hydroxyapatite formation and growth during bone mineralization [32], making these proteins valuable markers of osteogenesis.

The expression of collagen type 1, a predominant organic component of bone, was shown to be only increased by Ca^{2+} from either composites or supplemented media cultures, however, this induction is largely dependent on the cell source.

Although a concentration of 7.8 mM was previously shown to induce ALP down regulation in hMSCs [8], in our study, similar results were only observed

when cells were cultured on composite particles. Conditioning of the medium with either calcium or phosphate ions did not have an effect on the expression of ALP mRNA, which does not reflect the observed effect on the enzymatic levels discussed earlier; this discrepancy is likely related to the timing at which the expression was quantified since peak ALP expression at mRNA level is transient.

In general, analysis of osteogenic markers has led us to conclude that both calcium and phosphate ions instruct hMSCs to differentiate towards the osteogenic lineage. Signs of mineralization were observed in all experiments in which phosphate ions were increased and the supplemented media cultures suggested a dose-dependent effect. Elevated Pi concentrations have previously been shown to initiate ECM mineralization *in vivo* [20] and in hPDCs cultures [7]. Although in the study on hPDCs it was concluded that calcium ions do not affect mineralization [7], in the present study we did observe signs of mineralization in Ca8, PLA-Ca5% and PLA-Ca50%, indicating that Ca^{2+} also enhanced ECM mineralization by hMSCs in a dose-dependent manner. Besides the difference in the cell type used, a reason for a more pronounced mineralization observed in the current study may be a larger surface area of the composite particles as compared to the cell culture well plate, that enabled more ECM production, followed by its mineralization.

Taken together, the results of this study confirmed that there are individual effects of calcium and inorganic phosphate ions on proliferation, osteogenic differentiation and mineralization of hMSCs in basic medium, and thus they are effective in the absence of other stimulators of osteogenic differentiation or mineralization. Similar effects were observed when composites containing calcium or phosphate salts were used, although ion concentrations measured in the medium were lower compared to conditioned medium. It is however important to emphasize that these measurements were performed in bulk of the medium, and that local concentrations in the vicinity of cells may have been much higher. By varying the initial concentration of salts, but also by carefully selecting polymer properties, the release profile of the ions can be controlled.

Our approach can aid in elucidating the individual effects of a variety of soluble compounds, including bioinorganics, without interference from other material properties. Current approaches for the delivery of bioinorganics include incorporation in or adsorption on calcium phosphate bulk ceramics [33, 34], coatings [35] and bioactive glasses [36]. Although these strategies also allow the use of delivery vehicles as scaffolds, they generate a complex environment with other dissolution products that also affect cell fate. Besides being an interesting study model, this approach supports the concept of using bioinorganics as alternatives to growth factors, rather than just structural components of a ceramic or glass. Locally delivered, they have been shown to enhance mineralization [20], vascularization [2] and tissue regeneration in general, making bioinorganics potentially a cost-effective alternative to biological growth factors.

4.5 Conclusion

By carefully designing a polymeric based composite material we were able to release calcium and inorganic phosphate ions independently to one another and study the effects on cultured hMSC cells independently to other material properties. In doing so, and supported by cultures in which these ions were added exogenously, we have shown that both ions have a strong effect on the proliferation, osteogenic differentiation and mineralization of hMSCs in the absence of other stimulators of osteogenic differentiation or mineralization. Our approach can aid in elucidating the individual effects of a wide variety of soluble compounds, including bioinorganics, without interference from other material properties.

References

1. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: a review of current technology and applications. *J Appl Biomater.* 1991 Jan;2(3):187-208.
2. Habibovic P, Barralet JE. Bioinorganics and biomaterials: bone repair. *Acta Biomater.* 2011 Aug;7(8):3013-26.
3. Barrère F, Ni M, Habibovic P, Ducheyne P, Groot K de. Degradation of Bioceramics. In: van Blitterswijk CA, Thomsen P, Lindahl A, Hubbell J, Williams DF, Cancedda R, et al., editors. *Tissue Engineering*. Academic P. Amsterdam: Elsevier; 2008. p. 223-54.
4. Kokubo T. Bioactive glass ceramics: properties and applications. *Biomaterials.* 1991 Mar;12(2):155-63.
5. Le Huec JC, Clément D, Brouillaud B, Barthe N, Dupuy B, Foliguet B, et al. Evolution of the local calcium content around irradiated β -tricalcium phosphate ceramic implants: in vivo study in the rabbit. *Biomaterials.* 1998 Apr;19(7-9):733-8.
6. Dvorak MM, Siddiqua A, Ward DT, Carter DH, Dallas SL, Nemeth EF, et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc Natl Acad Sci USA.* 2004 Apr 6;101(14):5140-5.
7. Chai YC, Roberts SJ, Schrooten J, Luyten FP. Probing the osteoinductive effect of calcium phosphate by using an in vitro biomimetic model. *Tissue Eng Part A.* 2011 Apr;17(7-8):1083-97.
8. Barradas AMC, Fernandes HAM, Groen N, Chai YC, Schrooten J, van de Peppel J, et al. A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells. *Biomaterials.* 2012 Apr;33(11):3205-15.
9. Wen L, Wang Y, Wang H, Kong L, Zhang L, Chen X, et al. L-type calcium channels play a crucial role in the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells. *Biochem Biophys Res Commun.* 2012 Aug;424(3):439-45.
10. Meleti Z, Shapiro I., Adams C. Inorganic phosphate induces apoptosis of osteoblast-like cells in culture. *Bone.* 2000 Sep;27(3):359-66.
11. Beck GR, Zerler B, Moran E. Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci USA.* 2000 Jul 18;97(15):8352-7.
12. Shih Y-R V, Hwang Y, Phadke A, Kang H, Hwang NS, Caro EJ, et al. Calcium phosphate-bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling. *Proc Natl Acad Sci USA.* 2014 Jan 21;111(3):990-5.
13. Yamasaki H, Sakai H. Osteogenic response to porous hydroxyapatite ceramics under the skin of

dogs. *Biomaterials*. 1992 Jan;13(5):308-12.

14. Habibovic P, Yuan H, van Der Valk CM, Meijer G, van Blitterswijk CA, de Groot K. 3D microenvironment as essential element for osteoinduction by biomaterials. *Biomaterials*. 2005 Jun;26(17):3565-75.

15. Okamoto M, Dohi Y, Ohgushi H, Shimaoka H, Ikeuchi M, Matsushima A, et al. Influence of the porosity of hydroxyapatite ceramics on in vitro and in vivo bone formation by cultured rat bone marrow stromal cells. *J Mater Sci Mater Med*. 2006 Apr;17(4):327-36.

16. Yuan H, Fernandes H, Habibovic P, de Boer J, Barradas AM., de Ruiter A, et al. Osteoinductive ceramics as a synthetic alternative to autologous bone grafting. *Proc Natl Acad Sci USA*. 2010 Aug;107(31):13614-9.

17. Ripamonti U, Crooks J, Kirkbride A. Sintered porous hydroxyapatites with intrinsic osteoinductive activity: Geometric induction of bone formation. *S Afr J Sci. Bureau Scientific Publications*; 1999;95:335-343.

18. Habibovic P, Gbureck U, Doillon CJ, Bassett DC, van Blitterswijk CA, Barralet JE. Osteoconduction and osteoinduction of low-temperature 3D printed bioceramic implants. *Biomaterials*. 2008 Mar;29(7):944-53.

19. Danoux CB, Barbieri D, Yuan H, de Bruijn JD, van Blitterswijk CA, Habibovic P. In vitro and in vivo bioactivity assessment of a polylactic acid/hydroxyapatite composite for bone regeneration. *Biomater*. 2014 Jan;4:e27664.

20. Habibovic P, Bassett DC, Doillon CJ, Gerard C, McKee MD, Barralet JE. Collagen biomineralization in vivo by sustained release of inorganic phosphate ions. *Adv Mater*. 2010 Apr;22(16):1858-62.

21. Fernandes H, Mentink A, Bank R, Stoop R, van Blitterswijk CA, de Boer J. Endogenous collagen influences differentiation of human multipotent mesenchymal stromal cells. *Tissue Eng Part A*. 2010 May;16(5):1693-702.

22. Both SK, van der Muijsenberg AJC, van Blitterswijk CA, de Boer J, de Bruijn JD. A rapid and efficient method for expansion of human mesenchymal stem cells. *Tissue Eng*. 2007 Jan;13(1):3-9.

23. Barradas AM., Yuan H, van Blitterswijk CA, Habibovic P. Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms. *Eur Cell Mater*. 2011 Jan;21:407-29.

24. Barbieri D, Yuan H, Luo X, Farè S, Grijpma DW, de Bruijn JD. Influence of polymer molecular weight in osteoinductive composites for bone tissue regeneration. *Acta Biomater*. 2013 Dec;9(12):9401-13.

25. Kasuga T, Maeda H, Kato K, Nogami M, Hata K, Ueda M. Preparation of poly(lactic acid) composites containing calcium carbonate (vaterite). *Biomaterials*. 2003 Aug;24(19):3247-53.

26. Maeda H, Kasuga T. Preparation of poly(lactic acid) composite hollow spheres containing calcium carbonates. *Acta Biomater*. 2006 Jul;2(4):403-8.

27. Kanatani M, Sugimoto T, Kano J, Chihara K. IGF-I mediates the stimulatory effect of high phosphate concentration on osteoblastic cell proliferation. *J Cell Physiol*. 2002 Mar;190(3):306-12.

28. Golub EE, Boesze-Battaglia K. The role of alkaline phosphatase in mineralization. *Curr Opin Orthop*. 2007;18(5):444-8.

29. O'Neill WC. Pyrophosphate, alkaline phosphatase, and vascular calcification. *Circ Res*. 2006 Jul;99(2):e2.

30. Rosen V. BMP2 signaling in bone development and repair. *Cytokine Growth Factor Rev*. 2009 Jan;20(5-6):475-80.

31. Roach HI. Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralisation and resorption. *Cell Biol Int*. 1994 Jun;18(6):617-28.

32. Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. *Crit Rev Oral Biol Med*. 2004 Jan;15(3):126-36.
33. Luo X, Barbieri D, Davison N, Yan Y, de Bruijn JD, Yuan H. Zinc in calcium phosphate mediates bone induction: in vitro and in vivo model. *Acta Biomater*. 2014 Jan;10(1):477-85.
34. Barralet J, Gbureck U, Habibovic P, Vorndran E, Gerard C, Doillon CJ. Angiogenesis in calcium phosphate scaffolds by inorganic copper ion release. *Tissue Eng Part A*. 2009 Jul 20;15(7):1601-9.
35. Yang L, Perez-Amodio S, Barrère-de Groot FYF, Everts V, van Blitterswijk CA, Habibovic P. The effects of inorganic additives to calcium phosphate on in vitro behavior of osteoblasts and osteoclasts. *Biomaterials*. 2010 Apr;31(11):2976-89.
36. Hoppe A, Mouriño V, Boccaccini AR. Therapeutic inorganic ions in bioactive glasses to enhance bone formation and beyond. *Biomater Sci*. Royal Society of Chemistry; 2013 Feb;1(3):254.

Development of Highly Functional Biomaterials by Decoupling and Recombining Material Properties

Abstract

The majority of currently applied functional biomaterials are complex with properties that are largely intertwined, making it difficult to change one without affecting the others. Here, we present an approach to first decouple individual properties of such materials, and upon investigating their sole effect on a biological process, rationally recombine them into new biomaterials with improved functionality. As a proof of concept, chemical and geometrical properties of biomimetic calcium phosphates, widely used as bone graft substitutes, are decoupled by transferring their multiscale, three-dimensional (surface-)geometrical features into a polymer using nanoimprinting. In a next step, the chemical component is either reintroduced or replaced by another one by applying appropriate coating techniques. Individual and combined effects of chemical composition and geometrical features are determined by culturing clinically relevant human bone marrow-derived stromal cells on the different developed materials and assessing cell differentiation towards the osteogenic lineage. It is demonstrated that the proposed materials design-driven approach is a valuable tool to (1) increase fundamental knowledge of the relationship between a material property and a biological response and (2) develop new functional biomaterials with overall improved performance and/or applicability.

5.1 Introduction

Functional biomaterials are gaining importance in regenerative medicine as alternatives to classical treatments based on patient's own tissue, the limited supply of which is an important concern [1-3]. A range of biomaterials exists, varying from natural to fully synthetic, from polymers to metals, from monolithic to complex composites and mixtures. Regardless the type or complexity, a biomaterial is always designed and produced in such a way that it elicits a desired biological function [4]. Assessment of this function in response to the biomaterial should be reliable enough to serve as basis for further improvement, reverse engineering or combinatorial development of new materials. Recently, Murphy et al. asserted on the importance of co-varying inherent material properties in a controlled manner in order to obtain a better understanding of the relationship between individual properties [3]. This, however, can only be achieved by decoupling individual properties of a material and understanding their sole effect on a biological process. In the current study, we undertook an attempt to shift the development of functional biomaterials so far requiring a materials processing-driven approach to now a design-driven one, increasing not only fundamental knowledge of the interactions between biomaterials and biological systems, but also practical possibilities to develop advanced biomaterials in terms of both functionality and applicability. To this end, we employed a range of processing techniques to first isolate individual properties and, upon investigating their sole effect on the biological function of clinically relevant human mesenchymal stromal cells (hMSCs), recombine them into improved or completely new functional biomaterials (Figure 5.1). As a model material, we used calcium phosphate (CaP) ceramics, which are the most widely used synthetic bone graft substitutes, despite their intrinsic brittleness [5]. Independent of the production method, i.e. precipitation, sol-gel technique or sintering, CaP ceramics are generally crystalline materials with distinct chemical composition, crystallinity, crystal/grain size, surface roughness, specific surface area (SSA), etc. These different properties are largely intertwined, and it is difficult, if not impossible, to change one without affecting the others by applying conventional production techniques [6, 7]. It is therefore particularly challenging to define the contribution of individual properties of CaP ceramics to a biological response as well as to produce such materials with well-defined and controlled properties. This is somewhat in contrast to other biomaterials, such as some (amorphous) polymers, which allow, to a certain extent, control over a single parameter without changing the others. For example, mechanical properties such as stiffness of a hydrogel can be tuned in a relatively facile manner by varying the crosslinking density of the network [8]. Similarly, topography of polymeric surfaces can be closely controlled down to the nanoscale using a diversity of techniques including imprint lithography-based ones [9], whereby one can investigate the topographical effects while keeping the polymer chemistry constant [10]. Furthermore, polymeric surfaces can be chemically decorated in a highly controlled fashion, without significant changes

to their topography or mechanical properties [11].

While various properties of CaP-based biomaterials have been suggested to play a role in the processes related to bone formation and remodeling [12, 13], no consensus has been reached yet as of how an optimal ceramic-based bone graft substitute should look like in a given application. Here, we first explored to which extent a conventional technique to synthesize CaPs, such as precipitation, can be used to decouple and investigate sole effects of their chemical and geometrical properties. Instead of relying on the possibilities of precipitation as a synthesis technique, in a second step, a combination of exemplary techniques, namely nanoimprinting and sputtering or biomimetic deposition, was applied to further decouple the effects of individual properties and, based on this knowledge, to develop improved or fully new functional biomaterials.

5.2 Materials and Methods

Synthesis and Characterization of CaP Crystals: All chemicals were purchased at either Merck or Sigma. B and OCP crystals were synthesized by means of homogeneous crystallization in sterile solutions prepared in MilliQ water. To this end, each solution was filtered through a sterile disposable filter unit with SFCA membrane of 0.2 μm pore size (Nalgene) prior to mixing. BN crystals were produced by mixing equal volumes of 0.25 M CaCl_2 and 0.25 M $(\text{NH}_4)_2\text{HPO}_4$ solutions at pH 4.0. The resulting solution was diluted to a final phosphate concentration of 0.2 M and kept at 40°C for 48 hours. For BP crystals, equal volumes of 0.25 M $\text{Ca}(\text{NO}_3)_2$ and 0.25M $\text{NH}_4\text{H}_2\text{PO}_4$ solutions were mixed and kept at 40°C for 48 hours. OCP crystals were produced by mixing 450 ml 40 mM CaCl_2 -, 450 ml 30 mM Na_2HPO_4 - and 4.5 l 0.2 M NaOAc solution at pH 6.5 and keeping it at 45°C for 48 hours. Upon precipitation, B and OCP crystals were collected, rinsed with 70% ethanol and dried at 37°C overnight. M crystals were obtained by autoclaving B crystals at 120°C for 20 minutes. Crystals were characterized using SEM (XL30, ESEM-FEG, Philips, The Netherlands), FTIR (Spectrum100, Perkin Elmer Analytical Instruments, USA) and XRD (Miniflex, Rigaku, Japan). SSA of crystals was quantified with the Brunauer-Emmett-Teller (BET) method using a nitrogen adsorption volumetric technique (TriStar 3000, Micromeritics, USA). To determine chemical and morphological changes of the crystals in aqueous environment, 40 mg of crystals were immersed in 1.5 ml basic cell culture medium (α -minimal essential medium (Gibco), 10% fetal bovine serum (Lonza), 0.2 mM ascorbic acid (Sigma Aldrich), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco)) for 7 and 14 days, at 37°C in a humid atmosphere with 5% CO_2 , without refreshment. Upon ageing, Ca^{2+} and Pi ionic concentrations of the ageing medium were determined by using a quantitative colorimetric method (QuantiChromTM Calcium assay kit (DICA-500) and QuantiChromTM Phosphate assay kit (DIPI-500), respectively) as per manufacturer's protocol and the crystals

Figure 5.1: A new concept of design-driven approach toward functional biomaterials development, whereby individual properties are first decoupled to investigate their sole effects on a biological process, and, following this investigation, recombined in such a way that the overall performance and applicability of the biomaterial is improved. This is in contrast to classical, processing-driven biomaterials development, where the properties of a material are mainly determined by the possibilities of the technique used to produce it. In the current study, nanoimprinting was used in the first step to transfer complex, multiscale three-dimensional (surface) geometrical features of biomimetic CaPs into a polymer to subsequently study their effect on the differentiation of clinically relevant hMSCs, independently of CaP chemistry. Instead of nanoimprinting, other techniques could be used to isolate individual properties. In the second step, a biomimetic coating method was used to reintroduce the CaP chemistry, while retaining the structural features as well as bulk-mechanical properties of the polymer underneath the coating. In other words, a functional material was built having structural and chemical properties of a CaP ceramic, but not suffering from its intrinsic brittleness and low strength. Alternatively, DC sputtering was used to deposit a thin layer of titanium on the imprints, creating a material with the bulk-mechanical properties of a polymer, the crystalline structure of the ceramic and the surface chemistry of a metal. This ceramic-polymer-metal triad is an example of a material that does not occur in nature and with properties being a result of contributions from all three main classes of synthetic biomaterials. Like in the case of property isolation, in the recombinant step of materials development, other techniques can be used to design the properties of the final material.

underwent SEM and FTIR analyses.

Imprinting and Surface Chemistry Modification: The negative (inverse) PDMS molds were prepared by distributing and immobilizing the CaP crystals on a double-sided tape followed by casting of the silicone resin (Sylgard® 184, Dow Corning, USA), mixed with the curing agent (Sylgard® 184, Dow Corning, USA) at a 10:1 ratio, over the master structures. The samples were subsequently placed under vacuum to remove air bubbles, followed by incubation at room temperature for 2 days and subsequently at 50°C overnight to facilitate polymerization. After polymerization, molds were cleaned with 6 M HCl solution and deionized water. Crystal-mimetic imprints were produced by hot embossing of the PDMS molds onto a COC film (kindly provided by TOPAS, Frankfurt, Germany) with a thickness of 300 µm at 130°C for 90 minutes. The same imprinting procedure was used for reproducing the surface of decellularized human allograft chips. To introduce chemistry changes while retaining the surface structure, crystal imprints in COC were coated with either titanium by DC sputtering at 200 W (Sputterke, TCO, University of Twente, The Netherlands), which resulted in a titanium layer with a maximum thickness of 35 nm, or with CaP by immersion in a simplified fivefold concentrated simulated body fluid [46] for 12 hours at 37°C following air plasma (Plasma sterilizer, PDC-002, Harrick Scientific, USA) treatment for 6 seconds. After the coating process, samples were thoroughly washed with demineralized water and dried at RT. In order to examine the efficiency of the replication technique, a sample of MN crystals was selected to analyze surface morphology using SEM and to perform roughness quantification using white light interferometry (Contour GT-I, Bruker, USA). Elemental analysis of surfaces before and after chemical modification was performed using energy dispersive X-ray analyzer (EDAX; Apollo X, Ametek, USA).

In vitro Cell Culture and Assessment of Proliferation and Osteogenic Differentiation: Bone marrow aspirates from healthy patients were obtained after written informed consent, and hMSCs were isolated and expanded as described previously [47, 48]. After weighing, crystal samples were placed in ultra-low attachment 24-well plates (Costar® Corning, USA) to minimize the adhesion of cells to the plate. The samples were then rinsed twice with 70% ethanol at RT, followed by washing with sterile phosphate-buffered saline (PBS) upon complete evaporation. COC crystal imprints without additional coating were treated with air plasma for 1 minute prior to further sterilization. Individual samples of the imprints of each crystal type, without or with titanium or CaP coating were placed in ultra-low attachment 24-well plates and fixed with O-rings with suitable diameter. The samples were then rinsed three times with 70% ethanol and once with 100% ethanol, and, following complete evaporation, washed with sterile PBS. Crystals and imprint samples were pre-conditioned in proliferation medium (basic medium supplemented with 1 ng/ml basic fibroblast growth factor (FGF, Fisher Scientific)) overnight in a humidified atmosphere with 5% CO₂ prior to cell seeding.

Name	Primer Sequence
ALP	5'-GACCCTTGACCCCCACAAT-3' 5'-GCTCGTACTGCATGTCCCCT-3'
BMP-2	Purchased from SA Biosciences
GAPDH	5'-CGCTCTCTGCTCCTCCTGTT-3' 5'-CCATGGTGTCTGAGCGATGT-3'
Osteocalcin	5'-GGCAGCGAGGTAGTGAAGAG-3' 5'-GATGTGGTCAGCCAACTCGT-3'
Osteopontin	5'-CCAAGTAAGTCCAACGAAAG-3' 5'-GGTGATGTCCTCGTCTGTA-3'

Table 5.1: Primers sequences for human genes.

DNA content (CyQUANT® Cell Proliferation assay, Life Technologies) and ALP activity (CDP-Star® Reagent, Roche) were assessed after 7 and 14 days upon culturing 20,000 cells of passage 3 on 40 mg of crystals in 1.5 ml of proliferation medium, with refreshment every 2-3 days. To assess cell morphology using SEM, 20,000 cells were cultured on 40 mg of crystals or on each imprint for 3 or 7 days, followed by washing, fixation and dehydration steps. To investigate the osteogenic gene expression, 200,000 cells were seeded on either 120 mg of crystals or on an imprint sample in 1.5 ml of proliferation medium. Cells cultured in plasma-treated 6-well plates (Costar® Corning, USA) at a density of 50,000 cells per well in 3ml of medium served as controls. After 24 hours, cell culture continued in either basic or osteogenic medium (basic medium supplemented with 10 nM dexamethasone) for 7 or 14 days, with refreshment of medium every 2-3 days. RNA isolation and cDNA synthesis were performed as described previously [27]. Quantitative real-time polymerase chain reaction (qPCR) analysis was performed for a set of markers (Table 5.1) and fold induction was calculated using the comparative $\Delta\Delta CT$ method, upon normalization of CT values to the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Statistical analysis: Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison post-hoc test. The level of significance was set at $p < 0.05$. All quantitative data presented are expressed as mean $\pm$ standard deviation.

5.3 Results and Discussion

5.3.1 Decoupling Properties of CaPs by Using Precipitation as a Conventional Synthesis Method

In order to decouple the chemical effect of CaPs on the fate of hMSCs from the corresponding geometrical one, in a first part of this study, we synthesized biomimetic CaP crystals with three distinct phases: dicalcium phosphate dihydrate (brushite, B), dicalcium phosphate anhydrous (monetite, M) and octacalcium phosphate (OCP), all of which have shown the potential to be used as bone graft substitutes [14, 15]. By controlling the crystal precipitation process, both B and M were produced as needle- (N) and as plate-shaped (P) crystals with a size between 500 μm and 1 mm. While the crystal shape was modified, chemistry remained unchanged. This allowed us to investigate the individual effect of the CaP phase on the osteogenic differentiation of hMSCs on the one, and the corresponding effect of the crystals' geometrical (surface) features on the other hand. Phase composition of the three types of CaP, as well as chemical similarity between needle- and plate-shaped crystals of the same phase, were confirmed by Fourier transform infrared (FTIR) spectroscopy analysis [16-18] (Figure 5.2.A in the Supporting Information). X-ray diffraction (XRD) patterns (Figure 5.2.B) of BN and BP presented analogous profiles, and so did the XRD patterns of MN and MP, indicating that both shapes of B and M had very close to identical crystalline structures. The patterns of the three crystal types were in good agreement with the literature values [19], and suggested they were phase-pure.

Figure 5.2: FTIR spectra (A) and XRD patterns (B) of the five crystal types. The FTIR spectra of BN and BP were similar, confirming identical chemistry of both shapes of B crystals. B spectra exhibited peaks characteristic of tetrahedral PO_4^{3-} units at 526, 576 and 668 cm^{-1} (O-P-O bending modes), 986 cm^{-1} (P-O symmetric stretching mode) and 1060, 1136 and 1218 cm^{-1} (P-O asymmetric stretching modes). The presence of water in the crystal lattices was demonstrated by the peaks at 1650 cm^{-1} (H-O-H bending mode of water) and at 3488 and 3544 cm^{-1} (O-H stretching). Similar to B crystals, resemblance of FTIR spectra of MN and MP indicated that both crystal types were of the same chemistry. The peaks of tetrahedral PO_4^{3-} units were observed at 526 and 564 cm^{-1} (bending modes) and 1120 and 1068 cm^{-1} (stretching modes). Furthermore, the absence of peaks at around 3500 cm^{-1} and 1650 cm^{-1} confirmed the anhydrous nature of the M crystals. The FTIR spectrum of OCP exhibited two typical peaks at 860 and 912 cm^{-1} that can be assigned to HPO_4^{2-} units in the crystal structure. XRD patterns were typical of respectively phase pure B, M and OCP, and confirmed similarity in crystal structure between plate- and needle-shaped crystals of the same chemistry.

Low-magnification scanning electron microscopy (SEM) images (Figure 5.3, first row) confirmed that both B and M were synthesized as needles as well as plates, while OCP crystals possessed a distinct spherical shape with plate-like crystals radiating from the nucleus. Higher-magnification SEM micrographs (Figure 5.3, second row) showed a relatively smooth surface of as-prepared B crystals, in contrast to M crystals that exhibited a more rough, flake-shaped surface. OCP crystals showed a rough surface that was built of large, plate-shaped crystals with sharp edges. In accordance with SEM analysis, the SSA of M crystals was about two orders of magnitude larger than that of B crystals, whereas the SSA of OCP was about twice as large as that of M. While morphological differences were observed upon ageing of various crystals in cell culture medium (Figure 5.3, third and fourth rows), no significant chemical modification was observed by FTIR analysis (Figure 5.4). The analysis of the calcium (Ca^{2+}) and inorganic phosphate (Pi) ionic content of the ageing medium indicated different behavior of the crystals dependent on their chemistry (Figure 5.5). While both BN and BP crystals released Ca^{2+} and Pi over a 14-day ageing period, the ionic concentration of the incubation medium of the M crystals did not change over time. In OCP immersed solutions, however, a decrease of Ca^{2+} and Pi ions was observed, suggesting predominant precipitation. Based on theoretical solubility product constants at neutral pH, B is the most soluble of the three, followed by M and OCP [20], which is in accordance with the data observed here. Nevertheless, in solutions that are saturated with Ca^{2+} and Pi, it has been shown that not the release but an uptake of the ions occurs upon immersion, a phenomenon that is also dependent on the SSA [21]. It is known that both the chemical composition and structural properties are important in the process of ionic exchange with the aqueous medium [22].

The physico-chemical analysis of the five crystal types prepared here showed that, by controlling the synthesis parameters, we were able to produce three distinct CaP phases, of which two with two distinct crystal geometries. With this indirect control of material parameters, and with extensive characterization of the materials properties, a first step could be made towards relating a given material property to a biological response. To this end, bone marrow-derived hMSCs were seeded on each of the five crystal types to study their attachment, proliferation and differentiation towards the osteogenic lineage, the latter being an important indicator for the bone-regenerative potential of a material.

Qualitative analysis of cell morphology upon seeding on the crystals in basic medium (Figure 5.3, last row) showed that both the geometry of the crystal and its surface morphology affected attachment and spreading of hMSCs. For both BN and MN, the cells preferentially attached to the material in the corners of entangled crystals, while on the plate-shaped crystals of both types they were found on the planar surface. Spreading of cells was more pronounced on the rougher M crystals than on the B ones where the cell area retained relatively small. Surface-microstructural features have previously been shown to affect cell

Figure 5.3: SEM micrographs of BN, BP, MN, MP and OCP crystals and of hMSCs on different crystals after 7 and 14 days of culture in basic medium (scale bars 500, 50 and 10 μm). Needle-shaped crystals had an elongated three-dimensional geometry with a length of about 800 μm for BN and 550 μm for MN and a width and height ranging from 40 to 80 μm for both crystal types. On the other hand, crystal plates presented a flat three-dimensional geometry, with a relatively large area (about $2.5 \times 1 \text{ mm}^2$ for BP, and $2 \times 1 \text{ mm}^2$ for MN), but a limited thickness, in both cases below 50 μm . OCP crystals possessed a spherical shape with plate-like crystals radiating from the nucleus having a diameter of around 450 μm . Upon ageing, on B crystals, flower-shaped crystalline deposits were visible. While flake-shaped crystals that built M plates and needles became sharper with ageing, the edges of sharp OCP crystals appeared smoother. Cells cultured on B retained a somewhat rounded morphology, whereas on M, they tended to flatten and spread on the rougher surface. On needle-shaped crystals of either chemistry, the cells were preferentially located in corners or in between crystals, whereas on plates, they were found on the planar surface. HMSCs cultured on OCP formed a flat sheet covering the sharp crystal plates.

Figure 5.4: FTIR spectra of BN, BP, MN, MP and OCP upon 14-day ageing. None of the crystals showed significant chemical modification during the 2-week ageing study by immersion in basic cell culture medium.

Figure 5.5: Ca^{2+} (A) and Pi (B) ion concentration in the ageing medium. The lines represent the theoretical concentrations of Ca^{2+} and Pi ions in basic medium. Both BN and BP crystals released Ca^{2+} and Pi into the medium. An increased release of both ions was observed with extended ageing time of 14 days. The Ca^{2+} and Pi ion concentrations obtained for M crystals were close to the theoretical values of basic medium and did not change over time. In contrast, upon OCP ageing, Ca^{2+} and Pi concentrations measured were lower than those found in the medium itself, suggesting uptake of these ions by OCP crystals.

shape, cytoskeletal arrangement and cell adhesion [10, 23, 24]. Cell attachment and spreading was poor on OCP crystals, which is possibly related to the sharp morphology of OCP crystals and in accordance with previous studies in which either rat periosteal and bone marrow stromal cells [25] or mouse bone marrow stromal ST-2 cells were used [26].

This initial cell attachment was also reflected in the total DNA amount determined after 7 and 14 days of culture (Figure 5.6), which provides an indication of cell proliferation. While for B and M crystals a significant increase in DNA amount was observed between days 7 and 14, no increase was seen when cells were cultured on OCP. Poor proliferation of cells on OCP was in accordance with previous work [26]. During the initial 7 days, BN seemed to better support hMSCs' proliferation than other crystal types. However, after 14 days, the only significant difference left was that between OCP and other crystal types. Both Ca^{2+} and Pi ions were previously shown to affect proliferation of hMSCs [21, 27], however, also surface properties such as topography and surface energy may have an influence on proliferation [28, 29], emphasizing the difficulty of attributing a biological response to a single property in the case of complex biomaterials.

Figure 5.6: DNA content (A) and ALP activity corrected for DNA content (B) of hMSCs cultured on BN, BP, MN, MP, and OCP crystals, and tissue culture plates in basic and osteogenic medium for 7 and 14 days. For clarity, only the significant differences among different conditions analyzed at the same time point are indicated by asterisks on the graphs. Patterned lines are used to distinguish different comparisons. B and M crystals of both shapes sustained cell proliferation over the 14 days of the experiment, in both basic and osteogenic medium, while proliferation of cells cultured on OCP was limited. The amount of DNA measured after 7 days in cultures on BN was significantly higher than DNA level of cells cultured on the other types of crystals, while after 14 days the only difference observed was between OCP and other crystal types. ALP enzyme levels of hMSCs cultured on BP were in general highest among the different crystals.

Osteogenic differentiation of the cells was first assessed by alkaline phosphatase (ALP) enzyme production (Figure 5.6). ALP is considered an early marker of osteogenesis [30] that is known for its ability to convert extracellular pyrophosphate, a mineralization inhibitor, into Pi [31]. An increase in ALP production was shown on all crystal types between days 7 and 14, suggesting support of osteogenic differentiation. The levels of ALP were higher in the BP condition as compared to the other crystal types. We further investigated the expression of a panel of relevant osteogenic markers on the mRNA level by hMSCs of two donors (Figure 5.7 and Figure 5.8). While the expression of ALP was higher when cells were cultured in presence of dexamethasone, a known stimulator of osteogenic differentiation of hMSCs [32], the expression of bone morphogenetic protein-2 (BMP-2), osteopontin (OP) and osteocalcin (OC) was higher on cultures in basic culture medium, an observation that is in accordance with other studies combining hMSCs and CaP ceramics [27, 33]. Regarding differences among different crystal types, and in

accordance with the results on ALP enzyme production, the ALP mRNA expression was higher on BP than on the other crystal types. Regarding the expression of BMP-2, having a well-established role in the process of osteogenic differentiation [34], and OP, which is thought to inhibit hydroxyapatite formation and growth during bone mineralization [35], the general trend was a higher expression on the plate-shaped B and M crystals and OCP as compared to the two needle-shaped crystal types. Similar differences among the materials were also found for the expression of OC, known for its regulatory role in the growth of hydroxyapatite crystals during bone formation [35], although they were relatively small. It has been previously shown that OP, BMP-2 as well as OC are responsive to Ca^{2+} and/or Pi [36-38], and their expression observed here seems to be positively correlated with the SSA, and negatively with the amount of free Ca^{2+} and Pi ions in the solution. A lower amount of these ions in the medium, in the case of OCP even lower than the initial medium concentration, may actually mean precipitation of a new CaP phase, and thus a temporary supersaturation of these ions in the material vicinity. Since cells are found in contact with the material, it is proposed that the upregulation of BMP-2 and, to a lesser extent, of OP on OC is due to this increased local Ca^{2+} concentration.

Figure 5.7: QPCR data showing the expression of a panel of osteogenic markers by hMSCs after 7 and 14 days of culture on various crystal types in basic medium (black bars) and osteogenic medium (white bars) for Donor 1. Cells cultured on tissue culture plastic served as controls. For clarity, only the significant differences among different conditions analyzed at the same time point are indicated by asterisks on the graphs. Patterned lines are used to distinguish different comparisons. While ALP gene expression was down-regulated in cells cultured on crystals at both time points and in both media as compared to cells cultured on tissue culture plastic, BMP-2 and OP expression was higher on the crystals, in particular in basic medium. Regarding differences among the crystals, the general trend was that cells cultured on needle-shaped crystals showed a lower gene expression of osteogenic markers than cells cultured on their respective plate-shaped counterparts and OCP. Cells cultured in osteogenic medium on BP expressed significantly higher levels of ALP than cells cultured on the other types of crystals, at both 7 and 14 days. BMP-2 expression of hMSCs cultured on OCP crystals in basic medium was the highest among different crystals. In contrast, the culture on BN resulted in the lowest expression of BMP-2 among different crystal types, which was highlighted by statistically significant differences with MP after 7 and 14 days and MN after 14 days. Similarly, cells cultured in basic medium on BN expressed significantly lower levels of OP than cells cultured on the other types of crystals, at both 7 and 14 days, with the exception of BP at 7 days. Relatively small differences were observed in OC expression among different conditions.

Figure 5.8: QPCR data showing the expression of a panel of osteogenic markers by hMSCs after 7 days of culture on the crystals in basic medium for Donor 2. Cells cultured on tissue culture plastic in either basic or osteogenic medium served as controls. Analogous to the cells of the Donor 1, ALP gene expression was lower in cells cultured on crystals as compared to those cultured on tissue culture plastic in both media. OCP crystals, and to a lesser extent plate-shaped B and M crystals, were shown to induce a significantly higher expression of BMP-2 and OP than the needle-shaped crystals and tissue culture plastic.

5.3.2 Decoupling Individual Properties of Biomimetic CaP Ceramics by Combining Nanoimprinting and Chemical Surface Modification

The results presented so far showed the effect of both geometry and chemistry on the growth and differentiation of hMSCs, however, the extent of each effect was difficult to determine because all crystals consisted of CaP. Therefore, to further decouple the chemical effect of CaP (free Ca^{2+} or Pi ions in the vicinity of the surface, initial CaP as a substrate or reprecipitated CaP layer upon immersion in the cell culture medium) from the geometrical effect (crystal shape, surface micro- and nanostructure), a soft-embossing technique was used [39]. Thereby, inverse replicas of the crystals were first made in polydimethylsiloxane (PDMS), a widely used elastomer in various applications including microfluidic devices [40, 41] and soft lithography [42]. Subsequently, using this casting as a mold, imprints of crystals were made in cyclic olefin copolymer (COC), an amorphous transparent copolymer that is gaining attention for its optical clarity, biocompatibility and structural strength [43, 44]. A schematic of the entire process is shown in Figure 5.9.A. In order to test the ability to reliably reproduce the structure of the crystals in the polymeric substrate using a thermally induced plastic deformation of the flat substrate against a three-dimensional flexible mold, a MN sample was selected. As is shown in the SEM images of the original crystal and the imprint (Figure 5.9.B1 and 5.9.C1, respectively), it was possible to reproduce the needle-like shape of the crystal in its original size, and most of its surface features. High-magnification SEM images (insets in Figures 5.9.B1 and 5.9.C1) demonstrated that also the flake-like submicron structure of the surface was reliably reproduced in the polymer substrate. Surface roughness mapping of the crystal and the imprint using white light interferometry (Figure 5.9.B2 and 5.9.C2, respectively) indeed confirmed a good transfer of the shape and structure features, and the quantification of the roughness (Figure 5.9.D) showed minimal differences between the original sample and the imprint in COC. This technique appeared suitable for separating the geometrical properties of CaP crystals from their chemical ones, resulting in materials with identical complex multiscale geometrical features, but completely different chemistry and other associated properties, such as mechanical strength and ductility [45]. As a next step, and with the aim to retain these superior properties provided by the polymer as compared to the bulk CaP ceramic while recovering the chemical effect of CaP, the surface of the crystal imprints in COC was chemically modified with CaP by applying a biomimetic precipitation method at near-physiological pH and temperature [46]. Alternative to this chemistry-recovery procedure, to develop a new material, the imprint surface was chemically modified with a thin (maximum 35 nm thick) layer of titanium using DC sputtering. This artificial material combines the surface chemistry of titanium, the microstructure of a CaP the bulk mechanical properties of COC. Both coating methods resulted in a homogenous layer of CaP and titanium, respectively, with minimal effect on surface roughness, due to the low thickness and nanocrystalline or quasi-amorphous nature of the coatings (Figure

5.10 and Figure 5.11).

Figure 5.9: Schematic representation of crystal to polymer imprint replication process in COC using hot embossing (A); MN crystal (scale bar = 500 μm , scale bar inset = 100 μm) (B1) and its roughness map (B2); imprinted MN crystal in COC (scale bar = 500 μm , scale bar inset = 100 μm) (C1) and its roughness map (C2); their respective roughness quantification by R_a and R_q (D). The method used to transfer the structure of the crystals into the polymer proved to be reliable down to the submicron features of the crystal surface.

Figure 5.10: SEM micrographs of imprints of the five crystal types in COC without and with titanium or CaP coating (scale bar = 500 μm) with insets roughness map at the same magnification. Bottom row shows the results of quantification of roughness parameters R_a and R_q . While no apparent differences were observed in surface structure between uncoated imprints and those coated with titanium, regardless of the imprint type, biomimetic deposition of CaP resulted in the formation of mineral globules, with a size below 1 μm that only slightly affected surface roughness. SEM images furthermore confirmed a homogenous distribution of both coatings over the surface of imprints.

Figure 5.11: EDAX spectra of uncoated and titanium- or CaP-coated imprints of MN in COC. Elemental analysis of the titanium-coated imprint showed an additional small peak that is absent in the spectrum of the uncoated sample and that belongs to titanium, confirming the deposition of a thin metal layer. Similarly, the EDAX spectrum of the CaP-coated sample showed the presence of peaks belonging to calcium and to phosphorus, which were absent in the uncoated sample spectrum.

5.3.3 Decoupling and Recombining Properties as a Means to Control Biological Behavior of hMSCs

To test whether decoupling and subsequent recombination of the properties of materials can be used as cues to control the biological response to these materials, hMSCs were cultured on uncoated and coated imprints under conditions comparable to those used previously for cell culture on crystals. Regarding cell morphology upon initial attachment, interestingly, the effect of both the structural properties and the chemistry were observed (Figure 5.12). The location of the preferential cell attachment on different imprint geometries was in accordance with what was observed on the original crystals and largely independent of the surface chemistry. On needle-shaped crystal imprints, the cells were mainly found between the entangled needles, whereas on the plate-like imprints, attachment occurred on the planar area of the plates. In the case of OCP imprints, the cells were found on top of the spherical crystal imprints, bridging their sharp, plate-like extensions. When different surface chemistries were compared, the cells appeared more spread on the polymeric and metallic than on the CaP surfaces, independent of the imprint shape. Analysis of the expression of a panel of osteogenic markers at mRNA level confirmed the effect of both structural properties and chemistry (Figure 5.13). Remarkably, upon reintroduction of CaP chemistry by deposition of a thin, mainly amorphous CaP layer on the surface of imprints in COC, a significantly higher expression of the Ca^{2+} - and/or Pi-responsive genes BMP-2 and OP, and, to a lesser extent, OC was observed as compared to uncoated imprints, an effect that was also seen for the original CaP crystals. ALP expression, on the other hand, seemed more pronounced on uncoated than on coated imprints, with the expression level on coated imprints being in accordance with the data observed on crystals. When the imprint shape is considered, it was observed that in the CaP-coated group the expression of BMP-2, OP and OC was in general higher on plate-shaped than on needle-shaped imprints of both B and M, an effect that was also observed for the original crystals. The expression of these markers on OCP imprints was comparable to that on BP and MP imprints. It should be noted that while in the case of original crystals, both the shape, thus the SSA, and the CaP phase may play a role in surface/biological system interface dynamics, all imprints were coated using the same solution, and therefore had an identical surface chemistry. This implies that in CaP-coated imprints the effect observed on cell behavior was solely due to a difference in imprint geometry. Introduction of titanium also had a positive effect on the expression of OP and OC as compared to the uncoated imprints, with a comparable effect of imprint shape as observed for CaP-coated imprints. The data of the last part of the study demonstrate that it is indeed possible to decouple and subsequently recombine individual properties of biomaterials to achieve a very similar biological effect while improving other properties.

Figure 5.12: SEM images of hMSCs cultured in basic medium for 3 days on bare COC imprints of the five crystal types and on imprints coated with titanium or CaP (scale bar = 50 μm). Cells cultured on the crystals under the same conditions are shown for comparison. Cells cultured on needle-shaped crystals and respective imprints of both B and M retained a somewhat rounded shape and were mainly found in the corners between the “needles”. Cells cultured on plate-shaped crystals and on bare and Ti-coated imprints of both B and M appeared more spread, whereas on CaP-coated imprints, cell surface area seemed smaller. Finally, on both OCP crystals and their imprints, independent of surface chemistry, the cells showed a relatively rounded morphology, with adhesion points on sharp crystal edges and “connecting” the individual crystal flakes. The effect of surface chemistry was confirmed by cultures on flat samples, where a more pronounced spreading of cells was observed on uncoated and Ti-coated samples as compared to CaP-coated ones.

Figure 5.13: QPCR data showing the expression of a panel of osteogenic markers by hMSCs cultured for 14 days in basic medium on uncoated imprints of the five crystal types in COC and on the same imprints coated with titanium or CaP. Cells cultured on flat imprints and cells cultured on tissue culture plastic served as controls. Asterisks indicate the significant differences among different conditions analyzed for the same chemistry and patterned lines are used to distinguish different comparisons. As a general observation, hMSCs cultured on COC imprints without a coating showed a higher ALP expression than those cultured on Ti- or CaP-coated imprints or on tissue culture plastic controls. In contrast, the expression of BMP-2, OP and OC was the highest on CaP-coated imprints. Within the uncoated COC group, the expression of ALP was upregulated on plate-shaped and OCP crystals as compared to needle-shaped crystals, whereas the expression of BMP-2, OP and OC was only positively affected by OCP imprints. Within the CaP-coated group, the trend of a lower expression on needle-shaped than on plate-shaped and OCP imprints was observed for BMP-2, OP and OC, analogous to the culture on original crystals, although the differences were not always statistically significant. While in general relatively small differences among different structures were observed on Ti-coated imprints, cells cultured on BN always consistently showed the lowest values for the expression of osteogenic markers.

In this study, we presented a novel approach towards biomaterials understanding and development by first decoupling material properties, which are intertwined in most conventional materials, and by subsequently combining the desired properties into an improved material, for example with comparable bioinstructive potential, but better mechanical properties and potentially against lower costs. Here, we used synthesized crystals as a starting material. However, it is anticipated that a similar process can be applied to any type of complex, synthetic as well as natural material (see for example the reproduction of the surface microstructure of natural bone into a polymer; Figure 5.14). While in this proof-of-concept study we applied nanoimprinting to decouple chemical, and, indirectly, mechanical from geometrical properties, and coating techniques to introduce the original or a new, different chemistry, a great diversity of other techniques can be applied within the same concept to isolate and combine properties and design new biomaterials (Figure 5.1). For example, 3D imaging of a (surface) structure of a functional material (e.g. by nanotomography) could be used as input to build similar structural features in other materials, using microfabrication techniques such as laser lithography based on two-photon polymerization. Photo- or UV nanoimprint lithography could be used as an alternative to thermal nanoimprinting. Electroplating/galvanofarming could be used as a chemical modification technique, or, instead of coating methods, nanosized fillers with defined chemistry could be used to build composite materials. This modular approach toward biomaterials design that is not limited by the conventional processing parameters will not only generate new fundamental knowledge of the relationship between a material property and a biological response, but will also open new possibilities to develop functional biomaterials comprising instructive cues to regulate such a response. The freedom that such an approach offers will potentially enable us to develop a spectrum of new biomaterials ranging from fully biomimetic on the one side, to something that could be called “biometamaterials” on the other side. It is expected that such a shift in the design approach will have an important societal impact by enabling development of materials for biomedical applications with improved properties and/or in a more cost-effective manner, the latter making such materials also affordable in second- and third-world settings. Taking an example from the field of hard tissue regeneration, this would mean the development of bone graft substitutes with excellent bioactivity in terms of osteoconduction or osteoinduction while being load-bearing and cost-efficient both in terms of material and its processing. But the potential application field is obviously much broader since the process of isolation and recombination of properties is applicable to any type of functional biomaterials.

Figure 5.14: SEM micrograph of decellularized human allograft chips (bone Extracellular Matrix, bECM, EMCM, the Netherlands), which were produced by subsequent treatment of microorganisms-free trabecular bone pieces by CO_2 , H_2O_2 , NaOH and NaH_2PO_4 , followed by ethanol treatment and shaping into 1-2 mm large particles (scale bar = 1 mm) (A), zoom-in of the area in (A) delineated by the dashed lines showing the surface microstructure of decellularized bone (scale bar = 50 μm) (B) and SEM image of the bone imprint in COC (scale bar = 50 μm).

5.4 Conclusions

Understanding the relationship between the individual properties of a functional biomaterial and a biological response is a prerequisite for the rational design of new, improved, biomedical devices and synthetic substitutes for patient's own tissue, the need for which is rapidly growing. In this study, it has been demonstrated that both chemical composition and geometrical properties of CaP ceramics, being widely used bone graft substitutes, have a distinct effect on the growth and differentiation of clinically relevant bone marrow-derived hMSCs. While the use of a conventional synthesis method of CaPs suggested these individual effects, application of rationally selected nanoimprinting and surface chemistry modification techniques was required to reliably decouple the effects of the two properties and build new materials encompassing desired combinations of properties. This study demonstrates the advantages of a design-driven biomaterials development approach over a production process-driven one.

References

1. M. E. Furth, A. Atala, M. E. Van Dyke, *Biomaterials* 2007, 28, 5068.

2. J. A. Hunt, *Nature materials* 2008, 7, 617.
3. W. L. Murphy, T. C. McDevitt, A. J. Engler, *Nature materials* 2014, 13, 547.
4. D. Williams, *Essential Biomaterials Science*, Cambridge University Press, Cambridge 2014.
5. S. Dorozhkin, *BIO* 2011, 1, 1.
6. P. Habibovic, J. E. Barralet, *Acta biomaterialia* 2011, 7, 3013.
7. H. Lapczynska, L. Galea, S. Wust, M. Bohner, S. Jerban, A. Sweedy, N. Doebelin, N. van Garderen, S. Hofmann, G. Baroud, R. Muller, B. von Rechenberg, *European cells & materials* 2014, 28, 299.
8. A. Engler, L. Bacakova, C. Newman, A. Hategan, M. Griffin, D. Discher, *Biophysical journal* 2004, 86, 617.
9. S. Y. Chou, P. R. Krauss, P. J. Renstrom, *J Vac Sci Technol B* 1996, 14, 4129.
10. H. V. Unadkat, M. Hulsman, K. Cornelissen, B. J. Papenburg, R. K. Truckenmuller, A. E. Carpenter, M. Wessling, G. F. Post, M. Uetz, M. J. Reinders, D. Stamatialis, C. A. van Blitterswijk, J. de Boer, *Proceedings of the National Academy of Sciences of the United States of America* 2011, 108, 16565.
11. P. Murugan, M. Krishnamurthy, S. N. Jaisankar, D. Samanta, A. B. Mandal, *Chemical Society reviews* 2015, 44, 3212.
12. F. Barrere, C. A. van Blitterswijk, K. de Groot, *Int J Nanomed* 2006, 1, 317.
13. A. M. Barradas, H. Yuan, C. A. van Blitterswijk, P. Habibovic, *European cells & materials* 2011, 21, 407.
14. O. Suzuki, *Acta biomaterialia* 2010, 6, 3379.
15. F. Tamimi, Z. Sheikh, J. Barralet, *Acta biomaterialia* 2012, 8, 474.
16. M. S. Djosic, V. B. Miskovic-Stankovic, Z. M. Kacarevic-Popovic, B. M. Jokic, N. Bibic, M. Mitric, S. K. Milonjic, R. Jancic-Heinemann, J. Stojanovic, *Colloid Surface A* 2009, 341, 110.
17. M. Kumar, J. Xie, K. Chittur, C. Riley, *Biomaterials* 1999, 20, 1389.
18. Y. Liu, J. E. Barralet, P. R. Cooper, R. M. Shelton, *Key Eng Mater* 2008, 361-363, 1009.
19. J. C. Elliot, *Surface Chemistry of the Apatites and Other Calcium Orthophosphates*, Elsevier Sciences, Amsterdam 1994.
20. J. a. H. Black, G., Ed. *Handbook of Biomaterial Properties*, Chapman & Hall, London 1998.
21. A. M. Barradas, V. Monticone, M. Hulsman, C. Danoux, H. Fernandes, Z. Tahmasebi Birgani, F. Barrere-de Groot, H. Yuan, M. Reinders, P. Habibovic, C. van Blitterswijk, J. de Boer, *Integrative biology : quantitative biosciences from nano to macro* 2013, 5, 920.
22. F. Barrere, Ni, M., Habibovic, P., Ducheyne, P., de Groot, K., in *Tissue Eng.* (Ed: C. A. van Blitterswijk, et al.), Elsevier, Amsterdam 2008, 223.
23. D. O. Meredith, L. Eschbach, M. O. Riehle, A. S. Curtis, R. G. Richards, *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2007, 25, 1523.
24. M. Hulsman, F. Hulshof, H. Unadkat, B. J. Papenburg, D. F. Stamatialis, R. Truckenmuller, C. van Blitterswijk, J. de Boer, M. J. Reinders, *Acta biomaterialia* 2015, 15, 29.
25. Y. Liu, R. Shelton, U. Gbureck, J. Barralet, *Journal of biomedical materials research. Part A* 2009, 90, 972.
26. T. Anada, T. Kumagai, Y. Honda, T. Masuda, R. Kamijo, S. Kamakura, N. Yoshihara, T. Kuriyagawa, H. Shimauchi, O. Suzuki, *Tissue engineering. Part A* 2008, 14, 965.
27. C. Danoux, D. C. Bassett, Z. Othman, A. I. Rodrigues, R. L. Reis, J. Barralet, C. A. van Blitterswijk, P.

Habibovic, *Acta biomaterialia* 2015.

28. A. J. Dulgar-Tulloch, R. Bizios, R. W. Siegel, *Journal of Biomedical Materials Research Part A* 2009, 90A, 586.
29. U. Hempel, T. Hefti, P. Dieter, F. Schlottig, *Clin Oral Implan Res* 2013, 24, 174.
30. E. E. Golub, G. Harrison, A. G. Taylor, S. Camper, I. M. Shapiro, *Bone and mineral* 1992, 17, 273.
31. W. C. O'Neill, *Circulation research* 2006, 99, e2.
32. M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, D. R. Marshak, *Science* 1999, 284, 143.
33. Z. Tahmasebi Birgani, Klotz, B. J., van Blitterswijk, C. A., Habibovic, P., *Advanced Biomaterials and Devices in Medicine* 2014, 1, 1.
34. V. Rosen, *Cytokine & growth factor reviews* 2009, 20, 475.
35. H. I. Roach, *Cell biology international* 1994, 18, 617.
36. M. Ahlstrom, M. Pekkinen, U. Riehle, C. Lamberg-Allardt, *Bone* 2008, 42, 483.
37. A. M. C. Barradas, H. A. M. Fernandes, N. Groen, Y. C. Chai, J. Schrooten, J. van de Peppel, J. P. T. M. van Leeuwen, C. A. van Blitterswijk, J. de Boer, *Biomaterials* 2012, 33, 3205.
38. Y. C. Chai, S. J. Roberts, E. Desmet, G. Kerckhofs, N. van Gastel, L. Geris, G. Carmeliet, J. Schrooten, F. P. Luyten, *Biomaterials* 2012, 33, 3127.
39. A. P. Russo, D. Apoga, N. Dowell, W. Shain, A. M. P. Turner, H. G. Craighead, H. C. Hoch, J. N. Turner, *Biomed Microdevices* 2002, 4, 277.
40. C. S. Effenhauser, G. J. Bruin, A. Paulus, M. Ehrat, *Analytical chemistry* 1997, 69, 3451.
41. D. C. Duffy, J. C. McDonald, O. J. Schueller, G. M. Whitesides, *Analytical chemistry* 1998, 70, 4974.
42. Y. N. Xia, G. M. Whitesides, *Angew Chem Int Edit* 1998, 37, 550.
43. W. D. Niles, P. J. Coassin, *Assay and drug development technologies* 2008, 6, 577.
44. P. S. Nunes, P. D. Ohlsson, O. Ordeig, J. P. Kutter, *Microfluid Nanofluid* 2010, 9, 145.
45. A. Kasgoz, D. Akin, A. I. Ayten, A. Durmus, *Compos Part B-Eng* 2014, 66, 126.
46. P. Habibovic, F. Barrere, C. A. van Blitterswijk, K. de Groot, P. Layrolle, *J Am Ceram Soc* 2002, 85, 517.
47. S. K. Both, A. J. C. Van der Muijsenberg, C. A. Van Blitterswijk, J. De Boer, J. D. De Bruijn, *Tissue Eng* 2007, 13, 3.
48. H. Fernandes, A. Mentink, R. Bank, R. Stoop, C. van Blitterswijk, J. de Boer, *Tissue Eng Pt A* 2010, 16, 1693.

Independent effects of chemical and microtopographical surface properties of polymer/ceramic composites on proliferation and osteogenic differentiation of human MSCs

Abstract

Within the general aim of finding affordable and sustainable regenerative solutions for damaged and diseased organs and tissues, significant efforts are invested in developing synthetic alternatives to natural bone grafts, such as autograft. Calcium phosphate (CaP) ceramics are among widely used synthetic bone graft substitutes, but their (mechanical) properties and bone regenerative capacity is still outperformed by their natural counterparts. In order to improve the existing synthetic bone graft substitutes, it is imperative to understand the effects of their individual properties on a biological response, and to find a way to combine the desired properties into a new improved, functional biomaterial. To this end, here, we have studied the independent effects of chemical composition and surface topography of a poly(lactic acid)/hydroxyapatite (PLA/HA) composite material on the proliferation and osteogenic differentiation of clinically relevant bone marrow-derived human mesenchymal stromal cells (hMSCs). While the molecular weight of the polymer and presence/absence of the ceramic phase were used as the chemical variables, a soft embossing technique was used to pattern surfaces of all the materials with either pits or pillars with identical microscale dimensions. The results indicated that, while the cell morphology, in terms of spreading was affected by both presence and level of exposure of HA and by the surface topography, the effect of the latter on cell proliferation was negligible. The osteogenic differentiation

of hMSCS, and in particular the expression of bone morphogenetic protein-2 (BMP-2) and osteopontin (OP) were significantly enhanced when cells were cultured on the composite based on low molecular weight PLA, as compared to the high molecular weight PLA-based composite and the two polymers. The OP expression on the low-molecular weight PLA-based composite was further enhanced when surface was patterned with pits. Taken together, within this experimental set up, the individual effect of the chemistry, and in particular of the presence of CaP was more pronounced than the individual effect of the topography, although they combined effects were, in some cases, synergistic. The technique presented here opens new pathways to study interactions of biomaterials with the biological environment in greater depths, which can serve as a starting point for developing biomaterials with improved bioactivity.

6.1 Introduction

With a growing need for affordable regenerative strategies for damaged and diseased organs and tissues, recent research efforts have strongly focused on development of smart, or instructive synthetic biomaterials that can exert a desired biological function in the body [1-4]. In the case of musculoskeletal regeneration, and more specifically, regeneration of bone tissue, the promise of synthetic bone graft substitutes is to offer an inexpensive and in large quantities available replacement of natural bone grafts, i.e. auto-, allo- and xenografts. Calcium phosphate (CaP) ceramics are among the most widely used and clinically most successful bone graft substitutes, owing to their close chemical resemblance to the mineral portion of bone [5], and their bioactivity [6, 7] in terms of osteoconduction, bone bonding and occasionally osteoinduction [8]. However, despite the use of CaPs for over four decades in the fields of orthopedics and dentistry, in general, their bioactivity is still considered inferior to that of their natural counterparts. Furthermore, brittleness of CaP ceramics is an important limitation for their use, in particular in load-bearing locations.

Various strategies have been considered to improve the bioactivity of bone graft substitutes, while retaining their synthetic character. In the case of CaP-based bone graft substitutes, variations in chemical composition have often been used as a means to control the *in vivo* degradation of the bone graft substitutes, and therewith, hypothetically, the rate and the extent of new bone formation. For example, biphasic CaP ceramics in which the ratio between CaP phases with different *in vivo* degradability can be tuned were used to maximize their osteoinductive potential [9-11]. Also architecture of such materials has been suggested to play an important role, at different scales, varying from macroporosity down to nanoscale surface topography [12-15]. More in general, recently, surface topographical features of biomaterials have been elegantly shown to be a powerful tool to control cell fate [16-19]. Such studies commonly employ highly precise

methods stemming from the field of micro- and nanotechnology and materials that are amenable to processing using such techniques. Therefore, the translation of data obtained from such studies to the behavior of bulk functional materials is not always trivial. CaP ceramics, for example, are relatively complex materials, with an intertwined set of physico-chemical properties. This makes it difficult to modify one of such properties without affecting one or more of the others. As a result, correlating any type of biological response to one or a combination of properties is difficult, while this is a very important step in the process of designing new bone graft substitutes with improved functionality to the extent that it is equal to the autograft. In our earlier studies, we have elaborated on the individual effects of calcium (Ca^{2+}) and inorganic phosphate (Pi) ions, by developing composite materials consisting of a polymer and either a calcium [20] or a phosphate salt [20, 21]. More recently, an attempt was made to separate the chemical effect of CaP from the stiffness of the material [22].

In the present study, the focus was on microstructural surface properties on the one hand, and on local Ca^{2+} and Pi concentrations on the other. To this end, a composite material consisting of (poly)lactic acid (PLA) and nano-sized hydroxyapatite (HA) powder with controlled surface features at micrometer scale was used. Such composites are considered a promising family of biomaterials in which positive aspects of bioactivity of CaPs and versatile mechanical properties of polymers are combined. The specific PLA/HA composite used here contains CaP in the same phase as used for production of CaP ceramic bone graft substitutes. Furthermore, its degradation rate, and consequent release of Ca^{2+} and Pi, can be controlled in a relatively facile fashion by varying the molecular weight of PLA [23]. In a number of studies, composite materials based on PLA and HA have shown to support attachment, growth and osteogenic differentiation of mesenchymal stromal cells (MSCs) and (pre)osteoblasts [24-26]. In vivo, PLA/HA composites showed successful healing of a 6 mm femoral intercondylar defect in rabbits and a critical-sized cranial defect in rats [27, 28] and even osteoinductivity of PLA/HA composites was demonstrated upon intramuscular implantation in dogs or sheep [29-31].

Another reason for choosing PLA/HA composite in the present study is its amenability to surface micropatterning using soft lithography, in contrast to classical sintered CaP ceramics. We used micromolding to generate materials with distinct surface topographies at micrometer scale. Micromoulding is a common molding-based soft lithography technique that has a long history of use in fabrication of microelectromechanical sensors (MEMS) by precise control of ordered structures such as pits, pillars, grooves, and wells at the micrometer scale [32-34]. Soft-lithography techniques have been successfully used to transfer well-defined micro-sized patterns from silicon or stainless steel masters to surfaces of biomaterials [35-37], allowing recreation of controlled microenvironments and in depth study of the influence of surface properties on cell behavior [38, 39]. Here,

we hypothesized that both the molecular weight of PLA, known to affect composite degradation rate and release of Ca^{2+} and Pi, and the surface topographical features, influence the growth and differentiation of bone marrow derived human MSCs (hMSCs). To test this hypothesis, two distinct micro-scale topographies, pits and pillars with identical sizes were imprinted into two PLA/HA composites based on two molecular weights of PLA as well as in polymeric controls not containing CaP. The rationale behind the choice of these topographies was the earlier suggested effect of concavities on processes related to bone formation [40-42]. HMSCs were cultured on these materials, and their attachment, proliferation and osteogenic differentiation were assessed, along with the analysis of Ca^{2+} and Pi concentrations during culture.

6.2 Materials and methods

6.2.1 Preparation of PLA/HA composites and micropatterning of their surface

Two types of composite materials were prepared, one using low (55,000 - 59,000 Da) molecular weight poly(D,L-lactic acid) (L-PLA, ANaBior, France) and one using high (400,000 Da) molecular weight PLA (H-PLA, Purac, the Netherlands). HA powder was synthesized in house using a wet precipitation method and its properties were the same as we reported earlier [23, 43]. To produce the composite material, a mixture of 50 wt.% PLA and 50 wt.% HA was prepared and extruded using a conical non-converging twin-screw extruder (Artecs BV, Enschede, the Netherlands) at a temperature of 150°C and with a screw speed of 100 rpm as described previously [29, 30]. Control materials made of L-PLA or H-PLA without HA were produced using the same procedure. Then, PLA/HA composites and PLA controls were shaped into pellets with a diameter of 10 mm using a metal frame mold and a heating press at 120°C (for L-PLA/HA composite and L-PLA) or 150°C (for H-PLA/HA composite and H-PLA) and 30 kN for 5 min.

Pulsed femtosecond laser micromachining (Lightmotif, Enschede, the Netherlands) was used to produce a stainless steel master containing 25 different topographies, varying in shape, including the pits-topography used in this study. Inverse polydimethyl siloxane (PDMS) molds of the selected pits topography were prepared by casting the silicone elastomer (Sylgard® 184, Dow Corning, USA), mixed with the curing agent at a 10:1 ratio, over the stainless steel master. The elastomer and master were then placed together under vacuum to evacuate air bubbles and left at room temperature for 2 days, followed by incubation at 50°C to allow polymerization. Upon removal, the PDMS negative replica (thus with pillars) was used to produce a second PDMS replica (with pits) using the same method. Both PDMS replica's were subsequently used as molds to imprint surfaces of composites and polymers pellets by hot embossing at 120°C (for L-PLA/HA

composite and L-PLA) or 150°C (for H-PLA/HA composite and H-PLA) for 10 and 6 minute for composites and polymers, respectively. Samples embossed with flat PDMS mold without topography served as control. Gold-sputtered patterned surfaces were analyzed using scanning electron microscopy (SEM) in the secondary electron mode, with an acceleration voltage of 10 kV for composites and 5 kV for polymers. The SEM was equipped with an energy dispersive X-ray spectroscopy (EDAX; Apollo X, Ametek) that allowed elemental analysis of the surface. As a preparation for cell culture, the samples were placed in low-attachment 48-well plates and fixed with an O-ring. They were then immersed in 70% isopropanol for 30 minutes with two refreshments every 10 minutes, and after complete evaporation thoroughly washed with sterile phosphate-buffered saline (PBS).

6.2.2 Cell culture on imprinted PLA/HA composites and polymers

hMSCs were isolated from bone marrow aspirates obtained from a healthy donor after written informed consent and proliferated as described previously [44, 45]. In brief, bone marrow aspirates (5-20 ml) were resuspended with 20 G needles, plated at a density of 5×10^5 cells/cm² and cultured in proliferation medium comprising α -minimal essential medium (a-MEM; Gibco), 10% fetal bovine serum (Lonza), 2 mM L-glutamine (Gibco), 0.2 mM ascorbic acid (Sigma Aldrich), 100 U ml⁻¹ penicillin (Gibco), 100 μ g/ml streptomycin (Gibco), and 1 ng/ml basic fibroblast growth factor (bFGF) (Instruchemie, The Netherlands). Cells were expanded at 37°C in a humidified atmosphere with 5% CO₂. Medium was refreshed twice a week, and cells were subcultured until 80% confluency.

Prior to cell seeding, the PLA/HA and PLA pellets were incubated overnight in either BM or in osteogenic medium (BM supplemented with 10 nM dexamethasone) at 37°C in a humidified atmosphere with 5% CO₂. For the analysis of cell morphology, 4000 cells were seeded on each sample in BM. For the assessment of proliferation, osteogenic differentiation, gene expression, Ca²⁺ and Pi assay and surface topography analysis, 10000 hMSCs were seeded on each sample and incubated in either BM or OM. The media were refreshed every 2 to 3 days.

6.2.3 Cell morphology assessment

After culture in BM for 3 days, the samples were washed with PBS and fixed with 4% paraformaldehyde for 20 min. They were then divided into two groups, one for the fluorescent microscopy and the other for the SEM analysis. To perform immunofluorescence staining and analysis, the samples were permeabilized with 0.1% Triton-X 100 for 5 min and blocked using 1% bovine serum albumin (BSA) in PBS. Cell cytoskeleton was stained by Alexa Fluor 488 (1:60 dilution in 1% BSA in PBS, Invitrogen), and DAPI (1:100 dilution in 1% BSA in PBS, Sigma-Aldrich/Fluka) was used to stain cell nuclei. Cell images were acquired using fluorescence microscopy (BD Pathway 435, BD Biosciences) and analyzed using CellProfiler with

built-in modules (Measure Object Area Shape), as described previously [46]. 8 different areas of each sample and at least 1,500 cells were used for quantification.

The samples intended for the SEM analysis were rinsed with PBS, and then dehydrated through a graded series of isopropanol (70, 80, 90, 96 and 100%) for 20-30 min. The dehydration was completed in hexamethyl disilazane (HMDS) for 2 times 15 min. The samples were then air-dried, mounted onto aluminum stubs, and sputter-coated with gold.

6.2.4 Cell proliferation and osteogenic differentiation

Cell proliferation was assessed after 3, 7 and 14 days of culture (n=4). Total DNA was quantified using CyQUANT® Cell Proliferation Assay kit (Life Technologies) as a measure of cell number, according to the manufacturer's protocol. Measurement of fluorescence (excitation at 480 nm and emission at 520 nm) were performed using a spectrophotometer (Victor3, Perkin Elmer).

ALP activity, an early marker of osteogenic differentiation, was determined after 7 and 14 days of culture (n=4) by using CDP-star chemiluminescent substrate (Roche Applied Science), according to the manufacturer's protocol. The ALP activity was normalized for the DNA content.

To evaluate the influence of surface topography and chemical composition of the samples on the expression of the markers of osteogenesis at mRNA level, hMSCs were cultured for 7 and 14 days (n=3). Total RNA of the cells was extracted by using a combination of TRIzol® (Invitrogen) and a Nucleospin® RNA isolation and purification kit (Macherey-Nagel GmbH & Co.). One mL of TRIzol reagent was added to each well. Following one freeze/thaw cycle, 200 µL chloroform was added and after mixing and centrifugation, the aqueous phase of each sample was collected and mixed with 350 µL 70% ethanol before loading onto the RNA binding column of the NucleoSpin RNA II isolation kit. Subsequent steps were in accordance with the manufacturer's protocol. First strand cDNA was synthesized using iScript (Bio-Rad). Quantitative real time PCR was performed and CT values were normalized to the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene and fold induction was calculated using the comparative ΔCT method. Primer sequences of the selected markers Bone Morphogenetic Protein-2 (BMP-2), Runt-related transcription factor 2 (Runx2), osteopontin (OP), ALP and collagen Type 1 are given in Table 6.1.

6.2.5 Quantification of Ca^{2+} and Pi concentration in cell culture medium

Cell culture medium (n=4) was collected during refreshments at 3, 7 and 14 days of culture. Ca^{2+} and Pi content of the medium was determined using QuantiChrom™ Calcium assay kit (BioAssay, USA) and a QuantiChrom™ Phosphate assay kit (BioAssay, USA), respectively, according to the manufacturer's protocol. Medium containing cells but not materials, and medium without either cells or materials

Name	Primer Sequence
ALP	5'-GACCCTTGACCCCCACAAT-3' 5'-GCTCGTACTGCATGTCCCCT-3'
BMP-2	Purchased from SA Biosciences
Collagen type 1	5'-GAGGGCCAAGACGAAGACATC-3' 5'-CAGATCACGTCATCGCACAAAC-3'
GAPDH	5'-CGCTCTCTGCTCCTCCTGTT-3' 5'-CCATGGTGTCTGAGCGATGT-3'
Osteocalcin	5'-GGCAGCGAGGTAGTGAAGAG-3' 5'-GATGTGGTCAGCCAACTCGT-3'
Osteopontin	5'-CCAAGTAAGTCCAACGAAAG-3' 5'-GGTGATGTCCTCGTCTGTA-3'

Table 6.1: Primers sequences for human genes.

served as controls. The absorbance was read with a spectrophotometer (Multi-skan™ GO, Thermo Scientific) at 612 nm and 620 nm for Ca^{2+} and Pi, respectively. At day 14, the samples were evaluated using SEM and EDX as described above for the as-produced samples.

6.2.6 Statistical analysis

Statistical analysis of the data was performed using a univariate general linear model in (IBM SPSS Statistics 22). The model was selected via an iterative approach, first including all possible interactions and subsequently removing the non-significant interaction terms if the adjusted-R2 remained the same or decreased. The Bonferroni post-hoc test was used for pairwise comparisons between groups. The level of significance was set as $p < 0.05$. All quantitative data presented are expressed as mean $\pm$ standard deviation.

6.3 Results

6.3.1 Characterization of micropatterned PLA and PLA/HA

To investigate individual effects of surface microscale topography and chemical composition on the behavior of hMSCs, the surface of the four materials L-PLA/HA, H-PLA/HA, L-PLA and H-PLA was patterned by pits and by pillars, and flat materials served as controls. SEM micrographs (Figure 6.2) showed the all surfaces were successfully patterned with either pits or pillars. Both topographies had an oval shape and their dimensions were similar, with a major axis length of $19.9 \pm 1.0 \mu\text{m}$, minor axis length of $15.6 \pm 0.7 \mu\text{m}$, and center-to-center distance of $31.4 \pm 1.6 \mu\text{m}$. The height of the pillars was measured to be $4.0 \pm 0.1 \mu\text{m}$, which is expected to be the same as the depth of the pits, (not possible to measure reliably using SEM), as demonstrated before [47, 48]. No differences in topographical dimensions were observed between the composites and the polymers. EDX spectra of the two composite materials showed pronounced calcium and phosphorus peaks (Figure 6.1.a, 6.1.b), which were absent in the spectra of the PLA samples (Figure 6.1.c, 6.1.d). Elemental mapping of calcium and phosphorous demonstrated a homogenous distribution of the ceramic phase, independent of the molecular weight of the PLA (Figure 6.1.e-6.1.h).

Figure 6.1: EDX spectra (a-d) of flat L-PLA/HA, H-PLA/HA, L-PLA and H-PLA, respectively and calcium (e, g) and phosphorus (f, h) elemental maps of flat L-PLA/HA and H-PLA/HA respectively. These data are also representative for the patterned surfaces. Calcium and phosphorous peaks were only seen in the spectra of the two composites, but not in the spectra of the polymers. A homogenous distribution of both elements was observed for both L-PLA/HA and H-PLA/HA, suggesting a homogenous distribution of the ceramics phase within the composite.

Figure 6.2: Top view SEM images of pits-patterned, pillars-patterned and flat L-PLA/HA, H-PLA/HA, L-PLA and H-PLA, and side view SEM images of L-PLA of both patterns, which are representative of all materials. All materials were successfully patterned by both pits and pillars. Both topographies had an oval shape in the x-y plane, with the long axis of about 20 μm, short axis of approximately 15 μm and center-to-center distance between individual pits/pillars of about 30 μm. Their depth/height was about 4 μm.

6.3.2 Cell attachment and morphology

SEM (Figure 6.3) and fluorescent microscopy images (Figure 6.4) of cells cultured for 3 days revealed that all materials, independent of the chemistry or topography, allowed the attachment of hMSCs. On the non-patterned surfaces of both composites and polymers, hMSCs displayed a well-spread morphology with distinct cytoplasmic processes. Similarly, on the pits-patterned surfaces, the cells exhibited a spread morphology, with processes predominantly concentrated around the pits, or covering them at the surface level, but seldom entering inside the pits. The cells grown on pillars exhibited a less spread morphology. They seemed to preferentially attach over the pillars, with cytoplasmic processes “connecting” them. Based on qualitative observations, no obvious differences were seen in cell morphology when different material chemistries are considered.

CellProfiler-based analysis of fluorescent micrographs confirmed that the three morphology parameters, i.e. cell area, cell perimeter and minor axis length were affected by the surface topography (Figure 6.5). The strongest effect was observed on the composite materials, with the lowest value for the three parameters on pillars-patterned substrates. On the two polymers, the effect of topography was less pronounced. Nevertheless, in L-PLA the three parameters were in general higher on the pits-patterned substrate, which was in line with the findings on the composite materials. However, the cell area, cell perimeter and the minor axis length appeared high on pillars-patterned H-PLA as compared to the pits-patterned and flat substrate.

Figure 6.3: SEM images of hMSCs cultured for 3 days in basic medium on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA. Cells showed a spread morphology on both pits-patterned and flat substrate, whereas on the pillars-patterned substrate their spreading appeared less pronounced, with cytoskeletal processes “connecting” individual pillars.

Figure 6.4: Fluorescent microscopy images of hMSCs cultured for 3 days in basic medium on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA and stained with phalloidin (green, cell cytoskeleton) and DAPI (blue, cell nuclei). Cells cultured on pillars-patterned surfaces exhibited a somewhat less pronounced spreading as compared to pits-patterned and flat surfaces, irrespective of material chemistry.

Figure 6.5: Quantification of cell morphology parameters cell area, cell perimeter and minor axis length by CellProfiler using fluorescent microscopy images of hMSCs cultured for 3 days in basic medium on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA and stained with phalloidin (green, cell cytoskeleton) and DAPI (blue, cell nuclei). The three parameters were consistently lower on pillars- than on pits- patterned surfaces on L-PLA/HA, H-PLA/HA and L-PLA, but not on H-PLA, where the opposite was the case.

Taken together, these data suggested the effect of both surface topography and surface chemistry on the morphology of hMSCs.

6.3.3 Cell proliferation on micropatterned PLA and PLA/HA

DNA amounts of cells cultured on different substrates for 3, 7 and 14 days were used as a measure of cell proliferation. The results showed that all substrates supported the growth of hMSCs over the 14-day culture period in both BM (Figure 6.6) and OM (Figure 6.7).

Figure 6.6: DNA amounts of hMSCs cultured for 3, 7 and 14 days in basic medium on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA. At 3 days, the only significant difference was observed between L-PLA/HA and H-PLA. At 7 days, both composite materials showed higher DNA values than the two polymers. This was also the case at 14 days, when also L-PLA/HA showed more DNA than H-PLA/HA. No significant effects of the topography were observed.

Figure 6.7: DNA values after 3, 7 and 14 days of culture of hMSCs on H-PLA, L-PLA, H-PLA/HA and L-PLA/HA in osteogenic medium. Regarding changes in time, DNA content of cells increased between day 3 and day 7, independent of chemistry or topography and remained stable between day 7 and day 14, except in the case of L-PLA, where a decrease in cell number was observed. When the effect of chemistry is considered, at 3 days, no significant differences among different samples were observed, At 7 days, alike for measurements in BM (Figure 6.6), the two composites showed higher DNA values as compared to the polymers, with exceptions of few topographies. Similarly, at 14 days, cells cultured on the composite samples had higher DNA values than those cultured on the PLA substrates, except for the difference between the surface with pillars, which was not different between L-PLA/HA and H-PLA. Finally, regarding the independent topography effect, significant differences were observed within the composite groups, with pillars showing the lowest DNA values.

In BM, an increase in DNA amount was observed between days 3 and 7 on all materials, remaining stable between days 7 and 14. Regarding the effect of the chemical composition, at day 3, cells cultured on L-PLA/HA showed higher DNA values as compared to cells cultured on H-PLA, for all topographies. At 7 days, cells cultured on either composite showed higher DNA values than those cultured on the polymers, independent of molecular weight or topography. The same observation was made at 14 days, with as additional difference that between L-PLA/HA and

H-PLA/HA. No significant effect of topography on DNA amount was observed after 14 days. The trend of cell proliferation in OM was in general similar to that in BM (Figure 6.7).

These data suggested that chemistry, and more specifically, presence of CaP, had an effect on cell proliferation, whereas the effect of surface topography appeared negligible.

6.3.4 Osteogenic differentiation of hMSCs on micropatterned PLA and PLA/HA

Enzymatic ALP activity of cells cultured on different materials for 7 and 14 days was measured and normalized for DNA amount, as a marker of early osteogenesis (Figure 6.8 and Figure 6.10 for culture in BM and OM, respectively). Furthermore, the expression of a set of markers of osteogenesis (BMP-2, Runx2, ALP, Col-I and OP) at mRNA level was determined at the same time points (Figure 6.9 and Figure 6.11, for culture in BM and OM, respectively).

Figure 6.8: ALP activity, normalized for DNA amount, of hMSCs cultured for 7 and 14 days in basic medium on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA. While no significant differences among the materials were observed at 7 days, at 14 days, the enzymatic ALP activity on H-PLA/HA was higher than that on the other three materials. No significant effects of the topography were observed.

Figure 6.9: The normalized expression of a set of osteogenic markers at mRNA level of MSCs cultured for 7 and 14 days in basic medium on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA. A general trend observed was the expression of osteogenic markers was the highest when cells were cultured on L-PLA/HA, with the exception of Col-1, where no differences were observed between L-PLA/HA and the two polymers. Some topographical effects were seen as well, with the positive effect of pits on the expression of OP, in particular on L-PLA/HA being the most remarkable one.

Figure 6.10: ALP activity of hMSCs cultured on H-PLA, L-PLA, H-PLA/HA and L-PLA/HA for 7 and 14 days in osteogenic medium. When cells were cultured in OM at 7 days, no differences among different samples were observed, which was in line with the findings in the BM at the same time point (Figure 6.8). Between 7 to 14 days, ALP activity strongly increased, independent of the material used. At 14 days, the positive effect on ALP activity of composites as compared to polymers was only visible for H-PLA/HA. At 14 days, patterned, but not flat H-PLA/HA showed significantly higher ALP activity than L-PLA/HA and H-PLA. The exception to this was substrate with pillars, where no significant difference was found between H-PLA/HA and L-PLA. Finally, the effect of surface topography was observed for H-PLA/HA, with significantly higher ALP levels of cells cultured on patterned than on flat substrates, and for L-PLA, where pillars-patterned substrate showed a higher ALP activity than the substrate with pits.

Figure 6.11: mRNA expression analysis of the osteogenic markers BMP-2, Runx2, ALP, Col-I and OP of hMSCs cultured on H-PLA, L-PLA, H-PLA/HA and L-PLA/HA for 7 and 14 days in osteogenic medium. At 7 days in OM, and comparable to the culture in BM, the expression of BMP-2, Runx2 as well as ALP seemed somewhat higher when cells were cultured on L-PLA/HA as compared to the other three material chemistries, although the differential expression of BMP-2 and Runx2 remained very low. Regarding the BMP-2 expression, at 7 days, a significant difference was reached between L-PLA/HA and H-PLA/HA, for pillar-patterned and flat substrate. At 14 days, cells cultured on the patterned L-PLA/HA showed a significantly higher BMP-2 expression as compared to the other three chemistries, with the exception of pillars-patterned substrate where the expression was comparable between the two composites. On L-PLA/HA the expression was highest on the pits-patterned substrate. Regarding the Runx2 expression, cells cultured on flat H-PLA/HA appeared to have a somewhat lower expression than when cultured the other flat substrates. The only topography effect was observed at 7 days on pits-patterned L-PLA/HA, showing a higher expression as compared to flat and pillars-patterned L-PLA/HA. While no relevant effect of surface chemistry was observed on the expression of OP at 7 days, at 14 days, a downregulation of the expression was observed on pits-patterned H-PLA/HA, L-PLA and H-PLA as compared to L-PLA/HA with the same topography. A similar, but weaker effect was observed of the pillars-patterned L-PLA/HA. Finally, pits-patterned LPLA/HA showed a higher expression than pillars-patterned and flat substrate of the same chemistry. While no differences were found in the ALP expression among different samples at 7 days, at 14 days, the ALP mRNA expression on H-PLA/HA was significantly lower than what was found on the other three substrates. Furthermore, H-PLA showed a higher ALP mRNA expression than L-PLA/HA. Finally, the main observation regarding the Col-1 expression was downregulation on H-PLA/HA as compared to the other three substrates, at both time points, although the differences were relatively small.

in BM, at 7 days, the ALP activity of cells cultured on the two composites appeared somewhat higher as compared to the culture on the polymeric substrates, although no significant differences were observed. ALP activity increased between 7 and 14 days for all conditions. At 14 days cells cultured on H-PLA/HA showed significantly higher ALP values as compared to the other three chemistries. No significant topography effects were observed on ALP activity at 14 days. The ALP activity by the cells cultured in OM was in general higher than what was observed upon culture in BM, with an increase between 7 and 14 days. The results of culture in OM showed a similar trend in the activity as observed in BM; no significant differences among the materials at 7 days, and in general higher activity on H-PLA/HA as compared to the other three chemistries at 14 days. A small effect of topography was seen on H-PLA/HA and L-PLA substrates (for more detailed information see supplementary information).

Regarding the mRNA expression, in BM, it was in general observed that the expression of BMP-2, Runx2 and OP was upregulated on L-PLA/HA as compared to the other three chemistries, at both time points. At 7 days, the mRNA expression of BMP-2 was significantly higher when cells were cultured on L-PLA/HA as compared to the other three materials, and this difference was independent of the surface topography. The same effect was observed for the expression of Runx-2 (although the fold change was only about 1.5 times), with the exception of the flat substrate at 14 days. Also the expression of OP of cells cultured on pits-patterned L-PLA/HA was significantly elevated as compared to that of cells cultured on the other three pits-patterned substrates, at both time points. The effect of topography was only observed in L-PLA/HA, with significantly higher OP expression on pits-patterned substrate as compared to the flat control at both time points. In general, differential expression of ALP mRNA was relatively small on different substrates. At 7 days, lower expression on H-PLA/HA as compared to L-PLA/HA was observed for all topographies, and at 14 days, a higher expression on the flat as compared to patterned surfaces, for all chemistries. The cells cultured on H-PLA/HA showed a significant downregulation of Col-1 as compared to cultures on the other chemistries, and independent of the topography, however, also here, the absolute differences were relatively small. Mild effect of the topography was observed for Col-1 expression, although no clear trend could be detected.

When culture in OM is considered, a general observation was that the addition of dexamethasone strongly suppressed the expression of BMP-2 and OP at both 7 and 14 days, while it significantly increased the expression of ALP at both time points as compared to BM. No obvious effect of OM was observed on the expression of Runx2 and Col-1. Furthermore, the effect of biomaterials properties, with emphasis on chemistry, seemed less pronounced as compared to the culture in BM (for more detailed information, see supplementary information).

While a mild effect of surface topography was observed on the ALP enzymatic activity of hMSCs, the main effect was due to the presence of CaP. Similarly, the

expression of BMP-2, OP and to a lesser extent Runx2 and Col-1 at the mRNA level was mainly affected by the chemical properties of the materials, with the composite made from low molecular weight PLA having the strongest positive effect. The topography played a role, although a less pronounced one, on the osteogenic differentiation of hMSCs as well.

6.3.5 Ca^{2+} and Pi concentration profile and material surface changes during cell culture

Ionic concentration analysis (Figure 6.12) indicated that cell culture medium containing L-PLA/HA contained significantly lower Ca^{2+} concentrations as compared to the other three chemistries, independent of the topography. Regarding the changes in time, it was observed that L-PLA/HA showed an increase in the concentration between day 3 and 7, remaining stable thereafter. In contrast, the other three materials showed a drop in the concentration between the first two time points, with concentration remaining stable between day 7 and 14. In line with Ca^{2+} measurements, lower Pi concentration values were found in L-PLA/HA incubation medium as compared to the other three materials. Furthermore, H-PLA/HA sample also showed lower Pi concentrations than the two polymers, but higher than L-PLA/HA at days 3 and 14. Regarding chronological change, while the concentration of medium containing polymers did not change over time, in composites, an increase between 3 and 7 days and a decrease between 7 and 14 days were observed. Trends observed in OM were similar to those in BM, for both Ca and Pi (Figure 6.13).

SEM analysis of the materials after 14-day cell culture in BM (Figure 6.14.a) exhibited presence of extensive number of submicron pores in L-PLA/HA, on both flat and patterned surfaces, while no such pores were observed on H-PLA/HA surface. L-PLA samples exhibited cracks, and the surface appeared more flat, with decreased dimensions of pits and pillars. No cracks were observed on H-PLA surface, and although a small decrease of the surface feature dimensions was seen, the surface appeared little affected. No obvious deposits of a CaP were observed on any of the substrates. The EDX elemental analysis of the two composite materials (Figure 6.14.b) showed that P/C and Ca/C ratios increased for both L-PLA/HA and H-PLA/HA during 14-day cell culture, while the Ca/P remained fairly constant.

Figure 6.12: Ca^{2+} and Pi concentrations during cell culture in basic medium for up to 14 days on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA. Medium from cell culture on tissue culture plastic served as a control. At all time points, the Ca^{2+} concentration of the medium containing L-PLA/HA was consistently lower than that of the other three materials, and independent of the topography. Also the changes in time were different, with an increase in Ca^{2+} concentration between days 3 and 7, and a small decrease between days 7 and 14 in the case of L-PLA/HA and an opposite trend in the other three materials. Similar effects were observed for the Pi concentration, with significantly lower amounts measured in L-PLA/HA medium, followed by H-PLA/HA medium, while no differences between the two polymers and the control were observed. Also here, an obvious increase in the concentration between day 3 and day 7 and a decrease between days 7 and 14 was observed for both composite materials and independent of the topography.

Figure 6.13: Ca^{2+} and Pi concentrations during cell culture in osteogenic medium for up to 14 days on L-PLA/HA, H-PLA/HA, L-PLA and H-PLA. Medium from cell culture on tissue culture plastic served as a control. The Ca^{2+} and Pi concentrations of osteogenic medium showed a similar trend as was observed in basic medium (Figure 6.12) with concentration of the two ions being lower in the medium containing L-PLA/HA as compared to the other three materials. The Pi concentration on H-PLA/HA medium was also lower than that of the media containing polymers and the control. No obvious effects of topography were observed.

Figure 6.14: Top view SEM images of pits-patterned, pillars-patterned and flat L-PLA/HA, H-PLA/HA, L-PLA and H-PLA, (a) and the P/C, Ca/C and Ca/P atomic ratios of flat L-PLA/HA and H-PLA/HA based on the EDX analysis (b) after 14 days of immersion in the cell culture medium. The EDX quantification is representative for the patterned composites as well. L-PLA/HA exhibited cracks and micropores on the surface, giving it a rough appearance, which was not the case for H-PLA/HA. Similarly, L-PLA showed exhibited many cracks, and obvious decrease in size of the topographical patterns. No such changes were seen on H-PLA. While an increase in both the amount of phosphorus and calcium were observed for both composites, no significant changes in Ca/P ration were observed.

6.4 Discussion

The current study was an attempt to combine biomaterials that are already used as bone graft substitutes and tools from microtechnology to distinguish between the effects of the chemical composition and surface topography on the attachment, proliferation and osteogenic differentiation of clinically relevant hMSCs. Composite materials are commonly used with the aim to combine the bioactivity of CaPs and versatility in terms of mechanical properties offered by polymers. Micromolding, a soft embossing techniques was used to successfully transfer the topographies, initially created using laser machining, into both polymeric and composite substrates, regardless of the molecular weight of the polymer. Furthermore, it was possible to imprint a negative replica of one topography to study the effect of concavity and convexity, while retaining the other parameters. Here, we selected pits and pillars, both to test the direct effect of the topography on the cells but also because it was expected that local ionic concentrations of Ca^{2+} and Pi in the patterned composites may be different between the two topographies. This imprinting technique can, however, be used for a wide range of topographies, and can be applied to different polymers. As an example, we have used it to provide the surface of polystyrene with topographies of different scales and levels of order (Figure 6.15) as well as to copy the surface of ceramics into a polymer (data not shown).

The analysis of the parameters of cell morphology revealed that both the chemical composition and the topography features had an effect. While there is no definitive correlation between cell morphology and its fate, previous studies have shown that increased cell spreading facilitates the osteogenic differentiation [49, 50]. Regarding the surface topography effect on the tested cell morphology parameters, it was observed that, in general, the spreading of cells was more pronounced on the pits- than on pillars-patterned substrates. This observation was, however, dependent of the surface chemistry; while these differences were highly pronounced on the composite materials, and in particular on L-PLA/HA, on the polymers, they were smaller, and on H-PLA even an opposite trend was observed. This suggests that the cells morphology is also affected, to different extents, by the molecular weight of the polymer and by the presence of CaP.

Surface topographical features did not appear to have an effect on cell proliferation. Reports exist on the effect of surface topographies on the proliferation of hMSCs [51-53], however, in contrast to our, in these studies, either titanium or titania /alumina ceramics and nanoscale topographical features were tested. While we did not observe an effect of the topography, material chemistry effect was evident. DNA amounts of cells cultured on the composites, and in particular on L-PLA/HA, were higher than when cells were cultured on either polymer. Conflicting reports exist regarding the effect of PLA/HA composite on the proliferation of hMSCs [43, 54], which suggests that other properties, apart from the presence of the ceramic phase, are also of influence on cell growth. Regarding the more specific chemical effect,

Figure 6.15: SEM images showing examples of micron- and submicron topographical patterns in polystyrene obtained using soft embossing.

in recent studies, it was demonstrated that elevated concentrations of Ca^{2+} in cell culture medium, significantly enhanced the proliferation of hMSCs [55, 56].

The osteogenic differentiation of the cells appeared to be affected by both the chemical composition and the topography, although the latter effect was much less pronounced. Furthermore, the extent of the effect was dependent on the markers used. While the ALP enzymatic activity appeared to be somewhat affected by the chemistry of the substrate, with cells cultured on H-PLA/HA showing higher values than those on other substrates, at mRNA level, the differences observed had little biological significance. The only relevant effect was observed for the type of medium, where the addition of dexamethasone enhanced the expression and activity of ALP, which is a well-known effect [57-59]. The materials had a much stronger effect on the mRNA expression of BMP-2 and OP, where an upregulation of both markers was observed when hMSCs were cultured on L-PLA/HA as compared to the other three materials. Similar effect was also observed on the expression of Runx2, although the differences were much smaller. Interestingly, these effects of the chemistry were in particular observed when

cells were cultured in BM, and the expression was strongly downregulated upon the addition of dexamethasone to the medium. A remarkable effect of the topography was observed in the case of the OP expression, and to a lesser extent the Runx2 expression on L-PLA/HA where pits-patterned substrate had a significantly positive effect. This observation was in accordance with a more spread morphology of cells on pits-patterned L-PLA/HA as compared to the other topographies.

Regarding the effect of the chemical composition, previous studies have shown that both the amount of CaP [60] in a composite material and the molecular weight of the polymer used [23] are determinant for its bioactivity *in vivo*. A lower MW of the polymer has been suggested to lead to more swelling of the composite, an enhanced degradation of the polymer and more exposure of the CaP to the environment, eventually leading to ectopic bone formation [23]. In another study, it was shown that locally elevated levels of Pi lead to collagen mineralization, a process which is of great importance in bone regeneration [21]. *In vitro*, the expression of BMP-2 and OP was enhanced at elevated Ca^{2+} and/or Pi levels [55, 56]. Interestingly, the measurements of the concentrations of the two ions consequently showed lower values of both in the media containing L-PLA/HA as compared to the other three materials. Similar observations were made previously, when CaP containing materials were shown to deplete the medium of Ca and Pi [56, 61]. SEM micrographs after cell culture showed that this material exhibited a rough structure with cracks suggesting swelling, degradation and plausibly release of Ca and Pi ions. This process could lead to precipitation of a new CaP layer, which was not detected visually with the SEM analysis, although changes with the Ca/P ratio are suggestive of this. Another possibility would be accumulation of Ca^{2+} and/ Pi ions in the form of complexes with e.g. serum proteins, although without any evidence, this statement remains speculative. In any case, the concentration of the two ions in the bulk of the medium was lower in the case of L-PLA/HA, which cannot explain the enhanced BMP-2 and OP expression, known to be positively affected by elevated concentrations of these ions. It should however be noted that the ionic concentrations in the vicinity of the material may be higher than what is measured in the bulk, but measuring this is difficult to impossible to do.

Regarding the independent surface microtopography effects, previous studies have shown that surface microcavities supported a more pronounced osteogenic differentiation of MSCs derived from human dental pulp and induced superior bone formation as compared to cells cultured on a smooth substrate [40]. Another report demonstrated that mineral deposition by osteoblasts was enhanced on the surfaces containing pits *in vitro* and *in vivo* [62]. Although both these studies were performed on different materials and using different cells, they are in line with the mild positive effect of the pits observed in our study.

Taken together, our data demonstrated that combining composite materials with nanoimprinting is a successful way to decouple the individual effects of chemical composition and surface topography on the behavior of hMSCs. Clearly,

the materials and the topographies tested here are among many that could be investigated by applying this versatile technique, offering thereby the analysis of a range of material properties. This could be a first step towards rational design of synthetic biomaterials for biomedical applications, whereby optimal properties for a desired response are first identified and then combined to obtain improved functional biomaterials.

6.5 Conclusion

In this study, micromolding was successfully used to pattern the surface of PLA and PLA/HA materials with two different molecular weights of the polymer. Morphology of hMSCs in terms of cell spreading was affected by both the microtopography and the chemistry, with pits and low molecular weight PLA-based composite having a synergistically positive effect. Cell proliferation was mainly affected by the chemical composition, and in particular the presence of the ceramic phase. The effect on osteogenic differentiation depended on the markers tested, with strongest positive effect of L-PLA/HA on the expression of BMP-2 and OP. The effect on the OP expression was synergistically enhanced by the pits topography. Taken together, within the selected experimental set-up, it can be concluded that hMSCs more strongly responded to the presence of CaP ceramic than to the surface microtopography.

References

1. R. Balint, N. J. Cassidy, S. H. Cartmell, *Acta biomaterialia* 2014, 10, 2341.
2. S. J. Buwalda, K. W. Boere, P. J. Dijkstra, J. Feijen, T. Vermonden, W. E. Hennink, *Journal of controlled release : official journal of the Controlled Release Society* 2014, 190, 254.
3. Z. Li, X. J. Loh, *Chemical Society reviews* 2015, 44, 2865.
4. L. Moroni, A. Nandakumar, F. B. de Groot, C. A. van Blitterswijk, P. Habibovic, *Journal of tissue engineering and regenerative medicine* 2013.
5. A. Bigi, G. Cojazzi, S. Panzavolta, A. Ripamonti, N. Roveri, M. Romanello, K. Noris Suarez, L. Moro, *Journal of inorganic biochemistry* 1997, 68, 45.
6. C. J. Damien, J. R. Parsons, *Journal of applied biomaterials : an official journal of the Society for Biomaterials* 1991, 2, 187.
7. N. Dobelin, R. Luginbuhl, M. Böhner, *Chimia* 2010, 64, 723.
8. A. M. Barradas, H. Yuan, C. A. van Blitterswijk, P. Habibovic, *European cells & materials* 2011, 21, 407.
9. P. Habibovic, H. Yuan, C. M. van der Valk, G. Meijer, C. A. van Blitterswijk, K. de Groot, *Biomaterials* 2005, 26, 3565.
10. H. Yuan, C. A. Van Blitterswijk, K. De Groot, J. D. De Bruijn, *J. Biomed. Mater. Res. Part A* 2006, 78, 139.

11. H. Yuan, C. A. Van Blitterswijk, K. De Groot, J. D. De Bruijn, *Tissue Engineering* 2006, 12, 1607.
12. N. L. Davison, X. Luo, T. Schoenmaker, V. Everts, H. Yuan, F. BarrÃre-de Groot, J. D. de Bruijn, *Eur. Cells and Mater.* 2014, 27, 281.
13. H. Yuan, H. Fernandes, P. Habibovic, J. de Boer, A. M. Barradas, A. de Ruiter, W. R. Walsh, C. A. van Blitterswijk, J. D. de Bruijn, *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107, 13614.
14. P. Habibovic, M. C. Kruyt, M. V. Juhl, S. Clyens, R. Martinetti, L. Dolcini, N. Theilgaard, C. A. van Blitterswijk, *J Orthop Res* 2008, 26, 1363.
15. P. Habibovic, H. Yuan, M. van den Doel, T. M. Sees, C. A. van Blitterswijk, K. de Groot, *J. Orthop. Res.* 2006, 24, 867.
16. C. J. Bettinger, B. Orrick, A. Misra, R. Langer, J. T. Borenstein, *Biomaterials* 2006, 27, 2558.
17. A. Curtis, C. Wilkinson, *Biomaterials* 1997, 18, 1573.
18. M. J. Dalby, N. Gadegaard, R. Tare, A. Andar, M. O. Riehle, P. Herzyk, C. D. Wilkinson, R. O. Oreffo, *Nature materials* 2007, 6, 997.
19. H. V. Unadkat, M. Hulsman, K. Cornelissen, B. J. Papenburg, R. K. Truckenmuller, A. E. Carpenter, M. Wessling, G. F. Post, M. Uetz, M. J. Reinders, D. Stamatialis, C. A. van Blitterswijk, J. de Boer, *Proceedings of the National Academy of Sciences of the United States of America* 2011, 108, 16565.
20. C. B. S. S. Danoux, D. C. Bassett, Z. Othman, A. I. Rodrigues, R. L. Reis, J. E. Barralet, C. A. Van Blitterswijk, P. Habibovic, *Acta Biomaterialia* 2015, 17, 1.
21. P. Habibovic, D. C. Bassett, C. J. Doillon, C. Gerard, M. D. McKee, J. E. Barralet, *Advanced materials* 2010, 22, 1858.
22. G. Mattei, C. Ferretti, A. Tirella, A. Ahluwalia, M. Mattioli-Belmonte, *Scientific reports* 2015, 5, 10778.
23. D. Barbieri, H. Yuan, X. Luo, S. Fare, D. W. Grijpma, J. D. de Bruijn, *Acta biomaterialia* 2013, 9, 9401.
24. E. Nejati, V. Firouzidor, M. Eslaminejad, F. Bagheri, *Materials Science and Engineering: C* 2009, 29, 942.
25. X. H. Wang, S. Shi, G. Guo, S. Z. Fu, M. Fan, F. Luo, X. Zhao, Y. Q. Wei, Z. Y. Qian, *Journal of Biomaterials Science, Polymer Edition* 2011, 22, 1917.
26. S. I. Jeong, E. K. Ko, J. Yum, C. H. Jung, Y. M. Lee, H. Shin, *Macromolecular bioscience* 2008, 8, 328.
27. S. Hasegawa, J. Tamura, M. Neo, K. Goto, Y. Shikunami, M. Saito, M. Kita, T. Nakamura, *Journal of Biomedical Materials Research Part A* 2005, 75, 567.
28. M. O. Montjovent, L. Mathieu, H. Schmoekel, S. Mark, P. E. Bourban, P. Y. Zambelli, L. A. LaurentÃApplegate, D. P. Pioletti, *Journal of Biomedical Materials Research Part A* 2007, 83, 41.
29. D. Barbieri, A. Renard, J. Bruijn, H. Yuan, *European cells & materials* 2010, 19, 252.
30. D. Barbieri, H. Yuan, X. Luo, S. Fare, D. W. Grijpma, J. D. de Bruijn, *Acta biomaterialia* 2013, 9, 9401.
31. S. Hasegawa, M. Neo, J. Tamura, S. Fujibayashi, M. Takemoto, Y. Shikunami, K. Okazaki, T. Nakamura, *Journal of Biomedical Materials Research Part A* 2007, 81, 930.
32. A. Khademhosseini, R. Langer, J. Borenstein, J. P. Vacanti, *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103, 2480.
33. D. Falconnet, G. Csucs, H. Michelle Grandin, M. Textor, *Biomaterials* 2006, 27, 3044.
34. Y. Ito, *Biomaterials* 1999, 20, 2333.
35. Y. Xia, G. M. Whitesides, *Annual review of materials science* 1998, 28, 153.

36. M. Geissler, Y. Xia, *Advanced Materials* 2004, 16, 1249.
37. G. M. Whitesides, *Nature* 2006, 442, 368.
38. J. Le Digabel, M. Ghibaud, L. Trichet, A. Richert, B. Ladoux, *Medical & biological engineering & computing* 2010, 48, 965.
39. M. Nikkhah, F. Edalat, S. Manoucheri, A. Khademhosseini, *Biomaterials* 2012, 33, 5230.
40. A. Graziano, R. d'Aquino, M. G. Cusella-De Angelis, G. Laino, A. Piattelli, M. Pacifici, A. De Rosa, G. Papaccio, *PloS one* 2007, 2, e496.
41. D. W. Hamilton, B. Chehroudi, D. M. Brunette, *Biomaterials* 2007, 28, 2281.
42. U. Ripamonti, J. Crooks, A. N. Kirkbride, *South African J Sci* 1999, 95, 335.
43. C. B. Danoux, D. Barbieri, H. Yuan, J. D. de Bruijn, C. A. van Blitterswijk, P. Habibovic, *Biomatter* 2014, 4, e27664.
44. J. Zhang, X. Luo, D. Barbieri, A. Barradas, J. D. de Bruijn, C. A. van Blitterswijk, H. Yuan, *Acta biomaterialia* 2014, 10, 3254.
45. S. K. Both, A. J. v. d. Muijsenberg, C. A. v. Blitterswijk, J. d. Boer, J. D. d. Bruijn, *Tissue engineering* 2007, 13, 3.
46. A. E. Carpenter, T. R. Jones, M. R. Lamprecht, C. Clarke, I. H. Kang, O. Friman, D. A. Guertin, J. H. Chang, R. A. Lindquist, J. Moffat, P. Golland, D. M. Sabatini, *Genome Biol* 2006, 7.
47. R. Tewari, C. Friedrich, *Microsyst Technol* 2010, 16, 1601.
48. S. Belligundu, P. S. Shiakolas, A. Pandey, P. B. Aswath, *Journal of biomedical materials research. Part B, Applied biomaterials* 2008, 85, 469.
49. W. Song, N. Kawazoe, G. Chen, *Journal of Nanomaterials* 2011, 2011, 6.
50. R. McBeath, D. M. Pirone, C. M. Nelson, K. Bhadriraju, C. S. Chen, *Developmental cell* 2004, 6, 483.
51. G. M. de Peppo, H. Agheli, C. Karlsson, K. Ekstrom, H. Brisby, M. Lenneras, S. Gustafsson, P. Sjoval, A. Johansson, E. Olsson, J. Lausmaa, P. Thomsen, S. Petronis, *Int J Nanomedicine* 2014, 9, 2499.
52. A. J. Dulgar-Tulloch, R. Bizios, R. W. Siegel, *Journal of biomedical materials research. Part A* 2009, 90, 586.
53. U. Hempel, T. Hefti, P. Dieter, F. Schlottig, *Clin Oral Implan Res* 2013, 24, 174.
54. S. Morelli, S. Salerno, J. Holopainen, M. Ritala, L. D. Bartolo, *Journal of biotechnology* 2015, 204, 53.
55. A. M. Barradas, H. A. Fernandes, N. Groen, Y. C. Chai, J. Schrooten, J. van de Peppel, J. P. van Leeuwen, C. A. van Blitterswijk, J. de Boer, *Biomaterials* 2012, 33, 3205.
56. C. Danoux, D. C. Bassett, Z. Othman, A. I. Rodrigues, R. L. Reis, J. Barralet, C. A. van Blitterswijk, P. Habibovic, *Acta biomaterialia* 2015.
57. A. M. C. Barradas, H. A. M. Fernandes, N. Groen, Y. C. Chai, J. Schrooten, J. van de Peppel, J. P. T. M. van Leeuwen, C. A. van Blitterswijk, J. de Boer, *Biomaterials* 2012, 33, 3205.
58. J. N. Beresford, C. J. Joyner, C. Devlin, J. T. Triffitt, *Archives of oral biology* 1994, 39, 941.
59. S. Walsh, G. R. Jordan, C. Jefferiss, K. Stewart, J. N. Beresford, *Rheumatology* 2001, 40, 74.
60. D. Barbieri, A. J. Renard, J. D. de Bruijn, H. Yuan, *European cells & materials* 2010, 19, 252.
61. A. M. Barradas, V. Monticone, M. Hulsman, C. Danoux, H. Fernandes, Z. Tahmasebi Birgani, F. Barrere-de Groot, H. Yuan, M. Reinders, P. Habibovic, C. van Blitterswijk, J. de Boer, *Integrative biology : quantitative biosciences from nano to macro* 2013, 5, 920.
62. D. W. Hamilton, B. Chehroudi, D. M. Brunette, *Biomaterials* 2007, 28, 2281.

Discussion & Outlook

As is indicated in the introductory chapter of this thesis, the focus of this work was on synthetic bone graft substitutes that are needed to replace natural bone grafts, including autograft, allograft and xenograft in treating large, critically-sized bone defects. The reason for this need stems from a continuous improvement of life quality and associated increase of life expectancy in the developed countries that have resulted in a growing demand for successful replacement of damaged and degraded bone. As a result of orthopedic trauma and bone diseases, about 5 million orthopedic interventions are performed annually. In 3 million of these interventions, bone grafting is required to ensure bone growth at defect sites, which is an increase of 25% in the past 10 years. With such an increase, limited availability of natural bone grafts, with the total amount of bone that can be harvested from the iliac crest being only about 5 cc [1], is becoming a serious clinical challenge. This comes on top of other disadvantages which are associated with the use of natural bone grafts including the need for an additional invasive surgical procedure that may lead to donor site morbidity and chronic post-operative pain (in up to 18.7% of all patients after 2 years) [2], hypersensitivity and infection. Bone graft substitutes based on growth factors such as Bone Morphogenetic Proteins have been successfully used since their clinical introduction about a decade ago, but in particular their high cost, being almost 5000 EUR for one 5-ml vial, are an indication that growth factors cannot be a sole solution for a continuously increasing demand for bone regenerative strategies. Also classical tissue engineering approached,

combining a carrier material with (patient's own) cells and/or growth factors have not been able to claim a significant portion of the bone graft substitute market, owing to their complexity, and therewith associated logistic issues and high cost.

Not surprisingly, the focus is now moving from this complex and expensive bone graft substitutes to substitutes that can be produced in large quantities, are readily available and relatively inexpensive. Synthetic bone graft substitutes meet these requirements, as their production cost are comparatively low, and the issues with stability, thus storage are practically non existent. Among various synthetic bone graft substitutes, CaP-based ones have been extensively used in bone repair and regeneration since 1970s [3]. Despite this widespread use, and a great number of products available on the market [4], CaP-based bone graft substitutes have not yet reached the status of a full alternative to natural bone grafts. Owing to their resemblance to bone mineral, CaP bone graft substitutes are biocompatible, osteogenic and osteoconductive, but the majority of them lacks osteoinductivity, which is believed to be a prerequisite for reaching the same bone regenerative potential as natural bone grafts.

This description of the state-of-the art demonstrates the challenge materials scientists and engineers are facing when developing modern synthetic bone graft substitutes: their clinical performance needs to be significantly improved in order to be accepted as a full alternative to natural bone grafts, while, on the other hand, their cost need to be kept low and their availability practically unlimited. In order to succeed in this challenge, the approach toward (further) development of synthetic bone graft substitutes needs to change significantly, because the way they have been developed and evaluated so far has brought only incremental improvements, and has made little difference to the patient.

For example, in the last three decades, bone formation after heterotopic implantation, i.e. osteoinduction has been observed in a number of synthetic biomaterials, predominantly CaP ceramics of various types, but also in some polymers and metals [5]. The first evidence of osteoinductivity was a case of serendipity, involving a study in which (poly)HEMA materials, intended for breast implants, were implanted in soft tissue to test their biocompatibility. Upon explantation, bone formation was observed in contact with the polymer [6]. Although all other studies had the aim to find a material with intrinsic osteoinductivity, they had a somewhat trial-and-error character. A material was produced, characterized and then implanted in a heterotopic site, like muscle or subcutis, followed by a histological evaluation. Based on the volume of bone formed, the materials were comparatively evaluated, and conclusions regarding their osteoinductive potential were drawn. This result served as input for the development of a next material. However, a great number of factors affected the result on the occurrence and amount of newly formed bone. Not only the chemistry of the material, but also its physical properties, which are mainly the result of the production process applied, as well as the animal model, implantation site, implantation time, implant

size, etc. are determinant for the biological response to the material. Since no two studies are exactly the same, it is not surprising that this kind of studies remained mainly observational and did not bring much insight into the mechanism behind osteoinductivity. It should be emphasized that this mechanism is not solely needed to satisfy the scientific curiosity, but is imperative to know what an ideal osteoinductive biomaterial should look like in order to be able to make it.

Therefore, nowadays, research focus is slowly shifting from finding new osteoinductive materials to better understanding, controlling and optimizing the osteoinductive potential of biomaterials. In the past decade, expectedly relevant parameters have been listed and tested, such as chemical composition of the material, degradation properties, porosity, surface structure down to the nanometer level. This has unravelled a part of the complexity of this challenge. Traditionally, a rational approach to multi-parameter problems is to evaluate the effect of each parameter individually. This deconvolution usually simplifies the task while requiring a lot of time, work and resources. In the case of osteoinductive materials, and even more generally, of synthetic bone graft substitutes, the degree of complexity further increases as various material parameters are also interlinked. This makes it difficult to change one parameter of the material without affecting the others, in particular when one needs to rely to the conventional production techniques. This presents an additional challenge to materials scientists. Despite this difficulty, some experiments were successfully conducted providing a partial insight into the effect of individual parameters on osteoinductive, and therewith bone regenerative potential of synthetic bone graft substitutes. As an example, Habibovic et al. achieved to decouple the effect of microstructure of CaP ceramics, from their macrostructure and chemical composition by controlling the temperature at which the ceramics were sintered. While the chemistry and macrostructure were kept identical, ceramics with two different microstructures were tested in vivo in an intramuscular model in goats [7]. The ceramic with a microstructure presenting a significantly larger specific surface area induced heterotopic bone formation in all the animals, as opposed to the ceramic with a five times smaller surface area, where no bone formation was observed. This experiment demonstrated how a single parameter can affect the biological performance of a material, but it also emphasized the complexity of a material like CaP ceramic. Indeed, while a clear effect was observed of microstructure of the ceramic, it is still unknown whether this effect was related to the chemistry (an increase in specific surface area increases degradation rate of a ceramic, and therewith potentially the release of calcium and phosphate ions) or physical (the ceramic with large specific surface area contains smaller grains and more micropores which could directly affect cell behaviour). Furthermore, in sintered ceramics, sintering conditions are one of the few parameters than can actually be used to control the properties of the end product, clearly demonstrating the limitation of this method.

Facing this limitation, the approach taken in this thesis consisted of finding

practical solutions to decouple the material parameters, by not necessarily relying on the conventional processing parameters used to produce bone grafts substitutes. As was indicated in the introductory section, the general aim was twofold: (1) to explore strategies to isolate individual properties of complex biomaterials in order to investigate their independent effects on a biological response and based on this knowledge (2) to recombine individual properties into new, improved functional biomaterials.

7.1 Composites

While so far, CaP based ceramics have shown the strongest potential to become a comprehensive synthetic alternative to natural bone grafts, intrinsic properties of CaP ceramics, including their brittleness, chemical composition, crystalline structure and other associated properties makes these materials difficult to modify in a controlled manner and to study mechanisms behind their biological effects. Polymers, on the other hand, offer much more flexibility when it comes to processing and control over individual properties, but in general, polymers are less bioactive than CaP ceramics in bone regeneration applications. Composite materials, consisting of a polymer and a ceramic, offer interesting possibilities when it comes to combining positive characteristics of both material types, and their application in bone regeneration is also a logical one, considering that bone itself is a composite material. Indeed, recent developments in composite design and production open new opportunities for material design in terms of chemistry, structure and mechanical behaviour. For example, composites can be used as carriers and release vehicles of other compounds, whereby the polymeric component can be used to finetune the degradation and thus the release profile of the compound of interest. In this thesis, poly(lactic acid) (PLA), a biocompatible and clinically used aliphatic polyester, was shown to be a suitable matrix for the incorporation of hydroxyapatite (Chapter 3), as well as calcium- and phosphate salts (Chapter 4), creating composite carriers that can be assessed both *in vivo* and *in vitro*. It has been shown that such materials can also be produced as microcarriers (spheres, capsules) allowing solely the release of the (inorganic) compound of interest, without acting as a substrate for cell culture or tissue growth. For example, recently, it has been shown that PLA microspheres combined with a calcium salt can be used as carriers of calcium ions, to study their effect on cell behaviour [8]. In order to optimize the benefits of using composite materials as carriers, the degradation and consequently the release of active compounds are crucial parameters. It has been previously shown that the molecular weight of the matrix polymer controls in part the degradation and release mechanism [9]. However, other material parameters such as the size of the filler and the matrix-filler interface and interaction should also be taken into account to control the delivery mechanism from composite materials. In addition to their role as carriers of other compounds,

polymeric composite materials can also be used as structural materials, thus as bone graft substitutes. In Chapters 3, 4 and 6 of this thesis, this property was exploited to control individual material properties and to study their effect in vivo or in vitro.

7.2 Macrostructure: The effect of overall geometry of the implant osteoinductive properties of composite materials

In the first experimental chapter of this thesis, we investigated the effect of the overall implant geometry on the bioactivity of a potential bone graft substitute in terms of osteoinductivity. In previous studies on osteoinductive materials, the overall geometry of the implant, i.e. the presence of pores or predefined geometries, such as concavities and channels, or their formation post-implantation, has been shown to be important to facilitate osteoinduction [10-13]. It has been suggested that these “protected” spaces were needed to reach optimal conditions (e.g. calcium and phosphate ion concentrations) to trigger the process of osteoinduction [5]. Recently, Davison et al. demonstrated that osteoinduction indeed occurs on a planar surface of ceramics as well, i.e. in the absence of protective areas such as pores [14], although in lower amounts than with the macroporous counterparts [15]. This suggests that macrostructure is important but not an essential property in the process. Here, instead of using CaP ceramics like in these previous studies, we used a PLA/HA composite material that was produced by mixing and extruding, resulting in a homogenous, dense composite. In Chapter 3, a new implant design was developed and used in an in vivo study, whereby two of such composite plates were paired with a spacer between them to create an “artificial pore” in order to test (1) the importance of the presence of a CaP ceramic for osteinduction to occur in such a composite material and (2) the importance of the sheltered area for the location of new bone formation. The data confirmed that indeed, only the material that contained HA ceramic was osteoinductive upon implantation in paraspinal muscles of dogs and that bone formation only occurred inside the sheltered area between the two composite plates, in particular there, where the material was degraded. This demonstrated the importance of the protected areas, in particular in case of materials with relatively low osteoinductive potential. It should be mentioned that this implant design offers new practical tools to study other fillers, for example, as discussed below for Chapter 4, and to modify the surface of the material inside the “artificial” pore with well defined micro- and macrostructure, like for example in Chapters 5 and 6.

7.3 Chemistry: Individual effects of calcium and inorganic phosphate ions on osteogenic differentiation of hMSCs

As is discussed in detail in the introductory chapter of this thesis as well as in the introduction of this Discussion chapter, CaPs have been extensively used in orthopaedic and craniomaxillofacial applications for over 40 years now, mainly owing to their chemical resemblance to bone mineral. Interestingly, there is still no conclusive evidence for what makes these materials bioactive in the environment of bone. With other words, no definitive answer exists to the question whether it is their chemistry, their structure or both that is responsible for their bioactivity. The main reason for this lack of understanding is that all these properties are intertwined. Each CaP bone graft substitute possesses a certain chemical phase, crystallinity, surface topography, all of which dynamically change to different extents upon implantation. Focusing on chemistry, in Chapter 4 of this thesis, we chose not to use conventional composite materials, consisting of a polymeric matrix and a CaP filler. Instead, we decided to separate calcium from inorganic phosphate chemical effect by developing composite materials with a calcium salt (without phosphate) and a phosphate salt (without calcium). This study not only confirmed the important role of calcium, but also emphasized on the role of inorganic phosphate in the process of osteogenic differentiation of hMSCs. Although previously some attempts have been undertaken to study the effect of individual ions of hMSCs proliferation and differentiation [16-18], mainly by conditioning cell culture media, here we presented one of the first attempts to develop functional materials that could actually be implanted to release the ions of interest locally. The approach of using composite carriers could be used to optimize the effect of these compounds by controlling their release profile in space and time. This work also shows how powerful inorganics can be to influence the cell fate and how important the current research on trace elements use in bone regeneration is [19].

7.4 Surface topography: surface chemistry versus microstructure effect on osteogenic differentiation of hMSCs

Besides chemical composition, surface topography has also been shown as a powerful tool to control cell fate [20]. Surface structural properties including microporosity, grain size and therewith-related specific surface area have also been shown to play a determinant role in the process of osteoinduction by biomaterials. Concerning CaPs, important information about the effect of surface structural properties stems from studies in which sintering temperature was used as a tool

to control grain size and microporosity [7, 15, 21, 22]. Based on these studies, it was concluded that the submicrometer scale of grains and pores on the surface is the property that renders a ceramic osteoinductive [21]. This was specifically demonstrated for sintered ceramics, and although this finding cannot be directly extrapolated to all CaPs, the importance of surface-structural features is evident. To take this investigation a step further, in Chapters 5 and 6, a number of materials and technologies were combined and converged in order to separate their individual properties, and upon investigating their individual roles on a biological process, recombine them into biomaterial with improved functionality.

In Chapter 5, a novel approach was undertaken to isolate the effect of surface topography of natural materials from their chemistry. Nature has always been a potent source of inspiration for researchers and biomimetic strategies have often led to great success. Monetite, brushite and octacalcium phosphate, three different phases of CaP, all reported to naturally occur inside the body [23] were produced in such a way that their macro- and microstructure was controlled to a high extent. Then, these materials were used as templates for polymeric moulds that were then used to transfer the structure of these CaP crystals into a polymer, thereby isolating the role of the structure from that of chemical composition. To achieve this, the use of polymers was an absolute prerequisite, as only polymers are suitable for employing the techniques like nanoimprinting. The results of this study suggested that the role of chemistry, i.e. presence of CaP is more important in triggering the differentiation of hMSCs into the osteogenic lineage than the surface microstructure. We further showed that smart combination of different technologies, rather than relying on the conventional ways of producing a certain type of material is a very promising route to develop materials with improved properties.

The approach from Chapter 5 was further extended in Chapter 6 to functional composite materials. Here, rationally designed micro-sized topographies were produced and applied to surfaces of PLA and PLA/HA composites to study individual effects of surface chemistry and surface microtopography on cell behaviour. By replicating the same topography on substrates having different chemistry, this study confirmed a preponderant effect of chemistry on the differentiation of hMSCs, although some additional influence of the topography was also observed (Chapter 6). This approach can be exploited in a more systematic manner, with other natural or (rationally) designed topographies and in a great range of materials, clearly demonstrating the potential for development of new, improved biomaterials for regenerative medicine. Since in Chapter 6, clinically used biocompatible materials were used, it is evident that this technology is not solely useful for fundamental studies, but can also be readily translated into the clinic. Indeed, in the scope of this thesis, microstructuring of the surface as described in Chapter 6 was combined with the innovative implant design from Chapter 3 and these materials were used in an animal study. However, this entire *in vivo* study failed, (plausibly as a result of administration of NSAIDs that prevented heterotopic bone formation,

even in positive controls), which is why the results have not been discussed here. Nevertheless, this study confirms the potential of combining these innovative implant designs to produce improved bone graft substitutes.

7.5 Perspectives

This thesis was an exploratory journey aiming at identifying tools that can be used to change the way biomaterials for regenerative medicine, and more specifically for bone regeneration, are designed and developed. The initiator of this journey was a changing, more demanding role synthetic biomaterials are expected to play in regenerative processes, while remaining affordable. The approach taken was to step away from the conventional, processing-driven biomaterials development to a design-driven one, whereby combining and converging of materials and technologies is a logical step. This approach will not only bring answers to the missing questions regarding the interaction of various materials with the biological system, but will also result in new, improved materials.

The results shown in this thesis demonstrated that there is an enormous potential in this approach, which should now be further explored. Each of the techniques that were explored here, i.e. application of composites with various fillers, use of micro- and nanotechnology techniques for structuring surfaces, use of coating techniques, innovative implant designs are useful and should be expanded to other materials, and other application areas. This will undoubtedly bring new challenges but also new technologies that can bring this field another step further.

Eventually, one should have a toolbox with material properties, each with a well-known biological effect, which can be combined depending on the demand of a (clinical) application. This is clearly not trivial and many challenges will have to be overcome, but it is a promising way for synthetic biomaterials to make a true difference in biomedical applications. This will also require a tight interaction with biologists and bioinformaticians, because this approach of developing and combining properties and of coupling them to a biological response will result in an enormous amount of data. These data are only valuable when they provide answers to the fundamental questions regarding the material-biological system interactions, and when they can be used in the iterative process of new materials development.

References

1. Goulet JA, Senunas LE, DeSilva GL, Greenfield MLVH. Autogenous Iliac Crest Bone Graft. Complications and Functional Assessment. *Clin Orthop Relat Res*. 1997;339:76-81.
2. Ricciardi BF, Bostrom MP. Bone graft substitutes: Claims and credibility. *Semin Arthroplasty*. 2013 Jun;24(2):119-23.
3. Habraken W, Habibovic P, Epple M, Böhner M. Calcium phosphates in biomedical applications: materials for the future? *Mater Today*. 2015 Oct;

4. Van der Stok J, Van Lieshout EMM, El-Massoudi Y, Van Kralingen GH, Patka P. Bone substitutes in the Netherlands - a systematic literature review. *Acta Biomater.* 2011 Feb;7(2):739-50.
5. Barradas AMC, Yuan H, Blitterswijk CA van, Habibovic P. Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms. *European Cells & Materials.* 2011;21:407-29.
6. Winter GD, Simpson BJ. Heterotopic Bone formed in a Synthetic Sponge in the Skin of Young Pigs. *Nature.* Nature Publishing Group; 1969 Jul 5;223(5201):88-90.
7. Habibovic P, Yuan H, van den Doel M, Sees TM, Blitterswijk CA van, Groot K de. Relevance of osteoinductive biomaterials in critical-sized orthotopic defect. *J Orthop Res.* 2006 May;24(5):867-76.
8. Birgani ZT, Klotz BJ, van Blitterswijk CA, Habibovic P. Poly (lactic acid) microspheres for local delivery of bioinorganics for applications in bone regeneration. *Adv Biomater Devices Med.* 2014;1:1-10.
9. Barbieri D, Yuan H, Luo X, Farè S, Grijpma DW, de Bruijn JD. Influence of polymer molecular weight in osteoinductive composites for bone tissue regeneration. *Acta Biomater.* 2013 Dec;9(12):9401-13.
10. Habibovic P, Gbureck U, Doillon CJ, Bassett DC, van Blitterswijk CA, Barralet JE. Osteoconduction and osteoinduction of low-temperature 3D printed bioceramic implants. *Biomaterials.* 2008 Mar;29(7):944-53.
11. Ripamonti U, Crooks J, Kirkbride A. Sintered porous hydroxyapatites with intrinsic osteoinductive activity: Geometric induction of bone formation. *S Afr J Sci.* 1999;95:335-43.
12. Gosain AK, Song L, Riordan P, Amarante MT, Nagy PG, Wilson CR, et al. A 1-year study of osteoinduction in hydroxyapatite-derived biomaterials in an adult sheep model: part I. *Plast Reconstr Surg.* 2002 Feb;109(2):619-30.
13. Habibovic P, Sees TM, van den Doel MA, Blitterswijk CA van, Groot K de. Osteoinduction by biomaterials—physicochemical and structural influences. *J Biomed Mater Res A.* 2006 Jun 15;77(4):747-62.
14. Davison NL. Cell and tissue response to osteoinductive calcium phosphate architecture. Universiteit Twente; 2014.
15. Yuan H, Fernandes H, Habibovic P, de Boer J, Barradas AMC, de Ruiter A, et al. Osteoinductive ceramics as a synthetic alternative to autologous bone grafting. *Proc Natl Acad Sci U S A.* 2010 Aug 3;107(31):13614-9.
16. Chai YC, Roberts SJ, Schrooten J, Luyten FP. Probing the osteoinductive effect of calcium phosphate by using an in vitro biomimetic model. *Tissue Eng Part A.* 2011 Apr;17(7-8):1083-97.
17. Barradas AMC, Fernandes HAM, Groen N, Chai YC, Schrooten J, van de Peppel J, et al. A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells. *Biomaterials.* 2012 Apr;33(11):3205-15.
18. Shih Y-R V, Hwang Y, Phadke A, Kang H, Hwang NS, Caro EJ, et al. Calcium phosphate-bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling. *Proc Natl Acad Sci USA.* 2014 Jan 21;111(3):990-5.
19. Habibovic P, Barralet JE. Bioinorganics and biomaterials: bone repair. *Acta Biomater.* 2011 Aug;7(8):3013-26.
20. Biggs MJP, Richards RG, Gadegaard N, Wilkinson CD., Dalby MJ. The effects of nanoscale pits on primary human osteoblast adhesion formation and cellular spreading. *J Mater Sci Mater Med.* 2007 Feb;18(2):399-404.
21. Davison NL, Luo X, Schoenmaker T, Everts V, Yuan H, Barrère-de Groot F, et al. Submicron-scale surface architecture of tricalcium phosphate directs osteogenesis in vitro and in vivo. *Eur Cell Mater.* 2014 Jan;27:281-97.

22. Habibovic P, Krut MC, Juhl MV, Clyens S, Martinetti R, Dolcini L, et al. Comparative in vivo study of six hydroxyapatite-based bone graft substitutes. *J Orthop Res*. 2008 Oct;26(10):1363-70.
23. Elliott JC. *Structure and Chemistry of the Apatites and Other Calcium Orthophosphates*. Amsterdam: Elsevier Sciences; 1994. 404 p.

Publications

Book chapter

Danoux C., Tare R., Smith J., Bradley M., Hunt J.A., Oreffo R.O.C. and Habibovic P. (2013). Development of materials for regenerative medicine: from clinical need to clinical application. In J. de Boer and C.A. van Blitterswijk (Eds.), *Materiomics, High-Throughput Screening of Biomaterial Properties*. Cambridge University Press.

Journal papers

Danoux C., Sun L., Koçer G., Birgani Z., Barata D., Barralet J., van Blitterswijk C., Truckenmüller R., Habibovic P., Development of Highly Functional Biomaterials by Decoupling and Recombining Material Properties. 2015. *Advanced Materials*.

Sun* L., Danoux* C., Wang Q., Pereira D., Barata D., Zhang J., Truckenmüller R., Xin Xu C., Habibovic P., Independent effects of chemical and surface-structural properties of polymer/ceramic composites on proliferation and osteogenic differentiation of human MSCs. Ready for submission. *authors contributed equally

Danoux C., Bassett D.C., Othman Z., Rodrigues A.I., Reis R.L., Barralet J.E., van Blitterswijk C.A. and Habibovic P., Elucidating the individual effects of

calcium and phosphate ions on hMSCs by using composite materials. 2015. *Acta Biomaterialia*.

Danoux C., Barbieri D., Yuan H., de Bruijn J.D., van Blitterswijk C.A. and Habibovic P., In vitro and in vivo bioactivity assessment of a polylactic acid/hydroxyapatite composite for bone regeneration. 2014. *Biomatter*.

Chatterjea A., van der Stok J., Danoux C., Yuan H., Habibovic P., van Blitterswijk C.A., Weinans H., de Boer J., Inflammatory response and bone healing capacity of two porous calcium phosphate ceramics in critical size cortical bone defects. 2013. *Journal of Biomedical Materials Res.*

Conference Presentations and posters

Charlène Danoux, Clemens van Blitterswijk, Pamela Habibovic. In Vivo Assessment of Osteoinductivity of Polylactic Acid/Hydroxyapatite Composite. Poster presented at the conference NBTE, 2010, Lunteren, the Netherlands.

Charlène Danoux, Clemens van Blitterswijk, Pamela Habibovic. Combining Soluble and Insoluble Signals for Improved Osteoinductive Potential of Implants. Presentation at the conference NBTE, 2011, Lunteren, the Netherlands.

Charlène Danoux, Davide Barbieri, Huipin Yuan, Clemens van Blitterswijk, Pamela Habibovic. In vitro and in vivo Assessment of Osteoinductivity of a Polylactic Acid / Hydroxyapatite Composite. Presentation at the 9th World Biomaterials Congress, 2012, Chengdu, China.

Charlène Danoux, Jake Barralet, Clemens van Blitterswijk, Pamela Habibovic. Calcium-Phosphate Crystals and their natural Topographies: in vitro Effect on Cell Behavior. Poster presented at the 9th World Biomaterials Congress, 2012, Chengdu, China.

Charlène Danoux, Jake Barralet, Clemens van Blitterswijk, Pamela Habibovic. Calcium Phosphate Crystals: Natural Topographies and their Influence on Osteoinduction. Poster presented at the conference NBTE, 2012, Lunteren, the Netherlands.

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"The strongest of all warriors are these two - Time and Patience."

L. Tolstoï

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