Biomedical Materials

PAPER

Organically modified hydroxyapatite (ormoHAP) nanospheres stimulate the differentiation of osteoblast and osteoclast precursors: a co-culture study

Christiane Heinemann, Sascha Heinemann, Sina Rößler, Benjamin Kruppke, Hans-Peter Wiesmann and Thomas Hanke

Max Bergmann Center of Biomaterials and Institute of Materials Science, Technische Universität Dresden, Budapester Str. 27, D-01069 Dresden, Germany

E-mail: christiane.heinemann@tu-dresden.de

Keywords: calcium phosphate, hydroxyapatite, scaffold, co-culture, human bone marrow stromal cells, human monocytes

Abstract

Isolated nanospheres consisting of organically modified hydroxyapatite (ormoHAP), prepared by an electric field-assisted ion double migration process, were embedded in foamed gelatin to form a composite scaffold. Degradation rates have been demonstrated to correlate with the crosslinking degree (40%, 80%) as well as with the mineral content of the scaffolds (0%, 20%, 40%). A human co-culture model of osteoblasts and osteoclasts, derived from bone marrow stromal cells and monocytes, respectively, without external addition of the factors RANKL and M-CSF, was run for up to 42 d in order to characterize the action of the ormoHAP-gelatin scaffolds on the co-culture. Examination was performed by quantitative biochemical methods (DNA, LDH, ALP, TRAP5b), gene expression analysis (ALP, BSP II, RANKL, IL-6, VTNR, CTSK, TRAP, OSCAR, CALCR) and confocal laser scanning microscopy (cell nuclei, actin, CD68, TRAP). Results confirm that ormoHAP embedded in the gelatin matrix enhanced TRAP 5b activity. As a feedback, ALP activity and gene expression of BSP II of osteoblasts increased. Finally, a sequence of cell cross-talk actions is suggested, which can explain the behavior of the formed vital co-culture and moreover the influence of the presence and concentration of ormoHAP.

1. Introduction

Nowadays, biomaterials development—focused on bio-inspired or biomimetic materials. Bone as a natural composite is designed from complex structured mineralized collagen. Therefore, several attempts to develop inorganic/organic composites based on components of native mammalian bone, namely carbonated apatite and collagen, have been undertaken [1, 2]. However, the incorporation of mostly micron-sized mineral phases into polymer matrices can cause problems with brittleness and in vivo resorbability [3]. In the present study, a novel bioinspired mineralization process was used which we have recently published [4, 5]. It is based on electric field-assisted double migration of both calcium and phosphate ions into specially carboxymethylated gelatin acting as an organic template. The mineral phase obtained from this process is termed organically modified hydroxyapatite (ormoHAP), in order to emphasize the composite character of the mineral sphere and its structural organization. The basic hydroxyapatite (HAP) nanocrystals self-assemble in the organic environment of the mineralization matrix to form hollow spherical agglomerates with diameters in the range of 100–500 nm [4]. These spheres have been investigated and discussed with respect to their application as parts of a composite biomaterial for bone substitution—which is the focus of the present paper.

These composite biomaterial consists of the ormoHAP spheres isolated from the carboxymethylated gelatin that had served as a mineralization matrix and template. Afterwards the isolated spheres were embedded in gelatin again and subsequently freeze dried. Gelatin was chosen as the scaffold basic material.
because it is already a part of the electric field-assisted mineral formation process and thus the product of the ormoHAP spheres itself. Both gelatin and HAP are known to be cytocompatible towards several cell species at various in vitro cultivation conditions [6, 7]. The combination of both to form composites has been used in many studies. Especially, the simultaneous collagen fibrillogenesis and mineralization as well as characterization of products thereof was extensively discussed in terms of mechanical properties and biocompatibility [8]. Gelatin is a denaturation product of collagen and features some advantages like abundant availability, processibility, biological resorbability, and low antigenicity. Therefore, a large number of medical devices are based on gelatin processed to fibers, hydrogels, coatings, sponges, foams, fleece, and membranes [9–12]. Gelatin wound dressings and bone substitution devices are mainly produced by freeze drying of suspensions, followed by chemical cross-linking. The same methods have been used in previous studies to create composite scaffolds based on gelatin/alginate [13], gelatin/chitosan [14], gelatin/β-TCP [15] or gelatin/HAP [16–18]. It has to be noted that the use of the term composite is critical when coating procedures are used for the combination of the composites. In most cases the mineralized variants of samples demonstrated advantages in terms of cell proliferation compared to monophase references.

Most studies carry out monoculture cell experiments using cells of human origin. Particularly, the biological response of several bone cell types cultivated in the presence of HAP has been investigated [19, 20]. However, in vitro experiments making use of co-cultures of different cell types are attractive for biomaterials testing because they represent one step forward to natural conditions by taking into account the interactions between different cell types [21, 22]. The authors of the present paper previously reported on a human supplements-free co-culture model comprising both osteoclasts and osteoblasts, which is intended to provide a more accurate picture of what is likely to take place in vivo [23]. The cross-talk of bone forming osteoblasts and bone resorbing osteoclasts, either through soluble factors or by direct cell–cell contact, is a fundamental requirement for balanced bone remodeling. Additionally, cell–biomaterial interactions affect cell–cell interactions in cell monocultures as well as in co-cultures. The resulting system consisting of the biomaterial, the environment (especially supplements), and different cell types is very complex and a challenge for all studies in this field. On the other hand, taking advantages of these effects can generate success in developing new approaches for promoting tissue regeneration. Synthetic materials as well as natural biomaterials can modulate cell response via different material properties such as surface chemistry and ion balance [24]. In particular, this is true for bone cells, where previous studies demonstrated for example the biomaterial surface to play an important role in cell behavior [25].

Paper that simultaneously consider both aspects, namely—at first—co-cultivation of bone cells without addition of RANKL and M-CSF and—at second—a biomaterial as a substrate, are rarely to be found. Some of the few examples are studies on 2D surfaces, e.g. composite xerogels [26] and similar studies on nanotopographical polymer surfaces or on micropatterned ceramics [27, 28]. When switching to 3D constructs, the interaction between osteoblasts and osteoclasts was demonstrated to result in an enhancement of osteoblast differentiation and an earlier osteoclast differentiation, which led to a deposition of bone tissue like extracellular matrix.

The present study, for the first time is intended to consider all the previously mentioned aspects by co-cultivation of human bone marrow stromal cells (hBMSC)/human osteoblasts (hOB) and human monocytes (hMc)/human osteoclasts (hOC) without supplements on 3D composite scaffolds in order to evaluate the influence of organically modified HAP nanospheres on cell behavior and interaction.

2. Materials and methods

2.1. Preparation of organically modified HAP nanospheres

OrmoHAP was prepared as described previously [4]. In brief, carboxymethylated gelatin (native porcine gelatin, Heraeus®) was prepared as 10 wt% sol by swelling in deionized water for 30 min at 20 °C followed by heating to 50 °C for 24 h. Carboxymethylation of gelatin was achieved at 50 °C for 24 h by adding up to 250 μg sodium-D-glucuronate (D-glucuronic acid sodium salt, Sigma, Germany) to 1 mg gelatin. Carboxymethylated gelatin was poured between partition walls of a custom-made PMMA chamber. Gelatin was allowed to form a stable gel by storing at 4 °C for 24 h, followed by removal of the partition walls. Mineralization solutions of CaCl2 and Na2HPO4 were poured into the two reservoirs separated by the gelatin gel in the middle. An electric field (15 V, 40 mA) was applied to accelerate the opposed migrations of positively charged calcium ions and negatively charged phosphate ions into and within the gelatin gel. After up to 6 d the mineralized gelatin gels were removed from the chamber and liquefied with 60 °C-heated deionized water. The released mineral was harvested by centrifugation at 10 000 g for 5 min at 40 °C. By repeating alternating washing, heating and centrifugation steps, non-mineralized gelatin was successively removed and separated mineral was collected and finally freeze-dried.

2.2. Scaffold preparation

Composite scaffolds were prepared by adding separated mineral at an amount of 20 wt% and 40 wt% to
carboxymethylated gelatin followed by mixing. In order to increase pore sizes, gelatin or composite solutions heated at 40 °C were foamed by vigorous vortexing for 10 min. Foamed solutions were poured into precooled Petri dishes up to 3 mm height followed by fast cooling at 4 °C. During this step gelatin and composites formed a stable gel which was used to cut several single samples with 6 mm diameter. These samples were frozen at a cooling rate of 0.5 K min−1 to −20 °C in a SH-221 climate chamber (Espec, Osaka, Japan) followed by freeze-drying (Christ Alpha 1–4 Lab freeze-dryer, Osterode, Germany). The resulting scaffolds were chemically cross-linked by N-(3-dimethylaminopropyl)-N’-ethycarbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Sigma) in 40% ethanol over a period of up to 48 h. Cross-linker concentrations of 100 mM EDC, 50 mM NHS, 10 mM EDC/5 mM NHS and 1 mM EDC/0.5 mM NHS were used to investigate the influence on cross-linking degree. Finally, the scaffolds were rinsed in deionized water, freeze-dried again and gamma-sterilized at 25 kGy (Gammaservice, Radeberg, Germany).

For evaluation of the cross-linking degree, the ratio of free amino groups was determined by using the trinitrobenzensulfonic acid (TNBS) method [29]. Therefore, samples were incubated for 4 h at 40 °C in 4% NaHCO₃ and 0.5% TNBS-solution. After adding 6 N HCl and heating to 60 °C, the mixture was vortexed until samples were dissolved completely. Finally, 300 μl of this solution were pipetted in microtiter plates and absorbance was measured at 405 nm using a SpectrafluorPlus microplate reader (Tecan, Germany). A calibration line was obtained from a series of a gelatin reference.

2.3. Structural analysis of the scaffolds
Slices of scaffolds were mounted on aluminum stubs and coated with carbon. An ESEM XL 30 scanning electron microscope (Philips) working at 3 kV in high-vacuum and detecting secondary electrons was used for imaging.

For light microscopy, scaffolds were fixed in 0.1 M phosphate buffer containing 2.5% glutaraldehyde (Roth, Karlsruhe, Germany) and dehydrated in graded concentration series of ethanol. Subsequently, the specimens were transferred over propylene oxide (Merck) into Araldite (Agar scientific, Essex, UK). After polymerization for 3 d at 60 °C, 1 μm slices were cut off the embedded scaffolds using an EM UC 6 microtome (Leica, Vienna, Austria) followed by staining with toluidine blue (Sigma). Light microscopy was carried out using a Nikon Mikrophot FXA and Nis-Elements D software.

2.4. Degradation experiments
Degradation of the scaffolds was carried out in 1 ml phosphate buffered saline (PBS) or simulated body fluid (SBF) for up to 56 d [30]. At defined time points, scaffolds were removed, rinsed in deionized water and freeze-dried. The weight loss of the scaffolds was determined by measuring the value of every single sample related to its initial value determined before incubation. Furthermore, each PBS supernatant was analyzed in terms of gelatin degradation by applying the Lowry protein determination method. For that, 50 μl of supernatant was mixed with 250 μl of 98 vol% 2% sodium carbonate in 0.1 N NaOH and 2 vol% 0.5% copper sulfate in 1% sodium citrate. After reaction for 20 min, 25 μl of Folin-reagent was added and incubated for additional 90 min. Absorbance was measured at 700 nm. A calibration line was obtained from a series of a gelatin reference.

For calcium determination, 10 μl degradation supernatant was mixed with 300 μl of a 1:1 mixture of AMP buffer, pH 10.7, and o-kresolphthalein complexon, 8-hydroxyquinolin, HCl and Fluitest CA-CPC detergent (Analyticon, Germany) according to the manufacturer’s instructions. After reaction for 10 min, the absorbance at 570 nm was read using a microplate reader. A calibration line was obtained from a series of a calcium reference.

2.5. Cell culture experiments
hMSCs, isolated from bone marrow aspirates were kindly provided by Professor Bornhäuser and co-workers, Medical Clinic I, Dresden University Hospital [31]. The cells were expanded in cell culture medium (Dulbecco’s modified Eagle’s medium (DMEM), low glucose, supplemented with 10% fetal calf serum (FCS), 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin in a humidified atmosphere at 37 °C and 7% CO₂. Medium and all supplements were obtained from Biochrom, Berlin, Germany.

hMCs were isolated from human buffy coats using a modified method based on the OptiPrep™ (ProGen Biotechnik, Heidelberg, Germany) density-gradient medium technique described in detail previously [23]. Briefly,uffy coats were centrifuged at 450 g for 20 min and the leukocyte-rich fraction was collected. Using an OptiPrep-Working Solution (WS), the density of the LRP was adjusted to 1.1 g ml⁻¹ and was placed under a layer of 1.078 g ml⁻¹ lymphocyte-specific gradient solution. After centrifugation at 700 g for 20 min the peripheral blood mononuclear cell (PBMC) fraction was collected, washed with PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma) and 0.5% bovine serum albumin (BSA, Sigma), followed by centrifugation at 400 g for 10 min. The PBMC fraction and WS were mixed to obtain a density of 1.1 g ml⁻¹ and was covered by layers of 1.078 g ml⁻¹ gradient solution and 1.068 g ml⁻¹ gradient solution, followed by centrifugation at 600 g for 25 min. The monocyte-enriched PBMC fraction floating in the 1.068 g ml⁻¹ layer was collected and washed with PBS/EDTA/BSA. Finally, monocytes were purified via magnetic activated cell sorting by negative
selection using a Monocyte Isolation Kit II (Miltenyi, Bergisch Gladbach, Germany).

For the co-culture experiments, sterilized scaffolds were soaked in cell culture medium supplemented with 50 μM ascorbic acid 2-phosphate for 24 h before cell seeding. hBMSC in passage 5 were seeded at a density of 2 x 10^4 per scaffold in 96-well plates and cultured in cell culture medium containing 50 μM ascorbic acid 2-phosphate (Sigma). After adhesion for 24 h, the samples were transferred to fresh 96-well plates in order to exclude cells adherent to the TCPS. Osteogenic differentiation was started at day 3 by the addition of 10 nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), and 10 nM Vitamin D₃ (Calbiochem/Merck, Darmstadt, Germany) to the medium. On day 13, cell suspension with 4 x 10^5 monocytes per well were topped on the sample in the 96-well-plates. The medium was changed to DMEM supplemented with 7.5% FCS, 7.5% human serum 24 h, the samples were transferred to fresh 96-well plates in order to exclude non-adherent monocyes as well as monocytes adherent to the TCPS. Co-cultivation was performed for additional 4 weeks, with medium changes twice weekly. For monoculture reference experiments, hBMSC were cultivated identically to the method described above but without the addition of hMc. At specific time points, the medium was removed and the scaffolds were washed with PBS and frozen at −80 °C until biochemical analysis. Scaffolds for microscopic analysis were fixed with 3.7% formaldehyde (FA) in PBS and stored at 4 °C in 0.37% FA/PBS. After 28 d of co-cultivation, scaffolds were washed with PBS and used for gene expression analysis.

2.6. Biochemical analyses
All biochemical analyses were performed with cell lysates obtained with 1% Triton X-100 (Sigma) in PBS. For all colorimetric measurements, a SpectraFluor Plus microplate reader (Tecan, Crailsheim, Germany) was used.

DNA-Assay: examination of DNA amount was carried out by using the Quant-iT™ PicoGreen® dsDNA Reagent. The fluorescence intensity was measured at 485/535 nm excitation/emission wavelengths and was correlated with the defined number of cell nuclei from calibration samples.

Lactate dehydrogenase (LDH) activity assay: cell proliferation was determined through the total activity of LDH in the cell lysates using an LDH Cytotoxicity Detection Kit (Takara, Saint-Germain-en-Laye, France). An aliquot of cell lysate was mixed with LDH substrate buffer, and the enzymatic reaction was stopped after 30 min with 0.5 M HCl. The absorbance was read at 492 nm. The LDH activity was correlated with the cell number using a calibration line of cell lysates with defined cell numbers.

Alkaline phosphatase (ALP)-activity-assay: osteoblast differentiation was evaluated by the measurement of ALP activity. Cell lysates were added to ALP substrate buffer, containing 2 mg ml⁻¹ p-nitrophényl phosphate (Sigma), 0.1 M diethanolamine, 1 mM MgCl₂ (Sigma) and 0.1% Triton X-100 (pH 9.8), and the mixture was incubated at 37 °C for 30 min. The enzymatic reaction was stopped by the addition of 0.5 M NaOH, and the absorbance was read at 405 nm. A calibration line was obtained from different concentrations of p-nitrophenol.

Tartarate resistant acid phosphatase (TRAP)-activity-assay: osteoclast differentiation was evaluated by the measurement of TRAP5b activity using naphthol-ASBI phosphate (N-ASBI-P, Sigma) as a substrate according to a slightly modified protocol of Janicka et al [32]. An aliquot of cell lysate was added to the TRAP5b reaction buffer, consisting of 2.5 mM N-ASBI-P in 100 mM Na-acetate (Sigma) buffer containing 50 mM Na-tartrate (Sigma), 2% NP-40 (Sigma) and 1% ethylene glycol monomethyl ether (Sigma) adjusted to pH 6.1, and the mixture was incubated at 37 °C for 1 h. The enzymatic reaction was stopped by adding 0.1 M NaOH and fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. Relative fluorescence units were correlated to a TRAP5b standard.

2.7. Reverse transcription polymerase chain reaction
For analysis of gene expression by reverse transcription-polymerase chain reaction (RT-PCR), cells on the scaffolds were used for RNA preparation. Total RNA isolation was performed using the peqGOLD MicroSpin Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions. Total RNA concentration and purity were determined using a Nanodrop ND 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Transcription of complementary DNA (cdNA) was carried out by preparing a 20 μl reaction mix containing 200 ng of total RNA, 200 U of SuperScript II Reverse Transcriptase (Invitrogen, Darmstadt, Germany), 0.5 mM dNTP (Invitrogen), 12.5 ng μl⁻¹ random hexamers (MWG Biotech, Ebersberg, Germany) and 40 U of the RNase inhibitor RNase OUT (Invitrogen). For cDNA synthesis, the reaction mixtures were incubated for 50 min at 42 °C followed by 15 min at 70 °C in a Thermocycler (Peqlab). PCR was performed using 2 μl of cdNA as a template and adding 18 μl of master mix containing specific primer pairs (MWG Biotech) to detect transcripts of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ALP, bone sialo-protein II (BSP II), osteocalcin (OC), receptor activator of nuclear factor κB ligand (RANKL), interleukin-6 (IL-6), TRAP, osteoclast-associated receptor
(OSCAR), calcitonin receptor (CALCR), β3-subunit of vitronectin receptor (VTNR) and cathepsin K (CTSK), respectively for each sample. The primer sequences (MWG Biotech) and annealing temperatures are summarized in table 1. After the initial activation step at 95 °C for 4 min, 25–40 PCR cycles were run with each a denaturation step at 95 °C for 45 s, an annealing step at 50–59 °C for 45 s, and a synthesis step at 72 °C for 1 min followed by a final synthesis step at 72 °C for 10 min in a Primus 25 Advanced Thermocycler (Peqlab). For all genes, the same single-stranded cDNA was used to investigate the expression. The resulting PCR products were analyzed using the FlashGel Dock and documentation system (Cambrex Bio Science, East Rutherford USA).

2.8. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (cLSM)

For SEM, scaffolds were mounted on aluminum stubs and coated with carbon in a Balzers SCD 050 coater. A Philips ESEM XL 30 SEM working in HiVac mode at 3 kV was used for imaging by detecting secondary electrons.

cLSM was applied to distinguished cell types, evaluate adhesion and differentiation of the cells on the scaffolds. Fixed cells were permeabilized with 0.2% Triton-X-100 in PBS and blocked with 1% BSA for 30 min. In order to visualize precursor cells of the osteoclast lineage a monoclonal anti-human CD68 antibody (KP1, sc-20060, Santa Cruz) in combination with an AlexaFluor 546-conjugated rabbit anti-mouse IgG (Invitrogen) as the secondary antibody was used. The cytoskeletal actin was stained with AlexaFluor 488-Phalloidin (Invitrogen). For evaluation of the differentiation of hMc into hOc, a polyclonal anti-human TRAP (K-17) goat IgG (sc-30833, Santa Cruz) was used. Alexa Fluor 488-conjugated donkey anti-goat IgG was used as secondary antibody. Cytoskeletal actin was stained with AlexaFluor 546 phallolidin. In all cases, cell nuclei of the fixed cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

CLSM was carried out using an upright Axiocam 2 FS mot microscope equipped with an LSM 510 META module (Zeiss, Jena, Germany). Excitation of AlexaFluor 488 was carried out at 488 nm (argon ion laser), the excitation of AlexaFluor 546 at 546 nm (helium–neon laser). A near-infrared femtosecond titanium:sapphire laser was used for excitation of DAPI at 750 nm (two-photon excitation) and fluorescence was recorded at 461 nm.

2.9. Statistics

All measurements were collected in at least triplicates and expressed as mean ± standard deviations. ANOVA was employed to assess significance with p values less than 0.1 (*, significant), 0.01 (**, very significant), and 0.001 (***, highly significant).

3. Results

3.1. Scaffold preparation

The ormoHAP was embedded as a functional component in gelatin and the obtained composite was processed to a scaffold. Figure 1(a) shows a number of individual scaffolds used for cell culture experiments, obtained by cutting from a larger volume. In all variants, 10% carboxymethylated gelatin was used and various amounts of ormoHAP were added. Figure 1(d) shows a SEM image of the ormoHAP spheres separated from the gelatin gel after double migration. The spheres have a very uniform shape at diameters of about 100–500 nm [4].

Light microscopy and SEM were used in order to evaluate the microstructure as well as the nanostructure of the porous scaffolds. It becomes obvious from SEM that the applied foaming process is suitable to switch the scaffold structure from individually closed pores to interconnecting porosity (figures 1(b), (c)). This was observed for all compositions—indeed from addition of ormoHAP—with pore sizes varying in the range of 100–200 μm. Light microscopy images of semithin section of gelatin scaffolds without and with ormoHAP confirm the aforementioned pore sizes and the interconnectivity (figures 1(e), (f)). Moreover, scaffold wall thickening is

| Gene   | Forward primer (5′–3′)                      | Reverse primer (5′–3′)                      | Tm (°C) |
|--------|--------------------------------------------|--------------------------------------------|---------|
| GAPDH  | GGTGAGAAGCTGAGGATCAGAGG                    | GGTGATGAGCTTCCAGGAT                      | 55      |
| ALP    | CACGGGACACATGAAAGAA                       | ATTCCTGCGTGCAAC                         | 55      |
| BSP II | ATGAGCAGGACAGGACACAGG                      | ATCCATAGGACAGTCTTC                       | 55      |
| OC     | CAAGTGGCAACAGCTCAGC                       | TCACAGAGCCGTTAGGCTTA                     | 55      |
| RANKL  | CAGAGTCTGCTCAACATGACT                     | TACACCATTAGGATGACT                      | 50      |
| IL-6   | CTGCCTGTGGTGTGCT                        | CCTGACCTGGTAGGCTT                      | 57      |
| TRAP   | TTCACGCGCTGCACTCCAA                      | AGCTGATCACATGGCAA                      | 57      |
| OSCAR  | GATGACGCTAAGGAAGCAGGACG                  | CAGAGCTGATGGTGCTCACTTTTA               | 59      |
| CALCR  | GCAATGCTTCTACCTGTCAGAAC                  | CAGTAAACACAGGACAGGACGAGTAG              | 59      |
| VTNR β3| TGCCCTAACATTGGAGCTACCTCCT                | AGACACATGAGCACCAGAGGAGCCT              | 59      |
| CTSK   | GATACTGGACACCCACTGGA                      | CATTCTCAGACACAACTCACC                  | 57      |
identified to be a result of mineral embedding in the gelatin matrix.

The influence of the mineral phase onto the micro- and nanostructure was revealed at higher magnification (figure 2). Pure gelatin scaffolds show smooth surfaces of the pore walls. In contrast, the addition of ormoHAP leads to observable surface patterning of the scaffold. The ormoHAP particles present in the gelatin matrix are clearly visible. Remarkably, ormoHAP is homogeneously distributed to the greatest extend, whereas commercial HAP forms large agglomerates.

3.2. Influence of cross-linking on degradation
Chemical cross-linking of gelatin determines the stability as well as the degradation behavior of scaffolds in liquid media. The degree of cross-linking is represented by the amount of remaining free amino groups. The influence of the reaction time and cross-linker concentration on the degree of cross-linking was investigated. Detection of the amount of free amino groups indicates that cross-linker concentrations of 100 mM EDC, 50 mM NHS result in a cross-linking degree of 60% already after one hour reaction time, which is only slightly increased after 48 h (figure 3(a)). The influence of the cross-linker concentration is shown in figure 3(b). The results show...
approximate halving or quartering of the cross-linking degree when reducing the EDC/NHS concentration by factors of 10 and 100, respectively. In all cases, the increase of cross-linking degree between 2 and 24 h reaction time is marginal.

Hydrolytic degradation in PBS was investigated to examine differences between varying cross-linking degrees as well as differences between varying ormo-HAP concentrations each compared with pure gelatin scaffolds. In figure 4(a), mass loss is shown which was determined by measuring the value of individual samples related to their initial value before degradation. Scaffolds with low cross-linking degree (10 mM EDC, 5 mM NHS) degraded much faster (e.g. 60%–70% after two month) compared to scaffolds with high cross-linking degree (100 mM EDC, 50 mM NHS). Protein concentrations in the supernatant determined by Lowry method confirmed the results of the weight determination (figure 4(b)). More protein release was observed for low cross-linking degrees. In all cases, protein release increased with increasing mineral content of the scaffolds and, furthermore continuously with time during the experiment. At low cross-linking degrees, the influence of the mineral content on the protein release is remarkably higher than at high cross-linking degrees. The rate increases with increasing mineral content and with reducing cross-linking degree. Figure 4(c) shows the calcium ion concentrations in the supernatants determined for each time point. The results are in accordance with the degradation indicators mass loss and protein release and show higher calcium release at lower cross-linking degree. Obviously, the calcium was initially released, followed by a precipitation period and repeated release at the later time points of the experiment.

Comparative evaluation of bioactivity and degradation is visualized in figure 4(d). Low or high cross-linked gelatin scaffolds without mineral are not bioactive in the early stages of the experiment. In contrast, scaffolds with 20% ormoHAP are most bioactive on day 56 demonstrated by the calcium binding levels. The time course of the 40% ormoHAP samples also confirms a high bioactivity in the early stages of the experiment. However, this effect is increasingly compensated and finally overcompensated by the release of calcium from the scaffolds because of degradation, which is more distinct the lower the cross-linking degree of the gelatin is.

3.3. Co-culture
Two different ormoHAP amounts and pure gelatin scaffolds were compared in co-culture experiments. The timeline used for the presentation of the results represents the cultivation time of both cell lineages. From d14/d1 to d42/d28, both cell types were cultivated on the scaffolds, finally involving 42 d old hBMSC/hOB (human bone marrow stromal cell-derived human osteoblasts) and 28 d old hMc/hOC (human monocyte-derived human osteoclasts), with the last period representing the age of the co-culture. As a reference, hBMSC/hOB were cultivated as a monoculture on the scaffolds under similar conditions applying the same timescale.

The proliferation of the cells in monoculture as well as in co-culture was calculated from the results of DNA analysis. This method was preferred since the DNA amount per nucleus is similar for both cell types. This allows the evaluation of the total cell number for the co-cultures. Since several hMcs differentiate into multinucleated hOcs, the results were presented as number of cell nuclei instead of cell numbers. In figure 5(a) the cell nuclei numbers for the co-culture on the different types of scaffolds are shown. Series of experiments with monoculture hBMSC served as a reference. The results of day 1 are in line with the seeded cell number of $2 \times 10^4$ hBMSC. From day 3 to day 13, hBMSC were osteogenically induced resulting in a reduced proliferation rate. At the later time points and cultivation without osteogenic supplements in day 56 demonstrated by the calcium binding levels. The time course of the 40% ormoHAP samples also confirms a high bioactivity in the early stages of the experiment. However, this effect is increasingly compensated and finally overcompensated by the release of calcium from the scaffolds because of degradation, which is more distinct the lower the cross-linking degree of the gelatin is.

3.3. Co-culture
Two different ormoHAP amounts and pure gelatin scaffolds were compared in co-culture experiments. The timeline used for the presentation of the results represents the cultivation time of both cell lineages. From d14/d1 to d42/d28, both cell types were cultivated on the scaffolds, finally involving 42 d old hBMSC/hOB (human bone marrow stromal cell-derived human osteoblasts) and 28 d old hMc/hOC (human monocyte-derived human osteoclasts), with the last period representing the age of the co-culture. As a reference, hBMSC/hOB were cultivated as a monoculture on the scaffolds under similar conditions applying the same timescale.

The proliferation of the cells in monoculture as well as in co-culture was calculated from the results of DNA analysis. This method was preferred since the DNA amount per nucleus is similar for both cell types. This allows the evaluation of the total cell number for the co-cultures. Since several hMcs differentiate into multinucleated hOcs, the results were presented as number of cell nuclei instead of cell numbers. In figure 5(a) the cell nuclei numbers for the co-culture on the different types of scaffolds are shown. Series of experiments with monoculture hBMSC served as a reference. The results of day 1 are in line with the seeded cell number of $2 \times 10^4$ hBMSC. From day 3 to day 13, hBMSC were osteogenically induced resulting in a reduced proliferation rate. At the later time points and cultivation without osteogenic supplements in day 56 demonstrated by the calcium binding levels. The time course of the 40% ormoHAP samples also confirms a high bioactivity in the early stages of the experiment. However, this effect is increasingly compensated and finally overcompensated by the release of calcium from the scaffolds because of degradation, which is more distinct the lower the cross-linking degree of the gelatin is.
− medium, the measured cell number increased significantly up to the double of the seeded cell number. No differences were recognized between the different scaffold types. On day 13, hMc were added to the hBMSC-seeded scaffolds, what is reflected by the increased DNA values measured for the co-cultures on day 14. Approximately 25% of the seeded monocytes were recovered after 24 h cultivation on the scaffolds. Again, no differences were detected between the different types of scaffolds. From d28/d14 to d42/d28 of co-culture, the DNA amount decreased slightly at all scaffolds. In addition, LDH activity was measured in order to reveal the proliferation of hBMSC/hOB in co-culture, which was possible because of the negligibly low LDH activity of hMc compared to the LDH activity of the hBMSC [23]. In figure 5(b) the calculated number of cells is shown. For the hBMSC monoculture reference the same temporal course was detected as with DNA analysis. The LDH activities measured for the co-culture hardly differ from the hBMSC monoculture, i.e. the proliferation of hBMSC/hOB remains unaffected by the presence of the hMc/hOC.

The differentiation behavior of hBMSC in monoculture as well as in co-culture was characterized by means of the regulation of the ALP activity, which increases from day 1 to day 14 as a result of the osteogenic differentiation (figure 5(c)). After day 14, the ALP activity decreases because of increased cell maturation as well as the cultivation with (−) medium. This was observed for the monoculture of hBMSC/hOB as well as for the co-culture. The influence of the ormoHAP on the osteogenic differentiation becomes obvious on day 28. In monoculture the ALP activity decreases, because of increased cell maturation as well as the cultivation with (−) medium from day 14. A higher amount of ormoHAP results in a higher decrease of ALP activity. In contrast, in co-culture the relative ALP activity is twice as high for the cultivation on scaffolds with 40% ormoHAP compared to gelatin scaffolds without ormoHAP. The differentiation of the hMc to hOC in co-culture was characterized by means of the regulation of the
TRAP5b activity (figure 5(d)). As expected, at all time points no TRAP5b activity was detected for the monoculture of hBMSC/hOB—except for the basic level typical for the analysis method. In co-culture, hMc started with low TRAP5b level after day 1 of cultivation. Osteoclastic differentiation induced by hOB, results in significantly increased TRAP5b activity from d28/d14 of co-culture. The influence of the scaffold composition is remarkable, since with increasing ormoHAP amount a higher enzyme activity is detected. At d42/d28 of the co-culture, the scaffolds were examined by means of cLSM (figure 6). The green fluorescent actin skeleton and the blue visualized cell nuclei show both hBMSC/hOB and hMc/hOC. The hBMSC/hOB show the typical spreading resulting in a thick cell layer on all types of scaffolds. The red visualized monocytic surface marker CD68 allows better distinction because it is not available in hBMSC/hOB. On all scaffold types CD68 positive cells were detected with portions apparently decreasing with increasing mineral amount. The morphology of the CD68 positive cells was found to vary from spindle-shaped to spherical. TRAP was marked green on the right side of figures 6(b), (d), (f) in order to visualize osteoclastic differentiation. On samples without ormoHAP, TRAP was found in regular particular distribution. With increasing ormoHAP amount of the scaffolds, TRAP was found to be concentrated in individual cells, highlighted in an ortho-representation of these cells (figure 7).

In order to characterize the phenotypes of the differentiated hBMSC as well as the hMc in more detail, gene expression was analyzed by performing RT-PCR after d42/d28 of co-cultivation (figure 8). Markers assigned to hBMSC/hOB were ALP, BSP II, OC; markers assigned to hMc/hOC were TRAP, OSCAR, CALCR, VTNR and CTSK. The cytokines RANKL and IL-6 were examined, since they regulate osteoclastogenesis originated from hBMSC/hOB. Expression of
GAPDH was determined to verify the usage of similar amounts of RNA for RT-PCR (see table 1). Gene expression of the osteoblastic marker BSP II and the cytokine IL-6 were identified to increase with increasing ormoHAP amount of the scaffolds. In contrast, the gene expression levels of ALP, OC, and RANKL were similar for all scaffold types. Moreover, the markers characterizing osteoclastic differentiation TRAP, VTNR and CTSK were detected at all scaffold types. Gene expressions of the osteoclast marker OSCAR and CALCR II were highest on scaffolds with 40% ormoHAP.

4. Discussion

In this study, the effects of ormoHAP-gelatin scaffolds on cells of the bone remodeling system were investigated in vitro by means of an osteoblast/osteoclast co-

Figure 6. 3D reconstructions from cLSM image stacks at d42/d28 of hOB/hOC co-cultivation on gelatin scaffolds without (a), (b), with 20% (c), (d) and with 40% ormoHAP (e), (f). In the left column, actin (green), cell nuclei (blue) and CD68 (red) are visible; in the right column, actin (red), cell nuclei (blue) and TRAP (green) are visible.
culture. Scaffold properties that have been studied comprise pore sizes, mineral distribution, gelatin cross-linking, and degradation. Thereby, the ability of these scaffolds to actively influence the interaction between bone forming and bone resorbing cells, the so-called cross-talk, was evaluated.

Considering the individual pore sizes, foaming had a significant impact, also proven by reduced apparent density of the scaffold. The size of the individual pores was maximized by slow freezing with final diameters of 150–200 μm which is considered to be ideal for cell ingrowth [33, 34]. Structure and interconnectivity—which finally are similar to common tissue engineering collagen/mineral-scaffolds—support this parameter [35, 36]. Geometric cues are important for the direction of cell differentiation [37].

With regard to bone substitution materials, Bohner et al discuss the size and shape of pores to be the most important parameter of a scaffold’s architecture [38]. This is most obvious for cell behavior, e.g. adhesion, spreading, migration. In addition to the geometric cues, there are also chemical ones that are often difficult to separate from the former in experiments [39].

The mineralization method applied in the present study seems to be a smart way of chemical modification to support osteoblastogenesis and osteoclastogenesis by simultaneously retaining a beneficial pore structure. Many conventional mineralization methods, e.g. plasma processes, cannot be applied for composites containing organic substances since high
process temperatures are harmful or destroying [40]. A principal aim of the present study is the homogeneous embedding of mineral in gelatin at ambient conditions. The mineral did not influence pore formation, however, it was distributed differently in the organic matrix. Conventional HAP-powder tends to agglomerate and was irregularly distributed as agglomerates of different sizes, which is a common challenge described in literature [41]. Especially the presence of mineral phases with micrometer size in collagen scaffolds is discussed to be detrimental regarding resorbability and strength of the material [3]. In contrast, ormoHAP was much more evenly distributed as fine particles, which is considered favorable regarding mechanical properties and reproducibility of results in general.

In addition to the composition of the scaffolds, the degree of cross-linking is also one of chemical cues by determining how much of the scaffold material is released and in what form (biologically active or inactive) it is released. The biochemical investigations in the present study confirmed the influence of scaffold composition and cross-linking degree on bioactivity and degradation behavior. Some studies report on apatite precipitation on non-mineralized gelatin in SBF, where carboxy groups act as nucleation center [42, 43]. The low bioactivity of scaffolds without ormoHAP during 8 weeks of the present study can be a result of the cross-linking reaction which inactivates these nucleation points. Similar results were reported previously for collagen [44]. In contrast, the composite scaffolds with embedded ormoHAP exhibited high bioactivity. This can be attributed to the interaction of organic and inorganic phases in these scaffolds—for example the intermediate release of embedded ormoHAP particles out of the organic matrix and direct precipitation on the same matrix. These areas in turn act as nucleation centers for consecutive mineralization processes. Therefore, the overall bioactivity of the composite scaffold can be adjusted by the amount of ormoHAP embedded. Degradation behavior is predominantly defined by the cross-linking degree of the gelatin, which was also observed previously [45]. The cross-linking degree does not influence the bioactivity of the scaffold—indeed from the mineral concentration. This confirms the independence of the processes apatite precipitation, calcium release, and gelatin degradation.

The main purpose of the cell investigations in this study is to investigate the influence of scaffold geometry on the interaction of bone-forming and resorbing cells with each other and with the scaffold. This builds a bridge to the production conditions, which ultimately have a considerable influence on the geometry and thus the cell behavior. Some authors have already investigated and described geometric triggering stimuli, so-called geometric cues [37, 39]. In previous studies a human co-culture model was established in order to investigate the influence of biomaterials on interaction of bone-building and bone-resorbing cells of the remodeling process [23]. The same setup was used for assessment of the gelatin/ormoHAP scaffolds and is much more similar to the in vivo situation than other studies in this field, e.g.: Jones et al, which used a murine co-culture with an osteoblast cell line and primary monocytes on silk fibroin and PLLA films [25]; co-culture of hBMSC and human hMc from buffy coat on mineralized collagen [46, 47]. However, in these examples M-CSF and RANKL were added as cell differentiation inducing supplement, which is in contrast to the present study which is based solely on natural cell signaling. The focus of the present study was on the transfer of the cell culture model to the biomaterial environment and the investigation of the mineral concentration in the scaffolds on cell behavior and interaction. The first point was addressed by prolonging the overall cultivation time to 28 d co-culture including 42 d of hBMSC cultivation. A hBMSC/hOB monoculture was used as a reference. During the cultivation period, results suggest that the scaffold type seems not to affect adhesion and proliferation of hBMSC. The decreased DNA value in co-culture from d14/d1 to d42/d28 could be related to cell death of non-differentiated hMc. Detected cell numbers for hBMSC are similar with both methods. HMc/hOC do not contribute to LDH activity detected, and the presence of hMc/hOC does not affect the proliferation of hBMSC/hOB compared to mono-culture. In all setups, the addition of osteogenic supplements lead to osteoblast differentiation confirmed by the typical increase of ALP activity up to day 14 [48]. The following decrease is also typical, but could be amplified by the waiver of addition, previously discussed. Moreover, hBMSC/hOB exprimize osteoblastic marker genes ALP, BSPII, osteocalcin, and RANKL. The last one is essential for the osteoclastogenesis of hMc. During cultivation on the scaffolds, hBMSC/hOB alone induced osteoclastogenesis, confirmed by increased TRAP5b activity. This level decreased at the end of the experiment, which is related to the cyclic course of enzyme activity and limited lifetime of osteoclasts. Moreover, the highly specific marker genes TRAP, OSCAR, and CALCR were detected in all cases.

SEM revealed dense cell layers on all samples, making it difficult to differentiate cell type by morphological characteristics, and was therefore not used for further evaluation of co-culture samples. On the other hand, cLSM confirmed the regular dense hBMSC/hOB cell layer with individual CD68-positiv cells embedded. This marker is predominantly found on monocytes and reduces during osteoclastic differentiation [49]. Caused to the complex 3D complexity of the environment, it was difficult to identify large multinuclear osteoclasts by cLSM. The omnipresent TRAP activity suggest the presence of mononuclear osteoclasts which are completely functional in terms of bone resorption [50, 51].
A remarkable relation between scaffold composition and cell behavior becomes obvious by comparison of ALP and TRAP5b activity as well as BSP II-, OSCAR- and CALCR gene expression: especially on d28/d14 in co-culture with high scaffold ormoHAP concentration an increased ALP activity was determined. Since this effect was not observed in monoculture, it seems to be related to stimulating hMC/ hOC interaction. This is supported by recognition of the highest TRAP5b values at the same time point—also on scaffolds with 40% ormoHAP. Moreover, gene expression of OSCAR and CALCR increases with increasing scaffold mineral content. In turn, increased TRAP5b activity is attributed to increased numbers of active osteoclasts. These cells seem to stimulate osteoclastogenesis of hBMSC, which is confirmed by increased ALP activity and increased BSPII expression. These results suggest an additional feedback of the hBMSC/hOB on osteoclastogenesis. In addition to M-CSF and RANKL, hBMSC/hOB release cytokines like IL-6, IL-11, and LIF, which all can influence osteoclastogenesis independently from RANKL [52, 53]. In previous studies, this was presented by the authors for the present co-culture model [25]. In the present study, RT-PCR analysis revealed increasing IL-6 gene expression with increased ormoHAP content in the scaffolds, which previously was reported by Ninomiya et al for the influence of HAP particles on IL-6 expression in fibroblasts [54]. Moreover, the stimulating influence of HAP on IL-6 expression in monocytes was reported [55]. From all the experiments presented here, the following sequence of events becomes obvious: ormoHAP in the gelatin scaffolds stimulates IL-6 expression in hBMSC/hOB as well as in hMC, which in turn stimulates differentiation of hMC into hOC. That again results in increased TRAP5b activity, which ends up with increased ALP activity and BSPII expression by hBMSC/hOB.

5. Conclusion

The present study confirmed ormoHAP nanospheres to be suitable for embedding in gelatin resulting in a composite scaffold. From the point of view of materials science, advantages over conventional HAP obviously include particle size distribution and homogeneity of the inorganic component—demonstrated after applying a foaming process to obtain optimal pore sizes. Degradation is not influenced by ormoHAP, but by the cross-linking degree adjusted by the concentration of chemicals used.

In vitro experiments confirmed the development of a vital osteoblast/osteoclast co-culture, where osteogenically differentiated hBMSC were able to induce osteoclastogenesis of monocytes without external addition of RANKL and M-CSF. Vice versa osteoclasts stimulate BSP II gene expression of osteoblasts. Remarkably, increasing concentration of ormoHAP in the scaffolds stimulated the crosstalk of osteoblasts and osteoclasts as well as their precursors by pushing gene expression of IL-6, OSCAR, CALCR, and TRAP5b activity as well as ALP activity and BSPII gene expression.

Acknowledgments

We gratefully acknowledge the Deutsche Forschungsgemeinschaft Grants HA5284/2-1 and TRR79-TP-M03 for partial financial support.

ORCID iDs

Christiane Heinemann https://orcid.org/0000-0002-1343-4938
Sina Rößler https://orcid.org/0000-0002-3506-0455

References

[1] Bohner M, Galea L and Doebelin N 2012 Calcium phosphate bone graft substitutes: failures and hopes J. Eur. Ceram. Soc. 32 2663–71
[2] Barradas A M, Yuan H, van Blitterswijk C A and Habibovic P 2011 Osteoinductive biomaterials: current knowledge of properties, experimental models and biological mechanisms Eur. Cells Mater. 21 407–29 discussion 429
[3] Cuniffie G M, Dickson G R, Partap S, Stanton K T and O’Brien F J 2010 Development and characterisation of a collagen nano-hydroxyapatite composite scaffold for bone tissue engineering J. Mater. Sci., Mater. Med. 21 2293–8
[4] Heinemann C, Heinemann S, Krupke B, Woroch H, Thomas J, Wiesmann H P and Hanke T 2016 Electric field-assisted formation of organically modified hydroxyapatite (ormoHAP) spheres in carboxymethylated gelatin gels Acta Biomater. 44 135–43
[5] Krupke B, Heinemann C, Keroue A, Thomas J, Rößler S, Wiesmann H-P, Gemming T, Woroch H and Hanke T 2017 Calcite and hydroxyapatite gelatin composites as bone substitution material made by the double migration technique Cyst. Growth Des. 17 738–45
[6] Van Vlierberge S, Dubriel P and Schacht E 2011 Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review Biomacromolecules. 12 1387–408
[7] Heinemann S, Gelinsky M, Woroch H and Hanke T 2011 Resorbierbare knochenersatzmaterialien Der Orthopäde. 40 761–73
[8] Gelinsky M, Welzel P B, Simon P, Bernhardt A and Koenig U L 2008 Porous three-dimensional scaffolds made of mineralized collagen: preparation and properties of a biomimetic nanocomposite material for tissue engineering of bone Chem. Eng. J. 137 84–96
[9] Zhang S, Huang Y, Yang X, Mei F, Ma Q, Chen G, Ryu S and Deng X 2009 Gelatin nanofibrous membrane fabricated by electrospinning of aqueous gelatin solution for guided tissue regeneration J. Biomed. Mater. Res. A 90 671–9
[10] Rathna G V 2008 Gelatin hydrogels: enhanced biocompatibility, drug release and cell viability J. Mater. Sci., Mater. Med. 19 2351–8
[11] Kuttappan S, Mathew D and Nair M B 2016 Biomimetic composite scaffolds containing bioceramics and collagen/gelatin for bone tissue engineering—a mini review Int. J. Biol. Macromol. 93 1390–401
[12] Su K and Wang C 2015 Recent advances in the use of gelatin in biomedical research Biotechnol. Lett. 37 2139–45
Biomed. Mater. 14 (2019) 035015

[13] Yang C, Frei H, Rossi F M and Burt H M 2009 The differential in vitro and in vivo responses of bone marrow stromal cells on novel porous gelatin-alkinate scaffolds J. Tissue Eng. Regen. Med. 3 601–14

[14] Zhao F, Grayson W L, Ma T, Bunnell B and Lu W W 2006 Effects of hydroxyapatite in 3D chitosan-gelatin polymer network on human mesenchymal stem cell construct development Biomaterials 27 1859–67

[15] Takahashi Y, Yamamoto M and Tabata Y 2005 Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and beta-tricalcium phosphate Biomaterials 26 3587–96

[16] Rohanizadeh R, Swain M V and Mason R S L B-R 2006 Gelatin sponges as a scaffold for osteoblasts J. Mater. Sci., Mater. Med. 17 1173–82

[17] Zandi M, Mirzadeh H, Mayer C, Urch H, Eslaminejad M B, Baghiri F and Mivechi H 2009 Bio-compatibility evaluation of nano-rod hydroxyapatite/gelatin coated with nano-HAp as a novel scaffold using mesenchymal stem cells J. Biomed. Mater. Res. A 92 1244–55

[18] Kim H W, Kim H E and Salih V 2005 Stimulation of osteoblast responses to biomimetic nanocomposites of gelatin–hydroxyapatite for tissue engineering scaffolds Biomaterials 26 5223–30

[19] Bernhardt A, Dittrich R, Lode A, Despang F and Gelinsky M 2013 Nanocrystalline spherical hydroxyapatite granules for bone repair: in vitro evaluation with osteoblast-like cells and osteoclasts J. Mater. Sci., Mater. Med. 24 1755–66

[20] Muscalo M 2015 Hydroxyapatite (HAp) for biomedical applications (Amsterdam: Elsevier)

[21] Kirkpatrick C J 2014 Developing cellular systems in vitro to simulate regeneration Tissue Eng. A 20 1355–7

[22] Kirkpatrick C J, Fuchs S and Unger R E 2011 Co-culture systems for vascularization—learning from nature Adv. Drug. Deliv. Rev. 63 291–9

[23] Heinemann C, Heinemann S, Worch H and Hanke T 2011 Development of an osteoblast/osteoclast co-culture derived by human bone marrow stromal cells and human monocytes for biomaterials testing Eur. Cells Mater. 21 80–93

[24] Battiston K G, Cheung J W C, Jain D and Santerre J P 2014 Biomaterials in co-culture systems: towards optimizing tissue integration and cell signaling within scaffolds Biomaterials 35 4465–76

[25] Jones G L, Motta A, Marshall M J, El Haj A J and Cartmell S H 2009 Osteoblast: osteoclast co-cultures on silk fibroin, chitosan and PLLA films Biomaterials 30 5376–84

[26] Heinemann S, Heinemann C, Wenschis S, Alt V, Worsch H and Hanke T 2013 Calcium phosphate phases integrated in silica/collagen nanocomposite xerogels enhance the bioactivity and ultimately manipulate the osteoblast/osteoclast ratio in a human co-culture model Acta Biomater. 9 4878–88

[27] Halai M, Ker A, Meek R M D, Nadeem D, Sjostrom T, Su B, McNamara L E, Dalby M J and Young P S 2014 Scanning electron microsopic observation of an osteoblast/osteoclast co-culture on micropatterned orthopaedic ceramics J. Tissue Eng. 5 1–10

[28] Young P S, Tsimbouri P M, Gadegaard N, Meek R M D and Dalby M J 2015 Osteoclastogenesis/osteoblastogenesis using human bone marrow–derived cocultures on nanotopographical polymer surfaces Nanomedicine. 10 949–57

[29] Kakade M L and Liener I E L B-K 1969 Determination of available lysine in proteins Anal. Biochem. 27 273–80

[30] D’Orsi A, Kim H M, Furuya T, Kokubo T, Miyazaki T and Nakamura T I B-O 2003 Preparation and assessment of revised simulated body fluids Period. Inc. 65 188–95

[31] Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M and Werner C 2004 Mesenchymal stem cells can be differentiated into endothelial cells in vitro Stem Cells 22 377–84

[32] Jancikl A J, Takahashi K, Sun S Z and Yam L T 2001 Naphthol-ASB1 phosphate as a preferred substrate for tartrate-resistant acid phosphatase isomorphism 5b J. Bone Miner. Res. 16 788–93

[33] Murphy C M and O’Brien F J 2010 Understanding the effect of mean pore size on cell activity in collagen-glycosaminoglycan scaffolds Cell Adhes. Migr. 4 377–81

[34] Loh Q I and Choong C 2013 Three-dimensional scaffolds for tissue engineering applications: role of porosity and pore size Tissue Eng. B 19 485–502

[35] Karagorgiou V and Kaplan D 2005 Porosity of 3D biomaterial scaffolds and osteogenesis Biomaterials 26 5474–91

[36] Bernhardt A, Despang F, Lode A, Demmler A, Hanke T and Gelinsky M 2009 Proliferation and osteogenic differentiation of human bone marrow stromal cells on alginate–gelatine–hydroxyapatite scaffolds with anisotropic pore structure J. Tissue Eng. Regen. Med. 3 54–62

[37] Kilián K A, Bugarija B, Lahn B T and Meischke M 2010 Geometric cues for directing the differentiation of mesenchymal stem cells Proc. Natl Acad. Sci. 107 4872–7

[38] Bohner M, Loosli Y, Baroud G and Lacroix D 2011 Commentary: deciphering the link between architecture and biological response of a bone graft substitute Acta Biomater. 7 478–84

[39] Tan K Y, Lin H, Ramstedt M, Watt F M, Huck W T S and Gautrot J E 2013 Decoupling of biomimetic and chemical cues directing epidermal stem cell fate on polymer brush–based cell micro-patterns Integ. Biol. 5 899–910

[40] Lickorish D, Ramsaw J A, Werkmeister A J, Glattauer V and Howlett C R 2004 Collagen-hydroxyapatite composite prepared by biomimetic process J. Biomed. Mater. Res. A 68 19–27

[41] Supova M 2009 Problem of hydroxyapatite dispersion in polymer matrices: a review J. Mater. Sci., Mater. Med. 20 1201–13

[42] Bigi A, Boonini E, Panzavolta S, Roveri N and Rabin R 2001 Bonelike apatite growth on hydroxyapatite–gelatin sponges from simulated body fluid, J. Biomed. Mater. Res. 59 709–15

[43] Rhee S H, Lee J D and Tanaka J 2000 Nucleation of hydroxyapatite crystal through chemical interaction with collagen J. Arth. Res., Soc. 83 2890–2

[44] Li X and Chang J 2006 Preparation and characterization of bioactive collagen/wollastonite composite scaffolds J. Mater. Sci., Mater. Med. 16 561–5

[45] Lai J Y 2011 Evaluation of cross-linking time for porous gelatin hydrogels on cell sheet delivery performance J. Mech. Med. Biol. 11 967–81

[46] Domachick H, Gelinsky M, Burmeister B, Fleig R, Hanke T, Reinstorff A, Pompe W and Rosen-Wolff A 2006 In vitro ossification and remodeling of mineralized collagen I scaffolds J. Mater. Sci., Mater. Med. 17 485–98

[47] Bernhardt A, Thieme S, Domachick H, Springer A, Rosen-Wolff A and Gelinsky M 2010 Crosslink of osteoblast and osteoclast precursors on mineralized collagen–towards an in vitro model for bone remodelling J. Biomed. Mater. Res. A 95 848–56

[48] Robins S P I B-R 1997 Biochemical markers in bone turnover Methods Bone Biology ed T R Arnett and B Henderson (Boston, MA: Springer) pp 231–50

[49] Toyosaki-Maeda T, Takano H, Tomita T, Tsuruta Y, Maeda-Tanimura M, Shimaoka Y, Takahashi T, Itoh T, Suzuki R and Ochi T 2001 Differentiation of monocyes into multinucleated giant bone-resorbing cells: two-step differentiation induced by nurse-like cells and cytokines Arthritis Res. 3 306–10

[50] Hattenley G and Chambers T J 1989 Calcitonin receptors as markers for osteoclastic differentiation: correlation between generation of bone-resorptive cells and cells that express calcitonin receptors in mouse bone marrow cultures Endocrinology. 125 1606–12

[51] Takeshita S, Kaji K and Kudo A 2000 Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts J. Bone Miner. Res. 15 1477–88
[52] Kim D H et al 2005 Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell Cytokine 31 119–26
[53] Kudo O, Sabokbar A, Pocock A, Itonaga I, Fujikawa Y and Athanasou N A 2003 Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism Bone 32 1–7
[54] Ninomiya J T, Struve J A, Stelloh C T, Toth J M and Crosby K E 2001 Effects of hydroxyapatite particulate debris on the production of cytokines and proteases in human fibroblasts J. Orthop. Res. 19 621–8
[55] Laquerriere P, Grandjean-Laquerriere A, Jallot E, Balossier G, Frayssinet P and Guenounou M 2003 Importance of hydroxyapatite particles characteristics on cytokines production by human monocytes in vitro Biomaterials 24 2739–47