Bioinspired calcium phosphate mineralization on Net-Shape-Nonwoven chitosan scaffolds stimulates human bone marrow stromal cell differentiation

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Abstract
Chitosan fibers were processed using the Net-Shape-Nonwoven (NSN) technique in order to create porous scaffolds which were functionalized in two bioinspired ways: collagen type I coating and unique mineralization with organically modified hydroxyapatite (ormoHAP). While collagen is common to enhance cell attachment on surfaces, the electric-field assisted migration and deposition of ormoHAP on the surface of the NSN-scaffolds is a novel technique which enables sub-micrometer sized mineralization while maintaining the original pore structure. Microscopy revealed fast attachment and morphological adaptation of the cells on both, the pure and the functionalized NSN-scaffolds. Remarkably, the cell number of osteogenically induced hBMSC on ormoHAP-modified NSN-scaffolds increased 3.5–5 fold compared to pure NSN-scaffolds. Osteogenic differentiation of hBMSC/osteoblasts was highest on collagen-functionalized NSN-scaffolds. RT-PCR studies revealed gene expression of ALP, BSP II, and osteocalcin to be high for all NSN-scaffolds. Overall, the NSN-scaffold functionalization with collagen and ormoHAP improved attachment, proliferation, and differentiation of hBMSC and therefore revealed the remarkable potential of their application for the tissue engineering of bone.

1. Introduction
Bone tissue engineering is a remarkably fast developing research field, dominantly driven by the introduction of new manufacturing technologies and the use of different materials as well as their modifications. The present work combines several aspects of the mentioned points, by processing of chitosan short fibers to scaffolds with defined pore structure using the Net-Shape-Nonwoven (NSN) technology [1]. In order to mimic the organic and inorganic components of natural bone, the surface of the NSN-scaffolds are functionalized either with collagen or with organically modified hydroxyapatite (ormoHAP). Chitosan is soluble in dilute acids and can be processed by various techniques to form beads, films, gels, and sponge-like scaffolds [2]. Its capability to form fibers, obtained from electrospinning or wet spinning process, and processing into textile scaffolds makes it a promising and innovative biomaterial for tissue engineering applications [3, 4]. In this field the advantageous properties of chitosan like biocompatibility, biodegradability, and osteoconductivity are outstanding [5–8].

The NSN technology enables a layer-by-layer bonding of short fiber layers to form three-dimensional structures with adjustable pore size distributions. Thus, the production of individual and complex scaffolds containing material and structural grading,
anisotropic areas and cavities becomes feasible [1]. Because of their high porosity and interconnected pores, NSN scaffolds have essential advantages over foamed scaffolds, which enhance the positive interaction with cells and ensure the ingrowth of cells or blood vessels.

Cell behavior is affected by the chemical composition of the substrate, which raises significant interest in the design of bioinspired materials. In this context, coating tissue engineering scaffolds with collagen is a common procedure to enhance biocompatibility [9, 10]. This was confirmed by several studies in which different types of chitosan were modified by collagen: Wet spun chitosan-collagen fibers [11], electrosprun collagen-chitosan nanofiber [12] or collagen coating on fibrous scaffolds [13]. In vitro studies using hBMSC on collagen-functionalized fiber scaffolds revealed fast cell attachment, considerable increase of the cell number, and expression of the osteogenic phenotype [14].

The main inorganic component of bone is calcium phosphate—more specifically hydroxyapatite (HAP). The modification of chitosan with this mineral is an additional route to improve osteoconductivity and consequently to enhance the adhesion, proliferation, and differentiation of mesenchymal stem cells cultured on chitosan scaffolds [15]. Conventional methods to combine both functionalizing components are based on the addition of HAP to chitosan by blending [16–19] or precipitation [20, 21]. However, blending often yields in inhomogeneous distribution of HAP due to particle aggregation—especially for sub-micrometer particles—in the chitosan polymer solution [16–19]. On the other hand, precipitation is widely used because it leads to more homogeneous distribution of the particles in the chitosan matrix [22, 23]. For example Thien et al used chitosan nanofibers treated in simulated body fluid to induce HAP formation on their surface [24]. The results revealed cell viability and differentiation on chitosan/HAP nanofibers to be significantly higher than on pure chitosan nanofibers. Liveranini et al combined chitosan and HAP by using the electrospinning technique after dispersing HAP in the chitosan solution. As a result, HAP was homogeneously dispersed within the fibers and on the fiber’s surface, which subsequently improved bioactivity [25].

A novel approach for the synthesis of biomimetically inspired HAP by double migration technique was recently presented [26]. The process is based on mineral formation within gelatin gels which results in spherical HAP particles—so called organically modified hydroxyapatite (ormoHAP)—with 100–500 nm in diameter. Each of the hollow spheres is hierarchically structured by nanocrystals forming a shell surrounding a core of low mineralized gelatin. Subsequent studies on this technique demonstrated the variability of the double migration setup to produce gelatin-mineral composites and their application in a co-culture study with osteoblasts and osteoclasts precursors [27, 28].

The focus of the present study is the application of the double migration technique to achieve an electric field assisted migration and deposition of ormOHAP on the surface of the NSN-scaffolds. Furthermore, scaffold coating with fibrillar collagen type I is applied as an established way of mimicking the extracellular matrix of bone cells. Cell behavior on both types of functionalized NSN-scaffolds, compared to pure NSN-scaffold is investigated. Adhesion, proliferation, and osteogenic differentiation of hBMSC with and without osteogenic supplements were quantitatively and microscopically analyzed, to judge the scaffolds bone biocompatibility.

2. Materials and methods

2.1. Preparation of NSN-scaffolds
NSN scaffolds were prepared from wet-spin chitosan fibers as starting material [8]. Chitosan flakes with high purity and a degree of deacetylation (DA) of 90%, a viscosity of 50 mPas (1% wt. CTS in 1% wt. acetic acid) and a molecular weight of Mw = 350 kDa was kindly provided by Heppe Medical Chitosan GmbH, Germany. The spinning solution with 8.0 wt% chitosan in a acetic buffer solution is filtered and stirred for 5–10 h, degassed under vacuum, heated to 60 °C and spun into a 0.5 M NaOH/10% EtOH coagulation bath using a wet spinning machine from Fourné Polymer-technik GmbH, Germany. The spun fibers are then washed and dried, wound onto bobbins and cut into short fibers.

The fiber diameter and length, that define the pore-size and the overall porosity, were estimated using a modeling approach as described by Brünner et al [29]. To achieve pore-sizes of 100 μm and an overall scaffold porosity of 90%, suitable for hBMSC seeding and differentiation, a fiber diameter of 40 μm and a fiber length of 1 mm were used. The fibers were bonded with 1% acetic acid (Merck, Germany) by partial dissolution locally added by means of a piezo-driven nozzle applying 3 nl droplets in a 200 μm grid within the two-tier NSN process. Disc-shaped scaffolds with a diameter of 8 mm and height of 3 mm were printed.

2.2. Functionalization of NSN-scaffolds
Collagen: Bovine tropocollagen type I (GfN, Wald-Michelbach, Germany) was purified by salt precipitation (1 M NaCl) followed by dialysis against deionized water and mixed with 60 mM phosphate buffer at pH 7.4 and 37 °C to perform fibrillogenesis. Collagen fibrils were then resuspended at a concentration of 2 mg ml\(^{-1}\) in 0.1 M TRIS buffer pH 7.4 and NSN-scaffolds were coated by soaking the scaffolds in the collagen solution for 1 h. Collagen coating on the scaffolds was stabilized by immediate transfer of the
scaffolds into chemical cross-linking solution composed of 100 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS) (both Sigma, Taufkirchen, Germany). After crosslinking for 4 h, the scaffolds were rinsed in deionized water and were freeze-dried.

OrmoHAP: Mineralization of the NSN-scaffolds was performed using the double migration method [26, 27]. In brief, a gelatin gel was prepared as a barrier between reservoirs of CaCl₂ (40 mM, pH 8, Roth) and Na₂HPO₄ solution (24 mM, pH 12, Roth), respectively (figure 1). Each reservoir was equipped with a platinum wire acting as an electrode. A potentiostatic electric field (15 V, max. 40 mA) was applied to accelerate the opposed movement of calcium ions and phosphate ions into and within the gelatin gel. For NSN-scaffold mineralization, a polyacrylic barrier with 24 cylindrical holes of 6 mm in diameter was used instead of the gelatin barrier. An aqueous solution of 5% gelatin (native porcine gelatin, Heraeus®) was prepared by swelling in deionized water for 30 min at 20 °C followed by heating to 50 °C and was poured into the holes. Then, the NSN-scaffolds were embedded into the gelatin-filled holes, and the assembly was stored for 4 h at 4 °C for gelation. Subsequently, two different treatment conditions were investigated: NSN-scaffolds either pre-incubated in deionised water or in 24 mM Na₂HPO₄, pH 12 for 24 h at 20 °C before embedding into the chamber. After 6 d of mineralization, the scaffolds were separated from gelatin by applying 50 °C deionised water followed by air-drying at 37 °C. Gamma-irradiation with 25 kGy dose was applied to sterilize the pure and functionalized NSN-scaffolds before using for cell culture experiments.

2.3. Cell culture experiments

hBMSC, isolated from bone marrow aspirates of two donors (donor I: 19 years old, female; donor II: 24 years old, female), were kindly provided by Prof. Bornhäuser and co-workers, Medical Clinic I, Dresden University Hospital. Cells in passage 5 were used for the experiments. The cells were cultured in alpha medium (a-MEM), supplemented with 10% fetal calf serum (FCS), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. Medium and all supplements were obtained from Biochrom, Berlin, Germany.

Sterilized NSN-scaffolds were placed in 48-well-plates and soaked in cell culture medium for 24 h for equilibration. After replacing the medium, hBMSC were seeded with a density of 2 × 10⁵ (donor I) and 2.5 × 10⁵ (donor II) per 48-well, respectively. On the third day, cells were osteogenically induced by addition of 10 nM dexamethasone (Sigma), 50 mM ascorbic acid 2-phosphate (Sigma), and 5 mM β-glycerophosphate (Sigma) to the medium. The medium was changed twice weekly.

2.4. Scanning electron microscopy and confocal laser scanning microscopy

SEM was used to characterize the pure and functionalized NSN-scaffolds before and after cell culture experiments, respectively. Cell-seeded samples were washed and the cells were fixed. Dehydration was achieved by using a graded ethanol series followed by infiltration with hexamethyldisilazane (Fluka, Germany). All samples were mounted on stubs and coated with carbon in a Balzers SCD 050 coater. Microscopy was carried out using a Philips ESEM XL 30 in Hi-Vac mode by applying an acceleration voltage of 3 kV and detecting secondary electrons for imaging.

CLSM was applied to evaluate cell morphology, orientation, and growth. After washing and fixing, the cells were permeabilized with 0.2% Triton-X-100 in PBS and blocked with 1% bovine serum albumin (BSA, Sigma) for 30 min. Cytoskeletal actin was stained with AlexaFluor 488®-Phalloidin (Invitrogen) and cell nuclei with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Microscopy was carried out on an
upright Axioscop 2 FS mot equipped with a LSM 510 META module (Zeiss, Jena, Germany) controlling an argon-ion (Ar⁺) laser, helium-neon (HeNe) laser and NIR-femtosecond titanium-sapphire laser for 2-photon excitation (Coherent Mira 900F). Excitation of AlexaFluor 488® was carried out at 488 nm (Ar⁺ laser). The NIR-fs-laser laser was used for excitation of DAPI at 750 nm (2 photon excitation) and fluorescence was recorded at 461 nm.

2.5. GeoDict simulation

The voxel-based software GeoDict (Math2Market GmbH, Germany) was used to model the collagen coating in the NSN-scaffold. By combining the modeling approach from Brünler et al. [29] with thin, curved fibers and binder considering the different densities of chitosan and collagen, a replication of the collagen coated scaffold morphology with respect to the bridging of small gaps between the microfibers and the fibril formation of the collagen content was realized (see figure 2(f)).

2.6. Micro-computed tomography (μCT)

Evaluation of the mineralization degree of the NSN-scaffolds was carried out by fully micro-computed tomography (vivaCT 75, SCANCO Medical, Brüttsellen, Switzerland) using an isotropic voxel size of 20 μm (45 keV, 88 μA, 130 ms, 1000 projections). Calibration of the scanner was done weekly using HAP phantoms. For analysis, the Image Programming Language software (IPL) from SCANCO Medical was used. Contours were drawn manually around the sample’s surface to access the total volume (TV). The threshold of the mineral volume content (MV) of the samples was set to 10 and 80 mg HA cm⁻³.

2.7. Colorimetric measurements

Evaluation of cell proliferation and osteogenic differentiation was performed by lactate dehydrogenase (LDH) assay or ALP assay. After 1, 7, 14, 21 and 28 d, cell lysates were prepared in triplicates from 1% Triton X-100 (Sigma) in PBS. In addition to cell lysis, ultrasound (20 s, 80 W) was performed. A SpectraFluor Plus microplate reader (Tecan, Crailsheim, Germany) was used for the colorimetric measurements.

The LDH activity test was performed using the LDH cytotoxicity detection kit (Takara, Saint-Germain-en-Laye, France). An aliquot of cell lysate was mixed with the LDH substrate buffer in a 96-well tissue culture polystyrene plate and the enzymatic reaction was stopped with 0.5 M HCl after 30 min.
Absorption was measured at 492 nm and the obtained values were correlated with the cell number from a calibration series of known cell numbers.

For the ALP activity test p-nitrophenylphosphate was used as substrate. An aliquot of the cell lysate was mixed with the ALP buffer containing 2 mg ml\(^{-1}\) p-nitrophenylphosphate (sigma), 0.1 M diethanolamine, 1 mM MgCl\(_2\), 0.1% Triton X-100 (pH 9.8) in a 96-well tissue culture polystyrene plate. After an incubation period of 30 min the enzymatic reaction was stopped by the addition of 0.5 M NaOH. Absorption was measured at 405 nm and represented as ALP activity by means of a calibration series of different concentrations of p-nitrophenol and related to cell number.

Examination of calcium concentration was carried out using the Fluitest\textsuperscript{®} CA-CPC detergent (Analyticon, Germany). All measurements were performed with cell culture supernatants obtained after 1, 7, 14, 21 and 28 d of cultivation. According to the manufacturer’s instructions, 10 \(\mu\)l cell culture supernatant was mixed with 300 \(\mu\)l of a 1:1 mixture of AMP buffer (pH 10.7) and o-kresolphthalin complexon, 8-hydroxyquinolin, HCl. After reaction for 10 min in a 96-well tissue culture polystyrene plate, the absorbance at 570 nm was read. A calibration line was obtained from graded series of a calcium reference.

2.8. Reverse transcription polymerase chain reaction

Gene expression of the cells cultivated on the NSN-scaffolds was analyzed after 28 d as described previously [28]. Briefly, RNA was isolated with the peqGOLD MicroSpin Total RNA Kit (Peqlab) according to the manufacturer’s instructions and the concentration was measured with a Nanodrop ND 1000 (Peqlab). Transcription of complementary DNA (cDNA) was performed using a 20 \(\mu\)l reaction mix of 400 ng total RNA, 200 U of Superscript II Reverse Transcriptase (Invitrogen, Darmstadt, Germany), 0.5 mM dNTP (Invitrogen), 12 \(\mu\)l (cDNA) and 12 \(\mu\)l (cDNA), 5 ng \(\mu\)l\(^{-1}\) random hexamers (MWG Biotech, Ebersberg, Germany) and 40 U of the RNase inhibitor RNase OUT (Invitrogen) and incubated for 50 min at 42 °C, followed by 15 min at 70 °C in a Primus 25 Advanced Thermocycler (Peqlab). PCR was performed using 2 \(\mu\)l cDNA as a template and adding 18 \(\mu\)l mastermix with specific primer pairs to detect transcripts of ALP, bone sialoprotein II (BSP II), osteocalcium (OC) and the household gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The primer sequences and annealing temperatures used are summarized in table 1. The PCR products were analyzed with the FlashGel Dock and the documentation system (Cambrex Bio Science, East Rutherford, USA).

2.9. Statistics

All measurement data were obtained in triplicate and presented as mean \(\pm\) standard deviations. The analysis of variance was used to determine significance with \(p\)-values of less than 0.1 (significant), 0.01 (very significant), and 0.001 (highly significant).

3. Results

3.1. Scaffold morphology

Pure chitosan fibers with about 40 \(\mu\)m in diameter were processed to scaffolds by using the NSN technique. Figure 2(a) shows a photograph of a scaffold, which was used for analysis and as a substrate for functionalization. SEM images of the pure NSN-scaffold reveal the characteristic structure, which results in an inherently interconnected porosity (figure 2(b)). Higher magnification of single fibers confirm a smooth surface (figure 2(c)).

The morphological characteristics of the NSN-scaffolds functionalized by collagen include areas of collagen layers formed inside the scaffolds and collagen layers forming smooth surfaces by bridging two or more single chitosan fibers (figure 2(d)). As an important feature, the porous structure of the NSN-scaffold remains unaffected to a large extent. Higher magnification reveals single collagen fibrils as building blocks of the covering layer on the chitosan fibers (figure 2(e)). GeoDict simulation visualizes distribution of collagen and chitosan fibers (figure 2(f)).

The electric field assisted migration of calcium and phosphate ions in the gelatin gel with NSN-scaffold embedded leads to the deposition of ormoHAP on the surface of the chitosan fibers (figure 2(g)). The porous structure of the NSN-scaffolds remains unaffected. Two different conditions were applied by using either deionised water or phosphate buffer for pre-incubation before embedding the scaffolds in the chamber. While equilibration with deionised water leads to low precipitation of ormoHAP crystals on the fibers (figure 2(h)), equilibration with phosphate buffer leads to a highly mineralised fibers (figure 2(i)).
fibers are studded with the typical ormoHAP spheres [26].

Micro-computed tomography (μCT) visualizes collagen but does not detect chitosan due to its low material density. Therefore, only collagen and the mineral phases of investigated scaffolds were visualized. The images in figure 3 confirm uniform collagen coating (a) as well as uniform mineral deposition inside ormoHAP-functionalized scaffolds (b).

3.2. Cell adhesion and proliferation

Cell adhesion on the pure and functionalized NSN-scaffolds was investigated by performing confocal laser scanning microscopy 24 h after cell seeding. The morphological adaptation of the cells to the provided substrate is evaluated by the spreading of the green fluorescent actin skeleton (figures 4(a) and (b)). Cell nuclei are visualized by blue color. Adhered hBMSC are completely spread on the scaffolds and show elongated and spindle-shaped morphology on aligned chitosan fibers with their orientation being parallel to that of the fiber. Moreover, cells reveal remarkable flexibility of adaption by spanning over several single chitosan fibers. On functionalized NSN-scaffolds spreading towards the fiber’s alignment seems to be more closely than on pure NSN-scaffolds. This could be a result of the cell’s intention to achieve the maximal contact area to the substrate.

After 28 d of cultivation, osteogenically induced hBMSC proliferated on all functionalized NSN-scaffolds. The cLSM images confirm increase of cell density and the formation of dense layers on these scaffolds (figures 4(e), (h) and (k)). On the other hand, low cell numbers indicating low proliferation rates were recognized on pure NSN-scaffolds (figure 4(b)).

SEM images after 28 d of cultivation confirm cLSM results by visualizing the morphology of both, the cells and the fibers (figures 4(c), (f), (i) and (l)). Low cell numbers occurring as cell patches are easy to identify between the pure fibers of the NSN-scaffold. Identification of cells is difficult on collagen-functionalized scaffolds because of morphological equality. However, high magnification allows visual distinction. Similar results were obtained for the ormoHAP-functionalized NSN-scaffolds, where SEM confirms dense extracellular matrix covering the fibers.

Proliferation of hBMSC was determined quantitatively over a cultivation period of 28 d and is illustrated in figures 5(a) and (b). Despite differences in the absolute cell number, both donors showed similar trends regarding cell behavior. On the supposition of constant LDH activity per cell during the cultivation period, for all scaffold types the osteogenically induced cells showed higher proliferation rates compared to non-induced cells. Non-induced cells on pure and collagen-functionalized NSN-scaffold showed nearly constant cell numbers. In contrast, the initial cell numbers of non-induced hBMSC on NSN-scaffolds functionalized with ormoHAP increased by factors of about 7.5 (donor I) and of about 3.5–4.5 (donor II) after 28 days of cultivation. For both donors, hBMSC cultivated on collagen-functionalized NSN-scaffolds showed a slightly higher proliferation rate compared to cells on pure NSN-scaffolds. For example, the initial cell number of osteogenically induced donor I hBMSC increased by factors of 6 (CS/Coll) compared to 4 (CS) after 28 d of cultivation. In contrast, cell numbers of osteogenically induced hBMSC on ormoHAP-functionalized NSN-scaffolds increased about 14 fold (donor I) after 28 d of cultivation.

3.3. Cell differentiation

The course of relative ALP activity of hBMSC cultivated on pure or functionalized NSN-scaffolds is illustrated in figures 5(c) and (d). All cell fractions showed low ALP activity levels until addition of osteogenic supplements on day 3. During further cultivation, ALP activity of these osteogenically
induced hBMSC raised on all NSN-scaffold modifications.

Relative ALP activity of non-induced cell fractions remained at the low base level for the total cultivation time except for collagen-functionalized NSN-scaffolds. In this setup, the collagen coating seemed to have a stimulating effect on hBMSC differentiation, even without addition of osteogenic supplements. In general, osteogenic induction of hBMSC lead to increased ALP activity, while collagen functionalization lead to the highest ALP increase for both donors. In all cases ALP activity was lowest during cultivation on pure NSN-scaffolds, however continuing increase until day 28 was recognized. Except for these pure NSN-scaffolds, ALP activity showed the typical maximum representing osteogenic differentiation [30]. Additional functionalization with ormoHAP resulted in similar positive effects on differentiation compared to collagen-functionalization, with being more obvious for donor I cells compared to donor II cells.

Analysis of cell mineralisation was performed for the pure and the collagen-functionalized NSN-scaffolds using the colorimetric cresolphthalein assay. Calcium concentration in the cell lysates increased continuously from day 14 to day 28, and is significantly higher for the collagen-functionalized NSN-
In order to characterize the phenotypes of differentiated hBMSC, gene expression was analyzed by performing RT-PCR after 28 d of cultivation. Figure 6 shows the gene expression profile of donor I. Expression of GAPDH was determined to verify the usage of similar amounts of RNA for RT-PCR. For all scaffold modifications, the typical osteogenic markers ALP, BSP II, and OC was increased for osteogenically induced cells, whereas non-induced cells showed a low expression level. Gene expression analysis of osteogenically induced hBMSC after 28 d of cultivation revealed an ALP transcript level which was highest on the collagen-functionalized NSN-scaffold. Moreover, scaffolds (figure S1 of the supplementary material is available online at stacks.iop.org/BMM/14/045017/mmedia).

Figure 5. Cell number and specific ALP activity (normalized to the cell number) of non-induced (−OS) and osteogenically induced (+OS) donor I hBMSC (a) and (c) and donor II hBMSC (b) and (d) cultivated on pure chitosan (CS), collagen-functionalized (CS/Coll) and mineralized NSN-scaffolds (CS/ormoHAP_low, CS/ormoHAP_high). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001: CS/Coll + OS versus CS + OS, **p < 0.001: CS/ormoHAP_low + OS versus CS + OS, ***p < 0.001: CS/ormoHAP_high + OS versus CS + OS, †p < 0.05, ††p < 0.01: CS/Coll - OS versus CS - OS.

Figure 6. Gene expression of the osteoblast-related markers ALP, BSP II, OC, and the housekeeping gene GAPDH, after 28 days of cultivation of non-induced (−) and osteogenically induced (+) hBMSC (donor I) on pure (CS), collagen-functionalized (CS/Coll) and mineralized NSN-scaffolds (CS/ormoHAP_low, CS/ormoHAP_high).
BSP II expression was much higher on the collagen-functionalized NSN-scaffolds, whereas induced cells on pure NSN-scaffolds showed the lowest expression. The gene expression level of OC was similar for all scaffold types.

4. Discussion

The development of three-dimensional scaffolds—based on their similarity to extracellular matrices—that support the adherence, anchoring, growth, migration, and functional differentiation of cells is still a primary objective in biomaterials research. Several studies confirmed fibers to be suitable building blocks in order to create open-porous structures with large functionalized surface area. In this field, chitosan has demonstrated intrinsic properties that favor the application as a base material [3, 4, 14].

In the present study chitosan fibers were processed to NSN-scaffolds. As described in [29], the porosity and pore size of the scaffolds can be predicted based on an in silico modeling and simulation approach using Geodict software, which is validated by measurements in a capillary flow pore-measuring device using the liquid displacement method. Thus, the pore morphology can be precisely determined by varying the diameter and length of the fibers and tailored to the biomimetic requirements of the target tissue. A bioinspired functionalization of the NSN-scaffolds was applied in order to mimic the organic and inorganic components of natural bone.

Collagen coating is an established procedure to biofunctionalize surfaces of biomaterials, thus to improve their biocompatibility [9, 10]. The coating procedure modifies the open porous structure only slightly, due to the homogenous distribution of collagen layers inside the scaffold. The NSN-scaffold is not sealed-off by the collagen coating which is important regarding the useful porosity features. This is a remarkable difference to previous studies with conventional chitosan fiber scaffolds, where collagen spanning between several chitosan fibers dominated the scaffolds overall structure in contrast to superficial surface coverage [14, 31]. Owing to the chosen crosslinking technique after coating, the collagen fibrils were physically attached close to the chitosan fibers. As a result, cells cultivated on these scaffolds, had direct contact to both scaffolds components, collagen and chitosan.

In former studies we established a mineralization method for generation of organically modified hydroxyapatite (ormoHAP) using the double migration technique in a gelatin gel with an accelerating electric field [26, 27]. This innovative process was identified to be suitable to mineralize fibers in a bioinspired way. Microscopy analysis confirmed that the pore structure of the NSN-Scaffolds on a micro-level was not changed by the mineral formation, but that the originally smooth surface of the chitosan fibers was studded by the sub-micrometer sized mineral particles resulting in a comparably rough surface. Pre-incubating the scaffolds in water or in phosphate solution prior to the double migration process resulted in different degrees of mineral layer coverage on the fibers. The layers were much thicker in case of phosphate pre-incubation since phosphate ions initiate nuclei formation and support growth [32, 33].

Micro-computed tomography and SEM confirmed that the double migration method facilitates homogeneous distribution of equally sized mineral particles in the whole NSN-scaffold, due to self-assembling on the fiber’s surface. This is advantageous compared to conventional methods of mineral deposition, where heterogeneous particle size and distribution in a polymer matrix can be detrimental to mechanical properties [34].

In the present study all cell culture experiments were performed on pure, collagen-modified and both ormoHAP-modified NSN-scaffolds. Adhesion, proliferation, and differentiation of osteogenically induced and non-induced hBMSC of two donors were investigated and discussed.

Using both, cLSM and SEM is particularly suitable to visualize the morphology, distribution, and orientation of cells adhered to the pure and functionalized scaffolds. Only 24 h after seeding, cells showed good initial adherence by being spread along and between the fibers of all scaffold types without significant differences. It can be assumed that cells on functionalized scaffolds were closer to the fibers.

HBMSC and osteogenically induced hBMSC of both donors showed higher proliferation rates on functionalized NSN-scaffolds which was confirmed microscopically and quantitatively. Collagen-functionalization leads to improved conditions for cell proliferation compared to pure NSN-scaffolds. This result is consistent with the results of a study reported by Hild et al. where enhanced cell proliferation of MSC cultivated on collagen-modified substrates was investigated [1]. In a former study, comparing pure and collagen-coated chitosan fibers, we also observed similar proliferation rates of the cells for the collagen-functionalized and non-coated chitosan scaffolds during a cultivation time of 28 d. It has to be noted that higher cell numbers observed on the collagen-functionalized samples might be caused by slightly enhanced adhesion and therefore higher initial cell density than by improved proliferation [14].

Remarkably, the deposition of ormoHAP particles on fiber surface extensively supported cell proliferation. The rates increased up to day 14 for osteogenic induced as well as for non-induced hBMSC. This was confirmed by microscopy. While cell proliferation is hardly to observe on pure chitosan scaffolds, the fibers of ormoHAP-functionalized scaffolds were completely enveloped by a dense cell layer. The influence of HAP on cell proliferation was reported previously by...
several authors [24, 35]. Zhao et al demonstrated incorporation of HAP in chitosan scaffolds by dispersion in order to improve initial adhesion and proliferation of human mesenchymal stem cells [15]. The authors discussed more effective calcium ion adsorption and protein adsorption achieved by the mineral to be the reason for the effect. In the present study, measurements in the supernatant of cell culture medium showed increased calcium ion adsorption in case of ormoHAP-functionalization (data not shown), which confirms enhanced bioactivity. Similar results were shown in previous experiments with ormoHAP as a component in gelatin foams. Here also a higher bioactivity was detected due to calcium deposition during SBF [28]. Rogina et al detected a higher bioactivity of chitosan modified with HAP and furthermore reported an increased cell number on these composite scaffolds [36]. Liu et al demonstrated nanocrystalline HAP to promote cell growth in an in vitro model [37]. Clearly, it is the nano-HAP crystallite that plays the essential role in the proliferation behavior [38]. Moreover, the quantity of proteins adsorbed on HAP particles positively correlates with their specific surface area which in turn increased with reduced particle size—particularly in the case of nanosized HAP [39, 40]. Higher amounts of proteins were reported to induce enhancement of subsequent cell adhesion and proliferation [41].

Although it was reported that cell differentiation occurs at an expense of proliferation rate, in the present study on each scaffold modifications the values for osteogenically induced HBMSC were similar (donor II) or increased (donor I) compared to the non-induced hBMSC. Some studies also report enhanced proliferation rates due to osteogenic induction, which is attributed to the stimulatory effect of dexamethasone [42]. Furthermore the presence of ascorbate is known to promote cell proliferation of MSC [43]. Accordingly, Machado et al found higher proliferation rates of osteogenically induced MSC compared to non-induced MSC cultivated in chitosan-containing three-dimensional scaffolds [44].

Osteogenic differentiation of the hBMSC towards the osteoblast lineage was confirmed by the relative ALP activity of the cells. Low ALP activity was determined for non-induced cell fractions as expected. Only scaffolds with collagen functionalization showed positive impact on the ALP activity levels already without osteogenic supplements, which indicates a promoting effect of collagen coating on osteogenic differentiation of MSC [45, 46]. Increased ALP activity was observed for hBMSC of both donors during cultivation on all scaffolds in the presence of osteogenic supplements. The ALP activity passing a typical maximum for all functionalized NSN-scaffolds, characterizes cell mineralization at a late stage of differentiation. Especially for collagen, adhesion and differentiation are supported by RGD sequences of the collagen coating which are recognized by the cells [47], which in turn can enhance ALP activity as well as BSP II and OC expression [48]. Moreover, the increased differentiation of the hBMSC in the presence of collagen can be explained by the higher specific surface area and the biochemical as well as by structural analogy to extracellular matrix and bone.

In contrast, cells cultivated with osteogenic supplements on pure chitosan NSN-scaffolds do not show the discussed maximum of ALP, but a continuously increasing level up to day 28. This course suggests the osteogenic differentiation to be slow compared to functionalized scaffolds. However, achieving a maximum at a later time point is expected.

Mineralization behavior of the cells was tested for the pure and the collagen-functionalized NSN-scaffolds using a colorimetric assay (supplementary results). OrmoHAP-functionalized fibers were not analyzed due to misleading calcium background level caused by the mineral. The results correlated to matrix mineralization studies observed for osteogenically induced hBMSC cultivated on textile chitosan scaffolds [14].

5. Conclusion

NSN-scaffolds were processed from chitosan fibers and were functionalized by different bioinspired techniques: biological functionalization using collagen type I and mineralization with organically modified HAP. The second was achieved by embedding the NSN-scaffolds in a gelatin gel and subsequent electric field-based double migration. In this particular process, homogeneously distributed formation of uniform ormoHAP particles on the chitosan fibers even deep in the scaffolds keeps the pores open so that cells are not prevented from ingrowth. Our investigation on adhesion, proliferation, and osteogenic differentiation of hBMSC showed the suitability for a prospective application in bone tissue engineering. Other than the collagen coating, the mineralization step led to remarkably improved cell proliferation of hBMSC. Differentiation towards the osteoblast lineage was most significantly enhanced by the collagen coating functionalization. Consequently, the study demonstrates the important and synergistic functions of collagen coating and mineralization—especially by ormoHAP—in improvement of the biological performance of the scaffold by mimicking the bone matrix.

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