In vitro Evaluation of a 20% Bioglass-Containing 3D printable PLA Composite for Bone Tissue Engineering

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Abstract: Three-dimensional (3D) printing is considered a key technology in the production of customized scaffolds for bone tissue engineering. In a previous work, we developed a 3D printable, osteoconductive, hierarchical organized scaffold system. The scaffold material should be osteoinductive. Polylactic acid (PLA) (polymer)/Bioglass (BG) (mineral/ion source) composite materials are promising. Previous studies of PLA/BG composites never exceed BG fractions of 10%, as increase of bioactive BG component negatively affects the printability of the composite material. Here, we test a novel, 3D printable PLA/BG composite with BG fractions up to 20% for its biological activity in vitro. PLA/BG filaments suitable for microstructure 3D printing were spun and the effect of different BG contents (5%, 10%, and 20%) in this material on mesenchymal stem cell (MSC) activity was tested in vitro. Our results showed that all tested composites are biocompatible. MSC cell adherence and metabolic activity increase with increasing BG content. The presence of BG component in scaffold has only slight effect on osteogenic gene expression, but it has significant suppressive effect on the expression of inflammatory genes in MSC. In addition, the material did not provoke any significant inflammatory response in whole-blood stimulation assay. The results show that by increasing the BG content, the bioactivity can be further enhanced.

Keywords: Bone tissue engineering; Composite; Polylactic acid; Bioglass; Osteoconductive; Osteoinductive

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1. Introduction

Three-dimensional (3D) printing is considered a pioneering technology in the development of novel bone substitutes (scaffolds) in the context of bone tissue engineering (BTE). For the 1st time, the structural requirements of bone regeneration in every detail can be taken into account to develop scaffolds using 3D printing technology. Further development from purely biomimetic to bioaugmentative designs is possible. Highly complex, bioactive internal structures, beyond the classical grids, can be easily integrated into load-bearing frame structures. However, in addition to design, the scaffold material has a significant impact on bone regenerative potential. Common biopolymers (polylactic acid [PLA], polylactic glycolic acid [PLGA], polycaprolactone [PCL], etc.) exhibit excellent printing properties, high mechanic stability, and complex micron-scale structures that are feasible with conventional 3D printers. Their
degradation products are non-toxic and absorbable by the organism\[15,13\]. Despite that, these materials display some disadvantages for BTE applications, such as a low bioactivity, a hydrophobic surface, and a low degradation rate\[8\]. Polyhydroxylkanoates are now coming into focus as promising alternatives. These pro-osteogenic biopolymers can be naturally produced by bacteria in the bioreactor\[49\]. However, the mass production of these polymers still needs to be established\[60\].

Mineral alternatives, such as tricalcium phosphates and hydroxyapatite (and many others), with their bone-like composition and highly porous surfaces, improve cell attachment and bone formation. Calcium release during resorption has a stimulatory effect on bone regenerative cells\[7\]. However, the printing properties and low mechanical stability limit their application in BTE\[8,9\]. To generate a mechanical stability comparable to polymers, compact grid structures are necessary. Large pores in the scaffold walls, required for hematoma penetration, can thus be obtained only at the expense of stability. In addition, due to the very high melting point of mineral components, melting and subsequent molding, as it occurs with polymers, are not possible. In this case, a solvent or a binder is required. Only after its volatilization or pyrolysis and subsequent sintering connection between individual layers and single-strand structures gain stability\[10,11\]. Complex, ultrafine internal structures, such as interconnected pores and fibers with diameters of a few micrometers, important for cell attachment and vascularization, are, in turn, very difficult to obtain\[11\].

Composites are increasingly reported in literature in the attempt to combine the beneficial properties of polymer and mineral materials\[12,13\]. Incorporation of calcium phosphates in a composite can increase wettability and roughness of the material that, in turn, can lead to better cell adhesion and proliferation\[14\]. The combination of PLA and bioglass (BG) is very promising. PLA, commonly used in routine clinical applications (suture material, material for resorbable screws, and bone anchors), is characterized by its good biocompatibility, mechanical resistance, and resorbability\[15\]. In addition, it is widely used in 3D printing\[16\]. Even the finest structures down to the nanometer range can be produced\[1\]. However, PLA’s poor osteoconductive and osteoinductive properties, its hydrophobicity, which could inhibit cell adhesion and tissue regeneration and the cytotoxic acidity induced by its degradation products could limit its widespread use. These restrictions could be counteracted by the addition of BG on its composition. Literature shows that the brittle and very hard BG material forms a hydroxyapatite layer when in contact with physiological fluids that serve as a basis for bone formation by osteogenic cells\[17,19\]. The simultaneous release of ions, such as calcium, sodium, or magnesium, has a positive effect on proliferation, cell adhesion, and osteogenic differentiation (OD) of mesenchymal stem cells (MSCs)\[20,21\]. In addition, studies have shown inhibition of antimicrobial growth, especially of resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* or methicillin-resistant *Staphylococcus epidermidis*\[22,23\]. Finally, its weak alkaline nature has shown to reduce the strong acidic effect of PLA’s degradation products\[24\]. With these potential properties, composites could then also be used in the integrated tissue-organ printer, a bioprinting strategy\[25\]. Here, vascularized cell-loaded bone constructs with tunable mechanical properties are developed. They thus represent the next stage in BTE\[26,27\].

The use of composites in 3D printing to create complex structures is not yet routine. Even the production of the necessary filaments is a challenge. In most cases, PLA and BG are available as a powder or fine granules, which are melted together. Usually, the mass is then pressed into a mold or extruded through a nozzle. The resulting frameworks are usually compact and have amorphous internal structures\[7,28,29\]. PLA/BG composites with up to 10% BG have already been produced, but the printing results in terms of strand thickness and pore size are insufficient\[30,32\].

It has been shown that a high BG fraction has a beneficial effect on bioactivity. However, as the proportion of mineral components increases, the stability of the material decreases\[33\]. This increases the likelihood of strand breakage during the printing process and also reduces the mechanical stability of the printed products. This especially impedes the printing of ultrafine structures and limits the structure size.

Thus, the basis for the successful development of 3D-printed scaffolds for BTE is the production of a composite filament that serves the highest possible BG content without compromising the printing requirements. Finally, it should be possible to print ultra-fine structures with the material.

In view of this requirement profile, our working group developed a PLA/BG composite material with three different BG contents (5%, 10%, and 20%) which are suitable for high-resolution 3D printing. Despite the high proportion of BG, scaffolds with fine internal structures could be produced. The aim of this work was the *in vitro* characterization of the composite material. Its stimulatory influence on MSCs behavior (cell adherence and viability, OD), as well as the immunostimulatory and inflammatory potential were detailed investigated. Therefore, 3D-printed mesh specimens with a diameter of 5 mm and varying BG concentrations were successfully fabricated and thoroughly tested.
2. Materials and methods

2.1. Ethics

A MSC pool was obtained from residual bone marrow samples from five healthy donors. The use for research purposes is covered by an ethics vote (329/10 of the Department of Medicine of the Goethe University). For the whole-blood stimulation assay, the analysis of blood samples from volunteers is covered by ethics vote 89/19 of the same department. All donors signed informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University Hospital Frankfurt am Main, Goethe University (project no. 89/19).

2.2. Establishment and characterization of the MSC pool

Pooled MSCs were used in this study to minimize the influence of individual differences between individuals. Mononuclear cells were isolated by Ficoll density gradient centrifugation from EDTA-anti-coagulated bone marrow samples (approximately 1-2 ml volume) as previously described. Mononuclear cells were seeded at a density of 1 × 10^3 cells/cm² using MesenCult-supplements (hereinafter referred to as “complete medium,” Stemcell Technologies, Cologne, Germany) and cultured in 75 cm² culture flasks for two additional passages after reaching confluence. Cells were then harvested and 1 × 10⁶ MSC each per 1 ml of freezing medium consisting of 90% FCS and 10% DMSO was stored in liquid nitrogen until the target number of donors was reached. To create the pool, one vial of MSC from each donor was thawed and cultured in complete medium over another passage. Cells were then enzymatically detached and counted. 1 × 10⁶ cells from each donor were pooled, then centrifuged, resuspended in freezing medium, and stored in liquid nitrogen until use. Pooled MSCs were used in this study to minimize the influence of individual differences between individuals.

2.3. Filament fabrication

Pre-dried (12 h; 40°C) PLA granules (mean granule size of 2-5 mm) (PLA-Filament Kristall Natur, 3dk.berlin, Berlin, Germany) were mixed with pre-dried S53P4-BG (mean granule size 25-42 µm; BG composition: 53% SiO₂, 23% Na₂O, 20% CaO, and 4% P₂O₅, S53P4; BonAlive Biomaterials Ltd., Turku, Finland) according to weight with 0%, 5%, 10%, and 20%. Using a filament extruder (NEXT 1.0 Advanced, 3deo, Utrecht, Netherlands), the resulting granulate mixture was heated within the heating zone, extruded at 4 rpm and then cooled down using a fan. Automatic extrusion speed ensured a constant diameter of 1.75 mm for the subsequent printing process. This took place on a commercially available fused filament fabrication (FFF) 3D printer (i3 MK3S, Prusa Research, Prague, Czech Republic), whereby the slicing process was first carried out using the Cura Ultimaker software (V4.6, Ultimaker, Utrecht, Netherlands). The detailed filament manufacturing process is described in Schätzel et al.

2.4. Test specimens and scaffold fabrication

The prepared PLA/BG composite was now used to print two-layer test specimens (diameter 5 mm; height 0.3 mm) for the in vitro studies on a 3D printer (i3 MK3S, Prusa Research, Prague, Czech Republic) with a nozzle diameter of 0.4 mm. Design was performed with computer-aided design software NX 12 (Siemens NX 12, Siemens AG, Berlin and Munich, Germany) and preprocessed with Cura Ultimaker v.4.6 (Ultimaker, Utrecht, Netherlands). To verify whether complex 3D structures are also printable with the prepared composites, cylindrical porous scaffolds (height 6 mm; diameter 5 mm; macroscopic pores 700 µm, microscopic pores 150 µm, wall thickness 1.5 mm) were printed under same printing conditions and examined by light microscopy (Axio Observer Z1, Carl Zeiss, Göttingen, Germany). Filaments from 1.65 to 1.85 mm diameter were used. The detailed printing process is described in Schätzel et al.

2.5. Porosity

Porosity for the printed columns was estimated using slightly modified Archimedes principle as shown in various other works. A printed box with predetermined specific volume was first filled with agarose (A9539-500G, SIGMA, St. Louis, USA) and weighed with a high precision scale (S-234, Denver Instrument, Göttingen).
Deutschland) before and after submerging the printed parts (n = 3) and removing of the supernatant. Afterward, the submersed and with agarose filled parts were weighed, too. In addition, these values were compared to calculated parameters within a CAD tool (Fusion 360, v2.0.12665, Autodesk, San Rafael USA).

2.6. Adhesion and metabolic activity of MSC
MSCs were seeded at a density of 1 × 10^4 cells/test specimen and allocated in a well of a 12-well plate. For this purpose, cells were suspended in 15 μL culture medium, transferred in a droplet onto the test specimen, and incubated for 30 min at 100% humidity to allow the cells to adhere to the test material. The wells were then carefully filled with 500 μL of complete culture medium each.

Fluorescence micrographs were taken to directly detect adherent cells. For this purpose, MSCs on the test specimens were stained with Calecin-AM (BD Biosciences, Heidelberg, Germany) 24 h after seeding. Specimens with cells were transferred into individual wells of a 24-well plate and 1 ml of pre-warmed culture medium containing 20 μM Calecin-AM was added to each well. Cells were incubated for 40 min at 37°C, washed carefully 3 times with phosphate-buffered saline (PBS), and stained with DAPI (1 μg/ml in PBS, Sigma-Aldrich, Taufkirchen, Germany). After 10 min incubation, cells were again carefully washed 3 times with PBS. MSC adherence and distribution on the test specimen were then assessed by examining the samples with fluorescence microscopy using a Zeiss Axio Observer Z1 (Zeiss, Gottingen, Germany).

The metabolic activity of MSC adhering to the test specimens was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (MTT, Roche Diagnostics, Mannheim, Germany). For the determination of the metabolic activity, test specimens were carefully moved 24 h, 72 h, and 168 h after seeding into a new 48-well plate to prevent the adherence of cells to the bottom of the wells that could interfere with measurement results. MTT assay principle is based on the cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) to purple formazan crystals by metabolically active cells. All experiments were performed in duplicate. The MTT assay was performed according to the manufacturer’s instructions. Briefly, MTT reagent was incubated 2 h followed by overnight lysis of the formazan crystals. The supernatants were collected and transferred into wells of a new 96-well plate where its absorbance was measured at 570 nm using an ELISA reader (Infinite M200, Tecan, Mainz, Germany).

2.7. Scanning electron microscope (SEM)
Scaffolds seeded with cells were analyzed using SEM. Samples were fixed with glutaraldehyde (2% in −/− PBS) for 10 min and were immersed in a four-step ethanol gradient (50%, 75%, 96%, and 100%) for 5 min each. After a short passage in 1,1,3,3,3-hexamethyldisilazane (Merck Schuchard, Hohenbrunn, Germany) and draining overnight, gold was deposited on the samples by sputtering (5 × 60 s, Agar Sputter Coater; Agar Scientific Ltd., Stansted, United Kingdom). Analysis was performed using a SEM (Hitachi, Düsseldorf, Germany) and the Digital Image Processing System 2.6 (Point Electronic, Halle, Germany).

2.8. Determination of the osteogenicity of BG20
Osteogenicity of the composite BG20 was analyzed in a functional test. For this purpose, 2 × 10^4 MSCs were seeded into individual wells of a 24-well plate. After 24 h, the medium was changed and either 500 μL normal medium/well or 500 μL OD medium/well (PromoCell) were added in each well. In addition, a test specimen made of PLA or BG20 was coincubated contact-free through an insert (pore size 3 μm, Corning B.V. Life Sciences, Amsterdam, Netherlands) over a period of 14 days. The medium was changed twice a week. After 14 days, calcium deposition of MSC was analyzed by alizarin red staining (Merck, Darmstadt, Germany) as described in the previous study[36]. For evaluation, the percentage of alizarin red positive area per microscopic field of view was calculated using ImageJ software (https://imagej.nih.gov/ij/). The experiment was performed 3 times with two technical replicates each time.

2.9. Assessment of OD and inflammatory pathway gene expression
To evaluate the influence of the BG component on OD and induction of inflammatory pathways, the expression of the genes involved in osteogenesis (RUNX2 [transcription factor], alkaline phosphatase [ALP], collagen-1α [COL1A], and bone morphogenetic protein-2 [BMP2]) and inflammation mitogen-activated protein kinase ([MAPK]8 cJun-N-terminal kinase 1 [JNK-1], MAPK14 [p38α], and interleukin-6 [IL-6]) were measured. Analogous to the previously described experiments, 1 × 10^4 MSCs were seeded onto the different materials and cultured in osteogenic medium for 24 h, 72 h, and 168 h. At the respective time points, the medium was removed and total RNA was isolated using the RNeasy kit, according to the manufacturer’s instructions (Qiagen, Hilden, Germany). RNA concentration was measured photometrically using NanoDrop (ND-1000, NanoDrop Technologies, Wilmington, Delaware, USA).

Each 1 μg of RNA was reversely transcribed using an Affinity script QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer’s instructions. Contaminated genomic DNA was removed.
by digestion with the RNase-free DNase Kit following the manufacturer’s protocol (Qiagen).

Real-time RT-PCR was performed using primer assays (all obtained from Qiagen, first: gene accession number, 2. order number for primer set) for RUNX2 (NM_001015051, PPH01897B), AIP (NM_000478, PPH01311F), COLIA (NM_00088, PPH01299F), BMP2 (NM_001200, PPH00549C), MAPK8 (NM_002750, PPH00720B), MAPK14 (NM_001315, PPH00750B), IL6 (NM_000600, PPH00560C), and GAPDH (NM_002046.3, PPH00150F) as housekeeping gene. Quantitative RT-PCR over 40 cycles (94°C: 15 s, 70°C: 30 s) was performed on a Stratagene MX3005p qPCR system (Stratagene, La Jolla, CA, USA). A melting curve analysis was applied to ensure the specificity of the PCR reaction. Relative quantification of the mRNA levels of the target genes was determined using the Livak method (\(2^{-\Delta\Delta CT}\)) normalizing gene expression to GAPDH and MSC cultured under normal conditions\(^{[40]}\).

2.10. Effect of calcium ions released from the BG component

In this experiment, it was investigated whether calcium ions released from the BG component led to the observed changes in gene expression. The expression of the IL-6 gene marker was selected for this purpose, since its expression was strongly attenuated by incubation with BG20. From preliminary experiments, it is known that within 72 h, an average of 0.35 nmol/µL calcium is released from a test sample BG20\(^{[37]}\). Here, 200 µL of normal medium was conditioned over 72 h with one BG20 or PLLA specimen as control. Released calcium ions were bound by the addition of the specific calcium chelator EGTA (ethylene glycol tetraacetic acid, Sigma-Aldrich). In respective assays, EGTA was added equimolar to the putative released calcium in a volume of 2 µL. In each case, 1 × 10⁴ MSCs/well were incubated for 72 h in a 96-well plate with the conditioned media with or without EGTA. This was followed by analysis of IL-6 gene expression as described in the previous section. The experiment was conducted 3 times with two technical replicates each.

2.11. Immunostimulatory potential of the composite

Whole-blood stimulation assay was used to investigate the immunostimulatory potential of BG20. The assay was performed as previously described with the following modifications\(^{[41]}\). Blood was collected using lithium heparin-coated Monovette (Sarstedt, Nürnberg, Germany) from three healthy volunteers (N.S., S.A., and D.H., one female and two males, mean age 39 ± 14 years). In each case, 500 µL blood were mixed in endotoxin-free sealable tubes at a ratio of 1:4 with Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Bleiswijk, Netherlands) and three test specimens (PLA or BG20) were added to each mixture under sterile conditions. Only normal medium was used as negative control, and the positive control was stimulated with lipopolysaccharide (LPS) (5 µg/mL, Sigma-Aldrich). After 24 h incubation at 37°C, 5% CO₂, the cell supernatant was separated by centrifugation (15 min, 1100 ×g, 4°C), aliquoted, and stored at −80°C until analysis.

Analysis of secreted mediators in the supernatant was performed semi-quantitatively using the Proteome Profiler Human XL Cytokine Array Kit (RnD-Systems, Minneapolis, Minnesota, USA), according to the manufacturer’s instructions. Complete parameter list is available at https://www.rndsystems.com/products/proteome-profiler-human-xl-cytokine-array-kit_ary022b, (accessed on 09/24/2021). Membranes were photographed immediately after the addition of the chemiluminescent substrate using a Fusion FX7 gel scanner (Vilber Lourmat, Eberhardzell, Germany). The exposure time was 5 min for each membrane. The images were saved as uncompressed 16-bit TIFF files. The densitometric analysis of the spots was performed using ImageJ. For this, the product of spot area (pixel) and mean gray value was calculated for each spot. Subsequently, the results were normalized to the reference spots (n = 6/membrane).

2.12. Statistics

Statistical comparisons were made using the nonparametric Kruskal–Wallis test with Bonferroni-Holm corrected post hoc analysis according to Dunn. \(P < 0.05\) indicates statistically significant differences. \(P\) values between 0.05 and 0.1 were considered statistical trends. Statistical analyses were performed using Bias 11.12 software (Epsilon Verlag, Darmstadt, Germany). Unless otherwise indicated, results are presented as box plots of the median and interquartile ranges (median 50%, quartile 25%, and quartile 75%) in diagrams.

3. Results

3.1. Filament, test specimen, and scaffold properties

Filaments with a BG content of up to 20% can be produced. The BG is distributed homogeneously in the filaments and can be found both in the center of the strand and at the interfaces (Figure 1B, black arrows). The entire strand is interspersed with BG particles (Figure 1B and C). A more detailed characterization of the filament can be found in Schätzlein et al. (under submission).

With all four filaments, high-resolution 3D printing is possible on a standard FFF printer. Thereby, the strand thickness in the body seems to be independent of the BG
concentration at about 200 µm. It is noticeable that the number of cracks, dehiscence, and holes increases with increasing BG content (Figure 1B, white arrows). The flat test specimens used show differences with regard to the front and back sides. The side facing the print bed is smooth and flat (due to the surface properties of the heated bed), while the top side has the desired roughness achieved by the printing process. For the experiments, only the populated rough surfaces were pointing upward and were investigated. This was controlled for each specimen by a magnifier.

The core property of the material presented here is its use in high-resolution 3D printing. A scaffold design with complex internal structure based on PLA previously developed by our group served as a reference [1]. It turned out that this structure could also be printed with PLA/BG 20% composite. Porous structural elements with pore sizes of less than 150 µm are possible (Figure 2). Total porosity estimated by modified Archimedes principle was 32%, whereas the calculated value was 28%.

3.2. Characterization of the MSC pool

Characterization of pooled MSCs revealed that the cells were positive for the expression of surface markers such as CD73, CD90, and CD105, and were negative for the expression of hematopoietic markers such as CD34 and CD45. Thus, the cells exhibited a typical MSC phenotype. In addition, we investigated the potential for trilineage differentiation. After running appropriate differentiation protocols, calcium deposition as marker of OD was shown, accumulation of fat droplets was evidenced after adipogenic induction, and formation of a dimethylmethylene blue stainable MSC pellet, evidencing the presence of sulfated glycosaminoglycans, demonstrated successful chondrogenic differentiation (Figure 3).

3.3. MSC adhesion dependent on BG content in the scaffold specimen

Fluorescence microscopy images taken 24 h post-seeding revealed MSC in comparatively low density on PLA as BG material. Cell density increased with BG content and visually reached the highest densities at 10% and 20% BG content. Furthermore, Figure 4E-H shows BG-dependent colonization with MSC. The cell density increases with increasing BG content. The MTT assay revealed an increase in metabolic activity of cells with the higher BG contents. At 24 h time point, metabolic activity of BG10 and BG20 was significantly enhanced compared to PLA group, and cells in BG20 material showed higher activity as BG5. These differences decreased after 72 h and 168 h. Here, the highest median values were recorded in the BG10 and BG20 groups, despite that, no significant differences could be detected compared to the PLA control. It is also noted that metabolic activity does not continue to increase over time on any scaffold specimens and remains approximately at the levels detected at 24 h (Figure 4I).

The influence of the PLA/BG scaffolds on OD was investigated at different levels. In the coinubation experiment, the extent of calcium deposition by MSC was semi-quantitatively analyzed in pure PLA specimen and PLA/BG20 specimen under control and osteogenic culture conditions. The MSCs were seeded to the well 48 h before the experiment in normal growth medium (control) or OD medium. The test material was placed in inserts with 3 µm diameter pores in the respective wells, ions released from the test material could pass the

Figure 1. Light microscope images of the test specimens (diameter: 5 mm, height: 0.3 mm) with section magnification. (A) and (C) pure PLA, (B) and (D) PLA/BG 20%. White arrows in figure (B) show holes and dehiscence between the individual filament strands. Black arrows show BG particles at interface. Black arrows with white frame show BG particles in image (D).

Figure 2. 3D-printed scaffolds for bone tissue engineering with complex internal structure made of pure PLA (A) and the composite PLA/BG 20% (B). Even with a BG content of 20%, fine porous 3D structures in the sub-millimeter range can be realized.
Söhling, et al. Tissue engineering using bioprinting technology can improve tissue regeneration and function. This study aimed to evaluate the effectiveness of a specific bioprinting technique in promoting cell growth and differentiation. The researchers used a combination of poly-lactic acid (PLA) and bioactive glass (BG20) to create scaffolds with interconnected pores. There was no direct contact between test materials and cells. Cells were incubated for 14 days. Calcium deposition was visualized by Alizarin Red staining. To avoid staining artifacts on the PLA/BG20 material, cells were cultured without direct contact with scaffolds. Alizarin red staining revealed that BG20 did not induce

**Figure 3.** Representative FACS-analysis is shown in (A). Antigen expression (black line) and respective isotype controls (filled histograms) are presented. MSCs did not express hematopoietic markers CD45 (A) and CD34 (B) and were positive for established MSC antigens CD73 (C), CD90 (D), and CD105 (E). Differentiation potential is depicted in (B). MSCs were capable for osteogenic, adipogenic and chondrogenic differentiation after the application of corresponding differentiation protocols. Evidence for osteogenic (calcium precipitation visualized by Alizarin Red staining) (B), adipogenic (intracellular lipid droplets visualized by Oil Red O staining) (D), and chondrogenic (DMMB staining after 21 days in pellet culture) differentiation (E). (A, C) MSCs cultured in control medium.
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MSC calcium deposition after 14 days of incubation under control conditions. Furthermore, BG20 did not promote increase in calcium deposition under osteogenic conditions compared to the PLA test specimen (Figure 5).

Next, the effect of the BG component on the expression of prototypical osteogenic marker genes was analyzed over time for 7 days. Gene expression of the osteogenic transcription factor RUNX-2 was increased after 24 h on median in the BG10 and BG20 groups compared to PLA group, however, this was not statistically significant after alpha correction was applied ($p^* = 0.01$ BG10 vs. PLA, $p^* = 0.03$ PLA vs. BG20). In the further course, RUNX-2 gene expression in the BG10 and BG20 groups decreased, in some cases significantly, compared to the corresponding 24 h values. ALP gene expression was initially increased in the BG5 group, however, it was significantly elevated throughout time only in BG20 preparations. At time 168 h, ALP gene expression was significantly increased in BG20 group compared to BG10 group and in trend ($p = 0.08$) compared to BG5 and PLA preparations. COL1A gene expression increased significantly over the course to 72 h in some cases (BG10 group) and declined to the 24 h levels after 168 h. No significant differences on the relative expression of COL1A could be detected among the different groups at any time point (Figure 6).

3.4. Influence of BG scaffolds on inflammatory processes

The regulation of inflammatory signaling pathways in MSC, which are involved in cell differentiation processes and play an important role in inflammatory response in situ, was investigated at the gene expression level. Results demonstrate that IL-6 gene expression was significantly suppressed with increasing BG content (BG10, BG20) over the entire observation period. The MAP kinases (MAPK8 = JNK1, MAPK14 = p38) are involved in signal transduction, most of which lead to a pro-inflammatory response through activation of NFkB. In particular, relative expression of MAPK8 was significantly downregulated in MSC cultured on scaffolds containing high levels of BG. Thus, MSC cultured on BG20 exhibited the significantly lowest MAPK8 gene expression over the entire observation period. Gene expression of MAPK14 was not subject to this marked regulation, but significant suppression by BG20 was also measured for this gene at the 72 h and 168 h time points (Figure 7).

3.5. Calcium released from BG contributes to anti-inflammatory BG effect

To test the hypothesis that calcium ions from the BG component of the scaffold are responsible for the IL-6
suppression after its release, calcium was immediately complexed after release by the selective calcium chelator EGTA, proportionally added to the medium. MSCs were incubated for 72 h with control medium, with medium conditioned 72 h with BG20 or medium conditioned 72 h with BG20 and EGTA. IL-6 gene expression was evaluated as a readout parameter. Significant inhibition of IL-6 gene expression by BG20 conditioned medium was recorded. This suppression was almost completely absent by the addition of EGTA (Figure 8).

3.6. Immune Stimulating potential of BG20

Immune stimulating potential of BG20 was determined by whole-blood stimulation assay. Of a total of 105 different serum peptide mediators, 23 were detected in medium control, 24 in PLA control, 31 in LPS control, and 23 in BG20 group. Figure 9 shows the relative expression levels of the mediators depending on the kind of stimulation applied to whole-blood samples. Table 1 shows expressed proteins and their main functional assignment.

4. Discussion

In this study, a 3D printable biomaterial made of PLA and a BG content of up to 20% was investigated for cytocompatibility and immunoreactive potential. Pooled human MSCs were cultured on bilayer printed disk-shaped test specimens, provided with increasing BG fractions, and gene expression was determined. Furthermore, osteogenicity and inflammatory potential of the material were analyzed. Osteogenic genes underwent only slight changes in activity, with only ALP gene expression increasing significantly after 7 days. Further in vitro analyses showed correspondingly that an increasing BG fraction did not lead to an improved OD of the MSC, measured by calcium deposition. Interestingly, the BG fraction significantly inhibited prototypic inflammatory processes in MSC at the mRNA level. IL-6
gene expression was almost completely inhibited over the observation period of 7 days, as well as gene expression of the MAP kinases JNK-1 and p38 was significantly suppressed. The final whole-blood stimulation assays supported these results, as the material did not lead to significant stimulation of pro-inflammatory mediators. Mechanistic analyses using IL-6 gene expression as a readout parameter suggest that calcium ions released from the BG component are causative for the anti-inflammatory effect of the material.

4.1. Composite material

Pure PLA is used as the standard material in 3D printing. Due to its high melting point around 200°C and its beneficial rheological properties structures with feature sizes down to 100 µm, PLA can be readily fabricated using commercially available 3D printers. Furthermore, it is 100% biodegradable. Unfortunately, it has low affinity for cells such as MSC or human osteosarcoma cell line (Saos-2) and exerts no or only little osteoconductiv or osteoinductive influence on applied cells, such as MSC or endothelial progenitor cells (EPCs)[1,7]. Instead, a composite of PLA with BG showed a stimulation of EPC differentiation and function. Apoptosis rate compared to pure PLA was decreased[7,42].

For composite preparation, both components are mixed and melted together. The BG component used here is commercially available in various particle sizes. For the filament used in this study, BG granules in the size range of 25-42 µm were used. The integrated BG particles require the application of a nozzle with a diameter of at least 400 µm for printing to avoid clogging. Still, fine structures with a resolution below to one-fourth of the nozzle diameter could successfully be printed.

The material exhibits a homogeneous distribution of BG particles, which are localized both near the surface and centrally in the filament (Figure 1). This allows the continuous release of bioactive ions during the degradation process. The dose-dependent accumulation of calcium ions from the test particles in initially calcium-free PBS could be demonstrated by us[37]. The calcium concentration was about 0.35 nmol/µL after 7 days of incubation of a BG20 sample. Moreover, it is conceivable that the BG particles alter roughness of the PLA surface, which could facilitate the attachment of MSCs[43]. This is clearly indicated by the SEM images with the increased cell affinity with increasing BG content (Figure 4).

The core property of the composite material should be its use in high-resolution 3D printing. This is mandatory to construct complex scaffold designs with biologically active structural elements beyond the classical grid with its monotonous pore structure[1,31]. In addition to the test specimens used for the experiments, it was also possible to print scaffold designs with complex internal structure from the composite filaments with 20% BG content (Figure 2). This clearly shows that the BG content also influences the print result. A high BG content leads to lower strand adherence. Pores and crevices appear on a micrometer scale (Figure 1). Overall, there is also a loss of stability, which is quantified by Schätzlein et al. Furthermore, porosity slightly differs due to the fact that printed parts have slightly different surface, resulting from the layer topography by the printing process.

4.2. BG20: Influence on OD

It is well known that BG supports the differentiation of various progenitor cells (MSC, EPC, and osteoblasts)[41]. For example, Aguirre et al. showed that a composite...
material of BG (G5 glass) and PLA enhanced the differentiation of EPC and their angiogenic activity\cite{45}.

The present study shows the OD of MSC under the influence of BG.

The MSC gene expression profile shows a characteristic course during the different phases of OD. In this experiment, the focus was on the early phase of OD up to day 7. For this phase, a significant increase in the \textit{ALP} gene expression was proven\cite{46}. This is in line with our experimental outcomes. For MSCs cultured in standard medium on BG20, a significant increase in \textit{ALP} gene expression was measured at day 7 compared to the PLA control.

As an essential transcription factor for OD, \textit{RUNX2} is expressed in the early phase after induction of OD\cite{47}. Here, \textit{RUNX2} gene expression was increased when the cells were cultured on BG10 and BG20, albeit without statistical significance compared to PLA control. Significant decrease of \textit{RUNX2} gene expression in MSC cultivated on BG20 has been shown after 72 h and 168 h. The rapid decline of \textit{RUNX2} expression as one initial step of OD in MSC is also congruent with previously reported work on osteogenic MSC differentiation\cite{35}. Nevertheless,
this rather pro-osteogenic gene expression pattern did not result in a significant calcium deposition after 14 days of incubation. A significant calcium deposition could only be achieved by coincubation with OD medium whereas the extent of calcium deposition between PLA control and BG approach was at a similar level.

These findings suggest that the BG components are sufficient to initiate and/or support OD processes in MSCs, but that further signals are required to achieve complete OD. These signals could be exogenous factors such as BMP-2 and 7 or mechanical stimuli\[48,49\].

An interesting aspect that emerged in this work is the marked suppression of key pro-inflammatory genes when cells were cultured on BG20. MAP kinases (JNK1 and p38) are involved in signal transduction leading to a pro-inflammatory response mostly through activation of nuclear factor kappa B\[50\]. In particular, gene expression of JNK1 was significantly downregulated in MSC cultured on samples with high BG contents (10% and 20% BG). Gene expression of p38 was not subject to this significant regulation, but the highest median levels were always in the PLA group and the lowest median levels in the BG20 group. IL-6 gene expression was also attenuated in MSC cultured on BG10 and BG20. There is increasing evidence that MSCs are maintained in a proliferative state through endogenous IL-6 and components of the IL-6 pathway\[51,52\]. We believe that the observed decrease in IL-6 gene expression as well as key inflammatory signal transduction components indicate the onset of OD, induced by the BG component. Pricola et al. (2009) showed that “IL-6 gene expression is significantly higher in undifferentiated MSC compared with chondrogenically, osteogenically, and adipogenically differentiated MSC”\[52\]. Thus, ERK1/2 activation has been shown to act as a primary signaling pathway by which IL-6 regulates both MSC proliferation and inhibition of differentiation\[52\].

JNK-1 belongs to the MAPK family and is ubiquitously expressed in all cells. It is activated by various stimuli, such as inflammatory cytokines and growth factors, and phosphorylates various downstream proteins, including the transcription factors c-JUN, ATF-2, and ELK1\[53\]. However, their specific role in MSC has only been studied from certain aspects and suggests relevance

| Function                        | Medium | LPS          | PLA | BG20         |
|---------------------------------|--------|--------------|-----|--------------|
| Primary inflammation            | ---    | TNF-α        | --- | ---          |
| Anti-inflammation               | IL-18BPa | IL-18BPa     | IL-18BP | ---          |
| Chemotaxis                      | CXCL4 (PF4) | CXCL5 (ENA-78), CXCL8 (IL-8), MCP-1, MIP-1α, MIP-3α, CXCL4 (PF4), CCL5 (RANTES) | CXCL4 (PF4), CCL5 (RANTES) | CXCL4 (PF4), CCL5 (RANTES) |
| Cell activation (growth, proliferation) | ---    | IL-24       | --- | MIF          |
| Complement                      | C5a, Compl. Factor D | C5a, Compl. Factor D | C5a, Compl. Factor D | Compl. Factor D |
| Angiogenesis                    | Angiogenin | Angiogenin, Thrombospondin-1 | Angiogenin, Thrombospondin-1 | Angiogenin, Thrombospondin-1 |
| Tissue repair (remodeling)      | Chitinase 3-like protein 1, MMP9, Osteopontin, Serpin E1 | Chitinase 3-like protein 1, MMP9, Osteopontin, Serpin E1 | Chitinase 3-like protein 1, MMP9, Osteopontin, Serpin E1 |
| Peptide hormone                 | Adiponectin, Leptin | Adiponectin | Adiponectin, Leptin | Adiponectin, Leptin |
| Surface receptor                | Endoglin, CD31, VCAM | Endoglin, CD31, VCAM | Endoglin, CD31, VCAM | Endoglin, CD31, VCAM |
| Other (transport, osaponization, proteolysis, regulation) | Apolipoprotein-1, Vitamin D-BP, CRP, DPP-IV, IGF-BP2, IGF-BP3, Lipocalin-2, RBP4, SHBG | Apolipoprotein-1, Vitamin D-BP, CRP, DPP-IV, IGF-BP2, IGF-BP3, Lipocalin-2, RBP4, SHBG | Apolipoprotein-1, Vitamin D-BP, CRP, DPP-IV, IGF-BP3, Lipocalin-2, RBP4, SHBG |
| Sum                             | 23     | 31           | 24  | 23           |

Medium (DMEM) without serum served as negative control. Secreted factors were categorized in terms of their putative major function.
to MSC proliferation, induction of cell migration, and inhibition of cell differentiation processes\textsuperscript{[54,55]}. Thus, the strong suppression of \textit{JNK-1} gene expression might contribute to the reduction of proliferation and support of cellular differentiation processes.

IL-6-mediated activation of \textit{p38} is essential for adipogenic differentiation of MSC\textsuperscript{[56]}. In this way, the decreased \textit{p38} gene expression after incubation of MSC on BG20 could be interpreted as a suppression of adipogenic differentiation and as further evidence for preferential OD.

Taken together, these results indicate good cytocompatibility of BG20 for MSC. Cell adhesion is high and gene expression analyses indicate that OD processes are at least initiated by the material. The BG component is composed of 53\% SiO\textsubscript{2}, 23\% Na\textsubscript{2}O, 20\% CaO, and 4\% P\textsubscript{2}O\textsubscript{5}. The previous studies by our group on the effect of BG on EPCs showed that especially calcium ions released from BG supported cell differentiation\textsuperscript{[37]}. To investigate a possible influence of calcium ions released from BG20 on MSC, we performed an analogous experiment with BG20. Calcium ions were selectively bound by EGTA, and the amount of EGTA added was based on the expected calcium ion release, which was previously determined by colorimetric detection\textsuperscript{[37]}. \textit{IL-6} gene expression was chosen as the readout parameter because it is rapidly and significantly downregulated by BG20. This BG20 induced inhibition of \textit{IL-6} gene expression could be almost completely abolished by EGTA addition. This result is a clear indication that calcium ions released by BG20 are causative for the observed effects. It cannot be excluded that other ion species of the BG component also exert effects on MSC differentiation. However, this has not been analyzed further in this study.

### 4.3. BG20: Immunological compatibility

Another significant aspect is the reaction of the immune system to the implanted bone graft substitute. The initial step of the foreign body reaction consists of the deposition of proteins such as fibrin and the binding and activation of monocytes. These, in turn, secrete chemotactic factors, leading to the accumulation of further monocytes, granulocytes, and fibroblasts\textsuperscript{[57]}. Immunoreactive materials such as polymethyl methacrylate-based bone cements elicit a strong foreign body reaction, leading to encapsulation of the implanted material with a fibrous membrane\textsuperscript{[58]}. The onset of foreign body reactions has also been described for other bone graft substitutes\textsuperscript{[59]}. Whole-blood stimulation assay is an established method to predict the response of the innate immune system to various stimuli. In the previous work, our group was able to demonstrate that different bone substitutes can elicit characteristic and reproducible cytokine expression profiles in the whole-blood stimulation assay in terms of an immunological signature\textsuperscript{[60]}. In the present work, test specimens made of PLA or BG20 were used in the whole-blood stimulation assay. Subsequently, 105 different mediators were semi-quantitatively determined using a protein array. Of the 105 different mediators, which include interleukins, chemokines, and complement factors, 23 were detected in the medium control, 24 in

**Figure 9.** Inflammatory potential of BG20 (yellow) in whole-blood stimulation assay I (\textit{n}=3 subjects) in comparison to medium (blue), medium containing LPS (orange), and PLA (gray) measured by whole-blood stimulation assay I (\textit{n}=3 subjects). Proteins in the supernatant were detected semi-quantitatively by means of a Proteome Profiler Membrane assay. Median values are shown.
the PLA control, and 23 in the BG20 approach. In the LPS approach (5 \( \mu \)g/mL), the expression of 31 mediators could be induced, including primary immune activators such as TNF-\( \alpha \) and chemokines such as CXCL8 (IL-8), CCL2 (MCP-1), CCL3 (MIP-1\( \alpha \)), CCL20 (MIP-3\( \alpha \)), and CXCL5 (ENA-78), which exert potent chemoattractant and stimulatory effects on monocytes and/or granulocytes\[^{61-65}\]. The factors induced in the PLA and BG20 approaches are part of cascades such as primary inflammatory, anti-inflammatory, chemotaxis, cell activation (growth, proliferation), complement, angiogenesis, tissue repair, tissue remodeling, peptide hormone, surface receptor, and other (transport, opsonization, proteolysis, and regulation) and are similarly expressed compared to the medium control. The lack of induction of strongly inflammatory cytokines, as observed in the LPS approach, suggests a good immunological compatibility of BG20. Whether this assessment correlates with a low foreign body reaction, \textit{in vivo} remains to be analyzed in appropriate animal experiments, for example, implantation of the material in subcutaneous pockets in the rat\[^{59}\].

5. Conclusion

The present study demonstrated that a 3D printable material made of PLA with 20% BG content exhibits high cytocompatibility for MSC, probably supports OD, and has a low and thus beneficial immunostimulatory potential. However, whether 3D-printed bone graft substitutes made from this material support healing of large bone defects needs to be determined in appropriate \textit{in vivo} experiments and additionally also depends on factors such as scaffold design.

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Conflict of interest

The authors declare no conflicts of interest.

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