INTRODUCTION

Calcium phosphates are widely used as biomaterials and bone regeneration is one of their most important applications, being considered a main topic in regenerative medicine\(^1,2\). In general, scaffolds serve as basis component for the repair of bone defects. Three-dimensional printing allows manufacturing those scaffolds with defined and reproducible internal structures directly from computed-tomography (CT) data\(^3\). Tissue engineering (TE) is highly important for bone repair, so that repair of damaged and diseased tissue is the principal goal of all tissue engineering efforts\(^8,9\). TE is an interdisciplinary field of research, which tries to create artificial tissues or tissue replacement systems in vitro in order to support or substitute damaged tissue or organs. For TE it is necessary that cells can ingrow and interact with a printed scaffold. The requirements for the scaffolds are characterized by a complex internal structure with a pore diameter above 300 \(\mu\)m to improve a good vascularization and attachment of bone cells to guide their growth in all dimensions\(^10\). A highly porous, open pored, fully interconnected geometry has to be designed in order to ensure nutrient supply and waste removal of cells seeded on scaffolds. The technique of three-dimensional (3D) printing allows to fabricate scaffolds based on calcium phosphate with a complex internal structure similar to mimic bone structures and a high porosity between 50 and 90%\(^11\). Today, calcium phosphates are approved as promising materials to build scaffolds with the required properties\(^13\). Scaffolds can be generated from hydroxyapatite (HA) or tricalcium-phosphate (TCP). Both these calcium phosphates offer different degrees of osteoclastic resorbability and solubility\(^14\). TCP has also proven to be of good biocompatibility and biodegradability and is highly osteoconductive\(^15\).

Healing of bone defects requires cell proliferation and new matrix production to bridge the damaged tissue. Printed and sintered TCP scaffolds can be used for guided bone tissue engineering with patient cells, seeded onto the scaffolds for a better compatibility in the organism. Although small defects heal without substituting the lost material, in more extensive defect sites the natural healing process has to be supported in order to regain full functional capacity. Due to the high porosity 3D printed calcium phosphates scaffolds are brittle and do not have high mechanical stability. This fact considerably limits their clinical application, especially with respect to the repair of large bone defects. A possibility to achieve better mechanical properties is offered by the infiltration with biodegradable polymers\(^16\). Therefore, the aim of this study was to evaluate the influence of infiltration of 3D printed tricalcium phosphate scaffolds with biodegradable polymers significantly improved mechanical properties and biological properties were comparable to those of uninfiltred TCP scaffolds.

MATERIALS AND METHODS

3D printing process

The printing technique is a powder based process,
which starts with a stack of 2D bitmaps obtained from a slicewise 3D dataset bitwise divided. The three dimensional printer uses these two dimensional bitmap files, that represents the printing matrix, to build a 1:1 physical model of the computer data. The 3D printing process works as follows: A defined layer with a thickness of 250 µm calcium phosphate granulate is deposited on the building platform. According to the printing matrix a printhead ejects liquid binder into the powder bed surface. The aqueous binder of dextrin (20 wt%) and saccharose (2.5 wt%) bonds the ceramic powder in the selected wetted regions. The weight of the droplets ejected from the printhead is between 7–9 µg. The printing raster solution was chosen to be 0.25 mm for all specimens. After each printed layer the building platform moves down by the thickness of a layer and new powder is deposited upon the last layer. Afterwards the process restarts and prints the next layer. The scaffolds are surrounded by unbound powder, which supports the printed object. After completion, the 3D printed green scaffold is removed from the building platform. All unbound powder from the internal structure of the green scaffold is manually removed by airflow. Details of the process have been published previously19).

Granulate for the 3D printing
The calcium phosphate granulate TCP 4 was supplied by BioCer Entwicklungs (Bayreuth, Germany) and used for 3D printing of scaffolds. CaP based granulates with the desired properties are fabricated from CaP slurries by spray drying. Further details about the granulate have been published previously8). Furthermore, it is well known that tricalcium phosphate has a significantly better degradability compared to hydroxyapatite20,21).

Sintering
Finally, the 3D printed specimen is consolidated in a sintering process. The specimen is sintered for 2 h at 1,250°C in an electrically heated chamber furnace (Nabertherm, Lilienthal, Germany) in ambient air. The organic binder is removed by pyrolysis during the sintering process and 3D printed part obtains its final properties during sintering. Due to large voids between the particles in the green state, significant sintering shrinkage occurs by up to 30%8).

In order to characterize the crystalline phase of the tricalcium phosphate used in this study, TCP cylinders (diameter 7 mm, height 5 mm) before and after sintering were analyzed by X-ray diffraction, XRD Siemens D5000, (Siemens, Munchen, Germany) using Co Kα radiation (λ=1,78897 Å). Samples were powdered with agate mortar and pestle. Data were collected in the range of 2θ=20°–75° in increments of 0.05°.

Scaffold geometry
The fabricated scaffolds have highly defined characteristics and precise dimensions. The interconnecting cavities were measured with the light microscope Olympus SZX9. The internal structure and external shape can be fabricated by using Computer-aided Design and Computer-aided Manufacturing (CAD/CAM). The sintering process results in a significant shrinking of up to 30%9 of the printed scaffolds, so that the change in dimension needs to be precalculated. The outer dimensions of the sintered specimen for the mechanical characterization were 10 mm in diameter and 20 mm in height and for the biocompatibility tests 7 mm in diameter and 5 mm in height. All sintered cylinders have a complex internal structure comprised of horizontal and vertical channels. This typical structure was used in order to ensure nutrient supply and waste removal of cells seeded on scaffolds.

Biopolymers
Four different biodegradable polymers have been chosen for infiltration. Each polymer influences the mechanical stability of the infiltrated scaffolds. The biodegradable copolymers used in this study have a 50:50 molar ratio of poly(D,L-lactide-co-glycolide) (Resomer® RG 503H), 70:30 molar ratio of poly(L-lactide-co,D,L-lactide) (Resomer® LR 706 S), 80:20 molar ratio of poly(L-lactide-co-glycolide) (Resomer® LG 824 S) received from Boehringer Ingelheim (Ingelheim, Germany) and Polyhydroxybutyrate (PHB) obtained from Goodfellow (Bad Nauheim, Germany). Furthermore all poly(D,L-lactide-co-glycolide) have different degradation times. RG 503 H needs approximately 3 months, LG 824 S 18 months and LR 706 S 36 months to degrade according to the manufacturer. The degradation time of different varieties of PHB was described in detail by Freier22).

Method of infiltration
Biodegradable polymers can be dissolved in different organic solutions. In this study all polymers have been solved in trichloromethane (Roth, Germany). To dissolve the PHB, the polymer solution was heated to 45°C. Thus the solution process of the polymer is accelerated. For post processing TCP cylinders with 10 mm diameter, 20 mm height, horizontal and vertical cavities for the mechanical characterization and TCP tablets with 7 mm diameter, 5 mm height for biocompatibility tests were inserted into a glass tube. Afterwards every glass tube is filled with a one percent polymer solution of LG 824 S, LR 706 S, PHB and five percent solution of RG 503 H was added for 30 min. The glass tube was turned every 5 min. After 30 min all the TCP scaffolds were removed from the glass tube and placed on a paper towel. After 24 h of drying in the air, all samples were dried again for 36 h at 35 degrees in vacuum to reduce the boiling temperature and to make sure no trichloromethane is included in the sample.

Porosity and interconnectivity
Porosity and interconnectivity of infiltrated scaffolds (TCP infiltrated with the polymer RG 503 H) were studied. Based on scanning electron microscope (SEM), the average side length of the interconnecting channels with its square cross-section was assessed. For SEM evaluation, sputter coating was performed at a working pressure of 0.3 atmospheres (30.4 kPa) at 40 mA for
60 s. Subsequently, scaffolds were scanned with an applied acceleration voltage of 10 kV in the SEM (Leo DSM 982, Carl-Zeiss NTS, Oberkochen, Germany). Own preliminary experiments showed that the open porosity of uninfiltred TCP scaffolds was 75.05±0.74%. For this study, investigation of open porosity was also carried out for infiltrated scaffolds. In this case, 2.5, 5 and 10% polymer solutions of RG 503 H were prepared. The open porosity of the infiltrated scaffolds was measured by the liquid displacement method\(^\text{[12]}\).

**Mechanical characterization**
TCP cylinders with 10 mm diameter, 20 mm height were infiltrated with the different biopolymers as described before. To determine the average compressive strength of the uninfiltred and infiltrated printed scaffolds a uniaxial testing system (Zwicki-Line) from Zwick (Ulm, Germany) was used. Each of the five scaffold types (1 uninfiltred and 4 infiltrated scaffolds) was studied six times. For the compression test the following parameters were set: (i) force shutdown threshold: 80% of maximum force, (ii) speed: 2 mm/min, (iii) max load: 2,500 N. The printing cylinder with horizontal and vertical channels was put onto the center of the lower compression plate. The compression test was started and the upper compression plate moved down.

**Cell culture**
All experiments were performed with a mouse osteoblastic cell line (MC3T3) in passage 10. The cells were cultivated using Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), 100 IE Penicillin/mL, 100 lg Streptomycin/ml (PAA Laboratories, Pasching, Austria) in a humidified incubator at 37°C with 5% CO\(_2\). During passaging, cells were detached from 75 cm\(^2\) cell culture flasks using 5 mL of a 0.05% trypsin in Phosphate Buffered Saline (PBS). After 1:1 dilution of the cell suspension was counted using a Neubauer-hemocytometer (Brand, Wertheim, Germany) and cells were seeded in new flasks at a confluence of 70%. Medium change took place every 3 days.

**Biocompatibility tests**
Cell seeding on Gamma-Ray-sterilised scaffolds with 7 mm diameter, 5 mm height were set with sterile tweezers in a 24-well culture plate (NUNC, Langenselbold, Germany). MC3T3 cells were trypsinized and counted using a Neubauer-hemocytometer (Brand). \(3\times10^3\) cells in 100 \(\mu\)L suspension were seeded on top of every scaffold. After 2 h of incubation the seeded scaffolds were covered with culture medium. Cells were kept in culture for three days prior biocompatibility test using live/dead assay. Plastic surface (NUNC) were used as control wells and were set to 100% living cells in each run. The experiment was repeated three times in triplicates.

**Live/dead-assay**
A double staining with fluoresceindiacetat (FDA) and propidium iodide (PI) (both Fluka/Sigma-Aldrich) was performed to assess cell survival. A stock solution of 10 mg FDA solved in 2 mL pure acetone was prepared and diluted in a ratio of 1:500 in PBS. To produce the final working solution 1 mL FDA/PBS solution was dissolved in 1 mL PI solution (1 mg/mL). After 1 min the staining incubation is followed by 3 times washing in PBS. For image acquisition an Axiovert S100 (Zeiss, Jena, Germany) microscope equipped with a 75 XBO W/2 was used. Viable cells were imaged with appropriate filter sets (AHF, Tübingen, Germany). Images were acquired with a black and white digital camera (AxioCam MRm) (1,388×1,040 pixels), a Zeiss Fluar 10×/0.50 objective and processed with Axiovision software, AxioVs40 V4.7.1-08-2008 (all Zeiss, Jena, Germany). Large field images with a final resolution of 5,000×5,000 pixels were taken using an automated microscopy table (SCAN IM 130×100-1 mm; Märtzhäuser-Wetzlar, Steindorf, Germany) and cells were counted with ImageJ (Version 1.44e).

**Statistical analysis**
Statistics were performed using Microsoft Excel 2011 (Redmond, WA, USA) and SPSS version 21.0 (SPSS Inc, Chicago, IL, USA). Values are given as mean±standard deviation (SD). Significant values were calculated using the Kruskal-Wallis test and a value of \(p<0.05\) was considered significant. For all experiments, a minimum of three independent experimental runs was performed.

**RESULTS**

**Printed scaffolds**
Figure 1 demonstrates the XRD pattern of the TCP used in this study and is typical for beta-tricalcium phosphate (\(\beta\)-TCP). There is no considerable difference
between the characteristic peaks of the scaffolds that were sintered and those that were not sintered.

The printing technique in this study allowed building high-resolution structures required for tissue engineering. After infiltration with polymer (five percent solution of RG 503 H), the interconnecting channels with its square cross-section showed a mean side length of 463.6±25.3 µm depicted in Fig. 3.

The macroporosity of infiltrated scaffolds measured by the liquid displacement method was 74.21±0.81% (infiltrated with 2.5% polymer solution of RG 503 H) and 70.12±0.80% (infiltrated with 5.0% polymer solution of RG 503 H). Ten percent polymer solution was very viscous and, as a result, some channels in the samples were partially closed. Therefore, the 10% polymer solution was neglected for further analysis.

**Compression tests**

Uninfiltrated TCP had an average compression strength of 1.92±0.38 MPa. Further mechanical stability was accomplished with all infiltrated TCP scaffolds. The results of the compression were 2.38±0.34 MPa for LG 824 S, 4.49±0.69 MPa for RG 503 H infiltrated TCP scaffolds, 7.36±0.57 MPa for LR 706 S and 3.41±0.71 MPa for PHB. Results are summarized in Fig. 4 and Table 1. Furthermore it was observed that the infiltrated and uninfiltred samples show different fracture characteristics during mechanical characterization (Fig. 5). The uninfiltred scaffolds crumbled in many parts whereas the infiltrated samples were still intact and did not crumble after the compression test even under extensive mechanical loading.

**Assessment of cell vitality on scaffolds**

The cell behaviour on top of the scaffolds is shown in Fig. 6. After three days, all probes presented viable MC3T3 cells. The green color of the cells due to Fluorescein Diacetate (FDA) staining demonstrated their vitality on the TCP scaffolds, while there were red cells visible related to Propidium Iodide (PI) staining, indicating some dead cells in all groups. There was no significant difference (p=0.05 level) between the control surface.
Fig. 5 Uninfiltrated TCP scaffold (left) and an infiltrated TCP scaffolds (right) after compression test.

Fig. 6 Live/dead assay of MC3T3 cells on scaffolds. Cell viability (cell survival in %) of cells seeded three days on the scaffolds prior analyses. The control wells on a plastic surface were set to 100% in each run.

and the uninfiltrated TCP as well as the TCP scaffolds infiltrated with PHB, RG 503 H and LG 824 S. The infiltration with LG 824 S or LR 706 S respectively leads to a significant ($p<0.05$) induction of cell death within 3 days compared to the control surface (Fig. 6). The most suitable scaffold regarding cell survival was detected on TCP PHB scaffolds with 94±10% (mean±SD) living cells, slightly ahead of RG 503 H infiltrated TCP scaffolds. Also in the center of RG 503 H infiltrated TCP scaffolds, high level of cell survival was observable (Fig. 7).

DISCUSSION

Rehabilitation of large bone defects is still a considerable challenge both in orthopaedic surgery as well as oral and maxillofacial surgery. So far, autologous bone transplants are seen as the gold standard, however their use is associated with a number of shortcomings. Autografting strategies can result in severe complications, donor site morbidity and, especially important in the craniomaxillofacial area, inappropriate shape of the transplant$^{23,24}$. Bone tissue engineering (BTE) is a recently evolving alternative to autologous bone grafting. Among others, calcium phosphate scaffolds can serve as a matrix for BTE. By combining calcium phosphate ceramics with polymers, the mechanical properties of both entities can be greatly enhanced. Thus the problem of brittleness and the difficulty of shaping hard ceramics can be overcome. Different biodegradable polymers were tested as infiltration material for 3D-printed scaffolds.

The printing technique in this study allowed building high-resolution structures with interconnecting channels measuring around 450 to 500 µm as required for tissue engineering. Furthermore, the macroporosity of polymer infiltrated scaffolds was assessed in this study. In comparison to uninfiltrated TCP scaffolds (macroporosity of 75.05±0.74%), scaffolds with infiltration of 2.5 and 5% RG 503 H showed only slightly decreased porosity (74.21±0.81 and 70.12±0.80%) due to the fact that the polymer film is only coating the scaffold surface and does not infiltrate the microstructure itself. A macroporosity between 70 and 75% meets the requirements of TE applications$^{11,12}$. However, solutions with a high polymer concentration are viscous and therefore may obstruct the interconnecting pores.

During the manufacturing process, some typical effects of 3D printing could be observed. In order to reduce these effects, some arrangements were realized. A typical effect is shrinkage. There are two kinds of shrinkage that occur during manufacturing in 3D printed scaffolds. Firstly, printing shrinkage is due to gluing of the granules by the binder fluid. During shrinking, the effect of the curling of the first layers appears. To minimize the curling of the layers a droplet size of 6–9 µg/drop were used. To optimize the printing process each scaffold was placed on a base in order to reduce the effect of the curling. After printing the base can be removed from the scaffolds. Secondly, shrinkage can be observed during sintering. The sample can be sintered on the base...
to reduce a deformation with the sintering procedure. This procedure is to be particularly considered with large samples. In clinical applications it is important to take the overall dimensional change of about 30 % during the fabrication process into account. Therefore, a clinical CAD/CAM workflow has to be adjusted accordingly.

Superior mechanical properties can be realized by the infiltration of bioactive ceramics like TCP with biodegradable polymers\(^{16-18}\). Furthermore, there are alternative ways in order to fabricate composite scaffolds made from ceramics and biopolymers like fused deposition modelling of polycaprolactone/tricalcium phosphate or prepared nano-hydroxyapatite/poly(L-lactic acid) (nano-HAP/PLLA) composite using thermally induced phase separation techniques\(^{25-27}\). These composite scaffolds come along with favourable mechanical and biochemical properties, together with favourable degradation behaviour and resorption kinetics. In order to maximize the scaffolds’ compressive strength, concentrations of the polymer solutions were intended to be as high as technically practicable. In case of RG 503 H, solutions up to 5% showed a viscosity that was low enough to guarantee proper infiltration into the TCP scaffold. 2.5 or 5% solutions of all other polymers, however, were too viscous to allow infiltration of TCP and, consequently, only 1% solutions were used. In analogy to other studies, in this work it was apparent that infiltrated scaffolds demonstrated better average mechanical compression strength than uninfiltred samples. The average compression strengths were improved by infiltration of LG 824 S and PHB. A further improvement of mechanical stability could be achieved with RG 503 H. The best results were achieved with LR 706 S infiltrated samples, whereby the mechanical compression strength could be quadrupled from 1.92±0.38 MPa (TCP) up to 7.36±0.57 MPa (TCP infiltrated with LR 706 S). Mechanical characterization allowed for differentiating fracture characteristics of infiltrated and uninfiltred samples. The infiltrated samples were still intact after extensive mechanical loading. In contrast, the uninfiltred scaffolds crumbled in many parts. From a clinical point of view, this might be an advantage in terms of implant failure. Small particles separated from the main implant during the insertion process can pose an origin of infection.

Furthermore, cell survival was determined on all scaffolds. Three-dimensional scaffolds are often used in combination with biological factors such as cells or specific proteins\(^{28,29}\). The goal of this approach is to enhance tissue regeneration by initially maintaining mechanical function until the healing tissue can bear load itself and by supporting the natural healing process by providing osteoinductive growth factors and/or viable metabolically active cells respectively. The scaffold material as such should be biocompatible and resorbable so that it allows replacement by newly formed bone in the long term\(^{30}\).

TCP is known to have the property of complete biodegradation with no adverse biological response and also to possess a good biocompatibility. The advantage of appropriate porosity allows seeded cells to migrate and proliferate on top of the scaffolds\(^{31}\). PHB degradation and biocompatibility has been widely investigated over the last years. A good biocompatibility has been shown with seeded osteoblasts or epithelial cells. However, its high brittleness and low degradation have limited its application\(^{32}\). The review from Jendrossek et al. summarizes the biochemical and molecular-biological properties of polyhydroxalkanoic acid depolymerases, which initiate the biodegradation process\(^{33}\).

Composite scaffolds made from bioactive ceramics and polymers do not only show enhanced mechanical properties. These scaffolds also demonstrate good biocompatibility and bioactivity stimulating proliferation and differentiation of osteoprogenitor cells \textit{in vitro} and \textit{in vivo}\(^{34-36}\). Further enhancement of bioactivity can be achieved by growth factors like BMP-2 or VEGF as well as by seeding of stem cells\(^{36,35,37-39}\). With respect to cell behaviour on top of the scaffolds, no cytotoxicity was observed in this study, and cells on all scaffolds largely remained viable. Live/dead-assays highlighted best cell survival for PHB and RG 503 H infiltrated TCP scaffolds. In contrast, the two groups in which TCP was infiltrated with LG 824 S or LR 706 S respectively demonstrated statistically significant reduced cell survival compared to the control surface. Overall, the infiltrated scaffolds showed not only a significant improvement of compression strength but also concurrently good biocompatibility and maintained their osteoconductive or osteogenic potential.

**CONCLUSION**

In conclusion, infiltration of 3D printed TCP scaffolds with biodegradable polymers significantly improved mechanical properties compared to uninfiltrated TCP scaffolds. Moreover, the infiltrated scaffolds demonstrated appropriate biocompatibility. In consideration of the present research results, studying both mechanical stability and cell survival, TCP infiltrated with the polymers RG 503 H or PHB respectively were identified the most promising scaffolds for the reconstruction of extensive bone defects. Further experimental setups should evaluate feasibility of modelling complex craniofacial structures by CAD/CAM, seeding strategies for large constructs, seeding of stem cells and oxygen measurements within the scaffolds.

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