Biocompatibility of CAD/CAM biomaterials for bone tissue engineering application

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Large bone defects have so far mainly been treated with autogenous bone grafts. Owing to limited availability and donor site morbidity, research is ongoing into the development of various bone replacement materials. An advantage of CAD/CAM implants is the possibility of patient-specific engineering. Ceramics and polymers have been extensively investigated, but not all materials can be produced in a standardised and patient-specific way yet. In this study, a wide range of materials were investigated, all of which can be CAD/CAM manufactured and individually dimensioned in the clean room with standardised techniques using digital light processing, selective laser sintering and fused deposition modelling. The novelty of the materials is the compounding of these, including the special processing by 3D printing. Eight polymer and ceramic CAD/CAM materials—poly-L-lactic acid and calcium carbonate, poly-L-lactic acid and tricalcium phosphate, poly-L-lactic acid and polyglycolic acid and calcium carbonate, poly-D, L-lactic acid and magnesium, poly-D, L-lactic acid, beta-tricalcium phosphate (β-TCP) and hydroxyapatite, β-TCP and β-TCP'—were tested to evaluate the cytotoxic effects on human osteoblasts. Biocompatibility was tested using a proliferation assay, a cytotoxicity assay, an apoptosis assay and fluorescence microscopy. The ceramic-based scaffolds, in particular β-TCP, showed very high cell counts in the proliferation assay as well as rapidly falling apoptosis rates and offer significant potential for use for patient-specific bone replacement implants.

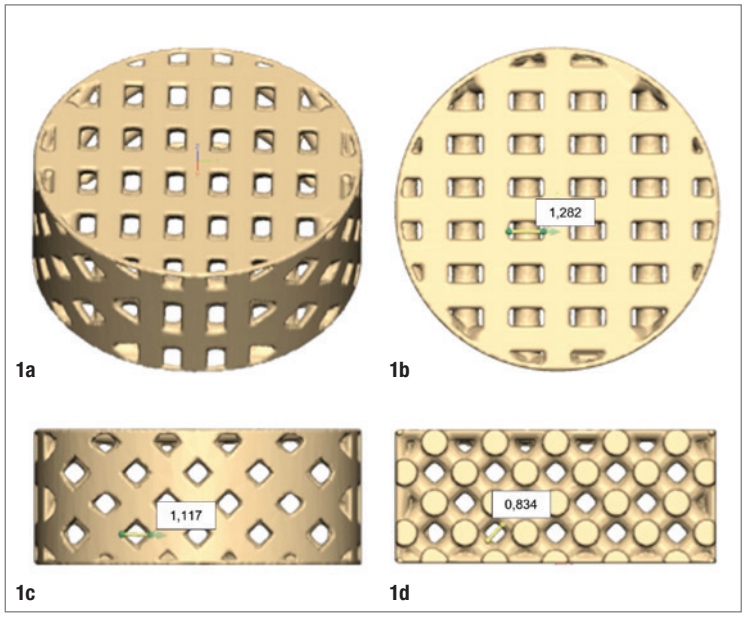

Fig. 1: Scaffold construction (sizes in mm). Scale bars = 1 mm. 3D view **(a)**, top view **(b)**, side view **(c)**, Cross section **(d)**.

Introduction

Bone defects often occur in the context of tumour resection, bone inflammation, malformation or trauma.[1] Autogenous bone transplantation continues to be the gold standard for the reconstruction of such defects. However, bone availability is limited in this case, and not inconsiderable donor site morbidity, including impaired wound healing, functional limitations, scarring and necrosis, can occur.[2] Research in the field of bone regeneration is steadily growing.[3] Of great interest are biomaterials, which being bone replacement materials, avoid the creation of donor sites and the associated complications and which, owing to their osteoconductive properties and suitable architecture, represent a viable alternative to autogenous bone transplantation.[4–6] In addition, materials that can be additively manufactured offer the advantage of being able to be individually dimensioned according to the defect. The growing demand requires bone replacement materials to possess improved mechanical and biological properties. An ideal biomaterial is characterised by biocompatibility and is replaced by regenerated new bone after the healing period. In terms of chemical composition and architecture, it should mimic the extracellular bone matrix so that cells can adhere, multiply and differentiate.[7, 8] Biomaterials that are very frequently used include ceramics such as beta-tricalcium phosphate (β-TCP) and hydroxyapatite (HA). Owing to their osteoconductivity and similar composition to that of bone, they play a crucial role in tissue engineering. In particular, β-TCP has a high degree

of solubility and is broken down more quickly, enabling replacement with new, regenerated bone.[9–11] Polymers such as poly-L-lactic acid (PLLA) or poly-D, L-lactic acid (PDLLA) have also shown promise in numerous studies. Their biocompatibility and biodegradability make them suitable for the regeneration of bone tissue. They have sufficient mechanical stability, and their modulus of elasticity is closer to that of the natural cortex than ceramic materials, which are more brittle.[12, 13] Composite scaffolds made of polymer and ceramic are also frequently used biomaterials in bone tissue engineering and are currently being investigated clinically. Ceramic and polymer components are combined to achieve good biocompatibility and stability.[14–19] Likewise, PDLLA or PLLA mixed with calcium carbonate (CC) or magnesium (Mg) is rated as promising.[20, 21] However, the comparability of materials has been limited by the different methodologies of the various studies on them, and most studies have only described one group of materials. Previous studies have shown that certain defined parameters, such as pore size, pore shape and porosity, in addition to certain defined mechanical properties and biocompatibility, are decisive for cell adhesion and bone ingrowth.[22, 23] Thanks to the 3D construction of a scaffold that is optimal with regard to these parameters, the bone metabolism can be positively influenced in a targeted manner. However, this complex construction can only be implemented with difficulty using conventional production techniques, since parameters such as pore size, porosity and pore distribution cannot be precisely controlled.[24] We examined such materials more closely, all of which can be additively manufactured in the clean room using standardised techniques. In this way, defined construction parameters can be implemented precisely for a wide variety of materials. We examined eight different biomaterials of PLLA–CC; PLLA–TCP; PLLA, polyglycolic acid and CC (PLLA–PGA–CC); PDLLA–Mg; PDLLA; β-TCP–HA; and β-TCP and β-TCP' for biocompatibility using the same methodology. The compounding of the materials, including special processing by 3D printing, represents an innovation in additive manufacturing. All eight materials were produced by digital light processing, selective laser sintering or fused deposition modelling (FDM). Both the respective processes and the pore structures were optimised accordingly in order to be able to produce comparable scaffolds using all technologies. This enabled us to objectively compare a wide range of materials and material combinations.

Material and methods

Biomaterials

The scaffolds were manufactured, packaged and then sterilised with gamma irradiation in cooperation with the medical technology company Karl Leibinger Medizintechnik under clean room conditions. All scaffolds were constructed with a diameter of 12 mm and a height of 5 mm (Fig. 1). In order to obtain comparable scaffolds, the wall was reduced in the first step. After an optimisation of the process parameters, sections of the wall were removed in a second optimisation, thus making the scaffolds permeable to liquids and cells in the edge structures. In a final step, the pore geometry was enlarged and rotated in order to achieve greater reproducibility and comparability between the various manufacturing methods (Figs. 2 & 3). The PLLA–CC scaffolds were manufactured on the FORMIGA P 110 (EOS), using selective laser sintering technology. The scaffolds made of PLLA–TCP, PLLA–PGA–CC, PDLLA–Mg and PDLLA were manufactured using FDM technology on the ARBURG AKF freeformer 200-3X (ARBURG). To achieve technical feasibility, technically pure Mg (99.8%, Alfa Aesar) was used. The β-TCP–HA and β-TCP scaffolds were manufactured using digital light processing technology on the

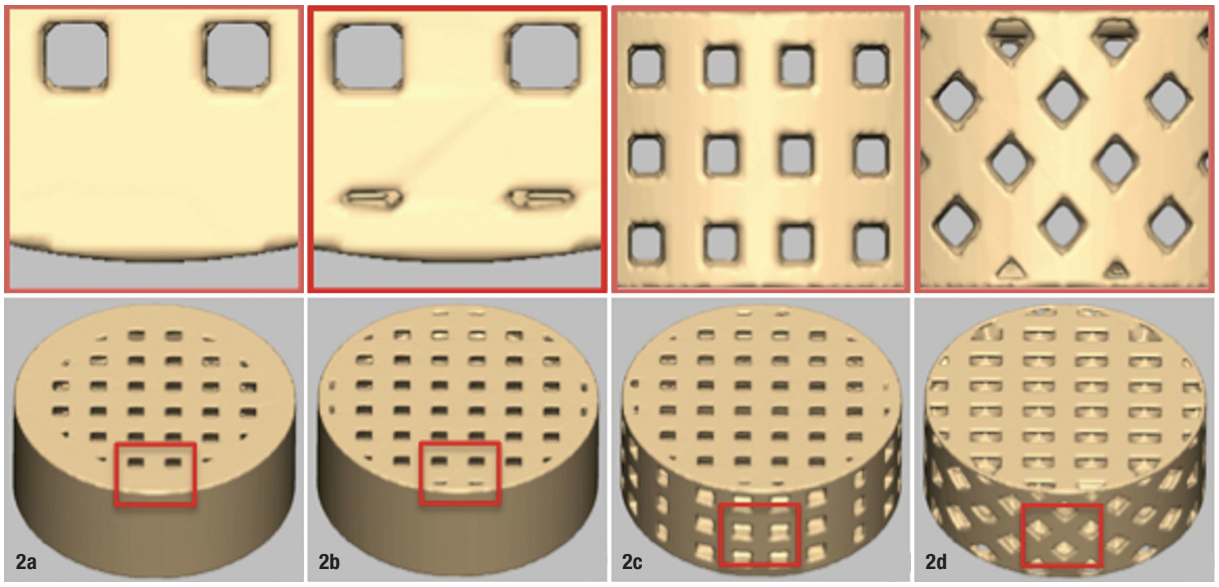

Fig. 2: Scaffold production. Initial state **(a)**, reduction of the wall **(b)**, removal of sections of the wall **(c)**, enlargement and rotation of the pore geometry **(d)**.

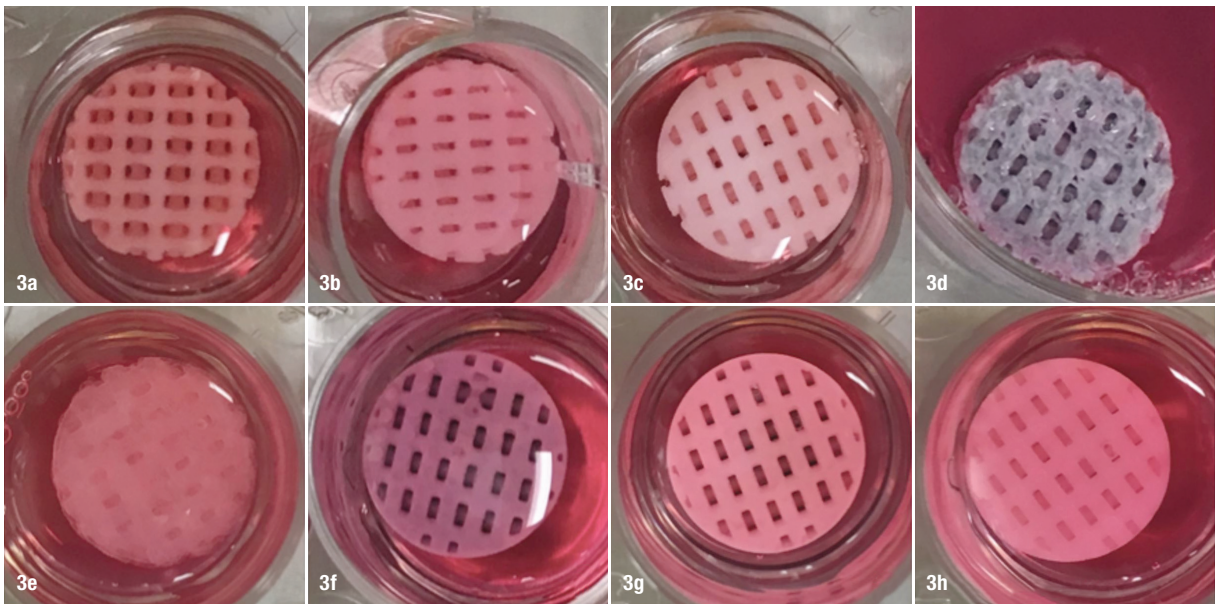

Fig. 3: Examined scaffolds in culture medium. Scale bar = 10 mm. PLLA-CC **(a)**, PLLA-TCP **(b)**, PLLA-PGA-CC **(c)**, PDLLA-Mg **(d)**, PDLLA **(e)**, β-TCP-HA **(f)**, β-TCP **(g)**, β-TCP' **(h)**.

CeraFab 7500 (Lithoz). For the β-TCP–HA scaffolds, sintering took place between 1,150 and 1,300 °C, and for the β-TCP between 1,050 and 1,200 °C. Two scaffold types with different mechanical properties were made from β-TCP (β-TCP and β-TCP'). To better differentiate between β-TCP and β-TCP', the flexural strength was determined in a flexural test of the samples. The flexural strength between β-TCP and β-TCP' increases with increasing sintering temperature. A flexural strength of 68 N/mm² was determined for β-TCP and of 120 N/mm² for β-TCP'.

Seeding of biomaterials and cultivation

The biomaterials were seeded with human osteoblasts (PromoCell). Before seeding, the scaffolds were incubated for 72 hours at 37 °C and 5% carbon dioxide (CO_2) in standard culture medium (Osteoblast Growth Medium, PromoCell) to hydrate the scaffold matrix in order to later facilitate the growth of the cells into the scaffold structure. In addition, the pores in the medium were de-aerated by applying a vacuum in a 100 ml syringe. The cells were amplified in monolayer culture with standard culture medium to a confluence of 80–90% and then passaged. Cells from the second passage were used. For seeding, the cells were detached by trypsinisation and resuspended in standard culture medium to obtain a cell suspension with a final cell concentration of 2×10^6 cells/ml. One scaffold was placed per well in a 24-well plate. For seeding, the cell suspension was pipetted on to the hydrated scaffolds. To ensure that the cells were homogeneously distributed, each batch was pipetted from a cell suspension and vortexed several times in between. For the apoptosis and proliferation assays, the scaffolds were seeded with 2×10^5 cells, each with a density of 3.54×10^5 cells/cm³. In order to enable cell adhesion, the seeded scaffolds were incubated for 30 minutes at 37 °C and 5% CO_2. The well was then filled with 1 ml of culture medium so that the scaffolds were covered by medium. During the course of this, the medium was changed every two days.

Fluorescence microscopy

In order to visually validate the success of culturing after 21 days, the scaffolds were evaluated using a fluorescence microscope. The scaffolds (n = 2) were seeded with ten million cells and cultured for 21 days in differentiation medium (StemMACS OsteoDiff Media, human, Miltenyi Biotec) and then fixed in 3% formaldehyde. They were covered in a 24-well plate with a Hoechst staining solution (Hoechst 33342, AppliChem, in phosphate-buffered saline; 1:2,000) and incubated for 10 minutes at room temperature, protected from light. They were then transferred to a well filled with phosphate-buffered saline and viewed there under a fluorescence microscope (BZ-9000 BIOREVO, Keyence) with a DAPI filter.

MTS assay

The number of metabolic cells growing on the scaffold surface and in the scaffold matrix was estimated using an MTS assay (CellTiter 96 AQ_{ueous} One Solution, Promega). This proliferation assay uses tetrazolium salt, which is converted by the cells into purple formazan. The amount of formazan dye produced is directly proportional to the number of proliferating cells in the sample. Since this assay is not cytotoxic, it is suitable for multiple measurements over long periods. To each scaffold, 200 µl of MTS assay was added in 1,000 µl of phenol-free medium and incubated for 1 hour at 37 °C and 5% CO_2. The absorption of each sample was then measured three times at 490 nm in a photometer (BioPhotometer plus, Eppendorf).

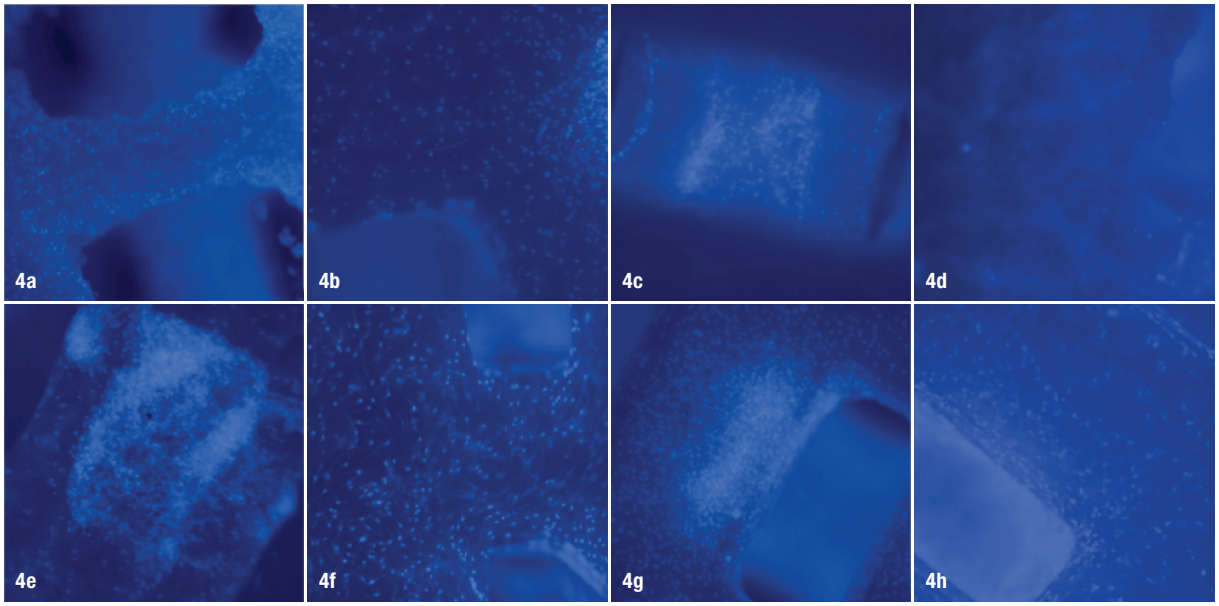

Fig. 4: Fluorescence microscopy of the scaffold surface with Hoechst staining solution on day 21 after seeding. Scale bar = 200 µm. PLLA-CC **(a)**, PLLA-TCP **(b)**, PLLA-PGA-CC **(c)**, PDLLA-Mg **(d)**, PDLLA **(e)**, β-TCP-HA **(f)**, β-TCP **(g)**, β-TCP' **(h)**.

An unseeded scaffold, medium and MTS served as control. To determine the number of cells, a calibration curve was carried out with human osteoblasts. The samples were analysed on days 2, 5, 7, 14 and 21 (n = 8).

Apoptosis assay

In order to assess the apoptosis activity of the cells on the scaffolds, an apoptosis assay (Caspase-Glo 3/7 assay, Promega) was carried out. A DEVD substrate was used which, in the presence of the apoptotic enzyme caspase -3 or -7, luciferase and adenosine triphosphate, results in the luciferase reaction and the production of light. This luminescence is directly proportional to the apoptosis activity of the cells. At room temperature, Caspase-Glo reagent was pipetted in a ratio of 1:1 on to the scaffolds in the medium. These were then agitated on the plate shaker (30 seconds, 300–500 rpm) and incubated at constant room temperature for 45 minutes. The luminescence of each sample was then measured three times in a plate-reading luminometer (Victor X2, PerkinElmer). The samples (n = 8) were analysed on days 2, 5, 7, 14 and 21. To determine the apoptosis activity based on the metabolic cells in the scaffold, the quotient of the apoptosis value (luminescence) divided by the cell count in the scaffold was generated. With the help of an establishment experiment, it was shown that the assay is not cytotoxic and is therefore suitable for a series of measurements over longer periods. For this purpose, the proliferation rate of cells incubated with the apoptosis assay was checked by means of the MTS assay.

Cytotoxicity assay

The cytotoxicity assay was performed according to ISO 10993-5. Extracts of the scaffolds were produced by hydrating them in 2 ml of serum-containing culture medium for 72 hours in order to accumulate potentially cytotoxic substances in the medium. Human osteoblasts were cultivated in 96 well plates with a density of 1,000 cells per well and, after addition of the extracts (in the dilutions 100%, 75%, 50% and 25%), incubated for 24 hours. Viability was assessed with the aid of the proliferation assay (CellTiter 96 AQ_{ueous} One Solution). The extract from ThinCert membranes (Greiner Bio-One), which are considered to be particularly cell-friendly, served as a negative control, and 100% dimethylsulfoxide (DMSO) was used as a positive control.

Sulforhodamine B assay

The sulforhodamine B assay allows conclusions to be drawn about the number of cells in the osteoblasts growing in the milieu of the scaffolds by measuring protein quantities. Human osteoblasts were seeded in six-well plates at a density of 100,000 cells per well. With the help of ThinCert inserts, the scaffolds were placed in the medium above the cells. It was thereby possible to investigate whether the materials release cytotoxic substances into the medium over longer periods and to what extent this affects the number of cells and therefore cell growth. The cells were fixed with methanol (99%; Carl Roth) on the measurement days and stored at –80 °C. For staining, the methanol was removed from the wells, and the cells were covered with sulforhodamine B staining solution (1% acetic acid solution and 0.4% w/v sulforhodamine B sodium salt, Sigma). Incubation was performed for 30 minutes at room temperature with continuous agitation. The sulforhodamine B staining solution was then removed, and the fixed cells were washed five times in a 1% acetic acid solution. The stained cells were dried and, after 24 hours,

dissolved in 2 ml of 10 mM Tris buffer. The absorption of each sample was then measured three times at 550 nm in a photometer (BioPhotometer plus). To determine the number of cells, a calibration curve was carried out with human osteoblasts. The samples (n = 8) were analysed on days 2, 5 and 7.

Statistical analysis

The data for the tests performed are presented as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software). To evaluate the differences between time points and groups, one-way ANOVA and Friedman Test were performed followed by Dunn's post hoc multiple comparisons. T-test and Mann–Whitney U test were performed for significance of viability in cytotoxicity testing. A P value < 0.05 was considered significant.

Results

Fluorescence microscopy

PLLA–PGA–CC, PDLLA, β-TCP–HA, β-TCP and β-TCP' showed the highest cell density (Fig. 4).

MTS assay

The initial cell count after seeding was 2×10^5 cells/scaffold. On day 2, the first measurement was performed. The cell count on day 21 was evaluated in comparison to days 2, 5, 7 and 14. In the group of PLLA-based polymers, the PLLA–CC scaffold contained 2.77 x 10^5 ($\pm$ 0.34×10^5) cells on day 2. After a slight decrease in cell count on day 5 ($2.01 \times 10^5 \pm 0.80 \times 10^5$) and day 14 ($2.36 \times 10^5 \pm 0.51 \times 10^5$), the cell count per scaffold increased to 4.20×10^5 ($\pm 0.63 \times 10^5$) after 21 days ($p < 0.05$). For PLLA–TCP, the cell count increased until day 7 ($8.28 \times 10^5 \pm 1.34 \times 10^5$), decreased to 3.17×10^5 ($\pm$ 0.93×10^5) on day 14 and increased to 4.49×10^5 ($\pm$ 1.03×10^5) on day 21. The difference in cell count between day 21 and day 14 was significant ($p < 0.05$). For PLLA–PGA–CC, the cell count increased from day 2 ($4.37 \times 10^5 \pm 0.56 \times 10^5$) to day 5 ($3.48 \times 10^5 \pm 1.16 \times 10^5$), day 14 ($4.07 \times 10^5 \pm 0.62 \times 10^5$) and day 21 ($5.74 \times 10^5 \pm 0.61 \times 10^5$; $p < 0.05$). Overall, the highest values were obtained on day 7 with 10.15×10^5 ($\pm$ 2.30×10^5) cells. PLLA–PGA–CC showed the highest cell counts over time in the group of PLLA-based polymers (Figs. 5a & d). For PDLLA–Mg, the total cell count decreased from 0.14×10^5 ($\pm$ 2.25×10^5) on day 2 to 0.00×10^5 ($\pm$ 0.26×10^5) on day 21, having only a temporary slight increase on day 5 ($1.44 \times 10^5 \pm 0.88 \times 10^5$) and day 14 ($0.89 \times 10^5 \pm 0.66 \times 10^5$). PDLLA increased steadily from day 2 ($1.67 \times 10^5 \pm 0.44 \times 10^5$) to day 5 ($2.65 \times 10^5 \pm 0.71 \times 10^5$), day 7 ($4.19 \times 10^5 \pm 1.00 \times 10^5$), day 14 ($4.13 \times 10^5 \pm 1.31 \times 10^5$) and day 21 ($9.39 \times 10^5 \pm 1.12 \times 10^5$; each $p < 0.05$). In the group of PDLLA-based polymers, PDLLA showed the best results over time (Figs. 5b & e). For the ceramics, an increase in cell count was observed in β-TCP–HA on day 21 to 6.54×10^5 ($\pm$ 1.26×10^5) compared with day 2 ($3.34 \times 10^5 \pm 0.68 \times 10^5$), day 5 ($3.22 \times 10^5 \pm 1.05 \times 10^5$) and day 14 ($4.83 \times 10^5 \pm 1.01 \times 10^5$; $p < 0.05$ for days 2 and 5). β-TCP increased steadily from day 2 ($1.67 \times 10^5 \pm 0.80 \times 10^5$) to day 5 ($3.93 \times 10^5 \pm 1.75 \times 10^5$), day 7 ($5.74 \times 10^5 \pm 1.49 \times 10^5$), day 14 ($5.68 \times 10^5 \pm 1.70 \times 10^5$) and day 21 ($7.35 \times 10^5 \pm 1.43 \times 10^5$; $p < 0.05$ for days 2 and 5). β-TCP' also showed a significant increase in cell count on day 21 ($7.46 \times 10^5 \pm 4.07 \times 10^5$) compared with day 2 ($1.87 \times 10^5 \pm 1.41 \times 10^5$), day 5 ($5.01 \times 10^5 \pm 2.55 \times 10^5$) and day 7 ($4.53 \times 10^5 \pm 2.62 \times 10^5$; $p < 0.05$). Day 14 showed the highest value with 8.26×10^5 ($\pm$ 2.98×10^5) cells (Figs. 5c & f). In summary, β-TCP–HA, β-TCP and β-TCP' showed the best results over time, and PDLLA–Mg showed the lowest cell counts.

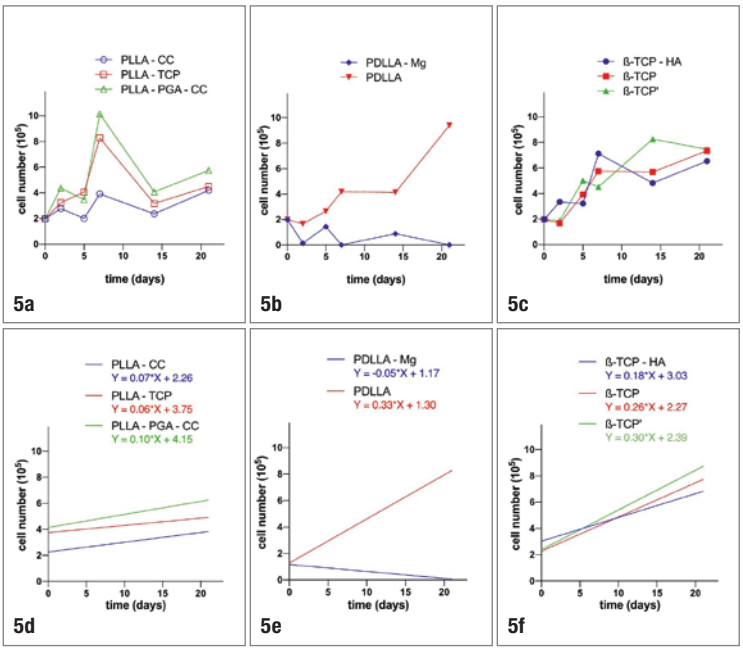

Fig. 5: Proliferation assay (MTS assay). Determination of the number of proliferating cells in the scaffolds after two, five, seven, 14 and 21 days **(a–f)**. Regression lines to determine growth tendency over time **(d–f)**.

Apoptosis assay

In order to determine the apoptosis activity in relation to metabolic cells, the quotient of the apoptosis value (luminescence) divided by the cell count in the scaffold was generated. The PLLA-based polymers all showed a similar course of apoptosis activity over the observation period. For PLLA–CC, apoptosis activity was significantly increased on day 2 to 118.57×10^{-5} ($\pm$ 19.60×10^{-5}) compared with days 5, 7, 14 and 21, when the value approached zero (range: 0.00–5.29×10^{-5}; $p < 0.05$). PLLA–TCP, with a value of 187.19×10^{-5} ($\pm$ 32.20×10^{-5}), and PLLA–PGA–CC, with a value of 107.32×10^{-5} ($\pm$ 21.90×10^{-5}), also showed increased apoptosis activity on day 2 compared with the other days ($p < 0.05$; Figs. 6a & d). After initially increased apoptosis on day 2 ($84.15 \times 10^{-5} \pm 53.80 \times 10^{-5}$), PDLLA–Mg decreased to 1.44×10^{-5} ($\pm$ 2.50×10^{-5}) on day 5 and increased again on day 7 ($971.03 \times 10^{-5} \pm 1{,}358.90 \times 10^{-5}$; $p < 0.05$). Subsequently, it remained slightly elevated at

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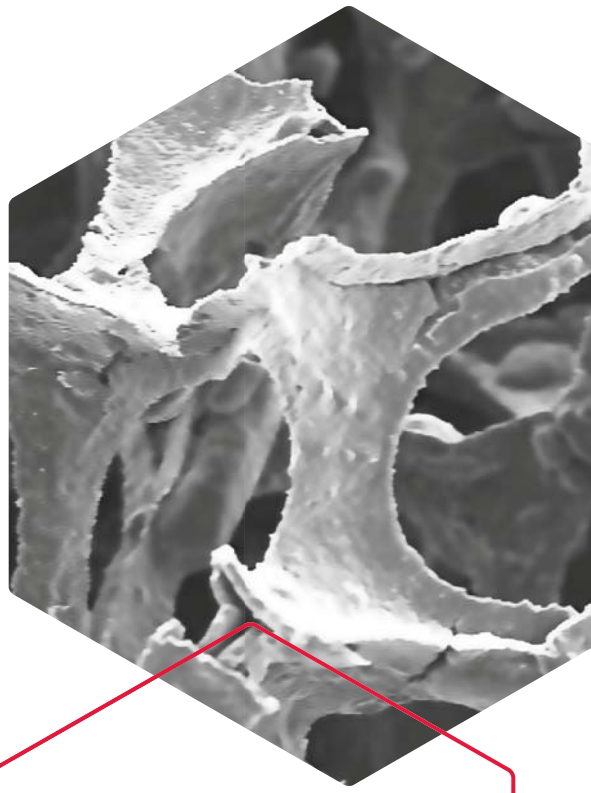

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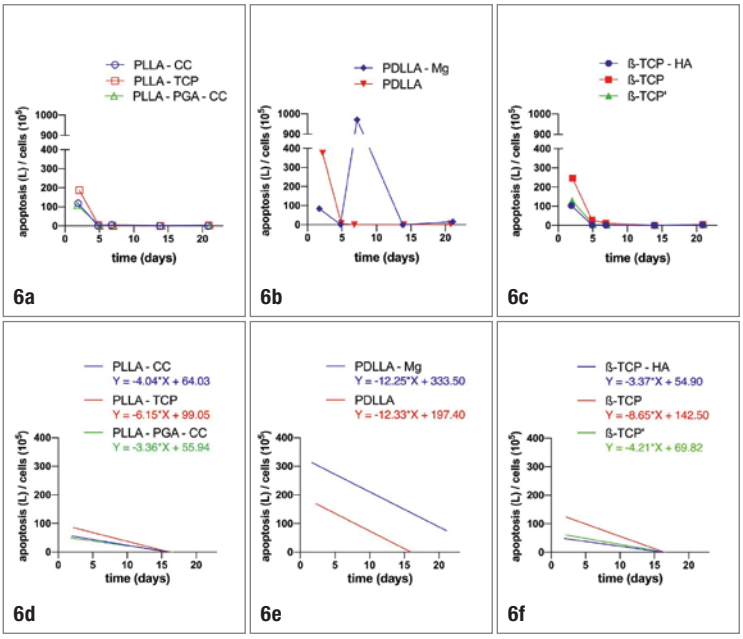

Fig. 6: Apoptosis assay. L = luminescence. Determination of apoptosis activity related to cell count on the scaffolds after two, five, seven, 14 and 21 days **(a–f)**. Regression lines to determine apoptosis tendency over time **(d–f)**.

15.40×10^{-5} (± 9.50×10^{-5}) on day 21 ($p < 0.05$ compared with day 5). For PDLLA, apoptosis activity was significantly increased only on day 2 ($375.48 \times 10^{-5} \pm 25.60 \times 10^{-5}$) and then decreased to low values between 0.00 and 7.01×10^{-5} (Figs. 6b & e). All ceramics showed a similar course of apoptosis activity. On day 2, β-TCP–HA, with a value of 103.52×10^{-5} (± 32.50×10^{-5}), and β-TCP', with a value of 129.85×10^{-5} (± 30.50×10^{-5}), showed significantly increased apoptosis activity compared with days 5, 7, 14 and 21 (0.00–4.79×10^{-5}). Also, β-TCP showed an increased value on day 2 ($246.27 \times 10^{-5} \pm 34.90 \times 10^{-5}$; $p < 0.05$). Furthermore, the curve flattened more slowly here (Figs. 6c & f). In summary, apoptosis decreased towards zero after having initially increased on day 2 for PLLA-based polymers, ceramics and PDLLA. For PDLLA–Mg, apoptosis activity peaked again on day 7 and moderately increased again after 21 days.

Cytotoxicity assay

The proliferation of human osteoblasts was not affected by the extracts (100% undiluted extract) of the biomaterials (Fig. 7). Cell growth and metabolism were unchanged compared with the non-cytotoxic control (negative control). The negative control value was set to 100%. The viability of extracts of PLLA–CC (156%) and β-TCP (151%) even exceeded that of the negative control. Cells incubated in 100% DMSO (positive cytotoxic control) reflected the cytotoxic effect of DMSO on viability (5.6 ± 4.8%). The viability of human osteoblasts cultured in DMSO was significantly reduced compared with the negative control and the scaffold extracts ($p < 0.05$). According to ISO guidelines, cell viability in the range of 0 to 50% reflects a strong cytotoxic effect of the tested extract, whereas values between 70 and 100% reflect the absence of cytotoxic components. The viability of 50% extract dilutions was at least as high as that of 100% extracts for all biomaterials, as required by the ISO guidelines.

Sulforhodamine B assay

The initial cell count after seeding was 1×10^5 cells/scaffold. On day 2, the first measurement was performed. After an increase of the cell count of PLLA–CC on day 2 to 4.46×10^5 (± 1.90×10^5), it decreased on day 5 ($2.57 \times 10^5 \pm 1.60 \times 10^5$) and remained almost unchanged on day 7 ($2.76 \times 10^5 \pm 1.01 \times 10^5$). For PLLA–TCP, the cell count after day 2 ($4.36 \times 10^5 \pm 2.63 \times 10^5$) decreased to 3.18×10^5 (± 0.97×10^5) on day 5 and to 2.79×10^5 (± 1.49×10^5) on day 7. PLLA–PGA–CC showed almost constant cell counts, having a value of 2.38×10^5 (± 0.74×10^5) on day 2 and of 2.20×10^5 (± 0.95×10^5) on day 5. On day 7, there was a significant increase to 2.84×10^5 (± 0.79×10^5) compared with day 5 ($p < 0.05$; Figs. 8a & d). Starting with 3.56×10^5 (± 0.66×10^5) on day 2, the cell count of PDLLA–Mg dropped to 1.45×10^5 (± 0.44×10^5) on day 5 and remained nearly unchanged ($1.52 \times 10^5 \pm 0.55 \times 10^5$) on day 7. For PDLLA, a value of 2.61×10^5 (± 0.23×10^5) was observed on day 2. On day 5, the cell count slightly decreased ($2.06 \times 10^5 \pm 0.49 \times 10^5$), and it also remained nearly unchanged on day 7 ($2.11 \times 10^5 \pm 0.39 \times 10^5$; Figs. 8b & e). After day 2 with a cell count of 2.22×10^5 (± 0.37×10^5), β-TCP–HA showed a slight decrease in cell count on day 5 ($1.84 \times 10^5 \pm 0.37 \times 10^5$). The count increased slightly on day 7 ($2.21 \times 10^5 \pm 0.52 \times 10^5$). β-TCP' showed a similar course, having 3.01×10^5 (± 1.82×10^5) cells on day 2, a slight decrease on day 5 ($2.64 \times 10^5 \pm 0.72 \times 10^5$) and an increase on day 7 ($3.08 \times 10^5 \pm 0.44 \times 10^5$) compared with day 5 ($p < 0.05$). β-TCP had a cell count of 2.37×10^5 (± 0.52×10^5) on day 2, a minimal decrease on day 5 ($2.08 \times 10^5 \pm 0.47 \times 10^5$) and a significant increase on day 7 ($6.72 \times 10^5 \pm 5.88 \times 10^5$) compared with day 5 ($p < 0.05$; Figs. 8c & f). PLLA–PGA–CC, β-TCP and β-TCP' showed a significant increase in cell count as well as the largest slope of the regression line over the observation period (Figs. 8d & f). The other materials showed only insignificant changes or decreasing cell count.

Discussion

Despite promising advances in tissue engineering, the treatment of large bone defects is still a challenge.[25] An optimal biomaterial should be biocompatible and have controllable biodegradability and architecture and optimal mechanical properties.[26] An interconnected pore system, porosity and optimal pore size are required, although opinions differ on this.[27, 28] In general, however, a pore size of over 300 µm is favoured.[29] We chose a pore diameter of 800 µm to allow osteogenesis, fluid exchange and subsequent vascularisation. While a complex scaffold design is difficult to implement using conventional

techniques, additive manufacturing processes allow for individual implant production.[24, 30, 31] This enables us to both individually adapt the scaffold shape to a bone defect and to construct the microscopic scaffold architecture. We evaluated the biocompatibility of various additively manufactured biomaterials using a proliferation, apoptosis, cytotoxicity and sulforhodamine B assay and were thereby able to objectively evaluate and compare a wide variety of materials and material groups. In the proliferation and apoptosis assays, multiple measurements could be made over longer periods owing to the lack of cytotoxicity of the assays. This also has the advantage of better comparability and fewer inaccuracies. TCP–HA is becoming an increasingly important biomaterial in bone tissue engineering. Owing to its similarity to the mineral phase of bone, HA plays an important role in cell adhesion and proliferation and, along with tricalcium phosphate, is one of the most frequently used ceramics.[32–34] *In vivo* studies have also shown that the combination of TCP–HA induces bone formation.[35–37] In this study, β-TCP–HA showed a significant increase in the number of cells growing on the scaffold over 21 days, but the cell count on days 14 and 21 was lower than that of β-TCP and β-TCP'. The apoptosis activity of β-TCP–HA was significantly increased on day 2 compared with the other days; over time, it decreased to zero. Initially increased apoptosis activity was observed in all the materials and is most likely explained by the trypsinisation and the passage when seeding the scaffolds. After day 2, hardly any cells were in apoptosis, evidence of the cell compatibility of the scaffold. Compared with pure β-TCP, the quotient of the apoptosis value divided by the cell count was significantly lower for the TCP–HA on day 2. Woo et al. describe suppressed cell apoptosis through the addition of HA to composite scaffolds.[38] This is in line with our results. In the cytotoxicity test in accordance with ISO 10993-5, the growth of the osteoblasts was not impaired by the scaffold extract either. The sulforhodamine B assay evaluated the number of osteoblasts that grew in the scaffold extract in the immediate vicinity of the scaffold for seven days. Good results were demonstrated here; the regression line had a positive gradient. However, the total number of cells was even higher for pure β-TCP and β-TCP'. Despite very good biocompatibility and low apoptosis values, TCP–HA showed somewhat poorer results than β-TCP and β-TCP' with regard to cell proliferation and growth behaviour. β-TCP is one of the most used biomaterials. Its osteoconductivity, rapid degradability and similarity to the composition of bone make it suitable for bone tissue engineering.[10, 11] This has also been shown by numerous *in vivo* studies. For example, Kondo et al. successfully implanted β-TCP into femur bones in the rat model.[39] The brittleness of the material usually makes it difficult to adapt to the individual,[40] but this is no longer necessary owing to the possibility of individual construction using additive manufacturing processes. Since, depending on the dimensions, classic fixation of ceramics with screws is not possible, alternative fixation techniques are necessary (e.g. a cage). Both β-TCP and the mechanically improved β-TCP' with higher flexural strength showed a significant increase in cell count from day 2 to day 21. In addition, both (with PDLLA) achieved the highest cell counts on days 14 and 21 compared with all other materials and therefore better cell proliferation. While β-TCP' showed a twofold drop in cells during the process, the growth curve of β-TCP demonstrated a consistent upward trend. After initially elevated values (day 2) for β-TCP and β-TCP', apoptosis activity decreased towards zero. On day 2, the quotient of the apoptosis value divided by the cell count of β-TCP was significantly increased compared with β-TCP' and TCP–HA. This agrees with the results of the proliferation assay, in which β-TCP had the lowest cell count among the ceramics on days 2 and 5, as the cells increasingly went into programmed cell death. However, the number of cells then rose steadily to very good values. Osteoblast proliferation was not negatively influenced in the cytotoxicity assay by the extract of β-TCP or β-TCP', also indicating good biocompatibility. In the sulforhodamine B assay, we observed a higher cell count on day 7 for β-TCP and β-TCP' compared with TCP–HA; for β-TCP', this difference was significant. Compared with all the materials, they also showed the best results here, having a regression line gradient of 67.5 (β-TCP) and 23.2 (β-TCP'). With regard to cell proliferation and growth behaviour, β-TCP and β-TCP' showed the best results in the ceramic scaffolds group. PLLA–CC was recently described in the literature as a bone replacement material.[20] CC has a beneficial effect in bone

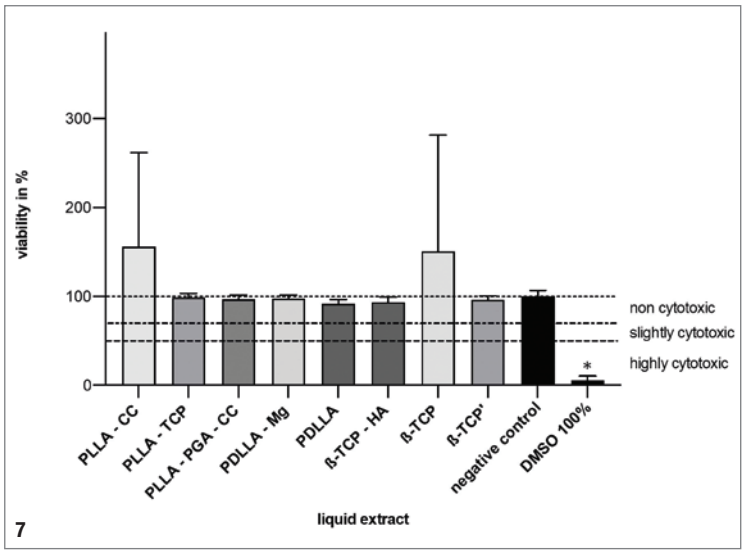

Fig. 7: *In vitro* cytotoxicity of 100% undiluted extract. Human osteoblasts cultivated in control extract (negative control = dotted line, 100%) or undiluted scaffold extract all showed high viability. The viability of osteoblasts cultivated in dimethylsulfoxide (positive cytotoxic control) was significantly reduced compared with the scaffold extracts ($p < 0.05$).

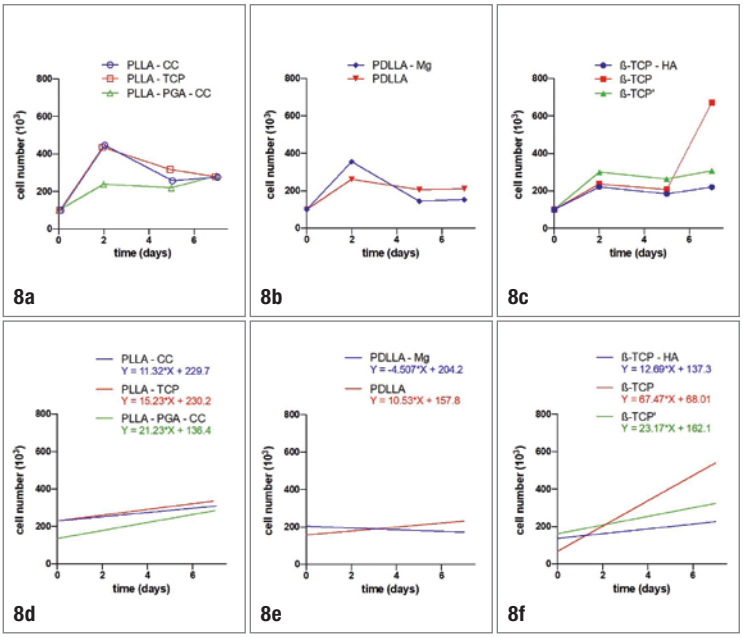

Fig. 8: Sulforhodamine B assay. Determination of the cell count of osteoblasts growing in the scaffold environment after two, five and seven days **(a–f)**. Regression lines to determine growth tendency over time **(d–f)**.

tissue engineering, as extracellular calcium enhances osteogenic gene expression and promotes bone regeneration.[41] CC was mentioned earlier as a suitable filler for polyester, because its pH-stabilising effect buffers the acidic degradation of polylactides.[42] In this study, PLLA–CC showed a significant increase in cells growing in the scaffold from day 2 to day 21, but the cell count was slightly lower than that of the other PLLA-based scaffolds at all measurement times. Apoptosis activity was significantly increased initially (day 2) and decreased towards zero over time, indicating the long-term cell tolerance of the scaffold. The increased apoptosis activity is probably related to differences in the degradation kinetics and initial water absorption of the polymeric scaffold systems. Different proteins also play a role as deposits on the scaffolds. In the cytotoxicity test, the growth of the osteoblasts was not impaired by the scaffold extract either. In the sulforhodamine B assay, the cell count on day 7 was not significantly different from that of the other PLLA scaffolds. However, the curve showed the smallest regression line gradient among the PLLA scaffolds. Gayer et al. described good cell compatibility of PLLA–CC, but there is no possibility of comparison with other materials.[20] In this study, PLLA–CC demonstrated overall good biocompatibility. In comparison with the poly-L-lactides PLLA–TCP and PLLA–PGA–CC, however, the latter can be assessed as even more promising in terms of cell proliferation and growth behaviour. Composite scaffolds made from PLLA–TCP are frequently used biomaterials in bone tissue engineering.[14–18] The aim is to overcome the shortcomings of the individual materials by combining PLLA and TCP. On the one hand, TCP counteracts the acidic environment that results from the breakdown of polylactide. On the other hand, the combination of PLLA and TCP improves the mechanical properties of a scaffold.[32, 43] We observed a non-significant increase in cells growing in the scaffold from day 2 to day 21; the increase from day 14 to day 21 was significant. The cell count over time was higher than for PLLA–CC, but lower than for PLLA–PGA–CC. For PLLA–TCP, the apoptosis activity was significantly increased on day 2, and over time, it also decreased to zero. The results in the cytotoxicity test reflected the absence of cytotoxic components. In the sulforhodamine B assay, the cell count on day 7 was not significantly different from that of the other PLLA scaffolds. However, the cell counts fell again after an increase on day 2, and the regression line showed a slightly lower gradient than PLLA–PGA–CC did. PLLA–PGA–CC in this composition has not yet been described in the literature as a bone replacement material. PLLA is already widely used in tissue engineering for the regeneration of bone tissue.[44–47] PGA is a less hydrophobic polymer with a relatively rapid degradation rate.[48] The co-polymer PLLA–PGA has been described for bioresorbable bone fixation in the form of screws, plates or orbital floor reconstruction plates.[49–52] There is also information on the good biocompatibility of the composite of poly (lactic-co-glycolic) acid (PLGA) and CC, but not on PGA–CC or PLLA–PGA–CC.[53] As a co-polymer of PGA, PLGA has similar properties in some cases. In our investigations, PLLA–PGA–CC showed a significant increase in cell metabolism from day 2 to day 21. The cell count of the osteoblasts growing on the scaffold was significantly increased on all measurement days compared with the other PLLA-based scaffolds, with the exception of PLLA–TCP on days 5 and 7. After initially increased values (day 2), the apoptosis activity decreased over time to zero, which is desirable. Furthermore, PLLA–PGA–CC sometimes showed the lowest quotient of the apoptosis value divided by the cell count compared with all the other materials, indicating good cell compatibility. In the cytotoxicity test too, the scaffold extract did not impair the growth of the osteoblasts. When evaluating the cell count in the sulforhodamine B assay, the cell count on day 7 was about the same as for the other PLLA scaffolds. However, PLLA–PGA–CC was the only material here that showed a significant increase in cell count over the course of the experiment and the largest gradient of the regression line. With regard to cell proliferation, growth behaviour and apoptosis activity, PLLA–PGA–CC showed the best results in the group of poly-L-lactides. PDLLA–Mg in this composition and Mg in this processing method have not yet been described in the literature. Mg is believed to have great potential in bone tissue engineering because of its biodegradability and its ability to promote new bone formation. In addition, the modulus of elasticity of Mg is comparable to that of cortical bone.[54–58] The problem, however, is the rapid corrosion of Mg, which can lead to a loss of structure and the release of degradation products.[59] The rate

of degradation of technically pure Mg is much faster than that of alloys such as WE34 which are already in clinical use. Because the degradation rates are significantly lower, these alloys also show very good biocompatibility, but other elements are also present here, for example rare earth elements that are not found in technically pure Mg. Our intention was to generate a polymeric matrix around the Mg material to create a polymer–metal composite to reduce the degradation rate of metallic Mg and thereby improve biological effects such as cell compatibility. Our results showed a comparatively low number of cells on the scaffolds. This was also confirmed by our electron microscopic examinations on day 21 (ongoing study). The quotient of the apoptosis value divided by the cell count was significantly increased in particular on day 7 compared with the other materials. The high value can be explained by the low cell count on the scaffold, and of these few cells, a large percentage were found to be in apoptosis. The high apoptosis levels are consistent with the low cell counts. In the sulforhodamine B assay too, the cell count was lower than that of the other materials, and the regression line decreased with a slope of –4.5. In contrast to this, osteoblast proliferation was not negatively influenced by the extracts, suggesting low cytotoxicity. Tavares et al. reported a lack of cytotoxicity of composite scaffolds to which Mg was added in the cytotoxicity test in accordance with ISO 10993.[60] This illustrates how important it is to test the material itself and not only to test an eluate produced from it, as here the effects of the scaffold architecture and other interactions are neglected. PDLLA–Mg showed less favourable results in this study compared with the other PDLLA-based materials. When looking at all the materials together, the other materials also performed better. This is presumably primarily due to the release of degradation products[59] and gas formation and was to be expected for pure Mg. It can be assumed that the slowdown in degradation, which we wanted to achieve with the composite material formulation, had occurred to an insufficient degree. However, we were able to show that the additive production of Mg-based implants using FDM technology is technically feasible. Further work is necessary to develop other material formulations that allow optimal degradation kinetics of technically pure Mg with a cell biologically compatible release of degradation products in order to fully exploit the material's potential for bone tissue engineering. PDLLA has been frequently described as a biomaterial.[42, 61–63] In this study, the cell count of the osteoblasts growing on the scaffold increased steadily up to day 21 and was significantly higher than that of the other PDLLA-based scaffolds on days 5, 7, 14 and 21. After initially strongly increased values (day 2), apoptosis activity decreased towards zero over the course of the experiment. In the cytotoxicity test too, the growth of the osteoblasts was not impaired by the scaffold extracts. In the evaluation of the cell count in the sulforhodamine B assay, the cell count on days 5 and 7 was significantly increased compared with PDLLA–Mg. In addition, PDLLA showed the most significant regression line gradient within the poly-D, L-lactide materials. With regard to cell proliferation, growth behaviour and apoptosis activity, PDLLA showed the best results in the group of poly-D, L-lactides. We evaluated the biocompatibility of the various additively manufactured biomaterials in the clean room and therefore had the opportunity to objectively evaluate and compare the different materials. The novelty of the materials is the compounding of these, including the special processing by 3D printing, to produce comparable scaffolds. Looking at all the materials together, the ceramic-based scaffolds proved to be the most promising. They showed the highest cell counts in the proliferation assay. They can be considered non-cytotoxic when used *in vitro*, and the apoptosis activity strongly decreased over the measurement period. β-TCP and β-TCP' exhibited particularly good results, showing the steepest growth curves in the sulforhodamine B assay. Among the poly-L-lactides, PLLA–PGA–CC performed best in terms of cell proliferation, growth behaviour and apoptosis activity. In the poly-D, L-lactide group, PDLLA showed the best results. The comparatively lowest cell counts and highest apoptosis values were observed for PDLLA–Mg. Further studies to improve the materials are planned, as these materials also demonstrated very promising properties that should be used for tissue engineering. A study is currently being carried out with regard to the behaviour of the materials *in vivo*.

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