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Towards bone replacement materials from calcium phosphates via rapid prototyping and ceramic gelcasting

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Abstract

Biomimetic porous scaffolds made of calcium phosphate mineral are promising structures to develop bone replacement materials. In order to fabricate scaffolds with a strut size of 450 μm, we used a stereolithographic technique which selectively polymerises photosensitive liquid resin by visible light to produce casting moulds for ceramic gelcasting. These moulds were filled with a water based thermosetting ceramic slurry which solidifies inside the mould. After burning the resin mould and sintering, hydroxylapatite structures with designed, fully interconnected macroporosity were obtained.

The preosteoblastic cell line MC3T3-E1, derived from mouse calvariae, was used to test for biocompatibility in cell culture experiments. The cells were seeded on the scaffolds immersed in the culture medium and cultured for 2 weeks. Thereafter the cells on the scaffold were fixed and investigated by histological methods. The osteoblast-like cells were found to cover the whole external and internal surface of the scaffold, they were embedded in collagenous extracellular matrix. The cells had in particular the tendency to fill any crack or opening and to generally smooth the exposed surfaces.

Keywords: Bone replacement materials; Implants; Rapid prototyping; Ceramic gelcasting; Hydroxylapatite; Hydroxyapatite; Scaffolds; Macroporosity; Cell culture; Osteoblasts; MC3T3-E1

1. Introduction

The development of biomimetic bone replacement materials is a growing field of research for application in medicine [1,2]. The potentially best bone replacement materials are grafts derived from the patient himself. They are biocompatible, osteoconductive and osteoinductive, and there is no danger of immuno-rejection. There is, however, only a limited amount of autograft available for each patient and the extraction induces additional trauma [3–5]. With allografts, derived from donators, or xenografts from animal tissue, there is an additional risk of immuno-rejection and disease transmission [6–9]. In addition to these biogenic materials, metallic implants [10] as well as ceramic [11–15], polymer and composite [16–18] biomaterials have been developed for bone replacement applications. These implants should be well integrated into the remaining bone, which implies not only full biocompatibility (to avoid immunoreactions) but also osteoconductive properties in order to ensure a tight connection with bone [19–21]. Polymeric and ceramic materials can also be resorbable, ceramic materials having the advantage of higher strength and stiffness compared to the polymeric materials [22], although the intrinsic brittleness of ceramics limits their applicability. Among the ceramic materials, the calcium phosphates are known to have promising biological properties [23], in that they can be biocompatible, resorbable, osteoconductive and even osteoinductive under appropriate

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conditions [24–29]. Moreover, they can be used for bone cements and fillers [11,12]. Ideally bone replacement materials should, in addition, be remodeled into native bone, which we expect to be possible if the material is bioresorbable and provides sufficient access to bone forming cells [19].

Cell ingrowth behaviour strongly depends on the pore size [24,29,30] and interconnectivity [11,31] within the implant. A pore diameter of 150 to 500 μm is referred to be best for cell access [25,30,32–34]. On the other hand, nutrition of the cells within the structure has to be ensured for viability, which is possible via the flow of serum through micro- and/or macro-pores. Considerable success has already been obtained with porous ceramics serving essentially as a scaffold for bone regeneration and produced via different routes, e.g. from corals [3]. One difficulty with producing porous calcium-phosphate scaffolds is to control the porosity in order to match the biological requirements but also to provide sufficient mechanical stability.

The mechanical properties of cellular solids such as porous ceramics depend mainly on three parameters: the apparent density, the properties of the base material and the architecture of the structure [35]. The possibilities of optimizing the properties of the base material are limited by the requirements of biocompatibility and bioresorbability. Moreover, due to the biological need for interconnected porosity with pore sizes in the range of a few hundred microns, the apparent density cannot be increased beyond a limiting value. Significant improvement of the mechanical performance at given apparent density is possible, however, by adapting the architecture. In previous experiments we built several periodic three-dimensional cellular solids with constant apparent density and showed by compression testing that a simple change in the architecture of the unit cell can account for variations by almost a factor of three in strength and, independently, in defect tolerance [36].

Based on these results, we pursued the route of designing porous calcium-phosphate ceramics with predefined architecture and sufficient accessibility for bone forming cells. In a first step, we investigated the potential of rapid prototyping (RP) and ceramic gelcasting to produce three-dimensional scaffolds suitable for growing bone-forming cells. We chose a methodology starting with a computer-aided design of the structure, which gives the full freedom to obtain various types of structures with different mechanical properties [36]. Then resin moulds were constructed using a stereolithographic technique [37], and filled with a thermosetting ceramic slurry. After temperature treatment, we obtained cellular ceramic scaffolds with designed macroporosity. We report first results for hydroxylapatite scaffolds with 450 μm pore diameter and fully interconnected pore morphology, which were manufactured and tested in cultures of a murine preosteoblastic cell line.

2. Materials and methods

The first step in the production of ceramic structures was to virtually design the desired structure with a computer aided design (CAD) software. We used Pro/Engineer (PTC, Needham, Massachusetts, USA). An example is shown in Fig. 1.

We constructed structures consisting of layers of parallel struts with quadratic cross-section and a side length with the same physical dimension as the distance between two of them. Each layer was turned 90° with respect to the previous one. In this manner, 20 layers were superimposed. The diameter of the whole structure was 10 mm, the side length of one of the struts and the height of one layer was 500 μm, hence the height of the whole structure was 10 mm, too. The porosity of the structure was therefore 50 vol.%. The limitation of the strut diameter within the mould was given by the RP machine and not by the mould filling capacity of the ceramic slurry. For the rapid prototyping machine used, the obtainable minimum strut size would be about 300 μm, that is in the range of the size of the trabeculae of natural bone material [38,39].

This virtual structure was imported by the software that controls the RP-machine and decomposed into thin layers to be built sequentially in the rapid prototyping process. The RP-machine used was a perfactory mini (Envisiontec, Marl, Germany).

This system uses liquid photosensitive resin (envisiontec perfactory® resin) that is selectively hardened by visible light. A micro-mirror array lets the light pass where the photosensitive resin should solidify and stops it where the resin should remain liquid. After the first layer has been exposed, and by this, the first layer of the future part (or structure) has been built, the stage moves for one layer thickness, new resin is applied and the procedure starts again (Fig. 2). When the whole part has been built layer by layer, the remaining liquid resin is removed with alcohol and the part is post cured with UV light [37]. This device provides a resolution of 32 μm for objects with outer dimensions of a few centimetres in each direction. This offers the possibility to

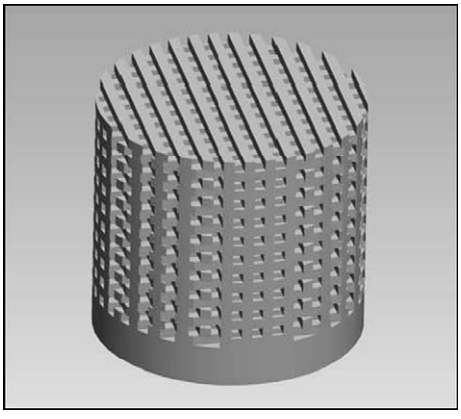

Fig. 1. Virtual structure designed with CAD (Pro/Engineer), for the description see text. This structure was reproduced in hydroxylapatite.

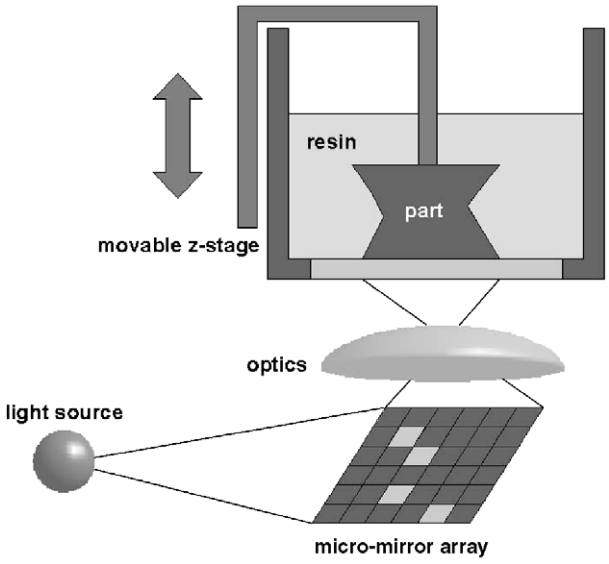

Fig. 2. Principle behind stereolithography, the rapid prototyping process we used to fabricate the casting moulds for ceramic gelcasting.

produce parts or casting moulds with struts in the order of a few hundred micron thickness.

The second step in the production process was to fill the casting mould in vacuum with a thermosetting ceramic slurry, a mixture consisting of water, ceramic powder, water soluble monomers and dispersion agent. Shortly before casting, a catalyst and an initiator were mixed into the slurry Thereafter, it was kept for an hour at a temperature of 60 °C, in order to polymerise the monomers and thus stabilise the green body. Then temperature was raised in steps to 1300 °C, which resulted in drying, burning of the resin and sintering of the ceramic particles [40]. The monomers used in our gelcasting experiments were methacrylamide and a crosslinking agent (*N*,*N*′-methylenbis(acrylamide); both Sigma-Aldrich). Monomer, crosslinker, water and dispersion agent were mixed in a bottle that slowly rotated on two powered rollers. The ceramic powder, artificial hydroxylapatite (Plasma Biotal, Tideswell, North Derbyshire, UK, with an average particle diameter of about 5 μm) was added in portions of decreasing amount, and the mixture was kept in motion for at least a week in order to fully homogenise the suspension. The composition of the ceramic slurry had to be optimized for viscosity and stability of the green body. Best compromise for the hydroxylapatite powder used was obtained with a mass ratio between ceramic powder and water of 5.5:1. The catalyst added shortly before casting was *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and the initiator ammonium persulfate (both Sigma-Aldrich). For infiltration of the mould, a vacuum of 10 mbar was used. During the temperature treatment, too fast temperature changes may lead to thermal expansion mismatch and a broken structure. Therefore we had to elevate the temperature very slowly in the range between room temperature and 600 °C. Above 600 °C, the resin mould was completely burnt and the sintering temperature (1300 °C) could be reached quickly. Also the cooling process was accomplished slowly and carefully.

After sintering, the hydroxylapatite material produced as described above was investigated by X-ray diffraction, to assess whether the material changed during the sintering process. Moreover, samples sintered at 1300 °C for various times (30 min up to 24 h) were embedded in resin, grinded and polished for investigation in scanning electron microscopy.

In order to assess the biocompatibility of the scaffolds, 4×10^5 preosteoblastic MC3T3-E1 cells [41] were seeded per scaffold in culture medium (αMEM containing 50 μg/ml ascorbic acid supplemented with 10% fetal bovine serum and 30 μg/ml gentamicin), and kept in culture for 2 weeks. Medium was changed twice a week. After this culture period the cells were fixed for morphological investigations. In a first set of experiments, they were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min at 4 °C, washed again with PBS for 15 min and permeabilised with acetone for 5 min at room temperature. Afterwards, the cells were incubated with propidiumiodide (4×10^{-4}M) for 45 min at room temperature and after washing with phosphate buffered saline (PBS) for 30 min, they were examined using a confocal laser scanning microscope. A second set of cultures were fixed in Schaffer's solution for 48 h, dehydrated with 70%, 80% and 100% of ethanol for 24 h each and embedded in poly-methylmetacrylate. 100 μm thin sections were prepared by sawing, grinding and polishing and surface stained with Giemsa for histological examination. In addition, several hard microtome sections were performed for Gömory staining to visualise the collagen matrix. During the microtome cutting process the scaffold part was lost. To study gene expression MC3T3-E1 cells were cultured for 4 weeks. Total RNA was isolated with 1 M guanidiniumthiocyanate, 0.1 M sodium acetate, pH=5.0, 5% glycerol, 0.4 M ammonium-isothiocyanate and 38% phenol. Gene expression was measured by reverse transcription-polymerase chain reaction (RT-PCR). 1 μg of total RNA was reverse transcribed by Moloney murine leukaemia virus reverse transcriptase in a volume of 20 μl and thereafter diluted to 40 μl. An aliquot of 5 μl was used in the PCR for osteocalcin and an aliquot of 2.5 μl was used in the PCR for glyeraldehyde-3-phosphate-dehydrogenase (GAPDH). Osteocalcin-PCR: 95 °C for 5 min followed by 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C, 24 cycles (primer: 5′-CAAGTCCCACACAGCAGCTT-3′ and 5′-AAAGCCGAGCTGCCAGAGTT-3′). GAPDH-PCR: 95 °C for 5 min followed by 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, 30 cycles (primer: 5′-CTGCACCACCAACTGCTTAGCC-3′ and 5′-GTCCACCACCCTGTTGCTGTAG-3′).

3. Results

In Fig. 3 one can see two of the structures obtained after sintering and in the background two resin moulds produced

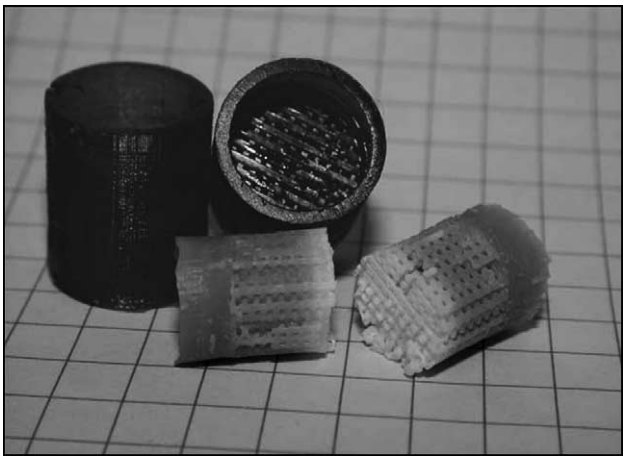

Fig. 3. Resin casting moulds and sintered structures made of hydroxylapatite, side length of the squares in the background is 5 mm. The excess length on top of the casting moulds works as reservoir for the ceramic slurry, which fills shrinking voids within the mould when the external pressure is applied.

by stereolithography. Although the structures are not free of cracks, they can easily be handled without outstanding attention.

In order to prove that the material (artificial hydroxylapatite) did not change during the sintering process, we performed X-ray investigations. Except for secondary properties like grain size, the experiments proved that the material remains unchanged up to a sintering temperature of 1300 °C at a sintering time of 1 h.

The microstructure of the sintered material has been investigated by scanning electron microscopy (SEM). Some specimens were sintered at a temperature of 1300 °C for various times. The results can be seen in Fig. 4: the longer the sintering times were, the higher the density of the material is. Due to the fact that there is no pressure applied during the sintering process, there is always a remaining porosity, independent from the sintering duration. As far as mechanical properties are concerned, the higher the density the better the material is.

Cell culture experiments showed that the scaffolds were a suitable substrate for growth and differentiation of MC3T3-E1 cells. After 2 weeks of culture, light microscopic analyses revealed that the cells formed multilayers covering the internal and external surfaces of the hydroxylapatite structure. First examination of the three-dimensional culture stained with propidiumiodide by confocal laser scanning microscopy showed an almost complete covering of the struts within the depth accessible to light microscopy (data not shown). Due to the opaqueness of the scaffolds, the full thickness of the structure could not be investigated by this method.

Some of the cultures were therefore fixed, resin embedded and Giemsa stained. The investigation of sections revealed that the cell coverage was similar in all regions of the three-dimensional scaffold (Fig. 5a,b). Typically all struts were covered by a 20–50 μm thick layer of cells with extracellular matrix, forming a tissue-like structure [42]. Moreover, the cells were rounding edges and filling cracks eventually present in the structures (Fig. 5b,c). A very typical picture in this respect is Fig. 5c. The cells seemed to cover preferentially inner edges by proliferating more in such positions while the coverage of outer edges was usually much thinner. The net result of this behaviour is a considerable smoothening of all the contours of the scaffold. Nevertheless, the fact that the cell matrix is covering even rather sharp corners (such as in Fig. 5c) indicates that there is a strong affinity of the cells to attach onto the hydroxylapatite substrate.

Finally, Gömöri staining of the sections (Fig. 5d) revealed 5 to 7 cell layers embedded in a dense extracellular collagenous matrix, which was obviously produced by the cells.

To assess the phenotype of the osteoblasts on the scaffolds RT-PCR for osteocalcin, a marker gene of the

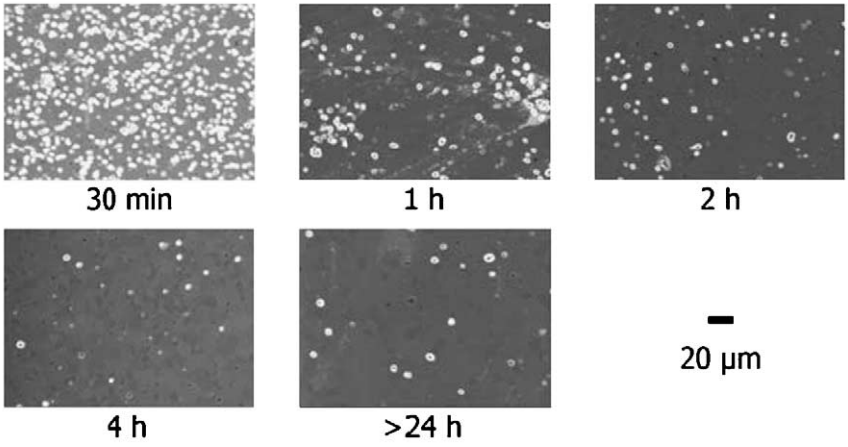

Fig. 4. Scanning electron microscopy images showing the influence of the sintering duration on the microstructure of the sintered hydroxylapatite. Pores in (dark grey) hydroxylapatite can be seen (light grey). Sintering temperature was 1300 °C for all samples.

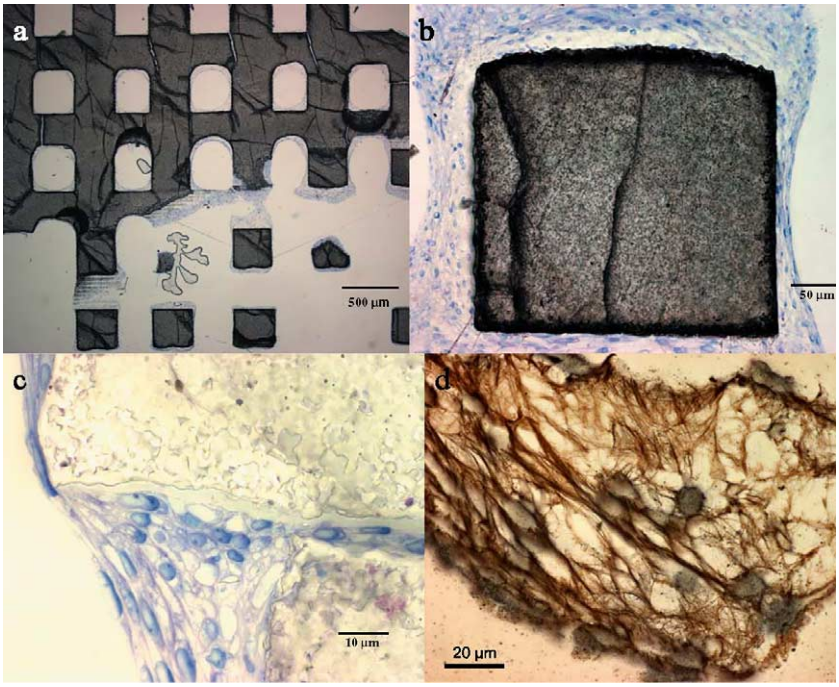

Fig. 5. (a) Light microscopic overview (objective lens 2.5×), showing a hydroxylapatite scaffold after culturing with MC3T3-E1 cells for 2 weeks, embedded in resin and stained with Giemsa. The scaffold is visible in dark grey and all surfaces are covered with cells (blue). (b) Further magnification (20×) showing: a single strut of a scaffold (grey), completely covered by cells (blue/pink). (c) High magnification of the Giemsa-stained section (100×) showing a crack between two struts of the scaffold (due to higher illumination here in pastel yellow) completely filled by MC3T3 cells (blue) and matrix generated by the cells (pink). (d) Gömöri stained section (100×) to reveal collagen in the matrix formed by the cells. Note that the mineral scaffold is not visible in this preparation because it was lost during the microtome sectioning process.

osteoblastic phenotype was performed. The GAPDH mRNA codes for a housekeeping gene and serves for normalization of the osteocalcin expression. As clearly shown in Fig. 6, the preosteoblastic MC3T3-E1 cells seeded on the scaffolds did not express osteocalcin (Fig. 6, lane 2). After a culture time of 4 weeks, a strong signal for osteocalcin (Fig. 6, lane 3) was found indicating osteoblastic differentiation of the precursor cells into osteoblasts on the scaffolds.

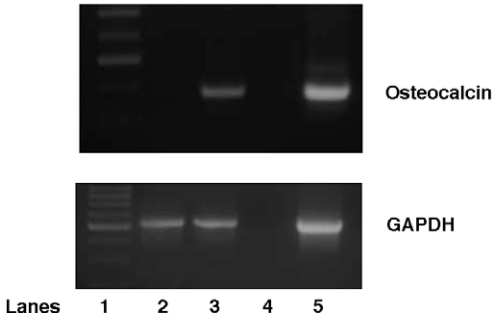

Fig. 6. MC3T3-E1 cells cultured on scaffolds express osteocalcin indicating a differentiated osteoblastic phenotype. Lane 1: 100 bp DNA-length standard. Lane 2: RT-PCR from RNA isolated from preosteoblastic MC3T3-E1 cells, which were seeded on the scaffolds; these cells did not express osteocalcin. Lane 3: RT-PCR from RNA isolated from osteoblastic MC3T3-E1 cells, which were cultured for 4 weeks on the scaffolds; a strong signal indicates osteocalcin mRNA expression. Negative control and positive control for the PCR is shown in lanes 4 and 5, respectively.

4. Discussion and conclusion

As a first step towards a new bone replacement material, the production of porous ceramic structures with controlled architecture via rapid prototyping and ceramic gelcasting has been shown to yield scaffolds, which support the multiplication of osteoblast-like MC3T3-E1 cells. These cells covered all internal and external surfaces, sometimes in several cell layers and generated an extracellular matrix forming a tissue-like structure. In this respect the behaviour of the cells on the surface of these three-dimensional scaffolds was very similar to their behaviour in (two-dimensional) culture dishes [42,43]. The cultured preosteoblastic cells formed new collagen and differentiated into osteoblasts, expressing osteocalcin, indicating that the system has the tendency to support the formation of new bone tissue. Surprisingly, they formed more cell layers at inner edges. It is well known that osteoblasts need cell–cell contacts and contacts to collagen matrix to proliferate and differentiate [44]. In the inner edge, the cells presumably find more possibilities to attach and come closer to forming three-dimensional contacts, thus promoting cell proliferation and differentiation, resulting in more cell layers in these areas.

Next steps in the development process will be to modify the material used (from hydroxylapatite to other calcium phosphates) in order to improve the biological resorbability [26–28,45–48], which also affects the

mechanical properties [49,50]. Indeed, one would eventually aim at a material which is resorbed by osteoclasts and gradually replaced by native bone material. Further, the architecture will be optimized to improve both the biological and the mechanical properties. This will make full use of the potential of rapid prototyping and ceramic gelcasting which allows to freely design the architecture of the porous structure.

Acknowledgement

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