Biofabrication of multi-material anatomically shaped tissue constructs

Visser, Jetze; Peters, Benjamin; Burger, Thijs J.; Boomstra, Jelle; Dhert, Wouter J. A.; Melchels, Ferry; Malda, Jos

Published in:
Biofabrication

DOI:
10.1088/1758-5082/5/3/035007

Publication date:
2013

Document Version
Peer reviewed version

Link to publication in Heriot-Watt University Research Portal

Citation for published version (APA):
Biofabrication of multi-material anatomically shaped tissue constructs

Jetze Visser¹, Benjamin Peters¹-², Thijs Burger¹, Jelle Boomstra³, Wouter Dhert¹-⁴, Ferry Melchels¹-⁵, Jos Malda¹⁵-⁶

1. Department of Orthopaedics, Netherlands Institute of Regenerative Medicine, University Medical Center Utrecht, Utrecht, The Netherlands.
2. University of Denver, Denver, Colorado, USA
3. Protospace, Fablab Utrecht, Utrecht, The Netherlands
4. Faculty of Veterinary Sciences, Utrecht University, Utrecht, The Netherlands
5. Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia
6. Department of Equine Sciences, Faculty of Veterinary Sciences, Utrecht University, Utrecht, The Netherlands
Abstract

Additive manufacturing in the field of regenerative medicine aims to fabricate organized tissue-equivalents. However, the control over shape and composition of biofabricated constructs is still a challenge and needs to be improved. The current research aims to improve shape, by converging a number of biocompatible, quality construction materials into a single three-dimensional fiber deposition process. To demonstrate this, several models of complex anatomically shaped constructs were fabricated by combined deposition of poly(vinyl alcohol), poly(e-caprolactone), gelatin methacrylamide/gellan gum and alginate hydrogel. Sacrificial components were co-deposited as temporary support for overhang geometries and were removed after fabrication by immersion in aqueous solutions. Embedding of chondrocytes in the gelatin methacrylamide/gellan component demonstrated that the fabrication and the sacrificing procedure did not affect cell viability. Further, it was shown that anatomically shaped constructs can be successfully fabricated, yielding advanced porous thermoplastic polymer scaffolds, layered porous hydrogel constructs, as well as reinforced cell-laden hydrogel structures. In conclusion, anatomically shaped tissue constructs of clinically relevant sizes can be generated when employing multiple building and sacrificial materials in a single biofabrication session. The current techniques offer improved control over both internal and external construct architecture underscoring its potential to generate customized implants for human tissue regeneration.
1. Introduction

Additive Manufacturing (AM) is the layer-by-layer construction of objects based on a three-dimensional (3D) model data set\textsuperscript{1,2}. In the field of regenerative medicine (RM), AM has the potential to produce customized tissue implants to replace damaged or eroded parts of the human body. In order to achieve this, a balanced combination of cells, growth factors, matrix and/or biomaterials needs to be processed into a 3D-construct with controlled architecture. Unlike conventional subtractive manufacturing, AM offers full control over internal and external architecture of the object and every deposited layer serves as a fundament for the next layer. Moreover, AM technologies, including 3D fiber deposition (3DF)\textsuperscript{1,2}, potentially allow for reproducibility and customization of complex tissue analogous with limited waste of the often expensive building biomaterials.

Scaffolds for tissue engineering have been fabricated with 3DF from biodegradable thermoplastic polymers, e.g. polycaprolactone (PCL)\textsuperscript{3-4} and PCL-derivatives\textsuperscript{5-7}, polylactic acid (PLA)\textsuperscript{8} or PolyActive\textsuperscript{9} allowing for direct implantation\textsuperscript{5,6} with the possibility of prior cell seeding\textsuperscript{8} or incorporation of growth factors\textsuperscript{3}. In addition, scaffolds have also been fabricated from hydrogels, where the hydrogel serves as a carrier for cells or bioactive factors, while simultaneously serving as a building block\textsuperscript{10-14}. Recently, the combined deposition of thermoplastic polymers and hydrogels was developed in order to reinforce hydrogel constructs with a thermoplastic polymer network\textsuperscript{15-17}.

For the translation of biofabrication to the clinic, tissue equivalents must be fabricated from digital blueprints of the anatomical structure that needs to be repaired or replaced\textsuperscript{1,20}. To this end, control over both shape and composition of implants needs to be improved\textsuperscript{1,2,18,19}, addressing complex internal and external architectures as well as multiple tissue types of the target structure. For example, when pursuing to fabricate a vascular network, the desired lumen diameter, branching pattern, vessel stiffness and possibly the different tissue types in the vessel wall need to be addressed. Similarly, when aiming to generate a construct with the shape of the external ear, the complex auricular shape, as well as the different tissue types (\textit{i.e.} cartilage, skin and blood vessels) need to be put in place. In order to achieve this, better control over the deposition is needed, requiring novel construction biomaterials that facilitate tissue regeneration. In addition, in order to process these materials in three dimensions with high resolution, biofabrication techniques need to be optimized.

The current research aims to improve the shape of constructs by integrating several quality construction biomaterials into a single fabrication session. This includes materials that temporarily support overhang geometries in complex structures, as layer-by-layer fabrication will fail in the absence of a base layer. These support materials need to be sacrificed after fabrication, a principle that has already been applied in microfluidics\textsuperscript{21,22,23}, but also for the casting of collagen scaffolds\textsuperscript{24} and for achieving internal porosity in tissue-engineered constructs\textsuperscript{25,26}. Moreover, sacrificial materials have been applied for realizing controlled internal architectures within hydrogel constructs, either by casting\textsuperscript{27} or combining printing and casting\textsuperscript{28,29}. For the current 3DF approach, sacrificial materials were selected so that they can be processed layer-by-layer and removed from the target structure without compromising its shape.
Furthermore, sacrificial materials should support, and form a stable interface with adjacent components, and should not compromise cellular survival.

Here, we demonstrate how several relevant-size models of complex anatomical structures, being a vascular network, an ear and a distal femur, can be fabricated by combining building and sacrificial materials in a multi-material 3DF setting. These include thermoplastic polymers and hydrogels that provide both mechanical support and a suitable environment for cells to regenerate tissue.
2. Materials and Methods

2.1 Materials

Polyvinyl alcohol (PVA) filament (diameter 3 mm, melting point 160-170°C, density 1.25-1.35 g/cm³) (Kuraray USA, Houston, TX, USA) was obtained from Makerbot Industries, NY, USA. PCL (average Mₘ 45,000; Mₘ 48,000-90,000) was obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands. Alginate hydrogel (10% w/v) was prepared by mixing sodium alginate powder (Sigma-Aldrich) overnight at 37°C in dH₂O. Gelatin methacrylamide (gelMA) was synthesized as described previously. GelMA-gellan hydrogel was prepared by dissolving gelMA (10% w/v) and gellan gum (Gelzan™ CM, Sigma-Aldrich) (1.1% w/v) at 90°C for 20 minutes in dH₂O containing 0.1% w/v photoinitiator Irgacure 2959 (Ciba, BASF, Ludwigshafen am Rhein, Germany). In case cells were mixed in the hydrogel, D-mannose (5.4% w/v) was added to the mixture to obtain isotonic conditions. GelMA-gellan hydrogels were stained with fast green (Merck, Whitehouse Station, NJ, USA) for visualization purposes when printing multi-layered constructs.

2.2 Experimental design

Several anatomically shaped constructs were designed using Rhinoceros computer aided design (CAD) software (McNeel, Seattle, WA, USA), including structures inspired by blood vessels, an ear and a distal femur (Table 1). Complementary structures were designed in order to temporarily support overhangs in the target structures on biofabrication. The CAD-files were loaded via computer-aided manufacturing (CAM) software (PrimCAM, Einsiedeln, Switzerland) in which a building material (PCL, gelMA-gellan hydrogel, or both) was assigned to the target structure and a specific sacrificial material (PVA, PCL or alginate hydrogel) was assigned to the support components. A stack of all these materials was fabricated, with subsequent removal of the sacrificial components, in order to evaluate the effects of the full fabrication process on the survival of cells embedded in the gelMA-gellan hydrogel component.

2.3 Cells: equine chondrocytes and mesenchymal stromal cells (MSCs)

Full-thickness cartilage was harvested (with consent of the owner) under sterile conditions from the stifle joint of a fresh equine cadaver (n=1; 7 years old) with macroscopically healthy cartilage that died of natural causes in the clinic. After overnight digestion in type II collagenase (Worthington Biochemical Corp) at 37°C the suspension was filtered and washed in PBS. The chondrocytes were seeded at a density of 5·10⁵ cells/cm² and expanded for 10 days in a monolayer culture in chondrocyte expansion medium consisting of DMEM (Dulbecco’s Modified Eagle Medium 41965, Invitrogen), 10% heat-inactivated fetal bovine serum (Biowhittaker), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen), and 10 ng/ml FGF-2 (R&D Systems). MSCs were obtained from a sternal bone marrow aspirate from a healthy, living equine donor (n=1), with approval of the institutional ethics committee. The mononuclear fraction (MNF) was isolated by centrifuging the sample on Ficoll-Paque. The MNF was seeded at a density of 2.5·10⁵ cells/cm² and
expanded in a monolayer culture till subconfluency in MSC expansion medium containing α-MEM (22561, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 0.2 mM L-ascorbic acid 2-phosphate (Sigma), 100 units/ml penicillin and 100 µg/ml streptomycin, and 1 ng/ml FGF-2.

2.4 Histology

MSCs were suspended in gelatin hydrogel (from bovine skin, type B, Sigma-Aldrich) (10% w/v, 5×10⁶ cells/ml), which was perfused through the lumen of a 3D-printed fiber reinforced tube. The tube was processed into cross-sectional 5 μm paraffin slides and a triple-stained with hematoxylin, fast green, and Safranin-O (all from Sigma). To visualize the cells, gelatin and the gelMA-gellan (vessel wall) component, stained sections were examined using a light microscope (Olympus BX51).

2.5 Three-dimensional fiber deposition

Constructs were fabricated using the BioScaffolder 3DF system (SYS+ENG, Salzgitter-Bad, Germany), which can build three-dimensional objects by coordinated motion of several dispensing heads, depositing on a stationary platform. Four dispensing heads were used, employing different types of extrusion: piston-driven extrusion for gelMA-gellan and alginate hydrogel; auger screw driven melt extrusion for PCL, and filament extrusion for PVA. The filament extruder was custom-built, based on the commercially available open-source Ultimaker 3DF machine (Ultimaker LTD, Geldermalsen, The Netherlands) and was adapted for use within the BioScaffolder multiple-tool dispensing setting. The BioScaffolder was located at room temperature in a custom-built laminar flow cabinet to ensure sterile conditions and to facilitate rapid cooling of deposited thermoplastic materials. Fabrication parameters were set for each printing material, as detailed in Table 2. Strand spacing for both hydrogels ranged between 0.8 and 1.8 mm, in which the first setting yields a solid - at a given ratio of spindle speed and translational speed - and the second a porous construct. Both strand spacings were used in order to represent respectively the cartilage and bone component of a structure inspired by a distal femur. To analyze internal shape fidelity, a four-strand-thick cross-section of the femoral condyle constructs was freeze-dried overnight and visualized with stereomicroscopy (SZ61/SZ2-ILST, Olympus, The Netherlands). Generated constructs were captured with a digital single lens reflex-camera (EOS 60D, Canon inc., Japan). Porosity of the fabricated PCL constructs was determined gravimetrically.

2.6 Sacrificing procedures

PVA, PCL and alginate hydrogel were used as sacrificial materials, each requiring a specific sacrificing procedure (Table 1). PVA served as a sacrificial support for PCL constructs. PVA dissolves easily in aqueous solutions and was, therefore, washed from the PCL construct with water on a rollerbank overnight. PCL and alginate hydrogel served as sacrificial materials to gelMA-gellan hydrogel constructs. The PCL was sacrificed by manually taking the hydrogel construct out of its PCL support structure. The alginate components were removed from the gelMA-gellan constructs by washing in a 130mmol sodium citrate solution on a rollerbank. The gelMA-gellan/alginate constructs were UV-
cured for 5 minutes after printing (Superlite S-UV 2001AV lamp (Lumatec, Munchen, Germany) at 320–500 nm, intensity of 6 mW cm\(^{-2}\) at 365 nm) in order to irreversibly crosslink the gelMA component. Printing the fiber-reinforced construct involved a PCL/GelMA-gellan target structure with alginate support. Temporary PCL stands were designed at both ends of the construct in order to anchor PCL strands that were deposited on top of different hydrogels. After UV-crosslinking the anchoring blocks were sacrificed manually with a scalpel and the alginate was subsequently sacrificed by dissolving in sodium citrate solution.

2.7 Viability assay

The effects of the printing process, as well as the sacrificing procedure on cell viability in a printed construct were investigated. Hereeto, a layered block (LxWxH = 20x20x6mm) was printed from PVA, PCL, gelMA-gellan (non-porous layer, containing \(5 \times 10^6\) chondrocytes/ml) and alginate. The printed block was cut in half and both parts were washed on a rollerbank at 37°C in two different isotonic, pH7.4 trisodium citrate solutions (65 and 130mmol) in the absence of divalent ions to prevent the alginate from crosslinking. For both concentrations, cytocompatibility and the ability to dissolve the alginate component were analyzed. After washing for one hour, the gelMA-gellan-chondrocyte layer was cultured for 1 and 3 days \textit{in vitro} at 37°C in chondrocyte expansion medium. A solid layer (LxWxH = 20x20x1.5mm) of gelMA-gellan containing chondrocytes was printed as a control to the washing procedure and a sheet (LxWxH = 8x8x1.5mm) was casted as a control to the printing process. All samples were UV crosslinked for 5 minutes. To visualize cell viability, a LIVE/DEAD Viability Assay (Molecular Probes MP03224, Eugene, USA) was performed according to the manufacturer’s instructions. The samples were examined using an Olympus BX51 light microscope and photomicrographs taken with an Olympus DP70 camera (both Olympus, United States). The excitation/emission filters were set at 488/530 nm to observe living (green) cells and at 530/580 nm to detect dead (red) cells. At least 300 live and dead cells per group were counted in at least four locations within the constructs.
3. Results

3.1 Cell viability after printing and sacrifice of support components

In order to analyze whether the printing process and the sacrificing procedure of temporary support materials described here is cytocompatible, all building and sacrificial materials were printed in a square stack (Figure 1A). By washing away the sacrificial layers, cells embedded in the gelMA-gellan hydrogel were exposed to low and high sodium citrate solutions containing an over-time increasing amount of dissolving PVA and alginate gel. After one hour the major part of the PVA and the alginate layers had dissolved in both solutions, resulting in separate PCL and gelMA-gellan sheets. From these macroscopic findings, both citrate concentrations were equally potent in dissolving the alginate component. After 1 and 3 days in vitro culture, chondrocytes were found 75-86% viable in all groups (Figure 1B). No significant difference in cell viability was observed for the printed group or for either the low or high citrate concentration as compared to the non-printed control.

3.2 Thermoplastic polymer scaffolds

Anatomically shaped PCL-based structures were successfully fabricated (Figure 2). Overhangs in these constructs were supported by co-deposited PVA components, which were sacrificed without compromising the quality of the PCL target structure. High shape-fidelity was achieved as the structures matched the dimensions of the design files. A structure inspired by a vascular tree was fabricated with an open vessel lumen decreasing in diameter from 4 to 2 mm; the strand spacing of 0.7 mm resulted in a porosity of 61% (Figure 2A-C). Next, a structure resembling a right ear was fabricated with relevant human dimensions in which a strand spacing of 1.0 mm resulted in a porosity of 74% (Figure 2D-F). PVA components were designed to support the tragus, located over a cavity representing the auditory channel and for overhangs up to 60° in the outer contour of the ear. Additionally, the model of the DNA-helix is a powerful demonstration of the successful co-deposition of support material, since 0 to 90° overhangs are present in this complex structure (Figure 2G-I).

3.3 Hydrogel constructs

A miniaturized model of a distal femur (Figure 3) was printed including a solid layer (green) representing the cartilage component and a porous bone component (yellow) (Figure 3B). GelMA-gellan hydrogel strands allowed for proper deposition onto the porous PCL support structure (Figure 3C). Moreover, their stiffness after deposition was sufficient to retain shape and to support the overlying layers. The shape-fidelity of the internal architecture was shown in a cross-section of the femoral condyles (Figure 3D, 3E). The hydrogel construct could be manually removed from the PCL support structure.

In a different approach, alginate hydrogel provided internal support for creating interconnected tubes within a gelMA-gellan hydrogel box (Figure 4). Both hydrogels were compatible with the co-deposition process and the high-viscosity alginate was found to be stable enough to carry the overlying structures without crosslinking. By sacrificing the alginate component, 4mm tubes were created within the box, matching the design geometry.
3.4 Fiber-reinforced hydrogel scaffold

A hollow tube was fabricated in a fiber-reinforced approach, inspired by a single blood vessel. Specific sacrificial components were integrated in supporting overhanging geometries of this hybrid construct (Figure 5). The inner and outer curvatures of the tube were supported by high-viscosity alginate. PCL fibers were anchored in temporary PCL stands (Figure 5A, B) that were successfully removed with a scalpel (Figure 5C). By sacrificing the alginate components in a sodium citrate solution, a 4mm diameter lumen was created in the tube. The lumen was shown to be open and perfusable, by injection of an MSC-laden gelatin hydrogel (green on safranin-O staining (Figure 5D and 5E)).
4. Discussion

Anatomically shaped constructs were fabricated by integrating biocompatible thermoplastics, hydrogels and sacrificial materials into a single 3DF-based biofabrication approach. The application of specific sacrificial materials was essential in supporting internal and external overhang geometries in these constructs. Co-deposition of multiple materials and the sacrificing procedures did not adversely affect the shape of the target structure or viability of embedded cells.

Overhangs are inevitable in the internal and external geometry of most anatomical structures, although, for AM purposes, they can be minimized through optimal 3D rotation of the digital blueprint. The remaining overhangs may require mechanical support components which need to be sacrificed by dissolving them in aqueous media or by manual removal from the target structure, as was recognized in a US patent in 1996\textsuperscript{32}. This patent has mainly been applied for fabricating solid objects from thermoplastic polymers with commercial filament-based 3DF-machines. Obviously, when aiming to support constructs for use in RM, the range of suitable support materials will be smaller, since the material and its removal needs to be biocompatible. Therefore, in the current study, several specific biocompatible support materials were implemented.

In the first place, clinically relevant structures were realized by co-deposition of PVA as a soluble support material to PCL. Importantly, PCL is a biocompatible and degradable polymer that has gained FDA-approved in a range of devices, and PCL scaffolds have proven to facilitate abundant formation of neo-tissue at orthotopic locations in animal models\textsuperscript{1, 39} and in humans\textsuperscript{6, 33}. In order to achieve this, they can be loaded with growth factors\textsuperscript{3} or cells\textsuperscript{33, 34}, or can be functionalized by mixing in other components\textsuperscript{6}. The current fabrication technique allow for customization of complex PCL-implants.

Printing complex structures from hydrogels is more challenging than printing them from thermoplastic polymers, since the large water fraction compromises the shape fidelity of hydrogel structures\textsuperscript{12, 35}. This may explain why hydrogel printed structures so far mainly have been fabricated using relatively simple architectures\textsuperscript{1, 2}. More complex, vessel-like constructs have been realized by co-printing hydrogels employing an ink-jet printer\textsuperscript{36} or a Fab@home dispensing system\textsuperscript{37}. Hereby, channels were created in controlled hydrogel structures; even so, the complexity in 3D-geometry was limited\textsuperscript{36} or the support component could not be sacrificed\textsuperscript{37}. Both issues were addressed in a combined approach of printing a sacrificial vascular structure as a framework for subsequent hydrogel casting\textsuperscript{28}. The carbohydrate glass vascular network was printed with high resolution, however casting offers limited control over the composition of the hydrogel construct, including gradients of different biomaterials, cell types or growth factors. For this reason, layer-by-layer assembly using multiple-materials, as demonstrated here, would be favorable for the fabrication of tissue equivalents.

Recently, gelMA-based hydrogels have shown to facilitate specific differentiation of different cell types\textsuperscript{30, 38}. In addition, gelMA hydrogel platforms have been tailored for better shape fidelity whilst
retaining their beneficial biological properties\textsuperscript{31, 39}. With the addition of gellan gum to gelMA, a hydrogel was created with beneficial rheological properties improving shape-fidelity in 3DF\textsuperscript{31}. By processing gelMA/gellan in combination with removable or soluble support materials the current research took a next step and the generated construct complexity goes beyond what has been achieved in 3DF with hydrogels so far\textsuperscript{11-14, 20, 39, 40}. A miniaturized model of a distal femur was constructed employing two hydrogel printing heads realizing structures representing a bone and cartilage component that can potentially contain specific cells, growth factors or bioactive peptides for tissue regeneration\textsuperscript{11, 41, 42}. The vertical porosity in the bone component facilitates diffusion of nutrients and oxygen\textsuperscript{43}, whereas chondrocytes are more likely to thrive under the relatively hypoxic conditions in the solid cartilage component\textsuperscript{44}. The femoral condyles were supported during fabrication by a PCL component, which could be manually removed afterwards, possibly facilitated by the hydrophobic character of PCL. Nevertheless, the approach of creating a removable joint between the building and sacrificial component is not applicable when complexity of the target structure demands an internal or embracing support. Therefore, sacrificial materials have been introduced that can be washed away from the target structure\textsuperscript{21-24, 27-29, 45}. Still, there is a lack of support materials that are soluble and allow for deposition along with the hydrogel target structure. High-concentration alginate hydrogel meets both these requirements as was illustrated here with the realization of a tubular network in a gelMA-gellan hydrogel box. It should be noted however, that the resolution of the current tubes was lower compared to casting techniques\textsuperscript{27, 28, 46}.

Future 3DF constructs may increasingly consist of multiple types of materials. For example, methods have been introduced recently to improve the mechanical properties of hydrogel constructs by co-depositing a PCL network\textsuperscript{15-17}. An additional advantage of this fiber reinforcement approach would be increasing the complexity of printed structures, as PCL can be processed with high shape-fidelity and its fibers can form a rigid network\textsuperscript{47}. Nevertheless, in contrast to earlier rectangle hybrid designs\textsuperscript{15-17}, integration of a reinforcing PCL network in complex structures may require deposition of PCL onto hydrogel in the absence of a fundamental PCL layer. As attachment of thermoplastic polymer strands onto hydrogel is poor, multiple-material designs should offer smart solutions. Therefore, in the present fiber-reinforced tube model, temporary PCL-stands were designed at both ends for anchoring the strands. Another solution could be implementation of a temporary support for PCL, as PVA was used in the current study. However, a material interface between PVA and hydrogel should be avoided, since the PVA would dissolve in contact with the hydrogel. This results in an unstable material interface compromising the shape fidelity of the target structure.

To increase the resolution of the fabrication technology, different technologies may need to be combined, as recently was achieved for solution electrospinning and inkjet printing\textsuperscript{48}. With inkjet printing, heterogeneous tissue constructs can be fabricated with high resolution\textsuperscript{49}. However, the droplet-driven technique may be less suitable for fabricating complex constructs of relevant size (particularly in height) as compared to 3DF. Moreover, for the fabrication of complex anatomical structures, melt electrospinning techniques may need to be integrated\textsuperscript{50}, allowing for control over high-
resolution fiber deposition\textsuperscript{11}, in contrast to traditional solution electrospinning where fibers are randomly deposited. In addition, interfacial bonding between the hydrogel and its reinforcing fibers should be addressed, in order to further improve the integrity of the construct.

In line with earlier reports\textsuperscript{15, 18, 22}, the present biofabrication process did not affect cell viability. Also, the sacrificing procedure of alginate in both low (65mmol) and high (130mmol) sodium citrate concentrations was not harmful to the cells. It was already known that cells are able to survive a 20-minute treatment with 55mmol citrate solutions for dissolving encapsulating alginate beads\textsuperscript{15}. Based on the current results, higher sodium citrate concentrations also seem to be compatible with cells embedded in gelMA-gellan hydrogel. Still, the longer-term effect on cellular behavior and the question whether or not higher sodium citrate concentrations would result in shorter dissolving times of alginate hydrogel, needs to be investigated.

Future research still needs to be performed in order to optimize the translation of a digital blueprint of the target anatomical structure into a printable CAD-file including sacrificial and building components. In addition, software and hardware have to be integrated for reproducible fabrication with multiple tools. Complexity of constructs should be further increased and cells and bioactive components should be integrated. However, with the current research, an important step was made in the biofabrication of complex anatomical tissue constructs for application in the field of RM.
7. **Acknowledgements**

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreements n°309962 (HydroZONES) and n°272286 (PrintCART). Jetze Visser was supported by a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant n°FES0908) and Jos Malda was supported by the Dutch Arthritis Foundation.
<table>
<thead>
<tr>
<th>Target construct</th>
<th>Permanent material</th>
<th>Sacrificial material</th>
<th>Sacrificing procedure</th>
<th>Printed structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermoplastic polymer</td>
<td>PCL</td>
<td>PVA</td>
<td>Wash in water</td>
<td>Models of a vascular branch, ear and DNA-helix</td>
</tr>
<tr>
<td>Hydrogel</td>
<td>GelMA-gellan</td>
<td>PCL</td>
<td>Manual removal</td>
<td>Model of a distal femur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alginate</td>
<td>Wash in citrate solution</td>
<td>Box with tubes</td>
</tr>
<tr>
<td>Fiber reinforced hydrogel</td>
<td>PCL + GelMA-gellan</td>
<td>Alginate</td>
<td>Wash in citrate solution</td>
<td>Single tube</td>
</tr>
</tbody>
</table>

*Table 1. Printing of a thermoplastic polymer (PCL), a hydrogel (gelMA-gellan) or a combination of both in a fiber reinforced construct required specific sacrificial materials (PCL, PVA or alginate hydrogel) in order to achieve optimal shape fidelity. Different procedures were applied to sacrifice these materials.*
<table>
<thead>
<tr>
<th></th>
<th>PVA</th>
<th>PCL</th>
<th>GelMA-gellan</th>
<th>Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Materials specifications</strong></td>
<td>Filament Granules, m_n 45,000</td>
<td>Hydrogel, 10%-1.1% w/v</td>
<td>Hydrogel, 10% w/v</td>
<td></td>
</tr>
<tr>
<td><strong>Extrusion mechanism</strong></td>
<td>filament auger screw</td>
<td>piston</td>
<td>piston</td>
<td></td>
</tr>
<tr>
<td><strong>Printing temperature</strong></td>
<td>185°C</td>
<td>90°C</td>
<td>35°C</td>
<td>25°C</td>
</tr>
<tr>
<td><strong>Needle type</strong></td>
<td>Ultimaker® Nozzle</td>
<td>DL Technology EZ-FLO™ (Haverhill, MA, USA)</td>
<td>Nordson EFD Precision Tips (East Providence, RI, USA)</td>
<td>Nordson EFD Precision Tips</td>
</tr>
<tr>
<td><strong>Needle internal diameter (mm, (Gauge))</strong></td>
<td>0.4 (22G)</td>
<td>0.32 (23G)</td>
<td>0.32 (23G)</td>
<td>0.5 (21G)</td>
</tr>
<tr>
<td><strong>Deposition speed (mm min⁻¹)</strong></td>
<td>350</td>
<td>150</td>
<td>1250</td>
<td>900</td>
</tr>
<tr>
<td><strong>Layer thickness (mm)</strong></td>
<td>0.20</td>
<td>0.17-0.20</td>
<td>0.17-0.20</td>
<td>0.19-0.20</td>
</tr>
<tr>
<td><strong>Strand spacing (mm)</strong></td>
<td>1.3-1.5</td>
<td>0.7-1.2</td>
<td>0.8-1.8</td>
<td>0.8-1.8</td>
</tr>
</tbody>
</table>

**Table 2.** Specifications of the BioScaffolder 3DF-system (SYS+ENG) for dispensing different materials in the current study.
A) In order to analyze cell viability in a multiple-material construct a square stack was printed of the four materials used in the present research, from bottom to top PVA, PCL, gelMA-gellan (containing chondrocytes) and alginate. B) Viability of chondrocytes in gelMA-gellan was not affected by the printing process after 1 and 3 days of in vitro culture, nor by the subsequent sacrificing procedure in 65 (low) and 130mmol (high) sodium citrate solutions. After one hour the alginate and PVA were considerably dissolved.
Figure 2. 3D-printed complex anatomical structures based on polycaprolactone (PCL) with polyvinyl alcohol (PVA) support. Vascular tree (A-C) (LxWxH = 67x42x8mm, vessel inner diameter 2-4mm); right ear (D-F) (LxWxH = 63x41x13mm); DNA helix (G-I) (LxWxH = 43x18x15mm). CAD designs (A,D,G) showing permanent (red) and sacrificial (gray) components; printed structures (B,E,H) showing PCL in bright white and PVA in off-white; PCL scaffold after sacrificing PVA support (C,F,I).
Figure 3. A) Distal femur from a human knee B) was designed in Rhino (LxWxH: 40x35x32mm) containing a bone component (yellow), a cartilage layer (green) and a support structure (white); C) the distal femur printed in hydrogel including PCL support structure; D) hydrogel component after manually removing support structure; E) freeze-dried four-layer thick cross-section (level marked by white lines in figure 2D) from the femoral condyles showing a solid cartilage component (marked by dashed boundary lines) and a porous bone component.
Figure 4. A) Perspective view of a gelMA-gellan (red) hydrogel box design (LxWxH = 15x15x10mm) containing an internal tubular structure (gray, diameter 4mm); B) a circular alginate component is marked by the dashed white line in a side-view of the gelMA-gellan box; C) by sacrificing the alginate hydrogel an internal tubular network was created as visible in side-view and D) top-view. All scale bars represent 5mm.
Figure 5. A) Design of a fiber reinforced single tube (inner diameter 4mm, outer diameter 6mm, length 20mm) including anchoring stands (white: PCL, red: gelMA-gellan, blue: alginate); B) construct directly after printing, scalpel cuts for removing stands represented by dashed lines; C) tube after removing PCL stands; D) cross-section of printed tube, right: design, left: after infusion with gelatin containing MSCs (safranin-O staining, red: gelMA-gellan tube wall; green: gelatin-MSC mixture). Reinforcing PCL fibers that were present in the tube wall were dissolved during the embedding process; E) magnification from picture D of gelatin hydrogel containing MSCs (blue dots) (scale bar represents 200μm).
5. References


engineering based on photo-curable gelatin, hyaluronic acid and chondroitin sulfate submitted. 2013


34. Park SH, Kim TG, Kim HC, Yang DY, Park TG. Development of dual scale scaffolds via direct polymer melt deposition and electrospinning for applications in tissue regeneration. Acta Biomater. 2008;4:1198-1207


49. Xu T, Zhao W, Zhu JM, Albanna MZ, Yoo JJ, Atala A. Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology. *Biomaterials*. 2013;34:130-139
