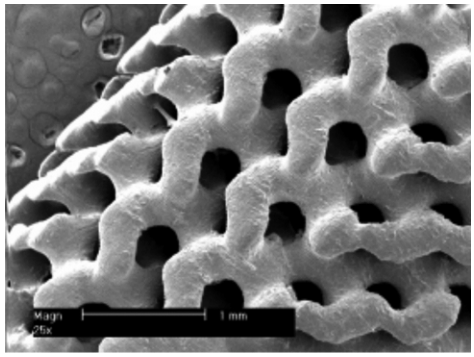
Flexible and Elastic Scaffolds for Cartilage Tissue Engineering Prepared by Stereolithography Using Poly(trimethylene carbonate)-Based Resins

Sigrid Schüller-Ravoo, Sandra M. Teixeira, Jan Feijen, Dirk W. Grijpma, André A. Poot*

The aim of this study is to investigate the applicability of flexible and elastic poly(trimethylene carbonate) (PTMC) structures prepared by stereolithography as scaffolds for cartilage tissue engineering. A three-armed methacrylated PTMC macromer with a molecular weight of 3100 g mol^{-1} is used to build designed scaffolds with a pore diameter of 350 ± 12 μm and a porosity of 54.0 ± 2.2%. Upon seeding of bovine chondrocytes in the scaffolds, the cells adhere and spread on the PTMC surface. After culturing for 6 weeks, also cells with a round morphology are present, indicative of the differentiated chondrocyte phenotype. Sulphated glycosaminoglycans and fibrillar collagens are deposited by the cells. During culturing for 6 weeks, the compression moduli of the constructs increases 50% to approximately 100 kPa.



1. Introduction

Due to the increase in life expectancy, degenerative disease and trauma, demands for strategies to restore the function of articular cartilage are increasing. Current methods for cartilage repair include the use of cell-based therapies, intended to regenerate the damaged tissue in a bioresorbable three-dimensional support structure. Under suitable culture conditions or after direct implantation into the patient, autologous chondrocytes should proliferate in the scaffold and deposit cartilage-specific extracellular matrix (ECM). Several efforts have been carried out in this direction, predominantly using hydrogels as substrates.[1,2] Although hydrogels can mimic a three-dimensional environment for cell attachment, their mechanical properties remain a challenge.[3]

Photo-polymerizable and photo-crosslinkable polymers that yield flexible and elastic materials receive increasing attention in the field of tissue engineering.[4,5] Processing of these polymers by stereolithography would produce flexible and elastic three-dimensional structures. By means of stereolithography, three-dimensional structures are created layer by layer, irradiating a photo-sensitive resin according to a given design.[6–8] The control of critical parameters such as porosity, pore size, pore size distribution, and pore interconnectivity, promotes the transport of

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nutrients and metabolic waste products throughout the scaffold.[7,9] Application of stereolithography in tissue engineering presents additional advantages over conventional approaches, since patient-specific implants can be built from scanning data of magnetic resonance imaging or tomography techniques.[10]

Only a few resins have been developed, that yield flexible and elastic networks by stereolithography. As reported by us, these resins are based on functionalized poly[(D,L-lactide)-*co*-(ε-caprolactone)] or poly(trimethylene carbonate) (PTMC) macromers.[11,12] The mechanical properties of photo-crosslinked PTMC depend on the molecular weight of the macromer, allowing the creation of structures with desired modulus, elongation, and strength.[13] In addition, PTMC networks show good compatibility with different cell types and are enzymatically degradable.[14–16] In contrast to aliphatic polyesters such as polylactides, which degrade by bulk erosion accompanied by the formation of acidic compounds, PTMC degrades by surface erosion without the formation of acidic degradation products.[14]

In view of the suitable properties of PTMC for tissue engineering and the ability to prepare flexible and elastic PTMC networks by stereolithography, the aim of the present study was to investigate the applicability of designed PTMC scaffolds built by stereolithography for engineering of cartilage tissue. Primary bovine chondrocytes were seeded in PTMC scaffolds with gyroid pore architecture. To evaluate cell morphology and deposition of ECM, microscopical and histological analyses were performed during a culture period of 6 weeks. Moreover, the effect of chondrocyte culturing on the mechanical properties of the constructs was investigated by dynamic mechanical analysis.

2. Materials and Methods

2.1. Preparation of PTMC Resins

A three-armed PTMC macromer was synthesized in a two-step reaction and formulated into a liquid resin to be applied in stereolithography. In the first step, hydroxyl-terminated PTMC oligomer was prepared by ring-opening polymerization (oligomerization) of 1,3-trimethylene carbonate (TMC, Boehringer Ingelheim, Germany) using glycerol (Sigma–Aldrich, USA; dehydrated by briefly heating at 180 °C) as initiator and 0.07 wt% $SnOct_2$ (Sigma–Aldrich) as catalyst on a 125 g scale. The TMC monomer and glycerol initiator were charged into a freshly silanized (silicone solution, Serva, Germany) and dried three-necked flask, and polymerized in an argon atmosphere at 130 °C for 5 d. Subsequently, the hydroxyl-terminated oligomer was functionalized by reaction with methacrylic anhydride (Sigma–Aldrich), applied at an excess of 50 mol% (per functional hydroxyl group) at 120 °C for 2 d. To avoid premature crosslinking, 0.06 wt% hydroquinone (Sigma–Aldrich) and 0.06 wt% D,L-α-tocopherol (Fluka, Switzerland) were added during this reaction. The obtained macromer was purified by distillation of the formed methacrylic acid and the excess methacrylic anhydride under vacuum up to a temperature of 180 °C ($T_{vapor} = 105–110$ °C). The monomer conversion of the oligomerization reaction, the molecular weight and the degree of functionalization were determined by ^{1}H NMR spectroscopy (Varian Innova 300 MHz, USA), using $CDCl_3$ (Merck, Germany) as solvent.

In order to apply a macromer in stereolithography, it has to be formulated into a liquid resin with a viscosity of approximately 10 Pa s.[11] The PTMC resin was prepared by adding 22 wt% propylene carbonate (Merck) to the macromer followed by addition of 5 wt% Lucirin TPO-L (BASF, Germany) and 0.15 wt% Orasol Orange G dye (Ciba Specialty Chemicals, Switzerland). The latter two weight percentages were relative to the macromer. It should be noted that films for tensile testing experiments were prepared from a resin without Orasol Orange G. The resin viscosity was determined at temperatures between 20 and 25 °C, using a Brookfield DV-E rotating spindle viscometer. The viscometer was equipped with a small sample adapter and the Brookfield s21 spindle, which was operated at shear rates of 2.79 and 3.72 s^{-1} (rotating at 3 and 4 rpm, respectively).

2.2. Preparation of PTMC Films and Scaffolds by Stereolithography

A Perfactory Mini Multilens stereolithography apparatus (EnvisionTec, Germany) was used to build flat films and three-dimensional scaffolds from the PTMC-based resins. The stereolithography apparatus was equipped with a digital micro-mirror device, allowing projections of 1280×1024 pixels, each measuring $32 \times 32\ \mu m^2$.[17] By applying Enhanced Resolution Module (ERM) technology, the x and y resolution of the apparatus were doubled, yielding pixels measuring $16 \times 16\ \mu m^2$. The wavelength of the blue light ranged from 400 to 550 nm, with a peak at 440 nm.[18]

Flat films for tensile testing were prepared by illuminating a rectangular area of the resin measuring $75 \times 60\ mm^2$ for 5 min at an intensity of approximately 10 mW cm^{-2} (lens $f = 60$ mm, without ERM). The layer thickness was 0.55 mm. The films were subsequently post-cured by irradiation for 30 min in a crosslinking cabinet (Ultralum, USA, operating at 365 nm and an intensity of 3–4 mW cm^{-2}) in a nitrogen atmosphere. To remove unreacted macromer, initiator and diluent, the resulting films were extracted with a 1:1 mixture of propylene carbonate and ethanol, which was refreshed twice after which the films were washed with ethanol and dried under vacuum until constant weight.

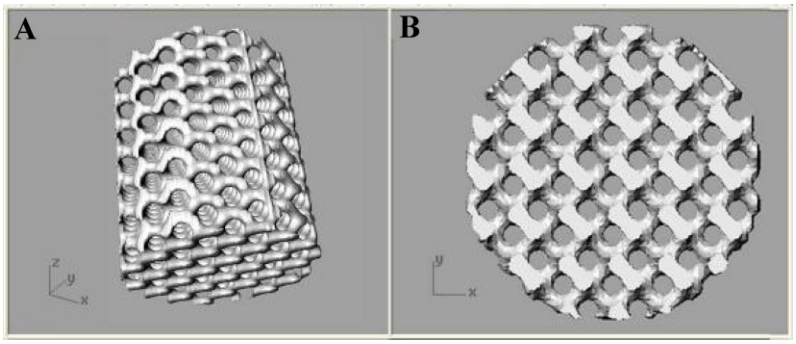

Figure 1. Design of the porous scaffold (diameter = 5.94 mm, height = 7.74 mm) with gyroid pore architecture having an average pore size of 547 μm and a porosity of 71%. A) Perspective and B) top-view.

A cylindrical three-dimensional scaffold with gyroid pore architecture was designed using K3dSurf v0.6.2 (freeware from http://k3dsurf.sourceforge.net) and Rhinoceros 3D (McNeel Europe) computer software. The scaffold was designed to have a porosity of 71%, an average pore size of 547 μm, a diameter of 5.94 mm and a height of 7.74 mm (Figure 1).

The three-dimensional structures were built by stereolithography at a pixel resolution of $16 \times 16\ \mu m^2$ and layer thicknesses of 25 μm. Subsequent layers were illuminated at an intensity of approximately 17 mW cm^{-2} for 65 s (lens $f = 85$ mm, with ERM). To be able to precisely control the penetration depth of the light when preparing the designed structure, a small amount of Orasol Orange dye was added to the resin, as described above. To remove unreacted macromer, initiator, dye and diluent, the built structures were carefully extracted with propylene carbonate for 1 week, after which small additions of ethanol allowed the structures to slowly shrink to their final dimensions in the course of several days. Finally, the scaffolds were washed with ethanol and dried under vacuum until constant weight.

2.3. Characterization of PTMC Films and Scaffolds

The network characteristics and the physical properties of the PTMC networks prepared by stereolithography were assessed using the flat film specimens after extraction and drying. To evaluate their crosslink density, the volume degree of swelling was assessed by swelling disk-shaped specimens (8 mm in diameter, punched out from the extracted films) in chloroform for 2 d at room temperature. The volume degree of swelling (q), which is inversely related to the crosslink density, was determined according to Equation (1). Values are reported as average ± standard deviation ($n = 3$

$$q = 1 + \left(\frac{m_{swollen} - m_{dry}}{m_{dry}}\right)\left(\frac{\rho_p}{\rho_s}\right) \quad (1)$$

where $m_{swollen}$ is the mass of the swollen network, m_{dry} the mass of the initially dry network and ρ_s and ρ_p the densities of chloroform (1.48 g cm^{-3}) and PTMC (1.31 g cm^{-3}), respectively.[12]

To determine their water uptake, disks 8 mm in diameter were punched out of the network films and equilibrated in ultra-pure water for 2 d at room temperature. The values were determined according to Equation 2 and are reported as average ± standard deviation ($n = 3$)

$$\text{water uptake} = \left(\frac{m_{wet} - m_{dry}}{m_{dry}}\right) \times 100\% \quad (2)$$

where m_{wet} is the mass of the water-swollen network and m_{dry} the mass of the initially dry network.

Static contact angles of ultra-pure water (MilliQ Plus, Millipore, France) on the surface of a dry PTMC network film were determined using a video-based OCA 20 contact angle system (DataPhysics, Germany), equipped with an electronic syringe module. The static contact angle was also determined on a wet PTMC network film after equilibration in water for 2 d. The measurements were performed at room temperature on five different regions of a film. The values are reported as average ± standard deviation.

To determine the glass transition temperature of the PTMC networks, the thermal properties of the films were assessed by differential scanning calorimetry using a Pyris 1 DSC (Perkin Elmer, USA) calibrated with cyclohexane, indium and lead standards. A sample of approximately 10 mg was heated from −100 to 100 °C at a heating rate of 10 °C min^{-1} and quenched rapidly (300 °C min^{-1}) to −100 °C. After 5 min, a second scan was recorded from which the glass transition temperature was determined as midpoint value of the heat capacity change.

The mechanical properties of the PTMC network films were determined at room temperature using a Z020 tensile tester (Zwick, Germany), equipped with a 500 N load cell according to ASTM D882-91 at a cross-head speed of 50 mm min^{-1} using specimens measuring 66 mm × 5 mm × 0.5 mm. The elongation of the specimens was derived from the grip-to-grip separation, which initially was 50 mm. The E-modulus was determined from the initial slope of the stress–strain curves. The yield stress and the elongation at yield were determined from the intersection of tangents to the stress–strain curves. The toughness of the specimens (in N mm^{-2}) was determined as the area under the stress–strain curves. Experiments were conducted in triplicate.

Micro-computed tomography (μ-CT) using an eXplore Locus SP μ-CT scanner (General Electric, USA, spatial resolution 8 μm) was employed to visualize the porous

structures built by stereolithography. Porosities, pore size distributions and average pore sizes of six different scaffolds were derived from the μ-CT data as previously described.[18] All values are presented as average ± standard deviation. In short, the pore volume and polymer phase of a 3D μ-CT scan were digitally filled with pore voxels and polymer voxels of 8 μm × 8 μm × 8 μm. Subsequently, the porosity was calculated as the percentage of pore voxels of the total number of voxels in the scaffold. To determine the pore size, pores were entirely filled with as large as possible overlapping spheres.[19] To every pore voxel, a pore size corresponding to the diameter of the largest sphere containing that voxel was assigned. From the pore size distribution, a volume-averaged pore size was then calculated. Humid constructs were also analyzed by μ-CT after seeding and culturing of chondrocytes. The porous scaffolds were also visualized by scanning electron microscopy (SEM) using an XL30 ESEM-FEG (Philips, The Netherlands). The specimens were sputter-coated with gold and analyzed at a voltage of 10 kV.

Dynamic mechanical properties of scaffolds were determined in the dry and wet state using a 7E dynamic mechanical analyzer (Perkin Elmer). A static force of 500 N was applied on which a dynamic force of 250 N was superimposed at a constant frequency of 1 Hz. The compression modulus was recorded between 20 and 40 °C at a heating rate of 1 °C min^{-1}. Dynamic mechanical properties of constructs were also determined in the wet state after seeding and culturing of chondrocytes.

2.4. Cell Culture

Bovine chondrocytes were isolated from the patellar–femoral groove of calf legs as previously reported and allowed to proliferate in culture flasks containing expansion medium consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10 vol% heat-inactivated fetal bovine serum (FBS, Sigma–Aldrich), 0.2 mM ascorbic acid 2-phosphate (Invitrogen), 0.1 mM nonessential amino acids (Sigma–Aldrich), 10 mM HEPES (Invitrogen), 0.4 mM proline (Sigma–Aldrich), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (both Invitrogen).[2]

Before cell seeding, the PTMC scaffolds were sterilized in ethanol for 30 min, washed three times in sterile phosphate-buffered saline (PBS, Gibco-BRL), placed in 96-well plates (1 scaffold per well) and incubated overnight in DMEM. After cell expansion, the cells were detached from the culture flasks with trypsin and the PTMC scaffolds were subsequently seeded by adding 100 μL expansion medium containing 250 000 cells on top of each scaffold. The culture plates containing the constructs were placed overnight on an orbital shaker in an incubator at 37 °C containing humidified air and 5 vol% CO_2. The next day, each well was supplemented with 100 μL differentiation medium consisting of DMEM containing 100 μg mL^{-1} sodium pyruvate (Sigma–Aldrich), 0.2 mM L-glutamine, 50 mg mL^{-1} insulin-transferrin-selenite (ITS + Premix, BD Sciences), 10 ng mL^{-1} transforming growth factor β-3 (TGF-β3, R&D Systems), 0.1 μM dexamethasone (Sigma–Aldrich), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.[2] Subsequently, culture media were changed every 2 d for a period up to 6 weeks by adding 200 μL fresh differentiation medium per well. Constructs were collected after 3 and 6 weeks of culturing and subjected to microscopical, histological and mechanical analyses.

2.5. Microscopical and Histological Analyses

After culturing for 3 and 6 weeks, PTMC constructs were analyzed by SEM. Specimens were fixed overnight in 0.14 M cacodylate buffer (pH 7.2–7.4) containing 1.5 vol% glutaraldehyde (Merck) followed by dehydration by immersion in sequential ethanol series. Subsequently, the specimens were critical-point-dried from liquid carbon dioxide using a Bal-Tec CPD 030 (Balzers, Switzerland). Samples were sputter-coated with gold and analyzed by SEM as described above.

PTMC scaffolds with and without cells were also collected after 3 and 6 weeks for histological analysis. The samples were washed in PBS and fixed in a 10 vol% buffered formalin solution for 1 h. Subsequently, the specimens were dehydrated in sequential ethanol series, embedded in Shandon Cryomatrix (Thermo, USA), and cut at −18 °C into 5 μm sections using a Shandon SME cryotome (Thermo). The sections were stained with 1 wt% Alcian Blue (Sigma–Aldrich) and 1 wt% Picrosirius Red (Polysciences) to visualize sulfated glycosaminoglycans (GAGs) and fibrillar collagens, respectively. The applied staining protocols were performed according to the manufacturers' instructions.

Mounted slides stained with Alcian Blue were examined under a light microscope (Eclipse E400, Nikon, Japan) and samples stained with Picrosirius Red were examined under the same microscope with a polarized lens. Representative images were captured with a digital camera (Sony, Japan).

3. Results and Discussion

3.1. Characterization of PTMC Network Films

A three-armed methacrylated PTMC macromer with a molecular weight of 3100 g mol^{-1} was synthesized in a two-step reaction using trimethylene carbonate, glycerol, and methacrylic anhydride. The monomer to oligomer conversion and the oligomer to macromer conversion were 95%

and 79%, respectively, as determined by ^{1}H NMR. However, as no peaks corresponding to unreacted oligomer chain ends could be discerned upon functionalization, it can be assumed that functionalization was essentially complete.[13] The PTMC macromer was formulated into a photo-crosslinkable resin with a viscosity of approximately 10 Pa s, applying propylene carbonate as non-reactive diluent.

First, flat PTMC network films with a thickness of 482 ± 16 μm, after extraction and drying, were characterized. The volume degree of swelling in chloroform (5.3 ± 0.2) was somewhat higher than that of a network of the same macromer, photo-crosslinked in the melt without non-reactive diluent (4.7 ± 0.2).[13] This indicates that formulation of the resin with the diluent results in a lower crosslink density of the PTMC network films. Networks formed with extended polymer chains due to the presence of the diluent, exhibit fewer entanglements and more elastically inactive network chains due to intra-molecular reactions, giving supercontracted network chains after deswelling and drying.[20] However, the PTMC network films prepared with the diluent-containing resin, still had a high degree of crosslinking as indicated by the relatively high midpoint glass transition temperature of -8.8 °C. Networks prepared from macromers of higher molecular weights, which have lower crosslink densities, show lower midpoint glass transition temperatures of approximately -15 °C.[12] Both glass transition temperatures indicate that these PTMC networks are in the rubbery state at room and body temperature. As compared to network films prepared by photo-crosslinking in the melt,[13] the PTMC network films formed in the present study had a lower E-modulus (3.7 ± 0.4 MPa vs 7.5 ± 0.2 MPa), maximum tensile strength (1.7 ± 0.1 MPa vs 3.5 ± 0.4 MPa) and toughness (76 ± 7 N mm^{-2} vs 131 ± 20 N mm^{-2}), and a higher elongation at break (85 ± 4% vs 78 ± 6%), which is in agreement with the effects of the non-reactive diluent discussed above.

With a water uptake of 1.0 ± 0.3%, the PTMC network films were quite hydrophobic. This complies with the static water contact angle of $93 \pm 2°$, measured on the dry network surface. However, conditioning in water led to a lower static contact angle of $61 \pm 3°$. This decrease is most probably caused by reorientation or diffusion of polymer end groups at the polymer–water interface due to the uptake of small amounts of water.[21]

3.2. Characterization of the PTMC Scaffold

The designed scaffold was built from the PTMC resin by stereolithography, applying a layer thickness of 25 μm. After extraction and drying, the scaffolds had shrunk 18.3 ± 0.6% to a diameter of 4.87 ± 0.03 mm and a height of 6.29 ± 0.03 mm. Figure 2 shows the gyroid pore architecture and pore interconnectivity of the built PTMC structures by μ-CT and SEM. As determined by μ-CT analysis, the scaffolds had an average pore size of 350 ± 12 μm and a porosity of 54.0 ± 2.2%, with 84% of the pores having diameters in the range of 300 to 415 μm (see also Figure 5 below). As reported by Lien et al., optimal pore sizes for chondrocyte proliferation and ECM formation in genipin-crosslinked gelatin scaffolds ranged from 250 to 500 μm.[22] Moreover, Jin et al.[23] showed that dextran-tyramine hydrogels with a pore size of 350 ± 70 μm facilitated chondrocyte proliferation and ECM production. Thus, the average pore size of our scaffolds (350 μm) is suitable for cartilage tissue engineering.

In contrast to the mechanical properties of photo-crosslinked PTMC network films, which are determined by the molecular weight of the macromer, the mechanical properties of photo-crosslinked porous PTMC scaffolds are determined by the porosity of the structures.[12] Scaffolds with a porosity of 54% as used in the present study, have a compression modulus in the dry state at room temperature of approximately 120 kPa (see also Table 1 below). Scaffolds of higher porosity have a significantly lower compression modulus (e.g., 20 kPa at 66% porosity).[12] In view of this effect of scaffold porosity on the compression modulus, we did not aim at a higher scaffold porosity than 54%. Although this is a relatively low porosity, the interconnectivity of the pores is very high, thus facilitating the transport of nutrients and metabolic waste products.[24]

The scaffolds were built using a resin based on a PTMC macromer with a molecular weight of 3100 g mol^{-1}. Macromers of higher molecular weight require more non-reactive diluent to be formulated into a suitable resin, yielding fragile scaffolds during the building process. Moreover, as discussed above, the use of higher molecular weight macromers will not increase the mechanical properties of the scaffolds. In the present study cylindrical scaffolds were used. For the final application, patient-specific scaffolds need to be built, for example, using MRI scanning data.[10]

3.3. Culturing of Chondrocytes in the PTMC Scaffolds

To evaluate the behavior of chondrocytes in the designed porous PTMC structures, 250 000 cells were seeded per scaffold and subsequently cultured under stationary conditions up to 6 weeks. As shown in Figure 3A, cells adhered to the PTMC scaffold after 3 weeks. The surface was almost completely covered with spread cells. After 6 weeks (Figure 3B), the porous structure was fully confluent with cells. Moreover, at this time point also cells with a round morphology were present on top of the spread cells (Figure 3C). In cartilage tissue engineering, chondrocytes should preferentially adopt a round shape

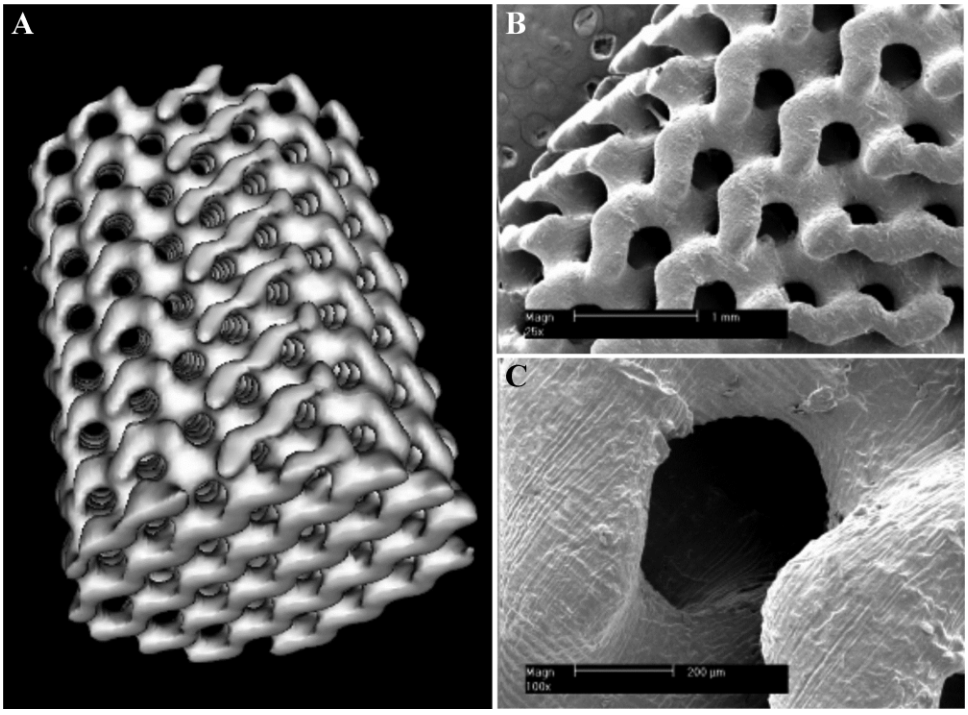

Figure 2. Visualization of scaffolds built from the PTMC resin by stereolithography. A) μ-CT scan and B,C) SEM images. Scale bar (B) 1 mm, scale bar (C) 200 μm.

Table 1. Compression moduli of PTMC scaffolds and constructs determined in the dry and wet state.

	State of scaffold	Culture period [weeks]	Compression modulus [kPa]	
			$T = 21\ ^{\circ}C$	$T = 37\ ^{\circ}C$
Without cells	Dry	–	119 ± 14	60 ± 7
	Wet	–	69 ± 15	47 ± 13
With cells	Wet[a)]	3	76	46
	Wet	6	102 ± 28	68 ± 22

Values are reported as average ± standard deviation of five samples; [a)]Values obtained from a single measurement.

since these are the differentiated cells producing cartilage-specific proteoglycans and collagen fibrils of high collagen type II over type I ratio.[25] The same cell behavior as shown in Figure 3 has been reported by Mahmood[26] using ceramic substrates. Chondrocytes were found to adhere and spread on the surface of the ceramic, whereas cells retained their round shape when they were attached to the spread cells. Moreover, they found that cell morphology was dependent on cell seeding density, with an increase in the number of round and aggregated cells at a higher seeding density. As we used a moderate seeding density, the number of round and presumably differentiated chondrocytes in our scaffolds could also be improved by increasing the seeding density of the cells.

Alcian blue staining of cross-sections of the PTMC constructs showed that the chondrocytes synthesized sulfated GAGs during culturing for 3 and 6 weeks (Figure 4A,B, respectively). Sulfated GAGs are main components of cartilage-specific proteoglycans, indicating appropriate matrix production by the cells. This is further substantiated by Picrosirius red staining for fibrillar collagens as shown in Figure 4C,D. Thin and loosely packed collagen fibrils stain green to yellow whereas thick and tightly packed fibrils stain yellow to red. Although this

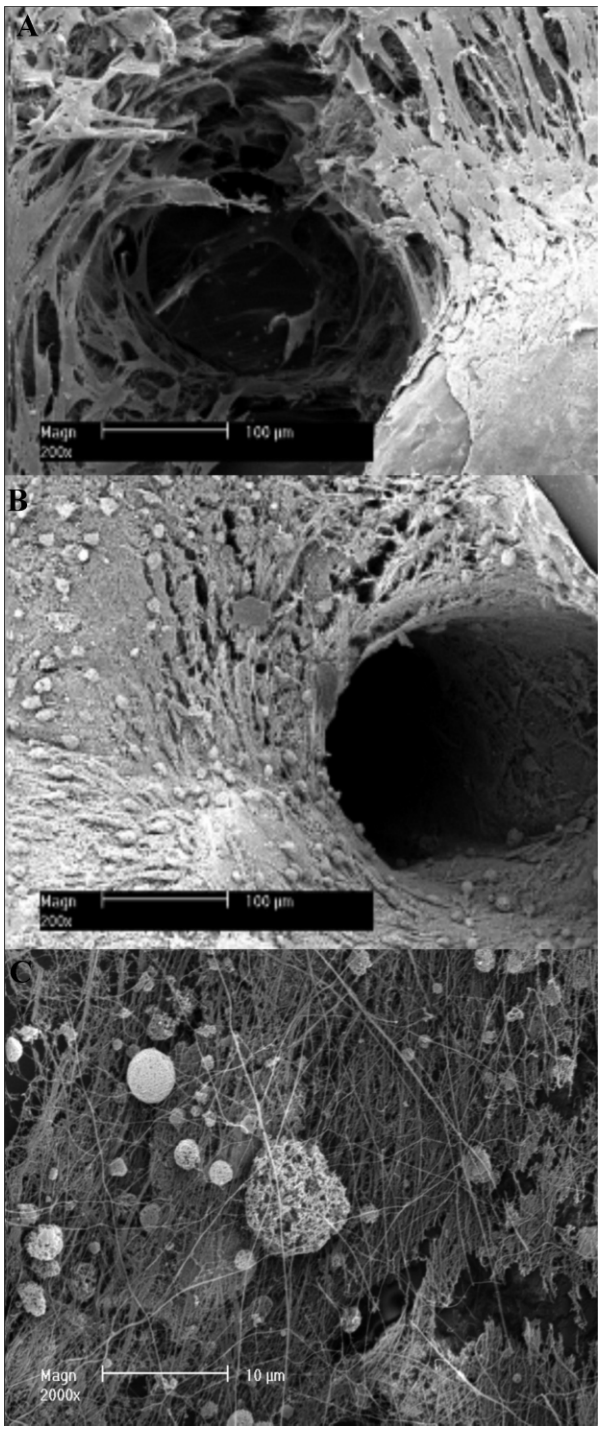

Figure 3. SEM analysis of constructs after culturing for A) 3 weeks and B,C) 6 weeks. Scale bars (A,B) 100 µm, scale bar (C) 10 µm.

staining protocol does not allow to distinguish collagen type II indicative of cartilaginous matrix, the cross-sections clearly show that fibrillar collagens were produced by the cells. Presumably, these fibrils are also visible in Figure 3C. Alcian blue and Picrosirius red did not stain the PTMC material. In future experiments, immunohistochemical staining for collagen type II, qRT-PCR analysis for expression of collagen type II over type I and determination of GAG/DNA content need to be addressed.

The chondrocyte-seeded scaffolds were also investigated by µ-CT, showing that after a culture period of 6 weeks the constructs were still porous. The average pore size and porosity were found to be decreased from 350 ± 12 to 325 ± 19µm and from $54.0 \pm 2.2\%$ to $39.2 \pm 9.3\%$, respectively (Figure 5). This indicates that after 6 weeks the pores were not yet completely filled with cells and extracellular matrix.

It has been reported that the hydrophilicity of scaffolds for cartilage tissue engineering is a key determinant to promote the round morphology of differentiated chondrocytes.[27–29] In these studies, porous poly(ethylene glycol)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) scaffolds were used, of which the hydrophilicity could be increased by using poly(ethylene glycol) (PEG) of a higher molecular weight and by increasing the weight percentage of PEGT in the copolymer. It was shown that chondrocytes seeded in hydrophilic scaffolds adopted a round morphology and expressed a high ratio of collagen type II over type I. This was correlated with a low adsorption of fibronectin to the substrates, resulting in little adhesion and spreading of chondrocytes on the surface of the scaffolds. The static water contact angle of flat films of the material was $39 \pm 1^\circ$. Although the PTMC network films used in the present study became more hydrophilic upon conditioning in water, the static water contact angle of $61 \pm 3^\circ$ was most probably too high to promote the differentiated chondrocyte phenotype. Photo-crosslinking of copolymers or blends of PTMC and PEG by stereolithography may be an alternative approach to prepare more hydrophilic PTMC-based scaffolds for cartilage tissue engineering. Yet another approach may be to use a chondrocyte-containing hydrogel to seed the cells in the designed scaffolds. Chondrocytes suspended in suitable hydrogels, e.g., mimicking the cartilaginous extracellular matrix, maintain the round chondrocyte morphology or differentiate to this phenotype.[2,25]

3.4. Mechanical Properties of PTMC Scaffolds and Constructs

To characterize the mechanical properties of scaffolds with and without cells, dynamic compression tests were performed at different temperatures. Table 1 shows the compression moduli of scaffolds without cells, tested in the dry and wet state, and of constructs with chondrocytes analyzed in the wet state after 3 and 6 weeks of culture.

Comparing scaffolds in the dry and wet state, showed that the compression moduli decreased due to water uptake and subsequent swelling of the network. The decrease was

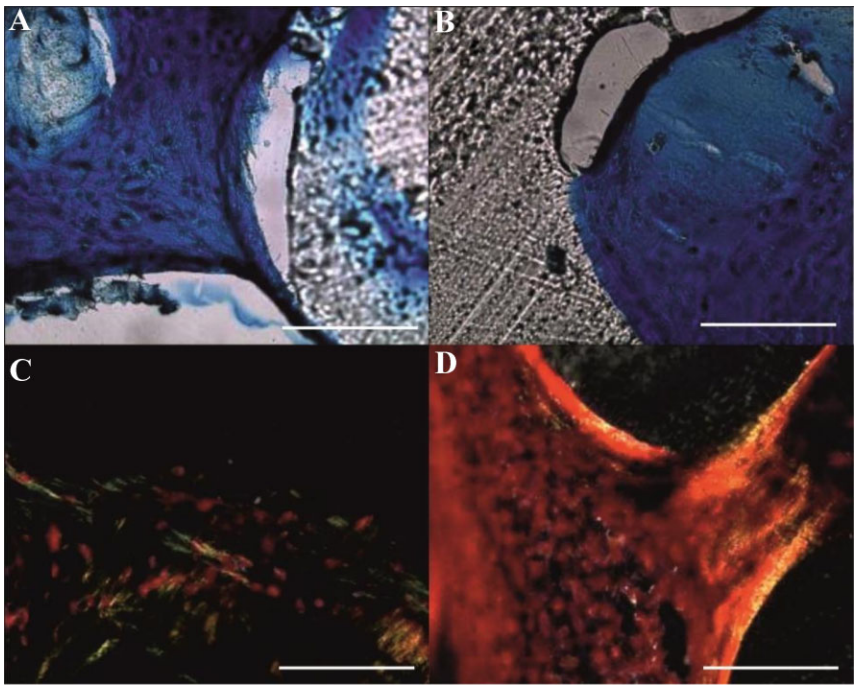

Figure 4. A,B) Alcian blue staining and C,D) Picrosirius red staining of constructs after culturing for 3 weeks (A,C) and 6 weeks (B,D). Scale bars: 100 μm.

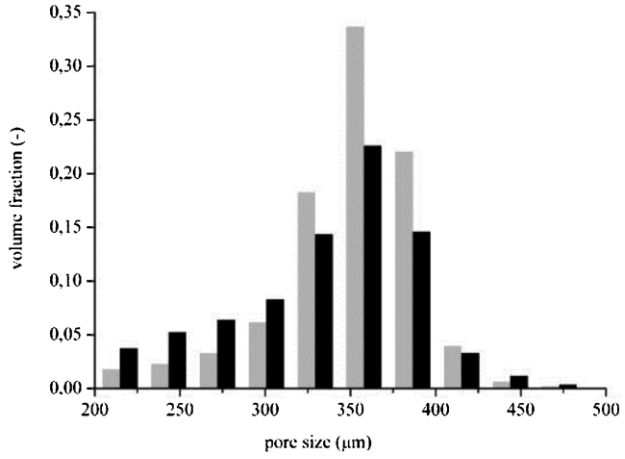

Figure 5. Pore size distributions determined by μ-CT analysis of PTMC scaffolds without cells (gray bars) and PTMC constructs after culturing for 6 weeks (black bars).

more pronounced at 21 °C than at 37 °C. Dynamic mechanical analysis further showed that the compression moduli increased approximately 50% upon culturing of the scaffolds with chondrocytes for 6 weeks. By improving the approach presented here in terms of stimulation of the chondrocytes to adopt the differentiated phenotype after seeding (e.g., by higher seeding densities, more hydrophilic scaffolds) and further maturation of the constructs (e.g., by dynamic culturing, mechanical stimulation), the moduli may approach the values of natural cartilage tissue which range from 450 to 800 kPa at room temperature.[30]

4. Conclusion

A three-armed methacrylated PTMC macromer with a molecular weight of 3100 g mol^{-1} was formulated into a photo-crosslinkable resin, using propylene carbonate as non-reactive diluent. By photo-crosslinking of the resin, flexible, tough and relatively hydrophobic network films were prepared.

The resin was used to build designed scaffolds with gyroid pore architecture by means of stereolithography. Highly interconnected porous structures with a pore diameter of 350 ± 12 μm and a porosity of 54.0 ± 2.2% were prepared. Bovine chondrocytes were seeded in the scaffolds and were found to adhere and spread on the PTMC surface. After culturing for 6 weeks, the porous structures were completely covered with cells. Also cells with a round morphology were present, indicative of the differentiated chondrocyte phenotype, adhering to the spread cells. Sulfated GAGs and fibrillar collagens were deposited by the cells. During culturing for 6 weeks, the compression moduli of the constructs increased 50% to approximately 100 kPa. Promotion of the differentiated chondrocyte phenotype after seeding, in terms of a shorter time required by the chondrocytes to adopt this phenotype, will further

increase the applicability of these constructs for cartilage tissue engineering.

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