Marica Markovic

Austrian Cluster for Tissue Regeneration,
Institute of Materials Science and Technology,
Technische Universität Wien (TU Wien),
Getreidemarkt 9,
Vienna 1060, Austria
e-mail: marica.markovic@tuwien.ac.at

Jasper Van Hoorick

Polymer Chemistry and Biomaterials Research Group,
Ghent University,
Krijgslaan 281 S4-bis,
Ghent 9000, Belgium;
Brussels Photonics Team,
Department of Applied Physics and Photonics,
Vrije Universiteit Brussel,
Pleinlaan 2,
Elsene 1050, Belgium
e-mail: jasper.vanhoorick@ugent.be

Katja Hölzl

Austrian Cluster for Tissue Regeneration,
Institute of Materials Science and Technology,
Technische Universität Wien (TU Wien),
Getreidemarkt 9,
Vienna 1060, Austria
e-mail: katja.hoelzl@tuwien.ac.at

Maximilian Tromayer

Austrian Cluster for Tissue Regeneration,
Institute of Applied Synthetic Chemistry,
Technische Universität Wien (TU Wien),
Getreidemarkt 9,
Vienna 1060, Austria
e-mail: maximilian.tromayer@tuwien.ac.at

Peter Gruber

Austrian Cluster for Tissue Regeneration,
Institute of Materials Science and Technology,
Technische Universität Wien (TU Wien),
Getreidemarkt 9, Vienna 1060, Austria
e-mail: peter.e308.gruber@tuwien.ac.at

Sylvia Nürnberger

Austrian Cluster for Tissue Regeneration,
Medical University of Vienna,
Department of Trauma Surgery,
Währinger Gürtel 18-20,
Vienna 1090, Austria
e-mail: sylvia.nuernberger@meduniwien.ac.at

Peter Dubrue

Polymer Chemistry and Biomaterials Research Group,
Ghent University, Krijgslaan 281 S4-bis,
Ghent 9000, Belgium
e-mail: peter.dubrue@UGent.be

Sandra Van Vlierberghe

Polymer Chemistry and Biomaterials Research Group,
Ghent University, Krijgslaan 281 S4-bis,
9000 Ghent, Brussels,
Photonics Team,
Department of Applied Physics and Photonics,
Vrije Universiteit Brussel,
Pleinlaan 2,
Elsene 1050, Belgium
e-mail: sandra.vanvlierberghe@UGent.be

Robert Liska

Austrian Cluster for Tissue Regeneration,
Institute of Applied Synthetic Chemistry
Division of Macromolecular Chemistry,
Technische Universität Wien (TU Wien),
Getreidemarkt 9,
Vienna 1060, Austria
e-mail: robert.liska@tuwien.ac.at

Aleksandr Ovsianikov¹

Austrian Cluster for Tissue Regeneration,
Institute of Materials Science and Technology,
Technische Universität Wien (TU Wien),
Getreidemarkt 9,
Vienna 1060, Austria
e-mail: aleksandr.ovsianikov@tuwien.ac.at

Hybrid Tissue Engineering Scaffolds by Combination of Three-Dimensional Printing and Cell Photoencapsulation

Three-dimensional (3D) printing offers versatile possibilities for adapting the structural parameters of tissue engineering scaffolds. However, it is also essential to develop procedures allowing efficient cell seeding independent of scaffold geometry and pore size. The aim of this study was to establish a method for seeding the scaffolds using photopolymerizable cell-laden hydrogels. The latter facilitates convenient preparation, and handling of cell suspension, while distributing the hydrogel precursor throughout the pores, before it is cross-linked with light. In addition, encapsulation of living cells within hydrogels can produce constructs with high initial cell loading and intimate cell-matrix contact, similar to that of the natural extra-cellular matrix (ECM). Three dimensional scaffolds were produced from poly(lactic) acid (PLA) by means of fused deposition modeling. A solution of methacrylamide-modified gelatin (Gel-MOD) in cell culture medium containing photoinitiator Li-TPO-L was used as a hydrogel precursor. Being an enzymatically degradable derivative of natural collagen, gelatin-based matrices are biomimetic and potentially support the process of cell-induced remodeling. Preosteoblast cells MC3T3-E1 at a density of 10×10^6 cells per 1 mL were used for testing the seeding procedure and cell proliferation studies. Obtained results indicate that produced constructs support cell survival and proliferation over extended duration of our experiment. The established two-step approach for scaffold seeding with the cells is simple, rapid, and is shown to be highly reproducible. Furthermore, it enables precise control of the initial cell density, while yielding their uniform distribution throughout the scaffold. Such hybrid tissue engineering constructs merge the advantages of rigid 3D printed constructs with the soft hydrogel matrix, potentially mimicking the process of ECM remodeling.

[DOI: 10.1115/1.4031466]

¹Corresponding author.

Manuscript received April 7, 2015; final manuscript received August 25, 2015;
published online September 29, 2015. Assoc. Editor: Ibrahim Ozbolat.

Introduction

The creation of functional tissues is a major aim of regenerative medicine and tissue engineering. One of the most popular approaches is to begin with a temporary artificial 3D cell culture matrix or a scaffold supporting the initial cell attachment, proliferation, and production of de novo ECM on the way to functional tissue. Such a matrix has to present an interconnected/permeable pore network to facilitate cell migration, and nutrient/waste exchange. The material has to be biocompatible and provide controllable degradation rates, suitable surface chemistry for cell attachment, proliferation, and differentiation. It should have mechanical properties that support or match tissues at the site of implantation, and an architecture which promotes the formation of native tissue structure and a reproducible architecture of clinically relevant size and shape [1]. In relation to the present work, two major options can be highlighted—porous 3D scaffolds and hydrogels. The latter are conventionally used to encapsulate the cells directly, resulting in high initial density and homogeneous distribution of cells throughout the construct. In rigid scaffolds, including the ones produced by 3D printing, the cells are usually introduced in a separate step, which might become challenging due to the fact that cell-seeding efficiency on polymer scaffolds is often low and results in their nonuniform distribution [2,3].

One among few synthetic polymers used for developing biodegradable scaffolds is PLA because of its excellent biocompatibility, biodegradability, and processing properties [4–7]. PLA is approved by Food and Drug Administration (FDA) for clinical applications [8] and has already proven its potential as a scaffold material for tissue engineering [6,7,9–13]. Especially investigations using PLA for engineering of cartilage and bone tissues are often reported [14–17]. In the present study, scaffolds were produced by 3D printing of PLA using fused deposition modeling technique. They were combined with photopolymerizable gelatin-based hydrogels. Being a derivative of collagen, which is one of the major ECM components, Gel-MOD is a hydrogel with excellent bio-interactive properties [18–23]. In addition, Gel-MOD is enzymatically degradable, which is advantageous for mimicking the natural process of ECM remodeling. Most importantly, combining rigid scaffolds with cell-containing hydrogels allows one to obtain constructs with adequate mechanical properties, while independently providing cells with a suitable environment for migration and proliferation. In a recent report, a multihead 3D printer was used to deposit strands of rigid poly- ϵ -caprolactone material and subsequently fill the intermediate space with cell-containing hydrogel in order to produce hybrid 3D constructs in a layer-by-layer fashion [24]. In our study, the objective was to combine the advantages of two approaches: the production of rigid highly porous 3D printed scaffolds with cell-laden hydrogels cross-linked by photopolymerization. The main advantage of this approach is that the cells are introduced in a separate step, which does not require specialized equipment and involves minimum cell manipulation. For this systematic evaluation of cell photoencapsulation in a model system containing only Gel-MOD and within the actual hybrid scaffolds were undertaken. The reported protocols present a proof-of-principle for a straightforward, rapid, and reproducible method allowing to obtain hybrid 3D constructs with homogeneous distribution of cells at high initial density.

Materials and Methods

Fabrication and Characterization of PLA Scaffolds. PLA scaffolds were produced via fused deposition modeling using an Ultimaker 1 (Ultimaker, Geldermalsen, The Netherlands) with a standard nozzle size of 400 μm in combination with a transparent PLA filament with molecular weight of 16 420 Da (Bitsfrombytes Ltd, Clevedon, UK). The printing instructions were generated in G-code using an in house developed program in Visual Basic for Applications (VBA) and Microsoft Excel 2010.

The G-codes were transferred to the device via Cura 13.06.4 (Ultimaker, Geldermalsen, The Netherlands). Scaffolds of $5 \times 5 \times 5 \text{ mm}^3$ were printed in meander at a speed of 11 mm/s at a temperature of 195 °C with a lay-down pattern of 0/90 deg and a layer height of 300 μm . The scaffolds exhibited an average pore size and strut diameter of 435 ± 24 and $385 \pm 15 \mu\text{m}$, respectively. Scaffolds were characterized using an Axiotech microscope (Zeiss, Oberkochen, Germany) and ImageJ software. The numerical molecular weight of the PLA filaments was determined using $^1\text{H-NMR}$ spectroscopy (Brüker WH 500 MHz). All samples were sterilized using ethylene oxide gas in a cold cycle (35 °C) at the Academic Hospital Sint-Jan, Bruges, Belgium.

Synthesis of Lithium (2,4,6-trimethylbenzoyl)Phenylphosphinate (Li-TPO-L). Photoinitiator lithium (2,4,6-trimethylbenzoyl)phenylphosphinate (Li-TPO-L) was prepared analogous to patent literature: 8.60 g (27.2 mmol) of (2,4,6-trimethylbenzoyl)-phenyl-phosphinic acid ethyl ester (commercial *Speedcure TPO-L* from Lambson) and 9.45 g (109 mmol) lithium bromide were dissolved in 150 mL butanone and stirred for 24 hrs at 65 °C. The resulting precipitate was collected by suction filtration, washed with petrol ether, and dried under vacuum at room temperature [25].

Cell Culture. Mouse calvaria-derived preosteoblast cells (MC3T3-E1 Subclone 4) were obtained from ATCC-LGC Standards. MC3T3 were expanded in Alpha Minimum Essential Medium (αMEM) with ribonucleases, deoxyribonucleases, 2 mM L-glutamine, without ascorbic acid (Gibco), supplemented with 10% fetal bovine serum (Sigma) and 1% of 10,000 U/mL Penicillin/Streptomycin (Lonza). Cells were cultivated in incubator in humid atmosphere with 5% carbon dioxide at 37 °C. Medium was refreshed every second day.

Evaluation of Photoinitiator Cytocompatibility. To evaluate cytocompatibility of photoinitiators, PrestoBlue Cell Viability Reagent (Life Technologies) was used. For these tests, 96-well plates were seeded with 10,000 cells per well and incubated overnight to let cells attach to the surface. Afterward, the cells were incubated with 100 μL of different dilutions of Li-TPO-L (2.23, 1.12, 0.625, 0.3, 0.15, and 0.075 mM) and 2.23 mM Irgacure 2959 (I2959, BASF) for comparison. The procedure was performed under the red light because of light sensitivity of the photoinitiators. One plate was placed in an incubator while the other was exposed to ultraviolet (UV) light for 10 mins (365 nm, 4 mW/cm²). After 24 hrs incubation with photoinitiators, cell viability was evaluated. Resazurin-based reagent PrestoBlue was diluted 1:10 with medium and 100 μL was applied per well and incubated for 1 hr. Because of the reducing environment of viable cells, this reagent is transformed and turns red, becoming highly fluorescent. The fluorescence was measured with a plate reader (Synergy Bio-Tek, excitation 560 nm, emission 590 nm). After correction for background fluorescence, the results of the cells exposed to different concentrations of photoinitiator were compared to each other and to the controls (nonstimulated cells, I2959 control) and dimethyl sulfoxide (DMSO, Sigma) control (cells stimulated with 50% DMSO and 50% medium for 1 hr to evaluate fluorescence signal of the wells containing dead cells). The cells were the same passage (P8) as the cells used for encapsulation.

Preparation of Hybrid Scaffolds and Hydrogel Controls. Gel-MOD with degree of substitution of 72% used in this experiment was prepared in accordance to a previously reported protocol [18].

Empty PLA scaffolds were prewetted with $\alpha\text{-MEM}$ in a desiccator using low pressure vacuum. Prewetted scaffolds were placed in 48-well plate and loaded with 20 μL of 10% (w/v) Gel-MOD solution in αMEM containing 0.6 mM Li-TPO-L and a cell density of 10×10^6 cells per 1 mL, which resulted in approximately 200,000 cells per scaffold. Control gel pellets with encapsulated

cells were produced using chambered coverslip molds (Sigma) with approximately same cell number per pellet as in the scaffolds. The gels produced with this molds were 6 mm in diameter and 1 mm height. Schematic overview of the scaffolds seeding procedure is presented in the Fig. 1. Both the seeded scaffolds and the control pellets were exposed to UV light in order to cross-link the gel, as previously described in the text. Subsequently they were soaked in α -MEM and left in incubator. Samples were maintained for 36 days.

Cell Viability. The LIVE/DEAD viability assay (Molecular Probes, Life Technology) was used to assess cell viability according to manufacturer's instructions. The culture media was aspirated, the scaffolds and pellets were rinsed three times in sterile phosphate-buffered saline (PBS, Sigma-Aldrich). The staining solution with 0.2 μ M Calcein AM (live stain) and 0.6 μ M propidium iodide (dead stain) was added for 20 mins at 37 °C. Samples were washed three times with PBS and imaged in 35 mm dish with glass bottom (ibidi) using laser scanning microscopy (LSM 700, Zeiss) with excitation/emission filter set at 488/530 nm to observe living cells (green) and 530/580 nm to detect dead (red) cells. Fluorescent cells were counted using image processing and analysis program ImageJ [26].

Scanning Electron Microscopy (SEM). Samples were fixed in formalin, rinsed in water, and dehydrated in a graded series of alcohol. Afterward samples were chemically dried with hexamethyldisilazane (Sigma), mounted on a stub, coated with palladium gold mixture (Emitech sputter coater SC7620), and imaged using SEM Jeol 6510 and SEM Jeol IT 300.

Statistical Analysis. Statistical evaluation of data was performed using software package IBM SPSS Statistic 22 (SPSS Inc., Chicago, IL) and Excel 2013 (Microsoft Office). Results are presented as average of repeated measurements $\pm$ standard deviation (SD). After verifying normal distribution and homogeneity of variance, a one-way analysis of variance was used to compare means of the samples. If a significant difference was observed ($p < 0.05$), Bonferroni post hoc multiple comparison tests were performed to detect significant difference between the samples.

Results

Cytocompatibility of Employed Photoinitiator. Due to its solubility in water and relative cytocompatibility, I2959 is a widespread photoinitiator often employed for cell encapsulation [27,28]. The common concentration of I2959 used for UV-encapsulation of cells is 2.23 mM. Therefore, this value was

selected as the highest concentration of Li-TPO-L photoinitiator for testing its effect on metabolic activity of the cells and indirectly its cytotoxicity at the following dilutions of 2.23, 1.12 and 0.6, 0.3, 0.15, and 0.075 mM. Observed values were compared to the corresponding control samples, which were cells untreated with photoinitiators. The metabolic activity (PrestoBlue Cell Viability Test) of the control not exposed to photoinitiators or UV light was assumed to be 100% (Fig. 2).

The metabolic activity of cells treated with I2959 and exposed to UV was 61% compared to untreated control. It showed no statistically significant difference to the samples treated with 1.12 mM Li-TPO-L or lower—confirming that for this concentrations Li-TPO-L is not cytotoxic. The metabolic activity of cells treated with 2.23 mM Li-TPO-L and UV was statistically decreased from the activity of UV and I2959 treated cells or compared to UV control ($p = 0.000$). Their metabolic activity was significantly lower ($17 \pm 5\%$), and similar to the DMSO control (dead cells control). Concentrations of Li-TPO-L lower than 0.6 mM showed less cytotoxicity, but this concentration of photoinitiator is not sufficient for UV polymerization of Gel-MOD in practical work. The results of these preliminary tests allow one to conclude that the concentration of 0.6 mM Li-TPO-L is most appropriate for performing cell encapsulation, as the resulting cell viability level is similar to that observed in controls.

Cell Viability and Distribution. Prewetting the scaffold with medium at low pressure ensures that no air bubbles are trapped inside of its pores. After this treatment the cell-loaded Gel-MOD can be distributed homogeneously throughout the pores of the scaffolds.

Cell viability and distribution within the scaffolds were regularly monitored over a period of 36 days using a laser scanning microscopy. Figure 3 shows confocal images of stained MC3T3-E1 on day 7, 14, 21, and 36. In the scaffolds loaded with a Gel-MOD/cell suspension, the cells were at first evenly distributed in the Gel-MOD, like in the control pellet. After a few days, the cells started to migrate to the PLA scaffolds and stretched along the structure. In the control pellet the cells formed small clusters. This is most likely due to proliferation of the cells as observed in previous reports [29]. One week after encapsulation, LSM revealed that 95% of the cells in the PLA scaffold were alive after live-dead staining. In the second week, the cell number in the pellet was 30% lower than one week after encapsulation, and stayed constant during the second and third week, just latter, 36 days after encapsulations, cell number dropped to 46% compared to the first week. Similarly, a substantial drop in cell number was observed within the Gel-MOD pellet. One week after encapsulation 95% of the cells in the pellet were alive, but cell number was

Fig. 1 Schematic overview of seeding the scaffolds with the 10% Gel-MOD loaded with cells. (a) Printing scaffolds with fused deposition modeling technique, (b) prewetting scaffolds with the medium using low pressure vacuum, (c) loading scaffold with cells embedded in 10% Gel-MOD, (d) polymerization of the Gel-MOD using UV light for 10 mins (365 nm, 4 mW/cm²), and (e) imaging the cells in scaffold using LSM.

Fig. 2 Influence of different concentrations of I2959 and LiTPO-L on metabolic activity of MC3T3-E1 after 24 hrs and exposed/not exposed to UV light (PrestoBlue Cell Viability assay). All values are presented as % of positive untreated control. The concentrations, which were not significantly different from the I2959 control after 24 hrs of UV treatment (1.12, 0.6, 0.3, 0.15, 0.075 mM Li-TPO-L) were considered to be not cytotoxic ($p > 0.05$). DMSO control represents cells treated with 50% DMSO for 1 hr (dead cells control), not showing any difference in metabolic activity from the cells treated with 2, 23 mM Li-TPO-L and UV.

Fig. 3 Distribution of MC3T3-E1 in the scaffolds and control pellets at different time points over 36 days. Living cells were stained with calcein and dead cells with propidium iodide. Scale bar represents 200 μm .

decreasing to 80%, 48%, and 40% for the second week, third week, and 36 days, respectively, compared to the first imaging. Moreover, the cells in the pellet had a more round morphology while the cells in the scaffolds were stretching along the pores. SEM image of 36 days old PLA/Gel-MOD scaffold (Fig. 4) shows that there are still cells stretching along the PLA structure, but also some Gel-MOD with cells embedded into it.

Discussion

Cell photoencapsulation in biodegradable hydrogels is a popular approach for tissue engineering applications [30]. However, it is critical to consider the cytocompatibility of the system being used. Free radicals formed in these processes are highly reactive and often cytotoxic, potentially damaging for DNA, proteins and

Fig. 4 SEM of PLA scaffold. Image (a) presents SEM of PLA scaffold and (b) PLA scaffold loaded with Gel-MOD. On images (c) and (d) is SEM 36 days after seeding with 10% Gel-MOD loaded with MC3T3-E1 cells. The overview image (c) shows the struts of the scaffold with the gel in between. Cells are growing on the surface of the scaffold and the gel and are embedded inside the gel (d). White arrow points to the cell encapsulated in the gel, black points to the cell stretching on the Gel-MOD.

lipids [31,32]. Furthermore, UV light applied to the samples can by itself cause DNA damage [33,34].

Li-TPO-L is a cleavable Type I photoinitiator, which after photofragmentation produces benzoyl and phosphinyl radicals that can initiate the polymerization of formulations containing acrylates or methacrylates. It has an absorption maximum at approximately 375 nm [35]. Therefore, at the wavelength of 365 nm, relevant for cell encapsulation, it provides better reactivity compared to I2959, whose absorption already tails out in this spectral range [36–38]. It was also previously demonstrated that Li-TPO-L exhibits a higher efficiency and quantum yield of radical formation and larger addition rate constants to double bonds for the phosphoryl radical than alternative water-soluble-cleavage type I radical photoinitiators including I2959 [35–37]. For additional information supporting superior performance of Li-TPO-L over I2959 for photopolymerization of Gel-MOD at 365 nm, please refer to the supplemental material available under the “Supplemental Data” tab for this paper on the ASME Digital Collection.

A preliminary cytotoxicity evaluation was performed to determine the optimal concentrations of photoinitiator. Established concentration of 0.6 mM of Li-TPO-L is almost three times lower than the concentrations of 0.05 wt. % (1.7 mM) used by other research groups [39,40]. Significantly lower concentrations of photoinitiators are cytocompatible, while still resulting in reasonable polymerization [35]. This reduction is highly desirable to minimize possible residuals, which can leach from the material after polymerization and impact the cells [31,35].

Having large and perfusable scaffolds are a prerequisite for tissue engineering in most clinical applications. However, as the size increases, uniform seeding of the cells throughout the scaffold becomes challenging [41]. There are several described methods for seeding 3D structures with cells, which can be roughly subdivided into passive and active. Seeding cells passively is achieved by putting the cells on top of the scaffold and allowing them to

infiltrate the scaffold over time. This is a simple approach in which cells are not damaged with the use of mechanical forces, but infiltration rate can be low often resulting in poor loading and uneven distribution of the cells within the scaffolds [42]. By applying an external force like centrifugation or external pressure gradients, the cells infiltrate into the scaffold at a faster rate and the distribution is more homogeneous, but the control of seeding procedure is rather complex making it impractical [42–44]. The use of the low pressure vacuum for prewetting in the experiment ensures that the scaffolds are free of air bubbles or residues of chemicals like ethanol frequently used in these procedures [45,46].

Cells encapsulated in Gel-MOD displayed rounded clustered morphology. It is observed that the single seeded cells can form 3D clusters in the hydrogel after multiple divisions, while the hydrogel matrix provides a scaffold environment on which the cells could settle, but within which they are also able to protrude into three dimensions when they divide [47]. While in the control pellets the cells encapsulated in Gel-MOD stay viable, but round in morphology, in the hybrid scaffolds they tend to move toward the PLA struts and stretch along them, presumably driven by local stiffness gradients. Previous reports confirm that the material properties and material rigidity may stimulate cell migration, influence their morphology and the structure of the cytoskeleton, the expression of specific genes, as well as the lineage [48]. Sunyer et al. showed that spreading of the cells seeded on hydrogels with the stiffness gradient correlates with the substrate rigidity [49]. The stiffness of the surface to which cells adhere can have a profound effect on cell structure and protein expression, but these mechanical effects vary with different cell types, and depend on the nature of the adhesion receptors by which the cells bind their substrate [50]. Substrates patterned with stiffness gradients revealed preferential migration of the cells toward stiff regions, e.g., durotaxis [51]. Most cell types spread more, adhere better, and appear to survive better on the stiffer matrices [48,52].

Our SEM images showed that even after 36 days cells that are attached to the rigid structure of the scaffold were present in sufficient number, stretching along the struts and some cells were embedded in the residues of the Gel-MOD, which lead us to the conclusions that this hybrid scaffolds are supporting longer survival of the cells. Survival in the hybrid scaffold was better compared to the control cells encapsulated in Gel-MOD even three weeks after encapsulation, possibly due to better perfusion of the scaffolds in comparison to the hydrogel pellet. Most of the studies with MC-3T3 cell line are conducted over the shorter time period. In three-dimensional apatite-coated PLGA scaffolds authors present good survival rate up to three weeks [53], on porous titanium scaffold after approximately two weeks, cell number started to decrease [54], the same as after delivery of MC-3T3 into injectable calcium phosphate cement through alginate-chitosan microcapsules [55].

Our study showed that prewetting of scaffolds with a medium using low pressure helps to avoid the formation of air bubbles or having the residues of substance used for prewetting like alcohols that can be potentially harmful for the cells. This is a straightforward approach that is not employing expensive equipment or time consuming procedures. Loading the cells in the viscous solution of Gel-MOD, and the subsequent UV-light induced polymerization of Gel-MOD results in the precise control of the seeding density and uniform distribution of cells throughout the scaffold.

We have developed a simple and fast protocol for seeding the solid 3D scaffolds with cells that results in uniform distribution and high seeding efficiency. Additionally, a combination of PLA scaffold and Gel-MOD also provided a hybrid scaffold that supported cell survival and proliferation over extended duration of the present study.

Acknowledgment

SEM has been performed at the University for Applied Science FH Technikum and at CIUS Cell Imaging and Ultrastructural Research Unit, Vienna, Austria. We would like to acknowledge support of Dr. Oscar Hoffmann (Department of Pharmacology and Toxicology, University of Vienna, Austria) and Dr. Tristan Fowler (present address: Department of Orthopedic Surgery, University of California San Francisco, CA). The authors acknowledge the financial support of the European Research Council (Starting Grant-307701, A.O.) and European Science Foundation (P2M network, R.L.). Sandra Van Vlierberghe would like to acknowledge the Research Foundation-Flanders (FWO, Belgium) for financial support under the form of a post-doctoral fellowship and Research Grants ("Development of the ideal tissue engineering scaffold by merging state-of-the-art processing techniques," "Inkjet printing as novel route toward the development of optical materials," FWO Krediet aan Navorsers).

References

- [1] Woodfield, T. B. F., Malda, J., de Wijn, J., Péters, F., Riesle, J., and van Blitterswijk, C. A., 2004, "Design of Porous Scaffolds for Cartilage Tissue Engineering Using a Three-Dimensional Fiber-Deposition Technique," *Biomaterials*, **25**(18), pp. 4149–4161.
- [2] Roh, J. D., Nelson, G. N., Udelsman, B. V., Brennan, M. P., Lockhart, B., Fong, P. M., Lopez-Soler, R. I., Saltzman, W. M., and Breuer, C. K., 2007, "Centrifugal Seeding Increases Seeding Efficiency and Cellular Distribution of Bone Marrow Stromal Cells in Porous Biodegradable Scaffolds," *Tissue Eng.*, **13**(11), pp. 2743–2749.
- [3] Bruinink, A., Siragusano, D., Ettel, G., Brandsberg, T., Brandsberg, F., Petitmermet, M., Müller, B., Mayer, J., and Wintermantel, E., 2001, "The Stiffness of Bone Marrow Cell-Knit Composites is Increased During Mechanical Load," *Biomaterials*, **22**(23), pp. 3169–3178.
- [4] Sin, L. T., Rahmat, A. R., and Rahman, W. A. W. A., 2012, *Poly(lactic Acid): PLA Biopolymer Technology and Applications*, William Andrew, New York.
- [5] Karst, D., and Yang, Y., 2006, "Molecular Modeling Study of the Resistance of PLA to Hydrolysis Based on the Blending of PLLA and PDLA," *Polymer*, **47**(13), pp. 4845–4850.
- [6] Zhu, Y., Gao, C., He, T., Liu, X., and Shen, J., 2003, "Layer-by-Layer Assembly to Modify Poly(L-Lactic Acid) Surface Toward Improving Its

- Cytocompatibility to Human Endothelial Cells," *Biomacromolecules*, **4**(2), pp. 446–452.
- [7] Pan, P., Zhu, B., Kai, W., Dong, T., and Inoue, Y., 2008, "Polymorphic Transition in Disordered Poly(L-Lactide) Crystals Induced by Annealing at Elevated Temperatures," *Macromolecules*, **41**(12), pp. 4296–4304.
- [8] El-Sherbiny, I. M., and Yacoub, M. H., 2013, "Hydrogel Scaffolds for Tissue Engineering: Progress and Challenges," *Global Cardiol. Sci. Pract.*, **2013**(3), pp. 316–342.
- [9] Richardson, S. M., Curran, J. M., Chen, R., Vaughan-Thomas, A., Hunt, J. A., Freemont, A. J., and Hoyland, J. A., 2006, "The Differentiation of Bone Marrow Mesenchymal Stem Cells Into Chondrocyte-Like Cells on Poly-L-Lactic Acid (PLLA) Scaffolds," *Biomaterials*, **27**(22), pp. 4069–4078.
- [10] Seppälä, J., Korhonen, H., Hakala, R., and Malin, M., 2011, "Photocrosslinkable Polyesters and Poly(Ester Anhydride)S for Biomedical Applications," *Macromol. Biosci.*, **11**(12), pp. 1647–1652.
- [11] Billiet, T., Vandenhaute, M., Schelfhout, J., Van Vlierberghe, S., and Dubrue, P., 2012, "A Review of Trends and Limitations in Hydrogel-Rapid Prototyping for Tissue Engineering," *Biomaterials*, **33**(26), pp. 6020–6041.
- [12] Meng, Q., Heuzey, M.-C., and Carreau, P. J., 2012, "Control of Thermal Degradation of Polylactide/Clay Nanocomposites During Melt Processing by Chain Extension Reaction," *Polym. Degrad. Stab.*, **97**(10), pp. 2010–2020.
- [13] Melchels, F. P. W., Feijen, J., and Grijpma, D. W., 2009, "A Poly(D, L-lactide) Resin for the Preparation of Tissue Engineering Scaffolds by Stereolithography," *Biomaterials*, **30**(23–24), pp. 3801–3809.
- [14] Schagemann, J. C., Chung, H. W., Mrosek, E. H., Stone, J. J., Fitzsimmons, J. S., O'Driscoll, S. W., and Reinholz, G. G., 2010, "Poly-Epsilon-Caprolactone/Gel Hybrid Scaffolds for Cartilage Tissue Engineering," *J. Biomed. Mater. Res. A*, **93**(2), pp. 454–463.
- [15] Endres, M., Huttmacher, D. W., Salgado, A. J., Kaps, C., Ringe, J., Reis, R. L., Sittlinger, M., Brandwood, A., and Schantz, J. T., 2003, "Osteogenic Induction of Human Bone Marrow-Derived Mesenchymal Progenitor Cells in Novel Synthetic Polymer-Hydrogel Matrices," *Tissue Eng.*, **9**(4), pp. 689–702.
- [16] Gloria, A., Causa, F., De Santis, R., Netti, P. A., and Ambrosio, L., 2007, "Dynamic-Mechanical Properties of a Novel Composite Intervertebral Disc Prosthesis," *J. Mater. Sci. Mater. Med.*, **18**(11), pp. 2159–2165.
- [17] Holloway, J. L., Lowman, A. M., and Palmese, G. R., 2010, "Mechanical Evaluation of Poly(Vinyl Alcohol)-Based Fibrous Composites as Biomaterials for Meniscal Tissue Replacement," *Acta Biomater.*, **6**(12), pp. 4716–4724.
- [18] Ovsianikov, A., Deiwick, A., Van Vlierberghe, S., Dubrue, P., Möller, L., Dräger, G., and Chichkov, B., 2011, "Laser Fabrication of Three-Dimensional CAD Scaffolds From Photosensitive Gelatin for Applications in Tissue Engineering," *Biomacromolecules*, **12**(4), pp. 851–858.
- [19] Van Vlierberghe, S., Samal, S. K., and Dubrue, P., 2011, "Development of Mechanically Tailored Gelatin-Chondroitin Sulphate Hydrogel Films," *Macromol. Symp.*, **309–310**(1), pp. 173–181.
- [20] Rodrigues, S. C., Salgado, C. L., Sahu, A., Garcia, M. P., Fernandes, M. H., and Monteiro, F. J., 2013, "Preparation and Characterization of Collagen-Nanohydroxyapatite Biocomposite Scaffolds by Cryogelation Method for Bone Tissue Engineering Applications," *J. Biomed. Mater. Res. A*, **101A**(4), pp. 1080–1094.
- [21] Dainiak, M. B., Allan, I. U., Savina, I. N., Cornelio, L., James, E. S., James, S. L., Mikhailovsky, S. V., Jungvid, H., and Galaev, I. Y., 2010, "Gelatin-Fibrinogen Cryogel Dermal Matrices for Wound Repair: Preparation, Optimisation and In Vitro Study," *Biomaterials*, **31**(1), pp. 67–76.
- [22] Vishnoi, T., and Kumar, A., 2012, "Conducting Cryogel Scaffold as a Potential Biomaterial for Cell Stimulation and Proliferation," *J. Mater. Sci. Mater. Med.*, **24**(2), pp. 447–459.
- [23] Chang, K.-H., Liao, H.-T., and Chen, J.-P., 2013, "Preparation and Characterization of Gelatin/Hyaluronic Acid Cryogels for Adipose Tissue Engineering: In Vitro and In Vivo Studies," *Acta Biomater.*, **9**(11), pp. 9012–9026.
- [24] Schuurman, W., Khristov, V., Pot, M. W., van Weeren, P. R., Dhert, W. J. A., and Malda, J., 2011, "Bioprinting of Hybrid Tissue Constructs With Tailorable Mechanical Properties," *Biofabrication*, **3**(2), p. 021001.
- [25] Noe, R., Henne, A., and Maase, M., 2003, "Acyl- und Bisacylphosphinderivate Acyl- und Bisacylphosphine," Patent Application, Germany.
- [26] Abramoff, M. D., Magalhães, P. J., and Ram, S. J., 2004, "Image Processing With ImageJ," *Biophotonics Int.*, **11**(7), pp. 36–42.
- [27] Fedorovich, N. E., Oudshoorn, M. H., van Geemen, D., Hennink, W. E., Alblas, J., and Dhert, W. J. A., 2009, "The Effect of Photopolymerization on Stem Cells Embedded in Hydrogels," *Biomaterials*, **30**(3), pp. 344–353.
- [28] Williams, C. G., Malik, A. N., Kim, T. K., Manson, P. N., and Elisseff, J. H., 2005, "Variable Cytocompatibility of Six Cell Lines With Photoinitiators Used for Polymerizing Hydrogels and Cell Encapsulation," *Biomaterials*, **26**(11), pp. 1211–1218.
- [29] Lee, B.-H., Li, B., and Guelcher, S. A., 2012, "Gel Microstructure Regulates Proliferation and Differentiation of MC3T3-E1 Cells Encapsulated in Alginate Beads," *Acta Biomater.*, **8**(5), pp. 1693–1702.
- [30] Nicodemus, G. D., and Bryant, S. J., 2008, "Cell Encapsulation in Biodegradable Hydrogels for Tissue Engineering Applications," *Tissue Eng. Part B Rev.*, **14**(2), pp. 149–165.
- [31] Rehmann, M. S., and Kloxin, A. M., 2013, "Tunable and Dynamic Soft Materials for Three-Dimensional Cell Culture," *Soft Matter*, **9**(29), pp. 6737–6746.
- [32] Kehler, J. P., 1993, "Free Radicals as Mediators of Tissue Injury and Disease," *Crit. Rev. Toxicol.*, **23**(1), pp. 21–48.
- [33] Cadet, J., Sage, E., and Douki, T., 2005, "Ultraviolet Radiation-Mediated Damage to Cellular DNA," *Mutat. Res.*, **571**(1–2), pp. 3–17.

- [34] Kappes, U. P., Luo, D., Potter, M., Schulmeister, K., and Rünger, T. M., 2006, "Short- and Long-Wave UV Light (UVB and UVA) Induce Similar Mutations in Human Skin Cells," *J. Invest. Dermatol.*, **126**(3), pp. 667–675.
- [35] Fairbanks, B. D., Schwartz, M. P., Bowman, C. N., and Anseth, K. S., 2009, "Photoinitiated Polymerization of PEG-Diacrylate With Lithium Phenyl-2,4,6-Trimethylbenzoylphosphine: Polymerization Rate and Cytocompatibility," *Biomaterials*, **30**(35), pp. 6702–6707.
- [36] Cheng, J., Jiang, S., Gao, Y., Wang, J., and Sun, F., 2014, "Tuning Gradient Property and Initiating Gradient Photopolymerization of Acrylamide Aqueous Solution of a Hydrosoluble Photocleavage Polysiloxane-Based Photoinitiator," *Polym. Adv. Technol.*, **25**(12), pp. 1412–1418.
- [37] Liu, M., Li, M.-D., Xue, J., and Phillips, D. L., 2014, "Time-Resolved Spectroscopic and Density Functional Theory Study of the Photochemistry of Irgacure-2959 in an Aqueous Solution," *J. Phys. Chem. A*, **118**(38), pp. 8701–8707.
- [38] Bahney, C. S., Lujan, T. J., Hsu, C. W., Bottlang, M., West, J. L., and Johnstone, B., 2011, "Visible Light Photoinitiation of Mesenchymal Stem Cell-Laden Bioresponsive Hydrogels," *Eur. Cell. Mater.*, **22**, pp. 43–55; Discussion 55.
- [39] Hammer, J., Han, L.-H., Tong, X., and Yang, F., 2014, "A Facile Method to Fabricate Hydrogels With Microchannel-Like Porosity for Tissue Engineering," *Tissue Eng. Part C Methods*, **20**(2), pp. 169–176.
- [40] Gandavarapu, N. R., Alge, D. L., and Anseth, K. S., 2014, "Osteogenic Differentiation of Human Mesenchymal Stem Cells on $\alpha 5$ Integrin Binding Peptide Hydrogels is Dependent on Substrate Elasticity," *Biomater. Sci.*, **2**(3), pp. 352–361.
- [41] Chen, Y.-C., Su, W.-Y., Yang, S.-H., Gefen, A., and Lin, F.-H., 2013, "In Situ Forming Hydrogels Composed of Oxidized High Molecular Weight Hyaluronic Acid and Gelatin for Nucleus Pulposus Regeneration," *Acta Biomater.*, **9**(2), pp. 5181–5193.
- [42] Solchaga, L. A., Tognana, E., Penick, K., Baskaran, H., Goldberg, V. M., Caplan, A. L., and Welter, J. F., 2006, "A Rapid Seeding Technique for the Assembly of Large Cell/Scaffold Composite Constructs," *Tissue Eng.*, **12**(7), pp. 1851–1863.
- [43] Pei, M., Solchaga, L. A., Seidel, J., Zeng, L., Vunjak-Novakovic, G., Caplan, A. L., and Freed, L. E., 2002, "Bioreactors Mediate the Effectiveness of Tissue Engineering Scaffolds," *J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, **16**(12), pp. 1691–1694.
- [44] Griffon, D. J., Sedighi, M. R., Schaeffer, D. V., Eurell, J. A., and Johnson, A. L., 2006, "Chitosan Scaffolds: Interconnective Pore Size and Cartilage Engineering," *Acta Biomater.*, **2**(3), pp. 313–320.
- [45] Lu, L., Peter, S. J., Lyman, M. D., Lai, H. L., Leite, S. M., Tamada, J. A., Uyama, S., Vacanti, J. P., Langer, R., and Mikos, A. G., 2000, "In Vitro and In Vivo Degradation of Porous Poly(DL-Lactic-Co-Glycolic Acid) Foams," *Biomaterials*, **21**(18), pp. 1837–1845.
- [46] Yang, J., Shi, G., Bei, J., Wang, S., Cao, Y., Shang, Q., Yang, G., and Wang, W., 2002, "Fabrication and Surface Modification of Macroporous Poly(L-Lactic Acid) and Poly(L-Lactic-Co-Glycolic Acid) (70/30) Cell Scaffolds for Human Skin Fibroblast Cell Culture," *J. Biomed. Mater. Res.*, **62**(3), pp. 438–446.
- [47] Huang, H., Ding, Y., Sun, X. S., and Nguyen, T. A., 2013, "Peptide Hydrogelation and Cell Encapsulation for 3D Culture of MCF-7 Breast Cancer Cells," *PLoS ONE*, **8**(3), p. e59482.
- [48] Georges, P. C., and Janmey, P. A., 2005, "Cell Type-Specific Response to Growth on Soft Materials," *J. Appl. Physiol.*, **98**(4), pp. 1547–1553.
- [49] Sunyer, R., Jin, A. J., Nossal, R., and Sackett, D. L., 2012, "Fabrication of Hydrogels with Steep Stiffness Gradients for Studying Cell Mechanical Response," *PLoS ONE*, **7**(10), p. e46107.
- [50] Yeung, T., Georges, P. C., Flanagan, L. A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P. A., 2005, "Effects of Substrate Stiffness on Cell Morphology, Cytoskeletal Structure, and Adhesion," *Cell Motil. Cytoskeleton*, **60**(1), pp. 24–34.
- [51] Trappmann, B., and Chen, C. S., 2013, "How Cells Sense Extracellular Matrix Stiffness: A Material's Perspective," *Curr. Opin. Biotechnol.*, **24**(5), pp. 948–953.
- [52] Marklein, R. A., and Burdick, J. A., 2009, "Spatially Controlled Hydrogel Mechanics to Modulate Stem Cell Interactions," *Soft Matter*, **6**(1), pp. 136–143.
- [53] Chou, Y.-F., Dunn, J. C. Y., and Wu, B. M., 2005, "In Vitro Response of MC3T3-E1 Preosteoblasts Within Three-Dimensional Apatite-Coated PLGA Scaffolds," *J. Biomed. Mater. Res. B Appl. Biomater.*, **75B**(1), pp. 81–90.
- [54] St-Pierre, J.-P., Gauthier, M., Lefebvre, L.-P., and Tabrizian, M., 2005, "Three-Dimensional Growth of Differentiating MC3T3-E1 Pre-Osteoblasts on Porous Titanium Scaffolds," *Biomaterials*, **26**(35), pp. 7319–7328.
- [55] Qiao, P., Li, F., Dong, L., Xu, T., and Xie, Q., 2014, "Delivering MC3T3-E1 Cells Into Injectable Calcium Phosphate Cement Through Alginate-Chitosan Microcapsules for Bone Tissue Engineering," *J. Zhejiang Univ. Sci. B*, **15**(4), pp. 382–392.