Tailored Melt Electrowritten Scaffolds for the Generation of Sheet-Like Tissue Constructs from Multicellular Spheroids

Rebecca McMaster, Christiane Hoefner, Andrei Hrynevich, Carina Blum, Miriam Wiesner, Katharina Wittmann, Tim R. Dargaville, Petra Bauer-Kreisel, Jürgen Groll, Paul D. Dalton,* and Torsten Blunk**

Melt electrowriting (MEW) is an additive manufacturing technology that is recently used to fabricate voluminous scaffolds for biomedical applications. In this study, MEW is adapted for the seeding of multicellular spheroids, which permits the easy handling as a single sheet-like tissue-scaffold construct. Spheroids are made from adipose-derived stromal cells (ASCs). Poly(ε-caprolactone) is processed via MEW into scaffolds with box-structured pores, readily tailorable to spheroid size, using 13–15 μm diameter fibers. Two 7–8 μm diameter “catching fibers” near the bottom of the scaffold are threaded through each pore (360 and 380 μm) to prevent loss of spheroids during seeding. Cell viability remains high during the two week culture period, while the differentiation of ASCs into the adipogenic lineage is induced. Subsequent sectioning and staining of the spheroid-scaffold construct can be readily performed and accumulated lipid droplets are observed, while upregulation of molecular markers associated with successful differentiation is demonstrated. Tailoring MEW scaffolds with pores allows the simultaneous seeding of high numbers of spheroids at a time into a construct that can be handled in culture and may be readily transferred to other sites for use as implants or tissue models.

Additive manufacturing (AM) has increasingly been used as a method to develop various tools within the biomedical sciences. In addition to well-established AM technologies, there are several emerging approaches that focus on increasing the resolution of fabrication to enhance their function. Continuous liquid interface production (CLIP),[1] two-photon polymerization (2PP),[2] and the technique used in this study, melt electrowriting (MEW),[3] are relatively new high-resolution technologies researched for numerous applications, including biomedical products.[2, 4] In a relatively brief period of time, MEW has been used to fabricate wave guides,[5] touch sensors,[6] biomaterials,[7] scaffolds for tissue engineering[4] and for cancer research.[8]

The high-resolution perspective for AM technologies is important, as it provides further design possibilities, particularly when combined with cells, or multicellular spheroids. The latter are considered an important building block in biofabrication and have been proposed as larger, assembled structures. These approaches include using bioprinting-assisted spheroid fusion,[9] microneedle-based (Kenzan) spheroid assembly,[10] caging within 2PP-fabricated “lockyballs”[11] and spheroids combined with direct polymer melt deposition.[12]

An advantage of MEW is a lower cost than CLIP or 2PP while morphological structures remain readily adaptable to various requirements simply by varying the position, shape, and size of the fibrous constituents all within a single print[13] to influence the mechanical and spatial properties. The principles of MEW, described in detail elsewhere,[14] sustain a molten fluid column at the mechanical and spatial properties. The principles of MEW, described in detail elsewhere,[14] sustain a molten fluid column at low flow rates by applying a potential difference between the nozzle and the collector upon which the jet is direct-written (Video S1, Supporting Information). The process results in well-defined fibers that can range from 820 nm[15] up to 130 μm[16] in size, depending on the manufacturing conditions. MEW is therefore distinct from electrospinning, where induced electrical instabilities result in only small diameter fibers that are both difficult to position and layer accurately. Many polymers used for MEW to date have a history of use as biomaterials, and scaffolds can have a high porosity[4] and volume,[18] and unique mechanical properties when embedded within matrices.[19] Thus, such MEW-based biomaterials could, among others, have applications in plastic and reconstructive surgery, including the reconstruction of soft tissue defects arising from trauma, tumor resections or congenital abnormalities.

Adipose tissue engineering aims to provide alternatives to current reconstructive procedures.[20, 21] Adipose-derived...
stromal cells (ASCs) are easily accessible through liposuction procedures and have been widely employed in tissue engineering approaches. However, they are almost exclusively used as suspensions of single cells combined with various biomaterials.\textsuperscript{[20,22]} When cultured as multicellular spheroids, ASCs have been shown to exhibit improved differentiation potential,\textsuperscript{[23]} and ASC spheroids hence appear as attractive building blocks for the engineering of tissue constructs.\textsuperscript{[24]} In this study, MEW scaffolds were fabricated from poly(\(\varepsilon\)-caprolactone) (PCL) for the seeding of multicellular spheroids, to establish constructs that can be readily manipulated, visualized and permit the adipogenic differentiation of ASC spheroids integrated in a sheet-like structure. These constructs may further serve as tissue models or implants in adipose engineering.

ASC spheroids were generated without any other additional material, and the scaffold dimensions could be tailored to match the spheroid size using a custom-made MEW printer, as previously described.\textsuperscript{[25]} The scaffolds had a “box-like” pore structure consisting of fibers with diameters between 13 and 15 \(\mu\text{m}\) with interlocking junctions, while two additional single fibers (herein termed “catching fibers”) with a diameter of 7–8 \(\mu\text{m}\) were introduced in the second layer and positioned approximately 130 \(\mu\text{m}\) apart within the pore (Figure 1A,B; Figure S1, Supporting Information). As the manufacture of the scaffold is a reiterative process, a nomenclature was established for this study, where the pore size (in \(\mu\text{m}\)) is followed by the number of fibers at a junction (arrowed; Figure 1B). For example, for initial experiments, 380 \(\mu\text{m}\) pore spacing with a total of 5 fibers in each direction (i.e., 10 fibers at the junction) was used, and the sample was referred to as 380-10.

In order to improve spheroid retention within the boxes, the scaffold design was optimized in subsequent experiments. This reiterative process led to an optimal pore size of 360 \(\mu\text{m}\) \(\times\) 360 \(\mu\text{m}\) pores for the 350 \(\mu\text{m}\) diameter spheroids (Table 1). Similarly, the optimal wall height of the boxes was achieved by depositing 30 or 40 fibers at the junctions with alternating 0° and 90° offset angles, hereafter referred to as 360-30 or 360-40, respectively. Accordingly, the walls of the pores for the 360-30 and 360-40 scaffolds consisted of 15 or 20 stacked fibers, respectively (Figure 1B). The scaffolds were fabricated as 48 \(\times\) 48 mm square sheets that were cut to size prior to spheroid seeding. The fiber diameter and spacing were measured from microscopic images.

Multicellular spheroids from human ASCs were generated by self-assembly in agarose-coated well plates using the liquid overlay technique (Figure 1C). Depending on the cell number seeded into the 96-well plates, ranging from 1000 to 20 000, spheroids with diameters varying between 190 and

![Figure 1](https://www.advancedsciencenews.com/)

**Figure 1.** SEM images of box-structured MEW scaffolds viewed from A) above and B) below, with catching fibers indicated with white arrows, while the blue arrow shows the point where the catching fiber is placed between two fibers within the box pore wall. C) Schematic of the spheroid manufacture in agarose-coated 96-well plates and seeding utilizing CellCrown96 (CC96) inserts. D) 350 \(\mu\text{m}\) diameter spheroid made from 5000 cells routinely used in this study. E) Variation of spheroid diameter via adjustment of cell number per spheroid.
S80 µm were achieved within one day (Figure 1D, E). In preliminary experiments inducing the ASC spheroids adipogenically in 96-well plates for 9 days, in spheroids made from up to 5000 cells, coherent tissue structures with triglyceride-filled cells throughout the cross-section of the spheroids were observed. By contrast, larger spheroids showed distorted tissue structures within the spheroids and gradients with stronger Oil Red O staining at the periphery (Figure S2, Supporting Information). As an alternative to the 96-well format, generation of spheroids using microwell arrays could be employed in order to upscale spheroid production.[26] How-

terestingly, initially empty pores started to become populated with cells after 4 days, and were completely filled by the end of the culture period (Figure 2B; Figure S5, Supporting Information). Live/dead staining showed high cell viability throughout the 14 day culture period for seeded spheroids cultured either in growth medium (Figure 2E) or under adipogenic induction conditions (Figure 2F). In order to demonstrate the scalability of the system and the overall ease of handling, larger 360-30 scaffolds (12 mm × 12 mm) were seeded with spheroids in a proof-of-principle experiment (Figure 2G, H).

Human ASCs in the spheroid-loaded 360-30 and 360-40 scaffolds successfully differentiated into the adipogenic lineage in response to hormonal induction. The characteristic accumulation of lipid droplets was observed only in the adipogenically induced samples, as detected in whole samples (data not shown) and histological sections by Oil Red O staining (Figure 3A, B). Likewise, substantial secretion of adiponectin, a major adipokine, was detected only in the supernatants of spheroid-loaded scaffolds that were adipogenically induced; no secretion of the protein was measurable in non-induced controls (Figure 3C).

To further confirm adipogenic differentiation on the molecular level, real-time qRT-PCR analysis was conducted for typically expressed adipogenic genes. Consistent with the histological data, strongly elevated gene expression of peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT enhancer binding protein alpha (C/EBPα), two key transcription factors of adipogenesis, and aP2 (fatty acid-binding protein 4), a late marker of adipogenesis, were observed in induced samples, as compared to controls (Figure 3D–F). Taken together, the analyses on the cellular and the molecular level underscored that the 3D MEW microfiber PCL scaffolds were suitable for supporting adipogenesis of ASC spheroids in vitro.

Sheet-like structures of engineered adipose tissue are desirable in reconstructive surgery, as, for example, many cases of complex injuries of the upper and the lower extremities leading to excessive scarring would benefit from the reconstruction of the subcutaneous fat layer.[27] Thus far, preformed ASC spheroids have been scarcely used in adipose engineering, and appropriate spheroid delivery remains challenging. In a previous attempt to utilize spheroid-like structures made from poly(l-lactic acid) (PLA) nanofilaments and bone marrow-derived stem cells for engineering adipose tissue, these composite spheroids were successfully combined with micro-structured PCL scaffolds.[28] However, due to the direct polymer melt deposition technique used to fabricate the scaffolds, the resulting constructs contained relatively thick PCL fibers (200 µm) in comparison to the spheroids (400 µm), resulting in a relatively high polymer fraction and possible obstacles for tissue formation within the construct. While adipogenesis of the applied cells was reported,[28] alternative constructs with

| Scaffold | Collector speed [mm min⁻¹] | Pressure [bar] | Box width [µm] | Wall fiber diameter [µm] | Catching fiber diameter [µm] |
|----------|-----------------------------|---------------|----------------|--------------------------|-----------------------------|
| 380-10   | 500                         | 1.2           | 379 ± 4        | 15 ± 3                   | –                           |
| 360-30   | Walls: 1000                 | Walls: 1.2    | 362 ± 2        | 13 ± 1                   | 8.5 ± 0.8                   |
| 360-40   | Catching fibers: 1500       | Catching fibers: 0.5 | 357 ± 12       | 14 ± 1                   | 7.5 ± 0.6                   |

Table 1. MEW parameters and corresponding scaffold dimensions determined from optical or SEM images.
distinctly thinner fibers such as those presented in the current study appear desirable. Furthermore, the long-term degradation associated with PCL can be potentially reduced by performing MEW with other thermoplastic polymers for the fabrication of such scaffolds. Medical polymers such as PLGA\(^{29}\) and PLA\(^{30}\) have already been melt electrospun.

Apart from adipose engineering, the concept of utilizing box-structured MEW scaffolds for seeding of multicellular spheroids...
may be readily transferred to other applications which aim to generate implants or tissue models, e.g., engineering cartilage tissue for which pellet culture is frequently used. For other tissues requiring sufficient blood supply, complementary strategies such as preseeding of the box structures with endothelial cells prior to spheroid insertion could be useful in order to support vascularization of the sheet-like structures.

For general tissue engineering purposes, a recent report presented a biofabrication concept for the automated assembly of preformed microtissues within a 3D plotted thermoplastic polymer scaffold. Using a layer-by-layer assembly process, alternating between 3D plotting and microtissue insertion in each layer, complex hierarchical structures could be achieved.\[^{31}\] While this approach relied on relatively thick thermoplastic fibers (220 µm),\[^{31}\] it appears attractive to also explore the concept of automated microtissue insertion in multilayered constructs for the spheroid-loaded thin-fiber MEW scaffolds. As an alternative approach, inkjet printing may be employed for placing spheroids into the box structures. The use of the MEW scaffolds not only provides a relatively high tissue-to-polymer ratio, but also readily enables spheroid contact and interaction through the thin fibers constituting the walls, so that the fusion capability of the spheroids\[^{32}\] may be exploited further for engineering complex implants or tissue models. Indeed, while after conventional spheroid fusion newly formed tissue is frequently reported to shrink, no shrinkage of the sheet-like structures was observed here, and due to the scaffold support the formed tissue sheets may maintain their long-term structural integrity. Furthermore, sheets of spheroid-laden MEW scaffolds may be used as customizable in vitro models, e.g., in cancer research to study tumor spheroid invasion using MEW scaffolds enhanced with hydrogel matrices or to assess the interaction of tumor and stromal cells such as ASCs in coculture.\[^{33}\]

Figure 3. Adipogenic differentiation of ASC spheroids in MEW scaffolds. Constructs were cultured for 14 days under adipogenic induction (“ind”) and non-induced control (“non”) conditions. A,B) Histological sections were stained for lipids with Oil Red O; lipid droplets appear red (blue arrows) and cell nuclei blue (hematoxylin staining); scale bars = 50 µm. Histological sections were readily achieved, however, fibers broke during the process. Due to its hydrophobic character, PCL was also stained red with Oil Red O (black arrows). C) Adiponectin secretion of ASCs. Cell culture media (conditioned between day 12 and 14) were evaluated by ELISA. Values are expressed as mean ± SD (n = 3). *Statistically significant difference (p < 0.05) between adipogenic induction (“ind”) and non-induced control (“non”) conditions. D–F) Adipogenic marker gene expression of ASCs as determined by qRT-PCR. Gene expression was normalized to EF1α; the obtained values were normalized to the values at day (d) 0; values are expressed as mean ± SD (n = 3). \(^{\ast}\)Statistically significant differences (p < 0.05) between adipogenic induction (“ind”) and non-induced control conditions (“non”) after 14 days, or \(^{\ast\ast}\)between adipogenic induction and day 0.
In conclusion, box-structured MEW scaffolds easily tailorable to spheroid size were seeded with ASC spheroids. Adipogenic differentiation within the sheet-like constructs was induced and characterized on the cellular and molecular level by histological staining of accumulated fat, and quantitative analysis of adipogenic marker gene expression and adipokine secretion. To our knowledge, this is the first long-term in vitro study combining MEW scaffolds with multicellular spheroids, and these constructs may be further utilized for adipose tissue regeneration research. Furthermore, the presented concept utilizing MEW scaffolds for spheroid seeding appears readily transferable and may be included in approaches to the engineering of other implants and tissue models.

Experimental Section

Materials: Medical-grade PCL (Corbion Inc, Netherlands, PURASORB PC 12, Lot# 1412000249, 03/2015) was used as received. Storage/handling of this polymer, for these experiments, is described elsewhere.[25]

Scaffold Fabrication: MEW was performed with a custom-built MEW 3D printer, as previously described.[25] Briefly, PCL pellets were loaded into a syringe fitted with a 22 G nozzle, and the heating controller was set to 85 °C. Positive (4.5 kV) and negative (~1.5 kV) high voltage were applied to the nozzle and the collector plate, respectively. The collector plate was driven in the X- and Y-axes by servos motors while the printing head, including syringe, heater, nozzle, and high voltage electrode, was in a fixed position, with a manually driven Z-axis used to adjust the collector distance to 4 mm. The printer parameters used to fabricate the scaffolds are shown in Table 1. The “box-pore” scaffolds were direct-written into 3D structures following stabilization of the electrified polymer jet.

Scaffold Imaging and Characterization: All scaffolds were imaged with a Zeiss StereOView V.20 microscope. Further imaging was performed using a Zeiss Crossbeam 340 scanning electron microscope equipped with a GEMINI e-Beam column. Samples were sputter-coated with a 4 nm layer of platinum. Imaging of seeded scaffolds was performed using an Olympus IX51 inverted microscope fitted with an XC30 digital camera at 0 and 24 h after seeding, and at various time points during the culture period. Furthermore, SEM images of the scaffolds were taken 24 h after seeding. Samples were prepared for SEM imaging by rinsing four times in PBS, then fixing in 6% glutaraldehyde for 15 min on ice. Dehydration was performed by a series of PBS, ethanol, and hexamethyldisilazane incubation steps, followed by drying of the samples overnight at room temperature. Prior to SEM imaging, the dried samples were sputter-coated with a 4 nm layer of platinum.

Live/Dead Staining: Cell viability in the spheroid-loaded scaffolds was assessed using the Live/Dead Staining Kit II from Promokine (Heidelberg, Germany), according to the manufacturer’s protocol (see Supporting Information for details).

Investigation of Adipogenesis: Histological analysis, and molecular investigation of adipogenesis on the mRNA and protein level by qRT-PCR and ELISA, respectively, was performed as previously described[35] (see Supporting Information for details).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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3D printing, additive manufacturing, adipose tissue engineering

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[1] J. R. Tumbleston, D. Shirvanyants, N. Ermoshkin, R. Janusziewicz, A. R. Johnson, D. Kelly, K. Chen, R. Pirschmidt, J. P. Rolland, A. Ermoshkin, E. T. Samulski, J. M. DeSimone, Science 2015, 347, 1349.
[2] A. Kuroleva, A. A. Gill, I. Ortega, J. W. Haycock, S. Schlie, S. D. Gittard, B. N. Chichkov, F. Claeyssens, Biofabrication 2012, 4, 025005.
[3] T. D. Brown, P. D. Dalton, D. W. Hutmacher, Adv. Mater. 2011, 23, 5651.
[4] M. L. Muerza-Cascante, D. Haylock, D. W. Hutmacher, P. D. Dalton, Tissue Eng., Part B 2015, 21, 187.
[5] Q. Chen, X. Mei, Z. Shen, D. Wu, Y. Zhao, L. Wang, X. Chen, G. He, Z. Yu, K. Fang, D. Sun, Opt. Lett. 2017, 42, 5106.
