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3D Scaffolds to Study Basic Cell Biology

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Mimicking the properties of the extracellular matrix is crucial for developing in vitro models of the physiological microenvironment of living cells. Among other techniques, 3D direct laser writing (DLW) has emerged as a promising technology for realizing tailored 3D scaffolds for cell biology studies. Here, results based on DLW addressing basic biological issues, e.g., cell-force measurements and selective 3D cell spreading on functionalized structures are reviewed. Continuous future progress in DLW materials engineering and innovative approaches for scaffold fabrication will enable further applications of DLW in applied biomedical research and tissue engineering.

1. Introduction

Controlling eukaryotic cells by tailor-made scaffolds is an intriguing goal, as it is an emerging way to "program" or instruct cell behavior without the need of genetic modifications. In the living organism, cell behavior is critically influenced by the actions of different cues from their microenvironment, which act cooperatively in three dimensions.[1] These microenvironmental cues include biochemical factors such as soluble growth factors, cell–cell interactions, and cell–matrix-binding. In addition, biophysical factors like mechanical properties of the surrounding extracellular matrix (ECM) can significantly influence cellular reactions. Likewise, alterations in the composition or the mechanical properties of the ECM are often linked to pathological conditions, as well as cancer progression and metastasis.[2,3] Concerning stem cells, including both embryonic stem cells or induced pluripotent stem cells, specific and often 3D culture conditions are necessary to either keep their pluripotent status or to differentiate them into the desired cell type.[4]

Consequently, a variety of experimental 3D cell culture models have been developed in recent years.[5] A prominent example is hydrogels that can mimic the different stiffness ranges of tissues. They are widely used biomaterials for tissue engineering and regenerative medicine.[6] In combination with techniques to obtain 3D scaffolds with tunable porosity, hydrogels proved to be suitable growth substrates for a variety of different cell types. For example, hematopoietic stem cells (HSCs) are highly demanding with respect to their cell culture conditions. On 2D substrates they rapidly differentiate. For clinical applications, however, it is necessary to expand HSCs and maintain the stem cell character. The Lee-Thedieck group successfully fabricated a macroporous hydrogel that mimics the spongy structure of the HSC-niche within the bone. They analyzed the stem cell character and proved that coculture with mesenchymal stem cells, used as support cells by HSCs in vivo, is more efficient in the 3D bone marrow analogs than in standard 2D culture.[7] The Cato group demonstrated the impact of dimensionality and biophysical properties on tumor cell proliferation by fabricating a mechanically stable superporous 3D cryogel for prostate tumor cell growth. They found that prostate tumor cells showed an increased response to growth promoting androgens when cultured in 3D as compared to 2D culture systems.[8] Thereby, they provide cryogels as a promising new platform for studies on prostate tumor models.

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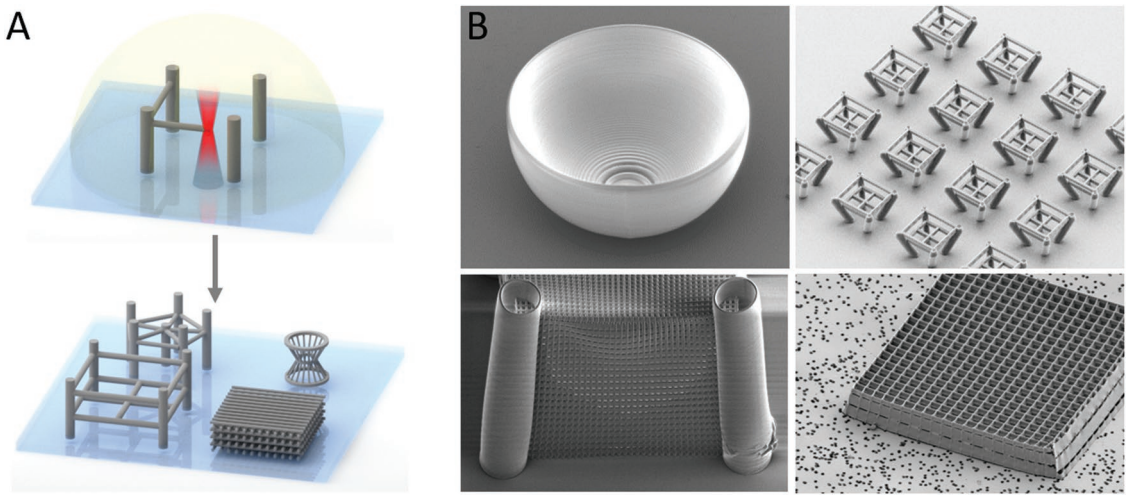

Figure 1. A) Schematic representation of the DLW technique. B) Scanning electron micrographs of selected DLW structures for cell biological applications ranging from simple bowl-like structures, an array of scaffolds for single cell culture, a porous cage-like structure to investigate cell invasion, and a grid on a porous surface for cell migration studies under the influence of a chemotactic gradient. Bottom right panels adapted with permission.[21] Copyright 2014, Elsevier.

Besides being useful growth environments to culture demanding cell types, the biophysical characterization of hydrogels is mainly performed on the bulk material and thus it is difficult to deduce the exact properties of the cellular microenvironment. Therefore, for both understanding and instructing cellular behavior, a controllable tailored 3D scaffold is highly desirable. Recent progress in additive manufacturing techniques has enabled the fabrication of scaffolds with defined geometries that can partly recapitulate the structure of the natural cellular environment.[9] Techniques that allow manufacturing of tailored scaffolds to study single cells or small cell assemblies are of particular interest. In this context, 3D direct laser writing (DLW) fulfills these requirements. This particular approach enables the fabrication of complex 3D cellular microenvironments with a well-defined geometry, a controllable stiffness range, the integration of multiple materials in the same structure, and a spatially resolved biofunctionalization. During the last ten years, DLW has rapidly evolved to be a valuable tool for bioengineering approaches. Several important aspects such as materials biocompatibility,[10–12] structural designs for tissue engineering,[13–15] and integration into microfluidic devices[16] have been thoroughly reviewed recently. On the other hand, 3D scaffolds with a tailored geometry also allow to systematically study cellular reactions (e.g., cell migration, cell mechanics, and cell differentiation) in response to a well-defined environment on a single cellular level. This will lead to a better understanding how a complex environment influences cell behavior and consequently, it will pave the way for novel cell culture devices to steer cell differentiation. In this Research News, we focus on the versatile applications of tailored direct laser written 3D scaffolds to answer basic biological questions.

2. Direct Laser Writing for Cell Culture

DLW is a lithography technique, which is based on two-photon absorption. In brief, due to the nonlinearity of the underlying process, the photon density to polymerize the photoresist is only sufficiently high in the focal plane of the objective. Thus, the voxel is additionally confined in the axial direction compared to conventional one-photon techniques. By relative movement of the laser focus to the sample in *x,y,z*-direction, it is possible to write arbitrary 3D scaffolds in the micrometer range (**Figure 1**A). For the chemical aspects of DLW, we refer to a recent review published by our groups.[17] The flexibility of this technique allows for the fabrication of cell culture scaffolds ranging from simple pillar-structures to more complex shapes. Examples include bowl-shaped growth substrates, arrays of box-ring like structures, cages with tunable porosity, and grid structures integrated on porous membranes (Figure 1B).

It was proven that 3D scaffolds produced by DLW can successfully instruct the behavior of different cell types with respect to proliferation and migration. The first studies on cells were performed on rather concise 3D scaffolds that consisted of a single material (**Figure 2**A). Hohmann and von Freymann investigated the proliferation of osteoblast-like cells on different 3D topographies and found that 3D culturing in square grids enhanced the proliferation up to 170% with respect to hexagonal structures or unstructured growth substrates.[18] This indicates that the 3D adhesion geometry can influence cell cycle progression. Furthermore, our groups used wheel-like structures to monitor the reaction of different fibroblast and epithelial cell lines with respect to proliferation, adhesion and cellular geometry. Although the proliferation and adhesion were similar to conventional 2D culture conditions, the cells largely differed with respect to their morphology. Strikingly, fibroblasts, but not epithelial cells, almost doubled their cellular volume when cultured in 3D.[19] This is consistent to their in vivo origin, where fibroblasts grow in a complex 3D environment whereas epithelial cells form a planar tissue.

Tayalia et al. demonstrated that 3D woodpile scaffolds increased the migratory speed of mesenchymal tumor cells in comparison to 2D substrates.[20] Our groups studied the effect

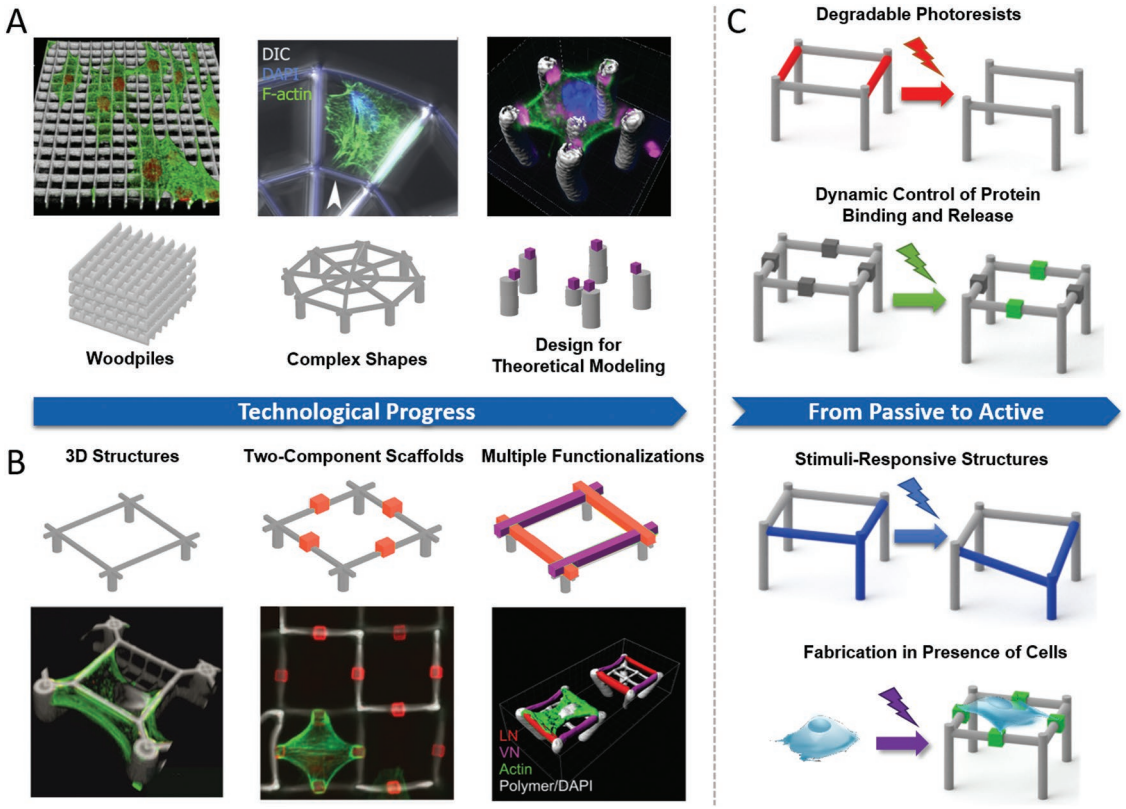

Figure 2. Overview of the technological progress of scaffolds for single cell studies obtained via DLW. A) 3D scaffolds ranging from basic geometries (e.g., woodpiles), to complex shapes and to sophisticated designs allowing the combination of experimental results with theoretical modeling. The left image is adapted with permission.[21] Copyright 2014, Elsevier. The center image is adapted with permission.[19] Copyright 2015, Elsevier. B) Evolution of biofunctionalization ranging from homogenously coated scaffolds, to structures with distinct adhesion sites and multiple spatially defined functionalizations. C) Current research is directed to active systems with tunable properties and towards the fabrication in the presence of living cells.

of different pore sizes on directed cell migration by writing 3D scaffolds onto porous membranes (Figure 1B). In brief, the invasive potential of mouse embryonic fibroblasts and epithelial cells with respect to a chemical gradient and the nuclear stiffness were monitored. Whereas in fibroblasts the biophysical property of the nucleus was crucial for the invading behavior, epithelial cells were triggered by the chemical gradient and invaded pores independently of the nuclear stiffness.[21]

The invasive behavior of tumor cell lines in relation to the mechanical properties of the fabricated 3D scaffolds was studied by Lemma et al. The authors investigated the capability of two different breast tumor cell lines to invade porous cage-like structures with different material stiffness. They showed that cells were more likely to cross scaffold facets with lower Young's modulus as compared to stiffer structures.[22] A similar cellular behavior was observed by the same group using substantially different architectures, i.e., cylinder scaffolds and an additional colorectal cancer cell line.[23] Together, their results suggest that not only the morphology but also the stiffness of the ECM surrounding tumors in vivo may play a key role in the resulting invasive behavior of cells.

The studies presented so far made use of relatively simple patterns fabricated via DLW to reveal that biophysical parameters like dimensionality and pore size are relevant for proliferation, migration, and maturation of different cell types. In order to address more complex biological questions, sophisticated 3D scaffolds with defined (bio-)functionalizations were needed (Figure 2B). Thus, we first developed two-component scaffolds with protein-repellent and protein-binding material properties.[24] Presenting spatially defined biofunctionalized spots for cell adhesion we obtained precise control over 3D cell shape. Only recently, we introduced a concept for scaffold functionalization with several proteins based on three different components.[25] The initial scaffold is built from a repellent photoresist. Subsequently, a second material is added which allows protein adsorption. In a third DLW step, a generally repellent photoresist with functional moieties that allow coupling to a specific protein-tag is applied. These scaffolds functionalized with different proteins, were used to study the adhesion of different cell types with respect to their protein affinity.

Until that point, it was possible to produce 3D DLW scaffolds that possess spatially defined areas of different stiffness. Although the stiffness cannot be varied during culture, the tailored 3D scaffolds allowed the investigation of complex cellular processes like force generation and mechanotransduction. In a first approach, we cultured chicken embryonic cardiomyocytes in scaffolds with deformable beams (**Figure 3**A). During spontaneous contraction cycles these cardiomyocytes were able to deflect the beams. Measuring the spring constant of the beams

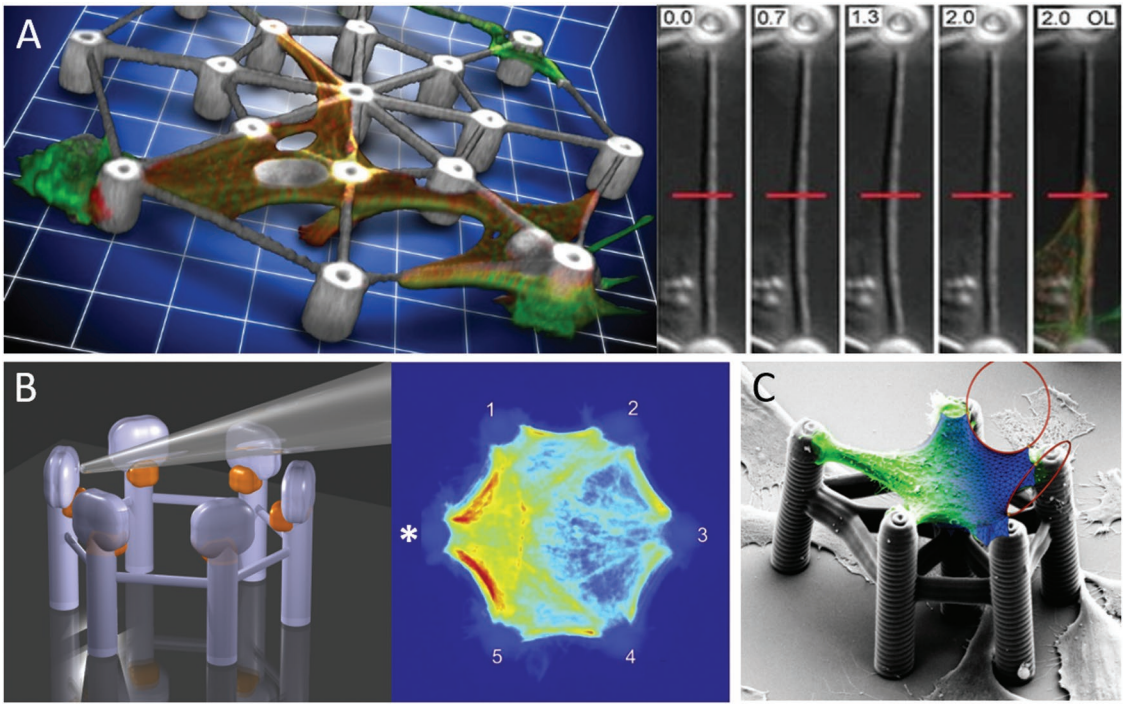

Figure 3. Scaffolds to study the mechanobiology of single cells. A) Wheel-like structures with thin elastic beams allow to measure forces exerted by beating cardiomyocytes. B) Hexagonal structures to mechanically stimulate single fibroblasts at one spatially defined adhesion site (asterisk). The heat map indicates the strengthening of actin filament bundles at the manipulated spot. C) SEM micrograph of a scaffold used for the combination of experimental data with theoretical modeling of 3D cell shapes. B) Adapted with permission.[27] Copyright 2015, Elsevier.

with atomic force microscopy allowed us to correlate the degree of deflection to traction forces generated by the cardiomyocytes.[26] Furthermore, 3D scaffolds can be applied to exert forces to specific adhesion sites of single cells. We analyzed the impact of mechanical stress on primary fibroblasts cultured in elastic, hexagonal 3D scaffolds (Figure 3B). The cells were either periodically or statically stretched by bending an individual post. Since the hexagonal scaffolds enable precise control over cell size and shape, they allow multiple experiments under the same conditions and thus averaging over results from individual cells. The resulting heat map unsheathes the subtle but persistent remodeling of cell–matrix adhesions and rearrangements of the actin cytoskeleton that would not be emphasized by analyzing individual cells.[27]

In collaboration with the group of Schwarz, we used scaffolds with spatially defined adhesion sites to control cell shape and growth in 3D (Figure 3C). The combination of high resolution 3D cell reconstructions with mathematical modeling revealed that not only actin contractility but also elastic stress determines the shape of fibroblasts.[28] With this work we pursued the model that described cellular mechanics during adhesion on 2D patterned surfaces.[29]

Over the last decade, DLW evolved to a powerful technique to produce well-defined, controllable cell culture growth substrates to instruct cell behavior and to answer basic biological questions. Whereas most of the experiments so far have been performed with commonly used cell lines, 3D scaffolds also have a high potential for the controlled culture of neurons and stem cells. First steps in this direction have been performed by the groups of Vieu and Malaquin, which demonstrated the biocompatibility of 3D scaffolds for neuroblastoma cells.[30,31] Concerning stem cells, Raimondi and coworkers improved the manufacturing of synthetic 3D niche structures for culturing various types of stem cells, including mesenchymal stem cells.[32] Along this line, Worthington et al. demonstrated the potential of DLW to study and control the fate of human induced pluripotent stem cells as a function of substrate interactions.[33]

An important step for future improvement will be the transition from passive scaffolds to active systems that can be tuned on demand (Figure 2C). One milestone toward tissue engineering for regenerative medicine will be the development of 3D scaffolds which slowly degrade after implantation without releasing toxic products. In this context, structures from proteins like chitosan[34] and BSA[35] have been fabricated by DLW. Another promising approach is the enzymatic degradation of gelatin-based scaffolds as demonstrated by the group of Ovsianikov et al.[36] in the presence of cells. Along this line, Baudis et al.[37] report on the development of biocompatible photoelastomers that allow for in vitro degradation. However, for well-defined experimental conditions, it would also be desirable to fabricate structures that can be degraded on demand via external biocompatible stimuli, e.g., illumination with visible light.

For the fabrication of layered structures composed of different cell types, it would be advantageous to manufacture structures in the presence of cells. In order to achieve this, water-soluble and nontoxic photoinitiators are indispensable. Pioneering work in that direction was performed by Ovsianikov and colleagues,[38] who fabricated gelatin-based hydrogels in the presence of cells. More recently, Qin et al.[39] demonstrated the

spatio-temporal control of cell invasion by modifying the cell-instructive properties of a hydrogel with DLW.

To precisely control tissue formation in 3D scaffolds, it is necessary to understand the underlying biological principles like the mechanical signals in the microenvironment. Accordingly, a dynamic control over cell adhesion and detachment with subcellular resolution is required. This could be achieved by scaffolds where protein adhesion and release can be triggered on the micrometer scale by external stimuli. Finally, stimuli-responsive scaffolds that change their stiffness or shape will allow to study the response of single cells growing in a defined 3D environment to local mechanical signals. In this direction, recent work of our groups demonstrates that 3D hydrogel microstructures can be controlled by temperature and light.[40]

The further development of DLW technologies, materials and techniques for bio-functionalization along with a deeper understanding of the basic biological mechanisms will pave the way towards rationally designed scaffolds for tissue engineering.

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Conflict of Interest

The authors declare no Conflict of Interest.

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