Micro-scaffolds as synthetic cell niches: recent advances and challenges

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Micro-fabrication and nano-fabrication provide useful approaches to address fundamental biological questions by mimicking the physiological microenvironment in which cells carry out their functions. In particular, 2D patterns and 3D scaffolds obtained via lithography, direct laser writing, and other techniques allow for shaping hydrogels, synthetic polymers and biologically derived materials to create structures for (single) cell culture. Applications of micro-scaffolds mimicking cell niches include stem cell self-renewal, differentiation, and lineage specification. This review moves from technological aspects of scaffold microfabrication for cell biological applications to a broad overview of advances in (stem) cell research: achievements for embryonic, induced pluripotent, mesenchymal, and neural stem cells are treated in detail, while a particular section is dedicated to micro-scaffolds used to study single cells in basic cell biology.

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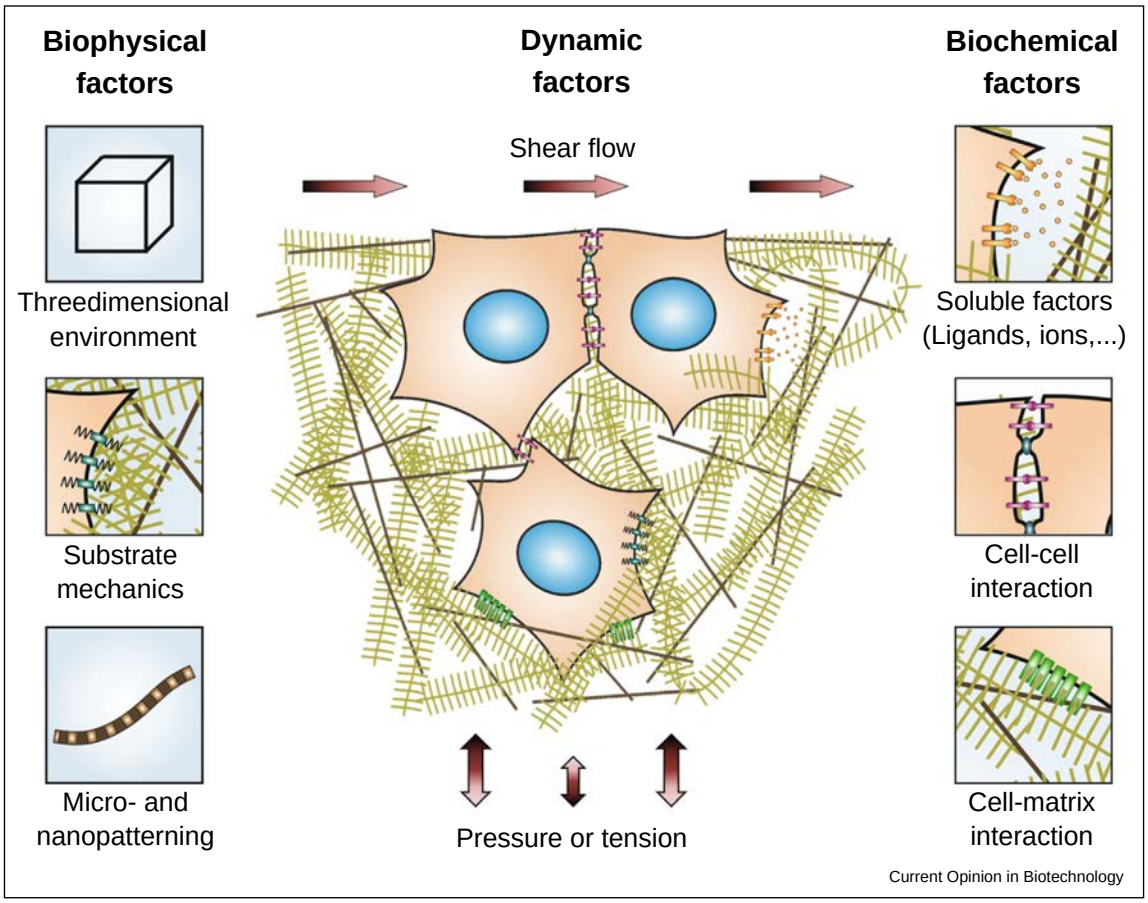
Introduction

The extracellular matrix (ECM) resembles an immensely complex three-dimensional (3D) structure. From a very general point of view, it is composed of three classes of biomacromolecules: collagens, proteoglycans, and glycoproteins. However, architectural and compositional variations define subsets of the ECM, reflecting cell-type specific microenvironments with a unique molecular, structural, and topographical footprint (Figure 1). The complexity of such cellular microenvironments is usually condensed in the term 'cell niche'. Especially fate decisions of embryonic and adult stem cells are drastically influenced by the molecular composition and the physical properties of the cell niche and the term is therefore also referred to as 'stem cell niche'. Despite being most prominently linked to stem cells, the cellular microenvironment also has an influence on differentiated cell types by facilitating tissue integrity and cellular physiology. Therefore, we will use the term 'cell niche' in the following as a general description for a cellular microenvironment. Crucial niche factors not only comprise molecular components but also the topography, geometry and stiffness of the ECM [1]. These physical cues are sensed by a complex interplay of several molecular programs that are activated by mechanical and biochemical stimuli. For further reading, we refer to detailed reviews, which specifically cover this fascinating topic [2,3]. Thus, to understand and control the fate decisions of cells and tissue formation, one also has to understand and mimic the physical properties of the cellular microenvironment [4]. In principle, this can be done on a single cellular level or on a macroscopic scale. Larger scale approaches enable harvesting sufficient cell quantities for the assessment of gene-expression (e.g. via PCR or mRNA sequencing) or protein-expression (e.g. via Western blotting). However, since fate decisions of early embryonic stem cells or adult stem cells are often controlled on a single cellular level, approaches to precisely mimic the environment of isolated cells are also important. Given this context, numerous studies set out to define the cellular microenvironments, raising great hopes for stem cell therapies and regenerative medicine.

This review briefly highlights the most widely used techniques to reproduce ECM-like environments based on scaffolds and presents several biological approaches for applied and basic research, with special reference to stem cell maintenance or stem cell differentiation and to micro-scaffolds with single cell resolution for basic cell biological research. The main publications addressing stem cell maintenance and differentiation are summarized in Table 1.

Figure 1



Microenvironment of cells.
In native tissues, cells are surrounded by a complex and heterogenous cell niche. Each cell niche is composed of a specific geometrical, topographical and molecular architecture, giving rise to an individual footprint. Additionally, dynamic factors like shear flow (e.g. in vessels), tension or compression act on the niche. Cells sense these individual properties via transmembrane receptors like ion channels, cell cell contacts or cell matrix contacts, and subsequently activate intracellular cascades that tune, for example, the cytoskeletal structure and the nuclear import of transcription factors. This relationship between the cells and their structural environment ultimately drives cellular fate decisions like cell migration, differentiation or proliferation.

Generating (semi-)synthetic *in vitro* cell niches: from 2D to 4D

Microfabrication for biological applications can be grossly divided in two main approaches: (i) to create a structural framework (either fully synthetic or from a biological source) called scaffold, able to host cells under investigation, or (ii) to mix cells with biologically compatible fluids or gels (e.g. Matrigel®) followed by deposition on a substrate. Both methods share the basic idea that the realized framework not only serves as the physical support, but can actively play a role in guiding cell behavior. Indeed, very often the physical properties of the scaffold and the spatial distribution of adhesion sites or cellular guidance cues are key factors to induce a specific cellular response. That said, the two approaches greatly differ from each other, and scaffold fabrication, as well as cell printing, can be obtained in a variety of different conditions. A classification of scaffold fabrication methods would be extremely complex, but in general, each technique should be considered according to its advantages and drawbacks, which cover the following aspects: (i) materials used; (ii) fabrication procedure; (iii) dimensionality and resolution achieved. These have also direct consequences on the kind of analyses that can be performed when culturing cells. Micro-scaffolds are suitable to study cell behavior with subcellular resolution and within well-defined geometrical parameters. In contrast, easily scalable technologies allow for the rapid fabrication of scaffolds with lower resolution and less defined geometries. These scaffolds are, however, useful to harvest sufficient cell amounts for high throughput 'omics' studies. The benefits and limitations of the most frequently

Table 1

Selection of scaffold based approaches to guide stem cell maintenance and differentiation

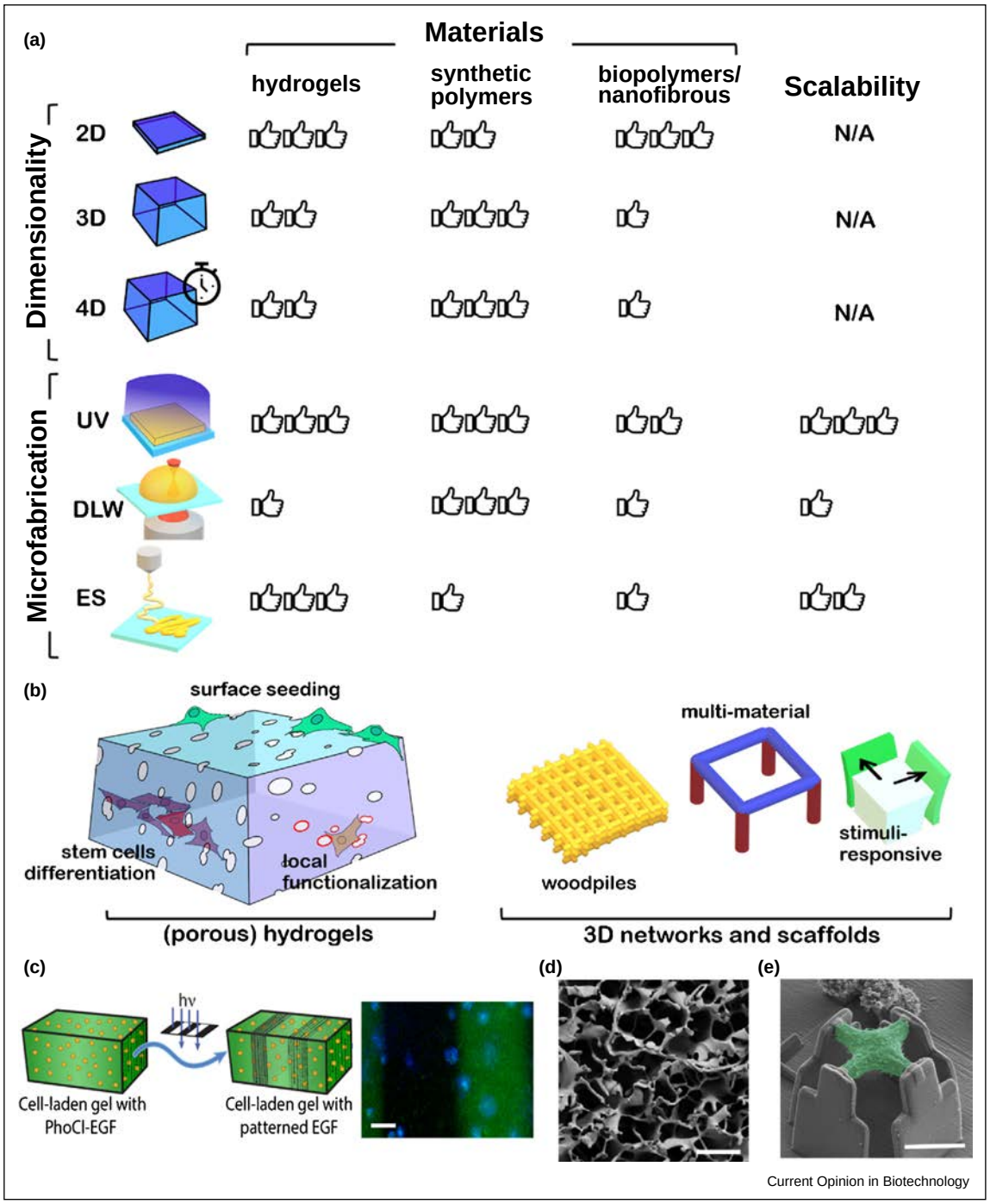
Cell type	Scaffold	Outcome/Application	Ref.
iPSC	Neutral charged PDMAAm and negatively charged PNaAMPS hydrogels	Both hydrogels showed higher proliferation and expression of pluripotency marker genes compared to Gelatin coated polystyrene scaffolds in the absence of feeder cells.	[17]
iPSC	Electrospun polystyrene scaffold	Pluripotency was maintained for 10 consecutive passages in a xeno free environment.	[18]
eSC	DLW fabricated micro scaffolds made from IP L photoresist	Stem cells showed higher proliferation rates when cultured in narrow 3D environments while the proliferation decreased on narrow 2D islands.	[20]
hSC	Porous polyHIPE foam scaffolds	The scaffolds support hSC and progenitor cell culture (erythroid progenitor cells and neutrophils) for 28 days.	[21]
hSC	Zwitterionic poly(carboxybetaine) based Hydrogel	The study achieved a 73 fold increase in long term hematopoietic stem cell frequency and the expanded HSPCs were capable of hematopoietic reconstitution for 24 weeks.	[22]
hSC	Magnetic PEG Hydrogel	Proof of concept for a perfusion system, showing controlled and contactless movement of cell laden magnetic hydrogel in culture media for hSC cultivation.	[23••]
hSC and mSC	Carboxymethylcellulose based cryogel	hSCs in co culture with supportive mSCs assemble a minimalistic and injectable 3D hematopoietic niche, which maintains over 12 weeks in immunodeficient mice.	[24]
mSC	Electrospun hydroxyapatite/collagen/chitosan nanofibers	The scaffold favors proliferation and attachment of mSCs over control substrates and enhances the expression of osteogenic marker genes.	[26]
mSC	Electrospun polycaprolactone microfibers, functionalized with collagen I, heparin and ceramic based bioglass	Proof of concept study that incorporates inorganic and biologically active compounds in the scaffold to serve as a multifunctional membrane during osteogenic differentiation and regeneration	[28]
mSC	Polypyrrole based array of electrochemically switchable Nanotubes	Multicyclic attachment/detachment of mSCs promotes osteogenic differentiation independent of surface stiffness	[31]
mSC	Hyaluronic acid based hydrogels of different molecular weights	The low strength hydrogel maintained mSC properties by activating the Wnt/β catenin pathway for 1 week, while the high molecular weight hydrogel with a higher mechanical strength promoted cartilage differentiation	[33]
mSC	Composite scaffold: Electrospun microfibers combined with a thermosensitive PEG PNIPAAm hydrogel	The reverse thermosensitivity of the hydrogel allowed its dissolution/ hydration upon cell seeding. It was demonstrated that the hybrid scaffold enhanced chondrogenic differentiation based on chondrocytic gene and protein expression.	[34]
nSC	Hydrogels, bifunctionalized with polylysine and the laminin binding motif IKVAV	Bifunctional hydrogels promote differentiation and attachment of embryonic cortical neurons and expansion of adult neurogenic clones.	[42]
nSC	Hyaluronic acid based hydrogel	The scaffold increased differentiation towards electrophysiologically active immature neurons. The fate towards oligodendrocytes was increased, while the differentiation into reactive astrocytes was reduced. The 3D cultures were maintained for at least 70 days in minimal medium	[43]

used materials and fabrication techniques are schematically compared in Figure 2.

From the materials perspective, hydrogels are widely used in scaffold fabrication due to their high water-content and tissue-like stiffness. Usually, these can be easily formed in two-dimensional (2D) or three-dimensional (3D) shapes, and eventually be processed via several techniques, ranging from lithography, to electrospinning, direct laser writing (DLW), and others [5•]. A key feature of some classes of hydrogel scaffolds is also represented by their capability of modifying their physical-chemical properties upon variation of external conditions (usually referred to as stimuli responsiveness). This widens the spectrum to investigate cell behavior, by adding a fourth dimension to biological experiments, that is, changing the properties of the environment.

4D scaffolds, however, are not only obtained using hydrogels. Indeed, complex dynamical systems can also be implemented with microfluidic technologies [6], or by using synthetic polymers which can change their properties over time depending on the environmental conditions. An advantage of synthetic polymers is their superior suitability to manufacture 3D scaffolds via DLW lithographic approaches [7]. Although the mechanical properties of these polymers usually differ from those of the physiological microenvironment, they have provided substantial insights in understanding cell behavior in artificial 3D niches [8,9]. In addition, micro-channels and nanopillars, fabricated from stiff polymers, have been used to investigate single cell migration [10••] and mechanotransduction events on a subcellular scale [11]. In summary, DLW allows to produce virtually any 3D geometry with subcellular resolution, however, at present the serial

Figure 2



Scaffolds for (stem) cell culture.
Panel **(a)**: Qualitative rating (👍 = poor, 👍👍 = medium, 👍👍👍 = good) of the suitability of the materials described in the main text with respect to the type of scaffold that can be realized (dimensionality) and to the microfabrication technique used for shaping and/or patterning (UV = ultraviolet mask lithography, DLW = direct laser writing, ES = electrospinning). Panel **(b)**: schematic view of cell seeding approaches for hydrogels based scaffolds and of structuring techniques for 3D synthetic polymers made or biopolymers made structures. Panel **(c)**: Light induced 3D patterning of cell laden hydrogels: photocleavable EGF is encapsulated in a PEG based gel and selectively cleaved via masked UV irradiation (left); spheroids of HeLa cells (right, blue) proliferate preferentially on regions with still tethered EGF (green). Scalebars 200 μm. Modified with permission from Ref. [17]. Panel **(d)**: Scanning electron micrograph of silk fibroin freeze dried scaffolds for cancer cells (LNCaP) cultivation. Scalebar 100 μm. Modified with permission from Ref. [13]. Panel **(e)**: 3D multi material scaffold fabricated via DLW for single cell controlled stretching. Scalebar 15 μm.

production of scaffolds is time-consuming and requires specific equipment.

Unsurprisingly, the previous strategies can be combined [12•] and enriched by the use of further biocompatible nanofiber-based materials, for example, silk [13], gelatin and gelatin-methacrylate (GelMA) hydrogels [14], and others [15]. In this case, precise fabrication of scaffolds is still challenging, but current methodologies tend to converge to structures with unprecedented mechanical, chemical and physical capabilities to mimic the physiological environment of cells [16•,17]. In the following, we will give an overview about several state-of-the-art approaches that use micro-scaffolds to direct the cellular behavior.

Scaffolds for stem cell self-renewal and homing

Micro-scaffolds are considered a promising tool to maintain and prolong the pluripotency of stem cells in a xeno-free environment. Pluripotent embryonic stem cells (eSC) and induced pluripotent stem cells (iPSC) possess enormous medical potential but their handling *in vitro* is highly demanding. Each stem cell type requires individualized culture conditions and animal-derived components to facilitate their self-renewal and as mentioned above, maintenance of these delicate pluripotency states requires highly defined growth conditions under controlled parameters. The use of porous hydrogels or microfiber-based scaffolds is a well-known approach for stem cell homing and allows xeno-free cultivation of eSC/iPSCs for up to two months or ten consecutive passages, as shown by the expression of pluripotency markers and the formation of all three germ layers in teratoma assays [18,19]. Fully synthetic structures also possess some potential in this regard. 'Nichoids' fabricated via DLW can maintain the pluripotency of mouse eSCs for two weeks in the absence of a feeder layer or exogenous conditioning factors [20]. Interestingly, it was recently shown that the cell division rate of single mouse eSCs increased in 3D scaffolds, when the adhesive area was more confined, while it decreased, when the adhesive area of 2D islands was smaller [21]. This demonstrates that the proliferation rate and gene expression profile of eSCs and iPSCs can be influenced simply by the size and adhesion geometry of the environment. Approaches with fully defined geometries therefore might bear great potential for future culture systems, allowing clonal expansion under comprehensible conditions.

Concerning adult SCs, promising results were recently achieved for hematopoietic stem cells (hSCs). Hydrogels or other porous polymer scaffolds that mimic the spongy structure of the hSC niche within bones (Figure 3a and f), have more effectively preserved the stemness of hSCs and progenitor cells as compared to standard 2D culture systems [22,23••,24], especially when hSCs were co-cultured with mesenchymal stem cells (mSCs) (Figure 3b) [25•]. Bai *et al.* achieved a 73-fold increase in long-term hSC frequency when using zwitterionic 3D hydrogels as a culture system [23••]. It was suggested that this culture system promotes the self-renewal of hSCs by inhibiting the excessive production of reactive oxygen species and the expanded hSCs were capable of hematopoietic reconstitution for at least 24 weeks in immunocompromised mice.

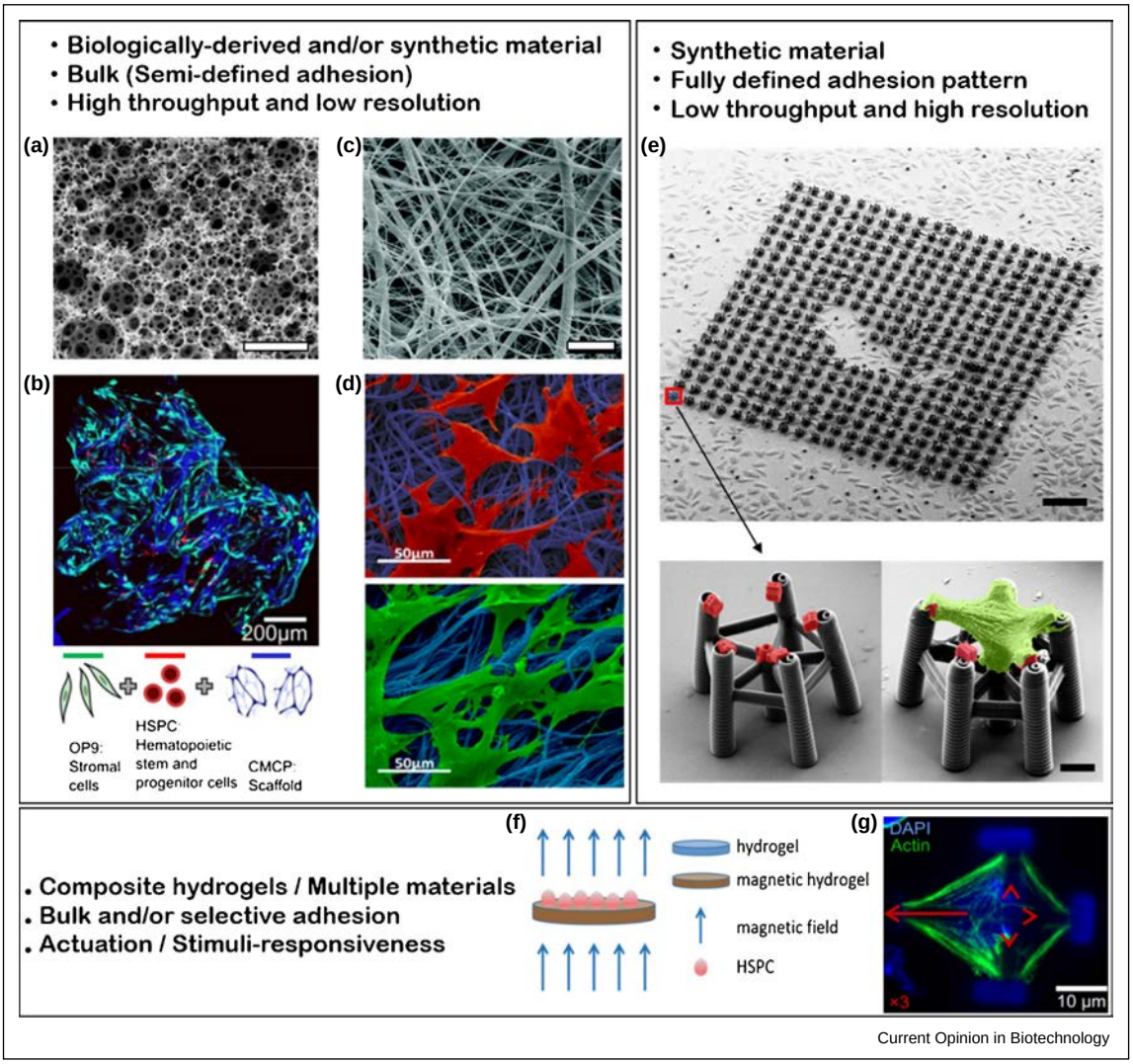
Scaffolds for mesenchymal stem cell differentiation and lineage specification

Many approaches try to harvest the power of biomimetic scaffolds to support and guide the differentiation of adult stem cells, that is, to ultimately provide an inexhaustible source of post-mitotic cells. mSCs are promising candidates in this regard. The differentiation of mSCs is a mechanosensitive process and although the biomolecular mechanisms are not investigated to the last detail, it is a well-established concept that the topography and stiffness of the substrate have a major impact on the mSC differentiation into adipose, myogenic or osteogenic precursor cells.

Of high impact are bioinspired scaffolds that support and enhance osteogenic cell differentiation. Among the most used are fibrous meshworks (Figure 3c and d), either directly derived from biological sources or fabricated from synthetic materials that are biologically functionalized subsequently. Biologically derived materials include decellularized and aligned ECM [26], collagen/chitosan-derived nanofibers [27] and nanofibrillar cellulose hydrogels [28], while polycaprolactone is a popular synthetic material [29•]. Additionally, the fiber orientation can be aligned or random (Figure 3d), and inorganic components have been successfully incorporated in the scaffold backbone to tune the rheological properties of the scaffold and to enhance the production of mineralized bone matrix [29•,30].

Further material improvements try to incorporate smart materials in the scaffold design. A recent study used a conductive material PEDOT:PSS and produced highly porous, collagen I functionalized scaffolds that support osteogenic differentiation [31]. Another study prepared a polypyrrole scaffold that can be reversibly switched between highly adhesive hydrophobic nanotubes and poorly adhesive hydrophilic nanotips through an electrochemical oxidation/reduction process. Here, multicyclic attachment/detachment of mSCs was shown to activate intracellular mechanotransduction and osteogenic differentiation independent of surface stiffness and chemical induction, as shown by increased BMP2 and bone sialoprotein expression [32]. Again, the use of fully defined synthetic scaffolds might be another approach that might bear great potential. Since DLW-fabricated scaffolds can be precisely tuned with regard to their rheological and

Figure 3



Examples of three basic scaffold types and exemplary applications.
Panel **(a)**: A thiol containing biomimetic porous hydrogel scaffold was polymerised by high internal phase emulsion and employed for human peripheral blood haematopoietic stem and progenitor cell expansion and proliferation. Scale bar 100 μm. Modified with permission from Ref. [22]. Panel **(b)**: A collagen coated, porous carboxymethylcellulose micro scaffold (blue) was used to assemble a 3D hematopoietic niche *in vitro* by co culture of supportive OP9 mesenchymal cells (green) and HSPCs (red). This set up maintained stromal and hematopoietic populations over 12 weeks after injection in immunodeficient mice. Modified with permission from Ref. [25•]. Panel **(c)**: Example of a nanofiber scaffold that was functionalized by exploiting host guest interactions between the electrospun cyclodextrin nanofibers and an adamantane conjugated laminin derived IKVAV epitope to support neurite outgrowth. Scale bar 10 μm. Modified with permission from Ref. [41]. Panel **(d)**: Randomly oriented or aligned polycaprolactone nanofiber scaffolds were fabricated by electrospinning and functionalized with collagen I and heparin, and a ceramic based bioglass, to analyze osteogenic induction of mesenchymal stromal cells (highlighted in red or green). Modified with permission from Ref. [29•]. Panel **(e)**: An array of fully synthetic 3D micro scaffolds was fabricated via 3D DLW, to guide the shape of single cells in a confined 3D environment. For the selective functionalization of the scaffolds, different photoresists with protein repellent or protein adsorbing properties can be used in sequential production steps. In the lower pictures, it can be seen that the cell (false colored in green) specifically adheres to the spatially defined adhesion sites (false colored in red). Scalebars represent 150 μm for the upper picture and 5 μm for the lower pictures. Panel **(f)**: To imply a dynamic system, the scaffold backbones can be further tuned, for example, by including a magnetic field in hydrogels for perfusion systems. Modified with permission from Ref. [24]. Panel **(g)**: Stimuli responsive materials can also be used for fabricating scaffolds able to stretch single cells symmetrically or non symmetrically. Modified with permission from Ref. [12•].

geometrical properties, bone-mimetic scaffolds could be individually adapted to the compact or spongy substructures of bones [33].

Importantly, such systems are not limited to osteogenesis. Hyaluronic acid-based hydrogels with adjustable stiffnesses [34] and electrospun PEG-based hydrogels [35] were successfully employed for cartilage differentiation and recent works improve the output/success rate by refining the microstructures of the scaffolds [36,37] or by including a dynamic dimension like degradability [38] or thermosensitivity [35]. Future studies might shed more light on the intracellular cascades and molecular mechanisms that drive the cellular fate decisions of mSCs growing in these different scaffold types.

Scaffolds for neural stem cell differentiation and lineage specification

Another cell type that displays high potential in this regard are neuronal stem cells (nSCs). Selective differentiation of neural progenitor cells on scaffolds functionalized with a laminin-derived peptide motif (IKVAV) is a well-established strategy for more than 15 years [39]. Today's material toolbox for electrospun nanofiber-scaffolds and hydrogel-scaffolds comprise (among others) silk fibroin [40], cyclodextrin/adamantane host-guest complexes [41], hyaluronic acid [42] or polyacrylamide [43]. Basically, all harvest the power to trigger neurite extension and depending on the functionalization can support certain neuronal fates [44•].

Another basic idea is to incorporate conductive materials for electrical stimulation and transmission in the scaffolds. Approaches include magnetoresponsive PEG-based microgels [45], conductive PEDOT composite materials [46 48], polypyrrole hybrid polymers [49] and graphene, which basically showed the upregulation of neuronal and/or glial marker proteins. Because of the myriad of graphene-based approaches, we refer to a review article specifically covering this topic [50].

Further approaches try to improve these materials by directing the neurite formation to spatially resolve the neuronal network formation. DLW was used to fabricate proof-of-concept scaffolds with micro-channels to spatially restrict and guide neurite outgrowth [51,52]. In contrast to 2D conditions, such 3D scaffolds supported the long-term culturing of neuronal networks for up to 120 days [53] and allow to principally guide the neuronal outgrowth, again highlighting the need for micro-scaffolds of cellular scale and defined growth parameters.

Micro-scaffolds with single cell resolution for basic research

Geometrically defined micro-scaffolds offer great possibilities for basic cell biological research by filling the gap between oversimplified experimental set-ups on conventional 2D substrates and the extremely complex *in vivo* situation. The opportunity to shape the scaffolds in the desired geometry thereby opens numerous ways for customized and nano-structured 3D substrates with single cell resolution (Figure 3e).

Since the migratory behavior of cells is strongly influenced by the properties of the ECM, cell migration is a prime example that can be investigated in more detail using micro/nano-structured substrates. Classic cell migration assays like wound healing or random migration assays, investigate the cell movement on plain coverslips with basically unlimited adhesive area and no directional orientation. *In vivo*, the migratory route and modus (mesenchymal, amoeboid, lobopodial) are, however, defined by environmental cues like chemogradients but also topography-gradients or stiffness-gradients. Therefore, microfabricated substrates can be precisely customized to investigate such cues in more detail. Renkawitz and colleagues fabricated micro-engineered pillar forests and obstacles to study how leukocytes find the way of least resistance in complex 3D microenvironments [10••]. In another approach polyacrylamide-based nanopatterns of different elastic rigidities have been used to study lymphocyte migration in structurally and mechanically defined microenvironments [54]. Here, it was shown that lymphocytes more effectively navigate through 3D microenvironments, when Rho pathway-dependent cortical contractility was increased. Similar platforms were used to investigate the contact guidance and mechanosensing of cancer cells [55]. Bioengineered scaffolds have also proven as a valuable tool to test the migration and invasiveness of cancer cells [56,57]. Tumorigenic tissue is often stiffer than healthy tissue and promotes cell migration and invasion into stromal tissue. Therefore testing the invasiveness/aggressiveness of cancer cell lines or patient-derived tumour cells in a standardized experimental set-up, for example, by tuning the mechanical properties of stent-like or cage-like micro-scaffolds [58], is a promising approach.

Besides migration and invasion studies, 3D micro-scaffolds can be customized towards various applications. Recently, rectangular 3D micro-scaffolds were used to improve the maturation of iPSC-derived cardiomyocytes. Directing the cellular shape of single cardiomyocytes towards the desired shape lead to improved structural maturation and faster Ca^{2+} transient kinetics [59]. In another approach, composite micro-scaffolds were combined with a stimuli-responsive hydrogel to mechanically stretch single cells in a defined 3D environment and their intracellular force response was traced at subcellular resolution by investigating actin cytoskeletal rearrangements under external stress (Figure 3g) [12•]. These approaches show the great versatility of nanofabricated scaffolds for basic cell biological studies.

Conclusion

Despite the fact that significant improvements have been achieved in the last years, the objective to create an *in vitro* environment, accurately mimicking the structure and functions of ECM is still far from being reached. Indeed, most of the reviewed approaches just try to shed light on the influence of one physical-chemical-mechanical characteristic of the fabricated micro-scaffolds on one cell behavior (e.g. differentiation or proliferation). However, since the natural cell environment is extremely complex and many of factors play a role in homeostasis, more complex models for cell investigation are required. In this direction, stimuli-responsive materials represent a significant advancement, as they allow for studying cell dynamics in controlled conditions, for example, vessel formation under dynamic flow or bone mineralization under cyclic stretch/compression. However, micro-scaffolds should be further improved, for example, to host more than one cell line at the same time, in order to better mimic the complexity of the ECM. In addition, more in-depth evaluations, will help to decipher the biomolecular pathways that trigger the cellular responses upon changing environmental cues.

Conflict of interest statement

Nothing declared.

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Declaration of Competing Interest

The authors report no declarations of interest.

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