Review

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Novel advances in the design of three-dimensional bio-scaffolds to control cell fate: translation from 2D to 3D

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Recreating the most critical aspects of the native extracellular matrix (ECM) is fundamental to understand and control the processes regulating cell fate and cell function. From the ill-defined complexity to the controlled simplicity, we discuss the different strategies that are being carried out by scientists worldwide to achieve the latest advances in the sophistication of three-dimensional (3D) scaffolds, stressing their impact on cell biology, tissue engineering and regenerative medicine. Synthetic and naturally derived polymers like polyethylene glycol, alginate, agarose, etc., together with micro- and nanofabrication techniques are allowing the creation of 3D models where biophysical and biochemical variables can be modified with high precision, orthogonality and even in real-time.

Cellular scaffolds: more than a platform

3D biosystems are evolving rapidly. Their continuous sophistication is accelerating our nascent understanding of cellular microenvironment and how the basic building blocks of biological systems are integrated into the dynamic landscape of tissue physiology. Traditionally, cellular scaffolds, from the typical 2D polystyrene surfaces to the first 3D constructs (natural or artificial), were intended as inert platforms that merely served as support for the cultured cells. Since then, more emphasis was given to provide these matrices with suitable physical (e.g. stiffness and mass transfer) and chemical (e.g. employed material type and degradation rate) properties for tissue engineering and cell transplantation [1]. More recently, the biology of the scaffolds is gaining the attention of scientists, including signals that cells receive via adhesion to the material or directly from soluble factors in the microenvironment [2,3].

It is demonstrated that cells are able to sense and interpret the information coming from the ECM responding and reorganizing in function of topography [4,5], mechanical properties (e.g. stiffness, viscosity and elasticity) [6–8], molecules presented by the ECM [9] and concentration gradients of both soluble and tethered growth factors [10]. Thus, cells receive and process a multiple combination of physicochemical and biological cues always within a spatiotemporal context and in three main ways: cell–cell contacts [11], cell–ECM interactions and cell-soluble/tethered factor interactions [12].

Interestingly, the inspiration that guides the design of new biomaterial approaches is always drawn from the observation on various length scales of the materials arranged naturally by the cells in the tissues [13]. Thus, gaining insight into so far unknown questions motivates the design of new models that allow for investigating more thoroughly the cell–ECM interaction and its effects in a feedback manner.

Glossary

Anoikis: cell apoptosis induced by lack of correct cell–ECM attachment.
Carbodiimide chemistry: compounds containing the carbodiimide functionality (RN=C=NR) are usually used to activate carboxylic acids towards amide or ester formation. Additives, such as N-hydroxybenzotriazole or N-hydroxysuccinimide, are often added to increase yields and decrease side reactions. Thus, it is a common way to attach peptides to the hydrocarbonated backbone of certain biomaterials.
'Click' reactions: simple orthogonal reactions starting from small molecular groups that do not yield side products and that give heteroatom-linked molecular systems with high efficiency under a variety of mild conditions.
Focused laser light-guided gel patterning (single- or multiphoton): patterning technique with micrometric resolution that enables full 3D control over the whole volume of the hydrogel. A laser beam (either single- or multiphoton) is used along with a confocal microscope to direct photoreactions at well defined points within the hydrogel matrix.
Ligand island spacing: distance between adhesion ligand clusters within patterned hydrogels.
Michael-type addition: 1,4-addition of a doubly stabilized carbon nucleophile to an α,β-unsaturated carbonyl compound. This reaction is one of the most employed methods for the mild formation of C–C bonds. It belongs to the larger class of conjugate additions.
Photolithographic patterning: masked light is directly focused onto the hydrogel, allowing photoreactions in precise regions within the hydrogel. These regions are defined by 2D patterns included in the photomask.
RGD: specific adhesion ligand epitope formed by arginine, glycine and aspartic acid, which is originally found in fibronectin, vitronectin, fibrinogen and osteopontin among other natural ECM proteins. It is used to promote integrin mediated cell–ECM attachment.
Thiol-ene chemistry: the thiol-ene reaction is an organic reaction between a thiol (–SH) and an alkene (–C=C) forming a thioether. One of its main advantages is that reactions can be readily induced by a photoinitiator. This reaction is considered to be cytocompatible, bio-orthogonal and is included in the group of 'click' reactions.
YIGSR: found in the laminin of natural ECMs and represents an integrin-mediated binding domain for cells. It is composed of tyrosine-isoleucine-glycine-serine-arginine.

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Biomaterial strategies are bridging the gap in many scientific fields because they have become a necessary tool in tissue engineering or regenerative medicine, among others. In fact, microfabrication, and more recently nanofabrication [14], are allowing the creation of suitable models where key factors may be studied from the nanometer to the supramillimeter length scale [15,16]. Moreover, the ability of the new bioinspired materials to be tuned in a wide range of biophysical and biochemical features is also optimizing the way scaffolds control the different biological properties of the cells.

Here, we present the latest advances in the sophistication of 3D scaffolds and its impact on cell biology, tissue engineering and regenerative medicine.

Translation from 2D to 3D

Recent findings suggest that cells often show a non-natural behavior when they are moved away from their natural niches and seeded onto flat substrates. Only to mention some examples, breast epithelial cells exhibited a tumoral trend when assayed in 2D (probably due to the unnatural extreme stiffness provided by the plate), but regressed to normal state upon transferal to 3D models resembling their natural niche [17]. In the same way, increased chondrogenesis has been noticed in embryonic stem cells (ESCs) cultured as 3D embryoid bodies when compared to the monolayer conformation [18]. Therefore, although 2D experiments represent a versatile and accurate way to screen the effects of isolated compounds of the ECM on cells (Box 1), 3D experiments are designed to direct a progressive and steady reconstruction of the complexity that entails the native ECM.

The disparities in cellular function described between 2D and 3D approaches are mainly given by the manner in which cells perceive their surrounding microenvironment. Cells plated onto 2D substrates are polarized, maintaining only part of their surface anchored and exposing remaining parts to the culture media. Moreover, the contact with neighboring cells is also limited to the flat edges that they share. This is in sharp contrast with the natural environment of the tissues, where each cell closely interacts with the nearby cells and the ECM [19]. Hence, 3D environment-based interplay reflects a more distributed integrin usage and enhanced biological activity [20]. Mass transport physics is also absolutely altered. Growth factors, morphogens, cytokines and so forth quickly diffuse in the media of 2D cultures, reaching cells uniformly, whereas native ECM produces chemical and biological diffusion gradients that play a key role in signaling and tissue development [21]. In addition, cell shape also has its influence on cell commitment. Once again, cells on 2D cultures are limited to a planar and spread morphology and do not experience the more complex morphologies found *in vivo* [18]. Furthermore, 2D surfaces offer almost undetectable resistance to cell migration, which contrasts notably with the mechanical interactions provided *in vivo*.

Therefore, the design of 3D models that resemble more or less accurately the native ECM is crucial in order to obtain reliable results that approximate to reality. Nonetheless, mimicking the ECM in the laboratory is not simple, especially because there is much we do not know yet about the cell–ECM crosstalk that occurs *in vivo*. As a consequence, the most frequently used models so far have been hydrogel scaffolds formed by animal ECM-derived proteins, Matrigel® or Vitrogen®, among others [22]. Even if these biosystems have provided seminal understanding for the cell biology field in the past few decades, they are far from being ideal. Limitations include: (i) reduced flexibility

Box 1. 2D approaches: overview

2D cell culture approaches have given rise to important advances, many of which have been pivotal in the understanding of cell–ECM interaction [66,75]. Deconstructing 3D complexity into 2D simple models is a smart way to perform univariable experiments to parse out the effects of isolated factors (e.g. matrix elasticity or adhesion ligand density) – either natural or synthetic – in cells [51]. Microarray technology and combinatorial and high-throughput screening (CTHS) approaches are powerful tools to make infinite combinations of structural, biophysical and biochemical parameters and thus elucidate some of the mechanisms that dictate cell biology (Figure I) [76,77]. Other advantages of 2D models include the facility to exert a precise control over chemical and topographical properties even at nanometer scale, the overall straightforward processing and the possibility to harvest the cells effortlessly.

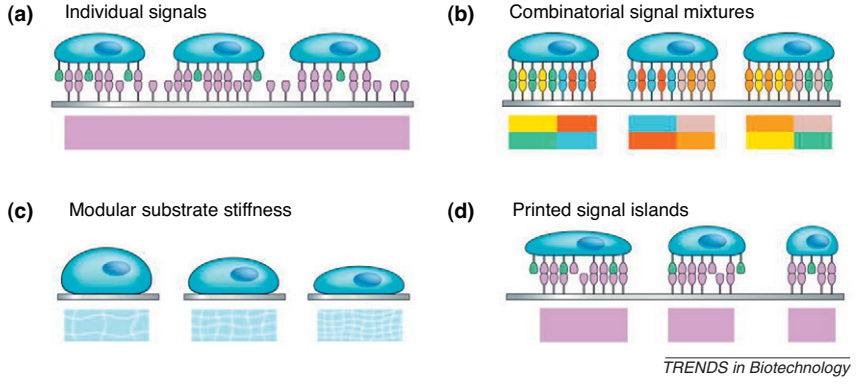

Figure I. Schematic summary of 2D microarrays systems to test the impact of multiple biophysical and biochemical variables on cell fate. Each panel is subdivided in two parts. The top part (side view) reflects cells seeded on uniquely engineered substrates (gray). Below, color blocks represent the different signals that are presented (view from above). **(a)** Substrate displaying individual molecular signals. **(b)** Different signal mixtures can also be displayed to explore their combinatorial effects. **(c)** Substrate stiffness is varied, for example, to study the influence of mechanical properties on cell fate. **(d)** Adhesion ligand peptides have been printed forming spots of different sizes to control cell shape and, consequently, cell fate. Reprinted and adapted, with permission, from [51].

to modulate their biophysical and biochemical properties (and furthermore, to control such variables independently), (ii) immunogenicity, (iii) batch-to-batch variability, and (iv) ill-defined complexity that provides too little mechanistic information [12,22].

Assuming some of these limitations, hydrogels formed by synthetic polymers like polyethylene glycol (PEG), and naturally derived polymers including alginate, agarose, chitosan, etc. have become the biomaterial of choice for artificial ECM reconstruction. Hydrogels are able to resemble the nature of most tissues due to their high water content and the presence of pores that facilitate the free diffusion of oxygen, nutrients and growth factors, morphogens, etc. [18,23]. Most importantly, many hydrogels offer the possibility to encapsulate cells under gentle and cytocompatible conditions, and furthermore, their physicochemical properties can be easily tuned [24]. Nonfouling polymers like PEG present the additional advantage of minimizing uncontrolled variables such as protein adsorption and their indirect effects, which makes PEG one of the most attractive biomaterials for 3D scaffold designs. By contrast, one of the reasons alginate has been so widely employed so far is that it can be further injected *in vivo* in a minimally invasive way, undergoing gelation *in situ*.

No single model can recapitulate the whole complexity of every tissue type ECM. Many authors agree on the fact that high levels of complexity are not necessary for many applications, and indeed simpler and practical models are enough to solve some specific questions [12,16,25]. In fact, cells enclosed within 3D matrices rapidly remodel their microenvironment depositing their own ECM molecules [22,26]. For that reason, it is possible to compensate the lack of such complexity with artificial systems capable of inducing desired effects to the hosted cells in a more efficient and rational way [27]. For example, presenting cells with tethered small-molecule chemical functional groups on the matrix backbone was enough to recreate unique chemical environments and induce multiple mesenchymal stem cell (MSC)-differentiated lineages [28]. Nonetheless, if the goal is tissue-like structures for regenerative medicine, for example, higher complexity levels in time and space are absolutely justified [22].

Sophistication of 3D biosystems

Adhesion ligand presentation

Cells may be provided with adhesion surfaces by using a variety of naturally derived ECM molecules such as collagen or fibrin, or using these molecules to decorate synthetic polymers to which adhesion is regulated by adsorbed proteins (Box 2). However, protein engineering allows us to isolate functional domains within large ECM molecules and incorporate them into otherwise inert substrates. Thus, epitopes that mediate cell adhesion can be mimicked using synthetic peptides. Among them, perhaps the most known are arginine-glycine-aspartic acid (RGD) (see Glossary), derived from fibronectin, and tyrosine-isoleucine-glycine-serine-arginine (YIGSR), derived from laminin. PEG hydrogels can also be modified by novel polymerization mechanisms such as thiol-ene [29] and thiol-acrylate chemistries [30], whereas other polymers like alginate are usually modified by means of carbodiimide chemistry [31].

Not only the adhesion moieties themselves but also their density and spatial distribution on micrometer and nanometer scales influence cell fate [32]. By manipulating the way adhesion moieties are presented to the cells, it is possible to induce major cellular processes such as migration, proliferation and differentiation [33]. With this idea, nanoscale patterns of RGD islands in hydrogels have been varied without altering the final ligand density. For instance, hydrogels with reduced island spacing were produced by uniformly distributing alginate chains containing a single ligand, whereas more increased island spacing was achieved by mixing unmodified chains and chains coupled with multiple peptides (Figure 1a). Thus, more closely

Box 2. Cell–ECM interaction through focal adhesions (FAs) and mechanosensing

FAs

One of the most relevant ways to establish cell–ECM interaction is given through integrin-mediated adhesions, which cells use to connect cell cytoskeleton to adhesion molecules, such as fibronectin or laminin, located on the fibers [78]. This phenomenon is known as focal adhesion (FA), which constitute specific types of large macromolecular assemblies through which both mechanical force and regulatory signals are transmitted. FAs serve to guide the cell through the ECM; these linkages induce the arrangement and polarization of cell cytoskeleton. Furthermore, FA is absolutely necessary to prevent anoikis in anchorage-dependent cells [33].

Mechanosensing

Mechanical properties of biomaterials can also influence cell commitment and lineage differentiation [79]. Mechanosensing is an active cellular process that entails a dynamic and reciprocal interaction between the ECM and the motor proteins that are connected to the cytoskeleton [13]. Cells do not only exert forces, but also respond to the resistance sensed through cytoskeleton organization/tension. These external forces trigger a series of intracellular signaling pathways that activate or inhibit gene expression [80]. In this context, elastic substrates with variable matrix rigidities can be used to study the traction forces exerted by cells and to establish correlations with triggered effects (Figure I).

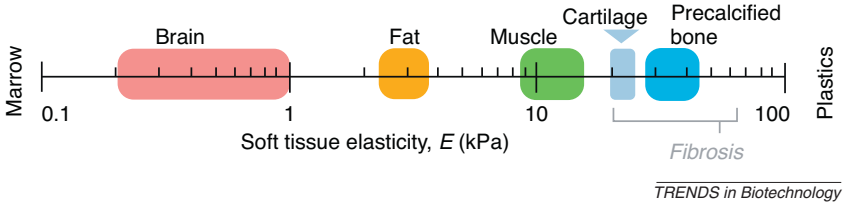

Figure I. The influence of matrix mechanical properties on cell fate behavior. Scheme illustrating the elastic modulus scale of different tissues ranging from the softest (brain) to the stiffest (bone). Reproduced, with permission, from [66].

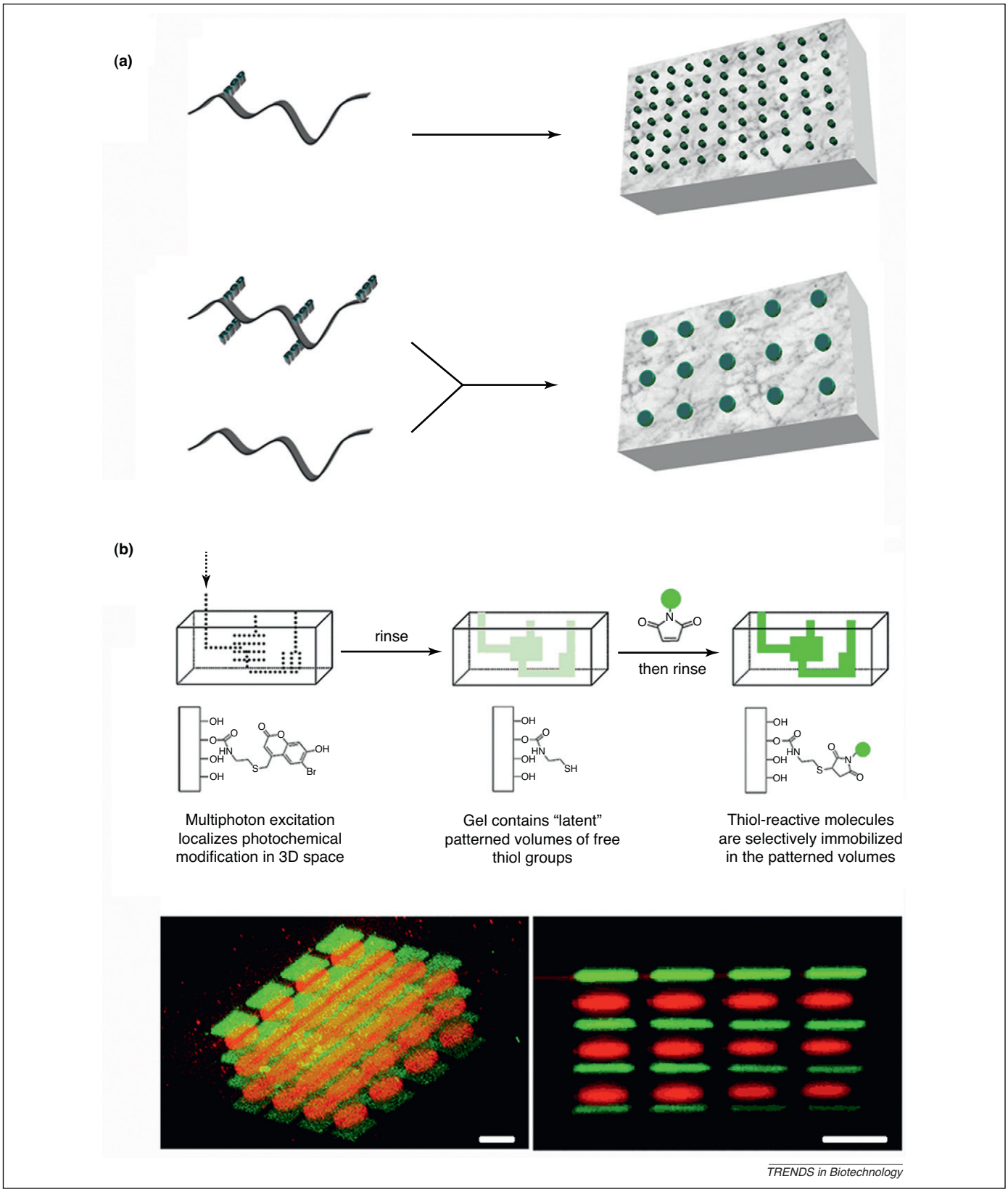

Figure 1. Adhesion ligand patterning. **(a)** Commonly used strategy to achieve well-characterized nanoscale patterns of arginine-glycine-aspartic acid (RGD) islands. Polymer chains modified with single adhesion moieties give rise to reduced island spacing (green dots). A blend of unmodified polymer chains and polymer chains with multiple adhesion ligands results in increased island spacing. **(b)** Schematized multiphoton chemical patterning in hydrogels and resulting oblique and side views of fluorescence images taken from 3D patterned squares and circle arrays (50 μm diameter). Agarose is covalently modified with a derivative of cysteine protected with a photocleavable group. The protection groups are removed in a patterned way by exposure with a laser beam. Thus, after rinsing, free thiol groups are left in the irradiated areas. Finally, the hydrogel is rinsed in a solution with desired oligopeptides (green fluorophore in this case), which are covalently immobilized via Michael-type addition (reacting with free thiol groups). As shown in fluorescence images, different biomolecules (represented with red and green fluorophores) can be patterned in desired spaces. Scale bars represent 50 μm in both fluorescence micrographs. Reprinted and adapted, with permission, from ref [35].

spaced islands favored cell spreading, whereas more widely spaced islands supported differentiation [34].

Beyond these approaches, much attention has been lately paid to the patterning of adhesive moieties. Biomolecules can also be immobilized in micropatterned volumes within agarose gels with a multiphoton laser [35]. When agarose or hyaluronic acid (HA) is covalently modified with a derivative of cysteine protected with a photocleavable group, the protection groups can be removed upon exposure with a laser beam (micrometric resolution). As a result, desired oligopeptides are covalently immobilized in patterned sites via Michael-type addition (Figure 1b). Using this procedure and by means of orthogonal physical binding pairs (barnasa–barstar and streptavidin–biotin), simultaneous patterning of multiple growth factors has been achieved to direct neural precursor cell differentiation [36]. Two-photon laser scanning (TPLS) photolithography in PEG diacrylate (PEGDA) hydrogels, can also be used to guide encapsulated dermal fibroblasts with precisely patterned RGD moieties [37].

Mechanical properties

The most common way to control scaffold stiffness is by using polymers and crosslinkers at different concentrations or varying the molecular weight of the polymers. In this way, hydrogels formed by macromers of PEG and poly(lactic acid) (PLA), modifying the initial macromer concentration from 10% to 20% result in gels with elastic moduli increased from 60 to 500 kPa. The latter is used to restore initial function in chondrocytes and facilitate the production of cartilaginous production [38]. The compressive modulus of hydrogels comprising HA–tyramine conjugates can be controlled by varying the concentration of hydrogen peroxidase (H_2O_2) employed to produce the covalent crosslinkage. Thus, lower crosslinked matrices enhance chondrogenesis in encapsulated MSCs. Moreover increasing crosslinking degree, and thereby matrix stiffness, MSCs differentiate towards fibrous phenotypes [39].

A blend of high MW and low MW alginates gives rise to highly crosslinked hydrogels; the mixture has a pre-gelled viscosity similar to that of pure high MW at low concentrations. Hence, rheological and mechanical properties can be decoupled: scaffolds can have a high elastic modulus but cells are not sheared during encapsulation [31]. Employing this procedure it has been demonstrated how MSCs are able to reorganize the adhesion ligands on the nanoscale as a function of the stiffness offered by alginate matrix where they were encapsulated. This adhesion ligand reorganization process may play an important role in MCS commitment [6].

Chemical signaling

The regulation of soluble molecule distribution within 3D scaffolds is difficult because the availability of biomolecules depends on (i) the total concentration in the medium, (ii) diffusion rate within the gel, and (iii) cellular metabolic activity [18]. In addition, artificial ECMs may also require the presence of growth factors and morphogens in a pharmacokinetic manner that resembles the natural cell niche. For example, during angiogenesis vascular endothelial growth factor (VEGF) promotes the proliferation of endothelial cells at the first steps of the process, whereas platelet derived growth factor (PDGF) regulates the maturation of these new created vessels *a posteriori*. Therefore, similar pharmacokinetics must be reproduced to succeed in the attempt to induce angiogenesis (Figure 2a). In this sense, different approaches have been carried out in the attempt to regulate the kinetics and distribution of soluble factors. In an attempt to mimic the native ECM, where glycosaminoglycans act as depots for growth factors, heparin was incorporated into the scaffold backbone for posterior sequestering and controlled release of growth factors [40]. Similarly, HA hydrogels have been designed for different degradation

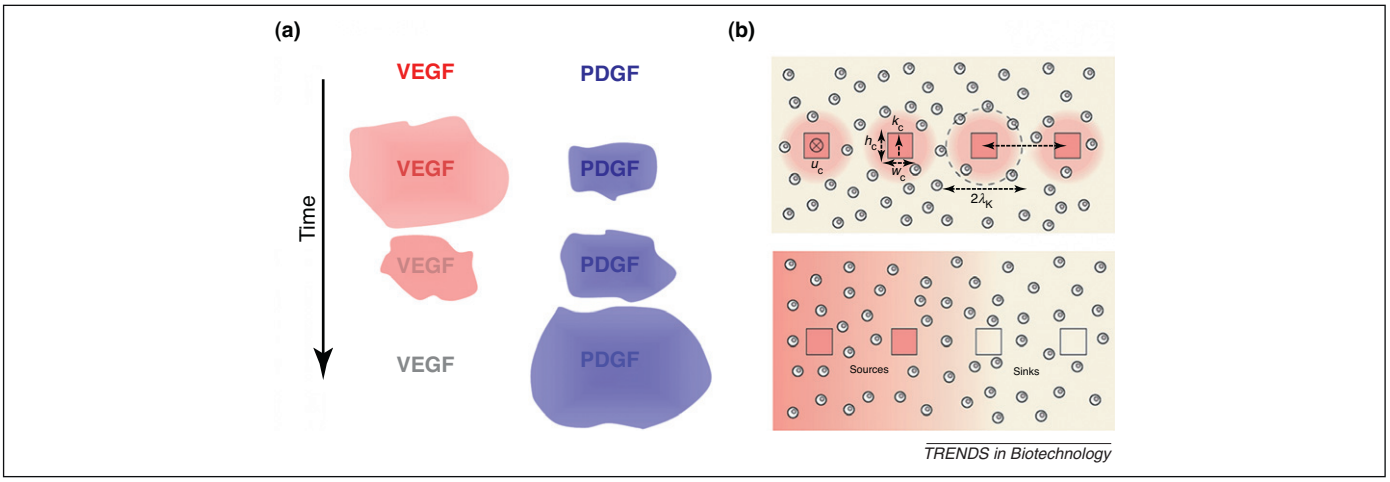

Figure 2. Spatiotemporal control of growth factor delivery. **(a)** Scheme illustrating a dual delivery of growth factors as a way to regulate the kinetics. Delivery systems are represented with capital letters indicating the growth factor encapsulated within their matrices. Vascular endothelial growth factor (VEGF; in red) and platelet derived growth factor (PDGF; in blue) contents are gradually released with different kinetics. The size of the spots represents the amount of delivered growth factor at each time point. Thus, there is a prompt VEGF burst, whereas PDGF burst is delayed in time. **(b)** Cross-sectional schematic depiction of cellular microfluidic scaffolds showing different manners to induce gradients of soluble factors. Encapsulated cells are shown as double circles. Microchannels are depicted as squares. The pink shading represents the gradients of soluble molecules at steady state. (Top) Solutes are delivered from the microchannels and are subsequently consumed by encapsulated cells as they diffuse into the matrix. (Bottom) Solutes are delivered via the channels on the left (sources) and removed by the channels on the right (sinks). λ_k (cm) = concentration variation over particular distance (Krogh length); λ_c (cm) = interchannel distance; w_c (cm) = microchannel width; h_c (cm) = microchannel height; K_c (cm s^{-1}) = mass transfer coefficient of the flow in the microchannels; U_c (cm s^{-1}) = flow speed in the microchannels. Reprinted and adapted, with permission, from [48].

rates and provide controlled release of cationic proteins such as bone morphogenetic protein-2 (BMP-2) and VEGF anchored to the matrix via electrostatic interactions [41]. Other approaches propose covalent linkage of specific ligands from the desired molecules to the scaffold (also known as phage display) [42]. By including multiple soluble factors within different encapsulation levels [e.g. polylactide-co-glycolide (PLG) spheres within alginate hydrogels], it is possible to sustain a simultaneous or sequential factor delivery. The significance of exerting control over growth factor availability in time and space has been probed, for instance, in stem cell differentiation or functional repair of segmental bone defects [16,43,44] or therapeutic approaches to induce angiogenesis [45].

Soluble biomolecules often show improved bioactivity when they directly attached to the hydrogel network [46]. In addition to improved stability, covalently immobilized growth factors can be used to spatially direct cell behavior (e.g. chemotaxis or differentiation) [47]. However, it is important to ensure that active domains of the molecules are accessible upon covalent linkage.

By contrast, cells are exposed to gradients of morphogens, growth factors and cytokines progressively in physiological tissue. They play a key role not only in morphogenesis, chemotaxis and axogenesis [48], but also during processes like wound healing or tissue homeostasis. Such gradients can be introduced into 3D models, for instance, using the same micropatterning techniques described above to attach ligand moieties [49]. In this way, endothelial cell (EC) tubule-like formation was guided through VEGF gradients patterned within RGD-modified agarose hydrogels [50].

Microfluidics-based systems are also increasingly being used to generate gradients within 3D models [15,51]. These platforms represent one of the most accurate and robust ways to reproduce morphogen gradients *in vivo* because they allow small amounts of expensive factors to be patterned into scaffolds with tight control [47]. Some approaches have already been carried out. For example, microfluidic channels have been embedded directly in cell-enclosing alginate scaffolds; the channels control the distribution and flux of solutes in the total volume by means of convective mass transfer (Figure 2b) [48]. Moreover, since biomolecules can also be tethered to the backbone of artificial ECMs, applying microfluidics technology with anchored proteins would give rise to more comprehensive and realistic ECM surrogates [51].

Mass transport and matrix permissiveness

One of the prime points that concerns scientists with 3D culture is the fact that cells may suffer the lack of gases and nutrients. This is especially evident *in vivo*, where encapsulated and transplanted cells rely on diffusion for oxygen and nutrients from surrounding blood vessels. The maximum thickness for these 3D biosystems has been reported to be limited to approximately 100–200 μm [52]. Moreover, 3D culture designs present physical constrains that hamper cell proliferation, migration and morphogenesis. In general, pore sizes of less than 1 μm are able to support free diffusion of molecules, but not cellular migration, whereas pores in the range of approximately 10–100 μm readily allow host cells to migrate through the entire volume of the scaffold [53]. Most chemically crosslinked polymer hydrogels form mesh-like structures with pores on the order of tens of nanometers, which means that they are small enough even to prevent cellular events such as the formation of filopodia [22]. Thus, cells remain literally trapped within their microvoids, showing round morphology. Nonetheless, using smart engineering tricks, researchers have managed to improve mass transport conditions of their scaffolds and increase the functionality of the enclosed cells.

By assembling a PEG hydrogel in the presence of crystal colloidal templates that could be further removed by solvent extraction ('leaching') scaffolds with a pore range of 20–60 μm were formed [54]. Another alternative approach is the use of CO_2 as porogen in the production of PEG scaffolds with interconnected pores ranging in size from 100 to 600 μm, which were used to promote osteogenesis in MSCs [55]. Similarly, two-photon lasers can be used to direct the patterned polymerization of multifunctional acrylate monomers. This technique uniformly produces pores of 12–110 μm, which can be used to study cell migration on the basis of pore size [56]. A more recent study showed that permeability can be readily improved in PEG hydrogels incorporating hydrophobic nanoparticles that induced partially looser crosslinking density. By these means, viability and functionality of encapsulated cells was improved without altering scaffold mechanical properties [57].

Matrix permissiveness also drastically influences tissue morphogenesis. Matrix degradation is fundamental, so that encapsulated cells can remodel the scaffold by secreting their own ECM molecules. In addition, degradation allows cell migration and regulates the release of matrix-tethered biomolecules that induce different cellular functions [58]. Apart from the scaffolds formed by ECM derived molecules, which present inherent degradability, it is possible to design inert matrices that can be degraded according to different strategies. For example, synthetic hydrogels can be designed to include degradable polymers within their network. Some studies describe the use of PLA [59] or polycaprolactone [60] blocks in combination with PEG backbone. Similarly, the scaffolds can be built by co-polymerization of different ratios of degradable and non-degradable macromers [61]. For all of these types of designs, the degradation rate is governed by the number of hydrolytically labile bonds in the hydrogel, although in general, normal cellular processes take place in less time than that required for these labile bonds to be degraded [18].

In alginate, a well known strategy to control the degradation rate of the scaffolds is partial oxidation of the main chains to create controllable numbers of functional groups in the backbone susceptible to hydrolysis [62]. Hydrolytically labile hydrogels have predictable degradation profiles, but the properties cannot be altered after gelling and the degradation rate is both uniform and independent of cellular interactions. In order to permit cellularly driven matrix degradation, synthetic hydrogels, such as those formed by PEG acrylate, can be modified by Michael addition and photoinitiated reactions to include specific sequences that are recognized and cleaved by proteases

like matrix metalloproteinases (MMPs) secreted by cells [18]. This design enables cells to locally remodel their surrounding matrix and deposit their own ECM proteins on the matrix, mimicking more realistically what occurs *in vivo* during wound healing, regeneration or tumor metastasis [22].

Nanofabrication

A typical cell size (≈7–15 μm) is similar to or smaller than the hydrogel microstructures described so far. Thus, some authors argue that the range of microporosities (≈10–100 μm) will effectively act as 2D surfaces with curvature for cell attachment [18,19]. One possibility to address this problem is the fabrication of nanofibrillar architectures. In fact, the necessity to understand in detail the nature of the native ECM has fueled new paths towards the fabrication of biomimetic scaffolds with nanoscale properties. Starting with the natural fibrous mesh of the ECM, it is possible to construct novel scaffolds with interconnected and porous structures formed by interwoven fibers with similar diameters to those presented by collagen fibers [14]. Thus, the forces exerted by cells in the scaffold influence further material structural reorganization [19]. In this sense, electrospinning [63] and molecular self-assembly [64] (Box 3) are increasingly growing nanofabrication techniques used to create 3D scaffolds of interwoven fibers that resemble collagen structures of the native ECM [14].

External control of spatiotemporal signal presentation

The native ECM is highly dynamic. Therefore, the temporal and spatial variability typical of ECM properties must also be introduced into 3D models in order to simulate contextually meaningful and realistic microenvironments. Local modifications of the environment at certain times can force a few cells to adopt decisions and develop new functionalities, which may give rise to the start of a hierarchical reorganization at the multicellular scale, reproducing those processes that take place in nature (Figure 3a,b) [65]. For example, it has been reported that cells tend to invade stiffer areas guided by a process known as 'durotaxis' [66–68]. A noticeable increase in the elastic modulus of fibrotic tissues formed as a result of processes like acute myocardial infarction has been observed [66]. To shed light on whether 'durotaxis' is the mechanism promoting MSC homing to these injured zones, an ideal 3D *in vitro* model would allow certain *in situ* manipulation to recreate such a physiological situation. Therefore, the creation of models that can be externally manipulated in time and space results are advantageous to study cell–ECM dynamic interplay.

With this aim in mind, a photodegradable PEG-based hydrogel model has been developed that has predictable degradation rate patterns and stiffness gradients in real-time under cytocompatible conditions (long-wavelength UV light). The gel can be further manipulated at the micrometer-scale resolution with light-guided gel patterning [69]. As a result, cell behavior can be conditioned *in situ* within a 3D environment, for example, by creating elastic modulus microgradients with well defined structures at desired times [65]. This technology can also be employed to dynamically alter other biophysical and biochemical properties. For instance, chondrocytes show an enhanced differentiation in a scaffold with photolabile RGD moieties when the moieties are removed at certain time points during 3D cell culturing [70].

'Click' reactions can also be used to attach varying concentrations of biomolecules (adhesion ligands in this

Box 3. Electrospinning and self-assembly: general concepts

Electrospinning

Elecrospinning is a technique in which different polymer fibers (natural and synthetic) are deposited on a defined substrate by means of an electric field [81]. The resulting scaffolds present continuous fibers with high porosity. The nanofibers can be orientated to recreate more or less arranged tissues [14]. Moreover, the structure can be designed to incorporate delivery systems for controlled release of cytokines, growth factors and drugs among others [82–84]. One important limitation of electrospinning is the harshness of the fabrication process: cells cannot be encapsulated *in situ* [19], the resulting scaffolds are weak, and the fiber diameters only emulate the thickest ranges found in the native ECM (50–500 nm) [14].

Molecular self-assembly

Molecular self-assembly is based on the spontaneous arrangement of individual building blocks into ordered and stable architectures by means of non-covalent bonds [14]. For example, one of the most broadly described nanofibers is formed by the amphiphile peptide (Figure I) [85]. These nanofibrillar matrices are very close in architecture to those composed of collagen in the native ECM. They have 10 nm oscillating fiber diameters, pores ranging from 5 to 200 nm, and high water content (>99.5%) [64]; the ability to retain water is fundamental to mimic the features of a real ECM, where water represents the highest percentage of the total weight. The amphiphile peptides can form hydrogels at near-physiological conditions, and in many cases the fiber morphology can also be controlled [86,87]. Furthermore, they can be designed to be sensitive to (easily degraded by) the actions of proteases and include adhesion moieties in their backbone structure to support cell migration or induce lineage differentiation [88]. Scaffolds presenting the laminin epitope IKVAV can prompt neural progenitor cells to differentiate into neurons [89]. Remarkably, some of these nanofibrillar constructs, such as PuraMatrix™, are now commercial products intended to be used in the fields of cell biology or tissue engineering [22]. Unfortunately, the nature of the crosslinkages (noncovalent bonds such as hydrogen bonds, electrostatic interactions, hydrophobic interactions, van der Waals interactions, etc.) does not offer flexibility in tuning the mechanical properties of the scaffolds or, at least, they have not been described yet [90].

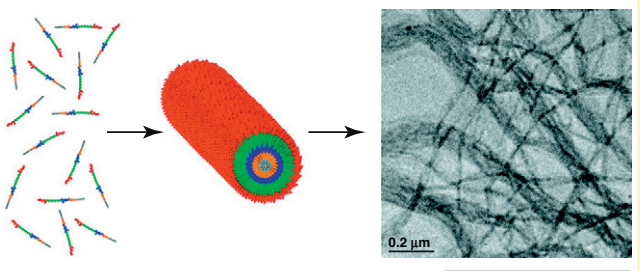

Figure I. Schematic representation of peptide amphiphile and cryo-transmission electron microscopy (TEM) images of resulting nanofibers. Reprinted, with permission, from [74].

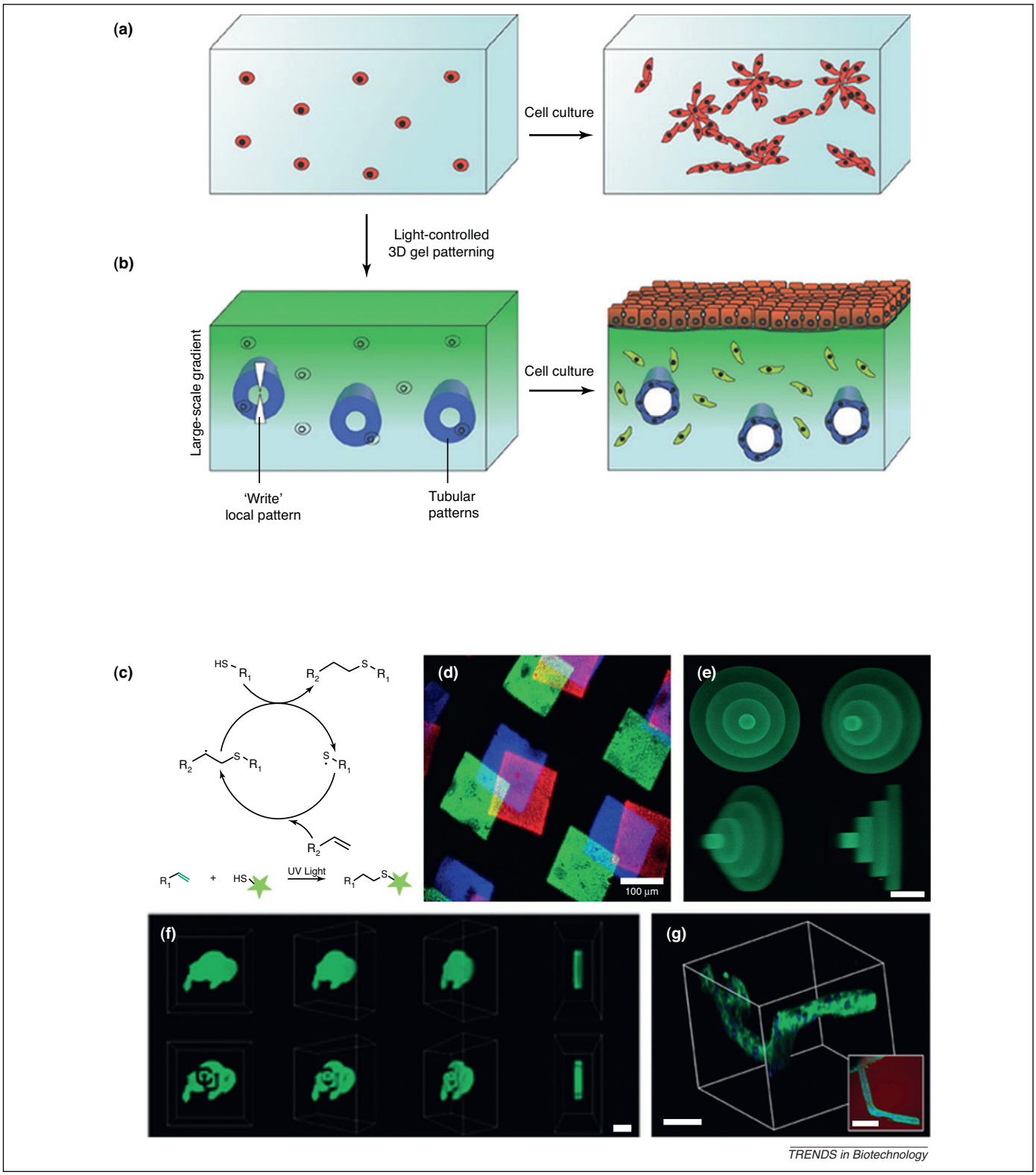

Figure 3. Creating spatiotemporal signals by *in situ* modification of matrix properties. **(a)** Cells within homogeneous hydrogels give rise to disorganized cellular structures with no functionality. **(b)** Light-mediated *in situ* patterned hydrogels may possibly prompt well-defined structures and, ideally, tissue-like cell function. Photodegradable hydrogels may be obtained introducing polymer chains that act as photolabile crosslinkers. Upon irradiation, the photolabile groups are cleaved and hydrogel crosslinking density is decreased. Thus, large-scale stiffness gradients may be created, for example, by flood irradiation with a highly absorbed wavelength, which gradually penetrates into the hydrogel creating continuous and linear degradation gradients in the z axis of the hydrogel. In addition, local patterns of degraded areas or 3D structures within the hydrogel may be formed using focused light directed by a single photon confocal laser scanner microscope. **(c)** Thiol-ene reaction employed to create *in situ* patterning of hydrogels. **(d)** The thiol-ene reaction mediated incorporation of biochemical cues is confined to user-defined spaces of the hydrogel by photolithographic patterning technique. This process can be continuously repeated to add new biochemical cues to the scaffold, and this is represented in the figure introducing different fluorophores within the hydrogel. **(e)** Full 3D control can be obtained by using focused laser light-guided gel patterning, with which micrometric special resolution is achieved. **(f)** Orthogonality of photocoupling and photodegradation reactions. Multiphoton visible light is first used to create user-defined shapes by coupling a green fluorophore-labeled peptide within the hydrogel (top: buffalo), and then desired regions are degraded with multiphoton ultraviolet light, thus removing the fluorophore from the irradiated areas (bottom: University of Colorado and horn). **(g)** The techniques described above can be employed, for example, to directed cell outgrowth through 3D functionalized channels. The inset depicts the hydrogel viewed from above. Scale bars represent 100 μm in all pictures. (a,b) Reprinted, with permission, from [65]. (c–e) Reprinted and adapted, with permission, from [71]. (f,g) Reprinted and adapted, with permission, from [72].

case) to a scaffold backbone by means of cytocompatible photolithographic patterning (Figure 3d) and/or focused laser light-guided gel patterning (micrometer resolution) (Figure 3e) after cell encapsulation [71]. Taking into account that photoreactive groups for patterning are coupled with enzymatically degradable sequences, this approach represents a valuable strategy to build artificial ECMs *in vitro* with the possibility to modulate a wide number of variables in a spatiotemporal way. More recently, a novel light-based strategy has been developed that enables the combination of mutually exclusive technologies to date. Thus, biophysical and biochemical properties of the hydrogel can be controlled in an independent way with orthogonal photoreactions in real-time (Figure 3f,g) [72].

Concluding remarks

There is a wide range of possibilities for building 3D scaffolds. Design considerations are varied according to the intended use and pursued goal. For instance, researchers interested in the study of cell migration through given biomolecular gradients *in vitro* will possibly prefer the use of synthetic hydrogels like PEG to create their own patterns. On the contrary, those more interested in forming bone-like tissue within scaffolds *in vivo* will probably choose polymers such as alginate that can be easily injected to form hydrogels once implanted. In any case, the ideal model should offer wide possibilities to tune and modulate structural and mechanical properties such as elastic modulus, pore size or topography. Moreover, all biophysical and biochemical properties should allow independent manipulability (orthogonality) from each other. For example, increasing polymer concentration to achieve a higher elastic modulus should neither affect adhesion–ligand density nor mode of presentation.

It should be considered if simplifying is the best strategy for intended scaffold utility. Lately, for example, the type of cell attachment that is provided to promote cell–substrate interactions is under debate, because it is not clear whether this biofunctionalization is better accomplished by short peptides like the so far well known RGD or, in contrast, by full ECM proteins like fibronectin or collagen [73]. Those who root for the use of short synthetic peptides base their arguments on the fact that these short peptides are chemically well defined and, thereby, much easier to isolate and understand the effects caused by their use. Indeed, this precise composition avoids the uncertainty of possible adverse effects providing a favorable ending through regulatory pathways. Finally, the possibility to alter the ligand type, density or presentation patterns results in a more interesting model. By contrast, researchers who are against this motion state that the integrin-mediated signaling mechanisms are much more complex and cannot be completely reproduced by isolated RGD moieties. In fact, the relevance of the synergistic sites found in natural ECM proteins to regulate cell fate has also been demonstrated.

Understanding the complexity of the whole tissue physiology by deconstructing its building blocks and studying their effects in an isolated way is challenging by itself because contextual meaning is lost. Perhaps, the real challenge lays on finding a balance that would allow us to study well defined and controllable variants while taking advantage of the biological mechanisms that we do not yet understand.

It is likely in the future that 3D models will replace much of those routine procedures so far performed on 2D flat surfaces. Indeed, as the technology advances and we gain new insights into the mechanisms that regulate cell–ECM interactions, we will be able to design more sophisticated and tailormade 3D scaffolds for the study of particular tissue physiologies, always having in mind that the only true results are those validated *in vivo*.

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