Chapter 1
Recent Advances in 3D Tissue Models

A. Kivelio and M. Ehrbar

Abstract Physiologically relevant tissue models that bridge the gap between 2D tissue culture and animal trials would be highly desirable to study the function of tissues in health and disease as well as for the validation of lead compounds during drug development. The field has made impressive advances in 3D culturing cells and organoids in naturally derived materials. Novel, rationally designed, biomimetic materials have been established, which allow the almost individual variation of matrix parameters, such as stiffness, cell adhesion, degradability, or growth factor binding and controlled release. The combination of innovative materials with novel technological platforms such as printing, microfluidics, and additive or preventive manufacturing provides a great potential to build unprecedented, complex tissue models. Here we review recent advances in the design of materials building blocks which allow the formation of 3D structured microenvironments. We will mainly focus on strategies to locally position cell-instructive molecular cues and discuss needs to generate models which would allow the investigator to controllably manipulate cells in their 3D context with the aim to generate complex but yet scalable tissue models.

Keywords 3D tissue models • Cell-instructive hydrogels • Growth factors • Patterning • Spatiotemporal control

A. Kivelio, M.Sc.
Department of Obstetrics, University Hospital Zurich, PATH,
G-48 Schmelzbergstr 12, Zurich, Switzerland

Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL),
Lausanne, Switzerland

M. Ehrbar, Ph.D. (✉)
Department of Obstetrics, University Hospital Zurich, PATH,
G-48 Schmelzbergstr 12, Zurich, Switzerland

Zurich Centre for Integrative Human Physiology, Zurich, Switzerland
e-mail: martin.ehrbar@usz.ch

M. Akashi et al. (eds.), *Engineered Cell Manipulation for Biomedical Application*, Nanomedicine and Nanotoxicology,
DOI 10.1007/978-4-431-55139-3_1

1.1 Introduction

For the understanding of fundamental biological phenomena and for development of novel drugs there is a large need for highly reproducible, reliable, and physiologically relevant test platforms. Over the past decades a wealth of detailed insight into cellular and molecular details has been acquired using 2D cell culture models. This knowledge has frequently been transferred to animal models, where among other techniques, genetic tools have helped to understand individual factors in the complex systemic context. However, though in vitro and in vivo experiments have been a very successful combination for many decades, both of them have clearly indefinable limitations. 2D tissue culture models provide limited information regarding physiologically relevant (1) tissue morphogenesis, (2) chemotaxis and haptotaxis, (3) cell morphology, (4) matrix remodeling, and (5) effects of matrix-mediated signaling.

In contrast, in vivo animal models are limited by (1) throughput, (2) systemic effects and compensation phenomenon, (3) differences in physiology between species, and (4) ethical concerns.

In order to study cells in a physiologically relevant environment, conditions nearly identical to the ones of the native tissue with respect to composition and 3D arrangement would have to be established in vitro. Cells in tissues are embedded in a microenvironment consisting of neighboring cells, extracellular matrix (ECM) components, and signaling molecules. Of course the cellular response is the result of an integration of these various signals. By dynamic changes of the microenvironmental composition, the cellular response can substantially adapt during both development and healing. To recapitulate developmental, physiological, or even pathological situations, ideally cells, matrix components, and signaling cues could be arranged in a rational and three-dimensionally controlled manner, such that single parameters can be individually varied.

In this chapter we will commence with a short discussion of 3D cell culture models which are based on matrix-free approaches. Since such assays are performed at high cellular density and rely on cell's own matrix production, the additional encapsulation of cells in a provisional matrix clearly offers the opportunity to provide and vary matrix signals. Thus, a summary of achievements using biologically derived materials to generate in vitro tissue models will be given next. Although with scaffold-based approaches impressive advances have been achieved, we will here focus our discussion mainly on hydrogel systems. For the establishment of fully defined, engineered tissue models the precise control over all components and their exact positioning in 3D would be desirable. Great advances in the engineering of naturally occurring and biomimetic extracellular hydrogel matrices towards the control of biological functions have been made. We will describe their fundamental design principles of currently available hydrogel platforms. Having done so, the achievements in design of functional building blocks and their spatial and temporal arrangement and release will be discussed. Finally, examples of sophisticated 3D tissue models which will be the basis for the development of in vitro tissue homologues will be given.

1.1.1 2D Cell Cultures

Many cell types are adhesion dependent and cannot be grown in suspension cultures without mechanical support. All freshly isolated, culture expanded, as well as immortalized cells have for many decades been cultured on tissue culture polystyrene plastic. In such cultures cells normally spread and form focal adhesions and stress fibers throughout the cytoplasm. Such cultures can relatively easily be used to determine gene and protein expression, biochemical pathways, and intracellular trafficking just to mention a few. However, 2D cultures do only to a certain degree represent the physiological environment with respect to cell shape, cell–matrix and cell–cell interactions, local chemotactic and haptotactic gradients, nutritional status, or interstitial flow [1, 2]. For example, tumor cells, when grown on 2D substrates, are flat, whereas in 3D they adapt a round morphology, much like seen in cancer biopsies. Also mesenchymal cells independent of substrate composition exhibit bipolar spindle-shaped morphology in 3D as compared to the observed artificial dorsal (upper side) ventral (lower side) polarity in 2D [3] as here illustrated for cells being cultured on top (Fig. 1.1a) or inside (Fig. 1.1b) poly(ethylene glycol) (PEG) hydrogels of identical composition.

Not surprisingly, recent findings suggest a strong correlation between cell shape and function. Impressively, the changes in morphology and cell–substrate interactions in 3D cultures translate to differential cell signaling [1] resulting among others functional differences in reduced sensitivity of tumor cells towards radiotherapy (cell adhesion-mediated radioresistance) and chemotherapy (cell adhesion-mediated drug resistance) [4]. The decreased sensitivity to doxorubicin or etoposide observed in small cell lung cancer cells cultured on fibronectin or laminin [5] and the increased radiosensitivity of lung carcinoma cells with altered cell shapes by destabilization of the actin filaments [6] point towards effects mediated by both matrix components as well as the 3D arrangement. Furthermore, in many organs and tissues, different

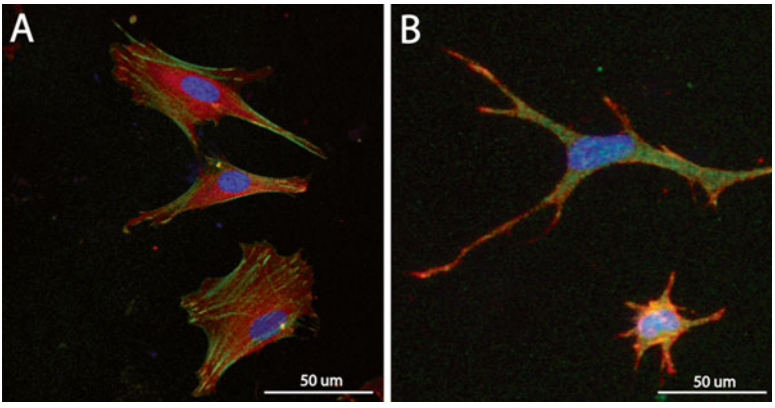

Fig. 1.1 Morphology of murine fibroblast cultured (**a**) on 2D substrates and (**b**) in 3D cultures

cell-types are arranged in spatially defined, repetitive manner and are structurally integrated with their neighbors. Together this might explain why some of the effects seen in 2D cultures are significantly different in the native tissue and thus findings from 2D cultures and even uniform 3D cultures likely have a limited predictive value [7]. Thus, the current workflow in pharmaceutical industry consists of screenings in 2D followed by validation in simple 3D models and animal models.

However, due to species-specific differences in physiology, metabolic activity, and cell function, also animal models have a restricted predictive value regarding drug therapeutic response. Thus, 3D tissue and organoid models partially representing functional properties of physiological or pathological human tissues are now seen as an interesting approach to bridge the gap between traditional 2D cultures and animal models [2, 8].

1.2 Top-Down Approaches (Matrix Free)

Typically tissue ECM undergoes tightly regulated remodeling through cells continuously secreting and subsequently remodeling their own ECM consisting of molecules such as collagens, fibronectin, elastin, and proteoglycans [9]. These secreted tissue-specific ECM components in turn will be sensed by cell integrin receptors and eventually influence the behavior of the cell itself as well as the one of neighboring or invading cell. When taken in culture, cells produce their own ECM [10] and are structurally and functionally integrated with their neighboring cells and the ECM [7]. During cell passaging, in order to permit the transfer of tightly adherent cells from one substrate to the next, cell–substrate interactions need to be disrupted by treatment with trypsin and EDTA by proteolytic cleavage and destabilizing of protein interactions. Due to cell surface-located proteins (e.g., growth factor receptors, syndecanes, integrins, proteases, and their regulators) engaged in receiving signals from the environment and providing modifying signals to the environment, passaging can have a significant impact on cell behavior.

1.2.1 Microtissues

Microtissues, also called spheroids, are 100–500 μm-sized multicellular clusters which can be formed on nonadhesive substrates by hanging drops or spinner flasks [11]. Microtissues can be assembled without the addition of matrix components and allow the formation of multicellular structures without the need of external signals. Additionally, within them cell–cell connections and connections of cells with their own ECM can be established. Due to 3D arrangement, cells adapt a more natural morphology and structure and function of deposited ECM takes the role of the natural environment. Microtissues present a straightforward approach to assemble a large variety of cell types as monotypic cultures or as cocultures in a mostly natural

environment; thus they have a great potential to be used in scalable drug screening platforms [12, 13]. They allow the determination of biochemical, morphological, functional, and morphogenesis-related parameters.

The major limitations of microtissues are the heterogeneity of the cellular environment and the limited ability to control the initial patterning of cells. The heterogeneity of cells within microtissues is largely due to the nonhomogeneous distribution of nutrients and oxygen, resulting in a hypoxic core with apoptotic cells and sufficiently nourished cells located on the outside. The difficulty to control the spatial arrangement of multiple cell types could potentially be overcome by varying the microtissue formation. The value of microtissues will rather be in understanding tissue self-organization processes and contact-mediated interactions between different cell types. For example in an attempt to create functional myocardial tissue rat cardiomyocyte-based microtissues were formed and exhibited coordinated beating after electrochemical coupling. When microtissues were coated with endothelial cells and assembled into macrotissues of mm^3 scale, they spontaneously formed microvessels with native vessel ultrastructural morphology. Transplantation of such prevascularized tissues into chick embryos or rat pericardium demonstrated both the functional integration of microvessels (60 h post transplantation) and the co-alignment of transplanted and host cardiomyocytes [14].

In order to take control over positioning of individual cell types DNA-programmed assembly of microtissues has been employed [7]. Using this approach the assembly of microsphere consisting of one MCF10A mammary epithelial cell with activated H-Ras and multiple control MCF10A cells could be achieved. The generated cell-to-cell variability in signaling was shown to lead cell mobility which is due to heterogeneity in pathway activation rather than in absolute pathway activity. Alternatively, polydimethylsiloxane (PDMS) molds have been generated in which by sequential seeding and sedimentation cells could be precisely positioned in 3D [15]. Such arrangement together with the stabilization of the structures within a hydrogel allowed the formation of liver-mimicking tissue structures in vitro. The arrangement of hepatic aggregates and liver endothelial cells demonstrated that the geometry of arrangement can have a large influence on cell function. If hepatocytes and endothelial cells were positioned in a compartmentally distinct localization allowing only paracrine signaling, albumin production could be sustainably enhanced compared to juxtaposed position. As such complex assays are clearly needed for the evaluation of tissue functions, such as that of liver, the combination of principles from bottom-up and top-down approaches as well as the use of patient's own cells will be necessary.

1.2.2 Cell Sheet Engineering

An interesting alternative to microtissues is cell-sheet engineering. This technology relies on a purely cell-based assembly of tissue structures. Again, cells are allowed to establish their own ECM and to form mature tissue structures. One of the first examples using this approach was the assembly of intermediate size blood vessels

(3 mm inner diameter) [16]. The construction of the vessel was initiated by first culturing fibroblast cell sheets, which were wrapped around a support and formed a tubular tissue. After decellularizing the remaining tissue matrix, forming an inner membrane, it was mounted on a perforated tubular mandrel before a sheet of smooth muscle cells was wrapped around to form the vascular media. This construct was maturated in a bioreactor before an additional sheet of fibroblasts, the adventitial layer, was wrapped around the vascular media. After another period of maturation in a perfusion bioreactor the tubular construct was removed from its support and seeded with endothelial cells which again were allowed to grow and maturate for 1 week. Histologically the constructs resembled a native vessel with intima, media, and adventitia and due to established ECM they were shown to resist physiologically relevant pressures (larger than 2,000 mmHg). Vessel constructs similarly produced using fibroblasts and endothelial cells were implanted as arteriovenous shunts in end-stage renal disease patients. In this study, the tissue-engineered constructs showed a patency rate of 78 % after 1 month and 60 % after 6 month of transplantation. Additionally, the constructs showed impressive resistance to intimal thickening and aneurysm formation [17].

Recent efforts towards the construction of engineered vessels concentrated on the development of production methods which give rise to constructs with better mechanical properties, are less time consuming, and are less dependent on cells' capacity to produce ECM. These aims were achieved by employing a single step assembly protocol [18] or the formation of fibroblast-derived, decellularized ECM which could be seeded with smooth muscle cells [19]. Together with the engineering of the vascular adventitia containing vasa vasorum, which was shown to improve graft integration and inosculation, engineered blood vessels hold great promise to become clinically applicable tissue-engineered products [20].

An alternative approach to harvesting of contiguous sheet of cells has been pursued by the development of surfaces where a simple shift of temperature (reviewed in [21]) or local charge [22] leads to a change of surface hydrophilicity or disintegration of the surface coating. The resulting sheading of the cell sheets without the use of proteolytic enzymes and EDTA allows cells to retain their structural and functional properties and to remain within the intact and functional ECM throughout the transfer [23]. The potential advantage of cell-sheet engineering lies in the ability to generate tissue constructs, which can be highly structurally ordered and allow the use of multiple different cell types. Initial attempts however have relied in single-cell-type sheets, which have even translated to clinical applications such as for the replacement of corneal tissue. Stratified epithelial cell sheets with normal cell profiles and functions were produced in human autologous serum and in the absence of feeder layers or bacterial or animal-derived products [24]. Later, using microcontact printing of fibronectin onto thermoresponsive surfaces, patterned cell sheets containing structurally arranged endothelial cells and hepatocytes could be produced [25]. In order to treat infarcted hearts, multiple myocardial cell sheets were stacked on top of a perfused vascular bed to generate functional 3D myocardial tissue constructs which contain a perfused vascular network [26]. With the advances in cell deployment using dispensing robots or microfluidics as well as the

layer-by-layer assembly of cell sheets could clearly help to generate more complex 3D tissue models. More likely, the integration of matrix-free and matrix-based approaches will be needed to generate models which recapitulate spatiotemporally regulated processes as they occur during tissue morphogenesis and healing.

1.3 Bottom-Up Approaches (Cells in Biomaterials)

In classical tissue engineering applications cells have been used in combination with biomaterials [27] which, with the aim to reconstruct or heal tissues, nowadays are often complemented with cell instructive factors provided with the biomaterial or with the cell culture medium. Biomaterials in this context are meant to substitute the native ECM and thus provide cells with an adequate provisional environment during tissue formation. ECMs in naturally occurring tissues consist mainly of fibrous proteins (collagens, fibrinogen, elastin, laminins) and proteoglycans. Both classes of molecules contribute to the mechanical (tensile and compressive) properties of tissues and are additionally involved locally in providing adhesion sites and molecular cues to embedded cells. Tissue engineering aims at replacing this naturally occurring matrix with (in most attempts) a provisional one to provide a template for the formation of novel tissue structures. A myriad of literature can be found on ceramic, polymeric, or biological materials which by different manufacturing processes give rise scaffolds with variable porosity, pore size distribution, and interconnectivity [28]. By providing structural support and a basis for the deposition of cell's own ECM, such porous scaffolds provide great platforms for both in vivo healing strategies and for the culture of cellular constructs in vitro. However, since the colonization with cells relies on their invasion from the outside or on dynamic seeding, porous scaffolds are not amenable for the exact positioning of cells as would be needed for advanced in vitro 3D tissue models. Therefore, within this chapter we will concentrate on hydrogel materials which are the generally used platform in advanced tissue models today.

1.4 Hydrogels

Hydrogels, comparable to glycosaminoglycans of the native ECM, are hydrophilic polymer networks which are highly swollen in aqueous solutions as they imbibe large quantities (often up to 99 %) of water [29]. Hydrogels, additionally to having similar mechanical properties to ECMs, permit the efficient diffusion of respiratory gasses, nutrients or waste products, and signaling molecules. Thus, they are often considered as good ECM models and broadly used for tissue engineering applications [29]. In all of these systems functional elements and means to control the materials function have been introduced over the years moving from simple hydrogels to hydrogels with more advanced properties. Functional elements can often be

employed in different materials and thus synthetic and biological elements become more and more integrated. However, in order to structure the following sections, we have made the distinction between biologically derived and synthetic materials.

1.5 Biologically Derived Hydrogel Materials

In early tissue engineering applications cells have been encapsulated and 3D cultured in a large variety naturally occurring, hydrogel-forming, protein-based materials such as collagens, fibrin, and matrigel or sugar-based materials such as alginate, agarose, hyaluronic acid, and chitosan [30]. Such 3D cultures have enabled the formation of relatively simple skin, bone, and cartilage tissue models, to name a few. Additionally they have allowed the study of morphogenetic events of, for example, intestine or mammary gland under tightly controlled culture conditions (reviewed in [31, 32]). They have also highlighted the influence of the dimensionality on the outcome of biochemical parameters. Due to the supportive biological properties of the naturally derived materials (e.g., their biodegradability and presentation of integrin ligands), such early approaches have led to impressive advances in the engineering of advanced tissue models.

1.5.1 Matrigel

Matrigel™ is a matrix that mainly consists of laminin, type IV collagen, entactin, and heparin sulfate proteoglycans [33]. Since Matrigel™ is isolated from Engelbreth–Holm–Swarm (EHS) mouse sarcoma, its composition is relatively ill defined, has large batch-to-batch variation, and can contain tumor-derived proteolytic enzymes and growth factors which can promote cellular function in an unpredictable manner. Despite the limitations of Matrigel™, this system has been successfully employed in a myriad of both tissue engineering applications and the formation of organoids in vitro. Impressive examples are the formation of small intestine organoids starting from intestine biopsies or even single intestine-derived leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) positive intestinal crypt stem cells [34].

1.5.2 Collagen and Gelatin

(Review on collagen in Lanza et al., 2011 [35]). Collagens are the most abundant proteins in the ECM of most tissues. Besides providing tensile strength to the tissue, by presentation of integrin binding sites in their native and proteolytically degraded form, they largely contribute to cell function and signaling. Due to their limited solubility and the presence of covalent intermolecular (Schiff base, aldimine)

cross-links in native tissues, collagens are commonly isolated using pepsin or acidic extraction conditions. Thermal denaturation of insoluble collagen results in gelatin, which dependent on the source can have variable properties.

Physical hydrogels formed by collagen type I provide excellent cell substrates both in vitro and in vivo. For example, plastic compressed collagen type I hydrogels have been used to engineer dermo-epidermal skin substitutes that can be formed at clinically relevant size and be transplanted and in a rat model successfully reconstitute full-thickness skin defects [36]. However, as during the common isolation process of collagens, the Schiff base is reversed to amines and aldehydes which further are converted to alcohols, collagen cross-links cannot spontaneously form [37]. Thus, in order to improve collagen and gelatin stability, cross-linking using glutaraldehyde or carbodiimide is needed, which is clearly not applicable for the delivery of proteins or encapsulation of cells.

1.5.3 Fibrin

Fibrin gels are formed by the thrombin-mediated cleavage or fibrinogen resulting in the release of the fibrinopeptides and the lateral aggregation of fibrin monomers to fibrin fibrils. This physical matrix is subsequently enzymatically cross-linked by the transglutaminase factor XIIIa (FXIIIa). Fibrin hydrogels are commonly used as surgical tissue glue. Due to its high biocompatibility Fibrin in a large number of in vitro culture cell and in vivo tissue applications led to impressive results. For example, fibrin in combination with knitted fabric has been used to create myocardial patches. Upon in vitro culture under cyclical stretch the provisional matrix was remodeled as shown by the increasing amounts of collagen after 1 week of culture. After subcutaneous implantation in rats cardiomyocyte survival and vessel ingrowth into these constructs were shown [38].

1.5.4 Alginate

Alginate is an unbranched, sugar-based material which consists of 1-4′-linked b-D-mannuronic acid (M) and a-L-glucuronic acid (G) derived from brown algae. Alginate hydrogels readily form by the cooperative binding of Ca^{2+} ions to the G-block [39]. By the association of two G-blocks, junctions are being formed, leading to the formation of a network structure. Alginate hydrogels are highly biocompatible but are largely devoid of biological function in mammalian tissues. Thus for tissue engineering applications they need the chemical integration of adhesion sites such as RGD [40]. Additionally, the loss of divalent cations by diffusion results in an uncontrolled disintegration of the hydrogels, which can be controlled by oxidation and covalent cross-linking [30]. Modified alginate hydrogels have indeed been shown to be very suitable for the delivery growth factors as well as tissue engineering applications. For review please refer to [41].

1.5.5 Hyaluronic Acid

Hyaluronic acid (HA) is a negatively charged matrix component which is present in the ECM of most tissues [42] and contributes to the compressive properties of tissues [43]. It is a linear polysaccharide of 100–8,000 kDa, consisting of repeating disaccharides of -1,4-D-glucuronic acid-b-1,3-*N*-acteyl-D-glucosamine. HA is not immunogenic and can easily be chemically modified [44]. Due to binding to CD44 HA is involved in many cellular processes [45]. Therefore, HA is also often combined and cross-linked with synthetic polymers to form semisynthetic hydrogels for protein and cell delivery. For review please refer to [46].

1.5.6 Engineering of Naturally Derived Biomaterials

However, the major drawback in the use of naturally occurring materials in engineering applications is their inherent biological properties such as the presentation of integrin ligands, the proteolytic degradability, and available specific and unspecific protein binding sites. To achieve prolonged materials stability, collagen hydrogels have been cross-linked by *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) [47]. To decrease plasmin digestion of fibrin hydrogels, aprotinin was engineered to be covalently incorporated in fibrin [48]. Additionally, the chemical or enzymatic coupling of functional groups such as heparin or growth factor-binding peptides to naturally occurring hydrogels have allowed the mimicking of naturally occurring ECM growth factor binding (Fig. 1.2a) [48–53]. The covalent enzymatic or chemical immobilization of engineered growth factors or the use of affinity linkers have provided another possibility to generate growth factor repositories for the sustained long-term delivery of minute quantities of highly potent growth factors such as vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMPs), or insulin-like growth factor (IGF) (Fig. 1.2b) [54–56]. The various strategies employed to immobilize growth factors in biological biomaterials are summarized in Table 1.1.

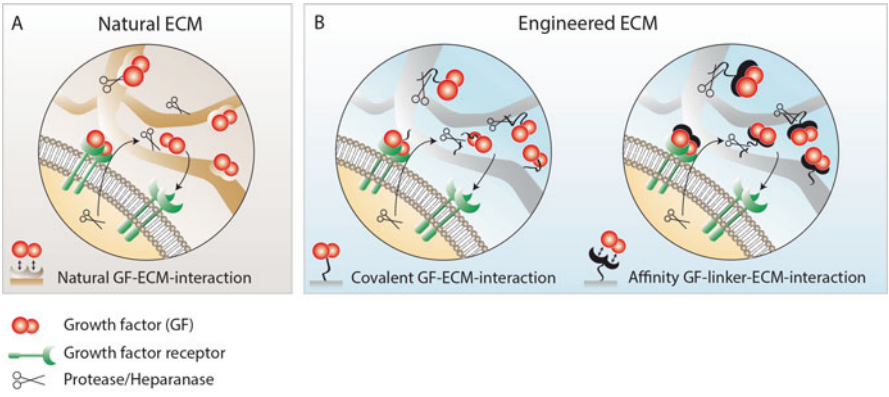

Fig. 1.2 Growth factor immobilization and release in (**a**) natural ECM, (**b**) engineered ECM with covalent interactions, and with affinity linkers

Table 1.1 Strategies for immobilization and release of growth factors in naturally occurring hydrogels (adapted from [57])

Matrix backbone	Linker	Ligand					References
		Native form	Modification	Immobilization	Release from		
					Matrix	Linker	
Fibrin	Heparin, Gln-HBP[a]	bFGF, β-NGF, PDGF-BB, NT-3	–	Affinity	Enz.	Dissoc.	[51, 58–65]
Collagen	Heparin, EDC/NHS[a]	VEGF, bFGF, SDF-1α	–	Affinity	Enz.	Dissoc.	[66–72]
Fibrin	FN-III9-10/12–14-Gln[a]	VEGF, PDGF-BB, BMP	–	Affinity	Enz.	Dissoc.	[53, 73]
Fibrin	–	bFGF	Ligand–Kringle fusion	Affinity	Enz./dissoc.	–	[74, 75]
Collagen	–	PDGF-BB, EGF, BDNF	Ligand–CBD fusion	Affinity	Enz./dissoc.	–	[76–78]
Collagen	SG-PEG-SG[a, b]	TGF-β2	Chemical	Covalent	Enz.	–	[79]
Collagen	SS-PEG-SS[a, b]	$VEGF_{165}$	Chemical	Covalent	Enz.	–	[80]
Collagen	EDC/sulfo-NHS[a, b]	$VEGF_{165}$	Chemical	Covalent	Enz.	–	[81]
Fibrin	SMCC-Gln[a, b]	KGF	Chemical	Covalent	Enz.	–	[82]
Fibrin	BTC-PEG-BTC[a, b]	SDF-1α	Chemical	Covalent	Enz.	–	[83]
Fibrin	–	β-NGF, BMP, $VEGF_{121}$, ΔAng-1, ephrin-B2, PTH, L1Ig6, IGF-1, Aprotinin	Ligand–Gln fusion[a]	Covalent	Enz.	–	[54–56, 84–93]
Fibrin	Gln-PIGF(123–144)	VEGF, BMP-2, PDGF-BB, multiple other ligands	–	Affinity	Enz.	Dissoc.	[94]

[a]Reacting with matrix
[b]Reacting with ligand
enz. enzymatic, *dissoc.* dissociation, *Gln* NQEQVSPL-peptide, *Gln-HBP* bifunctional NQEQVSPL-heparin binding peptide, *NHS N*-hydroxysuccinimide, *EDC N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide, *FN-III9-10/12–14* pieced together fibronectin III fragments from the 9th to 10th and 12th to 14th repeats, *SG-PEG-SG* disuccinimidyldiglutarate-polyethyleneglycol, *SS-PEG-SS* disuccinimidyldisuccinatepolyethyleneglycol, *SMCC* succinimidyl trans-4-(maleimidylmethyl) cyclohexane-1-carboxylate, *CBD* collagen binding domain, *ΔAng-1* truncated version of angiopoietin-1, *PIGF(123–144)* placental growth factor derived peptide sequence amino acids 123–144

All mentioned growth factor presenting materials can be engineered to instruct encapsulated or recruited cells to undergo differentiation or induce morphogenic processes in vitro and in vivo. For example the covalent immobilization of VEGF generated a growth factor repository for the sustained long-term delivery of minute quantities the highly potent growth factor [95]. Indeed this strategy has enabled the induction of large numbers of patent and morphological normal blood vessels in small animal models [85, 96]. Similarly, recently described linker peptides derived from fibronectin (FN) [53] have been shown to mediate the retention of growth factors and cytokines within fibrin hydrogels. Additionally to the efficient delivery of factors by the FN it also coordinated with growth factor receptors and integrin signaling. Delivery of platelet-derived growth factor-BB (PDGF-BB) was shown to improve in vitro mobilization of smooth muscle cells and in the presence of BMP-2 to improve bone healing in vivo.

The above examples illustrate those major achievements made by the engineering of biologically derived materials. However, though some of the inherent properties of biological materials can be overcome with engineering strategies, others such as proteolytic stability, presentation of integrin ligands, low affinity binding of proteins, or gelation kinetic cannot be so easily manipulated.

1.6 Synthetic Hydrogel Matrices

Ideally, for engineering applications the precise and independent control over following hydrogel parameters would be available: mechanical properties, gelation kinetics, micro- and/or nanoarchitecture, presentation of adhesion ligands, proteolytic degradability, specific protein binding and release. All of these parameters have to be considered when designing tissue engineering applications in vitro. Furthermore, mimicking dynamic changes throughout morphogenesis, materials should be available which allow trigger-inducible change in materials properties. Some of these materials have successfully been engineered to bind and locally present biological cues.

1.6.1 Backbone Design

Multiple (cationic, anionic, or neutral) polymeric molecules have been suggested for the use as biomaterials. For a more thorough review please refer to [29]. The architecture of the molecules giving rise to the hydrogel network can be varied regarding functionality and distance between the functionalities. Whereas the increase of functionality gives rise to higher cross-link density at constant polymer concentration, the increase in distance between functionalities will result in larger pore size. The polymer chemical composition largely influences the behavior of the material in aqueous environments. Whereas polymers containing ionizable pendant

groups such as copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol) P(MAA-g-EG) respond with shrinkage or swelling, uncharged polymers are indifferent towards changes in buffer pH [97]. The use of block copolymers consisting of hydrophilic and hydrophobic domains, such as poly(ethylene glycol)-bl-poly(propylene glycol)-bl-poly(ethylene glycol) (PEG-PPG-PEG), results in a hydrogel system, which due to a shift in temperature is swelling or shrinking [98]. The copolymerization of materials building blocks which are temperature or pH sensitive results in hydrogels that responded to both stimuli [99].

1.6.2 Cross-Linking Mechanisms

For in vitro and in vivo applications, cross-linking of hydrogels must be performed under conditions which are not affecting cell viability. Hydrogels can be created by establishing affinity interactions or by chemical polymerization and depending on the affinity of physical interactions the stability of hydrogels can be modulated. Relatively weak interactions can be employed to form hydrogel systems which are responsive to stimuli such as glucose or antibiotics. Additionally they can result in hydrogels which are self-healing. The stability of chemically cross-linked hydrogels can be modulated by the introduction of linkages which are sensitive or insensitive towards hydrolytic degradation, proteolytic digestion, or reducing conditions. Chemical cross-linking today is mostly done by radical polymerization using photoinitiators, step-growth polymerization (Michael-type reaction), or enzymatic reactions presenting a spectrum of possibilities to choose for specific applications. Problems associated with cross-linking are lack of substrate specificity, cytotoxicity, and reaction time. Although some photoinitiators have been associated with cytotoxicity, for a number of them, the concentrations used are in a range where they are not compromising cell viability [100, 101]. Michael-type reactions have been shown to have high substrate specificity and fast reaction kinetics under physiological buffer conditions. Recently, the even more selective native chemical ligation which involves a thioesther and an *N*-terminal cysteine has been used for the formation of hydrogels [102]. Also copper free click-reactions are now being developed, which have a good substrate specificity and improved reaction kinetics [103]. Alternatively, enzymatic reactions which are known to have a very high substrate specificity have been used to form hydrogels [102] being highly compatible with the preservation of active growth factors.

1.6.2.1 Mechanics

The mechanical properties of hydrogels rely on hydrogel architecture, cross-linking efficiency, swelling behavior, initial concentration of monomers, and stoichiometry of reactants. Generally, higher polymer concentrations with high branching and short arm length can potentially lead to highly cross-linked hydrogels. Such hydrogels,

with increasing hydrophilicity of the backbone polymer, can take up large amounts of water and thus will be mechanically strong. Factors that can weaken the mechanical properties of the hydrogel are limited cross-linking efficiency for example due to unbalanced stoichiometry of reacting groups, competing reactions, or suboptimal reaction conditions (pH, salt concentration). For a more thorough review please refer to [30].

1.7 Synthetic Hydrogels with Engineered Biological Functions (Static/Controlled by Cells)

As mentioned above, synthetic hydrogels are devoid of biological functions and thus provide a blank canvas for the engineering of materials with tightly controlled properties. Early experiments provided evidence that cells 3D encapsulated in such small porous (pore size is typically in the nm range) hydrogels cannot spread migrate, proliferate, and survive long term [104]. It has been demonstrated that the modification of the hydrogel backbone with biological building blocks such as cell adhesion sites and the introduction of proteolysis-sensitive backbone elements are necessary for the 3D culture of cells. These minimal modifications allow the encapsulated cells to locally digest the hydrogels, form small pores, and move via complex processes via substrate adhesion and retraction of cell extensions. Of course, cells from different lineages have different requirements regarding the presented biological functional building blocks, which is where the challenges arise but also where novel hydrogel platforms with tunable properties prove their worth.

1.7.1 Modularly Designed Platforms as Artificial Extracellular Matrices

Although synthetic hydrogels make it possible to study cellular response to isolated biological parameters, up to date only few hydrogels developed for 3D cell culture allow the independent tuning of properties such as biochemical signals and mechanical stiffness. Such materials have been created for example based on click chemistry [105], peptide self-assembly [106], and interpenetrating polymer networks [107].

In order to create a modular artificial ECM platform where matrix properties can be modified almost independent of each other, the factor XIIIa-catalyzed cross-linking scheme of fibrin clot involving the formation of a covalent isopeptide bridge between Gln and Lys residues by the enzymatic action of the transglutaminase factor XIIIa was employed [108]. Star-shaped PEG-vinylsulfone molecules were functionalized with two peptides acting as substrates for FXIIIa via a Michael-type addition reaction, thus creating a homogeneous synthetic hydrogel with fibrin-like biomolecular characteristics upon cross-linking with the enzyme. Simultaneously with the hydrogel cross-linking, growth factors, adhesion peptides, or other biological entities functionalized with either of the FXIIIa substrates can be incorporated in a controlled

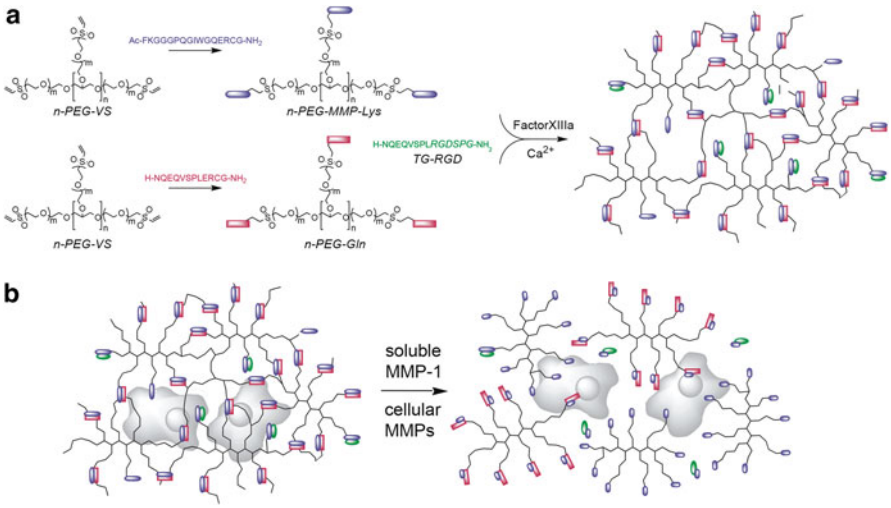

Fig. 1.3 Factor XIIIa-catalyzed PEG hydrogel formation and degradation. (**a**) The transglutaminase enzyme factor XIIIa was used to cross-link two multiarm *PEG* peptide conjugates, *n-PEG-MMP-Lys*, and *n-PEG-Gln* (here, $n = 8$), in combination with a cell adhesion peptide, *TG-Gln-RGD*, to form multifunctional synthetic hydrogels. (**b**) Gel degradation by cell-mediated proteolysis (reprinted with permission from [108])

manner (Fig. 1.3a). By engineering of the PEG backbone to contain matrix metalloproteinase (MMP) or plasmin-sensitive sites, the biomimicry of this system can be further extended (Fig. 1.3b). This platform (called TG-PEG from here on) has so far been used to elucidate the effect of physical and biochemical matrix properties on processes such as cell migration, proliferation, spreading, and angiogenesis and for creating structured microenvironments as discussed later.

Usefulness of modularity can be elucidated for example by the case of cell migration in 3D which has so far mostly been investigated in naturally occurring materials [109]. Findings from such experiments are limited in terms of studying the effect of biochemical and biophysical parameters since they cannot be decoupled from each other. For example, the effect of the ECM on the choice between proteolytic and nonproteolytic cell migration remains to be exhaustively answered [37]. Some light could be shed on the topic by using the TG-PEG hydrogels [110], which are devoid of microstructure therefore essentially nonporous for cells, as cross-linked polymers create pores in the range of tens of nanometer. By harnessing the modularity of this system in terms of protease sensitivity and stiffness, it was found that migration behavior was strongly dependent on matrix stiffness, with two regimes identified: a nonproteolytic migration mode dominating at relatively low matrix stiffness and proteolytic migration at higher stiffness. In nondegradable matrices with low stiffness, single cells could overcome the resistance of the matrix by engaging in a degradation-independent three-dimensional migration mode.

Similarly the TG-PEG system could be used for studying cell proliferation in 3D in response to selectively altered matrix characteristics [111]. To illustrate the

difference in cell behavior in naturally occurring materials and fully synthetic hydrogels, fibroblasts were studied in collagen and TG-PEG hydrogels. The main physical difference between the two systems was that the PEG gels are purely elastic, whereas collagen gels generally display viscoelastic behavior due to the physical entanglement of normally freely moving fibers. Cells are able to dislocate collagen fibers leading to predominantly physical modification of the matrix. The PEG gels on the other hand are mainly modified by biochemical processes, making it possible to systematically study such phenomena and their implications to other cellular processes, such as proliferation. By exploiting the modularity of the TG-PEG system, this study revealed that in spite of matrix sensitivity to proteases and the presence of cell integrin binding sites, proliferation in 3D was hindered by high stiffness (elastic modulus > 1,200 Pa).

1.7.2 Adhesion and Degradation Sites

Naturally occurring ECM contains numerous proteins and glycans which, dependent on the status of a tissue, are differently composed and thus provide different sets of adhesion domains. Ruoslahti et al. have found that a triple amino acid domain derived from fibronectin is sufficient for the integrin-mediated adhesion of cells [112]. Since then many cell-adhesive peptides, mostly derived from collagen, fibronectin, or laminin, to be linked into hydrogels have been described and used for the engineering of cell-instructive biomaterials (reviewed in [113]).

The polymeric backbone must provide space to cells in order to proliferate, deposit the cell's own matrix, and move. Degradability of the material can be achieved through spontaneous dissolution of the polymer as for example by the incorporation of poly(lactic acid) (PLA) within the polymer structure or the use of acryl–sulfhydryl bonds [114]. These modes of degradation are the material's autonomous properties. Materials stability is critical to appropriate tissue formation, as too fast degradation results in loss of structural and mechanical support and too slow degradation will inhibit cell, for example, cell ingrowth and function. To render materials with tunable, cell-responsive degradation properties, polypeptides derived from occurring naturally protease-sensitive sequences have been adapted and incorporated into linker molecules of the backbone [104]. Recently, a number of peptide sequences which are recognized and degraded with different efficiencies (kcat) have been described [115, 116]. The use of substrate with very well-defined degradation properties clearly will allow the fine tuning of materials properties towards specific applications.

1.7.3 Incorporation and Cell-Mediated Release of Growth Factors

Tissue development and regeneration depend on tightly coordinated spatial and temporal growth factor signals and recombinant growth factors have been widely proposed for therapeutic use in the regeneration and repair of diseased tissues.

Increased knowledge on growth factor signaling and advances in recombinant protein engineering and production have opened new possibilities is constructing artificial extracellular matrices. Strategies employed to immobilize growth factors in synthetic hydrogels are summarized in Table 1.1.

When growth factors are purely physically entrapped in the hydrogel matrix, availability for cells in space and time is determined by passive diffusion and coupled with hydrogel degradation. If not replenished in the culture medium, activity decreases over time. Diffusion can be controlled via modifications either on the hydrogel network properties or the growth factor by which the affinity to the matrix, bioactivity, stability, and bioavailability can be modified [117].

Effect of soluble factors on cells cultured in 3D can be studied in any hydrogel material, either by encapsulation to the material along with the cells or by addition to the growth medium, but for more sophisticated biomimicry, systems enabling the tethering and controlled release of growth factors are needed. Immobilization strategies allow the construction of gradients or localized areas where the factor is present, making it possible to more accurately recapitulate physiological situations. Natural ECM acts as a reservoir for growth factors from which they are released by cellular remodeling, which is often used as a release strategy in synthetic systems in addition to temporally controllable triggered release (Fig. 1.2a). In the following paragraphs, growth factor immobilization strategies for synthetic hydrogels are discussed first focusing on covalent tethering and then moving to affinity-based systems (Fig. 1.2b).

1.7.3.1 Covalent Immobilization

Covalent tethering of growth factors to synthetic or biologically derived hydrogels has been achieved by either chemical modification or genetic engineering of the factors to contain functional groups such as thiols, acrylates, azides, and Gln-tags (Table 1.2). Initial chemical conjugation approaches utilized, for example, homobifunctional PEG-based cross-linkers with terminal and primary amine selective succinimidyl groups [80, 148], which could serve both as hydrogel cross-linking entities and as means to incorporate growth factors. Another chemical conjugation strategy was based on hetero-bifunctional *N*-hydroxysuccinimide (NHS)-PEG-acrylate linker, which could be used by first modifying the factor of interest with the amine-specific NHS group reaction and subsequently coupling the acrylated biomolecules into PEG-diacrylate networks by photopolymerization [118, 119]. Also click chemistry has been successfully used for covalent immobilization of growth factors into synthetic hydrogels [123].

The downside of these broadly applicable strategies, such as the reaction of NHS with any accessible lysine or the *N*-terminus, is their lack of specificity. The exact site and number of modifications are difficult to control and may have drastic effects on the growth factor bioactivity [149]. More site-specific strategies have been realized by engineering recombinant proteins with additional cysteines as the abundance of reduced cysteines is inherently low in proteins and such modifications render them more susceptible to, for example, Michael-type reaction with vinylsulfone

Table 1.2 Strategies for immobilization and release of growth factors in synthetic hydrogels (adapted from [57])

Matrix backbone	Linker	Ligand			Release from		Reference
		Native form	Modification	Immobilization	Matrix	Linker	
PEG-DA	NHS-PEG-Acryloyl[a, b]	TGF-ß1, bFGF, EGF	Chemical	Covalent	Enz.	–	[118–121]
PEG-DA	SMC-PEG-Acryloyl[a, b]	PDGF-BB, FGF-2	Chemical	Covalent	Enz.	–	[122]
PLEOF	PEG-azide[a, b]	BMP peptide	Chemical	Covalent	Enz.	–	[123]
MAC	EDC/sulfo-NHS[a, b]	IFN-γ	Chemical	Covalent	Enz.	–	[124]
PEG-VS	–	$VEGF_{165}/VEGF_{121}$	Ligand-Cys fusion[a]	Covalent	Enz.	–	[125, 126]
PEG-TG	–	$VEGF_{121}$	Gln-Ligand fusion[a]	Covalent	Enz.	–	[127]
PEG-DA, (Hep/HA/Gtn)-SH	Heparin	VEGF, bFGF, Ang-1, HGF, KGF, PDGF-BB	–	Affinity	Enz./hydr.	Dissoc.	[50, 128–130]
PEG-DA, (Hep/CS)-SH	Heparin	bFGF	–	Affinity	Enz./hydr.	Dissoc.	[131, 132]
PEG-SBA, Hep-ADH	Heparin	VEGF		Affinity	Enz./hydr.	Dissoc.	[133]
PEG-NH2	Heparin, EDC/sulfo-NHS	FGF-2	–	Affinity	Enz.	Dissoc.	[134]
PEG-peptide, heparin	–	VEGF	–	Affinity	Enz.	Dissoc.	[135]
PEG-LMWH, $PEG\text{-}PF4_{ZIP}$	Heparin	bFGF	–	Affinity	Enz.	Dissoc.	[136]
PEG-LMWH, PEG-HIP	Heparin	bFGF	–	Affinity	Enz.	Dissoc.	[137]
PEG-SH, HMWH	Heparin	bFGF	–	Affinity	Enz.	Dissoc.	[138]

PEG-LMWH, VEGF	Heparin	VEGF	–	Affinity	Enz.	Dissoc.	[139]
PEG-DM, heparin-MA	Heparin	bFGF	–	Affinity	Enz.	Dissoc.	[140]
PEG-VS, Cys_3-peptide	Heparin	BMP-2	–	Affinity	Enz.	Dissoc.	[141]
PEG-DA	poly(AAC)-Cys-bFGF-bpa	bFGF	Cys-bFGF-bp	Affinity	Enz./hydr.	Dissoc.	[142]
PEG-TG	Gln-ZZ[a]	IL-4	Ligand–Fc fusion	Affinity	Enz.	–	[143]
PEG-TG	bead-Novo. GyrB-ZZ	PDGF-BB	Ligand–Fc fusion	Affinity	Enz.	Novo.	[144]
PEG-TG	Gln-GyrB[a]/Novo./GyrB-ZZ	FGF-7	Ligand–Fc fusion	Affinity	Enz.	Novo.	[145]
PEG-TG (Caged-Lys)		PDGF-BB, $VEGF_{121}$	Ligand–Fc fusion	Light/covalent	Enz.		[146]
PEG-TG		$VEGF_{121}$	Ligand–GyrB fusion	–	[c]		[146]
PEG-TG	$Fg\beta15–66_{(2)}$	FGF-2, PlGF-2	–	Affinity	Enz.	Dissoc.	[147]

[a]Reacting with matrix
[b]Reacting with ligand
[c]Coumermycin induced ligand dimerization
enz. enzymatic, *hydr.* hydrolyzed, *dissoc.* dissociation, *Novo.* novobiocin, *Gln* NQEQVSPL-peptide, *NHS N*-hydroxysuccinimide, *EDC N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide, *poly(AAC)-Cys-bFGF-bp* poly(acrylic acid) modified FGF-2 binding peptide, *PEG-NH_2* PEG-amine, *PEG-SH* PEG-thiol, *Hep* heparin, *HA* hyaluronan, *CS* chondroitinsulfate, *GEL* gelatine, *PEG-SBA N*-hydroxysuccinimidyl ester PEG-bis-butanoic acid, *$PF4_{ZIP}$* heparin binding, coiled-coil peptide, *HIP* peptide sequence from heparin interacting protein, *DM* dimethacrylate, *Cys* cysteine, *VS* vinyl sulfone, *PEG-TG* transglutaminase FXIII formed PEG hydrogel, $Fg\beta15-66_{(2)}$, *N*-terminus of the Fibrin(ogen) β-chain, *MAC* methacrylamide chitosane, *Coum.* coumermycin

groups of PEG macromers [125]. Another possibility for achieving high site specify while conserving bioactivity is employing enzymatic reactions as discussed next.

The TG-PEG system was developed having in mind the goal of a flexible and modularly designed artificial ECM allowing site-specific and stable immobilization of growth factor proteins into the gel network, without compromising the protein's bioactivity. The most straightforward way of doing this is by producing growth factors bearing a substrate for FXIIIa, as has been shown for VEGF [127]. $VEGF_{121}$ was engineered to contain the glutamine acceptor substrate NQEQVSPL, derived from the *N*-terminus of α_2-plasmin inhibitor (α_2PI_{1-8}) (termed Gln). This Gln-$VEGF_{121}$ could be covalently incorporated into the hydrogel and released in controlled manner by the cleavage of the MMP sites in the PEG backbone during cellular matrix remodeling. The ability of the matrix-bound Gln-$VEGF_{121}$ to stimulate angiogenesis was evaluated in the embryonic chick chorioallontoic membrane (CAM) assay and the response to it was found comparable to that of native, diffusible $VEGF_{121}$, indicating that this sequence-specific mode of morphogen affixation in aECM does not compromise a morphogen's activity, at least not in the case of VEGF. In contrast to conventional chemical bioconjugation schemes, the site-specificity of the enzymatic reaction gives precise control over the conformation of the immobilized molecules and results in no significant loss of its bioactivity. This may be crucial for applications involving sensitive transmembrane proteins (e.g., Notch ligands or cadherins) in order to recapitulate cell–cell interactions via aECMs.

1.7.3.2 Affinity Immobilization

In addition to covalent immobilization strategies, affinity interactions are another possibility for growth factor incorporation. These strategies take advantage of the interactions naturally occurring between growth factors and ECM components, such as heparin, chondroitin sulfate, hyaluronic acid, and fibronectin. Thus they do not require direct chemical or genetic modification of the protein offering high degree of flexibility. Synthetic hydrogels have been modified with heparin via for example EDC/sulfo-NHS chemistry [134] or with streptavidin to allow the immobilization of biotinylated factors [150]. Also recombinant affinity linkers, allowing simultaneous addition of growth factors and cells during cross-linking [143, 151], as well supramolecular peptide gels with affinity sites, have been engineered [152].

Recently a very interesting approach based on the functional domains of fibrinogen was presented [147]. Recombinantly produced heparin-binding domain of fibrin(ogen) located at the N terminus of the fibrin(ogen) β chain (Fg β) was covalently linked into TG-PEG hydrogels and then used for the immobilization of various growth factors, for example, FGF-2 and PIGF-2. Overall, interaction of the linker with 15 different growth factors were established which all displayed K_D values in the nM range. The fibrinogen fragment functionalized TG-PEG gels were successfully used to deliver growth factors into full-thickness skin wounds in mice which lead to faster wound closure and increased development of granulation tissue.

In order to develop a flexible affinity-based strategy for the conjugation of growth factors to the previously described synthetic TG-PEG platform, a linker peptide consisting of the TG-domain and two repeats of the synthetic protein A analog (Z) was designed [143]. The TG-domain of this TG-ZZ peptide can be covalently bound to the Lys-PEG component of the TG-PEG system by the factor XIII-mediated reaction. Due to the high affinity of the ZZ peptide (4.8×10^{-8} M^{-1}) to the fragment crystallizable (Fc) region of antibodies, Fc-tagged growth factors can be immobilized within the hydrogel network. Taking advantage of the high affinity of this interaction, the linker can be used to directly capture Fc-tagged proteins produced by mammalian cells encapsulated in the hydrogels. This creates a versatile platform to concentrate and purify large panels of recombinantly expressed growth factors for cell-based assays to identify novel regulators of cell behavior.

The fusion peptide Gln-ZZ was expressed in bacterial cultures and in its functionality demonstrated by capturing FITC-labeled IgG antibody from a solution into a linker containing hydrogel and retaining it throughout extensive washing. With the ultimate goal of growth factor immobilization in mind, the functionality of growth factor or cytokine-tethered hydrogels as cell-instructive matrices was demonstrated using the anti-inflammatory cytokine interleukin-4 (IL4). Protein-capture hydrogels and control hydrogels were patterned and placed in cultures of human embryonic kidney (HEK)-293 T cells expressing IL4-Fc and after washing, the activity of the captured IL-4 was demonstrated by a reporter cell line. ZZ-linker-modified hydrogels could be used for providing a reservoir of biomolecules, which can actively stimulate cellular responses, either in their matrix-immobilized form or after cell-mediated proteolytic degradation of the hydrogel in a soluble form.

1.7.4 Local 3D Structuring of Hydrogels

Homogeneous biomimetic hydrogels can only be used to address biological questions in a simplified manner, which for certain situations, such as high throughput screening platforms, can be sufficient or even desired. By creating spatially defined heterogeneous microenvironments, more sophisticated models recapitulating higher levels of biological complexity can be realized, helping to bridge the gap between current in vitro and vivo models. Bulk functionalization of synthetic hydrogels has been widely described in literature, but this alone is not sufficient for mimicking the natural ECM. Thus, efforts have been made for evolving hydrogels for cell encapsulation from physically and biochemically homogeneous materials into highly spatially defined environments structured in multiple size scales.

Heterogeneity can be introduced in 3D in terms of local biochemical composition (e.g., adhesion ligands and growth factors) or by introducing structural patterns (e.g., variation hydrogel composition, empty, or cell-filled spaces). Hydrogels with such features have been produced using techniques such as self-assembly of prefabricated building blocks (e.g., microscale hydrogels or fibers) [153–157],

casting [158], additive manufacturing (e.g., printing and layer-by-layer deposition) [159, 160], photo-patterning [161, 162], and microfluidics [163, 164]. Problems with spatial patterning of hydrogels in 3D arise from their thickness and extensive swelling.

Next, two approaches, namely the combination of printing with layer-by-layer deposition for modelling vascularized bone and electrochemical control of polymerization for creating channel structures and gradients of biomolecules, are discussed in the context of the TG-PEG system.

Generation of functional vascularized tissues remains the holy grail of tissue engineering, and in order to address related questions using in vitro models, structuring ECM-mimicking materials and cells comes into play. In the context of bone, combining the TG-PEG hydrogel system as an artificial ECM with matrix structuring technologies, namely robotic printing and layer-by-layer deposition, the role of biological constituents in the vascular bone microenvironment could be systematically evaluated [159].

Feasibility of layer-by-layer deposition of TG-PEG was demonstrated by combining 200 μm cell containing hydrogel blocks with confluent layers of cells (Fig. 1.4a). Since gel layers can be formed in the presence of cells or used as surfaces for the seeding of cells, even such a simple layering approach allows for a large variety of cell and matrix combinations. For introducing more heterogeneity with each gel layer, robotic printing platforms using a contact-spot arraying technology with a lateral precision of approximately 5 μm and robotic pipetting with a lateral precision of approximately 100 μm were used. With these systems spatially segregated hydrogel drops of ca. 100 μm of diameter and 20 μm height could be formed (Fig. 1.4a). The size of the droplets can be modified by adjusting different parameters such as the pin-head size and the amount of dispensed liquid or using repetitive printing steps.

Initially, conditions supporting the viability and spreading of osteoblasts (MC3T3-E1) and osteocytes (MLO) or blocking their migration were defined in terms of hydrogel stiffness and presentation of RGD ligand. The effect VEGF and mono-, co-, and tri-cultures on the formation of capillary like network in 3D was then assayed. Using a fully synthetic hydrogel platform, it was possible to provide evidence on the importance of single component on angiogenesis. It was demonstrated that besides the adhesion ligand RGD and the pro-angiogenic growth factor VEGF, the presence of both stromal cells appears to be critical for endothelial cells (ECs) to efficiently form tube-like structures in artificial environments.

Once the culture conditions were defined, the three cell types were assembled together to create spatially organized vascularized bone tissue-like constructs. A supportive layer of 4 % PEG hydrogel covered with a monolayer of MC3T3-E1 was used as a basis for the construct, and on top of this, human umbilical vein endothelial cells (HUVECs) were subsequently positioned in 1 μl-sized hydrolytically and proteolytically degradable PEG-acrylate gels. As PEG-acrylate degrades rapidly the drops were formed without RGD ligands to prevent high concentrations of soluble peptides which could potentially interfere with HUVEC viability. As a final step, a covering layer of PEG containing human osteocytes was added and the

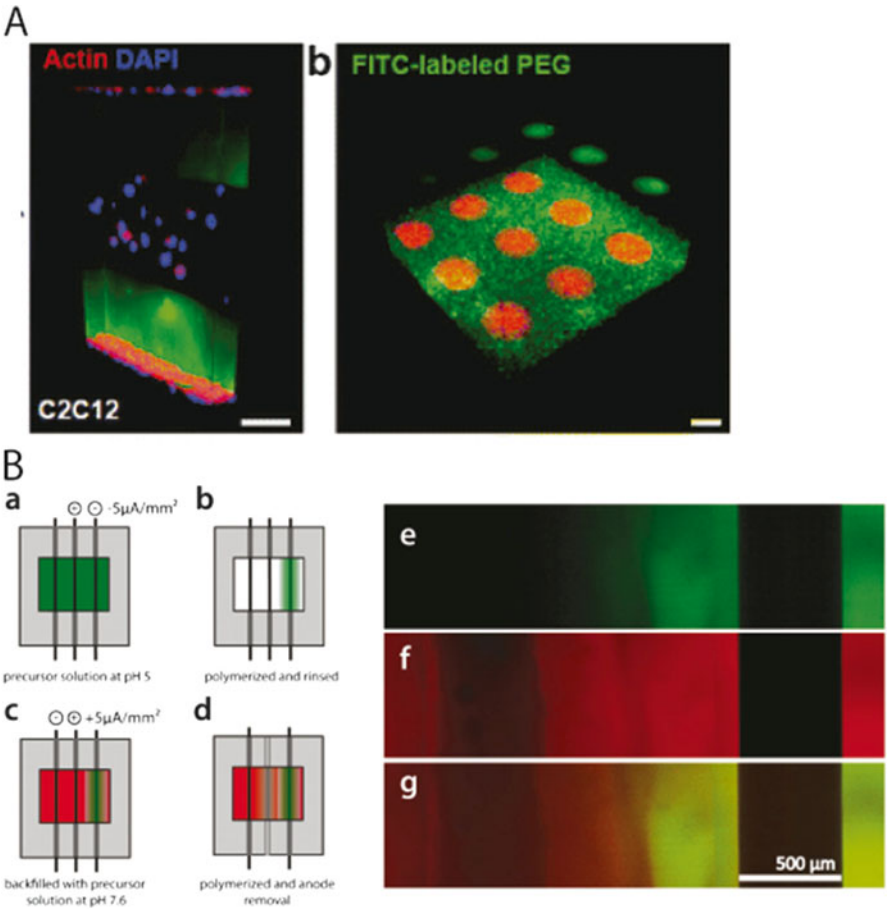

Fig. 1.4 Structured microenvironments in TG-PEG hydrogels. (**a**) Homogeneous layers of C2C12 cells and cell-containing or FITC-labeled gels (gel mass either in *green* or unstained) and different fluorescently labeled hydrogels arranged by robotic printing in defined positions on top of different layers of hydrogel (scale bar 100 µm). (**b**) Schemes depicting the production of an engineered microenvironment containing two different fluorophores using electrochemical structuring and confocal microscopic images of the resulting microenvironment. Reprinted with permission from [159, 160]

constructs were allowed to remodel for 10 days under the previously optimized conditions. Analysis of the constructs revealed a dense and interconnected network of HUVECs and preosteoblastic cells surrounding the endothelial structures.

These findings demonstrate that simultaneous patterning of matrix components, ligands, and cells to direct cellular spreading and to form artificial tissue-like constructs allows for the dissection of biological questions related to tissue remodeling and morphogenesis and their independent study in a fully controllable artificial environment.

In addition to the strategies described for biological functionalization of TG-PEG hydrogels, electrochemical polymerization has been employed for eliciting spatial control over the cross-linking in 3D [160]. This is based on the FXIII-catalyzed cross-linking reaction being pH-dependent and occurring with highest efficiency at pH 8 while being damped at more acidic or basic conditions [127]. The proteolysis of water induced by electric current was used to locally decrease (at the anode–electrolyte interface) or increase pH (at the cathode–electrolyte interface) and thereby prevent or promote hydrogel formation. This method was successfully used for creating microchannels within TG-PEG hydrogels in a PDMS mold using an anodically polarized tungsten wire as a template. By locally controlling the polymerization around the wire, it could subsequently be removed, while in absence of the current the TG-PEG hydrogel strongly adhered to the metal surface and was disrupted.

Electrochemical control of gel polymerization could also be used to form complex, locally functionalized 3D microenvironments in TG-PEG by a sequential approach (Fig. 1.4b). This was demonstrated by first polymerizing FITC functionalized gel around a cathode wire, followed by thorough washing to remove the unreacted components, and finally backfilling the mold with a TRITC functionalized gel while creating a second channel. The area between the two channels was thus functionalized with both molecules and the sides with one molecule alone. When injected into the preformed channels, various cell types such as bone-marrow-derived mesenchymal stem cells (MSCs), preosteoblasts, and fibroblasts were able to invade into the surrounding hydrogel mass, which will enable the study of cellular responses to different (graded) microenvironments. HEK cells expressing a yellow fluorescent protein (YFP) upon being exposed to IL-4 were used for demonstrating such spatial specificity of the channel surrounding microenvironments after functionalization with hydrogel bound IL-4. In conclusion, by electrochemically controlling the enzymatic cross-linking of TG-PEG hydrogels, it was possible to create spatially defined 3D microenvironments containing various tethered biomolecules and thereby control local cell functions.

Further understanding on how to best structure artificial microenvironments and automation of these procedures could lead to large-scale production of reproducible tissue-like constructs suitable for high-throughput screening approaching in vivo complexity, with yet a simple readout for evaluation.

1.8 Spatiotemporally Controllable Dynamic Microenvironments (External Control)

Native ECMs undergo tightly controlled, constant remodeling resulting in liberation of growth factors or other bioactive entities as well as changes in matrix mechanical properties. Although the above-presented synthetic hydrogel matrices have largely overcome the limitations of naturally occurring materials by handing over the control of the material's physical and biochemical properties to the scientists, they still do not

allow modulating the availability of extrinsic signaling molecules and material mechanics in time. Possible trigger mechanisms include changes in temperature [165–167], pH [22, 168], electromagnetic stimulation [169], or taking advantage of bioactive compounds [96, 170–172]. Most feasible in vitro approaches reported so far are based on light or using diffusible molecules interfering with interactions within the hydrogel, so the focus in this chapter is on such approaches. Light is an attractive candidate for spatiotemporal manipulation of hydrogels because its temporal patterning resolution can be controlled at single cell or even subcellular scale and in the visible and long-wave UV region has little effect on cell viability and behavior.

1.8.1 Dynamic Presentation of Biological Cues

Bioactive molecules such as growth factors can have cross-reactive effects on different cell types, making timing of their presentation in vivo as important as spatial control. This has promoted the development of experimenter-controlled culture matrices, in which adhesion sites and growth factors can be patterned and released on demand to change the biochemistry of the microenvironment to mimic processes such as differentiation.

Light-sensitive molecular building blocks can be attached to hydrogel networks to generate artificial ECMs in which the properties of microenvironments could be specifically modulated by light exposure [173]. Previously only small synthetic peptides have been the target of photopatterning approaches because the employed chemical cross-linking or bioconjugation reactions, using nonspecific chemistries, often damage full-length proteins. Photochemical caging strategies have emerged as a means to use light for controlling the temporal presentation of larger biological compounds without impairing their bioactivity.

Reversible presentation of biological entities has been reported based on the combination of two orthogonal, biocompatible wavelength-specific photoreactions, namely thiol-ene reaction for photocoupling and photoscission of an *o*-nitrobenzyl ether for later release from PEG hydrogels [174]. Spatial control of both reactions by controlling the introduction and removal of functional groups was achieved at a subcellular scale in 3D by focusing pulsed laser light within the hydrogel volume with resolution of 1 μm in the *x*-*y* plane and 3–5 μm in the *z*-plane. Mouse embryonic fibroblast (NIH 3 T3) cells were seeded on gels with photopatterned adhesive regions from where they could subsequently be detached by the removal of the adhesive ligand after being first allowed to adhere and spread for 24 h.

The effect of temporal ligand presentation on stem cell phenotype is an important question that could be studied by incorporation photocleavable adhesion molecules. A nitrobenzyl ether-derived moiety was acrylated by a pendant hydroxyl group [photodegradable acrylate (PDA)] and was subsequently attached with a pendant carboxylic acid to PEG-*bis*-amine to create a photocleavable cross-linking diacrylate macromer. The cross-linker was the copolymerized with PEG monoacrylate (PEGA)) by redox-initiated free radical polymerization to create photodegradable hydrogels [175].

Matrix-bound photolabile RGDS peptide was used for investigating the effect of persisting versus temporally modulated RGD environment on hMSC viability and chondrogenic differentiation. Mimicking native chondrogenic differentiation, during which fibronectin is initially produced but subsequently downregulated and replaced by glycosaminoglycans (GAGs) and type II collagen (COLII), was achieved by removing RGD sites after initial culture.

By utilizing multiple modes of cross-linking of acrylated hyaluronic acid in a sequential manner, degradability of 3D matrices could be regulated temporally [176]. During the primary polymerization step hydrogels with adhesive sites and MMP-cleavable dithiol cross-linkers were formed via an addition mechanism, which still left a portion of the acrylate groups unconsumed. At this stage the matrices were permissive to remodeling and migration but by localized light exposure (using a photomask or a focussed laser) could be further cross-linked rendering them unfavorable for such cellular functions. Robust spreading of mesenchymal stem cell was observed within the permissive areas and found to be dependent on the relative amount of each cross-linking mode whereas in highly cross-linked "inhibitory" hydrogels cells remained rounded. Such differences in cellular morphology could be useful for studying signaling mechanisms during spatially controlled differentiation of encapsulated stem cells.

Spatiotemporal regulation of biological compounds in synthetic microenvironments, trapping them in cages and subsequently uncaging them the site of interest, has been shown to allow for a nearly instantaneous manipulation of the bioactive compound concentration [177]. Small signaling molecules and chemically synthesizable peptides have been successfully caged based on chemical modification of the molecules using a photo-removable protective group. This strategy is in most cases not compatible with large proteins, such as growth factors, which require a tailored caging procedure. Also caging is often complicated due to difficulties in achieving site-specific chemical modification.

FXIII cross-linkable TG-PEG hydrogels could be rendered photosensitive by masking the active site of one of the FXIIIa substrates with a photolabile cage group [146], namely nitroveratryloxycarbonyl (Nvoc), which by its broad absorption in ultraviolet/visible around 350 nm is advantageous in terms of penetration depth and minimal DNA and biomolecule damage [177]. Caged Lys-substrates within the polymerized hydrogel network could be subsequently released enabling highly localized enzymatic biomolecule tethering. This system was exploited for light-activated enzymatic gel patterning to manipulate the behavior of live cells within the hydrogel microenvironment. 3D invasion of human MSCs was chosen as a physiologically relevant model, illustrating injury-induced cell recruitment. RGD, the recombinant fibronectin fragment FN_{9-10}, and platelet-derived growth factor B were engineered to contain the TG-peptide for cross-linking into the matrix. Microtissues of MSCs were encapsuled into MMP- and photosensitive TG-PEG hydrogels and a cuboidal pattern of uncaged TG-peptide was then created on one half of the microtissue. The enzymatically immobilized, fluorescently labeled RGD and FN_{9-10} permitted increased MSC migration within the patterned areas.

This demonstrated that light-activated enzymatic gel patterning can be exploited for manipulating the behavior of live cells in three dimensions directly in culture. The same strategy was employed on hyaluronic acid hydrogels [178].

To create a generic retain and release system applicable to any arbitrary protein with an fc-tag, a strategy based on pharmacological cage was employed [144]. The cage was formed by covalently coupling novobiocin to an epoxy-activated agarose matrix via a nucleophilic addition reaction. An adaptor protein consisting of the novobiocin-binding domain of the bacterial protein gyrase subunit B (GyrB) fused to the IgG-binding domain ZZ (derived from Staphylococcus aureus protein A) was used for anchoring proteins via an fc-tag. Protein contained in the cage could be subsequently released by addition of free novobiocin competitively inhibiting the binding between the adapter and the cage. Caging and rapid uncaging were demonstrated by regulating MSC migration out of microtissues in 3D hydrogels by controlled release of caged Fc-PDGF.

Another pharmacochemical approach based on controlling the activity of a growth factor via dimerization was used as a basis for an inducible on–off regulation for cysteine-knot growth factors such as VEGF [179]. The switch consisted of a chimeric protein with an engineered monomeric variant of the protein of interest fused to the inducible dimerization domain of the bacterial protein gyrase B (GyrB). The default state of the switch was off as the monomeric structure prohibits dimerization and thereby activation of the protein receptor. The switch could be turned on upon the addition of coumermycin which binds to GyrB, leading to dimerization of the protein and thus the activation of the receptor and downstream signaling processes. The off state can be restated by the administration of novobiocin, upon which the single coumarin ring of the novobiocin competitively inhibits binding of coumermycin to GyrB, returning the protein to its monomeric state. On–off regulation of the system was demonstrated by controlling the VEGF-induced migration of HUVECs in PEG hydrogels in 3D. As coordination of endothelial cell migration is a key component in angiogenesis, methods to study its regulation are highly valuable for both basic research and tissue engineering.

1.8.2 Mechanically Dynamic Materials

Cells embedded in the ECM of tissue encounter and respond to ECM stiffnesses in the range of 0.2–1 kPa (brain) to 30–45 kPa (osteoid). They actively exert pushing and pulling forces on their surroundings, which results on the activation of intracellular mechanotransduction pathways [180]. For example, vascular endothelial cells experience different types of flow which directs their behavior and stiffening of the ECM in the liver drives liver fibrosis. Mechanics are also tightly coupled with stem cell faith demonstrated by the finding that mesenchymal stem cells are driven towards osteogenesis by a stiffer hydrogel environment and towards adipogenesis by a softer hydrogel environment [181]. Despite the biological relevance of mechanically

dynamic systems, there are only few examples described in the literature and almost none based on synthetic hydrogels. Some work has been done on culturing cells in 2D on mechanically dynamic hydrogels which circumvents cell-induced changes in the matrix properties over time complicating the situation in 3D.

Hydrogel mechanics can be controlled for example by introducing chemical groups that can be cleaved by ultraviolet light leading to matrices that soften upon light exposure [175]. By copolymerizing photocleavable diacrylate macromer cross-linkers with PEG monoacrylate (PEGMA)), using redox-initiated free radical polymerization, photodegradable hydrogels were created. The cross-link density of hydrogel networks could be reduced (by light) in the presence of viable human mesenchymal stem cells allowing their transition from a round to elongated morphology. This same system could be used to direct cell migration by 3D patterning paths for encapsulated cells in real time.

In contrast to dynamically softening hydrogels, matrix stiffening, taking place for example during development and wound healing, might actually be a more biologically relevant phenomenon to mimic as it has been implicated to regulate many cellular processes. A sequential cross-linking approach was used to create dynamically stiffening hyaluronic acid hydrogels [182]. Methacrylate functionalized hyaluronic acid was in the first step cross-linked via a Michael-type addition reaction using dithiothreitol (DTT). The remaining initial hydrogel could be further stiffened by radical polymerization of the remaining methacrylate groups using a photoinitiator and ultraviolet light exposure. The system could be used to investigate cellular response to substrate stiffening in terms of cytoskeletal rearrangement and differentiation, though it was currently only reported on cells grown on top of the hydrogel instead of 3D.

The interaction of GyrB protein with novobiocin and coumermycin was utilized for synthesizing stimulus-responsive PEG hydrogels [145]. Thiol-containing GyrB was covalently grafted to multi-arm PEG-vinylsulfone molecules using a Michael-type addition reaction. Stable hydrogels could be formed by addition of the GyrB-dimerizing substance coumermycin. These hydrogels could again be dissolved in a dose-adjustable manner by the antibiotic novobiocin. This matrix could be used for cell growth either in vivo or in vitro, where the stimulus-responsive characteristics can be used to control the release of growth factors or for dynamic tuning of the matrix mechanical properties.

With the incorporation of RGD motifs the hydrogel could support the adhesion and growth of human primary cells derived from gingival epithelial and connective tissue. No cytotoxic effects could be observed upon gel dissolution with novobiocin, which at 50 μM concentration took place in 4 h. In order to add biological functionality with cell-instructive biomolecules like growth factors, the protein A-derived ZZ-domain was fused to GyrB (ZZ-GyrB), thereby allowing for the immobilization of proteins with an Fc-tag. Fibroblast growth factor 7 (FGF-7), a protein acting in a paracrine manner and being a key player in epithelial tissue regeneration, was used as a model protein. Fc-tagged FGF-7 could be released in a dose and time-dependable manner and it was also shown to retain its activity as demonstrated by the dose-related induction of proliferation of gingival mucosal keratinocyte.

1.9 Outlook and Needs

In the previous sections we have described some of the currently available mostly homogeneously engineered hydrogel systems to control cell function in 3D. However, morphogenetic processes (during tissue formation and regeneration) are highly orchestrated events, mastered by chemotaxis, differentiation, or proliferation of multiple cells from different lineages. These complex spatiotemporally regulated processes are driven by spatially restricted microenvironmental cues, consisting of growth factors, matrix components, and mechanical properties. In order to form 3D tissue mimetics and reproducible organoid culture systems such cues need at least to be partially recapitulated to initiate cell-autonomous tissue morphogenesis. Whereas in biologically derived, complex, and not easily amenable to engineering matrices, impressive tissue formation was observed, for fully defined hydrogels systems, optimal matrix properties for many applications still remain elusive. Clearly, much has to be learned about the function of the native ECM and to integrate more specific integrin ligands into synthetic materials. There will also be a need for more and highly specific, modular building blocks for the selective incorporation of multiple different growth factors, cross-linking elements, degradation sites, and cell adhesion sites.

Nonetheless the many existing, sophisticated materials building blocks generated in recent years provide a growing toolbox for the creation of tailor-made synthetic hydrogels and their integration with biological materials (Fig. 1.5a). Such platforms are an exciting starting point for the assembly of complex 3D structured tissue constructs by combining with emerging, highly sophisticated micromanipulation techniques such as cell and materials printing devices, microfluidics, layer-by-layer assembly (Fig. 1.5b), micromolding techniques, or preventive manufacturing. For manufacturing conditions using gel systems many critical issues will have to be solved, for example, the evaporation of water and the consequent shift in hydrogel properties (due to change in polymer cross-link density caused by increased concentrations) during printing. Also building blocks which are formed individually will likely suffer from different materials properties in the bulk and at the periphery. Such boundaries can largely influence the trafficking of cells and could restrict cell interactions and need to be carefully addressed.

Furthermore, in order to allow temporal, operator controlled, site and individual parameter-specific manipulation of the system, different building blocks would ideally respond highly specific to triggers such as light with different wave length, shift in temperature, or presence of minute quantities of small chemicals (Fig. 1.5c). Of course, improved precision of 3D culture systems must go along with the development of monitoring tools for cells and matrix components. Not only cell-based reporter systems but also high-throughput automated image acquisition and image analysis algorithm systems as well as means to understand the remodeling of the provisional matrix components and the deposition of cell´s own ECM need to be followed.

In conclusion, we believe that “advanced” cell culture models hold great promise for the establishment of physiological meaningful 3D tissue mimetics and the

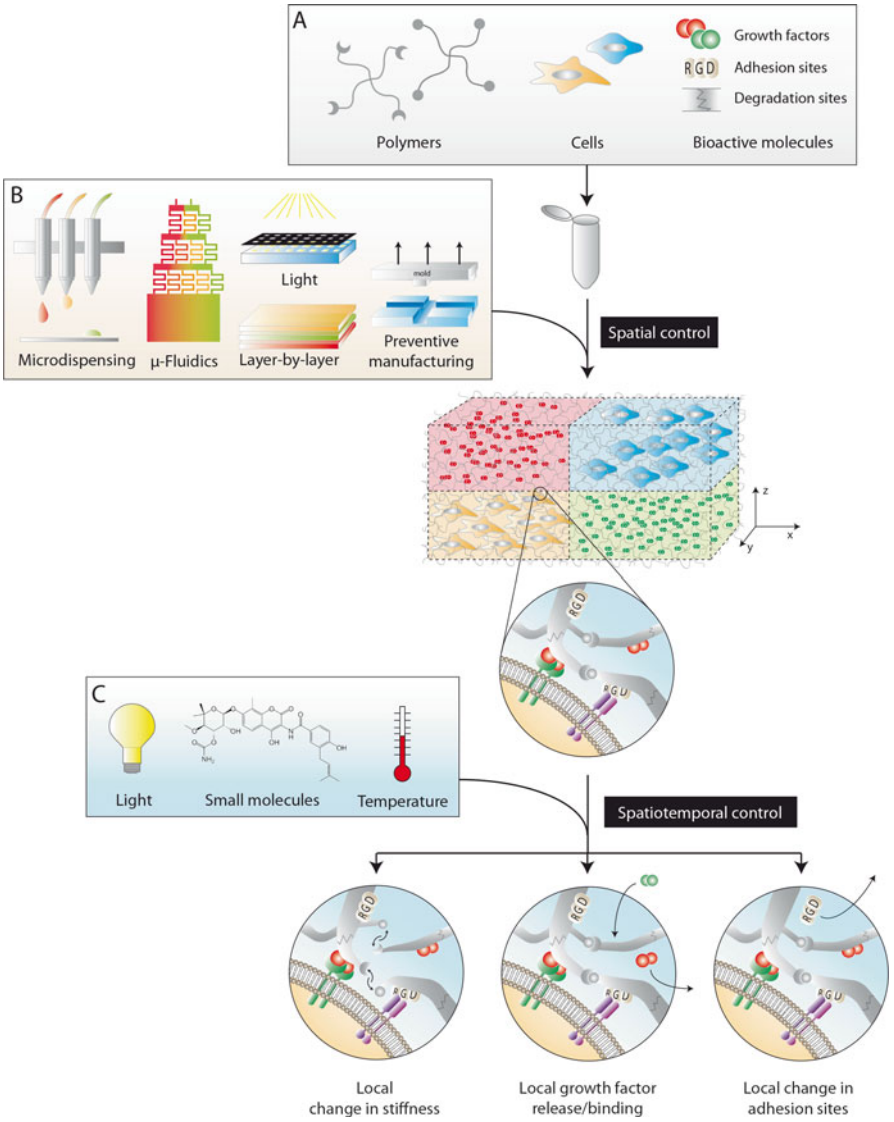

Fig. 1.5 Strategy for creating 3D tissue models starting from (**a**) combining biological elements with synthetic hydrogels, (**b**) structuring these elements, and finally (**c**) eliciting spatiotemporal control over the system

reproducible culture of organoids under highly defined conditions and amenable to investigator-controlled manipulations. Such systems in the future will not only be important intermediates between homogeneous 2D and 3D cultures and in vivo systems and thus could become tools applied to study basic biological questions as well as to validate lead compound during drug screening.

Acknowledgements We would like to express our gratitude to Dr. Philipp Lienemann for producing the illustrations and for helpful discussions.

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