Cell-Instructive Multiphasic Gel-in-Gel Materials

Sebastian Kühn, Jana Sievers, Aukha Stoppa, Nicole Träber, Ralf Zimmermann, Petra B. Welzel,* and Carsten Werner*

Developing tissue is typically soft, highly hydrated, dynamic, and increasingly heterogeneous matter. Recapitulating such characteristics in engineered cell-instructive materials holds the promise of maximizing the options to direct tissue formation. Accordingly, progress in the design of multiphasic hydrogel materials is expected to expand the therapeutic capabilities of tissue engineering approaches and the relevance of human 3D in vitro tissue and disease models. Recently pioneered methodologies allow for the creation of multiphasic hydrogel systems suitable to template and guide the dynamic formation of tissue- and organ-specific structures across scales, in vitro and in vivo. The related approaches include the assembly of distinct gel phases, the embedding of gels in other gel materials and the patterning of preformed gel materials. Herein, the capabilities and limitations of the respective methods are summarized and discussed and their potential is highlighted with some selected examples of the recent literature. As the modularity of the related methodologies facilitates combinatorial and individualized solutions, it is envisioned that multiphasic gel-in-gel materials will become a versatile morphogenetic toolbox expanding the scope and the power of bioengineering technologies.

1. Introduction

Living matter is typically heterogeneous, of hierarchical structure and highly dynamic with cells of different phenotypes and extracellular matrix (ECM) components acting in concert across different length scales in multifunctional, adaptive tissues and organs. Mammalian tissues are characterized by specific architectures, cell (pheno)types and ECM variants, and emerge in embryonic development or regeneration through bottom-up regimes by assembling sets of building blocks across length scales from molecular subunits over micrometer-sized units up to macroscopic dimensions. Importantly, tissue formation involves the interplay of endogenous cellular programs and cellular stimulation by exogenous (ECM-based) cues, which are reciprocally connected in being cause and consequence of the process. In general, functionality is gained with increasing levels of structural complexity of tissues. Additional heterogeneity can be generated out of existing tissues as, for example, in the response to altered environmental conditions or stimuli such as immune reactions.

Following nature’s lead (see Figure 1), multiphasic biomaterials receive more and more interest as they can enable the more faithful reconstitution of complex living tissues both in tissue-engineered biomedical constructs and in tissue/disease models for mechanistic biological studies and drug screening. The multiphasic constructs can be obtained by a range of bottom-up and top-down methodologies using cell- and tissue-instructive materials that mimic selected features of tissues in spatiotemporal arrangements such as structure, biochemical composition, mechanical properties or degradation/remodeling. Heterogeneity is achieved by the distribution of cells—directly embedded into multiphasic materials during processing or seeded to intermediate or final structures—or by spatiotemporally varying cell-instructive material properties. In either type of approach, ensembles of exogenous cues acting on cells create the starting point for the emergence of complex biological systems. Thus, cell-instructive multiphasic materials require dynamic features such as the susceptibility to cell-secreted enzymes—to effectively template the formation of tissue-like structures. Importantly, as recently pointed out by Brassard and Lutolf, tissue development can be directed by exogenous cues—including engineered matrix materials—at multiple stages. Using this option can elevate the physiological relevance of the resulting constructs.

Polymeric hydrogel networks are an increasingly prominent class of biomaterials and critical enablers of many tissue engineering approaches, creating both permissive and instructive conditions for the survival, growth, assembly and differentiation of cells. The respective gel systems include reconstituted assemblies of natural ECM components (collagen and gelatin, hyaluronate, alginate, and cellulose derivatives), synthetic (often poly (ethylene glycol)(PEG)-based), and several semisynthetic
or biohybrid hydrogels (such as glycosaminoglycan(GAG)-containing materials). Tremendous recent advances in the design and functionalization of engineered hydrogel systems were achieved targeting the recapitulation and systematic tuning of physical and biomolecular key features of natural ECMs. In that context, biology-inspired concepts and (components of) biopolymeric structures were shown to be instrumental. However, Matrigel, a basement-membrane matrix extracted from Engelbreth–Holm–Swarm mouse sarcomas, is still very widely used as it was demonstrated to enable a plethora of organotypic and organoid culture studies despite of its complex, ill-defined composition and limited tunability. Recently, a number of more simplistic, defined, highly liberal and reproducible synthetic gel types have shown equivalent or superior results. Current trends in the design of biomedical hydrogels aim at implementing advanced cell- and tissue-instructive characteristics through rational design approaches, i.e., further expand cell-instructive (rather than permissive) properties of thoroughly tunable hydrogel platforms. Important requirements to be met by successful gel systems are the independent modulation of different ECM-derived matrix characteristics and the implementation of tissue-mimetic dynamic and stress relaxation characteristics through reversible crosslinking schemes. Moreover, in efforts to further expand the tissue-templating capabilities of hydrogel matrices, multiphasic systems with hydrogel components have been introduced. Composites consisting of a hydrogel phase and a nonswelling scaffold or particles to create structure or provide reinforcement are already rather well established and applied for bone and cartilage tissue modeling and engineering.

Against this background, the current review article focuses on multiphasic cell-instructive materials, however, restricts itself to systems which are solely composed of highly hydrated hydrogel phases. Multiphasic soft and highly hydrated materials are thought to be particularly powerful for the templating of complex tissue-like systems. As the progression of developmental processes of tissues and organs in vivo is characterized by the coexistence and dynamic variation of locally differing and highly interactive environmental signals, multiphasic hydrogel systems containing spatiotemporally graded cell-instructive cues provide particularly powerful analogues and enable effective tissue-templating for both in vivo and in vitro tissue engineering applications. We summarize methods for creating multiphasic hydrogel (gel-in-gel) biomaterials, discriminating various types of spatio (macro, micro, nano)-temporal heterogeneity in composition/(bio)chemistry, mechanics and structural features, as well as their level of complexity. The discussed approaches are categorized according to the formation by i) assembly of distinct gel phases, ii) the embedding of gels in other gel materials, and iii) the patterning of preformed gel materials. Capabilities and limitations of the respective methods will be discussed. The review will not only cover engineered multiphasic biomaterials with stepwise changes in properties between discrete phases but include as well anisotropic materials with continuous transitions of properties as they are frequently occurring in living tissues (e.g., biosignal distribution and matrix mechanical gradients). Subsequently, we will highlight the potential of multiphasic gel-in-gel materials with some selected examples of the recent literature. These

Sebastian Kühn received his M.Sc. degree in biomedical engineering from the Imperial College London in 2016 and joined the group of Prof. Molly Stevens where his research was directed at the delivery of nanoparticles from hydrogels for therapeutic purposes. He is now a doctoral researcher in the group of Prof. Carsten Werner at the Leibniz Institute of Polymer Research in Dresden, Germany. His Ph.D. thesis research is focused on the development of glycosaminoglycan-based hydrogel microparticles for biomedical applications.

Jana Sievers received her M.Sc. degree in molecular bioengineering from Technische Universität Dresden in 2015 developing thermoresponsive cell-instructive hydrogels. She is now a doctoral researcher in the group of Prof. Carsten Werner at the Leibniz Institute of Polymer Research in Dresden, Germany, and works in close collaboration with the group of Prof. Peter Fratzl (Max Planck Institute of Colloids and Interfaces Potsdam, Germany) and the group of Prof. Claudia Fischbach-Teschl (Cornell University, Ithaca, NY, USA) on in vitro models of the invasion of breast cancer cells into bone using mineralized macroporous cryogels.

Carsten Werner is professor of biofunctional polymer materials at the Technische Universität Dresden Center for Regenerative Therapies and directs the biomaterials program at the Leibniz Institute of Polymer Research Dresden e.V./Max Bergmann Center of Biomaterials, Germany. His research aims at recapitulating functionalities of living matter in engineered polymer materials and includes studies on electrosurface phenomena, hemocompatible interfaces, and cell-instructive material platforms for regenerative therapies and tissue models. Carsten Werner has organized several scientific conferences and is cofounder of successful spin-off enterprises.
examples are ordered according to the dominating feature of their heterogeneity being either spatiotemporal variations in biomolecular composition, compartmentalization, mechanical properties, and structure. In a concluding section, we will give a perspective on the further sophistication of multiphasic gel-in-gel materials, highlighting options for adaptive spatiotemporal control of morphogenetic signals in systems integration approaches to guide multiple stages of development. Moreover, we will discuss how multiphasic hydrogels can enable combinatorial and individualized solutions for tissue-templating materials [2,48].

2. Methodologies for Producing Multiphasic Gel-in-Gel Materials

The subsequent section provides an overview of methods suitable for the formation of gel-in-gel materials. The described approaches can be distinguished according to their general mode of operation (Figure 2): they either rely on the assembly of distinct gel phases (A), the embedding of gel units in other gel materials (E), and the patterning of preformed gel materials (P). Techniques to be discussed include photopatterning (P), microfluidic-based methods (P), embedding and assembling of macroporous hydrogels (A, E) or hydrated fibers (A, E), hydrogel layering (A), microgel assembly and embedding (A, E), and bioprinting (A, E, P).

2.1. Hydrogel Layering

Multilayered hydrogels can combine physically and (bio)chemically different environments in cross-scale tissue-mimetic arrangements. Several manufacturing principles are available for the production of engineered multiphase 3D constructs resembling the native tissue architecture. In most of the cases, the basic structure of multiphase systems is achieved by bidirectional (sandwich systems) or coaxial layering which is presented in the following sections.

2.1.1. Bidirectional Layer Systems

Bidirectional multilayer constructs offer a variety of possibilities for the reconstitution of the spatial organization of tissues over several length scales. To produce sandwich systems, hydrogel building blocks consisting of microgel particles or successively
polymerized networks of various properties are stacked in layers.

Classical particle-based multilayered structures have often relied on electrostatic interactions that led to the self-assembly of controllable films. Over the past few decades, this classic layer-by-layer technology has been developed further and extended to other areas. Hydrogel particles offer the opportunity to be stacked and thus to form scaffolds with defined pore sizes. This very recent technique is discussed more in detail in Section 2.5.3.

Alternatively, polymer networks of different composition or properties can be layered directly. Parratt et al. have introduced multilayered constructs with a spatially varying material composition of poly(ethylene glycol) diacrylate (PEGDA) to mimic the hierarchical structure of human articular cartilage. Individual biomaterial combinations were partially polymerized and layered on top of each other in order to generate trilayered scaffolds for human bone marrow stromal cell differentiation.[50]

Physical stability of the individual layers can be achieved by the introduction of support materials. Derda et al. established a multizone paper platform to mimic multistructured 3D tissue.[51] Cell-laden and cell-free hydrogel slabs of different material composition and biofunctionalization are separated and mechanically supported by cellulose. These various options provided by the platform were used to compare the growth of 3D tumor models of different spatial composition and to examine the migration of cells in these structures.

Multilayered systems not only provide a platform for combining different compositions, but are also particularly suited for cocultures to spatially separate cells that require different growth and environmental conditions. Marchioli et al. have established bilayered composites made of PEG and PEGDA hydrogels. Here the PEG layer containing vascularized cells was pregelled and crosslinked on top of the PEGDA layer containing cell aggregates.[52] In another report, rat hepatocytes were sandwiched by two prevascularized PEG layers that were crosslinked via UV light.[53] Multilayered hydrogels prepared by photopolymerization with spatially varying mechanical properties and compositions were employed, for instance, to mimic the articular cartilage with its spatially varying structure.[54] Similarly, spatially varying biochemical and mechanical properties for osteochondral tissue engineering were realized by sequential photopolymerization of a PEG-based hydrogel.[55] Further examples as well as technical requirements of photopolymerization reactions can be found in Section 2.7. Another common method of producing multilayered hydrogels is 3D printing technology, where an object is built up in a layer-by-layer fashion.[56] Section 2.6 introduces a variety of multiphase systems that have already been developed using this technique.

2.1.2. Coaxial Layer Systems

Coaxial arrangement of hydrogel layers, in particular, round, and tubular shapes are being used for spheroid cultures or for the imitation of blood vessels.

Singh et al. encapsulated tumor cells in enzymatically cleavable peptide-functionalized PEG hydrogels. Subsequently, they added a collagen layer cultured with human dermal fibroblasts to study tumor progression.[57] The fabrication of multilayered outside-in-constructed chitosan hydrogels of various architectures have been reported by Nie et al.[58] Chitosan polymerizes when it comes in contact with hydroxyl ions. The contact area of the tubular chitosan structure turns to the first hydrogel layer immediately after contact with the coagulation bath. Hydroxyl ions diffuse into the structure and induce a gradual gelation that creates multiple hydrogel phases. The layer-oriented characteristics of the radial pattern of concentrically encircled gels were achieved by varying the gelation behavior and the entanglement of the polymer chains.

2.2. Assembly and Embedding of Macroporous Hydrogels

For tissue engineering applications, materials are usually considered macroporous when their pore size is >100 µm to facilitate cell infiltration.[59] Most of the reported macroporous hydrogels have a pore size of around 100–300 µm. Monophasic macroporous hydrogels are readily used for tissue engineering applications, due to their high porosity. Compared to conventional bulk hydrogels the macroporous architecture provides a high surface area accessible for cells to attach and at the same time allows for better supply of oxygen and necessary nutrients and removal of metabolic waste products.[59,60]

Several different types of techniques exist to fabricate macroporous hydrogel scaffolds. Common methods are salt/porogen templating (removal of porogen via leaching), gas foaming, cryogelation, electrospinning and solid free form fabrication such as 3D printing or assembly of microparticles. A detailed description of the different methods, including their advantages and limitations, is for example given in a review by De France et al.[59]

This section focuses only on multiphasic materials where all the different phases consist of highly hydrated networks. Therefore, we do not consider multiphasic macroporous scaffolds which are based on hydrophobic polymer materials, such as polyactic acid,[61] polyactide-co-glycolid,[62,63] polycaprolactone[64,65] or scaffolds made from calcium phosphates[66–68] as commonly applied for bone or cartilage tissue applications. The fabrication of these type of scaffolds is often based on salt/porogen templating, which still represents one of the most extensively used techniques to produce macroporous scaffolds,[59] however it is not that commonly applied to form macroporous hydrogels.[69] One example of a macroporous multiphasic hydrogel fabricated based on salt templating is the work of Chwalek et al.[70] Porous silk sponges were fabricated, serving as an anchoring scaffold for neuronal cells, and then filled with a softer collagen gel matrix to permit axonal outgrowth. A simple and versatile technique to obtain macroporous hydrogel scaffolds presents cryogelation, since the production of cryogels is generally based on an aqueous reaction mixture.[71] Cryogels, in turn, provide a powerful base for the fabrication of multiphasic materials, including gel-in-gel systems highlighted in this review.

2.2.1. Cryogel Formation

Cryogelation is a technique in which the polymerization of an aqueous monomer solution is performed under sub-zero temperatures.[66,71] Freezing of the reaction mixture results in
the formation of ice crystals containing the major fraction of the water and no reactants and thus serve as porogens. Cryo-
topic gelation of the hydrogel network occurs in the unfrozen
regions around the ice crystals where the reactants are con-
centrated (cryoconcentration effect). The ice crystals are later
removed via thawing or freeze-drying and result in scaffolds
with highly interconnected 3D macroporous structures.[60,72]
Cryogels are fabricated using natural biopolymers such as algi-
nate, gelatin or chitosan, but also synthetic polymers including
for example PEG or PEGDA are utilized.[71] Additionally, biohy-
brid approaches for example based on PEG and heparin exist.[72]
The majority of cryogel networks are covalently crosslinked
(e.g., via carbodiimide chemistry or free radical polymerization)
since this results in higher reproducibility and mechanical sta-
bility as compared to physical linkage.[73]

One major advantage of the cryogelation technique is the
rather easy and low time-consuming preparation.[71,73] As com-
pared to salt templating, the porous structure is usually more
interconnected and the production usually does not require the
use of organic solvents. Furthermore, by adjusting the freezing
conditions (cooling rate and time), their pore sizes and geom-
etries can easily be tailored. Moreover, relatively big scaffolds
can be produced in a short time frame showing a wide variety
of shapes, e.g., discs, blocks, sheets, or beads. For example,
Savina et al.[74] introduced a method for production of large
volume cryogels (400 mL) by the introduction of a prefreezing
step. Zhang et al.[75] applied a stage cooling method to fabricate
scaffolds with different pore sizes and structures, including
cryogels with pores on the vertical axial direction or scaffolds
with a gradient pore structure. In addition to macroscopic scaf-
diffs, micrometer-sized cryogels can also be produced using for
example water-in-oil emulsions, or flow focusing.[76,77]

Polymeric cryogels are generally considered to have superior
mechanical stability since they can withstand large deforma-
tion.[78] As a bulk material, cryogels are a rather soft material
(low bulk stiffness) because of their highly porous architecture.
However, due to the cryoconcentration effect, they contain thin
but dense pore walls that provide structural stability.[72,79]

For tissue engineering applications, the highly intercon-
ected pore structure enables rapid cell ingrowth and expansion
due to the available surface area inside the scaffolds. Moreover,
the scaffold protects housed cells from mechanical stress.[60,73]
As a result, cryogels are often used as a delivery vehicle for
cells and signaling molecules.[76,78,80–82] Cells delivered via cry-
ogels were found to show superior cell viability as compared to
simple injection or infusion.[83] However, one drawback of the
highly interconnected porous structure of cryogels is that
the available surface area is lower as compared to macroporous
scaffolds formed by other techniques.[84]

In summary, different design parameters of cryogels can be
adjusted to obtain macroporous scaffolds with highly tunable
cell-instructive properties (Figure 3, left).

2.2.2. Multiphasic Cryogels

Formation of various types of multiphasic cryogels are reported
in literature. Depending on the technique that is used and
the type of matrices that are combined, additional design
parameters arise from the multiphasic nature of the material
and thus allow for the fabrication of materials with higher
complexity (Figure 3, right). This enables to further extend
the applicability of cryogels for more sophisticated hydrogel-
based engineering approaches. As compared to bulk hydrogels,
macroporous cryogels show superior mechanical properties,
favor cell infiltration and provide superior supply with oxygen
and nutrients. However, the major limitation of the rather
macroscopic scaffolds remains their structural resolution. Even
though cryogels can also be processed into microparticles, pre-
cise tuning of their internal architecture is limited compared to
other methods such as 3D printing. However, their rather low-
cost and straightforward manufacturing process, as well as the
ability for upscaling, still make them very appealing for various
biomedical applications. Therefore, multiphasic approaches
which connect different types of cryogels and more importantly
those that combine cryogels with conventional hydrogel mate-
rials could be of high interest for future biomaterial design.

The four most common techniques to form multiphasic
cryogels are illustrated in the center of Figure 3. Cryogels can
either be embedded into other matrices by filling them or
coating them with another hydrogel-based material or they can
be assembled via layering. Furthermore, preformed hydrogel
structures can directly be incorporated in the production pro-
cess of cryogels (heterotypic reaction mixtures).

**Embedded Cryogels:** One method to form multiphasic cryogels is by filling the cryogel with another polymeric hydrogel solu-
tion (Figure 3, center).[85,86] In this technique, cryogels often
serve as a scaffolding structure that provides physical support
(reinforcement), which are then used in combination with
rather soft matrices that are filled inside. For example, Pustlauk
et al.[85] combined a soft hydrogel alginate with a mechanically
stiffer, porous collagen scaffold to induce chondrogenic differ-
entiation of mesenchymal stromal cells (MSCs), which requires
a soft matrix that alone is however difficult to handle. Kin-
neberg et al.[87] used a macroporous fibrous collagen scaffold
and filled it with soft and/or stiff PEG hydrogels to either form
monolayered or bilayered scaffolds. The incorporation of the
collagen scaffold in particularly improved the tangent modulus
and toughness of the PEG hydrogel.

**Coated Cryogels:** Cryogels can be coated with other polymer
matrices (Figure 3, center).[88,89] This can improve the biofunc-
tionality of the cryogel matrix and thus increase cell attach-
ment and proliferation. For example, Damania et al.[88] used
decellularized liver ECM and crosslinked it to the surface of
P(NIPAAm)–chitosan cryogels with glutaraldehyde.

**Layered Cryogels:** One of the most commonly applied strate-
gies for the formation of multiphasic cryogels is the construc-
tion of multilayered structures (Figure 3, center middle row).
With this method, the aim is often to mimic the layered nature
of tissues in the human body, such as the skin,[90–93] articular
cartilage,[93–97] or tendon.[98–100] Layered structures are thereby
formed either by layering a cryogel with another cryogel (homo-
typic layering) or by combining a cryogel with a different type
of scaffold, such as a hydrogel[101] or a 3D printed structure[102]
(heterotypic layering). Regarding the preparation process, one
can furthermore distinguish between two possible options:
i) postsynthesis assembly of cryogels or ii) simultaneous syn-
thesis of different types of cryogels (presynthesis assembly).
For postsynthesis assembly, the two different scaffolds need to be joined by gluing or suturing. For example, Saha et al. used a commercially available fibrin glue to bring different types of macroporous silk fibroin scaffolds together. Chen et al. applied medical alpha-cyanoacrylate adhesive for joining a chitosan membrane with a chitosan porous sponge. Bray et al. reported an alternative option, by using an additional PEG–heparin hydrogel solution as a “glue” to attach a preformed PEG–heparin hydrogel containing breast cancer cells on top of a PEG–heparin cryogel seeded with osteoblasts to create a biphasic scaffold for the study of breast cancer cell invasion into bone.

A “glue-free” method for the joining of cryogels that is often applied is the formation of the cryogel in the presence of an already preformed network. For example, Levingstone et al. used this technique to fabricate a trilayered scaffold for osteochondral repair via sequentially forming one cryogel on top of each other. The different layers hereby consisted out of collagen type I plus hydroxyapatite (bone layer), collagen type I and type II plus hydroxyapatite (intermediate layer), and collagen type I and type II (cartilage layer). A similar approach was used by Caliari et al. to form a core–shell scaffold consisting out of a high-density collagen–GAG membrane wrapped around a low-density macroporous collagen–GAG scaffold. The assembly was performed by placing the preformed membrane in a mold and then forming the macroporous scaffold with aligned pores in the middle. The high-density membrane hereby provided the mechanical integrity needed for a material to be used in tendon tissue engineering, while the macroporous core facilitated cell infiltration. Tellado et al. used this technique to fabricate a biphasic silk fibroin based scaffold with integrated anisotropic and isotropic porosities that was designed to create topographical cues to stimulate tendon/ligament-to-bone tissue regeneration. A priorly formed macroporous scaffold with isotropic pores was placed into a mold, on which the reaction mixture of the cryogel was added. A second polystyrene mold around the first mold then acted as a heat insulator along the lateral surface to create anisotropic pores within the cryogel. An overview of the cell application of the designed bilayered cryogel is given in Section 3.

One disadvantage of postsynthesis assembly is the formation of an abrupt hard interface between the different scaffolds that is often mechanically weak. Therefore, Harley et al. established a “liquid-phase co-synthesis” in which they simultaneously formed one mineral-containing and one nonmineralized layer with a stable coherent interface between the two phases. They sequentially poured a collagen type I and a chondroitin-6-sulfate solution into a polysulfone mold and let the layered
assembly is presented by Golunova et al.\cite{104} In their work, with distinct regions of mineral content and pore anisotropy forming aligned pores at the bottom of the scaffold. Thus, scaffolds mismatch throughout the layered reaction mixtures in order to other, they further extended the system by introducing a thermal fusive nonmineralized and mineralized phases on top of each other. However, in addition to forming scaffolds with interdiffusive nonmineralized and mineralized phases on top of each other, they further extended the system by introducing a thermal mismatch throughout the layered reaction mixtures in order to form aligned pores at the bottom of the scaffold. Thus, scaffolds with distinct regions of mineral content and pore anisotropy were obtained. Another method to circumvent postsynthesis assembly is presented by Golunova et al.\cite{104} In their work, electron beam initiated polymerization was used to form continuously layered acrylamide-based cryogels. This was achieved by sequential layering of reaction mixtures with and without propargylacrylamide at subzero temperature. A new layer was added when the previous layer had frozen. Due to superficial thawing of the previous layer when a new unfrozen solution was added on top, the procedure allowed for partial mixing at the layer interface. The final fully frozen samples were irradiated by electron beam to induce the polymerization and subsequently thawed to obtain continuously layered cryogels, which contained phases with and without alkyne groups. The latter were used for further modifications of the scaffolds using click-reaction.

**Heterotypic Reaction Mixtures:** Multiphasic cryogels can also be formed by incorporation of another already preformed hydrogel into the formation process of cryogels (heterotypic reaction mixture; see Figure 3, center bottom row). For example, Quinlan et al.\cite{105} added growth factor loaded alginate microparticles into the cryogel reaction mixture and thus formed collagen-hydroxyapatite cryogels containing alginate microgels in their matrix. Furthermore, Plieva et al.\cite{106} introduced a method for the formation of a double-continuous cryogel by synthesizing a new cryogel inside the interconnected macro pores of a preformed primary cryogel. The double-continuous macro porous cryogels were prepared from various different precursor solutions, including for example polyacrylamide or PEG. Similar to this, Yetiskin et al.\cite{107} also formed new cryogels within the pores of a precursor cryogel and thus developed triple-network cryogel scaffolds based on silk fibroin containing three generations of pores that showed improved mechanical performance.

### 2.3. Assembling and Embedding of Fibers

Matrix-embedded fibers are ubiquitous in living tissue with muscle fibers and tendons being prominent examples. Fibrillar architecture is a basic feature of many ECM assemblies and important for 3D mechanosignaling events such as focal adhesion formation.\cite{108-110}

Neither nanoporous, nor macroporous hydrogel scaffolds can mimic the sub-microscale fibrillarity of the natural ECM. Thus, several nanofiber-based systems were developed besides the well-known hydrogel scaffolds formed via self-assembly of ECM-based proteins, such as collagen or fibrin that often lack the required stiffness or stability and mechanical tunability necessary for many applications. Nanofiber scaffold fabrication based solely on such self-assembly methods is reported elsewhere\cite{111,112} and not considered in this review.

Multiphasic fiber-based materials can be classified in hybrid systems of bulk hydrogels with embedded nanofibers and systems that are solely composed of nano- or larger microfibers, whereby different fibers (building blocks) can be assembled to multiphasic materials for instance by simple layering or by textile techniques, like knitting, weaving or braiding. Additionally, the fiber itself can be multiphasic.

While most of the reported materials are based on nonhydrated polymer fibers,\cite{113} we herein focus on assembled or hydrogel-embedded highly hydrated proteinaceous or carbohydrate nanofibers and hydrogel microfiber assemblies.

Multiphasic scaffolds based on assembled or embedded fibers where heterogeneity is introduced via post-treatment, e.g., photopatterning (compare Section 2.7) or diffusion-controlled reaction of functional groups\cite{114} are not subject of this section.

#### 2.3.1. Fiber Production

Besides self-assembly of nanofibers, various approaches exist for fabricating of nano- and microfibers from naturally derived or synthetic materials. These include electrospinning, wet spinning, biospinning, interfacial complexation, microfluidic spinning and melt spinning as reviewed for instance in.\cite{115}

Electrospinning has been used to produce nanofibers, made of natural polymers, like collagen,\cite{116} silk,\cite{117,118} chitosan or hyaluronic acid.\cite{119} Coaxial electrospinning is an upcoming technology that has emerged from the conventional electrospinning process in order to realize also the production of nanofibers of less spinnable materials with potential applications. Jalaja et al.\cite{120} fabricated biphasic nanofibers with highly spinnable gelatin as core and chitosan as shell.

Pure or cell-laden hydrogel microfibers with diameters in the range of tens to several hundred micrometers were mainly obtained by wet spinning, microfluidic spinning, and interfacial complexation. For wet spinning, the polymer precursor solution is injected into one or multiple coagulation baths. Alginate, collagen and chitosan fibers have been produced among others. Cell encapsulation is possible, however long exposure of the cells to toxic crosslinkers should be prevented. Also, multiphasic fibers were produced by a wet-spinning approach that employs a coaxial spinneret. Mirabedini et al.\cite{121} thereby obtained chitosan/alginate core-sheath fibers for the first time. Microfluidic fiber spinning enables the production of single or multiphasic hydrogel microfibers with precise control in their size, shape, chemical composition and cell distribution.\cite{122}

Very often alginate was used and physically crosslinked via calcium or barium ions. Using a double coaxial laminar flow device, Sugimoto et al.\cite{123} obtained biphasic microfibers that combined mouse pancreatic beta cells encapsulated in a collagen core with an alginate sheath that covers the core to realize a mechanically robust hydrogel wire. Another example of multiphasic hydrogel microfiber production using a capillary microfluidic chip\cite{124} will be described in Section 2.4.2.
2.3.2. Nanofiber–Hydrogel Hybrid Systems

Nanofibers include natural ECM proteins, recombinant proteins, self-assembled peptide amphiphiles, or peptide-modified polymers, but can also be obtained by biospinning or electrospinning as described above.

Such nanofibers exhibit advantageous mechanical properties and can therefore be used to reinforce soft hydrogel materials. The fibers, fiber aggregates or various constructs of the fibers (nanofiber assemblies) are usually added to the hydrogel precursor solution that can also contain cells. After curing fiber-decorated hydrogels with improved mechanical properties are obtained. The fibers additionally determine the micro-mechanical properties of the system that can also influence the behavior of encapsulated cells and usually differ from its bulk mechanical properties.[125] In the context of mimicking fibrous tissues or the fibrillary nature of the ECM, the incorporation of fibers into soft hydrogel materials is moreover highly attractive for the design of structurally complex hydrogels. Fibers are known to align and guide cells, like myoblasts, cardiomyocytes and neuronal cells. The presented strategy is especially interesting for introducing structural attributes in in situ forming injectable hydrogel materials that usually form isotropic homogeneous matrices. As a complementary technique to micro-particle-based approaches (compare Section 2.3) it may help to overcome the formation of guiding structures in situ that is still a major challenge. Fibers can be just physically embedded, form an interpenetrating network with the hydrogel network or be chemically linked to the hydrogel network. For several applications defined alignment of the fibers in a hierarchical manner would be interesting, for instance for mimicking tissues with anisotropic and heterogeneous features, like the meniscus with its highly heterogeneous and complex ECM.

Nanofiber–hydrogel hybrid materials have been reported to be advantageous for tissue engineering in load bearing cartilage tissue in order to provide structural support to the commonly used natural hydrogel materials and to improve their mechanical properties. Silk fibers were embedded in silk matrices[126,127] or thermosensitive chitosan hydrogels.[118] Encapsulated chondrocytes showed enhanced deposition of GAGs and localization of collagen II indicating support of the chondrogenic phenotype. In a similar manner guiding structure and functionality was introduced in a set of semisynthetic PEG–heparin hydrogels by adding collagen I fibers during gelation[128] as demonstrated by local variations in stiffness and adhesion ligand density. Embedded MSCs aligned to the collagen microstructures in vitro. Zhan and Lowik[129] embedded self-assembled peptide amphiphile fibers containing a cellular binding peptide sequence (RGD) in a PEG hydrogel to mimic the natural fibrous structure of the ECM and to stimulate cell adhesion. Peptide amphiphile fibers also contributed to the trapping of cells homogeneously throughout the gel matrix avoiding cell sedimentation.

Recently, an interpenetrating network hydrogel system based on hyaluronic acid crosslinked via dynamic covalent bonds and collagen I was developed that recapitulates both the viscoelasticity and fibrillar architecture of ECM in tissues.[110] These composite materials did not only promote cell spreading and focal adhesion formation when compared to corresponding materials with a hyaluronic acid matrix crosslinked by static covalent bonds, but additionally enhanced fiber remodeling. These results demonstrate that stress relaxation might be important also for physical remodeling.

2.3.3. Fiber Assemblies

Nanofiber Assemblies: Nanofibers have also been investigated for generation of layered or gradient biomaterials, e.g., for interfascial tissue engineering as summarized in.[131] Electrospinning can be utilized as described to produce nanofibers with diameters ranging from tens of nanometers to a few micrometers that can be deposited or processed to pure nanofiber scaffolds with an ECM-like porous and fibrillar structure. Nanofibers possess a large surface area, which is favorable for cell attachment. Fiber material and topography have been shown to play a major role in regulating differentiation and proliferation of neural progenitor cells.[122–124] Moreover, neuronal differentiation of human embryonic stem cells was facilitated by aligned fibrin nanofibers with 400 nm diameter.[135] By varying the physical and chemical parameter of the fibers or their orientation during fabrication and deposition gradient features can be implemented. Extrusion and electrospinning can be combined to generate gradient nanofiber composites. This hybrid technique facilitates the incorporation of various ingredients in a time-dependent way during the electrospinning process. Electrospun fibrous scaffolds often lack complete cellularization and vascularization due to their dense packing and thus limited porosity. Sundararaghavan and Burdick[136] presented a modified electrospinning protocol to drive infiltration of cells into electrospun hyaluronic acid scaffolds by creating durotactic (mechanical) and haptotactic (adhesive) gradients. Besides enhanced infiltration, the reported approach potentially enables the fabrication of graded tissue structures, for instance those occurring at interfaces.

Hydrogel Microfiber Assemblies: As already mentioned, hydrogel fibers produced by wet spinning, microfluidic spinning, or interfascial complexation have rather large diameters (ten to several hundred micrometers). Similar as microgels they can be assembled to larger constructs. However, due to their high aspect ratio these fiber building blocks have been often tested for engineering of fibrous tissues. Assembling methods for pure hydrogel fibers but also for fibers containing biological factors and cells into constructs with biomimetic properties include random fiber deposition or various textile processes, like weaving, direct writing, and winding.[115,117,138] In the majority of the reported studies, fibers fabricated from a variety of natural polymer-based hydrogels, like alginate, collagen, chitosan, or hyaluronic acid were used. As especially cell-laden hydrogels are typically fragile, their processing by textile processes is not trivial. Many approaches aimed to design special setups to reduce exerted mechanical forces or used core-sheath fibers to improve the mechanical strength of the fibers.[139] Such controlled spatial arrangement of different hydrogel microfibers or of multiphasic microfibers into 2D and 3D constructs would allow for the production of materials with higher complexity and more hierarchical levels. So far, this is limited due to the already mentioned lack of mechanical stability.

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2.4. Microfluidic-Based Methods

Engineered cellular microenvironments that mimic the complexity and dynamics of living tissues can be obtained by the control of fluid dynamics at the micrometer scale. Microfluidics enables the fabrication of biomaterials with locally varied compositions and defined microarchitectures. Therefore, microfluidic-based approaches can provide cell-instructive materials with precise spatiotemporal resolutions and require rather small reaction volumes, making them particularly attractive for use in high-throughput applications.

2.4.1. Signal Molecule Gradients

A plethora of methods was developed to create cell-instructive concentration gradients of soluble signaling molecules in microfluidic experiments.

Similar microfluidic approaches have been applied for the formation of signaling molecule gradients in hydrogel matrices. The classical design of microfluidic chips developed for that purpose involves three parallel channels. The inner channel or cell culture chamber is separated from the outer channels by grid or (overhanging) pillar structures and can be filled with a cell-laden hydrogel. The outer channels can be used to supply/exchange the cell culture medium and to form a concentration gradient across the hydrogel.

Kobel and Lutolf reviewed several different approaches that were developed to combine hydrogels with a microfluidic supply of signaling molecules to mimic different cell niches, particularly those of stem cells. Microfluidic channels were directly integrated into hydrogels and used for the continuous supply of cell-instructive factors. The described platforms provide the basis for screening stem cell fate in dynamic microenvironments of different levels of complexity. For example, Kunze et al. used microfluidics to create a multilayered scaffold for the study of B27 (neuronal media supplement) gradients on neurite outgrowth. In the microfluidic device a central main channel is connected with four inlet channels to create a four-layered agarose-alginate hydrogel (two cell layers interlaced by two cell-free layers), which were in part loaded with B27 to create a gradient between the different layers.

Cosson and Lutolf developed hydrogel microfluidic inserts for standard 24-well plates to deliver biomolecules under controlled conditions to stem cells cultured in a conventional, macroscale format. The inserts are placed in individual wells and connected to a perfusion system for precise biomolecule delivery and cell-based assays. The design allows the decoupling of cell culture from microfluidic manipulation and thus allows to overcome shear stress and material related problems in microfluidic long-term cell cultures.

In a recent work by Manfrin et al., a microfluidic-based system was designed to emulate signaling centers that secrete different types of morphogens during early human development in a spatiotemporal varying manner. A more detailed review of this work can be found in the Section 3. Overall, the design of their setup allows the formation of spatially and temporally controlled morphogen gradients and even to study the effect of two opposing gradients on stem cell patterning.

2.4.2. Spatially Graded/Layered Materials

Hydrogels with tunable physical, chemical, and biofunctional properties are widely used to mimic the ECM of cells. The laminar flow in microfluidic channels in combination with in situ crosslinking schemes provides versatile options for the fabrication of spatially graded hydrogel-based microenvironments for cell culture experiments.

Burdick et al. used a microfluidic gradient maker to fabricate photo-crosslinked hydrogels with laterally varying crosslinking densities. Two macromer/initiator solutions were injected into a poly-(dimethylsiloxane) (PDMS) channel system that produces a hydrogel precursor gradient that was subsequently polymerized into a hydrogel upon exposure to ultraviolet light exposure. The technique was also applied for the fabrication of adhesive ligand gradients in order to modulate spatial distribution of attached cells.

Inspired by the knowledge about the hematopoietic microenvironment, Mahadik et al. developed a microfluidic mixing platform to create small volume 3D hydrogel constructs containing overlapping patterns of cell and matrix constituents. The platform was used to generate hydrogels containing opposing gradients of fluorescent microspheres, osteoblasts, primary murine hematopoietic stem, and progenitor cells, and combinations thereof in a manner independent of hydrogel density and cell/particle size.

Kobayashi et al. demonstrated the fabrication of stripe-patterned heterogeneous hydrogel sheets with a thickness of \(\approx 100 \mu m\) and varying stiffness for high-density 3D cocultures by using microchannel-combined micronozzle devices. Hepatoma cells and fibroblasts were embedded inside the hydrogel matrix and cocultured, to form heterotypic microorganoids mimicking in vivo hepatic cord structures. Besides, the platform allows to fabricate relatively large but precisely controlled 3D microenvironments for the high-density cocultures of multiple cell types.

Yu et al. designed a capillary microfluidic chip for the production of cylindrical multiphasic hydrogel-based microfibers in the range of 20–250 \(\mu m\) in diameter. Depending on the experimental conditions, multicompartiment microfibers with different microstructures and geometries (e.g., core–shell or spindle-knotted structure) were produced based on alginate hydrogels. Different types of cells can directly be loaded inside the fibers or later on top, for example to design templates for construction of artificial muscle fibers or blood vessels.

Yoshida and Onoe developed a method for the synthesis of core-shell alginate hydrogel microsprings. Encapsulation of several functional materials including an agarose gel and collagen encapsulating HepG2 human liver carcinoma cells was demonstrated to functionalize the materials. The core-shell hydrogel microsprings have application potential in various fields, including biological/chemical sensing and tissue engineering.

Leng et al. developed a one-step, continuous process for the scalable formation of mosaic hydrogels using a microfluidic system. In the device, solutions of two distinct biopolymers with the option of preloading the second with microparticles, biomolecules, or cells are organized into a planar fluid network that is crosslinked at the exit of the device. The mosaic hydrogel
properties (e.g., elasticity and diffusion of biomolecules) can be
tuned by the molecular composition of the precursor solutions. 
Bottom-up stacking or continuous collection of the hydrogel 
sheets onto a rotating drum enabled the formation of multi-
layered soft materials with compositional control in 3D with 
dimensions ranging from millimeter to centimeter scales. 
According to Leng et al., the introduced method provides the 
basis for a fully automated and continuous format for culturing 
cells in physiologically relevant microenvironments, the sys-
tematic analysis of cell–cell and cell–matrix interactions, and 
the fabrication of functional tissues.[144]

Microfluidics methods are further used to fabricate 
multiphasic hydrogel particles.[145,162–164] Dicker et al. intro-
duced a method for core–shell patterning of synthetic hydrogels 
via interfacial bio-orthogonal chemistry for local control of stem 
cell behavior by spatial variation of enzymatic degradability, cell 
adhesivity, and mechanical properties.[145] The platform allows 
straightforward patterning of cellular microenvironments to 
trigger desired responses or to promote the formation of multi-
layer tissues. Fu et al. developed methacrylated gelatin (GelMA) 
hydrogel encapsulated core–shell photonic crystal barcode par-
ticles for 3D cell aggregation culture and drug screening.[165]
The GelMA shells enable the creation of 3D ECM micro-
environments for cell adhesion and proliferation while the 
photonic crystal cores provide stable diffraction peaks for the 
identification of different cell spheroids during culture and to 
distinguish their biological response during drug testing. 
Furthermore, the encodability of the particles can be used for the 
localized readout of cellular responses in multiphasic microgel-
based materials (Section 2.5).
The different approaches for the formation of physical and 
biochemical gradients can also be combined to fabricate more 
physiologically relevant cell culture platforms that help to dis-
sect the impact of physical and chemical cues on cell fate 
decisions in complex microenvironments.[166] Park et al. used a 
curved microchannel design to study the impact of chemical 
concentration and mechanical shear stress gradients on the 
morphology, migration, and proliferation of L929 mouse fibro-
blast cells. The integration of different microenvironments 
on one chip further provides compact, cost-effective, high-
throughput platforms for multiparameter screening.[167]

2.5. Microgel Assembly and Embedding

Microgels have emerged as highly modular platforms in the 
field of tissue engineering and regenerative medicine covering 
a broad range of applications from delivery of cells and ther-
apeutics over scaffold materials[168–170] to studies on a single cell 
level,[171] cellular mimics,[172] bioprinting,[168,169] combinatorics,[173] and sensing.[174–178] The many sidedness of microgels 
lies within the plethora and versatility of available hydrogel 
systems and the large number of techniques for microgel fab-
rication[169,179,180] allowing for precise engineering and cus-
tomization of mechanical and biochemical properties as well 
as size and morphology (Figure 4). This section will focus on 
the potential of microgels as building blocks for multiphasic 
hydrogel materials and the assembly of complex and cell-
structive hydrogel composites where the performance of the 
system can be described as a function of its components and 
their combination allowing for improved functionality and 
complexity compared to conventional single-phase system. 
While the size of hydrogel building blocks can range down to 
the nanometer scale[181] this section emphasizes on the more 
commonly applied microscaled hydrogels discussing general 
design principles and how the combination of different phases 
offer new directions in tissue engineering.

2.5.1. Microgel Fabrication

A variety of methods has been utilized for the microfabrica-
tion of hydrogels allowing for different degrees of tunability 
with respect to size, morphology, structure, complexity, and 
throughput. The most prominent techniques encompass 
emulsification, micromolding, lithography, extrusion and 
electrospraying.[169,180,182] While simple emulsification and 
extrusion techniques are generally straightforward and easy 
to implement, they are limited to specific hydrogel systems 
and offer a lesser degree of control over size, shape and func-
tionalization than more advanced droplet based microfluidic 
approaches. A sophisticated channel design enables the high-
throughput and combinatorial fabrication of microgels as well 
as the implementation of on-chip functionalization, sorting and 
analysis.[147,179,183–185] Emulsion based techniques also allow for 
the application of cryogelation, yielding macroporous microgels 
with an interconnected pore structure.[179] Due to their geometric 
flexibility, molding and lithography techniques are widely used 
to create microgels with elaborated morphologies.[168,169,186–188]
Additionally, electrospraying and subsequent crosslinking of 
polymer solutions has been employed for microgel fabrication 
of various sizes, shapes and structures.[182]

2.5.2. Microgel Embedding—Gel-in-Gel Materials

In order to provide the essential signaling cues for successful 
cell guidance many hydrogel systems are limited due to a 
lack of anisotropy with respect to chemical, mechanical and 
haptic properties. Following a rather straightforward but effec-
tive approach this challenge can be overcome by the forma-
tion of gel-in-gel materials simply embedding micro scaled 
hydrogel building blocks (microgels) within a hydrogel matrix 
(Figure 4). This method takes full advantage of the versatility 
and malleability of hydrogel systems as highly tunable scaf-
fold material, cell carriers, factor delivery vehicles, etc., and 
enables the formation of complex tissue engineering con-
structs with functions beyond those of conventional homoge-
neous hydrogel systems arising from the composite nature. 
In analogy to cell encapsulation, microgel embedding relies 
on a supporting hydrogel matrix that can either be covalent-
ly or noncovalently crosslinked as reviewed in.[189] The 
benefit of such multiphasic microgel-based materials stems 
from the ability to engineer a system on three different levels 
where i) the microgels and ii) the embedding matrix can 
be modulated independently giving rise to iii) a composite 
whose properties can be tuned through the combination of the 
previous (Figure 4).
Hydrogels have long been proven as excellent vehicles for local administration of therapeutics and the incorporation of additional hydrogel phases can be harnessed to tune the release characteristics and overcome undesirable features such as burst release.[190,191] Chen et al. used covalently crosslinked degradable hyaluronic acid microgels embedded within a hyaluronic acid matrix crosslinked through host–guest interactions between adamantane and cyclodextrin. In addition to showing improved rheological properties for injection, a more sustained and controlled release of therapeutics was achieved through the composite nature of the material.[192] Providing the correct soluble signaling cues is essential to control stem cell fate and trigger/ensure the desired development of artificial tissue constructs. However, many approaches rely on factors supplied through the surrounding medium, covalently bound, physically linked or simply incorporated into the culture scaffold. While the first approach reaches diffusion limits with increasing size of the scaffold, the incorporation of the factors within the matrix requires a specific choice or functionalization of the matrix material which could conflict with the requirements for optimal cell culture. Microgel-based multiphasic materials can offer a valuable option to uncouple these design criteria, thus, extending the range of applicable materials. Although several approaches utilize nonhydrated polymeric microspheres for this purpose,[193,194] microgels offer a greater degree of tunability and better mimic soft components of natural ECM with respect to structure and mechanical properties. Bian et al. formed a multiphasic gel-in-gel material by embedding nanofilm-coated TGF-β loaded alginate microbeads and MSCs within a hyaluronic acid matrix in order to combine the sustained supply of growth factors with a stable 3D environment for cells, respectively.[195] In a similar fashion, Patel et al. merged the demand for an injectable material that provides a mechanically stable matrix as well as ensures sustained supply with essential growth factors for cell development. Using alginate microspheres for BDNF and NGF delivery, PEG-poly(l-alanine) served as a thermos-responsive injectable matrix material for neuronal differentiation of tonsile-derived MSCs.[196] In another example, Wang and Irvine integrated

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**Figure 4.** Design parameters of multiphasic microgel-based materials. Microgel units (left): Microgel building blocks can be designed, fabricated and modified individually, taking full advantage of the plethora of available hydrogel chemistries, functionalizations and processing techniques. Multiphasic (middle and right): The combination of the individually designed modular units within a supporting matrix (embedding) or with each other (assembly) creates complex gel-in-gel materials with additional structural, mechanical and biochemical design parameters allowing for higher dynamic, complexity and functionality compared to single-phase hydrogels. Due to the modularity of the microgel building blocks, the advantages of multiple complementary materials can be blended into one system while the system can also be decoupled with regard to its relevant design parameters.
chemokine-loaded alginate microgels within a collagen matrix and were able to establish local signaling gradients within the system.\cite{197} For more adequately controlling the signaling environment of individual cell types within more realistic tissue models, cell-embedding microgels can be incorporated into a cell-laden hydrogel matrix. Lee et al.\cite{198} as well as Husman et al.\cite{199} demonstrated this concept for a gelatin-based and a PEG–heparin biohybrid system, respectively, as discussed in more detail in Section 3. The programming and decoupling of complex dynamic behavior through multiphasic microgel-ingel materials is not only limited to biochemical signaling but can also be addressed on a structural level as demonstrated by Huebsch et al.\cite{200} and discussed in more detail in Section 3. They were able to develop an injectable cell-laden hydrogel with dynamic pore formation by incorporating microgels made from hydrolytically labile alginate as porogens into an alginate matrix with lower susceptibility to hydrolysis, effectively decoupling the pore formation from degradation of the overall bulk matrix.\cite{200} In a similar fashion, Zhong et al. provided room for cell spreading and migration through embedding of degradable collagen microcarriers within an alginate matrix.\cite{201} As demonstrated by Wang et al. before, a multiphasic approach further allows for the integration of cell adhesion sites without additional modification of the matrix.\cite{202} From a design point of view, such multiphasic materials possess higher degrees of freedom compared to homogeneous hydrogel systems which allows to increase complexity as well as functionality and to overcome limitations of conventional single phase systems.

Whereas the random distribution of the microgels within the matrix is sufficient for many applications, an important aspect to consider for the design of hierarchically structured tissue architectures is how to control the distribution and orientation of the microgels within the matrix. Lee et al. have considered this aspect for cardiac tissue engineering purposes studying the effect of scaffold geometry on cell behavior in a three-phase system of fibrin, gelatin and Matrigel as discussed more thoroughly in Section 3.\cite{203} A different example for the control of anisotropy was demonstrated by Rose et al. They reported an injectable system to embed anisometric PEG-based microgels within a PEG or fibrin-based hydrogel matrix with a great degree of control over the microgel orientation. The microgels could be aligned in a magnetic field during the in situ crosslinking of the cell laden matrix due to the functionalization of the microgels with superparamagnetic iron oxide nanoparticles.\cite{204} Kampermann et al. demonstrated the versatility of multiphasic microgel-based gel-ingel materials from a fabrication point of view giving rise to a highly modular system for tissue engineering applications. They combined cell-laden UV-crosslinked PEGDA microgels with various matrices such as PEGDA, dextran-tyramine conjugates, collagen, alginate/collagen methacrylol, and alginate and applied a variety of hydrogel fabrication techniques such as photolithography, emulsification, injection molding, extrusion printing, and wet spinning.\cite{205} Regarding these systems, however, current approaches mostly rely on random uniform distribution of the microgels within a matrix, and although several examples have successfully manipulated/arranged microgels their position remains fixed once the composite is casted.

### 2.5.3. Granular Hydrogels

A completely new class of materials for cell instructive matrices in tissue engineering has risen over the last decade from the inter-crosslinking of preformed microgels that subsequently serve as macroporous scaffolds (Figure 4). From an engineering perspective, they offer a plethora of opportunities for the construction of complex and multiphasic tissue mimicking constructs due to the modularity of the individual building blocks which enables the combination of different materials into one system as well as individual preconditioning of the modules resulting in a high degree of tunability. However, the challenge of these systems lies in the formation of mechanically stable assemblies of the building blocks. If not bypassed otherwise, this requires two orthogonal crosslinking strategies, for microgel synthesis and the annealing of the building blocks into stable constructs, respectively. Although complex microgel geometries can be generated\cite{179,205,206} and microgel morphology constitutes an important design parameter that governs structural features such as porosity, predominantly spherically shaped microgels are employed to form granular hydrogels. Microporosity adds a structural component to the otherwise nanoporous hydrogel system resulting in superior nutrient supply, cell attachment, improved cell mobility and ECM deposition.\cite{200,207,208} Moreover, due to their unique rheological properties granular hydrogels are excellently suited as printable/injectable scaffold materials that are shape filling and possess an interconnected microporous scaffold architecture at the same time.\cite{209} The microsphere-based systems take advantage of the jamming effect for the formation of such tissue constructs. At high concentrations and being densely packed, microgel suspensions slurries show a yield shear stress and shear thinning behavior caused by physical interactions between the particles and the jamming effect brings the beads in close enough contact for self-assembly and subsequent crosslinking (further referred to as “annealing”).\cite{210,211} Similar to the formation of conventional hydrogel materials, different methods for annealing of the building blocks have been applied relying either on physical interactions (hydrogen bonds, electrostatic or host–guest interactions etc.) or chemical crosslinking strategies such as click chemistries, photo (click)-chemistries, or enzymatic reactions. The advantages and drawbacks of the individual crosslinking strategies are reviewed in detail in\cite{189} and also apply to granular hydrogel systems.

**Microgel Crosslinking and Scaffold Annealing Using Orthogonal Strategies:** If the physical interactions between microgels provide enough stability a second chemical crosslinking is not required as demonstrated by Riederer et al. who covalently crosslinked chitosan with genipin to form stable microgels that assembled into mechanically integer microgel constructs through hydrogen bonding and hydrophobic interactions.\cite{212} Mealy et al. formed microgels through photo-crosslinking (also click chemistry) of dithiol crosslinkers and hyaluronic acid functionalized with norbornene as well as adamantane. The addition of cyclodextrin functionalized hyaluronic acid to the preformed microgels as a crosslinker allowed for subsequent formation of self-healing granular hydrogels through host–guest interactions between adamantane and cyclodextrin. Tunable release and degradation kinetics were governed at the
building block level, by the type for microgel crosslinker or at the composite level, by the combination of building blocks.[211] Zhong et al. obtained multiphasic osteon-mimicking tissue constructs by mixing precultured cell-laden collagen microgels with pure gelatin microgels without the need for secondary chemical crosslinking. While the collagen microspheres served as cell carriers, the smaller and quickly degradable gelatin microspheres used to establish micropores during cultivation of the constructs.[214] Hsu et al. developed an injectable self-healing granular hydrogel through physical crosslinking to establish signaling gradients in vivo. Growth factor loaded microgels of opposite charge were synthesized from gelatin methacrylate and chitosan methacrylate, respectively through photo-crosslinking which formed stable microporous gels upon mixing through electrostatic interactions. The application of this concept is highlighted in Section 3.[207]

However, most approaches rely on secondary chemical crosslinking to form stable scaffolds and to ensure proper mechanical integrity. Segura and co-workers developed a system based on hyaluronic acid microgels that were preformed through different thiol-based click chemistries. The functionalization of the hyaluronic acid with two different factor XIIIa substrates (K- and Q-peptides) enabled the annealing of the construct upon addition of factor XIIIa and thrombin at 37 °C within 90 min.[208,215,216] The same material could be annealed by light based radical polymerization or through amine/carboxylic acid based crosslinking using N-hydroxysuccinimide functionalized PEG (PEG-NHS).[217] In a similar manner, Hu et al. highlighted the versatility of granular hydrogels by crosslinking alginate microgels through biotin-streptavidin and different click chemistries using Diels–Alder reactions.[218] Using the same material system as Segura and co-workers, de Rutte et al. have demonstrated the scalability of the approach by developing a method for high-throughput fabrication of such microgels.[147] The same group also designed physically crosslinked microgels from gelatin modified with methacrylic anhydride that were subsequently annealed through photocrosslinking.[219,220] Also using gelatin as a precursor Li et al. formed microgels from gelatin modified with norbornene and photo-crosslinked with PEG-dithiol. The primary amines of the gelatin served as means for covalent bonding with PEG-NHS to anneal the microgels.[221] Scott et al. used microgels prepared from PEGDA and PEG-glycine monomers through precipitation polymerization and further crosslinked them into modular hydrogels using PEG-amine as a crosslinker. By applying the carbodiimide based chemistry the group was able to further integrate collagen into their scaffold and anchor it through covalent bonds.[222] Zhou et al. formed microgels from PEGDA and tetra-thiol through UV-mediated thiol-acrylate Michael-type addition where the excess thiol groups served as anchor points for annealing the microgels with PEG-vinylsulfone as well as for the incorporation of collagen.[223]

Feng and co-workers could show that apart from chemical and physical crosslinking also cells can be utilized as crosslinkers for microgel annealing and scaffold formation. Granular hydrogels from MSC-laden gelatin/hyaluronic acid microgels formed stable tissue constructs in vitro and in vivo which was mediated by cell–cell interconnectivity between MSCs locating to the surface of the microgels over time.

Microgel Crosslinking and Scaffold Annealing Using One Strategy: Bypassing the requirement for two orthogonal crosslinking strategies for microgel formation and annealing, respectively, Xin et al. and Zhou et al. took advantage of the UV-triggered thiol-norbornene click reaction both, for microgel crosslinking and annealing to assemble PEG-based granular hydrogels.[223,225] In a different example, Anseth and co-workers applied the same reaction for microgel crosslinking and annealing by synthesizing two different types of microgels both, crosslinked through the triazole formation of PEG-dibenzocyclooctyne and PEG-azide. Using an excess of PEG-dibenzocyclooctyne or PEG-azide for the synthesis of each type of microgel, respectively, prevented prebonding of the microgels during the production and enabled annealing upon mixing of the two types.[226] Also, Visser et al. annealed the scaffold through physical interactions and bypassed the requirement for orthogonal secondary crosslinking by depositing the microgels right after the synthesis. They developed an in-air microfluidic approach to form microgels of different shapes using alginate and Ca2+ by controlling the in-air impact of two liquid microjets. The microgels were formed upon impact of the two jets and the deposition of the microgels on a surface formed a stable construct through the same electrostatic interactions used for crosslinking the microgels.[227]

2.5.4. Microgels as Inks for Bioprinting

The development of suitable hydrogel materials as inks for extrusion-based bioprinting is directed by the requirement for materials showing shear-thinning behavior with a yield stress high enough to ensure sufficient stability of the printed construct. The use of microgel-based inks has recently emerged as a promising strategy for 3D bioprinting enabling the assembly of microporous hydrogel scaffolds.[168,169,228] Due to their unique rheological properties, densely packed microgel suspensions can be described as liquid-like solids behaving like an elastic hydrogel at low shear stresses but yielding and showing a shear-thinning behavior at high shear stresses.[210,211] Applying this approach for bioprinting, the important rheological properties are not primarily controlled through the choice of hydrogel material but the format of the particulate matter that is printed (size, shape, surface functionalization, and mixture) which extends the range of printable materials significantly. The group around Burdick has developed a range of injectable/printable microgel-based systems.[174,209,213] Demonstrating the applicability of microgel-based bioinks for different hydrogel systems and highlighting the modularity, Shin and co-workers fabricated cell-encapsulating microgels from hyaluronic acid, PEGDA or agarose and printed the previously jammed microgel ink using layer-by-layer extrusion as well as gel-in-gel printing. The method proved to be cell compatible and if required, secondary crosslinks could be introduced to chemically anneal the microgels and provide sufficient stability of the printed constructs.[109] Several examples directly combine both, microgel synthesis and 3D printing into one system/device.[227,229] For example, Ma et al. directly generated microrods of gelatin methacrylate and Matrigel in a simple polytetrafluoroethylene tubing and subsequently deposited the
rods emerging from the collection tubing using a micromanipulator.[230] Although not the focus of this review, microgel slurries cannot only serve as bioinks but also as supporting material for cell-only and free form bioprinting, due to their behavior as a liquid-like solid.[230–233] Jeon et al. have adapted this approach for the printing of cell-encapsulating oxidized methacrylated alginate microgels using gelatin microspheres as a supporting bath. The alginate microgel free forms could be photo-crosslinked into stable architectures and released from the gelatin supporting bath upon heating.[231]

2.5.5. Assembly of 3D Modular Structures

While granular hydrogels highlight the modularity of multiphasic systems from a material point of view, they are often constructed through random self-assembly. The potential of microgel-based systems can be extended when the assembly of the desired structures/patterns is controlled through direct or indirect manipulation of the microrods by external driving forces (magnetic and acoustic fields, mechanical and capillary forces, surface tension, polarity and molecular pair recognition) (Figure 4).[137,234] To reach a more thorough understanding of the explored techniques and strategies for microgel assembly that go beyond the selected examples in this section, the interested reader is referred to a more comprehensive review from Guven et al.[244] The group of Khademhosseini exploited the interface-directed assembly of the hydrophilic cell-laden microgel units within a hydrophobic oil or at the liquid–air interface. Structural organization was controlled through microgel geometry while photolithography could subsequently be used to crosslink the individual building blocks into stable tissue construct with specific patterns.[235–237] More recently, Wang et al. have exploited hydrodynamic interactions to self-assemble cell-laden microgel modules in a geometry guided manner into 3D tissue constructs enclosing a vessel-mimicking lumen by using a micromanipulator setup. The surface tension driven process allowed for precise alignment of the individual building blocks following their geometric outline.[206] In a synergistic approach, Yang et al. combined optofluiddic maskless lithography and optically induced dielectrophoresis into one setup for the fabrication of complex architectures. The lithography approach allowed forming cell-laden microgels with complex geometries which could then be spatially organized and assembled in a TETRIS-like manner.[205]

2.6. Bioprinting

Biomaterials that are hierarchically structured across length scales ranging from micrometers to millimeters are considered key to mimicking and directing the formation of tissue structures.[230,238] Addressing the complexity, additive manufacturing, often referred to as bioprinting, has rapidly evolved into a main fabrication technology for tissue engineering, regenerative medicine as well as advanced cell culture based studies.[48,239–241] Bioprinting comprises the application of computer-aided processes for patterning and assembling of cell-free and cell-laden materials into scaffolds with a defined 2D or 3D architecture.[48] Most frequently applied are methods based on laser-assisted material transfer and/or crosslinking, inkjet technologies, and/or robotic dispensing.[239,241] Each method has specific requirements on the physicochemical parameters of the processable bioinks[241] and limitations with regard to the spatial resolution, fabrication speed, as well as cell density and viability.[239] Although huge progress was achieved in the 3D bioprinting of tissue-like materials, the integration of the biological and functional complexity of naïve tissues into printed materials and the printing of full functional organs remain key challenges in the field.[239,242]

2.6.1. Spatially Graded Materials

The development of tissues and organs is controlled by spatially and timely orchestrated biophysical and biochemical signals. 3D printing provides versatile options to grade the properties of materials for cell and organoid cultures. Motealleh et al. printed step-gradient nanocomposite alginate based hydrogels with varying nanometer-scale topography for controlled cell migration.[243] The triphasic nanocomposite hydrogel has cell-adhesive properties and was used to direct the migration of fibroblasts toward the higher concentration of biopolymer-coated silica-based nanomaterials within the 3D network of the hydrogel. The authors further demonstrated the application of the material for stimulating the migration and subsequent osteogenic differentiation of human bone marrow derived MSCs.

Recently, we have developed a multicomponent inkjet bioprinting method that allows for spatially varying composition and network properties in cell-instructive hydrogels.[244] The method relies on the covalent crosslinking of different polymeric precursors through a very rapid bio-orthogonal Michael-type addition during the fusion of bioink droplets prior to and upon contact with the target. Vertically and laterally structured scaffolds, with and without cells, were printed from GAG-based biohybrid and pure PEG hydrogels with a resolution of 50 and 300 µm, respectively. Chemotactic molecular gradients produced by this approach within printed GAG-gels of defined zonal architecture were shown to effectively direct the migration and morphogenesis of embedded human bone marrow MSCs. The introduced methodology is expected to enable a new, holistic level of control over reductionistic tissue and organoid models.[244]

Schöneberg et al. developed a drop-on-demand bioprinting technique for the fabrication of a multilayered hydrogel that more closely resembles the natural structure and cellular arrangement of larger blood vessels.[245] The droplet-based approach allowed a modular assembly of the structure based on different hydrogel materials. A gelatin-based ink was used as a core around which a dense layer of fibrogenin- and thrombin-based hydrogel droplets were printed that provided mechanical support. Cells could directly be incorporated inside of the printed droplets without the need for manual injection of endothelial cells after printing.

Coaxial bioprinting enables the formation of multiphasic core–shell filaments, e.g., for the formation of vascular structures or nerve regeneration. Liu et al. reported a novel strategy
to fabricate cell-laden constructs with tunable 3D microenvironments by extrusion of GelMA/alginate core/sheath microfibers using a coaxial nozzle. The alginate sheath was physically crosslinked and served as a template to confine the GelMA in the core and to allow for subsequent UV crosslinking. Applying this strategy, it was possible to print cell-laden GelMA constructs at extremely low concentrations (1.0 to 2.0%) and fiber diameters between 400 and 800 μm. The introduced method allows a high degree of control over the 3D microenvironments for the encapsulated cells, which is difficult to achieve with conventional bioprinting methods.

2.6.2. Embedded Printing

Scaffolds that mimic the complex structures and functions of the ECM can also be obtained by embedding particles, strands or more complex architectures of a different material in a 3D matrix. Senior et al. report the fabrication of complex hydrogel structures using suspended layer additive manufacturing. The method enabled the successful fabrication of bulk, intricate, dual phase, and phase-encapsulated hydrogels from a variety of biopolymer materials with controlled spatial gradients in mechanical and chemical properties and integrity between different materials. Embedded printing methods further enable the creation of sacrificial structures within supporting gel materials that can be used for the vascularization of 3D printed tissues and organs. Kolesky et al. reported the fabrication of engineered tissue constructs that contained a dense perfusable vasculature, multiple cell types, and ECM with potential applications for drug screening and fundamental studies of wound healing, angiogenesis, and stem cell niches. The constructs were formed with structure sizes above 200 μm by sequential extrusion of different materials (PDMS, fugitive Pluronic F127, and two different cell-laden GelMA inks). After further refinement of the method, the 3D microengineered environments open new avenues for the rapid manufacturing of functional 3D tissues and organs. Burdick and co-workers reported a printing process where a shear thinning and self-healing hydrogel “ink” is injected directly into a “support” hydrogel with similar properties to create complex microchannels with diameters of several hundred micrometers for angiogenesis studies under different geometric constraints. The support hydrogel was additionally functionalized to undergo stabilization through a thiol-ene reaction, enabling the washing of the ink to form microchannels and tunable properties depending on the crosslinker design. The functionalization of the support hydrogel included RGD peptides to provide adhesion sites for embedded cells and protease-cleavable crosslinkers for cell-mediated degradation. The application of this method for the fabrication of vascularized scaffolds is reviewed in the Section 3. The mechanically induced fluidization of gels made from soft microscale particles by an injection tip provides further options to create multiphasic materials. Applying this principle, Bhattacharjee et al. created complex large aspect ratio 3D objects of various shapes, such as highly branched tubular networks inside a soft granular gel medium using silicones, hydrogels, colloids and living cells as writing material. The approach has huge application potential in different areas, including tissue engineering, flexible electronics, particle engineering, smart materials, and encapsulation technologies. Although 3D bioprinting enables researchers to mimic certain aspects of functional tissues and to print organoids and organs, numerous challenges remain. Bio-printing of complete tissues and organs requires large numbers of various mature or multipotent cells, the local deposition of tissue-specific ECM, including signaling molecules, and the formation of cell-instructive gradients. Further challenges include the sufficient vascularization and innervation of the engineered tissues and organs.

2.7. Photopatterning

Photoinitiated postmodification of hydrogels, commonly referred to as photopatterning, is a versatile methodology to introduce heterogeneity into a previously formed hydrogel. With this technique, creation and cleavage of (bio) chemical or physical bonds can be initiated with high precision and specificity, also during the course of a running 3D culture of already embedded cells. Small volumes can also be ablated with a focused high intensity pulsed laser to create pre-designed cavities like perfusable channels into hydrogels. Photopatterning allows space and time resolved external manipulation over the spatial distribution of cell-instructing biomolecules or crosslinks via control of dose, location and time profile of the applied light. A large variety of photochemistries is available for the modification of hydrogel materials, as reviewed elsewhere.

An important factor to consider when choosing the chemistry is the energy that needs to be locally introduced into the sample to initiate the desired reaction. Cells and biomolecules can be susceptible to damage when lower wavelengths and higher doses are applied. Also, some photochemical reactions, particularly those involving photoinitiators, produce radicals that can be toxic to cells and react with biomolecules, however careful design of the reaction conditions can restrict such undesired side effects.

2.7.1. Irradiation Technologies

Patterning 2D Projections: 2D patterns that project into the gel volume are typically achieved with photolithography systems that illuminate the sample with collimated light through a photomask. Since such illumination systems can be very costly, Huntington and Odom presented a low-cost construction as an alternative. Photomasks with high resolution need to be produced with special equipment and are commonly ordered from industrial manufacturers.

Optical systems applying digital micromirror devices offer a more flexible alternative to photomasks. They consist of a 2D array of multiple hundred thousand tiny mirrors that are individually switchable to either redirect light from a collimated source toward the sample (“on”) or not (“off”). As a technology commonly used in digital light processing video projectors, they can project computer generated binary images in a fast switching manner onto the sample, allowing “grayscale” values of locally projected...
light doses via pulse-duration modulation or varying “on” times for different pixels. This can allow the generation of graded densities of photocleavable or photogenerated bonds. With photomasks, it can also be achieved at lower resolution using half-tone masks[273,274] or by moving a mask relative to the sample.[275] Even simple linear increments of functionalization by sliding an opaque mask subsequently across two axes can create potent tools to gauge cell responses to multivariate stimuli. Vega et al.[276] created a gradient of the N-cadherin motif HAV orthogonal to a second gradient of RGD functionalization in a hyaluronic acid-based hydrogel. This yielded a combinatorial hydrogel that they used to probe the combined effect of these cell adhesion peptides at multiplexed densities. To yield graded or grayscale functionalization, also different masks could be applied sequentially; however, changing masks requires careful alignment and the number of grayscale levels is limited by the number of masks that can be applied. The digital nature of digital mirror devices also allows facile sequential application of different reagents with different masks.[277]

Focused light can also be applied using microlens arrays.[278] Alternatively, a focused laser beam can be applied.[257]

3D-Resolved Photoinitiation: While these techniques allow only highly resolved 2D projections along the light path through the sample, multiphoton techniques enable additional high resolution in the third dimension. They rely on the two-photon effect that yields a photon in the UV range in a distinct small volume in the focal plane while using a pulsed laser with much higher wavelength in the visible or infrared regime.[279] Owing to this effect, the sample volume, including sensitive cells and biomolecules along the path of the beam, remains virtually unaffected while a high 3D resolution can be achieved. Also, low absorption outside the focal plane allows deeper penetration into the sample.[261] By varying the dose depending on the irradiated location, the density of photochemical events can be controlled locally. One limitation of the technique is the long time required to pattern relatively small volumes, which can last up to several hours for volumes in the micrometer range.[280] Hence, it may be required to work in appropriate incubation chambers if live cells are present.

Bernal et al.[281] recently demonstrated a very different approach to achieve 3D resolution where they used a computed tomography-inspired technique to initiate photochemical crosslinking of cell-laden gelatin methacryloyl hydrogels. Although the smallest feature that could be resolved measured 145 µm, photochemical events could be initiated within large volumes of several mm³ within seconds.

### 2.7.2. Patterns of Crosslinking Densities and Biofunctionalization

When using photo-crosslinkable gels, one can make use of the dose dependency of crosslinking density by first creating a homogeneous material with a “background” density via bulk irradiation, and then locally decreasing the mesh size by targeting remaining unreacted functional groups with selective irradiation.[276] In a study of Khetan et al.,[252] background crosslinking of acrylated hyaluronic acid functionalized with cell-adhesive peptides was achieved with a matrix metalloprotease (MMP)-cleavable peptide as crosslinker. Using a photomask, remaining unreacted acrylates were crosslinked, yielding a patterned hydrogel with MMP-cleavable and noncleavable regions. On the other hand, using photocleavable bonds, crosslinking densities can be locally reduced upon irradiation.[255–257,262] Kloxin et al.[255] presented a photodegradable PEG-based gel that allowed migration of encapsulated cells along channels with lowered crosslinking densities created via irradiation.

Photochemical linkages can also be used to locally alter the biochemical properties of the material, most commonly by establishing bonds between the polymer and cell-adhesion peptides or growth factors.[251,252]

A different approach is to deprotect or uncage bioactive moieties upon irradiation.[260,283–286] Since many biomolecules like growth factors can be sensitive to the relatively high light doses usually, methods were developed where first reactive binding sites are photoactivated and the whole material is subsequently loaded with biomolecules to bind to them.[259,287,288] For example, Wosnick and Shoichet[288] deprotected thiols groups in an agarose-based hydrogel using multiphoton laser patterning, allowing Michael-type addition of a variety of subsequently added maleimide-conjugated species.

### 3. Applications of Multiphasic Gel-in-Gel Materials

Multiphasic hydrogel materials offer unprecedented possibilities to recapitulate, study and engineer the function of native ECM and tissue. The necessity of engineered systems to account for the dynamic and spatial heterogeneity of living matter is the driving force behind the technological advances in the field and defines the relevant design parameters. The available fabrication and processing methods (as described in the previous section) provide a large kit of tools for the assembly, embedding and patterning of hydrogel constructs across multiple length scales ranging from the nanoscale (nanofibers and photopatterning with nm resolution), over the microscale (microgel embedding and printing) to the macroscale (macroporous cryogels, layering and building block assembly). In the following section, five main characteristics will be highlighted which can be emulated through multiphasic gel-in-gel systems in analogy to nature, i.e., i) establishing biochemical and biomolecular gradients, ii) compartmentalization, iii) providing graduated mechanical properties, iv) establishing structural heterogeneity and v) accounting for the dynamical characteristics of nature. While multiphasic hydrogels stand out due to their ability to implement and control multiple functions in one system, the selected examples are classified with respect to the eminent feature governing their functionality. By discussing a few selected approaches in detail, this section aims to emphasize how gel-in-gel approaches can foster the development of more realistic tissue and disease models, inspire new treatment options and mediate between biology, medicine and material engineering.

#### 3.1. Biochemical and Biomolecular Gradients

**3.1.1. Assembled Microgel Scaffolds for Neuroregeneration**

Combining different hydrogel materials into one system allows for decoupling of relevant design parameters (structure,
chemistry, functionalization, etc.) and can even offer additional functionalities that reach beyond those of the individual single-phase systems. Hsu et al. were able to develop an injectable, self-healing, and degradable granular hydrogel with propagating NGF gradients and a microporous scaffold architecture for nerve regeneration (Figure 5).[207] The three different requirements could be resolved by means of a multiphasic and modular microgel-based approach. Using chitosan (positively charged at neutral pH) and gelatin-based microgels (negatively charged at neutral pH) as building blocks allowed for the assembly and layering of a stable self-healing construct through electrostatic interaction. The reversible annealing in combination with microgel jamming provided the desired shear-thinning properties for injection. Exploiting the modular nature of microgels, different amounts of NGF could be loaded to the gelatin particles in order to establish propagating gradients. Both, scaffold porosity as well as the propagating NGF gradient were demonstrated to significantly improve nerve generation in vitro and in vivo, i.e., axon outgrowth, myelination, and functional recovery.

3.2. Compartmentalization

3.2.1. Flexible Microgel-in-Gel-Systems for In Vitro Reconstitution of Distinct Tissue Features

Another key feature of complex hierarchical tissue comprises the distinct spatial arrangement of functional units and their compartmentalization on the macroscale. A way of how multiphasic and modular microgel-based materials can recapitulate this function of the ECM was demonstrated by Lee et al.[198] Macrophages were precultured in gelatin microgels and the polarization through lipopolysaccharide stimulation was dependent on the stiffness/network properties of the encapsulating microgels. Subsequently, the crosstalk between macrophages and fibroblasts or hematocarcinoma cells was investigated by embedding the microgels in a gelatin matrix containing one of the other cell types, establishing a more physiologically relevant condition compared to conventional coculture systems. This example highlights how the modularity of such methods, allowing for independent conditioning of the building blocks, can be harnessed to mimic the heterogeneous structure of complex tissues and path the way toward multiplex tissue models. Furthermore, multiphasic hydrogel materials represent a powerful toolbox to increase the complexity of scaffolds not only through the embedding of cells but their compartmentalization.

In another microgel-in-gel approach, we recently demonstrated the variation of cell-instructive properties (stiffness, degradability, presentation of adhesion moieties, and growth factors) as well as the localization of specific cell types in the microgel and the surrounding bulk gel compartment using a versatile platform of cell-degradable multifunctional PEG–heparin hydrogels (Figure 6).[199] As a proof of concept, prostate cancer cells were grown in microgels providing a stiffer spheroid-supportive matrix. The microgels...
were surrounded by a soft hydrogel matrix that contained heparin-bound growth factors and adhesion ligands to induce the formation of a capillary network by the embedded human umbilical cord endothelial cells. The new culture format allowed the formation of spatially resolved micro-tissue structures and was shown to overcome limitations of 3D in vitro tissue models based on monophasic hydrogel materials. The approach furthermore enabled the combination of cell assemblies (cancer spheroids and endothelial networks) into microgel-in-gel cocultures at different time points, i.e., at different levels of maturation while keeping their microenvironment.
3.2.2. Multilayered Cryogels for In Vivo Osteochondral Tissue Engineering

In the advanced stage of cartilage lesions, not only the cartilage needs to be repaired but often also the underlying subchondral bone. Kang et al. developed a functionally graded trilayered hydrogel scaffold based on PEGDA and N-acryloyl 6-aminocaproic acid covalently crosslinked via radical polymerization.[97] As mentioned by Kang et al. one of the challenges in designing scaffolds for osteochondral defect repair is to form discrete zones of chondrogenic and osteogenic tissue within one hydrogel, especially when using MSCs as a source for both types of tissues. The layer-by-layer assembly strategy, chosen here, has the advantage that discrete compartments within one scaffolds can be created. Furthermore, the bottom compartment of the multiphasic construct, which represents the bone niche, was intentionally left acellular and should later upon implantation promote cell infiltration and bone tissue formation due to the high porosity and the presence of minerals. This allowed an exogenous preconditioning of MSCs seeded in the top and bottom compartment toward chondrogenic phenotype, whereby the vertically oriented pores of the middle hydrogel promoted the alignment of the MSCs, similar to the structure found in natural cartilage and the dense structure of the nanoporous hydrogel of the top layer promoted lubricin formation in vivo. Even though the in vivo experiments were only performed subcutaneously in this work, the study nicely illustrates how different compartments within a multilayered hydrogel can help to guide the formation of distinct tissue types within one scaffold. Moreover, the study is a great example for showing the advantage of the highly porous cryogels, in that they provide a great tool for enabling rapid recruitment and infiltration of engineered hydrogels with host cells when implanted in vivo.

3.2.3. Bilayered Hydrogels for the Study of Tumor Vascularization

A bilayered hydrogel platform for studying tumor angiogenesis was introduced by Rousari et al.[289] The authors used a cell-adhesive proteolytically degradable PEG-based scaffold for the coculture of adenocarcinoma cancer cells and endothelial cells, which allows both compartmentalization and mass transfer. The layered scaffold structure was realized by the sequential addition of vascular and cancer cell-laden polymer solutions into PDMS wells. Cancer cells encapsulated in the PEG-based hydrogel were demonstrated to form lumenized spherical cell clusters with epithelial morphology and secreted angiogenic growth factors. Human umbilical vein endothelial cells were cocultured spatially separated in the second PEG-hydrogel layer. In the vascular layer, tubular networks of endothelial cells were formed and shown to secrete ECM components. This study introduced the first synthetic dual-layer approach that facilitated tubulogenesis of vascular cells and tumor spheroid formation in separate compartments of a system but allowing for the diffusion of growth factors. Moreover, the cell-mediated degradability of the polymer network facilitates the migration of vascular cells and their interaction with tumor cells. Due to the spatially separated coculture of cancer and vascular cells further insights into their crosstalk were obtained including angiogenic signaling.

3.3. Graduated Mechanical Properties

3.3.1. Stiffness Contrast Directing Differentiation in Layered Hydrogels

While tailoring the mechanical characteristics of cell-embedding hydrogels is a powerful way to guide cell behavior in homogeneous phases, sites of transition of their quantity—as abundantly present in complex multifaceted organs and developing embryos—can only be recapitulated using heterogeneous material systems. In early embryonic development, amniogenesis occurs in a niche that involves a soft tissue bed given by the uterine wall and tropoblast, as well as a 3D matrix provided by the epiblast during implantation. Shao et al. demonstrated that by mimicking this mechanical heterogeneity using a thick bed of Geltrex (a basement-membrane product) and a diluted matrix of the same material, 2D layers of human embryonic stem cells (hESCs) in between these phases could develop into amnion-like squamous cysts (Figure 7).[290] By comparing with hESCs cultured only on the Geltrex bed or only in the diluted Geltrex matrix, they demonstrated that the presence of two distinct phases was necessary for the environment to function as a biomimetic implantation-like niche. When the thickness of the bed layer was reduced from 100 to 60 or 20 μm, development of amnion-like cysts was inhibited as the apparent substrate rigidity increased with decreasing distance to the underlying stiff glass plate. This illustrates the importance of considering not only the local stiffness of phases in direct contact with cells, but that cells can also sense the rigidity of an underlying “hidden” phase if in close proximity. When designing heterogeneous materials it is important to keep in mind that small features or stiffness patterns of only a few tens of micrometers can be susceptible to this effect, such that variation of only the feature size in these length scales may significantly alter the perceived rigidity.[291]

3.4. Structural Heterogeneity

3.4.1. Embedded Printing of Hydrogels for the Study of Angiogenic Sprouting

The formation of a connectable vasculature is an important prerequisite for the long-term cultivation of tissues and organoids. Embedded printing methods can be advantageously used to fabricate template structures for the formation of a vasculature in hydrogel scaffolds for tissue engineering. Burdick and co-workers used this approach to investigate the influence of channel curvature on angiogenic sprouting in hydrogels functionalized with RGD peptides for adhesion and protease-cleavable crosslinkers for cell-mediated degradation.[249] In this system, the hydrogels were assembled through host-guest interactions by modifying hyaluronic acid with adamantane and β-cyclodextrin. For the support hydrogel, adamantane-hyaluronic acid was further modified with norbornene to
enable the introduction of RGD peptides and to permit covalent crosslinking with di-thiol crosslinkers via thiol-ene click chemistry. With this approach, endothelial cells were effectively stimulated to adhere at the walls of microchannels printed within the support hydrogel and to form confluent monolayers. Upon stimulation with angiogenic factors, the cells enzymatically degraded the support and formed sprouts into the material. By modifying the microchannel design, the method was then used to investigate the influence of channel curvature on angiogenic sprouting. The proposed concept is concluded to provide multiple options to print complex 3D structures for angiogenesis and tissue formation in various biomedical applications.

3.4.2. Three-Phase Cardiovascular Tissue Model Based on the Modular Assembly of Microgel Units

The high degree of spatial organization of cardiac tissue remains a great challenge within the field. Applying a multiphasic modular approach, Lee et al. have successfully demonstrated how microgel-based systems can serve as a valuable means to study the effect of geometry and overall tissue architecture on cell fate within models of complex tissues (Figure 8).[203] Their three-phase cardiac tissue model consisted of i) gelatin-based cardiac modules of various aspect ratios precultured with cardiomyocytes that were embedded within ii) a fibrin-based grid...
containing endothelial cells and iii) sealed with Matrigel. The modularity of the system allowed for independent precondi-
tioning/culturing of the individual cell-laden building blocks prior to the tissue assembly as well as merging complementary materials into one tissue engineering scaffold, hence, increasing its functionality. While fibrin and gelatin could meet the require-
ments of the two cell types for different scaffold materials, the Matrigel seal provided the essential signaling cues to support endothelial network formation. Furthermore, high aspect ratios of the cardiac modules and a high density of the modules within the fibrin grids lead to improved cellular function, i.e., cardiomyocyte alignment, anisotropic contraction and electromechanical coupling. This work highlights the potential of multiphasic gel-in-gel systems, with microgels as modular building blocks, as powerful tools to build and study functional tissue architectures of high complexity with respect to structure and organization.

3.4.3. Bilayered Cryogels with Graded Pore Structures for Tendon/Ligament-to-Bone Regeneration

A biphasic macroporous silk fibroin scaffold was engineered to promote tendon/ligament-to-bone regeneration.100,101 The interface between tendon and bone is a highly heterogeneous and specialized transition zone, with the ECM composition and cell type varying throughout the tissue, enabling the transfer of mechanical stresses between tendons/ligaments and bone. A biphasic macroporous silk scaffold was designed with graded isotropic and anisotropic pores to provide topological cues for alignment and differentiation of human adipose-derived MSCs. Pore morphology and alignment significantly affected cytoskeletal alignment and gene expression of MSCs, and a combination of pore alignment and growth factors stimulated enthesis regeneration (Figure 9). Local differentiation toward tenogenic/ligamentogenic, fibrochondrogenic, or chondrogenic pathways in the different zones of the

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Figure 8. a) Composite cardiovascular tissue formed by sorting of cardiac modules (red) within the endothelial module (green) and sealed with a thin layer of Matrigel (purple). b) Cardiac modules (red) sorted within endothelial modules (green). (scale: 500 µm). c) Synchrony score (average ± SEM) from all eight composite tissue designs, four shape aspect ratios in two loading densities. Composite tissue architecture was found to define cardiac beat synchronization. Reproduced with permission.203 Copyright 2016, Wiley-VCH.

Figure 9. Heparin functionalized biphasic silk fibroin scaffold with graded anisotropic and isotropic pore structure and local delivery of growth factors for tendon/ligament-to-bone tissue engineering. The combination of spatially graded pore structures and biochemical signals were found to induce a locally varying differentiation response of MSCs. Adapted with permission.101 Copyright 2018, Elsevier.
scaffolds was achieved by loading of the hydrogel matrix with TGF-beta and/or GDF5. Covalent functionalization of the silk hydrogel matrix with heparin ensured sustained delivery of the factors. MSC differentiation and ECM deposition was shown to depend on the combination of growth factors and the pore structure of the scaffold. In summary, this study is a great example for demonstrating the combined impact of local differences in structural and soluble cues on therapeutically relevant stem cell decisions.

3.5. Dynamical Characteristics

3.5.1. Dynamic Void-Forming Microgel-in-Gel System to Promote and Study Osteogenesis

Huebsch et al. investigated the effect of matrix stiffness and matrix chemistry on MSC osteogenesis in vitro and in vivo by means of an injectable void-forming alginate microgel-in-gel system. Tuning the degradation rate of the pore-forming microgels and the bulk matrix independently, the requirements for a degradable macroporous matrix and a more stable scaffold material were combined to study the effect of material stiffness. While pore formation promoted cell motility, remodeling and de novo tissue deposition, a slowly degrading scaffold was found to maintain the mechanical properties over the desired time span. Furthermore, allowing for injection and a microporous architecture, the material combined two features which are hardly reconciled by conventional approaches. The molecular weight of the alginate was used to alter the mechanical properties of the bulk phase, whereas the crosslinking of the porogen microgels through Ca\(^{2+}\) and the concentration of hydrolytically labile groups of the alginate was harnessed to control the rate of pore formation. Bone formation and material resorption upon implantation of cell-laden scaffolds was improved by the dynamic pore formation acting in concert with mechanotransduction at the cell–matrix interface. This work highlights the value of multiphasic hydrogels to decouple complex systems which entails a higher degree of control and tunability and makes it possible to gain mechanistic insight behind the driving forces of tissue development.

3.5.2. Microfluidic-Based Cell Culture Platform with Spatiotemporally Controlled Morphogen Gradients

In early human development spatial cell patterning occurs. This patterning is guided by the presence of signaling centers, which are usually localized groups of cells that secrete morphogens in a time and space dependent manner. To emulate this highly complex and dynamic signaling that drives stem cell patterning, Manfrin et al. developed a microfluidic cell culture device for in vitro germ layer patterning of human pluripotent stem cells (hPSCs) (Figure 10). In contrast to

![Figure 10](image-url)
conventional cell culture setups, microfluidic-based approaches offer a greater control over establishing defined morphogen concentrations and most importantly enable the formation of spatiotemporally controlled gradients. The microfluidic cell culture device designed by Manfrin et al. consists of a central Matrigel coated cell chamber that is flanked by two PEG hydrogel compartments, which are each in direct contact with a perfusion channel. The PEG hydrogels serve as barriers to prevent unwanted convective flow that could disturb the formed gradients, as well as help to keep the cells confined in the middle chamber. Morphogens are added to the perfusion channels, which then diffuse throughout the hydrogels into the cell chamber, resulting in the formation of morphogen gradients that serve as artificial signaling centers for the stem cells. Using this setup, Manfrin et al. show that they could change human pluripotent stem cell patterning in vitro by spatiotemporally controlling a morphogen gradient (e.g., BMP-4) from a localized source. Moreover, due to the biphasic setup of having two hydrogel channels flanking the inner cell chamber, not only one type of spatiotemporally controlled morphogen gradient can be created, but two. This allows studying the effect of an opposing gradient on stem cell patterning, for example of BMP-4 with its corresponding inhibitory partner NOGGIN.

In summary, the development of this microfluidic device is a great example that shows us one possible design strategy of how to develop advanced hydrogel-based cell culture systems in which morphogen gradients can not only be created but also temporarily controlled, for example to emulate the highly dynamic process of stem cell patterning in early embryonic development.

3.5.3. Directed Invasion of Embedded Cells upon Photopatterning During Culture

Mosiewicz et al. demonstrated how cells can be instructed to invade controlled volumes at defined starting times while being in culture. For this purpose, they created MMP-sensitive PEG-based hydrogels that were functionalized with photocaged FXIIIs. Upon irradiation, the photolabile caging group unmasks the active site of FXIIIs that is then free to bind to subsequently added recombinant proteins or peptides engineered with an exogenous complementary binding domain. By mimicking the natural binding mechanism involved in fibrin polymerization in vivo, and the possibility of adding the functional species only after irradiation, vulnerable full-length proteins can be readily grafted to the PEG hydrogel structure while conserving functionality.

First microtissues assembled from MSCs were embedded into MMP-cleavable PEG gels functionalized with photocaged FXIIIs. After photopatterning a cuboidal volume adjacent to a microtissue via laser scanning lead to a functionalization of the gel volume with RGD, fibronectin fragment FN9–10 and PDGF-BB, respectively, directed migration into these volume was initiated (Figure 11).

4. Perspective

The current stage of developing, testing and applying multiphasic gel-in-gel materials as covered by this review article demonstrates the feasibility and the potential of the related materials but remains far from exploiting the potentialities of the approach in full. However, the above-described available technologies for fabricating multiphasic systems already provide a rather broad range of basic options for implementing and exploring spatiotemporally gradiated hydrogels in in vitro and in vivo tissue engineering schemes. At the next stage, more specific applications enabled by dedicated multiphasic systems will have to be elaborated. For that purpose, we see primarily the necessity for increasing the complexity and integration, expanding the options to implement adaptive spatiotemporal control and to establish scalability and parallelization of multiphasic gel systems. Dedicated translational efforts will be required to benefit from the advantages of multiphasic gel-in-gel systems in an array of biomedical applications. Subsequently, some aspects of these proposed future extensions will be briefly discussed.

Figure 11. In situ manipulation of 3D MSC invasion by light-activated enzymatic patterning. a) Schematic depiction of directed 3D MSC migration after local two-photon initiated functionalization of a PEG-based hydrogel with cell binding moieties. b) Confocal micrographs showing a cross section of a cuboid volume functionalized with fluorescent RGD in direct contact with an MSC microtissue (left panel, top view, microtissue highlighted by white arrow) and progression of MSC invasion into the patterned gel region (subsequent panels). Scale bars: 200 µm. Adapted with permission. Copyright 2013, Nature Publishing Group.
4.1. Increasing Complexity and Integration

Progress in molecular, cell and developmental biology enables and motivates more and more sophisticated engineering platforms to recapitulate dynamic exogenous signals throughout developmental processes for supporting and directing tissue formation, growth and maturation in physiological and pathological conditions. Addressing this challenge, classical materials science approaches will have to emerge into systems integration strategies to meet the increasingly intricate requirements in a holistic manner. In particular, multiphasic hydrogels providing mechanical and structural signaling cues will have to be complemented with microfluidic technology for the dynamic, localized supply of soluble signaling factors.

Although the complexity of scaffold-free structures (organoids) relying on cellular self-assembly clearly outruns engineered material-based approaches, the resulting assemblies remain limited with respect to reproducibility and degree of control. As an overarching challenge in all these approaches, it will be critically important to identify the most effective balance between self-organization and cellular guidance. Integrated systems based on multiphasic hydrogels could bridge the two strategies allowing for a higher degree of control and standardization while accounting for the spatiotemporal complexity. Similar to what has been proposed by Kratochvil et al. for organoid engineering, cell-sized hydrogels (microgels) can be integrated into, and further guide the process of cellular self-organization while providing a high degree of functional flexibility due to their multiphasic nature. The versatility of multiphasic hydrogel materials can be furthermore expected to provide a comparable efficacy to commonly used biopolymeric matrix preparations, in particular Matrigel, while offering high reproducibility and thoroughly defined and broadly adjustable signaling characteristics.

Scalable and more functional tissue constructs, for better drug screening, more realistic disease models as well as more successful therapeutic constructs requires perfusion and connectivity to, e.g., vascular structures, external circulation and circuits. Various patterning approaches have been developed to tackle this challenge. Dynamic multiphasic materials in combination with bioprinting were demonstrated to facilitate the integration of perfusable channels through embedding of sacrificial structures allowing for connected vasculature. While the results obtained are truly impressive, the capabilities of multiphasic hydrogels go far beyond such fabrication oriented strategies. Besides progress toward more sophisticated fabrication and cultivation methods, increasing the size and complexity of tissue constructs further demands the development of advanced analytical methods. For example, imaging, to provide in-depth structural information across different length scales, and deciphering the molecular composition of complex and dynamic systems can hardly be adequately implemented by means of the currently available techniques.

The effect of ECM characteristics such as mechanical properties (e.g., stiffness, viscoelasticity, strain stiffening), structure (e.g., porosity and fibers), degradation, and biochemical composition on cellular behavior has been widely studied and considered for the design of biomaterials. However, the interplay of the different characteristics can alter the cellular response and it often remains challenging to elucidate this by means of monophasic systems. Although some approaches have proven very useful to decouple a manageable set of parameters, it becomes increasingly difficult the more parameters have to be taken into account. The modular nature of multiphasic gel-in-gel materials offers a means to investigate the effect of individual ECM properties on cellular behavior at the building block level and the interplay of multiple factors at the composite level as demonstrated by several examples discussed in this review. Furthermore, multiphasic hydrogels provide powerful options for cocultures to create and independently tailor different cellular microenvironments for the contained cell. The defined modulation of several ECM properties on one hand and the adjustment of microenvironments for different cell populations on the other hand is seen as an obvious advantage of gel-in-gel systems toward the implementation of more realistic cell-instructive conditions in engineered materials offering the ability to decouple and individually study the different parameters.

4.2. Expanding Spatiotemporal Control

Obviously, spatiotemporally controlled signaling is critically important for successful tissue development and adaptation and the tunability of hydrogel systems provides powerful options to address the resulting requirements of engineered living matter. In particular, a multiphasic setup of precisely positioned microgels serving as source- and sink-like signaling centers are envisioned to guide cell migration, differentiation, assembly and morphogenesis. This strategy may offer a smart, biology-inspired way to decouple the supply of cells with soluble cues from the requirements of supportive bulk matrix materials and effectively expands the range of applicable materials multifold. Recapitulating the dynamic nature of tissue development, homeostasis and adaptation requires effective principles of temporal control over material positioning and organization. The design of programmable and adaptive gel-in-gel materials is envisioned to converge engineered systems with the dynamic properties of living tissues and provide appropriate sets of signaling cues at every stage of development and condition. As already demonstrated for a number of exemplary cases—such as MMP3, or coagulation enzyme-cleavable materials—responsiveness of hydrogels can be effectively utilized to engineer programmable materials and feedback loops, linking materials properties directly to biomolecular cascade reactions and cellular response patterns. Regarding the challenge to match cell-mediated matrix degradation and de novo ECM deposition, Bryant and Vernery have emphasized the importance of computational modeling, which could greatly advance biomaterials research. Multiphasic gel-in-gel setups could also be harnessed for the programming of integrated systems to implement sensors, which continuously provide valuable insight to tissue development. Such hydrogel sensors could be used to establish materials-based tissue-instructive feedback loops. Gaining insight into the dynamic changes of material properties during development and adaptation will also be critical for the future design of more effective cell-instructive systems.
4.3. Enabling Scalability and Parallelization

Recent advances in the fabrication and modification techniques of hydrogels with resolution ranging down to the nanometer scale allows to downscale scaffold materials (e.g., through microfluidics) while maintaining high degrees of control over composition, structure, morphology and functionalization. Cells are known to sense and respond to structural features in their surrounding and can be guided through biomaterial architecture.\(^\text{[19]}\) The key challenge lays the hierarchical design of hydrogel materials across several length scales as it will enhance the functionality of engineered constructs by accounting for the structural complexity of natural tissue.\(^\text{[20–22]}\) While the majority of currently explored systems focuses on multiphasic hydrogels as scaffold materials and delivery vehicles, engineering gel-in-gel materials with sub-micrometer multiphasic features and cell-sized multiphasic hydrogels, complex enough to serve as cell mimics, may allow for targeting biology in yet another unprecedented way.\(^\text{[23,24]}\) On a different note, downsizing paths the way for high-throughput production. Recent examples of miniaturization and automation of conventional well-plate based approaches through liquid handling systems highlight the importance of parallelization and combinatorics for future design of bioengineered living matter.\(^\text{[25–27]}\) The high-throughput fabrication of multiphasic hydrogel materials will allow for meeting the demands for reproducibility, standardization and—most importantly enables rational and holistic biomaterial engineering schemes based on combinatorial approaches.\(^\text{[28]}\) Although hydrogels are highly versatile, the compatibility with available fabrication and processing techniques for scaling and high-throughput fabrication is tied to very specific material requirements (gelation time, viscosity, etc.) which effectively leaves us with only a fraction of their modularity in the particular case.\(^\text{[29]}\) Besides the development of novel hydrogel chemistries to keep up with the changing requirements these limitations can be overcome through synergistic strategies for hydrogel fabrication and assembly.\(^\text{[30]}\) While the modularity of hydrogel composites lays the foundation of superior functionality and dynamics of engineered tissue constructs, disease models and treatment approaches, dedicated analytical concepts will be essential to trace the complex interplay between material and biology in such settings.\(^\text{[31]}\) Adaptation of dedicated mathematical algorithms and massive computational support will be required to exploit the modularity of multiphasic hydrogels and the wealth of biological response patterns evoked by applying them.\(^\text{[32,33]}\)

4.4. Translation into Biomedical Applications

We believe that in the near future the malleability of multiphasic hydrogel systems and the multitude of processing techniques, synergistically combined and tailored for specific applications, will not only provide mechanistic insight into the convoluted processes driving tissue development, homeostasis and disease but greatly enhance the potentialities of tissue engineering and regenerative medicine. At present, the application of multifunctional complex materials and engineered tissue constructs in a clinical setting is relatively limited owing to high medical requirements regarding safety and regulation as well as biological restrictions due to improvable reproducibility, lifespan and low throughput/limited availability.

The transplantation of macro encapsulated islet for endogenous insulin production is one of the few examples where tissue engineering could reconstitute the physiological key function of an organ and translate into the clinic.\(^\text{[33,34]}\) Despite this remarkable success, it is still critical for the lifetime and effectiveness of such transplants to control the inflammation, prevent immune rejection and ensure integration into the host tissue through innervation and vascularization for sufficient nutrient and oxygen supply.\(^\text{[35]}\) Multiphasic gel-in-gel materials, integrated into an implantable device, are predestinated to address all these requirements within one system.

As another example, advanced bioprinting technology for fabrication of 3D biofunctional hydrogel constructs, eventually mechanically reinforced by degradable polymer scaffolds, is being used to implement the biomimetic reconstitution of the zonal organization of natural cartilage. Multiphasic constructs—including gel-in-gel systems—are optimized for cell-free application and also for combination with chondrogenic cells (chondrocytes and/or MSC). Preclinical in vivo testing of the constructs is currently employed to explore the validity and clinical potential of the approach.\(^\text{[36,37]}\) A challenge for extrapolation such approaches is the adaptation of processing techniques to build and sustain larger functional constructs.

As yet another example, implantable immunoregulatory systems such as gel-based systems that act as artificial lymph nodes by activating and multiplying cancer-fighting immune system T-cells\(^\text{[38]}\) or living, implantable delivery systems for therapeutic bioactives, such as bispecific antibodies for cancer immunotherapies,\(^\text{[39,40]}\) are expected to massively benefit from the capabilities of multiphasic gel-in-gel materials—such as multiphasic gels containing macroporous compartments—to maximize the integration and functionality of the constructs.

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Conflict of Interest

The authors declare no conflict of interest.

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