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REVIEW ARTICLE

3D-printed microfluidic cell culture devices and hydrogel integration: Trends, challenges, and solutions

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Abstract

Three-dimensional (3D) cell cultures are increasingly being used in a variety of contexts (e.g., drug discovery, disease modeling, and tissue engineering), as they offer the potential to increase physiological relevance compared to traditional monolayer cultures, while simultaneously reducing cost and time compared to *in vivo* models. Taking a cue from nature, researchers often create 3D cell cultures using hydrogels that can closely mimic the extracellular matrix that most mammalian cells are surrounded by *in vivo*. However, aside from the collective physical 3D arrangement itself, the physiology of the culture depends highly on the microenvironment, which is defined by the 3D cell culture shape and the complex combination of biochemical, biophysical, and biomechanical stimuli. Microfluidic devices offer researchers the tantalizing opportunity to precisely define and influence this microenvironment. Furthermore, they additionally enable the integration of external functional components for active stimulation and monitoring of cultured cells. Pushing for ever-more-realistic culture conditions has, however, increased the complexity that is required of these microfluidic culture systems, making their fabrication more difficult. In this regard, 3D printing is becoming an increasingly popular solution, as it offers researchers not only the ability to fabricate highly complex structures but also to benefit from rapid prototyping and customization of existing designs. This review discusses common challenges that researchers currently face when integrating hydrogel-embedded cells into 3D-printed microfluidic cell culture devices and seeks to offer a comprehensive overview of recent advancements aimed at addressing these challenges.

Keywords: 3D cell culture; 3D printing; Hydrogel; Microfluidics; Microphysiological system; Organ-on-chip

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1. Introduction

In an effort to reduce cost and time compared to *in vivo* models, while simultaneously increasing physiological relevance *vis-à-vis* traditional monolayer cell cultures of adherent cells, researchers have increasingly relied on 3D cell cultures. Traditional monolayer cultures have helped scientists gain substantial insight into cellular processes; but in recent years, it has become increasingly apparent that individual cells are also significantly influenced by their collectively generated 3D environment.[1,2] As a result, simplistic monolayer cultures behave significantly different from cells that exist within a more complex 3D environment—also in terms of their morphology, differentiation, protein expression, functionality, migration, apoptosis, and response to drugs.[1–4] Particularly in the field of drug development and testing, 3D cell cultures offer researchers an invaluable opportunity to reduce animal testing, thereby increasing throughput and reducing costs, while addressing lingering ethical concerns.[4–6] Furthermore, by working with human cells/tissues and integrating interactions between minimal functional units of different organs, *in vitro* models can mimic human pharmacokinetics and pharmacodynamics more closely than animal models.[7,8] *In vitro*, a 3D cell environment can be achieved either by promoting the aggregation of cells into spheroids/organoids or by cultivating cells using a 3D scaffold. Scaffold-free techniques tend to result in multilayers of cells that are comparatively much more variable in terms of their size, shape, and composition.[1,2,4] Furthermore, as the position of cells within this construct may change over time, analyzing specific cells over an extended period becomes a challenge.[9] Mammalian cells proliferate *in vivo*, surrounded by a complex scaffold formed primarily of hydrated proteins called the extracellular matrix (ECM).[1,4] Hydrogels can be used to mimic the ECM *in vitro*.[1,10] Compared to other scaffold-based cultivation methods that rely on rigid scaffolds (e.g., built from fibrils), hydrogels are suitable for culturing a wider range of cell types and allow for more homogenous seeding of cells, leading to more standardized results between experimental runs.[4] Another advantage of utilizing hydrogels for the implementation of 3D cell cultures lies in their ability to provide viscoelastic strength, which can be fine-tuned depending on the desired application.[11,12] Furthermore, the porosity, stiffness, and/or degradation of the hydrogel can be modified to optimize cell proliferation and better mimic organ-specific ECMs.[10–12] Degradable hydrogels also enable matrix remodeling—a process in which cells actively degrade and form new ECM[13]; thus allowing for even closer alignment of the matrix with the *in vivo* ECM over time. Hydrogels thus present an exciting opportunity to closely mimic the *in vivo* 3D arrangement of cells in *in vitro* experiments.

Apart from this collective 3D arrangement, the regulation of an individual cell's function and behavior is also influenced by the specific microenvironment in which that cell develops (e.g., soluble factors, pH, oxygen supply, temperature, and osmolality).[6,14] Microfluidic cell culture systems enable researchers to take a further step towards more *in vivo*-like conditions by allowing them to precisely define and influence the cellular microenvironment. They can facilitate the patterning of hydrogels in a fully 3D manner, for example, by guiding the hydrogel into a specific compartment of the device through design features like micropillars. The precise patterning is crucial for defining molecular exchanges and reconstructing physiological structures. Using these devices, it is possible to promote tissue–tissue communication, dynamic fluid flow, and integration of external functional components to actively stimulate and monitor the cultured cells.[8,15–17] The combination of 3D cell culture and microfluidics has, therefore, laid the foundation for the development of organ-on-chip (OOC) devices capable of mimicking complex microarchitectures and functions of human organs or diseases.[10,18]

However, when integrating hydrogels into microfluidic devices, specific challenges arise from their unique material properties. For example, their flow dynamics within microchannels are affected by their rheological properties, potentially leading to clogging or non-uniform distribution. Furthermore, requirements of the hydrogel (e.g., temperature, pH, and osmolarity conditions), as well as interactions of the hydrogel with the device material, must be carefully reflected upon.[19–21] Additionally, hydrogels may require specific curing or crosslinking processes to stabilize within the device,[10,11,21] which must be considered during device design.

These challenges related to the integration of hydrogels, as well as striving for ever-more-realistic culture conditions, have substantially increased the complexity of the required culture systems. 3D printing has become an increasingly popular solution to address this problem, as it not only offers researchers the opportunity to fabricate highly complex customized structures, but also to benefit from rapid prototyping.[22,23] The various manufacturing methods grouped under the term "3D printing"[23] such as fused deposition modeling (FDM), multijet printing, and stereolithography (SLA), including two-photon polymerization (2PP), each have distinct advantages and disadvantages in the context of manufacturing culture systems. Several articles in the literature provide an overview of the strengths and weaknesses of each specific

technique.[22–25] Unlike conventional methods for fabricating microfluidic devices, such as soft lithography with polydimethylsiloxane (PDMS), these techniques can create complex 3D structures in a single fabrication step without the need for a unique master mold for each design.[23] Even cavities required for fluid flow can be directly produced with these techniques, whereas their integration with traditional techniques is often challenging.

Since its invention in the 1980s, techniques to facilitate 3D printing have rapidly improved in terms of printing resolution, time, and material options.[22–24] Indeed, some modern 3D printing techniques already enable micro- to nanometer resolution,[22,24,26,27] and multi-material printing allows direct integration of components like gaskets, valves, and actuators into microfluidic devices.[28–30] This enables researchers to influence and control the microenvironment of cultured cells more precisely, and to ensure convenient handling of the device through a chip-to-world interface.[14,31,32] Modern 3D printers can also be operated without extensive training, making this technique viable for a wide range of users and enabling the sharing, reproduction, and modification of promising designs within the larger research community.[23]

Many review articles have been published on the growing applications of 3D-printed microfluidic devices in the field of biotechnology[23,24,33] and on hydrogels as scaffold materials for 3D cell culture applications.[10,11,34] Therefore, the present review merely aims to provide a concise summary of some common challenges that researchers face when integrating hydrogels into 3D-printed microfluidic cultivation systems for mammalian cells that hinder the wider application of these otherwise promising systems and to present an overview of relevant developments aimed at addressing these challenges (with a focus on publications from the last 5 years).

2. Addressing the challenges of integrating hydrogels into 3D-printed microfluidic cultivation systems

In most cases, hydrogels are used as a scaffold for cell cultivation in a 3D environment,[10,35–37] or as a barrier to separate different compartments of the cultivation system to regulate the diffusion of nutrients, oxygen, and signaling molecules within the microfluidic system.[38] In addition, hydrogels can also be functional parts of a microfluidic system (e.g., as part of sensors or valves), or the microfluidic chip can be printed entirely from hydrogel using bioprinting.[39–41] However, this review article focuses solely on the challenges researchers face when integrating hydrogels into 3D-printed devices for mammalian cell culture.

Some of these challenges are general to handling microfluidic cell culture devices, while others arise specifically from integrating hydrogels into 3D-printed devices (Figure 1). General challenges in microfluidic cell

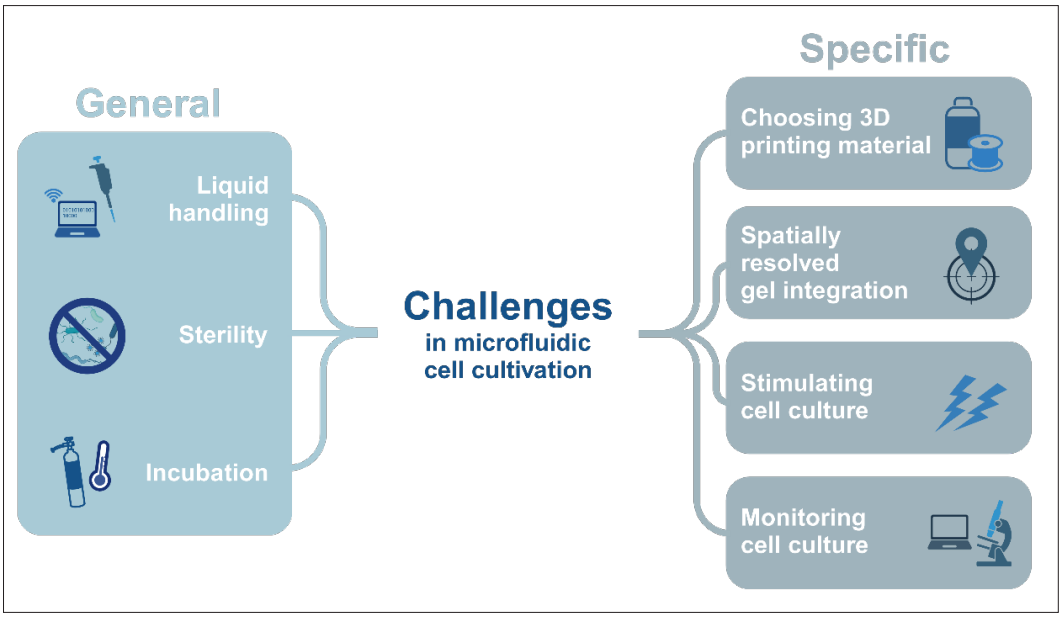

Figure 1. Main challenges faced when integrating hydrogels into 3D-printed microfluidic cultivation systems. General challenges when handling microfluidic cultivation systems independent of hydrogel integration are summarized on the left side; challenges specific to integrating hydrogels into 3D-printed devices are displayed on the right side.

cultivation often relate to liquid handling, ensuring sterility, and appropriate incubation. Specific challenges related to hydrogel integration include selecting an appropriate 3D printing material, achieving spatially resolved hydrogel integration into the device, and enabling effective stimulation and monitoring of the cell culture. 3D printing as a fabrication process for microfluidic cultivation devices offers potential solutions for all of these challenges.

For all microfluidic devices, reducing manual intervention and simplifying handling are desirable refinements that ensure reliable, repeatable performance of experiments, thus providing comparable results regardless of the experimenter. Therefore, it is unsurprising that the development of strategies to automate and miniaturize liquid handling has been at the forefront of recent literature. By using 3D printing as a fabrication technique, researchers can benefit from the availability and ease of adaptation of existing designs, for example, for miniaturized solutions to achieve fluid flow[8,42–44] and liquid dosing,[45] to integrate valves[9,30] and micro-controllers,[9] and to tackle the issue of air bubbles inside the device by integrating bubble traps.[46–48] In the context of microfluidic cell cultivation, another challenge is that a sterile cell environment must be maintained throughout the handling of the system to avoid contamination. Ensuring sterility is particularly challenging at the chip-to-world interface. Researchers can benefit from established designs for convenient 3D-printed chip-to-world interfaces, such as integrating 3D-printed Luer- and/or Luer-lock systems into the device design.[14,31,32,49] The use of these connections can also ensure compatibility with commercially available tubing and external pumps.[14,31,32]In addition to culture sterility, incubation conditions must also align with the desired parameters. A fundamental distinction exists between systems designed for operation in a cell culture incubator and systems that do not require external incubation. Independence from an incubator entails the need to control gas supply and temperature. The design freedom offered by 3D printing enables easy integration of components to make this possible. For example, Khan et al.[9] achieved temperature control in their device by integrating thermistor ports, an isolating cover, and on-device preheating of the culture medium via a heating plate.

The following sections discuss the specific challenges of integrating hydrogels into 3D-printed microfluidic cell culture systems and describe strategies to successfully overcome these challenges.

2.1. Selection of a suitable 3D printing material and 3D printing technique for device fabrication

To fully exploit the advantages of 3D printing as a fabrication process, printing technique and materials must be carefully selected from the growing variety of options. The use of microfluidic devices for the cultivation of hydrogel-embedded cells imposes specific requirements on the material, in terms of its mechanical, surface, optical, thermal properties as well as properties such as acid/base resistance, and cytocompatibility properties (Figure 2). The exact requirements for the material used depend on

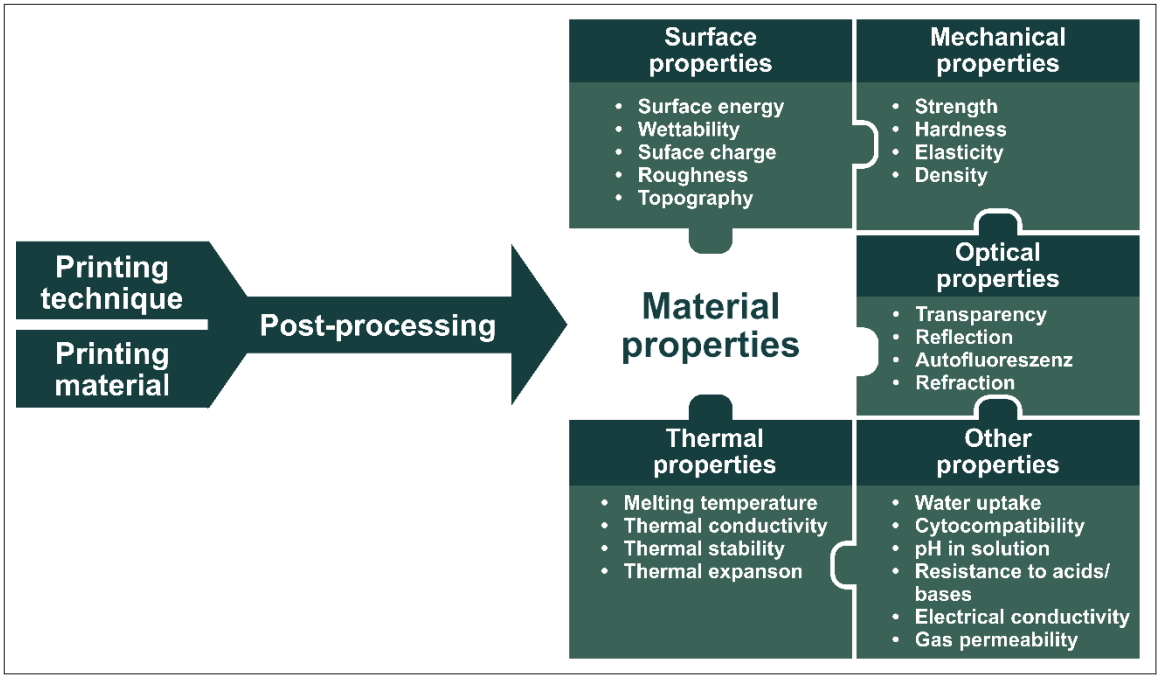

Figure 2. 3D printing material and technique, as well as post-processing of the printed devices, determine the material characteristics that influence the suitability of a 3D printing material for use in microfluidic hydrogel-based cell culture applications.

the specific purpose of the cultivation system. The general requirements that each cell culture device places on the material for the integration of hydrogels are discussed in the following section.

As aforementioned, device sterility is an absolute prerequisite. Therefore, any material used for this purpose must be able to withstand the sterilization/disinfection process without undesirable degradation or deformation. In heat steam sterilization (i.e., autoclaving), the material must withstand high heat and humidity, while alternative sterilization techniques, such as solvent (e.g., ethanol), plasma, or radiation treatment, have their specific requirements for the material.[22,50] Furthermore, researchers must consider that beyond the choice of printing material, specific printing parameters and sterilization conditions (e.g., temperature and duration of autoclaving) can have a significant influence on the material's ability to withstand the sterilization/disinfection process.[51,52] The acceptable degree of change in material properties and geometry induced by the sterilization/disinfection method depends on the cultivation system and its specific application. For an overview of the effect of various sterilization/disinfection methods on commonly used materials in additive manufacturing technologies, readers can refer to the literature by Told et al.[53] and Valls-Esteve et al.[52] Cytocompatibility is another key prerequisite for cell culture devices. Most manufacturers of 3D printing materials do not differentiate between cytocompatibility and the broader concept of biocompatibility in their material specifications. To our present knowledge, several 3D printing materials are currently marketed by manufacturers as biocompatible in their cured form.[54,55] Biocompatibility is generally defined as the ability of a material to exhibit an appropriate host response in a specific application.[54,56,57] This definition implies that the response of a particular host with respect to the specific material in question must be tested in each individual application, as it often depends on both the nature and duration of the interaction.[50,54,56] Therefore, the manufacturer's declaration can only provide a preliminary indication of a material's potential cytocompatibility in hydrogel-based culture applications and rigorous testing is necessary for each specific device to confirm its cytocompatibility under the intended culture conditions. In the context of cytocompatibility, all potential additives used to modulate material properties, like plasticizers, have to be critically evaluated, as leachable substances have reportedly affected cellular viability and behavior.[58,59] For example, Trebuňová et al. investigated how two plasticizers used to modify a lactic acid/polyhydroxybutyrate/thermoplastic starch blend for extrusion 3D printing impacted its cytocompatibility with 7F2 osteoblasts, observing differences in cell morphology, growth, and metabolic activity with varying plasticizer concentrations.[59] In microfluidics, even small quantities of leached chemicals can drastically alter the cell environment, impacting the cells and potentially introducing experimental inconsistencies.

In addition, the intended cultivation conditions that are envisioned within a device may place specific requirements on the material in terms of its long-term resistance to deformation due to mechanical forces or water absorption and/or swelling caused by high humidity and temperature in an incubator, as well as constant contact with the culture medium and hydrogel.

In contrast, the intended (microscopic) monitoring of the cells may depend on suitable optical properties of the material, such as adequate absorbance, low autofluorescence, and transparency. The potential effects of the interaction between the hydrogel, cultured cells, and the device material must also be carefully studied and considered, as the surface properties of the material can impact the interactions between hydrogel-device and cell-device.[19,20,22,60] In addition to the 3D printing material, the selected 3D printing technique can also play a critical role in determining the material properties of a 3D-printed device and its interaction with the hydrogel-based cell culture. The choice of 3D printing material and technique are closely linked, as the former can limit the latter. In particular, the selection of the 3D printing process depends on the required printing resolution, specific design elements of the device, and whether more than one material is to be used during the printing process. Each 3D printing technique has its strengths and weaknesses.[22,23,61] When choosing the printing technique for the production of microfluidic devices for hydrogel-based cell cultures, the strengths and weaknesses of various techniques must be carefully considered. For example, higher resolution and significantly smoother surfaces (and thus higher transparency) can be achieved with SLA compared to FDM.[22–25,62] Inkjet-based processes offer relatively high resolution and enable the combination of different materials in one printing process. This facilitates the direct integration of functional parts (e.g., gaskets) and influences hydrogel-device interaction, e.g., by spatially modulating the device's wettability.[28,63] Likewise, multi-material printing has its related challenges. For instance, when materials need to be manually exchanged (e.g., in SLA printing), the process becomes more time- and labor-intensive. In inkjet-based systems, technical limitations arise in controlling material deposition without unintended mixing before curing. Additionally, ensuring strong interlinking between materials is essential to prevent delamination of device components.[28,29] Photopolymers used in SLA and inkjet-based techniques also have a drawback: residual unreacted monomers and photoinitiators can impair the biocompatibility of the product.[22–25,62] In contrast,

FDM processes use a thermoplastic material, in which the polymer itself is already formed and no longer contains monomers or radical initiators. However, the melting of individual layers in FDM can also lead to a higher risk of water permeability[23] and subsequently leakage of medium and impaired sterility in cell culture applications. 2PP offers the best printing resolution and enables the printing of surface structures in the microphysiological size range. For example, Sharaf et al. fabricated biomimetic 2.5D micro- and nano-pillar arrays (diameter: 0.29–1.06 μm) in an attempt to mimic native brain tissue using 2PP. Compared to microglia cultured on flat substrates, cells grown on the pillar arrays exhibited increased expression of their *in vivo* phenotype.[64] However, the fabrication of large-scale structures, such as whole cultivation devices, using 2PP can be extremely time-consuming. Several comprehensive review articles explained the fundamental principles of established 3D printing techniques and compared their strengths and weaknesses,[22–25,62] providing an invaluable resource for researchers addressing this complex issue.

Finally, the post-processing of the 3D-printed cultivation device must also be accounted for,[54,65] as it can have a significant impact on the material properties of the device and its interaction with the hydrogel-based culture.[61,62,65–67]

2.2. Spatially resolved integration of hydrogels into 3D-printed microfluidic cultivation systems

The integration of a hydrogel into the microfluidic cultivation device has a crucial influence on its final shape and the microenvironment of the embedded cells. The supply of nutrients and gases and the removal of metabolites depend on the interaction between hydrogel-embedded cells and the culture medium. Therefore, the hydrogel must be placed within the cultivation device in a position that facilitates contact between the medium and the hydrogel, while simultaneously ensuring that the perfusion channels are not obstructed by misplaced hydrogel structures. In addition, precise positioning of the hydrogel within the cultivation device is essential for accurate monitoring of the embedded cells. Furthermore, researchers must consider that cells will inevitably exert forces on their surrounding matrices to spread, proliferate, and migrate,[12,36] which can potentially lead to hydrogel deformation and detachment from the cultivation device if not properly addressed. Finally, swelling/shrinkage of the hydrogel (e.g., in response to chemical administration) and shear stress induced by medium/fluid flow can also lead to detachment of the hydrogel.

Several distinct concepts for achieving the spatially resolved integration of a hydrogel into the 3D-printed microfluidic cultivation system are identified and discussed in detail in the next section. When selecting an integration strategy, researchers should consider that a specific hydrogel can often be integrated using multiple methods. The choice of strategy depends not only on the hydrogel's properties but also on device design requirements for cell monitoring, stimulation, and device handling during experiments.

2.2.1. Strategies for integrating hydrogels at the desired position

The methods for integrating hydrogels can be classified according to whether the hydrogel is incorporated into a monolithic or non-monolithic system, with each method offering specific advantages and disadvantages. In monolithic systems, the hydrogel can be integrated using techniques, such as photopolymerization, capillary forces, surface tension, and shape-memory effects. These approaches enable efficient and direct incorporation of the hydrogel, minimizing time and complexity associated with device assembly, but typically require subsequent sealing of channels to prevent contamination, leakage, and evaporation. In contrast, non-monolithic systems offer the advantage that cells and components can be more easily recovered after experiments. In these cases, various techniques (e.g., mold casting, shape-recovering hydrogels, and bioprinting) are employed, providing a flexible and often less complex method for hydrogel integration. These methods are particularly advantageous when samples or device components are to be recovered after the experiment (further discussed in **Section 2.4.2**). The different approaches for integrating hydrogels at a desired position are presented in Figure 3 and described in the following sections. These methods can be combined to achieve even more complex hydrogel patterns or structures composed of more than one hydrogel.

(i) Photopolymerization: Some crosslinking strategies can be used directly to achieve crosslinking of the hydrogel only in the desired area of the cultivation device. For example, when using hydrogels that are crosslinked by ultraviolet (UV) light, photomasks can be used to crosslink only the desired hydrogel structures.[11,68] The remaining hydrogel solution can then be removed to ensure no hydrogel remains in the perfusion channels of the cultivation device. To enable photopolymerization, the selected device material must be translucent, and it may be necessary to adjust the radiation dose compared to polymerization without the device. In addition, light scattering or diffraction at the edges of the microfluidic device may lead to distortion of the light pattern structured by the mask. Conversely, a significant advantage of light-based systems is their ability to achieve very high resolution. Another benefit is that they can

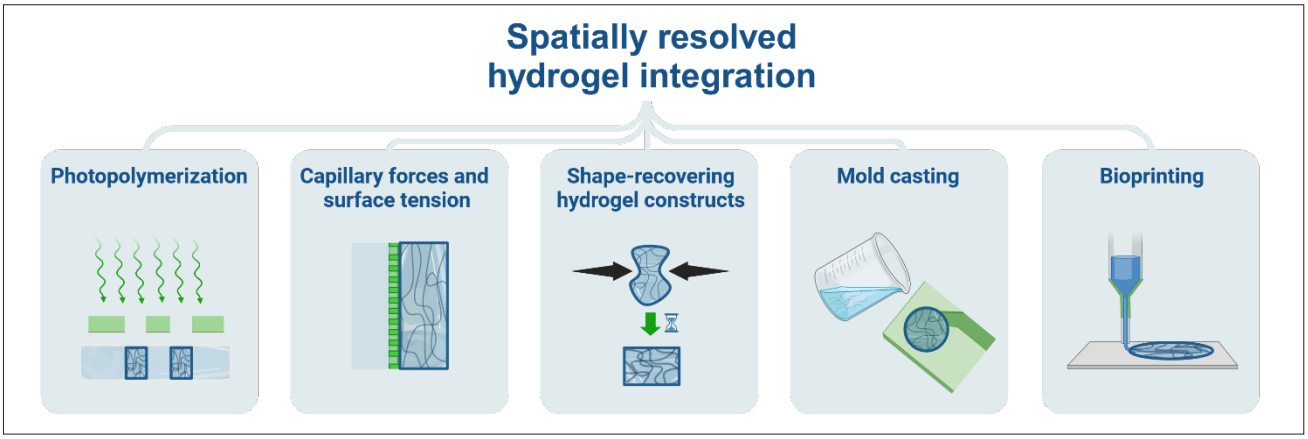

Figure 3. Concepts for spatially resolved hydrogel integration into 3D-printed microfluidic devices include: photopolymerizing the hydrogel within designated compartments; using capillary forces and surface tension to position the hydrogel; incorporating shape-recovering hydrogels; employing the device as a mold for hydrogel shaping; and bioprinting the hydrogel directly into or onto the device.

create large interfaces with the medium, which can be advantageous for gas and nutrient exchange.

(ii) Capillary forces and surface tension: Another approach is to utilize capillary forces and surface tension to guide the hydrogel solution to the desired position within the cultivation device.[38] This can be achieved by integrating specific design features into the device, such as those presented by Virumbrales-Muñoz et al.[69] The authors integrated parallelogram-shaped pillars between a central hydrogel channel and two parallel culture medium channels. These pillars ensured that their hydrogel for 3D cell culture only filled the central microchamber, facilitating perfusion of the culture through the lateral microchannels, without disturbing the confined hydrogel.[69] Micropillars have also successfully confined different hydrogels within distinct compartments in more complex organ models. Shanti et al.[70] used micropillars spaced 180 µm apart, each with a radius of 250 µm, to precisely position various matrix-embedded immune cell types within specific compartments of their device. Another option is the integration of a permeable membrane to enclose the hydrogel compartment of the device.[32] In this approach, the choice of membrane material and pore size can also influence which substances, macromolecules, or particles can reach the hydrogel compartment.[71] Another strategy to control the hydrogel solution is the use of specifically located hydrophobic and hydrophilic areas of the device. This can be achieved either by combining 3D printing materials with different material properties or by using surface modification strategies.[63,72] Employing thermoresponsive polymer coatings, developed for the controlled and spatially resolved detachment of cell sheets, in the cultivation device could also offer interesting opportunities. These coatings undergo a thermally triggered transition from a more hydrated state to a less hydrated state upon heating, and could potentially be used to alter hydrogel-device interaction during gel integration in a time-dependent manner.[73]

(iii) Shape-recovering hydrogel constructs: Ying et al.[74] presented an interesting approach to avoid crosslinking within the device by introducing an injectable, hierarchically patterned, porous gelatin-methacryloyl (GelMA) hydrogel that could recover its original shape after compression. Due to the macro-, micro-, and nanoporous structures present, the cell-laden hydrogel constructs were able to sustain high cell viability, proliferation, spreading, and differentiation even after compression and injection.[74]

(iv) Mold casting: Another approach to achieve a defined volume and shape of the hydrogel is to use the cultivation device as a mold. If the device is designed as a non-monolithic system, the hydrogel solution can be added to a disassembled device. In this case, one part of the device can be used as a mold for the hydrogel, while another part (containing the perfusion channels) can be added only after the hydrogel has polymerized to mitigate the risk of clogging the channels with the hydrogel.[32] Furthermore, sacrificial structures can be integrated into the device before hydrogel incorporation. After gelation, the sacrificial template can be removed, leaving behind the structured hydrogel.[75]

(v) Bioprinting: Hydrogel structures can be bioprinted using the 3D-printed cultivation device as a substrate. Hydrogel bioprinting offers researchers an opportunity to create complex hydrogel structures and assist with incorporating more anatomically accurate tissue architecture features.[76,77] A wide range of different

hydrogels suitable for bioprinting are now available on the market, and there are several comprehensive review articles discussing the advantages and challenges of the approach for structuring hydrogels.[78–80] For structuring hydrogels inside a microfluidic cultivation device, researchers must consider that extrusion-based printing techniques require an open device as a substrate, whereas techniques such as 2PP can also be used with monolithic devices. For a detailed discussion of the advantages and disadvantages of various bioprinting techniques for direct printing into or onto the culture device, readers can refer to the literature by Rothbauer et al.[81] 3D bioprinting and the combination of different crosslinking strategies can also be used to achieve complex hydrogel-based co-cultures. For example, da Silva et al.[82] generated their 3D *in vitro* model of breast tissue by first 3D printing alginate hydrogels into porous scaffolds, and then inoculating this scaffold with human mammary fibroblasts; subsequently, a peptide-modified alginate pre-gel loaded with mammary gland epithelial cells is introduced to fill the pores of the scaffolds. This pre-gel was introduced to form a hydrogel *in situ* by ionic crosslinking.[82]

2.2.2. Preventing hydrogel detachment

During the cultivation process, cells exert forces on the surrounding matrix and remodel it, which may result in hydrogel deformation and detachment from the cultivation device.[12,36] In addition, fluctuations in hydrogel volume due to various factors (e.g., chemical stimuli and/or shear forces from the fluid flow) may also cause detachment. Consequently, securing the hydrogel within the culture device is crucial.

One strategy to prevent hydrogel detachment is to incorporate retaining features into the hydrogel compartment when designing the cultivation device. Kim et al.[38] reported a comb-like structure to integrate a hydrogel channel between two fluid channels as a perfusion barrier in their device. The central hydrogel channel (1.0 mm [height] × 500 μm [width]) was connected to the neighboring fluid channels through 27-μm-wide capillary channels, which pinned the hydrogel within the barrier using surface tension.[38] Similarly, Virumbrales-Muñoz et al.[69] integrated parallelogram-shaped pillars between a central hydrogel channel and two parallel culture medium channels in their microdevice. These pillars ensured the confinement of a hydrogel for 3D cell culture in the central microchamber and allowed the perfusion of culture media through the lateral microchannels without disturbing the confined hydrogel.[69] Shanti et al. used micropillars spaced 180 μm apart, each with a radius of 250 μm, to precisely position hydrogels in specific compartments of the device[70] (Figure 4A). Khan et al.[9] utilized small indents of 0.5 × 0.5 mm^2 on the inner surface of culture wells (Figure 4B), thereby preventing the detachment of Matrigel-embedded organoids from the 3D-printed wells.

Another strategy to ensure a defined position of the hydrogel is to modify the surface of the culture chamber to enhance hydrogel attachment. Unfortunately, most established strategies for bonding hydrogels to a surface involve toxic chemicals and/or harsh reaction conditions that may change the mechanical and/or chemical properties of the hydrogel itself[83] and are incompatible for use with living cells. Depending on the hydrogel and cultivation device material, however, some methods developed for hydrogel coating or improving the attachment of hydrogel cultures to glass coverslips might provide a helpful starting point. For example, Qiao et al.[84] prepared amino-silanated coverslips to attach their polyacrylamide hydrogel used to culture neurons. This method might be helpful in instances where glass slides are incorporated into the device to serve as optical windows for better monitoring of the cultures (further discussed in **Section 2.4**). Another research field that could inspire hydrogel bonding to 3D-printed cultivation devices is the development of mussel-inspired strategies for bonding wet, soft materials, such as those used for fixing implantable devices and wound sealing.[85,86] Gao et al.[86] have reported a mussel-inspired synthetic adhesive based on catechol-modified polymers that could topologically entangle with the hydrogel network and chemically interact with an adherent surface (Figure 4C). The authors demonstrated the adhesion of hydrogels to various substrates, such as metals, glasses, elastomers, plastics, and living tissues.[86] Likewise, Sun et al.[85] reported negligible inflammation response and superior biocompatibility with L929 cells and in mouse models for their mussel-inspired adhesive.

Additionally, the incorporation of a porous membrane to confine the hydrogel compartment may prevent hydrogel detachment from the device.[71] An advantage of this approach is that the incorporated membrane can also be used to ensure a defined height of the hydrogel. However, a consequence of this strategy is that the direct contact between the hydrogel culture and the medium is interrupted, which alters the shear force of the medium and may affect the diffusion of metabolites and gases between the medium channel and the hydrogel compartment. Furthermore, depending on the characteristics of the membrane used, the migration pattern of the cultivated cells could also be altered. Similarly, researchers should carefully consider whether such a membrane can and should be reversibly integrated into the device, for example,

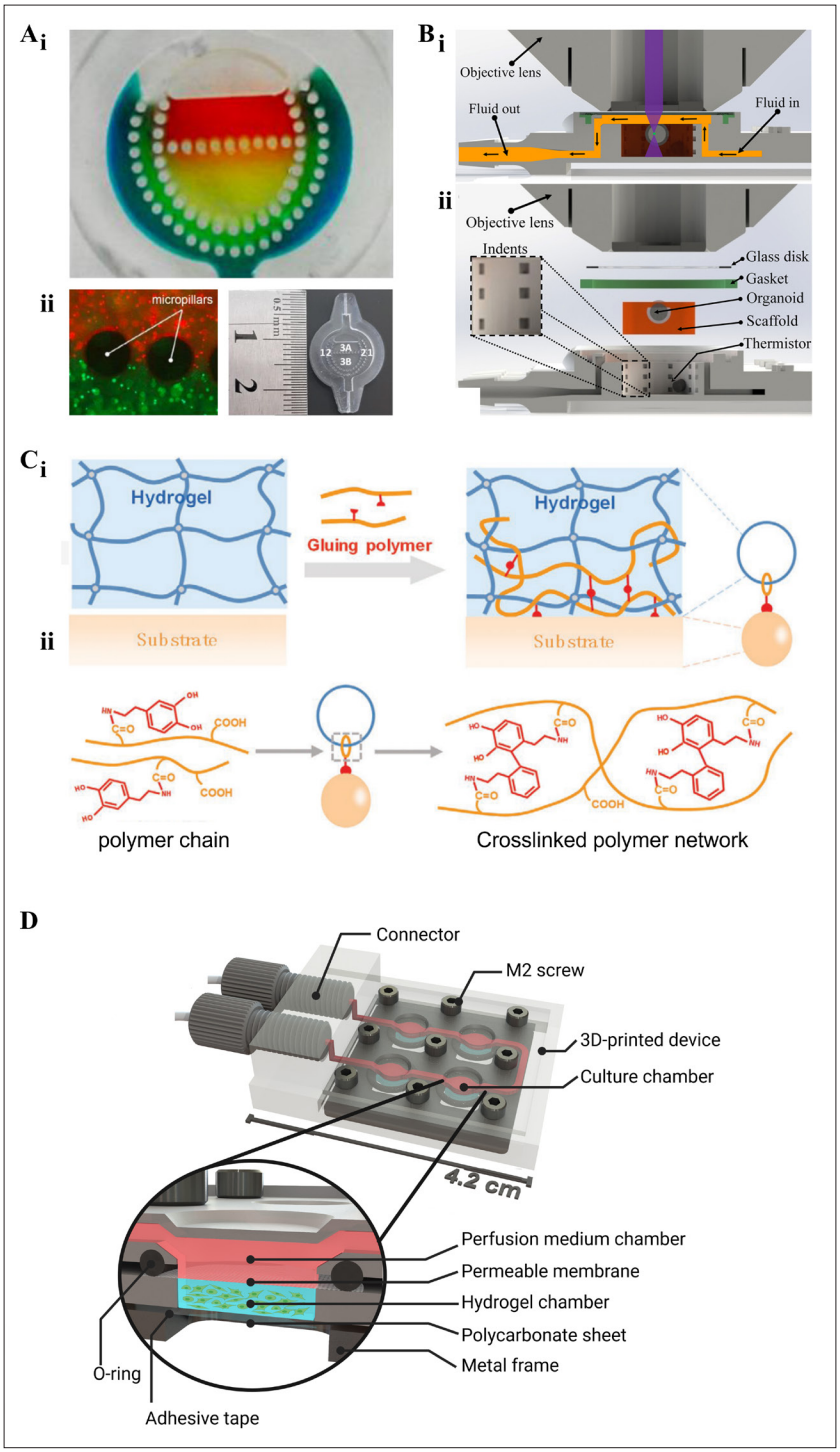

Figure 4. Strategies for preventing hydrogel detachment. (A) Utilizing micropillars for confining hydrogel in specific compartments of the device. (A, i) Illustration of pillar efficiency in providing separation between the different compartments. (A, ii) Different fluorescent-labeled immune cells (Jurkat cells [red]; THP-1 [green]), each confined to a distinct compartment of the device (left) and size representation of the entire device (right).[70] Adapted from ref.[70] (B) Microfluidic cell culture device with micro-indents in the culture well to avoid hydrogel detachment. (B, i) Schematic of the imaging setup with fluid flow. (B, ii) Explosion view of the components of one culture well with enlargement of the micro indents for hydrogel fixation. Adapted with permission from ref.[9] Copyright 2021, AIP Publishing. (C) Principle of a universal strategy for hydrogel adhesion. (C, i) Schematic of the stitch-bonding mechanism. (C, ii) The principle is illustrated with a dopamine-grafted polyacrylic acid (PAA) as the glue polymer and $NaIO_4$ as the trigger for intermolecular crosslinking. Adapted with permission from ref.[86] Copyright 2020, WILEY-VCH Verlag GmbH & Co. KGaA. (D) Hydrogel fixation by integrating a porous membrane between the hydrogel compartment and the fluid flow. Adapted from ref.[32]

to facilitate cell recovery from the device. Moreover, the selection of integration techniques will also depend, at least in part, on the timing of membrane integration. In cases where the membrane is integrated after the cells have already been seeded into the device, techniques that require potentially toxic chemicals (e.g., adhesives or solvents) or high temperatures (e.g., thermal fusion bonding or laser welding) should be avoided. Hernández-Castro et al.[87] reported the use of four screws and bolts distributed along the corners of their 3D-printed device for the capture and release of white blood cells to clamp a membrane between the two device parts. Likewise, Meyer et al.[32] used screws and an O-ring to secure a membrane in position between the hydrogel chambers and the perfusion channels (Figure 4D). Another increasingly popular strategy for microfluidic cell culture devices is using biocompatible adhesive taps for integrating functional units or bonding device parts.[32,88,89] With the appropriate adhesive tape, this strategy even allows for reversible bonding, for example, in response to UV light irradiation.[90] Porous structures, such as membranes, can also be integrated into microfluidic devices using multi-material printing approaches by printing the porous barrier directly into the devices.[91]

2.3. Stimulation of hydrogel-embedded cells

Cells are constantly subjected to a wide array of mechanical, physical, chemical, and biological stimuli in their *in vivo* environment, making it crucial for researchers to accurately replicate these conditions in experiments to develop realistic biological models and enhance cell growth and differentiation. For example, endothelial cells are greatly affected by the level of shear stress they are exposed to,[92,93] and the elastic modulus of hydrogels used as synthetic ECM for 3D cell culture has a significant influence on cell behavior, including cell spreading, migration, proliferation, and differentiation.[12,36,94] In microfluidic devices for culturing hydrogel-embedded cells, cell stimulation can be achieved by defining the hydrogel's shape and tuning its mechanical properties, as well as by integrating specific design features and components into the device for external stimulation. Examples of both approaches are described in the following paragraphs. Given the limited examples of fully 3D-printed microfluidic devices enabling stimulation of hydrogel-embedded cells, concepts that can be easily adapted to 3D-printed devices are also considered.

The native ECM exhibits dynamic mechanical properties and displays time-dependent responses to deformation or mechanical lading in terms of viscoelastic behavior, and there is a growing number of hydrogels currently being developed to fine-tune viscoelastic properties and imitate tissue-specific ECM.[12,36,94] It is also recognized that stiffness gradients in the cell microenvironment can act as signals that influence the regulation of cell function and behavior.[95,96] To achieve the fabrication of hydrogels with such stiffness gradients inside a cultivation system, the micromixer presented by Lavrentieva et al.[95] could potentially be integrated into a monolithic design as part of the loading unit for the hydrogel solution. In that work, the authors successfully established the desired gradients in GelMA hydrogels via dynamic mixing of low degree of functionalization (DoFs) and high DoF hydrogels, with no discernible effect on cell viability of adipose tissue-derived mesenchymal stem cells (AD-MSCs) and human umbilical cord vein endothelial cells (HUVECs) by the micromixer[95] (Figure 5A). The technique they described could also be adapted to introduce chemical gradients into a hydrogel. Furthermore, stimuli-responsive hydrogels can be used in cell culture devices to manipulate cells. These hydrogels organize their internal architecture in response to an external stimulus.[10] To trigger this response, different types of physical and biochemical stimuli—such as light, electric field, temperature, change in pH, ultrasonic waves, and magnetic fields—can be applied and leveraged[10,37,97] (Figure 5B). This approach allows for a timed release of chemical stimuli (e.g., drug treatments).[98,99] If the hydrogel is incorporated into the cultivation device prior to full assembly, its surface topography can also be used to influence protein adsorption, cell adhesion, and morphology.[19] The design of the device itself can also influence the embedded cells. For example, gradients can result from the position and course of media channels in the device. Virumbrales-Muñoz *et al.*[69] stated for their device, featuring a central hydrogel channel flanked on two sides by lateral microchannels, that the highest oxygen level and nutrient availability were observed close to the lateral microchannels, with a decrease towards the center of the microchamber due to cell consumption. It is important to consider these effects, as they may be desirable in certain circumstances (e.g., cancer research),[69,100] but are generally undesirable for the prolonged culture of non-cancerogenic cells. The channel layout and different channel dimensions in various parts of the device can also be used to achieve different perfusion rates in compartments of the device with only one pumping unit.[8] Shinha et al.[8] achieved a physiological flow ratio between the liver and lung compartments of their device by integrating a bypass channel with a different height in the lung cancer compartment (Figure 4C). In addition, the shear stress from the flowing fluid inside these channels should be taken into account, as some cells (e.g., endothelial cells) have reportedly exhibited enhanced differentiated cell phenotypes and functions when exposed to shear stress in their physiological range, while other cells (e.g., embryonic stem cells and primary neurons) respond much more poorly.[18,92] Another reason to avoid unnecessary high shear stresses is that it increases the need to fix the hydrogel

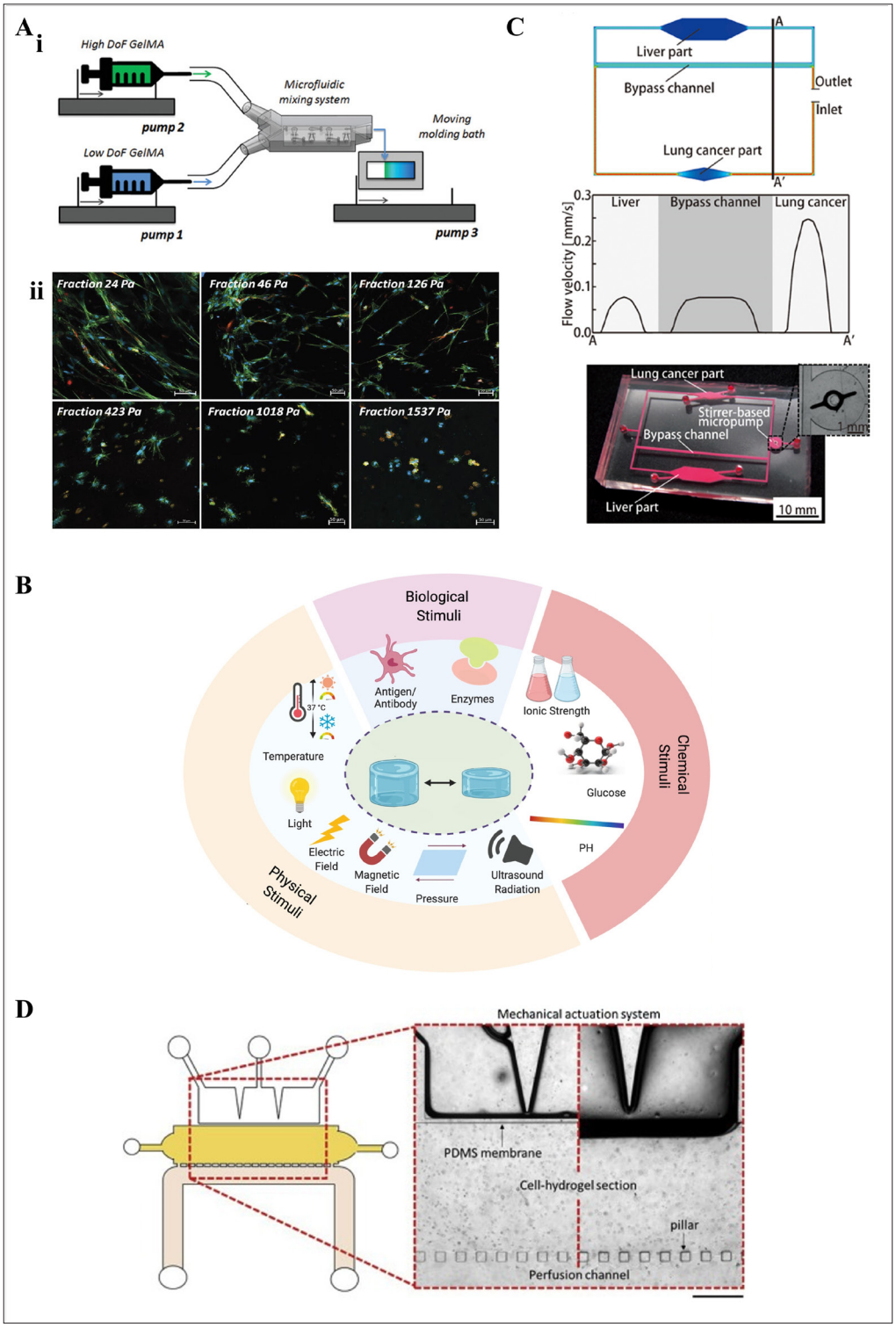

Figure 5. Strategies for enabling stimulation of hydrogel-embedded cells. (A) Gradients for cell stimulation. (A, i) Stiffness gradients in the hydrogel can be generated using a 3D-printed microfluidic mixing device. (A, ii) Their influence on hAD-MSCs and HUVECs encapsulated in GelMa hydrogel was analyzed using confocal microscopy on day 7 of cultivation; green staining: actin filaments; red staining: CD31; blue staining: DAPI. Adapted from ref.[95] (B) Overview of stimuli that can be applied to trigger stimuli-responsive hydrogels. Adapted from ref.[37] (C) Geometry and layout of media channels used to achieve different perfusion rates in the same fluid circuit with only one pump. Adapted from ref.[8] (D) Mechanical stimulation applied to hydrogel-embedded cells by pressure-controlled displacement of a membrane neighboring the hydrogel. Scale bar: 500 μm. Adapted from ref.[16] Abbreviations: DoF, degree of functionalization; GelMa, gelatin-methacryloyl; hAD-MSCs, human adipose tissue-derived mesenchymal stem cells; HUVECs, human umbilical cord vein endothelial cells.

within the device (described in **Section 2.2.2**). Although this is a challenging task, successful implementation has, for example, been demonstrated by Lyu et al.,[101] who were able to expose a layer of endothelial cells to shear stress in the physiological range while confining hydrogel-embedded neural and glial cells in the designated compartment of their neurovascular-unit-on-a-chip.

The device design can also be used for chemical stimulation of cells if the incorporation of chemical stimuli directly into the hydrogel is undesirable. When administering chemicals via medium flow, they can either be pre-loaded onto the device at the start of cultivation, or they can be directly applied to the hydrogel culture at the time of treatment. The first option can be achieved by integrating specific fluid reservoirs and individually controllable pumps into the device, while the second option can be implemented as presented by Khan et al[9]. In their work, the authors successfully integrated a designated drug delivery port into their hydrogel chamber design, inserted a standard cannula, and sealed it with biocompatible silicone or UV-curing adhesive[9].

In addition to the device layout, the integration of components can also be used to actively exert stimuli on the cells. For example, the exertion of mechanical forces can be achieved through the integration and controlled deformation of a membrane by vacuum or pneumatic pressure.[18,22,71,102] Such mechanical forces play an important role in tissue development, organ function, and disease pathology and can be used to mimic breathing-induced stretching, peristalsis-like motions, or the contractility of cardiac tissues.[16,18,103] For example, Paggi et al.[16] achieved compressive stimulation of hydrogel-embedded chondrocytes by deflecting a PDMS membrane adjacent to the hydrogel compartment (Figure 5D). In their device, defined membrane deflection was achieved by three actuation chambers connected to a commercially available pressure controller.[16] The components required for actuation could also be fully 3D-printed as demonstrated by Hinnen et al.[104] with their 3D-printed one-way valves and pumps fabricated using digital light processing SLA using a poly(ethylene glycol) diacrylate (PEGDA) resin. Another way to exert mechanical forces could be the approach presented by Yang et al.,[105] where they incorporated arrays of functional droplets into an elastomer matrix. These composited elastomers can be induced to perform precise deformations based on, for example, solvent-triggered droplet swelling, temperature-triggered solvent vaporization, and light-triggered phase transition.[105] However, in the context of cell culture devices, it must be ensured that the stimulus used to deform the composite material does not also directly affect the hydrogel or cells themselves.

Furthermore, integrating components can be used to simulate a changing cell environment. For this purpose, Arik et al.[106] developed an enzymatically degradable collagen-I-based membrane. In contrast to conventional polymer-based membranes, their membrane could be induced to allow cell migration only after a specific time point.

2.4. Monitoring of hydrogel-embedded cells and their environment

For obvious reasons, the ability of researchers to readily monitor their cultured cells is one of the most important requirements for any cultivation system. In this context, it can be differentiated between *in situ* monitoring during cultivation and subsequent analysis of the culture after cultivation. Where further analysis or recultivation of the cells is desired, recovery of the cells or the whole 3D culture from the cell cultivation device should be possible.

2.4.1. Enabling in situ monitoring

In situ monitoring offers the advantage of capturing dynamic changes and providing real-time feedback, thereby enhancing the understanding and control of experimental processes compared to endpoint measurements. Microscopic imaging remains one of the most popular analytical techniques for *in situ* cell analysis.[107] For the microfluidic cell culture system to enable such microscopic imaging, sufficient light incidence must be ensured. This influences both the device design and the material choice. With respect to the device design, both an appropriate geometry of the cell culture region, as well as an appropriate focal distance, must be assured. The choice of 3D printing material directly determines imaging feasibility through the printed device. For instance, with transparent printing materials (e.g., commercially available resins used by Fritschen et al.[49] for manufacturing their device for perfused hydrogel cultures via digital light processing [DLP]), imaging can occur directly through the device. For non-transparent devices, a transparent material can be strategically incorporated at the cell culture site to allow for imaging. For example, Meyer et al.[32] included a 250 µm polycarbonate sheet as the bottom part of the cultivation chambers of their microfluidic cell culture device. Likewise, Khan et al.[9] incorporated a 150-µm glass disk as an optical window above the culture region in their system (Figure 6A). Furthermore, the transparent material can also directly be used as a substrate for 3D printing. Kim et al.[38] reported using a 1.0-mm-thick glass slide as a substrate for building their device from commercially available resin using a DLP-SLA printer.

When even more information about the cultured cells is required, fluorescent imaging techniques can be deployed. At this point, however, the autofluorescence

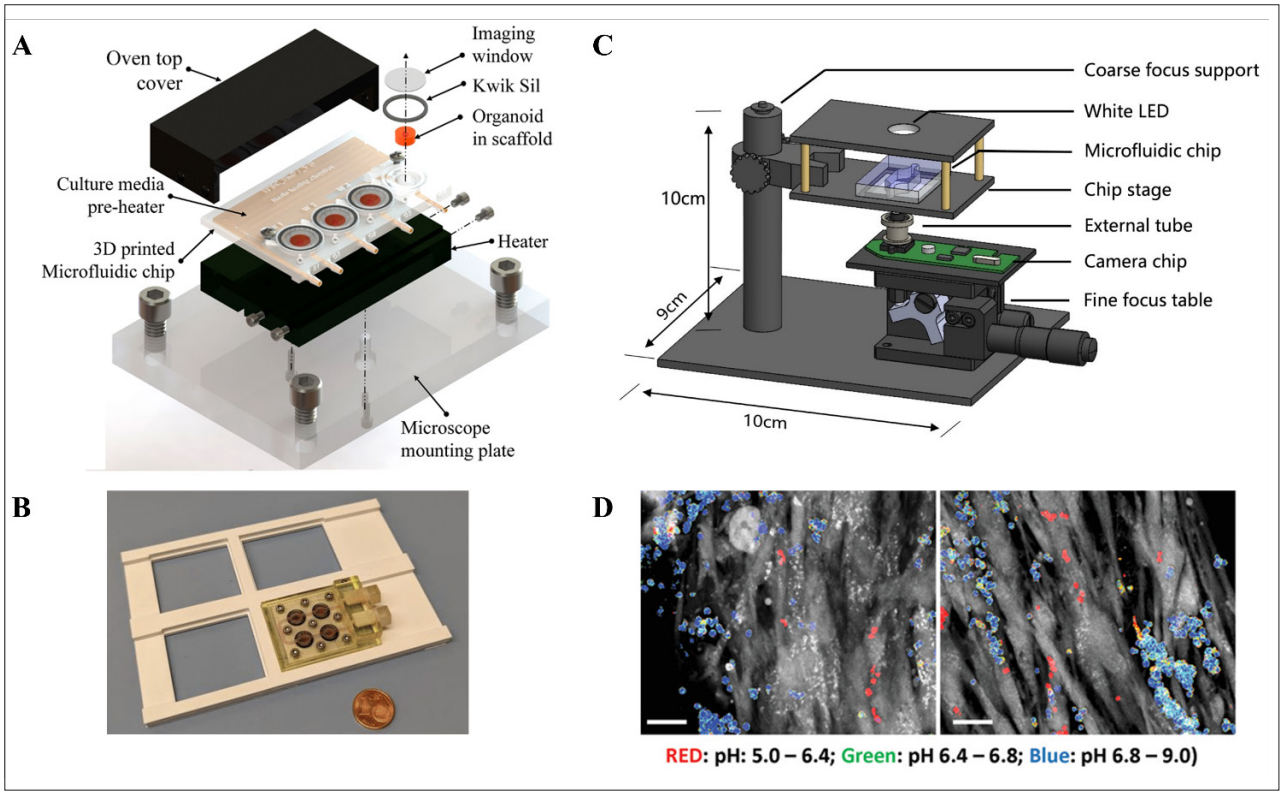

Figure 6. Strategies for enabling *in situ* monitoring of hydrogel-embedded cells. (A) 3D-printed microfluidic cell culture device with incorporated transparent imaging window for microscopic analysis of hydrogel-embedded organoids.[9] Adapted from Khan et al., copyright 2021, with the permission of AIP Publishing. (B) 3D-printed adapter for achieving compatibility of the 3D-printed microfluidic hydrogel culture perfusion device with standard imaging systems. Adapted from ref.[32] (C) Mini-microscope manufactured from readily available components for continuous *in situ* observation of cell growth and optoelectronic sensing-based pH measurement. Adapted from ref.[118] (D) pH-sensing capsules based on the fluorescent dye seminaphtharhodafluor (SNARF-1) enable spatially-resolved pH measurements in 3D cell cultures. Scale bars: 20 μm. Adapted from ref.[17]

of the 3D-printed material also becomes relevant. Autofluorescence can be challenging in imaging processes because many 3D printing materials exhibit some degree of autofluorescence, which can potentially interfere with and impede the desired imaging spectra.[67] To avoid this challenge, glass is a promising material in microscopic applications.[108] Additionally, manufacturing microfluidic devices made of glass at room temperature is becoming more practical with the use of glass-printed precursors processed using technologies adapted from polymer 3D printing. These printed precursors can be transformed into transparent glass through a heat treatment process.[24,108,109]

Another step towards facilitating ready and continuous imaging of cultivated cells within a device is found in achieving compatibility of the 3D-printed cultivation device with commercial live cell imaging systems. For example, Meyer et al.[32] fabricated an adapter in well plate format for their cultivation device to allow for easy imaging of the cultivated cells using a Cytation5 Cell Imaging Multimode reader (Figure 6B). Other authors have reported using smartphone-based approaches to allow for low-cost *in situ* monitoring.[107]

Furthermore, the hydrogel itself can be employed for monitoring the embedded cells. For example, it has been demonstrated that Förster resonance energy transfer (FRET) sensors can be successfully incorporated into synthetic hydrogels.[13] Yan et al.[13] presented a FRET sensor-modified synthetic hydrogel for real-time monitoring of cell-derived matrix metalloproteinase (MMP) activity using fluorescence lifetime imaging. Using a human breast cancer cell line, they demonstrated that their system allowed for real-time monitoring of MMP activity within the synthetic hydrogel.[13]

Quantifying cell numbers represents yet another challenge that arises from *in situ* monitoring. Currently, standard methods for assessing cell proliferation in 3D scaffolds usually rely on changes in metabolic activity or total DNA.[110] However, these approaches do not allow direct quantification of single cells and instead rely on

drawing comparisons against standard curves that often require degradation of the scaffold prior to analysis.[110] Recent suggestions offered by Zavadakova et al.[110] and Eschweiler et al.[111] to enable cell counting from 3D microscopic images might point researchers on a path toward a more direct approach. Additionally, artificial intelligence may very well aid in the standardized analysis of experiments conducted in 3D microfluidic cell culture devices.[112] Aside from microscopic analysis of the cells, monitoring the cell culture microenvironment (like temperature, pH, or local oxygen concentrations) or cell activity and behavior (e.g., via Transendothelial Electrical Resistance [TEER] sensors or microelectrodes for calcium signaling) by integrating sensors directly into the cell culture device can facilitate a deeper understanding of underlying processes.[88,113] However, the integration of sensors for these measurements into the cultivation device entails several challenges. Device design and sensing scheme usually have to be iteratively optimized, bearing in mind that different sensor types can be used to monitor the same readout.[88] Non-invasive/ minimal invasive measurements in 3D hydrogel-based tissue models are most promisingly realized by measuring alterations in the optical properties of a suitable indicator. Several comprehensive review articles have discussed the challenges that researchers face when integrating sensors into microfluidic devices/ OoCs[88,114,115] and monitoring microfluidic/ 3D cell cultures,[1,18,116,117] and they may provide both guidance and inspiration for analyzing 3D-printed cell culture systems. A fundamental decision that must be made with respect to every parameter is whether a measurement averaged over the cultivation area or only recorded at one point is sufficient, or whether a spatially-resolved analysis is required. For instance, a pH measurement can either be conducted for the medium within the cultivation chamber, or by taking the local pH value at various points in the 3D cell culture. For bulk measurement in the culture medium, the approach adopted by Li et al.[118] can be considered, as they developed a mini-microscope for *in situ* continuous observation of cell growth and optoelectronic sensing-based pH measuring inside a PDMS-based microfluidic cell culture system. The presented pH measurement required phenol-red in the culture medium, since it was based on the analysis of its pH-dependent light absorption. Their mini-microscope was made from a readily available camera, and a Bluetooth module was used for wireless data transmission to the computer outside the incubator[118] (Figure 6C). For a spatial resolved pH measurement, it can be turned instead to the example provided by Moldero et al.[17] Those authors were able to detect time-spatial pH variations and gradient formation utilizing pH-sensing capsules based on the fluorescent dye seminaphtharhodafluor (SNARF-1) (Figure 6D). Applying confocal fluorescence microscopy, they successfully spatially resolved monitored the pH across 3D cultures of human mesenchymal stromal cells.[17]

2.4.2. Recovery of the hydrogel and embedded cells for further analysis

After an experiment, the recovery of the hydrogel-based culture is often desired for further analysis. Retrieving hydrogel-embedded cells from entirely enclosed systems is frequently a difficult task to accomplish without causing damage to the hydrogel.[18] Where the 3D structure of the culture is intended to be retained during recovery, such as for preparing and analyzing cryosections for spatially resolved analysis of cell morphology or interactions, this must be considered during the device design stage. A solution is to implement a modular device design to facilitate its disassembly after cultivation. Meyer et al.[32] designed a cultivation device that consisted of two 3D-printed parts forming the cultivation chambers and medium supply channels, reversibly connected via a metal frame and screws. When desired, the device could be readily disassembled, allowing the hydrogel to be recovered intact.[32] McLennan et al.[119] also used a modular approach for their 3D-printed microfluidic culture device to facilitate cell retrieval after cultivation. Their design, which was fabricated by 2PP, consisted of a chip with 10 channels that delivered sub-microliter volume flowrates to 10 individually retrievable cell culture units that interlocked within the chip to create the microfluidic device[119] (Figure 7A).

Other commonly used techniques for the analysis of cultured cells include cell lysis for gene expression analysis and the analysis of protein content, as they often offer more information than *in situ* methods.[120] When using these methods, only the cells themselves need to be recovered. Therefore, controlled hydrogel degradation can be considered to facilitate the recovery of the cells, such as with the use of stimuli-responsive hydrogels.[37] Mahboubian et al.[121] demonstrated the use of a temperature-responsive hydrogel made of cellulose and hyaluronic acid to support *in vitro* 3D cell culture, where they were able to recover the embedded cells after 7 days of cultivation from the hydrogel by placing their hydrogel for 5 min at room temperature, before adding an excess of cell culture medium at room temperature. Another option would be enzymatic degradation of the hydrogel. Virumbrales-Muñoz et al.[69] presented a collagenase-based protocol for cell recovery from a collagen hydrogel in their microfluidic device within 10 min (Figure 7B). This protocol demonstrated no influence on cell viability and enabled cell extraction through the microfluidic

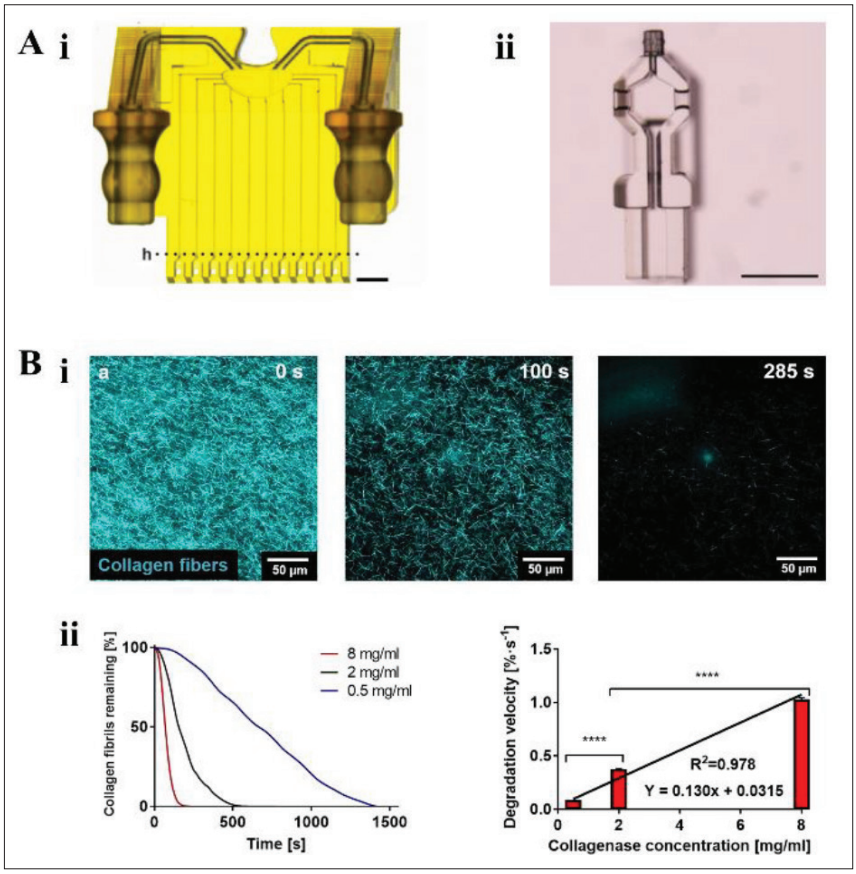

Figure 7. Strategies for enabling the recovery of hydrogel-embedded cells. (A) Modular approach for 3D-printed microfluidic culture device to facilitate cell retrieval after cultivation. (A, i) Main device. (A, ii) Separate insertable part containing the cultivation chamber. Adapted from ref.[119] (B) Enzymatic degradation of collagen hydrogel to facilitate cell retrieval. (B, i) Confocal reflection microscopy of the degradation dynamics of a collagen hydrogel. (B, ii) Quantification of hydrogel degradation dynamics for different collagenase concentrations using area quantification for 0.5, 2, and 8 mg/mL, and characterization of the degradation speed for the different collagenase concentrations. ****$p < 0.001$. Scale bars: 1 mm (A, i); 300 µm (A, ii); 50 µm (B, i). Linear fitting of the degradation speeds resulted in a linear equation ($Y = 0.130x + 0.0315$, $R^2 = 0.978$). The graph shows the average ± SEM (p-values < 0.001 in both cases). Adapted from ref.[69]

channels of the device.[69] Some commercially available hydrogels (e.g., TrueGel3D™ and VitroGel®) also offer the opportunity for defined hydrogel degradation and recovery of the cells.

3. Conclusion

The integration of hydrogels for mammalian cell culture into 3D-printed microfluidic devices presents diverse challenges that are highly dependent on the specific application of each device. Typically, these challenges revolve around ensuring a spatially resolved hydrogel integration into the device, effectively monitoring and precisely stimulating the cultured cells, as well as facilitating easy device handling. Given the interconnected nature of the described challenges, developing a reliable cultivation device is usually an iterative process. As a result, researchers can greatly benefit from the rapid prototyping and easy design adaptions enabled by 3D printing as a manufacturing technique. To date, relatively few fully 3D-printed microfluidic systems for culturing hydrogel-embedded mammalian cells have been reported in the literature. Particularly, fully 3D-printed approaches for stimulating hydrogel-embedded cells remain underexplored. However, with the ongoing advancements in 3D printing technology (particularly in 2PP, multi-material printing, and the printing of cell-containing hydrogels), along with the development of specialized 3D printing materials and optimized hydrogel properties, a significant increase in such systems can be anticipated. Looking ahead, the development of miniaturized sensors with enhanced spatial resolution and their reliable integration into these systems, alongside further automation of on-device liquid handling and monitoring of cultures, may unlock the full

potential of these advanced *in vitro* systems for addressing open questions in various fields of research.

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Conflict of interest

The authors declare they have no competing interests.

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Data curation: Katharina V. Meyer
Funding acquisition: Janina Bahnemann
Project administration: Janina Bahnemann
Supervision: Janina Bahnemann
Visualization: Katharina V. Meyer
Writing - original draft preparation: Katharina V. Meyer
Writing - review and editing: Katharina V. Meyer, Steffen Winkler, Janina Bahnemann

Ethics approval and consent to participate

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Consent for publication

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Availability of data

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