Functionalization, preparation and use of cell-laden gelatin methacryloyl-based hydrogels as modular tissue culture platforms

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ABSTRACT

Progress in advancing a system-level understanding of the complexity of human tissue development and regeneration is hampered by a lack of biological model systems that recapitulate key aspects of these processes in a physiological context. Hence, growing demand by cell biologists for organ-specific extracellular mimics has led to the development of a plethora of three-dimensional (3D) cell culture assays based on natural and synthetic matrices. We developed a physiological microenvironment of semi-synthetic origin, namely gelatin-methacryloyl (GelMA)-based hydrogels, which combine the biocompatibility of natural matrices with the reproducibility, stability, and modularity of synthetic biomaterials. We describe here a step-by-step protocol for the preparation of the GelMA polymer, which takes 1–2 weeks to complete, and can be used to prepare hydrogel-based 3D cell culture models for cancer and stem cell research, and tissue engineering. We also describe quality control and validation procedures, including how to assess the degree of GelMA functionalization, plus mechanical and diffusive properties, to ensure reproducibility in experimental and animal studies.

INTRODUCTION

In multicellular organisms, cells are embedded in a pericellular and/or an extracellular matrix (ECM). Structurally, the ECM of native tissues is subdivided into two general types: filamentous protein networks, as found in connective tissues[1], and thin layers with sheet-like organization, which are found in basement membranes[2]. It is now thought that the ECM represents more than just a structural architecture that provides adhesion sites for cell surface receptors[3]. ECM homeostasis is a critical factor in preserving normal tissue function and tissue-specific mechanical and biochemical properties[4]. The interaction between cells and the surrounding ECM regulates a variety of physiological cellular processes, including motility, migration, invasion, and proliferation[5,6]. On the other hand, the crosstalk of cells with the local microenvironment promotes the development and progression of various diseases, including cancer[7-9].

The physico-chemical properties of the cellular microenvironment directly influence the state of differentiation of various cell types. For example, with regard to chondrocytes, the properties of the native or tissue-engineered ECM play a key role in the successful development of a functional cartilage matrix[10-12]. These effects of the extracellular microenvironment on cellular behavior are well-known; thus, experimental organ-specific model systems need to mimic physiological conditions in humans, and their development has, therefore, become a major focus of biomedical research[13-15].

Two-dimensional (2D) cell culture systems, which are based on cells propagated as monolayers and are routinely utilized in research, differ greatly from the native microenvironment and often fail to adequately model normal tissue and disease processes[16]. To address this fundamental drawback of 2D cell culture systems, tissue-engineered *in vitro* platforms have become the preferred model system for experimental organ-specific studies[17]. These platforms can rely on different technologies, so that they consist of a three-dimensional (3D) modular culture system, rather than a stand-alone model. These modular systems can, therefore, enable researchers to adequately engineer complex tissue-specific niches. Several 3D culture approaches based on natural, synthetic and semi-synthetic biomaterials are available to serve as physiologically relevant mimics of the ECM for cell biology, tissue engineering, and regenerative medicine applications[18]. Most of these 3D cell culture systems consist of hydrogels, which are highly hydrated matrices of crosslinked polymer chains that mimic the 3D networks observed in native connective tissues[19,20].

Current 3D cell culture systems

A wide variety of natural, synthetic, and semi-synthetic hydrogels have been successfully used as 3D cell culture systems that mimic the extracellular microenvironment during disease development and progression[21,22] or that provide a cell delivery vehicle for animal experiments[23]. Biomaterials utilized for these purposes include natural hydrogel-forming proteins, like collagen type I[24], and more complex mixtures based on reconstructed basement membrane proteins, such as Matrigel[25], as well as synthetic polymers, like polyethylene glycol[13,23]. Although collagen type I gels effectively recapitulate the properties of connective tissues and Matrigel enables researchers to conduct cell growth and differentiation studies[17], such hydrogels derived from natural proteins often lack consistent properties and contain impurities resulting in high batch-to-batch variations[16]. Hence, researchers in the field of materials science have developed an array of synthetic hydrogels that are widely explored as 3D ECM mimics[26]. Although these synthetic polymers have the advantage of reproducible, well-defined, and tunable physico-chemical properties, they lack the cell-binding and protease cleavage motifs that are naturally found in ECM. This limitation can be overcome by adding cell-responsive sites, such as integrin cell-binding motifs, to synthetic polymers, thus engineering biomimetic materials[27].

Interestingly, other 3D cell culture approaches utilize scaffolds instead of hydrogels. For example, the work by the Cukierman group introduced cell-derived matrices as 3D culture and assay system that have the characteristics of basement membrane-like substrates in order to analyze cell morphology and behavior[28]. To create a specific type of basement membrane, the Lengyel team developed an *in vivo*-like tumor microenvironment by layering mesothelial cells onto an omental basement membrane. This omental basement membrane is generated by mixing ECM proteins,

including collagen type I, with primary fibroblasts to study ovarian cancer cell functions during metastasis[29]. Such a 3D culture approach for ovarian cancer represents an organotypic model that can be further expanded with other tissue-specific cell types present in the tumor microenvironment and enables the high-throughput screening of novel therapeutics[30]. Other approaches include the combined silk–collagen scaffolds developed by the Kaplan group to build a brain-like tissue, in which the biphasic architecture enables the spatially controlled growth of a neuronal network, thus mimicking the 3D morphology of the brain cortex[31]. Other scaffold technology platforms enable researchers to monitor oxygen levels and determine hypoxia in a 3D cell culture set-up[32] and to investigate mechanisms that are altered during bone metastasis in patients with prostate cancer[33].

Gelatin-methacryloyl-based hydrogels as cell culture platforms that mimic ECM

Our 3D cell culture technology platform, as described here, focuses on a semi-synthetic biomaterial that integrates the advantages of the aforementioned hydrogel types by harboring both integrin cell-binding and protease cleavage sites, as well as chemically active substituents that ensure that the biomaterial has reproducible and tunable properties. These properties in combination enable cell adhesion and proteolytic degradation to take place and afford the ability to control the mechanical and biochemical characteristics of the hydrogels. Using gelatin-methacryloyl (GelMA)-based hydrogels, we established a 3D ECM mimic that can be used to conduct a wide range of experimental and animal studies, as well as to implement quantitative cell biology applications.

Here, we describe a step-by-step protocol for the production of GelMA and the preparation of cell-laden hydrogels that can be utilized as 3D culture systems for cancer and stem cell research, as well as for tissue engineering applications. We also describe validation protocols to characterize the degree of GelMA functionalization and the mechanical properties of differently cross-linked hydrogels, which are key factors in influencing the behavior of encapsulated cells. Finally, we demonstrate various applications for cell biological assays, including visualization of cell morphology, detection of cell viability and proliferation, as well as total RNA extraction. We also demonstrate the use of these hydrogels as cell delivery vehicles for different animal studies and as cardiomyocyte microchannel approach.

Development of the protocol

GelMA is prepared from gelatin, a soluble polypeptide mixture of denatured and partially hydrolyzed collagens derived from animal tissues, like skin, tendons, and bones[34]. The main component of gelatin is collagen type I[34]. Depending on the hydrolysis processing conditions and tissue sources, a variety of gelatin products exist, which have slightly different compositions. According to our unpublished qualitative proteomic analysis, gelatin and GelMA mainly contain

collagen types I and III, but they also contain traces of other collagen types. Although this approach is not covered in this protocol, collagen type IV (and its methacryloyl-substituted derivative) can be added to GelMA-based hydrogels with control over the collagen concentration, enabling researchers to implement a variety of cell-specific applications.

Pure gelatin is water-soluble and forms thermo-reversible transparent hydrogels via physical interactions between collagen molecules[35]. However, this type of physical hydrogel is not stable at body temperature and does not allow researchers to control and fine-tune its properties. To obviate to these drawbacks, gelatin has been chemically functionalized with unsaturated methacryloyl groups to result in GelMA, which forms covalently crosslinked hydrogels by photo-initiated polymerization under mild conditions[35,36] and enables cell encapsulation with high viability[10,37]. Recent data from our research group suggest that GelMA prepared following the protocol described herein contains a mixture of methacrylamide and methacrylate groups, although the methacrylamide groups are the majority (> 90% of all the methacryloyl groups present in the hydrogel)[38]. As a result, terms that were widely used to describe this material in literature, such as gelatin methacrylamide, gelatin methacrylate or methacrylated gelatin, are not chemically accurate. Instead, we suggest using the term 'gelatin methacryloyl' to reflect the coexistence of both methacrylamide and methacrylate groups in GelMA[38].

The mechanical properties of GelMA-based hydrogels are readily tunable by varying the degree of methacryloyl substitution, polymer and photo-initiator concentrations, as well as photo-crosslinking time[35,39]. Specific physical or otherwise hydrogel characteristics are discussed in the Experimental design. The quantitative links between these factors and the predictable mechanical characteristics of the resulting hydrogels[10,37] are taken into consideration in our detailed relevant validation procedure. This protocol demonstrates a user-friendly and highly reproducible procedure to prepare GelMA-based hydrogels using a custom-made Teflon casting mould, which enables matrices with reproducible biochemical compositions, sizes, and mechanical properties to be obtained. The resulting hydrogels are suitable for different biological assays and as cell delivery vehicles (Figure 1). In addition, we describe how the covalent incorporation of methacryloyl-functionalized ECM components, such as hyaluronic acid methacrylate, yields complex ECM-mimicking matrices that can be adapted to act as tissue-specific model systems.

Applications

Our group's modular tissue culture technology platform system is of prime interest, not only to biomedical engineers and biomaterial scientists, but also to cell and molecular biologists working on applying novel biomaterial-based technology platforms to cancer research, and more generally *in vitro* disease models, as well as tissue engineering and regenerative medicine. We have

demonstrated that GelMA-based hydrogels prepared following our protocol (Figure 2) can serve as 3D model systems for studies on ovarian (Figure 3), breast (Figure 4), and prostate cancer[33], and for cartilage tissue engineering applications[10]. The GelMA concentrations recommended for *in vitro* chondrogenesis are within the physiological range of the collagen content of human articular cartilage. The mean (range) content of collagen type II in normal human articular cartilage is 14% (9.2–20.8%; weight per wet weight)[40], which is very similar to the 10% typically used in our experiments[41].

In expanding the applicability of the GelMA tool box, we developed different hydrogel formulations to generate hybrid hydrogels with varying chemical, biological, mechanical, and electrical properties that enable the differentiation of stem cells to desired cell types and the formation of vascular networks (Figure 5)[42]. Integration of microchannels into these hydrogels can further stimulate vascular network formation[43]. Moreover, GelMA-based hydrogels have been used as an injectable gene delivery system to stimulate vasculogenesis for myocardial therapy[44].

In the context of cancer research, cell–ECM interactions mediate critical steps in cancer progression and drug responses[18]. GelMA-based hydrogels were successfully used to develop a 3D cell culture model that allows formation of multicellular spheroids from single ovarian cancer cells, the initiating process during peritoneal metastasis[37]. Variation of hydrogel stiffness resulted in changes of the cell morphology, metabolic activity, and proliferation rate[37]. Additionally, this spheroid-based approach was applied to an ovarian cancer animal model (Figure 3). Cell-laden hydrogels were implanted intraperitoneally into NOD-*scid* mice. Tumor formation occurred 4 weeks post implantation, which enabled the administration and screening of a clinically used chemotherapeutic, paclitaxel, over 4 weeks. A paclitaxel response of 63% was detected (Figure 3), a value that is comparable to that determined in our previously published study using GelMA-based hydrogels[37] as cell delivery vehicle of the same cell type *in vivo*. Similarly, metastatic breast cancer cells have been embedded within GelMA-based hydrogels and showed high cell viability over the duration of 3D cell culture (Figure 4). These cell-laden hydrogels were then implanted in close proximity to a humanized tissue-engineered bone construct to mimic invasion of the humanized bone by the breast cancer cells (Figure 4). Hence, this approach provides an approach to study breast cancer bone colonization *in vivo*[45].

In the context of tissue engineering and regenerative medicine, co-culture of human blood-derived endothelial colony-forming cells (ECFCs) and human bone marrow-derived mesenchymal stem cells (MSCs) encapsulated in GelMA-based hydrogels supported extensive formation of a 3D capillary-like vascular network *in vitro* (Figure 5). The co-culture of ECFCs and MSCs can be further expanded by co-culturing endothelial cells with pericytes or smooth muscle cells. Importantly, functional anastomoses between the newly formed vascular network *in vitro* and the

native vasculature upon implantation into male athymic nude (*nu/nu*) mice were observed, indicating potential regenerative applications[42]. By combining microfluidic systems with GelMA-based hydrogels, GelMA prepolymers can be readily used to coat these microchannels. After photo-crosslinking, the hydrogel coating layer stimulates functions like cell adhesion and alignment within the microchannels, suggesting that these hydrogels are uniquely qualified for organ-on-a-chip applications, such as for cardiomyocyte cultures (Figure 6). Cardiomyocytes are elongated along the flow direction growing with cardiac fibers aligned inside the microchannels (Figure 6). This cardiomyocyte microchannel approach represents an *in vitro* model for diagnosis and drug screening applications, due to the widely known advantages of organ-on-a-chip models, including feasible control of the cellular microenvironment, delivery of nutrients and chemical cues to the cells through continuous media perfusion, as well as reduction in the amount of reagents and cells during experiments[46].

Many studies described in the literature are stand-alone demonstrations of a specific biomaterial or a combination of different methods into a 3D model for a single application, but a modular 3D tissue culture technology platform is still lacking[16]. Our protocol covers the complete manufacturing process, including polymer functionalization, standardized preparation of 3D cell cultures, and the application and characterization of cell-laden hydrogels for cancer and stem cell research, as well as tissue engineering applications. This broad spectrum of biomedicine-based research provides the much-needed continuity that will enable researchers to cross-validate different applications — a feature desperately needed in the area of 3D ECM mimics[26].

Limitations

GelMA-based hydrogels can cover a wide spectrum of physico-chemical properties by varying key parameters and factors in the hydrogel formulation, UV crosslinking conditions, and incorporation of additional ECM components to generate a library of hydrogels. The protocol described herein thus serves as a basis for further optimisation, depending on the encapsulated cell type and targeted culture model.

The surface properties of a wide variety of hydrogels commonly employed for 3D cell culture applications, including GelMA, are highly dependent on the properties of the substrate it is crosslinked on. Even for the same chemical structure, a hydrogel prepared on a hydrophobic substrate, such as Teflon, exhibits a lower crosslinking density at its surface than a hydrogel prepared on a hydrophilic substrate[47]. This effect has been largely neglected in the literature, yet it can be observed in a wide variety of hydrogels, including those commonly used for 3D cell culture applications, such as polyethylene glycol, and, to a far lesser extent, GelMA. Although the effects of the crosslinking substrate on the bulk hydrogel physico-chemical properties are negligible for

thick constructs (e.g. 2 mm), thin GelMA-based hydrogels (e.g. 1 mm) prepared on Teflon show increased swelling and lower compressive moduli compared to thick constructs (Figure 7). As this substrate effect only occurs at the immediate surface of the hydrogel, the bulk mechanical and swelling properties of thick hydrogel constructs remain unaffected by the properties of the substrate. Therefore, it is recommended using consistently a standardized protocol and identical experimental conditions to ensure reproducibility.

Another limitation is the necessity to optimize the hydrogel composition to encapsulate more biologically sensitive cells, such as endothelial cells, with high viability. As described herein, these cell types require a low degree of GelMA functionalization and a low crosslinking density of the GelMA-based hydrogel to achieve physiological cell behavior.

Experimental design

A GelMA-based hydrogel experiment can be designed by combining some of, but not necessarily all, the procedures described below. GelMA functionalization, dialysis and lyophilization will need to be included in any experimental study, as well as preparation of GelMA precursor solution and hydrogel construct preparation (with or without encapsulated cells). Characterization of GelMA functionalization is highly recommended to perform on every newly synthesized and purified batch, to ensure the intended degree of methacryloyl-substitution has been obtained. Mechanical testing is highly recommended as a functional quality control test to ensure agreement between batches and experiments. In addition, mechanical testing can be an assessment of degradation or tissue formation studies. Microscopy imaging will be part of most 3D cell cultures, as well as the assessment of cell viability and proliferation. Total RNA extraction from GelMA-based hydrogels can be performed when interested in the gene expression of encapsulated cells grown in 3D.

Hydrogel casting mould. Photo-polymerization of the GelMA precursor solution is performed in a casting mould made of Teflon (Polytetrafluoroethylene). In our laboratory, we use a mould to produce hydrogel strips with the dimensions of 50 mm x 4 mm x 2 mm (length x width x height), which are cut into smaller units of 4 mm x 4 mm x 2 mm (length x width x height) using custom acrylic cutting guides and a scalpel after photo-polymerization (Figures 1 and 2). Before commencing with the procedure outlined in this protocol, it is recommended manufacturing Teflon moulds and laser-cutting of cutting guides needed for the preparation of GelMA-based hydrogels as per technical drawings provided in the Supplementary materials. Note that the design of both mould and cutting guides may be customized to produce hydrogels of different shapes and sizes, depending on your experimental needs.

Tailoring mechanical properties of GelMA-based hydrogels. As indicated earlier, the mechanical properties of GelMA-based hydrogels can be tailored by variation of one or more of the

following factors: the degree of functionalization (DoF), the polymer and photo-initiator concentrations, as well as photo-crosslinking time. For example, the DoF directly influences the highest achievable crosslinking density of the hydrogels matrix, and ultimately the hydrogel porosity and mechanical properties. When other factors remain unchanged, increasing the DoF leads to stiffer hydrogels with a higher crosslinking density and improved shape fidelity[39], while the pore size decreases[42]. Increasing the polymer concentration or photo-crosslinking time generally yields stiffer hydrogels[39]. These factors can be varied within a large range without affecting handling or compromising cell viability and function, but should be optimized for each cell type. Changes to the photo-initiator concentration will affect the crosslinking kinetics, and a shorter photo-crosslinking time may be required to yield hydrogels with comparable mechanical properties when the initiator concentration is increased. Increasing the photo-crosslinking time leads to a higher crosslinking density and stiffness, both of which plateau when the hydrogel is fully crosslinked. For a detailed description of how the above mentioned factors influence hydrogel properties, refer to our previously published work[39]. It is recommended using the parameters summarized in Table 1 as a basis for further optimization.

Avoiding oxygen inhibition of photo-initiated polymerization. Oxygen is known to affect the photo-initiated polymerization of acrylate and acrylamide-based chemistries[48]. Thus, the degree of UV crosslinking and mechanical properties of the hydrogels can be compromised by the influx of oxygen during hydrogel photo-polymerization. It is therefore necessary to remove existing air bubbles and cover the hydrogel precursor solution with a glass slide during UV-exposure/photo-polymerization as outlined in the following protocol, as well as depicted in Figures 1 and 2. Degassing of the hydrogel precursor solution is not necessary.

Photo-polymerization of GelMA-based hydrogels. UV-initiated crosslinking of cell-laden hydrogels is a commonly applied method, and, to our knowledge, no significant adverse effects on cell viability and function have been reported, despite its extensive use with diverse cell types, including primary cells. The damage UV light may cause is highly dependent on its wavelength and the nature of the radicals it creates. Since DNA has an absorption maximum of 260 nm, it is recommended using a wavelength of 365 nm to minimize the risk of direct DNA damage. Our most recent results suggest that radicals generated by UV exposure preferentially react with the unsaturated methacryloyl groups of GelMA, which therefore have a cell-protective effect[49]. As a result, cell viability and function are not compromised by UV light-initiated crosslinking[49].

Co-polymerization of methacryloyl-substituted ECM components for tissue-specific models. To yield tissue-specific 3D models, methacryloyl substituted ECM components may be co-polymerized with GelMA. For example, for human articular chondrocyte cultures, hyaluronic acid can be functionalized with methacrylate groups by reaction with methacrylic anhydride to yield

hyaluronic acid methacrylate (HAMA) which enables the covalent and stable incorporation of hyaluronic acid into the hydrogel matrix and was shown to enhance chondrogenic cell differentiation, accumulation of cartilaginous ECM, and mechanical properties in articular cartilage tissue engineering applications when compared to GelMA alone[41]. While not specifically outlined in the following protocol, this principle can also be applied to other relevant ECM components to study their effects on cellular behavior.

Mechanical testing of GelMA-based hydrogels. To perform unconfined compression tests (Figure 7), in our laboratory, we are using a custom-manufactured immersion bath comprised of an aluminum block with a rectangular recess, which is capable of holding up to 25 ml of buffer for the mechanical testing of hydrogels. Beneath this recess, the aluminum block features a longitudinal feed-through with tubing attachments on either side, allowing perfusion with temperature-controlled water using an aquarium water pump, tubing and a conventional laboratory water bath.

MATERIALS

Reagents

! CAUTION When handling the chemicals used in this protocol, always wear suitable personal protective equipment, including a lab coat, nitrile gloves, safety goggles, and, where indicated, a face shield and respirator. For any chemical listed in this protocol, appropriate institutional and governmental safety guidelines must be followed. Please refer to the respective material safety data sheets.

GelMA functionalization, dialysis and lyophilization

- Gelatin from porcine skin, type A, gel strength 300 bloom (Sigma Aldrich, cat. no. G2500, store at room temperature (RT) at 23–25 °C)

  ▲ CRITICAL Other types of gelatin can also be used to prepare GelMA following the same protocol, if necessary. This may, however, affect the properties of the materials and resulting hydrogels.
- Methacrylic anhydride (Sigma Aldrich, cat. no. 276685, store at RT)

  ! CAUTION Methacrylic anhydride is a volatile and toxic organic compound, which may cause skin irritation, serious eye damage, and respiratory irritation if inhaled. Always wear a face shield and respirator. Avoid any direct contact.
- Phosphate-buffered saline (PBS), pH 7.4 (Life Technologies, cat. no. 100100, store at RT)
- $NaHCO_3$ (Sigma Aldrich, cat. no. S6014, store at RT)
- Irgacure® 2959 (IC2959; BASF, cat. no. 029891301PS04, store at RT)

- Hyaluronic acid (Hyasis®; Novozymes, molecular weight 0.86 MDa, CAS no. 9067-32-7, store air-tight in a dry place at 4 °C)

  ▲ **CRITICAL** If necessary, hyaluronic acid from other manufacturers can also be used. However, the source and molecular weight may influence cell behavior.
- Liquid nitrogen

  ! **CAUTION** Contact of liquid nitrogen with the skin or eyes may cause serious frostbite injury. When handling liquid nitrogen, always wear appropriate personal protective equipment, including a long sleeve lab coat, thermo-insulated gloves, safety goggles and a full face shield.

Characterization of GelMA functionalization

- Ninhydrin (Sigma-Aldrich, cat. no. 151173, store at RT)
- Sodium citrate monobasic (Sigma-Aldrich, cat. no. 71498, store at RT)
- HCl (Sigma-Aldrich, cat. no. 84435, store at RT)
- Glycerol (Sigma-Aldrich, cat. no. G5516, store at RT)
- UltraPure™ DNase/RNase-free distilled water (Life Technologies, cat. no. 10977-015, store at RT)
- Fluoraldehyde™ *o*-phthaldialdehyde reagent solution (Life Technologies, cat. no. 26025, store at 4 °C)

Human ovarian cancer cell culture

- OV-MZ-6 cells[13] or any other cell line of interest and the appropriate medium in which to culture these cells

  ! **CAUTION** The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- DMEM, high glucose, no sodium pyruvate (Life Technologies, cat. no. 10566-016, store at 4 °C)
- HEPES (Life Technologies, cat. no. 15630-080, store at 4 °C)
- Penicillin–Streptomycin, 10,000 U/ml (Life Technologies, cat. no. 15140-122, store at –20 °C)
- Gentamycin (Life Technologies, cat. no. 15710-064, store at 4 °C)
- Fetal bovine serum (FBS, Lonza, ca. no. 14-501F, store at –20 °C)

  ▲ **CRITICAL** Always use the same batch of FBS for replicate experiments.
- L-Arginine (Sigma-Aldrich, cat. no. A-5131, store at RT)

- L-Asparagine (Sigma-Aldrich, cat. no. A-4159, store at RT)
- Versene (Life Technologies, cat. no. 15040-066, store at 4 °C)

Human chondrocyte culture

- Human articular chondrocytes (see Reagent setup)

  ! CAUTION Informed consent must be obtained for experiments involving human tissues and cells. Experiments must conform to all relevant institutional and governmental regulations.
- DMEM, high glucose, sodium pyruvate, no glutamine (Life Technologies, cat. no. 10313-021, store at 4 °C)
- HEPES (see above)
- Penicillin–Streptomycin (see above)
- MEM non-essential amino acids solution, 100x (Life Technologies, cat. no. 11140-050, store at 4 °C)
- GlutaMAX™ supplement (Life Technologies, cat. no. 35050-061, store at 4 °C)
- L-Proline (Sigma-Aldrich, cat. no. P5607, store powder at RT, store solution at –20 °C)
- L-Ascorbic acid (Sigma-Aldrich, cat. no. A92902, store powder at RT, store solution at –20 °C)
- Insulin-Transferrin-Selenium (ITS-G), 100x (Life Technologies, cat. no. 41400-045, store at 4 °C)
- Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A9418, store at 4 °C)
- Human recombinant transforming growth factor beta 3 (TGF-β3; GroPep, cat. no. BJU100, store at 4 °C)
- Dexamethasone (Sigma-Aldrich, cat. no. D4902, store powder at 4 °C, store solution at –80 °C)
- Trypsin (Life Technologies, cat. no. 15090-046, store at 4 °C)

Co-culture of human ECFCs and MSCs for vascular network formation

- Human ECFCs (see Reagent setup)

  ! CAUTION Informed consent must be obtained for experiments involving human tissues and cells. Experiments must conform to all relevant institutional and governmental regulations.
- Human MSCs (see Reagent setup)

! CAUTION Informed consent must be obtained for experiments involving human tissues and cells. Experiments must conform to all relevant institutional and governmental regulations.

- Endothelial basal medium (EBM-2; Lonza, cat. no. CC-3156, store at 4 °C)
- SingleQuots (Lonza, cat. no. CC-4176, store at 4 °C)
- MSC growth medium (MSCGM; Lonza, cat. no. PT-3238, store at 4 °C)
- HEPES (see above)
- Penicillin/Streptomycin (see above)
- GlutaMAX™ supplement (see above)
- FBS (see above)
- Basic fibroblast growth factor (bFGF; R&D System, cat. no. 4826/10, store at 4 °C)

Laser scanning confocal microscopy (CLSM)

- Fluorescein diacetate (FDA; Life Technologies, cat. no. F1303, store powder at 4 °C, store solution at –20 °C)
- Propidium iodide (PI; Life Technologies, cat. no. P3566, store at 4 °C)
- Triton™ X-100 (Sigma-Aldrich, cat. no. X100, store at RT)
- Glycine (Sigma-Aldrich, cat. no. G8898, store powder at RT, store solution at 4 °C)
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. No. 158127, store powder at 4 °C, store solution at –20 °C)

  ! CAUTION PFA is a potent fixative and hazardous. Avoid any direct contact and wear appropriate personal protective equipment.
- Rhodamine 415–conjugated phalloidin (Life Technologies, cat. no. R415, store at –20 °C)

  ! CAUTION Rhodamine 415–conjugated phalloidin is toxic and harmful. Avoid any direct contact and wear appropriate personal protective equipment.
- 4´,6-diamidino-2-phenylindole (DAPI; Life Technologies, cat. no. D1306, store powder at RT, store solution at –20 °C)

Cell viability and proliferation assays

- AlamarBlue® cell viability reagent (Life Technologies, cat. no. DAL1025, store at 4 °C)
- CyQuant® cell proliferation assay kit (Life Technologies, cat. no. C7026, store at –20 °C)
- Proteinase K (Life Technologies, cat. no. 25530-015, store at 4 °C)
- Na_2HPO_4 (Merck Chemicals, cat. no. 106586, store at RT)
- NaH_2PO_4 x H_2O (Merck Chemicals; cat. no. 106342, store at RT)

- Na_2EDTA (Ajax Finechem, cat. no. 180-500G, store at RT)
- NaCl (Merck Chemicals, cat. no. 106406, store at RT)
- EDTA (Merck Chemicals, cat. no. 108418, store at RT)
- RNase A (Life Technologies, cat. no. 12091-021, store at RT)
- UltraPure™ DNase/RNase-free distilled water (see above)

Total RNA extraction from GelMA-based hydrogels

- TRIzol® Reagent (Life Technologies, cat. no. 15596-026, store at 4 °C)

  ! CAUTION TRIzol® Reagent contains phenol (toxic and corrosive) and guanidine isothiocyanate (an irritant), and may be hazardous if not handled properly. Follow the manufacturer's instructions and always work in a chemical safety fume hood and wear appropriate personal protective equipment.
- RNaseZap® RNase decontamination solution (Life Technologies, cat. no. AM9780, store at RT)
- Chloroform (Sigma-Aldrich, cat. no. C2432, store at RT)
- 2-Propanol (Sigma-Aldrich, cat. no. I9516, store at RT)
- Ethanol, 200 proof (Sigma-Aldrich, cat. no. E7023, store at RT)
- UltraPure™ DNase/RNase-free distilled water (see above)
- RNeasy Micro Kit, including RLT buffer (Qiagen, cat. no. 74004, store at RT and 4 °C)
- Borosilicate beads, 3-mm diameter (Sigma-Aldrich, cat. No. Z143928, store at RT) or stainless steel beads, 5-mm diameter (Qiagen, cat. No. 69989, store at RT)
- β-mercaptoethanol (Sigma-Aldrich, cat. No. M3148, store at RT)

  ! CAUTION β-mercaptoethanol is a hazardous and toxic. Follow the manufacturer's instructions, work in a chemical safety fume hood and wear appropriate personal protective equipment.
- Agarose, low electroendosmosis (Sigma-Aldrich, cat. no. 11685678001, store at RT)

EQUIPMENT

GelMA functionalization, dialysis and lyophilization

- Round bottom flask with magnetic stir bar

  ▲ CRITICAL Use a stir bar of sufficient size with a powerful stirrer to ensure good dispersion of methacrylic anhydride. Alternatively, an overhead stirrer with propeller agitator can be used instead of a magnetic stir bar.

- Freeze-dryer
- Sterile 50-ml centrifuge tubes with vented caps (0.2-µm pore size), for example Corning® 50-ml Mini Bioreactor (Sigma-Aldrich, cat. no. CLS431720)
- Sterile syringes (50-ml volume)
- Syringe filter units or disposable vacuum filtration units with polyethersulfone (PES) membrane (0.2-µm pore size), for example Nalgene™ Rapid-Flow™ sterile disposable filters (Thermo Scientific, cat. no. 595-3320)
- Dialysis membrane with a 12 kDa molecular weight cut-off
- CL-1000 UV crosslinker (UVP; Upland California; or similar) with 365 nm wavelength tubes

  ▲ **CRITICAL** Ensure a UV intensity of 2.2–2.8 mW/cm^2 in the crosslinker center. The intensity will vary throughout the crosslinker, with intensities in the corners only half as high as that in the center.
- Teflon casting mould (see Supplementary material)

  ▲ **CRITICAL** Clean Teflon mould with warm water and sterilize with 70% (vol/vol) ethanol before usage; autoclaving may damage the mould.
- Un-treated microscope glass slides

  ▲ **CRITICAL** Ensure usage of the same brand or type of glass slides to exclude variation of hydrogel properties due to different light permeation through different glass slides.
- Hydrogel cutting guides, laser-cut poly(methyl methacrylate)

  ▲ **CRITICAL** Do not autoclave; clean with warm water and sterilize with 70% (vol/vol) ethanol before usage. Distance of individual cuts of cutting guides can be modified to allow hydrogel preparation of certain length.
- Scalpel holders
- Scalpel blades (size 11 or 22)

Mechanical testing of GelMA-based hydrogels

- Instron MicroTester 5848 (Instron), or similar mechanical tester, with a 5 N-load cell
- Temperature-controlled water bath
- Fluidic chamber (hydrogel immersion bath)
- Aquarium water pump

Brightfield microscopy and CLSM

- Eclipse (Nikon) or similar fluorescence microscope

- SP5 (Leica) or similar confocal microscope

Cell viability and proliferation assays

- Black 96-well plates for fluorescence measurements
- POLARStar OPTIMA plate reader (BMG, Labtech) or similar fluorescent plate reader; wavelength measurements of fluorescent signals: excitation 544 nm, emission 590 nm (AlamarBlue® assay), excitation 485 nm, emission 520 nm (CyQuant® assay)

Total RNA extraction from GelMA-based hydrogels

- DNase/RNase-free, 2-ml conical microcentrifuge tubes
- Standard laboratory bench top centrifuge for 2-ml conical microcentrifuge tubes
- Nanodrop-ND-1000 photo-spectrometer
- Bullet Blender tissue homogenizer (Next Advance), Mini-Beadbeater (BioSpec Products), TissueLyser LT (Qiagen) or a similar mechanical tissue homogenizer
- Bioanalyzer (Agilent Technologies)

Other laboratory equipment

- Cell culture incubator
- Sterile 24-well and 48-well plates for 3D cell culture
- Opaque 96-well plates for photo-spectroscopy
- Photo-spectrometer (plate reader)
- Hemocytometer or cell counter
- Positive-displacement pipette and sterile capillary piston tips (1-ml nominal volume)
- Water bath
- Heat block
- Shaker/rocker plate
- Sterile 1.5-ml, 2-ml, 15-ml, and 50-ml conical (micro)centrifuge tubes
- Spatula
- Forceps

REAGENT SETUP

▲ **CRITICAL** Culture cells on tissue culture plastic to ~ 80% confluency in suitable culture media (see below) before encapsulation in GelMA-based hydrogels.

Medium for human ovarian cancer OV-MZ-6 cell culture. Prepare high glucose (4.5 g/l) DMEM supplemented with 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 20 μg/ml gentamycin, 10% (vol/vol) FBS, 0.550 mM L-Arginine, and 0.272 mM L-Asparagine. This medium can be stored at 4 °C for 4–6 weeks.

Human articular chondrocytes. Isolate these cells from the full-thickness cartilage of patients undergoing total knee replacement surgery for osteoarthritis as described elsewhere[10]. Cells encapsulated in GelMA-based hydrogels are cultured in high glucose (4.5 g/l) DMEM supplemented with 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM MEM non-essential amino acids, 2 mM GlutaMAX™, 0.4 mM L-Proline, freshly added at each media change, 0.1 mM L-Ascorbic acid, ITS-G (1:100), 1.25 mg/ml BSA, 10 ng/ml TGF-β3, and 0.1 μM dexamethasone. This medium can be stored at 4 °C for 4–6 weeks.

Human ECFCs. Isolate these cells from the mononuclear cell fraction of human cord blood samples as described elsewhere[42]. Cells are expanded in EBM-2 supplemented with SingleQuots, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM GlutaMAX™, and 20% (vol/vol) FBS. This medium can be stored at 4 °C for 4–6 weeks.

Human MSCs. Isolate these cells from the mononuclear cell fraction of human bone marrow samples as described elsewhere[42]. Cells are expanded in MSCGM supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM GlutaMAX™, 10% (vol/vol) FBS, and 10 ng/ml bFGF. This medium can be stored at 4 °C for 4–6 weeks.

Sodium citrate buffer for ninhydrin assay. Prepare a 0.5 M sodium citrate monobasic solution in demineralized or ultrapure water (107 mg/ml) and adjust to pH 5.5 using HCl 1.0 M. Prepare the solution as required and store according to the manufacturer's instructions.

Solutions for CLSM. Prepare a 10-mg/ml stock solution of FDA in PBS by dissolving 1 g of FDA in 1 ml of PBS and store protected from light at –20 °C for at least 1 year. Prepare a 4% (wt/vol) solution of PFA dissolved in PBS and store at –20 °C for at least 1 year. Prepare a glycine 0.1 M solution in PBS always fresh and store at 4 °C for 1–2 months. Prepare a 1% (wt/vol) solution of BSA in PBS by dissolving 1 g of BSA in 100 ml of PBS and store at 4 °C for 1–2 months. Prepare a 5-mg/ml stock solution of DAPI in PBS by dissolving 10 mg of DAPI in 2 ml of PBS and store protected from light at –20 °C for at least 1 year. It is recommended that the following solutions are

sterile-filtered using 0.2-µm syringe filter units prior to usage: 4% (wt/vol) PFA, glycine 0.1 M solution, and 1% (wt/vol) BSA in PBS.

Solutions required for CyQuant® assay. Prepare phosphate-buffered EDTA (PBE) buffer containing 20 mM Na_2HPO_4, 30 mM NaH_2PO_4 x H_2O and 5 mM Na_2EDTA in 500 ml of demineralized or ultrapure water; adjust pH to 7.1 and autoclave at 121 °C for 15 min. Make a stock solution of 100 mg of proteinase K in 10 ml of PBE buffer and prepare a final concentration of 0.5 mg/ml of proteinase K in PBE buffer always prepared fresh. Prepare 5 ml of RNase A solution per 96-well plate (180 mM NaCl, 1 mM EDTA, 4.5 ml of ultrapure™ DNase/RNase-free distilled water, 0.5 ml of 20x CyQuant® lysis buffer and 1.4 U/ml RNase A stock). Prepare 10 ml of CyQyant® GR dye solution per 96-well plate (9.5 ml of ultrapure™ DNase/RNase-free distilled water, 0.5 ml of 20x CyQuant® lysis buffer and 50 µL of CyQyant® GR dye stock). Prepare the solutions as required and store according to the manufacturers' instructions.

PROCEDURE

GelMA functionalization, dialysis and lyophilization ⏲ TIMING 1–2 weeks

! CAUTION Perform GelMA functionalization in a chemical safety fume hood and wear appropriate personal protective equipment, including a lab coat, nitrile gloves and safety goggles.

▲ CRITICAL Ensure cleanliness of all glassware and laboratory equipment to avoid chemical contamination during GelMA functionalization and purification steps.

▲ CRITICAL GelMA is light-sensitive; maintain lyophilized GelMA and solutions containing GelMA in the dark, for example by wrapping the dialysis setup and conical centrifuge tubes in aluminum foil.

▲ CRITICAL Maintain the GelMA solution at 37–50 °C to avoid thermal gelation between the functionalization and purification steps, for example in a water bath or oven.

1| Soak gelatin to a final concentration of 10% (wt/vol) in demineralized or ultrapure water at RT in a round bottom flask with a magnetic stir bar. Stir the resulting mixture for 10–60 min to facilitate gelatin dissolution. Alternatively, PBS can be used as a solvent for gelatin. For a large reaction volume, an overhead stirrer should be used instead of a magnetic stir bar. In our laboratory, we have tested reaction volumes of up to 300 ml, corresponding to 30 g of gelatin[35].

2| While moderately stirring, heat the mixture to (and keep at) 50 °C in a water bath until the gelatin is fully dissolved and the solution becomes clear.

3| While stirring vigorously, slowly add 0.6 g of methacrylic anhydride per 1 g of dissolved gelatin for a high degree of methacryloyl-functionalization using a glass pipette as organic solvents

may dissolve plastic pipette tips. Continue stirring vigorously for 60 min. If mixing is sufficient, the solution will turn homogeneously opaque due to dispersion of methacrylic anhydride. Alternatively, this reaction can be run for up to 3 h; however, the reaction time and temperature, as well as the mass ratio of methacrylic anhydride to gelatin are used determine the degree of GelMA functionalization.

▲ CRITICAL STEP Ensure adequate stirring during GelMA functionalization while minimizing air uptake. Insufficient stirring will lead to visible phase separation.

▲ CRITICAL STEP DoF can be varied by changing the ratio of methacrylic anhydride to gelatin. Use 0.6 g of methacrylic anhydride per 1 g of gelatin for a high DoF (75 ± 9% DoF[39] as assessed using a ninhydrin assay or fluoraldehyde assay, step 13), or 0.06 g of methacrylic anhydride per 1 g of gelatin for a low DoF (31 ± 7% DoF as assessed using a ninhydrin assay, step 13). Ultimately, the DoF will influence the crosslinking density, and hence, porosity and mechanical properties of hydrogel constructs and needs to be chosen based on the cell type used (see Experimental design and Table 1).

? TROUBLESHOOTING

4| After the reaction period, transfer the solution into 50-ml tubes and remove unreacted methacrylic anhydride by centrifugation at 3,500 g for 3 min at RT. Decant the GelMA-containing supernatant and discard the unreacted methacrylic anhydride deposited at the bottom of the 50-ml tubes (opaque and viscous 'pellet').

? TROUBLESHOOTING

5| Dilute the supernatant solution with two volumes of pre-heated (40 °C), demineralized or ultrapure water.

6| Transfer the solution to a dialysis membrane with a 12 kDa molecular weight cut-off and dialyze at 40 °C against a large volume of demineralized or ultrapure water for 5–7 days in a chemical safety fume hood. Change water at least once daily. Alternatively, dialysis can be performed at 4 °C in the cold room to minimize degradation. However, this may require extended dialysis time due to reduced diffusion at lower temperatures.

▲ CRITICAL STEP Methacrylic anhydride and acid are cytotoxic and may influence viability of encapsulated cells. It is therefore critical to fully remove these contaminants by dialysis before proceeding to step 7. Dialysis is completed when the GelMA solution appears clear, and the odor of residual methacrylic anhydride or methacrylic acid (byproduct) is no longer noticeable.

? TROUBLESHOOTING

7| Adjust the pH of the GelMA solution to 7.4 using 1 M $NaHCO_3$.

? TROUBLESHOOTING

8| In a class II biological safety cabinet, sterile-filter the GelMA solution using 0.2-µm syringe filter units or disposable vacuum filtration units with a PES membrane.

? TROUBLESHOOTING

▲ CRITICAL STEP Treat GelMA and GelMA solutions as sterile after this step.

9| Aliquot the GelMA solution into 50-ml tubes and snap-freeze them in liquid nitrogen.

■ PAUSE POINT Samples can be stored at –80 °C for at least 1 month.

10| Transfer all aliquots to the freeze-dryer without allowing the solutions to thaw, and lyophilize until GelMA is fully dehydrated (typically 4–7 days). To maintain a sterile barrier during lyophilization, the 50-ml tubes need to be sealed with vented screw-top caps or press-fitted with 0.2-µm syringe filter units prior to lyophilization. Exchange vented caps or filters with standard screw-top caps after lyophilization is completed to avoid hygroscopic absorption of water during storage specified in step 11.

11| Store lyophilized GelMA protected from light and moisture at –20 °C until usage.

■ PAUSE POINT Lyophilized GelMA can be stored safely in the mentioned conditions for at least 1 year.

Characterization of GelMA functionalization ⏲ TIMING 1–2 h

12| Soak duplicate samples of GelMA and gelatin at 3.5 mg/ml in ultrapure water. Although only ~0.2 mg are needed, weigh in at least ~10 mg of these duplicate samples for increased accuracy.

13| Determine the DoF of GelMA implementing the ninhydrin assay (option A), or the fluoraldehyde assay (option B). Both options are equally valid.

(A) Ninhydrin assay to determine the DoF

(i) Prepare a mixture of one part 0.5 M sodium citrate monobasic and two parts of glycerol, for example 1 ml of sodium citrate monobasic 0.5 M and 2 ml of glycerol.

(ii) Heat GelMA samples from step 12 to 50 °C and mix until the solution becomes homogenous and transparent.

(iii) For gelatin solutions, prepare 50% (0.5 ml of gelatin solution and 0.5 ml of ultrapure water), 20% (0.2 ml of gelatin solution and 0.8 ml of ultrapure water) and 10% (0.1 ml of gelatin solution and 0.9 ml of ultrapure water) dilutions to obtain the standard curve.

(iv) Dissolve ninhydrin in the sodium citrate monobasic/glycerol mixture prepared in step 13A (i) to a final ninhydrin concentration of 2.5 mg/ml.

(v) Prepare 2-ml tubes containing 0.950 ml of the ninhydrin solution prepared in step 13A (iv).

(vi) Heat a water bath to 50 °C.

(vii) Transfer 50-µl aliquots of the GelMA and gelatin solutions prepared in steps 13A (ii) and 25A (iii), respectively into the tubes containing ninhydrin solution prepared in step 13A (v) and include two blanks (50 µl of ultrapure water).

(viii) Incubate the closed tubes floating in the heated water bath for 12 min.

(ix) Remove tubes and let them cool at RT for 1 h.

(x) For absorbance measurements, transfer 250 µl of each solution in triplicates into a 96-well plate and measure the optical absorbance at 570 nm with a photo-spectrometer (plate reader).

(xi) Calculate the average absorbance of the triplicate measurements and draw a linear regression line from the values obtained from the gelatin dilution series, plotting absorbance against relative gelatin concentration (from 0% for blank to 100% for non-diluted gelatin). The absorbance at 570 nm corresponds to the concentration of free amine groups; any reduction in free amine concentration is assumed to be due to methacryloyl-functionalization. The absorbance of each GelMA sample corresponds to a gelatin concentration of X% on the standard curve; the DoF is then calculated as DoF = (100–X)%.

(B) Fluoraldehyde assay to determine the DoF

(i) Dissolve 0.5 mg/ml of GelMA in PBS and prepare gelatin solutions in PBS to draw a standard curve (0.02, 0.1, 0.5, and 1.0 mg/ml). Warm GelMA and gelatin samples to 50 °C and mix them until they turn homogenous and transparent. Allow all the solutions to cool to RT.

(ii) Warm the fluoraldehyde reagent solution to RT before usage.

(iii) Mix 300 µl of each of the GelMA and gelatin solutions with 600 µl of the fluoraldehyde reagent solution in separate test tubes; also prepare a sample containing 300 µl of PBS and 600 µl of the fluoraldehyde reagent solution as a control. Mix thoroughly all samples for 1 min to ensure completion of the reaction.

(iv) Pipette 250 µl of each solution in triplicates into a 96-well plate and determine the fluorescence intensity at 450 nm using an excitation wavelength of 360 nm.

▲ **CRITICAL STEP** For optimal results, measure the fluorescence intensity of the samples within 3 min at the same time interval after mixing. Use opaque 96-well plates designed for fluorescence assays only.

(v) Take the average fluorescence intensity and subtract the fluorescence intensity of the PBS control from the sample and standard solutions to determine the net fluorescence. Prepare a linear calibration curve based on the standard solutions. Determine the fluorescence intensity of the GelMA sample corresponding to a gelatin concentration of X mg/ml on the calibration curve and the residual amine concentration. The DoF is then calculated as DoF = (0.5–X) / 0.5 × 100%.

Preparation of GelMA precursor solution TIMING **1–2 h**

▲ **CRITICAL** Perform this preparation 1 day prior to cell encapsulation

▲ **CRITICAL** For cell culture applications, treat GelMA and GelMA solutions as sterile: work in a class II biological safety cabinet and use sealed vessels to transport GelMA-containing solutions. Clean all working surfaces with 70% (vol/vol) ethanol before commencing any procedure.

▲ **CRITICAL** Use a positive displacement pipette for viscous GelMA preparations that have concentrations higher than 5% (wt/vol).

14| Prepare at least 10-ml of fresh photo-initiator stock solution by weighing in 25 mg IC2959 in a 50-ml centrifuge tube and add PBS to a final concentration of 2.5 mg/ml. Dissolve IC2959 by placing the centrifuge tube in a water bath heated to 70 °C for 5–10 min, or until fully dissolved. Let solution cool to RT, transfer it to a class II biological safety cabinet and sterilize it using 0.2-µm syringe filter units. The stock concentration of IC2959 can be increased depending on the final concentration needed in step 17.

15| Determine the mass of GelMA required (m_{GelMA}) for your application based on the desired final concentration (c_{GelMA}) and volume (V_{GelMA}) using the equation $m_{GelMA} = c_{GelMA} \times V_{GelMA}$, and allow for ≥ 30% excess to account for losses during hydrogel preparation and to facilitate ease of pipetting. Note that V_{GelMA} is dependent on the nominal volume of your casting mould. For example, the nominal volume of each of the four recesses of the mould used in this protocol is 400 µl (50 mm x 4 mm x 2 mm; see Supplementary materials for mould and cutting guide design) which produces a total of 12 hydrogel constructs after cutting (step 25). To prepare 24 hydrogel constructs, two recesses of the mould need to be filled completely with hydrogel precursor solution (step 23) which requires the preparation of $V_{GelMA} \geq$ 1,040 µl (two recesses × 400 µl × 130%).

▲ **CRITICAL** The minimum total volume of the GelMA hydrogel precursor solution (V_{GelMA}) per hydrogel preparation should be ≥ 1-ml to facilitate ease of pipetting and mixing.

16| Weigh in the required amount of lyophilized GelMA and add PBS to 4/5 of the total final volume to obtain 1.25x the final desired concentration; for example, prepare a 12.5% (wt/vol) solution of GelMA if you desire a final GelMA concentration of 10% (wt/vol).

▲ **CRITICAL** If other functionalized (methacryloyl-substituted) ECM components, such as HAMA, are to be co-polymerized in the hydrogel matrix, they may be dissolved in this mixture at 1.25x the desired final concentration (if present in solid or lyophilized form). Alternatively, a concentrated stock solution of the component may be prepared in PBS and added to the mixture to obtain 1.25x the desired final concentration. Take care to adjust the volume of PBS to be added to the lyophilized GelMA accordingly, so that all functionalized components are present at 1.25x the final concentration after this step.

17| Add the IC2959 stock solution prepared in step 14 to a final concentration of 0.05% (wt/vol), in 1/5 of the total reaction mixture volume (see also step 16). Optionally, the final concentration of IC2959 can be varied to 0.5% (wt/vol) as desired, which will have an influence on the crosslinking kinetics and mechanical properties but not on cell viability. For recommended IC2959 concentrations refer to Table 1.

18| Allow the GelMA/IC2959 mixture to soak into the solvent protected from light at 4 °C overnight.

▲ CRITICAL STEP GelMA foam needs to be fully immersed into the solvent to enable the complete hydration of GelMA.

Cell encapsulation and hydrogel construct preparation ⏲ TIMING 3–5 h

19| Sterilize the Teflon casting mould, acrylic hydrogel cutting guides and glass slides by spraying them with 70% (vol/vol) ethanol in a class II biological safety cabinet.

▲ CRITICAL STEP Ensure equipment has fully dried before usage.

20| Transfer the tube containing the soaked GelMA foam with IC2959 to a water bath or cell culture incubator at 37 °C and allow GelMA to fully dissolve over ~3 h or until the mixture turns clear. Place tube on a shaker/rocker or manually agitate solution occasionally to accelerate the dissolution of GelMA.

? TROUBLESHOOTING

21| When GelMA is fully dissolved and the Teflon casting mould, cutting guides and glass slides have dried entirely, detach the cells of choice, e.g.: OV-MZ-6, chondrocytes, ECFCs, or MSCs following your laboratory's standard protocol, wash cell pellet with PBS to remove traces of trypsin or versene, resuspend the pellet in a suitable volume of media (5-ml or 10-ml), and determine the cell concentration ($c_{suspension}$) using a hemocytometer or cell counter.

22| Determine the required cell number (n_{cells}) based on the desired final cell concentration (c_{cells}) and the total volume of GelMA precursor solution prepared in steps 14–18 (V_{GelMA}) using the equation $n_{cells} = c_{cells} \times V_{GelMA}$. Aspirate a volume ($V_{cells}$) containing n_{cells} from the cell suspension prepared in step 21 and resuspend with $V_{cells} = n_{cells} / c_{suspension}$ and transfer to a fresh 50-ml tube. Pellet the cells by centrifugation and discard the supernatant.

▲ CRITICAL The cell concentration (c_{cells}) is dependent on the cell type and specific application, and needs to be optimized for cell types not listed in this protocol. For recommended cell concentrations refer to Table 1.

23| Wet the pipette tip and carefully aspirate the warm hydrogel precursor solution prepared in step 18 with a pipette, by immersing its tip to a depth of 2–5 mm from the solution surface. Resuspend the cell pellet in the hydrogel precursor solution at the desired cell concentration by

carefully pipetting up and down repeatedly, leaving some cell suspension in the tube at all times to avoid introduction of air bubbles. Remove existent air bubbles from the cell suspension by centrifugation at 200 g for 30 s and reheat suspension to 37 °C, if necessary. Once the cell pellet is dislodged and the suspension appears homogeneous and free of air bubbles, transfer the cell suspension into the Teflon casting mould using 10% volume excess over the nominal volume of the casting mould (here: 440 µl per recess). Cover with a glass slide in a single movement.

▲ **CRITICAL STEP** Avoid introducing air bubbles, as entrapped oxygen inhibits radical polymerization of the hydrogel precursor solution and leads to incomplete crosslinking.

? TROUBLESHOOTING

24| Photo-crosslink cell–hydrogel precursor solution in a UV crosslinker at 365 nm for 10–15 min, depending on cell type and required hydrogel mechanical properties (see Table 1). UV exposure time can be significantly reduced to 30–60 s if a higher concentration of IC2959 is used, such as 0.5% (wt/vol), depending on required hydrogel mechanical properties.

? TROUBLESHOOTING

25| Remove the crosslinked cell–hydrogel constructs from the Teflon mould by carefully lifting the glass slide, and cut the hydrogel strip(s) thus obtained into smaller units using a cutting guide and sterile scalpel.

26| Transfer individual cell-laden hydrogel constructs into a suitable cell culture medium (dependent on cell type, see Reagent setup) in a multi-well plate (Figure 2) and maintain in a cell culture incubator at 37 °C.

? TROUBLESHOOTING

▲ **CRITICAL STEP** If IC2959 concentrations above 0.05% (wt/vol) were used for photo-crosslinking, replace the cell culture medium three times at 30-min intervals to remove traces of the photo-initiator.

Mechanical testing of GelMA-based hydrogels ⏲ **TIMING 1–3 h**

27| Prepare cell-laden hydrogels as described in steps 19–26 and/or cell-free hydrogels (without the inclusion of cells) and incubate them in a suitable buffer, such as PBS or cell culture media, at 37 °C overnight to allow for swelling equilibration or culture cell-laden hydrogels for a desired period of time.

28| Determine the hydrogel cross-sectional area by direct measurement of hydrogel spatial dimensions (option A) or indirectly by measurement of the hydrogel wet weight and height (option B). Both options are equally valid and interchangeable for the measurement of the compressive moduli of GelMA-based hydrogel cultures by using unconfined compression tests.

▲ **CRITICAL** Implementing step 28, and those that follow it in the present procedure, compromises sterility of the tested hydrogel constructs, so this procedure must be performed as an endpoint analysis.

(A) Direct determination of hydrogel cross-sectional area

(i) Use a (digital) caliper to measure the hydrogel's length and width (rectangular hydrogel constructs) or diameter (cylindrical hydrogel constructs) and calculate the cross-sectional area.

(B) Indirect determination of hydrogel cross-sectional area

(i) Remove excess buffer by carefully blotting the equilibrated hydrogel against a laboratory wipe, and determine the hydrogel's wet weight using an analytical balance. Calculate the cross-sectional area using the equation $A = m / (p \times h)$, in which A is the hydrogel cross-sectional area (cm^2), m is the hydrogel wet weight (g), ρ is the hydrogel density in g/cm^3, and h is the hydrogel height in (cm); hydrogel height will be assessed in step 35.

▲ **CRITICAL STEP** This method is commonly used, in particular for soft and odd shaped hydrogels that deform strongly when applying a caliber, but the hydrogel density needs to be measured separately. For 5% (wt/vol) and 10% (wt/vol) GelMA-based hydrogels, the density is approximately 1.03 and 1.05 g/cm^3, respectively.

29| Prepare mechanical testing equipment and the hydrogel immersion bath. Equip a vertically configured mechanical testing system (e.g. an Instron MicroTester 5848) with a non-porous indenter and a suitable load cell (e.g. 5 N-load cell) and follow the manufacturer's load cell calibration and general user instructions. The cross-sectional area of the intender must be larger than that of the hydrogel.

30| For immersed unconfined compression tests, prepare a suitable hydrogel immersion bath with a flat-bottom surface that allows heating to 37 °C by filling with buffer as appropriate.

31| Determine the contact point of the indenter with the bottom surface of the immersion bath and set this position as zero gauge length (position 0 mm).

32| After the zero gauge length has been reset to the bottom level of the immersion bath in step 31, raise the indenter well above the expected hydrogel height.

33| Place the hydrogel in the immersion bath underneath the indenter.

? TROUBLESHOOTING

34| Using a ramp displacement command, lower the indenter at a rate of 0.01 mm/s, until a compressive strain of ≥ 15% of the hydrogel height is reached, and record the force-displacement data.

▲ **CRITICAL STEP** Initiate the linear displacement well above the hydrogel (e.g. 0.3 mm above the contact point) to allow for accurate determination of the hydrogel height and compressive strain.

▲ **CRITICAL STEP** Ensure hydrogel surfaces are flat and parallel to the bottom of the immersion bath.

▲ **CRITICAL STEP** For some applications including engineering of load-bearing tissues, which undergo large amounts of physical deformation like articular cartilage, the ultimate strength of hydrogel constructs may be of interest. To determine the fracture stress and strain, compress the sample until it breaks and obtain the stress (Pa) and strain (%) values from the stress-strain curve at the point of failure.

35| Determine the hydrogel height from the force-displacement curve, as the point where the force starts deviating significantly from zero.

36| Determine the compressive modulus E_c (Pa) as the slope of the linear region of the stress-strain curve between 10 and 15% (wt/vol) hydrogel compressive strain using a linear regression analysis or the formula $E_c = (\sigma_2 - \sigma_1) / (\varepsilon_2 - \varepsilon_1)$, where σ_2 is the stress at ε_2, σ_1 is the stress recorded at force (N) divided by cross-sectional hydrogel area (m^2) at ε_1, ε_2 is a strain of 0.15, and ε_1 is a strain of 0.10.

? TROUBLESHOOTING

Brightfield microscopy and CLSM ⏲ TIMING 3–5 h

37| The following microscopic procedures can be performed to visualize the cell morphology and viability of cell-laden GelMA-based hydrogels[10,37]. One cell-laden hydrogel is collected after days 1, 7 and 14 of 3D culture (or any other desired time point) for each replicate experiment. Perform microscopy of cell-laden GelMA-based hydrogels according to option A, if a low contrast image is sufficient (Figure 2), or option B, if a live–dead assessment is desired (Figure 4), or option C, if a high contrast image is required (Figure 2), or perform all determinations in sequence during the same experiment using the same cell-laden GelMA-based hydrogel sample (the order in which the assays are performed is relevant).

(A) Brightfield microscopy

▲ **CRITICAL STEP** Don't open the lid of your ongoing sterile hydrogel culture 24-well plate; tape lid to the plate when taking out of the cell culture incubator.

(i) To visualize the cell morphology of encapsulated cells grown within hydrogels in a 24-well plate, take brightfield images of the hydrogel samples using a standard microscope with a 10/20x air objective.

(B) CLSM of live hydrogel samples using live–dead staining

▲ **CRITICAL** Perform fluorescent staining of live and PFA-fixed samples protected from light.

(i) Wash hydrogel samples in an individual well of a 24-well plate in 1-ml of PBS for 5 min in a cell culture incubator at 37 °C.

(ii) Incubate samples from step 37A (i) in 0.5-ml of PBS containing 2 µg/ml FDA to stain living cells for 15 min at 37 °C.

(iii) Wash samples with PBS for 5 min at 37 °C as described in step 37B (i).

(iv) Incubate samples in 0.5-ml PBS containing 20 µg/ml PI to stain dead cells for 15 min at 37 °C. Alternatively, instead of being subjected to two sequential incubations, hydrogel samples can be incubated in PBS containing both FDA and PI for 15 min at 37 °C using the same concentration as reported above.

(v) Image samples with a standard fluorescent microscope with a 10/20x air objective or CLSM with a 10/20x oil objective. Please note that at this point, hydrogel samples may be discarded or utilized to achieve fixation and permeabilization according to the instructions in the following steps that are implemented to perform CLSM of PFA-fixed hydrogel samples. In the present context, therefore, option C is optional if researchers require a high contrast image of 3D-cultured cells.

(C) CLSM of PFA-fixed hydrogel samples

(i) Perform fixation and permeabilization of samples with 4% (wt/vol) PFA dissolved in PBS and 0.2% (vol/vol) Triton X-100 in PBS into a 24-well plate on a shaker/rocker for 30 min at RT. Alternatively, store samples in 4% (wt/vol) PFA in PBS at 4 °C for up to 1 month. When ready to proceed with the fluorescent staining, permeabilize samples in 0.2% (vol/vol) Triton X-100 in PBS for 30 min at RT.

(ii) Wash samples with glycine 0.1 M in PBS, then twice for 5 min each at RT in a similar fashion as done in step 37B (i).

(iii) Incubate samples in rhodamine 415–conjugated phalloidin 0.3 U/ml diluted in 1% (wt/vol) BSA in PBS for 1 h at RT to stain F-actin.

(iv) Wash samples twice with PBS for 5 min each at RT in a similar fashion as done in step 37B (i).

(v) Incubate hydrogels in DAPI 2.5 µg/ml in PBS for 40–50 min at RT to stain cell nuclei.

(vi) Wash samples twice with PBS for 5 min each at RT (see above for instructions).

(vii) Image samples with a 10/20x oil objective at three (or more) different positions and acquire z-stacks with a constant thickness of 2 µm, reconstructing a cross-section profile of 100–150 equidistant XY-scans, which will cover a distance of 200–300 µm and spheroids of 50–300 µm diameter, using a confocal microscope and generate maximal projections of each image [23]. If

required, larger z-stacks (4-µm slice thickness) can also be acquired for bigger spheroids with a diameter > 500 µm to cover the complete thickness of the hydrogel.

Cell viability and proliferation assays TIMING **3–4 days**

38| Collect cell-laden hydrogels after days 1, 7 and 14 of 3D culture in triplicates in biological replicate experiments[23]. Optionally, depending on the cell type, hydrogel cultures can be set up for additional time points after days 4 and 11 of 3D culture or for long-term cultures up to 4 weeks[23]. If required, quintuplicates can be measured per time point and replicate experiment. Set up cell-free hydrogels to obtain the baseline fluorescent signal for each culture medium depending on the cell type and time point.

39| Determine cell viability in the cell-laden GelMA-based hydrogel sample, option A, or cell proliferation in the same sample, option B, or perform both determinations in sequence during the same experiment (the order in which the assays are performed is relevant).

(A) AlamarBlue® cell viability assay

(i) Culture each individual cell-laden GelMA-based hydrogel in an individual well of a 24-well plate for the desired period of time, for example 2–4 weeks as mentioned above, in a cell culture incubator at 37 °C.

(ii) Prepare a fresh, sterile 24-well plate with at least 0.5 ml of the specific culture medium for the cell type listed in the reagent setup containing 4% (vol/vol) AlamarBlue® reagent and place each hydrogel replicate into individual wells 6 h prior to each time point and incubate in a cell culture incubator at 37 °C. Alternatively, 48-well plates can be used.

(iii) After 6 h, transfer individual hydrogels into an individual well of a new 24-well plate in 1-ml of PBS per well on a shaker/rocker for 30 min to destain the AlamarBlue® reagent-colored hydrogels (blue or pink) and measure fluorescent signals of the AlamarBlue® reagent-containing media in triplicates into a black 96-well plate using a fluorescent plate reader.

(iv) Transfer each individual destained hydrogel to a 2-ml conical tube using a spatula and freeze at –80 °C for at least 48 h.

■ **PAUSE POINT** Samples can be stored at –80 °C for at least 1 month.

(B) CyQuant® cell proliferation assay

(i) Implement step 39A (i) of the present procedure as described above. Alternatively, if AlamarBlue® cell viability assay has already been performed, thaw hydrogel samples set aside at step 39A (iv).

(ii) Incubate cell-laden GelMA-based hydrogel samples with 300 µl of proteinase K solution (0.5 mg/ml in PBE buffer) in 2-ml conical tubes overnight for at least 16 h at 65 °C using a heat block.

▲ **CRITICAL STEP** The optimal digestion temperature for proteinase K is 65 °C. Make sure that the temperature is kept constant over the incubation time for optimal hydrogel degradation.

(iii) Centrifuge samples at 8,000 g for 30 s at RT and pipet 50 µl of each hydrogel replicate in triplicates into a black 96-well plate.

(iv) Prepare a DNA standard curve using the λDNA standard provided with the CyQuant® assay kit over eight concentrations ranging from 0 to 2,000 ng/ml in 1x cell lysis buffer, and pipet 50 µl of each DNA standard concentration in triplicates into a black 96-well plate.

▲ **CRITICAL STEP** If the sample number exceeds 24, the number of wells in a 96-well plate, prepare a DNA standard curve for each individual plate.

(v) Add 50 µl of RNase A solution to each well (containing samples and DNA standards) to degrade any RNA reassuring DNA specificity and incubate for 1 h at RT on a shaker/rocker.

(vi) Add 100 µl of CyQuant® GR dye solution to each well and incubate for 5 min at RT on a shaker/rocker before measuring the fluorescent signals using a fluorescent plate reader.

▲ **CRITICAL STEP** Ensure that the CyQuant® GR dye solution is added in a light-protected room and incubated protected from light to ensure the integrity of the fluorescent signals.

(vii)Overlay the standard curve with a linear regression line and determine the DNA content of individual samples.

▲ **CRITICAL STEP** Subtract the fluorescent signal from the cell-free hydrogel sample for each cell type and time point measured.

▲ **CRITICAL STEP** If the fluorescent signal of the sample with the highest cell number exceeds the fluorescent signal of the DNA standard with the highest concentration, repeat the assay by diluting all cell-containing samples with 1x cell lysis buffer 1:5–1:10.

Total RNA extraction from GelMA-based hydrogels ⏲ **TIMING 2–4 h**

▲ **CRITICAL** RNA is sensitive to degradation by ubiquitous RNases and precautions need to be taken if working with RNA. If possible, perform RNA isolation in a designated area and treat bench and equipment surfaces with commercially available RNase-inactivating agents, for example an RNase decontamination solution. Clean benches with 100% (vol/vol) ethanol. Change gloves regularly and do not touch surfaces and equipment to avoid reintroducing RNases. Always use certified RNase-free disposable plastic ware, including filtered pipette tips and conical microcentrifuge tubes, and designated RNase-free reagents. Avoid cross-contamination between samples.

▲ **CRITICAL** It may be necessary to pool multiple cell-laden hydrogels, if cells were cultured at densities below 1.0×10^5 cells/ml in the GelMA precursor solution.

40| Culture cell-laden GelMA-based hydrogels for a desired period of time, for example 2–4 weeks as mentioned above, in a cell culture incubator at 37 °C.

41| Proceed with RNA extraction implementing the directions in option A, if extracting RNA from samples with relatively high cell numbers (> 1.0×10^6 cells per individual or pooled hydrogel sample) or option B, which is optimal for low cell numbers (< 1.0×10^5 cells per individual or pooled hydrogel sample)[13,23].

(A) Total RNA extraction using TRIzol® reagent

(i) Transfer each individual hydrogel to a 2-ml conical tube with 1 ml of TRIzol® reagent using a spatula. If possible, disrupt hydrogels by pipetting up and down several times. Incubate at RT for 5 min while gently shaking occasionally.

(ii) Snap-freeze samples in liquid nitrogen (freezing facilitates the homogenization of hydrogels in the following step) and store at –80 °C until further usage.

■ **PAUSE POINT** Samples can be stored at –80 °C for at least 1 month.

(iii) Thaw samples at RT and homogenize hydrogels either manually, by carefully passing hydrogels through a 19-gauge needle with a 5-ml syringe 15–20 times or until hydrogels look completely homogeneous, or mechanically, by adding five borosilicate beads or three stainless steel beads to each sample and homogenizing the resulting mixtures in a Bullet Blender tissue homogenizer, Mini-Beadbeater, TissueLyser LT, or a similar mechanical tissue homogenizer. Proceed by spinning twice for 1 min or 2–5 min at 50 Hz or until hydrogels look completely homogeneous. While manual homogenization is suitable for rather soft hydrogels (< 5 kPa), mechanical disruption needs to be used for stiff hydrogels (> 5 kPa).

▲ **CRITICAL STEP** When implementing manual homogenization, hydrogel pieces may clog the needle occasionally. Take care to avoid spillage and contamination when clearing clogged needles by gentle pressure.

▲ **CRITICAL STEP** Ensure best possible sample homogenization to maximize cell disruption and RNA yield.

(iv) Proceed to phase separation following the TRIzol® reagent manufacturer's instructions for the isolation and purification of total RNA using a chloroform and 2-propanol precipitation.

▲ **CRITICAL STEP** Ensure RNA integrity by agarose gel analysis or using a bioanalyzer and probe for absence of contaminants using a nanodrop photo-spectrometer (make sure that $A_{260nm/280nm}$ ratio is over 1.8 and $A_{260nm/230nm}$ ratio is in the 2.0–2.2 range) before proceeding to

downstream applications, such as reverse transcription to cDNA. If necessary, perform additional RNA clean-up using commercial column-based RNA purification kits.

(B) Total RNA extraction using RNeasy Micro Kit

(i) Transfer hydrogels into 0.35-ml RLT buffer containing 1% β-mercaptoethanol in 2-ml tubes using a spatula. If possible, disrupt hydrogels by pipetting up and down several times.

(ii) Snap-freeze samples in liquid nitrogen (freezing facilitates the homogenization of hydrogels in the following step) and store at –80 °C until further usage.

■ **PAUSE POINT** Samples can be stored at –80 °C for at least 1 month.

(iii) Thaw samples at RT and homogenize hydrogels either manually, as described in step 41A (iii), or mechanically, by loading the lysate onto a QIAshredder spin column placed in a 2-ml conical tube and centrifugation at 12,500 g for 2 min at RT using a standard bench top centrifuge.

▲ **CRITICAL STEP** Ensure best possible sample homogenization to maximize cell disruption and RNA yield.

(iv) Proceed following the RNeasy Micro Kit manufacturer's instructions for the isolation and purification of total RNA.

TABLE 1 | Recommended parameters for 3D cell cultures in high DoF GelMA. Of note, for enhanced chondrogenic differentiation, 9.5% (wt/vol) GelMA and 0.5% (wt/vol) HAMA can be used, instead of the 10% (wt/vol) GelMA reported in the Table[41].

Cell type	Cell density (cells/mL)	GelMA concentration [% (wt/vol)]	IC2959 concentration [% (wt/vol)]	UV exposure time (min)	UV intensity (mW/cm^2)	Expected compressive modulus (kPa)	Reference(s)
OV-MZ-6	2.8×10^5	5	0.05	10	2.7	3.2–5.2	[37]
Chondrocytes	1.0×10^7	10	0.05	15	2.6	25–30	[10,41]
ECFCs	1.0×10^6	5	0.5	0.33	6.7	4–5	[42]
MSCs	1.0×10^6	5	0.5	0.33	6.7	4–5	[42]

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

TABLE 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
3	Stirring not possible	Stir bar too small and/or reaction volume too large	Use a larger stir bar or an overhead stirrer with propeller agitator or reduce reaction volume
		Gelatin not dissolved	Fully dissolve gelatin at 50 °C
	Phase separation occurs	Insufficient stirring and/or mixing	Increase stirring speed and use a larger stir bar, or use an overhead stirrer with propeller agitator
4	Ineffective separation	Thermal gelation occurred	Maintain GelMA solution at 37–50 °C and use a temperature-controlled centrifuge (if possible)
6	Solution is cloudy	Incomplete dialysis	Extend dialysis with daily water changes until the solution turns clear
	Solids are present	Incomplete removal of methacrylic anhydride and/or acid	Remaining solids can be removed by filtration (step 8)
		Spontaneous crosslinking	Maintain GelMA solutions in the dark and remove solids by filtration (step 8)
7	pH > 7.4	Too much $NaHCO_3$ was added	Reduce pH with HCl 0.1 M or 1 M
8	Filters units clog	Unsuitable filter membrane	Use filters with PES membrane, such as Nalgene™ Rapid-Flow™ sterile disposable filter units
		Thermal gelation occurred	Maintain GelMA solution at 37–50 °C

20	GelMA does not dissolve	GelMA has crosslinked	Maintain GelMA solutions in the dark during and after functionalization, and store the lyophilized GelMA protected from light and moisture at –20 °C
23	Air bubbles in GelMA–cell suspension	Volume too small and/or unsuitable pipette	Increase excess volume and/or use positive displacement pipettes; air bubbles can be removed by short centrifugation (at 200 g for ~30 s); re-heat suspension to 37 °C and mix carefully before transferring into the casting mould
24	GelMA is not forming covalent hydrogels	Functionalization unsuccessful	Follow functionalization protocol and determine the degree of functionalization before cell-encapsulation
		Problems with photo-initiator	Always prepare and use a fresh photo-initiator solution
		UV polymerization parameters incorrect	Ensure a wavelength of 365 nm for photo-crosslinking and aim for a consistent UV dosage
		The casting mould used is oxygen-permeable	Use casting moulds made from non-permeable materials, such as Teflon
26	Hydrogels stick to glass slide	Glass slide was treated or coated	Use non-treated or hydrophobic glass slides; release sticking hydrogels using a sterile scalpel by gently pressing the blade flat against the glass slide and carefully sliding underneath the hydrogel to lift it off
33	Hydrogel does not remain underneath indenter	Too much buffer or hydrogel unconfined	Remove excess buffer until hydrogel is just covered by it; it may help to confine the

			hydrogel loosely with, for example, a large stainless steel washer
36	Inconsistent results between technical replicates	Hydrogel surfaces not flat or aligned in a parallel fashion	Ensure hydrogels are flat by using a suitable casting mould
		Air bubbles trapped inside hydrogels	Avoid introducing air bubbles during cell encapsulation

TIMING

Steps 1–11, GelMA functionalization, dialysis and lyophilization: 1–2 weeks

Steps 12–13, Characterization of GelMA functionalization: 1–2 h

Steps 14–18, Preparation of GelMA precursor solution: 1–2 h

Steps 19–26, Cell encapsulation and hydrogel construct preparation: 3–5 h

Steps 27–36, Mechanical testing of GelMA-based hydrogels: 1–3 h

Step 37, Brightfield microscopy and CLSM: 3–5 h

Steps 38–39, Cell viability and proliferation assays: 3–4 days

Steps 40–41, Total RNA extraction from GelMA-based hydrogels: 2–4 h

Anticipated results

This protocol can be used for a variety of applications in cancer research, as well as tissue engineering and regenerative medicine. This modular technology platform provides a universal and easily adaptable 3D cell culture system that combines natural binding sites with controllable properties. All components of GelMA-based hydrogels are affordable, broadly available and easy to handle, whereas the chemical modification enables researchers to control reproducibility, physico-chemical and mechanical properties, and cellular behavior. Additionally, GelMA-based hydrogel constructs can be molded into wide-ranging shapes and are easy to produce, which has proven to be a great advantage over systems like Matrigel and collagen type I gels, and allows for a greater variety of experimental procedures and analysis.

Following the GelMA functionalization procedure described herein, by using the recommended 0.6 g of methacrylic anhydride per 1 g of dissolved gelatine, an estimated DoF of 80% can be achieved. An estimated DoF of 50% is the least in order to generate 5% (wt/vol) GelMA-based hydrogels. The mechanical properties of GelMA-based hydrogels are largely dependent on the DoF, polymer and photo-initiator concentrations, thermal gelation state of the GelMA precursor solution, as well as the UV exposure (intensity and time) during photo-crosslinking of the polymer

network[39]. As hydrogel stiffness and crosslinking density are directly related to and strongly influence cellular behavior, mechanical characterization represents a cost- and time-effective measure of reproducibility between different GelMA batches and hydrogel preparations. Furthermore, it can also be performed to assess changes in the physical properties of the hydrogels following ECM deposition or enzymatic degradation during 3D cell culture and tissue engineering experiments. The practically achievable range of stiffness is approximately 1–200 kPa[39].

Although various 3D matrices are increasingly employed for cell-based studies, there are difficulties in adapting routinely used cytological methodologies to 3D cellular microenvironments. No consideration is given to the different cell architecture, cell surface area exposed or interference of the 3D matrix. Our team has optimized the application of two quantitative assays to 3D cell cultures in which cells are embedded within different hydrogel materials[13,23]. Cell viability and proliferation of 3D-cultured cells can be determined performing the AlamarBlue® and CyQuant® assays, respectively. Although the AlamarBlue® reagent enables researchers to measure a cell's metabolic activity, results of the CyQuant® assay provide indication of cell proliferation, based on the DNA content of the cell-containing sample. Both procedures can be performed individually and independently from each other using the same cell-laden GelMA-based hydrogel sample.

Both total RNA extraction procedures, TRIzol® reagent or a RNeasy Micro Kit, can be employed to isolate high quality total RNA from cells encapsulated in GelMA-based hydrogels for a variety of downstream applications, including reverse transcription real-time PCR, (micro)array analysis, whole transcriptome analysis using next generation sequencing, digital PCR, northern analysis and cDNA library constructions.

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AUTHOR CONTRIBUTIONS

D.L., C.M., E.K., A.K. and D.W.H. conceived and designed the experiments. D.L., C.M. and E.K. performed the experiments and analyzed the data. D.L., C.M. and D.W.H. wrote the manuscript; E.K. and K.Y. partially wrote the manuscript. L.C.M. performed the breast cancer bone colonization animal study. C.M., P.A.L. and T.J.K. established the cartilage tissue engineering. F.P.W.M. established the GelMA polymer production and physico-chemical characterization. A.K. and D.W.H. supervised this project. All authors read and critiqued the manuscript extensively.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interest.

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FIGURES

Figure 1 | Overview of the GelMA-based hydrogel preparation protocol. GelMA is dissolved in PBS at 37 °C and mixed with the photo-initiator to obtain the precursor solution. Cells, and optionally hyaluronic acid methacrylate (HAMA; indicated by a dashed line), are added to the precursor solution before casting it into a custom-made Teflon mould. The casting mould is then covered with glass slides and transferred into a UV crosslinker to enable formation of the hydrogel by photo-polymerization. Using a custom-made cutting guide, the hydrogel stripes thus obtained are cut into constructs of equal size (4 mm x 4 mm x 2 mm) and can be used for biological assays and as a cell delivery vehicle for animal studies.

Figure 2 | GelMA-based hydrogel preparation and 3D cell culture. The custom-made and sterilized Teflon mould is filled with cell-laden precursor solution under sterile conditions and covered with glass slides. After UV crosslinking, a custom-made cutting guide is required to cut the hydrogel strip into equal squares (4 mm x 4 mm x 2 mm), which are then transferred into culture media to enable cell growth and spheroid formation. Brightfield microscopy indicates the appearance of spheroids after 14 days of 3D culture of ovarian cancer OV-MZ-6 cells, which can be visualized by CLSM and maximal projections depicting cell nuclei (DAPI, blue) and F-actin (rhodamine 415–conjugated phalloidin, red).

Figure 3 | Application of GelMA-based hydrogels as cell delivery vehicles for an intraperitoneal animal model. **A.** Ovarian cancer OV-MZ-6 cells were transfected with a luciferase vector[23] and encapsulated within GelMA-based hydrogels [5% (wt/vol) polymer concentration]. After 2 weeks of 3D cell culture, bioluminescent signals ($3.15 \times 10^5 \pm 9.32 \times 10^4$ photons/s/cm^2/steradian; n = 12) of cell-laden GelMA-based hydrogels were confirmed prior to intraperitoneal implantation into NOD-*scid* mice. **B.** Bioluminescent imaging confirmed tumor growth 8 weeks after implantation ($1.80 \times 10^6 \pm 1.70 \times 10^6$ photons/s/cm^2/steradian; n = 6), and *ex vivo* bioluminescent imaging of the peritoneal organs indicated tumor spread. Human-derived tumor load was immunohistochemically detected by positive nuclear mitotic apparatus protein 1 (NuMA; Epitomics, cat. no. s2825, 1:100 dilution) staining as shown by representative images from two different mice per group. **C.** 4 weeks post implantation, mice were treated with intraperitoneal paclitaxel injections (10 mg/kg, twice/week) over 4 weeks, leading to a decreased tumor load, as detected by bioluminescent imaging ($6.59 \times 10^5 \pm 1.01 \times 10^6$ photons/s/cm^2/steradian; n = 6). The effect of paclitaxel was also confirmed via *ex vivo* bioluminescent imaging, with only minor tumor spread within the peritoneum. Smaller tumors were formed upon paclitaxel treatment as indicated by NuMA staining.

D. The average radiance indicated a paclitaxel response of 63%. All animal experiments conformed to Queensland University of Technology animal ethics approval.

Figure 4 | Application of GelMA-based hydrogels as breast cancer bone colonization animal model. **A.** Metastatic breast cancer MDA-MB-231BO (MDA-BO) and MDA-MB-231 (MDA) cells and non-tumorigenic epithelial MCF10A cells were encapsulated within GelMA-based hydrogels [5% (wt/vol) polymer concentration]. After 1 week of 3D cell culture, cell viability was assessed by CLSM by live/dead staining (FDA, green/PI, red). After 2 weeks of 3D cell culture, cell proliferation was measured with an Alamar Blue® assay (mean ± standard error; n = 5). **B.** Melt electrospun scaffolds seeded with primary human osteoblastic cells were cultured over 8 weeks *in vitro* before subcutaneous implantation together with recombinant human bone morphogenetic protein 7 into NOD-*scid* mice. After 8 weeks of ectopic bone formation in the humanized tissue-engineered bone, cell-laden hydrogels were implanted in close proximity to mimic invasion of the humanized bone by human breast cancer cells. Development of breast tumors in contact with the engineered bone was observed macroscopically (explant images) and microscopically [hematoxylin and eosin (H&E) staining] for both metastatic groups, but not for the control group. Human tumor and bone cells were detected immunohistochemically by positive NuMA (Epitomics, cat. no. s2825, 1:100 dilution) staining, as shown by representative images from each group. All animal experiments conformed to Queensland University of Technology animal ethics approval. NB, new bone; T, tumor; BM, bone marrow; CT, connective tissue; AT, adipose tissue; BV, blood vessel; hTEBC, humanized tissue-engineered bone.

Figure 5 | Formation of a humanized vascular network in GelMA-based hydrogels. **A.** Human ECFCs and MSCs were co-cultured within GelMA-based hydrogels [5% (wt/vol) polymer concentration; DoF: 50%] at a 1:1 ratio, as indicated by CLSM images of DsRed-ECFCs (left) and CMFDA-MSCs (right) after 6 days of co-culture. **B.** DsRed-ECFCs were imaged in the complete hydrogel construct by CLSM, and a 2D projection (XY-plane) was collected along the Z-axis (left). A 3D reconstruction with a cross-section covering a thickness of 400 μm in the direction of the white arrow was acquired (right). **C.** MSCs differentiated into perivascular cells surrounding the ECFC capillaries (left). A CLSM image shows the spatial distribution of the DsRed-ECFC-lined capillaries surrounded by alpha smooth muscle actin-expressing (αSMA; Abcam, cat. no. ab9465, clone EA-53,1:200 dilution; anti-mouse Alexa Fluor® 488, Life Technologies, cat. no. A-11001, 1:1,000 dilution) MSCs (right, top). The zoom-in view depicts details of a capillary and a cross-section image taken in the direction of the yellow arrow (right, bottom). **D.** CLSM image showing

that lumens were lined exclusively by DsRed-ECFC and surrounded by αSMA-expressing MSCs (yellow arrowhead); adapted from[42].

Figure 6 | Microfluidic channels coated with GelMA-based hydrogels for utilization of a cardiomyocyte culture model. **A.** Representation of the coating procedure, with cross-sections of a single microfluidic channel perpendicular to the direction of the flow (top) and channel cross-sections along the direction of the flow (bottom). GelMA prepolymer [5% (wt/vol) polymer concentration; DoF: ~80%] is perfused through the microfluidic channel and exposed to UV crosslinking. Non-crosslinked prepolymer is then washed away with PBS. The channel can be subsequently seeded with cells and perfused with culture media. PDMS, polydimethylsiloxane **B.** CLSM images of cardiomyocytes stained for F-actin (rhodamine 415–conjugated phalloidin, red) and cell nuclei (DAPI, blue) show an elongated morphology and alignment with the GelMA-coated microchannel on day 6 after seeding. **C.** CLSM images show immunostaining of cardiomyocyte markers troponin I (red, top; Abcam, cat. no. ab10231, clone 4C2, 1:200 dilution; anti-mouse Alexa Fluor® 594, Life Technologies, cat. no. A-11005, 1:1,000 dilution), αSMA (green; Abcam, cat. no. ab9465, clone EA-53, 1:200 dilution; anti-mouse Alexa Fluor® 488, Life Technologies, cat. no. A-11001, 1:1,000 dilution) and connexin-43 (red, bottom; Abcam, cat. no. ab11370, 1:200 dilution; anti-rabbit Alexa Fluor® 594, Life Technologies, cat. no. A-11012, 1:1,000 dilution) inside the GelMA-coated microchannel, with cell nuclei counterstaining (DAPI, blue); adapted from[46]. Scale bars, 50 μm.

Figure 7 | Effect of Teflon casting mould depth on mechanical and swelling properties of GelMA-based hydrogels**.** GelMA was dissolved to a final concentration of 10% (wt/vol) in PBS and photo-crosslinked in the presence of 0.05% (wt/vol) Irgacure 2959 by 15 min exposure to 365 nm light at an intensity of 2.6 mW/cm^2 in a CL-1000 crosslinker (UVP). **A.** Compressive moduli of cell-free GelMA-based hydrogels prepared in Teflon moulds with either 1-mm or 2-mm depth were determined after incubation in PBS at 37 °C overnight (mean ± standard error; n = 6) and differ significantly (p<0.001). **B.** Effective hydrogel swelling was determined by weighing GelMA-based hydrogels immediately after crosslinking and again after swelling in PBS at 37 °C overnight and changes significantly (p<0.001) with the casting mould depth. The difference of wet weights was expressed as percentage (mean ± standard error; n = 6).