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Gelatin-methacrylamide hydrogels: towards biofabrication-based tissue repair

Barbara J. Klotz¹, Debby Gawlitta¹, Antoine J.W.P. Rosenberg¹, Jos Malda²,³, and Ferry P.W. Melchels²,⁴

¹Department of Oral and Maxillofacial Surgery & Special Dental Care, University Medical Center Utrecht, P.O. 85500, 3508 GA, Utrecht, The Netherlands.
²Department of Orthopaedics, University Medical Center Utrecht, P.O. Box 85500, 3508 GA, Utrecht, The Netherlands.
³Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584 CM, Utrecht, The Netherlands.
⁴Institute of Biological Chemistry, Biophysics and Bioengineering, School of Engineering and Physical Sciences, Heriot-Watt University, EH14 4AS Edinburgh, UK.

Corresponding author: Malda, J. (j.malda@umcutrecht.nl)

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Hydrogels and the paradigm shift to the third dimension

In the last decade, cell culture research has witnessed a paradigm shift into the third dimension. Three-dimensionally (3D) cultured cells behave differently compared to those cultured in monolayers (2D) and their responses better resemble those in the native tissue [1]. In this shift from the second to the third dimension, hydrogel-based approaches are driving current biomaterial research in tissue engineering. Hydrogels ideally combine resemblance of the natural extracellular matrix (ECM) with its mechanical integrity. While current synthetic hydrogels are often still too reductionistic compared to biopolymers [2, 3], the biological materials generally lack the precise mechanical tunability. In present-day biomaterial research, there is a strong need for a merger of both biologically active and physico-chemically tailorable hydrogels [3]. For these reasons, gelatin-based hydrogels, modified by methacrylamide side groups (gelMA), have recently gained increasing attention [4-12]. By employing this 3D cell culture platform, not only the natural extracellular environment is represented, it also provides the possibility to generate well-defined 3D tissue constructs [13-15]. In this respect, conventional 3D casting techniques for cell-laden hydrogels are replaced by advanced fabrication techniques. The emerging field of biofabrication (see Glossary) allows for the generation of architecturally complex tissue analogs [16], which comprise a spatially organized assembly of various cell types potentially mimicking the native situation (Figure 1).

In the present review, we will provide an overview of the uses of gelMA as a cell-encapsulating hydrogel, serving as a base-material for a multitude of tissue engineering strategies. The trends in gelatin-based biomaterial research – and the place of gelMA
therein – are detailed in a compiled picture of diverse modifications of gelatin and its crosslinking systems. Particularly, we will describe the use of gelMA in state-of-the-art biofabrication approaches to obtain complex tissue-analogs and their functional aspects are highlighted. By this, the potency of gelMA-based engineered constructs is put in perspective of the translational aspect of regenerative medicine.

Gelatin-based hydrogels for cell encapsulation

Gelatin (Box 1) is a versatile biomaterial with several advantages for widespread applications in biomedicine. To employ gelatin as a biomaterial, its instability at body temperature is overcome by covalent crosslinking methods that allow for enhanced physico-mechanical properties [17]. Gelatin can be crosslinked either directly, without prior modification (Box 2) or after functionalization of its side groups. Application of gelatin-based hydrogels based on this latter approach are the main focus of this review. In order to allow for simultaneous cell encapsulation and crosslinking of gelatin hydrogels, cytocompatibility of these methods must, however, be ensured. Therefore, a multitude of cell-friendly crosslinking systems have been developed (Table 1).

Box 1. Gelatin, a versatile biomaterial

Gelatin is a proteinaceous substance that is obtained from denatured and partially hydrolyzed native collagen [18]. Typically, collagen is obtained from bovine or porcine skin or bone as a by-product of the meat processing industry [19]. Extracts from collagen are commonly obtained under acidic or basic conditions [20], which is referred
to as type A or type B gelatin, respectively. In contrast to collagen, gelatin exhibits very limited antigenicity due to the heat denaturation process [21]. Moreover, advantageous biological features of collagen are preserved in gelatin. In the form of a hydrogel, it can provide the residing cells with natural binding motifs. As such, essential cellular functions such as migration, proliferation and differentiation can be facilitated via integrin-mediated cell adhesion [22, 23]. Importantly, the bioactive sequences of collagen (e.g., the arginine-glycine-aspartic acid (RGD) peptide) for cell attachment and the matrix metalloproteinase (MMP)-sensitive degradation sites are retained in the gelatin backbone [24, 25]. Gelatin is extensively used in widespread applications ranging from the food industry [26] to medicine and pharmaceutical processing [27, 28]. For instance, in food preparation, gelatin’s gelling properties upon cooling are often taken advantage of. In the field of tissue engineering and regenerative medicine, gelatin is an attractive base material for engineering “smart” hydrogels for drug delivery (eg. [18, 29]). The increasing interest in the use of gelatin in this field, stems from its desirable features including biocompatibility, biodegradability, low cost and ease of manipulation [30]. Additionally, gelatin is a material that is “generally recognized as safe” (GRAS) by the U.S. Food and Drug Administration for food processing. Further, gelatin is routinely used in the clinics as a plasma expander and as stabilizer in several protein formulations including vaccines [29].

**Box 2. Direct crosslinking strategies of gelatin**

Unmodified gelatin can be directly crosslinked in various ways to form a covalent network, e.g., by chemical or enzymatic crosslinking. Aldehydes are well-known crosslinking agents for proteins [31-33], but are typically not suitable for simultaneous
cell encapsulation due to cytotoxicity, immunogenicity and inflammatory effects of their degradation products [34]. Also genipin, a natural crosslinking agent, which is considered less cytotoxic compared to aldehydes, must be used at a low dose when the hydrogel is employed to encapsulate cells [35]. Overall, most crosslinking agents that enable generation of gels with high mechanical stability exhibit considerable cytotoxicity [33]. In contrast, enzymatic crosslinking of gelatin under physiological conditions by means of transglutaminases or tyrosinases provides a more cell-friendly approach [36-40]. However, this crosslinking system exhibits limited tailorability in the design of the hydrogels. A major disadvantage of direct crosslinking methods is the poor control over the crosslinking density and the resulting stiffness of the hydrogel. For these reasons, employing functionalized gelatin has become a favored approach over the direct crosslinking of gelatin.

Covalent crosslinking after chemical modification

The addition of functional groups to the gelatin backbone, allows for a crosslinking strategy with a high degree of control over hydrogel design and properties, compared to direct crosslinking techniques. Crosslinking of the functional groups can be initiated using various systems. However, only a minority of them is suitable for simultaneous crosslinking and cell-encapsulation [41] (Table 1). Both, (photo)radical initiating systems and enzymatic crosslinking of functionalized gelatin are frequently used. In contrast to indirect enzymatic crosslinking, photo-initiation provides good temporal and spatial control over the crosslinking process, which is essential for creating an architecturally complex tissue analog [42-44]. For this, both ultraviolet light (UV) and visible light (VIS) are used for photo-initiation [13, 45].
Table 1. Modifications of gelatin and crosslinking systems used for cell-encapsulation

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Initiating system</th>
<th>Biological response after cell-encapsulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acrylamide</strong></td>
<td>Irgacure 2959</td>
<td>&gt; 90% viability after one day (HepG2)</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>(UV-A, 365nm)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Ferulic acid</strong></td>
<td>Laccase + O₂</td>
<td>&gt;91% viability (fibroblasts, ECFCs&lt;sup&gt;a&lt;/sup&gt;), angiogenesis</td>
<td>[47]</td>
</tr>
<tr>
<td><strong>Furfurylamine</strong></td>
<td>Rose Bengal</td>
<td>87% viability after one day (MSCs&lt;sup&gt;b&lt;/sup&gt;), used for osteochondral tissue formation</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>(VIS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methacrylamide</strong></td>
<td>APS/TEMED&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;80% viability after one day (chondrocytes)</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>Irgacure 2959</td>
<td>70 to &gt;90% viability depending on e.g. crosslinking conditions, e.g. [3, 5, 7-cell type, macromer 11, 13, 14, concentration; 22, 46, 49- various differentiations 64] investigated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(UV-A, 365nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LAP&lt;sup&gt;j&lt;/sup&gt;</strong></td>
<td>VA-086&lt;sup&gt;d&lt;/sup&gt; (UV-A, 365nm)</td>
<td>MSC/HUVEC&lt;sup&gt;h&lt;/sup&gt; co-culture, vascularization; &gt;97% viability after extrusion printing (HepG2&lt;sup&gt;i&lt;/sup&gt;)</td>
<td>[6, 15]</td>
</tr>
<tr>
<td><strong>LAP&lt;sup&gt;j&lt;/sup&gt;</strong></td>
<td>(VIS 430-490nm)</td>
<td>&gt;96% viability after one day (MSCs), chondrogenic</td>
<td>[12, 65, 66]</td>
</tr>
<tr>
<td>Method</td>
<td>Reagents</td>
<td>Description</td>
<td></td>
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<td>------------------------</td>
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<td>-----------------------------------------------------------------------------</td>
<td></td>
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<tr>
<td>Differentiation; cell proliferation</td>
<td>increase of 23% over two weeks (MSC); adipocyte culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2CK or P2CK(^k) (near-infrared femtosecond laser 800nm)</td>
<td>26% viability (MG63(^l) cells) after two-photon-polymerization [67]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methacrylamide and acetylation</td>
<td>Irgacure 2959 (UV) Chondrocyte encapsulation, used for inkjet printing [51]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methacrylamide galactosylation</td>
<td>Irgacure 2959 90% viability (HepG2), functional testing of hepatocytes [68]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norborene DTT(^m) or LAP (UV 365nm)</td>
<td>&gt;91% viability (MSCs) [69]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolation</td>
<td>HRP(^n) + H(_2)O(_2) &gt;94% viability or not quantified; various differentiations investigated (e.g. neurogenesis, osteogenesis, vascularization) e.g. [70-77]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Styrenation</td>
<td>Camphor-quinone (VIS 400-520nm) 26% viability (chondrocytes) [78]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)ECFC, endothelial colony forming cell; \(^b\)MSC, mesenchymal stromal cells; \(^c\)APS/TEMED, ammonium persulfate/ tetramethylethlenediamine; \(^d\)VA-086, 2,2'-Azobis[2-methyl-N-
In order to make use of (photo)radical initiating or enzymatic catalyzing systems for crosslinking, a variety of functional groups have been used (Table 1). Moreover, some double modifications were employed for improved cell-behavior [68] or enhanced processability [51]. Yet, the vast majority of the reported literature employs gelMA with the photo-initiator Irgacure 2959. Interestingly, deviations from this approach, except for the use of styrenated gelatin with camphorquinone (which showed very low cell viability), are all fairly recent and one-off demonstrations. The introduction of acrylamide, furfurylamine and norborene-substituted gelatin may present specific advantages compared to gelMA, which will have to be demonstrated by thorough further research.

The use of VIS light (with suitable initiators) has a strong rationale, since UV light is known to have detrimental effects on biological components. Although cell viability is generally assessed 1 day after crosslinking, more subtle damage may be incurred by the UV that could affect cell functionality and tissue formation on the longer term [79]. Moreover, long-term effects of Irgacure 2959, albeit relatively cell-friendly compared to other UV photo-initiators [80], have not yet been studied properly.
Gelatin-methacrylamide hydrogels for cell-encapsulation

GelMA was first introduced in 2000 by Van den Bulcke and coworkers [52]. Subsequently, it gained considerable interest in the tissue engineering community due to its inherent bioactivity and physico-chemical tailorability [81]. The first step in the hydrogel design of gelMA, is comprised of the selection of an appropriated degree of functionalization (DoF) of gelatin (Box 3). The biomaterial can be further tailored to accommodate formation of specific tissues by designing the desired physico-chemical properties.

**Box 3. GelMA synthesis**

GelMA is generally prepared by reacting gelatin with methacrylic anhydride in phosphate buffered saline (PBS) at pH 7.5. Methacrylic anhydride is added slowly to the gelatin solution while vigorously stirring at 50°C. During the reaction methacrylic acid is formed. After one hour, the reaction is diluted with water. To remove unreacted methacrylic anhydride from the reaction mixture, it is dialyzed against distilled water. The obtained reaction product, gelMA, is freeze-dried to a white porous foam [52]. The degree of functionalization (DoF) of the synthesized gelMA batch can be tailored by varying the ratio of methacrylic anhydride to gelatin [22, 52]. The DoF was characterized by van den Bulcke et al. as the percentage of the functionalized primary amine groups over the total primary amine groups [52] and is generally determined by the Habeeb method [82].

As an example, spreading of cartilage cells needs to be prevented within the gel. This can be achieved by employing increased polymer concentrations (conventionally 10%) that
may sterically hinder cells to spread. Also highly functionalized gels with a DoF ranging from 60-80% can hamper cell spreading, possibly by extensive crosslinks throughout the hydrogel. Generally, relatively higher percentages of methacrylation lead to hydrogels that are stiffer and more durable, with smaller pore sizes [53]. Typically, by varying the macromer concentration from 5-20%, hydrogels are generated with compressive moduli in the range of 5-180 kPa [13]. Next to the DoF of a synthesized gelMA batch and its macromer concentration, the parameters of photo-crosslinking critically influence the properties of the resulting hydrogel. These parameters include the light exposure time, light intensity and initiator concentration. Over time, these parameters can be affected by degradation. The gelMA network is susceptible to local degradation by enzymes, most notably by matrix metalloproteinases (MMPs) that are secreted by the (embedded) cells [3].

All design parameters need to be carefully balanced for each specific application. These include the stiffness, degradation profile and intended cellular behavior in the resulting hydrogel. For a detailed summary on the design parameters, the reader is referred to a recent review from Khademhosseini and colleagues [81]. Due to this tunability of gelMA properties, it can be employed for a broad variety of approaches in tissue engineering. Indeed, gelMA has been applied in approaches aiming for the regeneration of neural tissue, for vascularization, cartilage, bone, skin, skeletal muscle, cardio, liver, and kidney [81], underscoring the versatility of this material.
Biofabrication techniques

Conventionally, research on cell-encapsulating gelMA hydrogels is often based on casted or molded disk-shaped micro-tissues that serve as models to study cell-material interactions. To obtain a tissue-like construct with a defined 3D structure, more advanced technologies have now emerged. The excellent spatial and temporal control over gelMA crosslinking, and its rheological behavior enable deposition by various biofabrication techniques (Figure 2).

Fabricating cell-laden modules by microfluidic strategies

Microfluidic strategies were developed to encapsulate cells in gelMA droplets (Figure 2a) for a bottom-up tissue engineering approach or as micro-module for advanced assembling strategies to build more complex tissues [50]. Furthermore, a microfluidic spinning technique was introduced to fabricate photo-crosslinkable gelMA fibers with encapsulated cells [54]. It was shown that the engravement of gelMA fibers induced cell alignment on its surface [54]. In order to enhance the potential of these cell-laden fibers, encapsulation within a bulk hydrogel may be beneficial. Such 3D patterns in the fibers can be used as templates for creating tissues that exhibit preferential cell orientations, such as blood vessels or muscle fibers. Recently, an alternative set-up was introduced to create highly viable cell-laden microfibers in a straightforward and high-throughput manner. Upon stretching of the loaded fibers, cell alignment within the constructs was achieved [83].

Employing soft- and stereolithography for cell encapsulation

GelMA is also employed in various soft lithography techniques to fabricate micropatterned tissues that involve cell encapsulation. Construct features on the
micrometer scale reaching down to 100 microns in resolution were successfully fabricated resulting in robust cell-laden gelMA microtissues [22]. Such a micropatterning procedure was also used in a “layer-by-layer” bottom-up approach by means of masks to build an osteon-like hydrogel with microchannel networks based on gelMA [55, 84]. These approaches demonstrate a localized deposition of cells to form the vasculogenic and osteogenic parts of bone tissue. However, when moving towards creating larger constructs for tissue repair strategies, such a mask-based approach in micromolding is limited due to high costs, its time consuming nature and lack of automation.

In contrast to micromolding, stereolithography circumvents these challenges since it can be performed as a maskless photopatterning technique able to directly build up 3D structures. The design is processed by software and sliced into a number of layers. By a dynamic stereolithographic technique 100–250 μm thin slices with various shapes could be fabricated (figure 2b) with high cell survival of about 80% after eight hours of cell encapsulation [56]. Overall, stereolithography is a valuable means to create complex 3D architectures to guide cell alignment and behavior within a generated construct. However, stereolithography is limited to one resin-composition containing one biomaterial (mixture) and homogeneously distributed cells.

**Bioprinting of tissues with cell-containing gelMA-based inks**

In addition to lithographic approaches, tissue analogs can be also generated in a layer-by-layer fashion with bioprinting. Tissue construction by 3D-printing of cells by means of a hydrogel-based ink has recently become an attractive approach in the field of tissue engineering [13, 57]. By a direct-write bioprinting strategy, the feasibility was shown to build gelMA-based constructs with varying architectures [49]. To embrace the
complexity of a tissue in a printed analog, a bioprinting approach was proposed that is composed of heterogeneous subunits [57]. In this approach, a poloxamer gel was employed as a sacrificial material to create the vascular luminal space for seeding of endothelial cells. Around the vascular bed, fibers were co-printed containing heterogeneous cell types with high cell viability and the bulk material was molded using gelMA [57]. Furthermore, for engineering bone, a microcarrier technology was combined with printing technology. Mesenchymal stromal cells (MSCs) were seeded on polylactide microspheres for extensive cell expansion and these multicellular aggregates were printed within a gelMA-gellan gum ink [10].

Several strategies were introduced to allow for a well-defined deposition of cell-laden gelMA. In order to improve the rheological characteristics of gelMA for printing, viscosity-enhancing components were mixed into the bioink. For instance, adding gellan gum [14] or hyaluronic acid [13] to the gelMA-precursor solution optimized the ink rheological properties for dispensing. Another method for improving biofabrication of gelMA is co-deposition with reinforcing biomaterials. Thermoplastics, such as polycaprolactone (PCL), can here serve a dual role. Firstly, the deposition of gelMA is more defined as the PCL can delineate the boundaries of the gelMA compartment, and secondly, constructs can be generated with enhanced mechanical properties [13, 58]. A third approach to improving the printability of cell-laden gelMA hydrogels relies on gelatin’s inherent temperature-dependent sol-gel transition and not on viscosity-enhancing materials or co-deposition techniques [15]. Cooling of the printed fibers on the collecting plate to 5°C, immediately after deposition, enhanced physical crosslinking of gelMA and provided sufficient mechanical integrity to build up layers. However, the rapid change in temperature may affect the behavior of some more fragile cell types. This approach was suitable for high gelMA concentrations between 10 and 20% and
allowed encapsulation of a liver cell line (HepG2) with high viability.

In order to generate complex anatomically shaped constructs, sacrificial components like poly(vinylalcohol) (PVA) and alginate have been co-deposited with gelMA and PCL. These sacrificial materials that were removed in aqueous solution after the fabrication process were used as temporary structures for the support of overhanging geometries. By this approach porous constructs were obtained of clinically relevant sizes without affecting cell viability during the fabrication process [58].

**GelMA composite structures for enhanced tissue-specific functionality**

Analogous to the use of gelatin [85], also gelMA is increasingly employed in combination with other materials. In composites, a synergistic effect of the materials can be achieved that enhances the (bio)functionality of gelMA-based hydrogels. GelMA composites were for example developed with calcium phosphates [55], polysaccharides [86], hyaluronan [13, 59], silk [61], and ECM particles [62]. Furthermore, synthetic polymers such as PCL [48, 58] and poly(ethylene glycol) (PEG) [8, 87, 88], and nanoparticles have also been combined with gelMA [63].

**Engineering vascular networks in gelMA**

A major hurdle in tissue engineering is the limited supply of oxygen and nutrients in generated tissue constructs. This limitation is addressed by introducing a minute vascular network in tissue-engineered constructs. If perfused successfully, the formation of a necrotic core in the construct can be prevented. The feasibility to engineer vascular-
like networks in gelMA constructs has been investigated mainly by two approaches. First, by a scaffold-based strategy, relatively large diameter vascular beds are engineered that are seeded with endothelial cells after fabrication of the construct [89]. Second, smaller, capillary-like structures are generated by encapsulation of endothelial cells within the bulk material [89]. The latter approach is based on the intrinsic capability of endothelial cells to self-assemble de novo into capillary-like structures.

These vascular beds can be introduced in gelMA constructs by bioprinting of artificial, channeled structures from materials such as poloxamer [57], PVA [90] or agarose [5]. Following removal of the artificial templates, endothelial cells were seeded onto the channel walls of the cell-laden gelMA scaffolds [5, 22] to cover the channels with a monolayer [5]. These fabricated microchannels facilitated improved mass transport of nutrients compared to a non-channeled bulk hydrogel. This was demonstrated by significantly higher cell survival and osteogenic differentiation of an osteoblastic cell line (MC3T3) within the bulk hydrogel [5]. While engineering of vascular beds via sacrificial fibers seems to be an adequate tool for relatively thin (2mm) constructs [90], its effectivity remains to be demonstrated for clinically-relevant construct dimensions.

Capillary-like structures have been formed by self-organization of MSCs and endothelial colony forming cells (ECFCs) [9, 53] or human umbilical vein endothelial cells (HUVECs) [6] that were combined within a gelMA bulk hydrogel. By this approach, extensive capillary-like networks with lumens were detectable [53]. The next step in engineering vasculature-like structures within a tissue engineered construct could be taken by offering appropriate (blood) flow conditions for improved cell maturation. Accordingly, a co-culture of ECFCs and MSCs, embedded in a pure gelMA carrier, was implanted subcutaneously in immunodeficient mice [9, 53]. After 7 days, an evenly
distributed endothelial network was formed throughout the construct. This provided proof of concept of functional anastomoses of bioengineered vascular-like structures in gelMA [9].

Tissue specific differentiation in gelMA

Next to general approaches for vascularizing cell-laden constructs, gelMA has been employed towards a broad spectrum of tissue specific applications. For the engineering of cardiac patches, gelMA was combined with carbon nanotubes and graphene oxide microspheres, for introducing electrical conductivity [63, 64, 91]. Functional assessment of neonatal rat cardiomyocytes on a 2D composite patch, highlighted higher and synchronous beating rates and a lower excitation threshold compared to a culture on pure gelMA [64]. These 2D patches are thought to be rolled up or folded in order to form 3D tissues [64]. For a direct 3D approach that encapsulates cells within the composite, a cell line (NIH-3T3 fibroblasts) was employed that demonstrated good cellular functions [91].

In liver tissue engineering, hepatocyte micro-aggregates were generated in a high-throughput manner and encapsulated in gelMA [92]. Analysis confirmed that the encapsulation did not interfere with cell viability, and primary hepatocytes could be maintained with a stable phenotype during 21 days. Furthermore, gelMA containing the cell aggregates could serve as a bioink for 3D liver printing [92].

In bone tissue engineering, the combination of cells, mineral and protein such as occurring in the native tissue, is increasingly employed [85]. GelMA has been combined with calcium phosphates and human osteoblast-like cells (MG63) [55]. Although the
addition of the ceramics resulted in higher mechanical strength, no significant effect on osteogenicity has been shown so far.

Besides via the direct intramembranous route, bone can also be formed via the indirect endochondral route, with cartilage tissue as an intermediate stage. Endochondral ossification was shown in gelMA constructs in a subcutaneous rat model [62]. First, gelMA-encapsulated MSCs were cultured *in vitro* for 2 weeks to provide a cartilage template that was subsequently remodeled *in vivo* into mineralized bone tissue harboring bone marrow cavities. The gelMA hydrogel was almost completely degraded during this process, while the newly formed matrix assured construct integrity [62].

Cartilage is another load-bearing tissue, which requires prolonged mechanical performance of tissue-engineered constructs. It is of utmost importance that reinforcing strategies do not impede tissue formation. Hyaluronic acid has shown to be a valuable additive to gelMA-based constructs for cartilage tissue engineering, as it directly influences chondrocyte differentiation in a concentration dependent manner [13, 59, 60]. Moreover, for cartilage engineering a sophisticated construct was designed with high mechanical strength. In this approach, methacrylated PCL was 3D printed and covalently crosslinked with chondrocyte-laden gelMA [7].

*Tissue architecture*

The architecture of a tissue analog is mainly dictated by (bio)functional and mechanical aspects. Currently, the main challenge lies in up-scaling microtissues to clinically relevant sized constructs. This cannot be achieved by simply applying the same methods and creating a larger tissue. The complexity of the construct is increased together with the number of challenges. Up-scaling of a construct comprises for instance nutrient transfer throughout the construct and providing the required mechanical stability.
Whereas gelMA has proven its potency in creating microtissues, in future research yet more focus is expected on up-scaling.

Furthermore, an essential aspect of tissue architecture is to embrace the complexity of a tissue in its engineered analog. For example, Kolesky and co-workers divided different tissue components (vasculature, extracellular matrix and specific cells) over multiple bioinks [57]. However, this biofabrication approach, like most others, was focusing on short term cell-behavior rather than on long-term features such as matrix remodeling and tissue maturation. Such long-term outcomes of biofabricated tissues will be of great value to determine what level of architectural complexity will need to be imposed to obtain functional human tissue analogs (see outstanding questions).

GelMA from a (pre-)clinical perspective

Promising results were obtained in a pre-clinical study, demonstrating the potential of gelMA for clinical application. The aforementioned endochondral bone regeneration [62] is a great example of an impressive balance between degradation of a biomaterial and replacement by neo-tissue, which is one of the key and most challenging goals in tissue engineering.

For clinical translation, gelMA as a base-material has to meet several requirements. First of all, the in vitro and in vivo biocompatibility of gelMA and its degradation products, oligo methacrylic acids, have to be considered. An extensive in vitro study showed good biocompatibility for gelatin type B-based gelMA, while type A-based gelMA elicited inflammatory reactions [93], possibly caused by high levels of endotoxins in later
material. So far, only one immunocompetent animal (mouse) model was used to test gelMA biocompatibility. The absence of a pro-inflammatory activity provided a first proof of immunocompatibility for type B-based gelMA [93]. While in this study endotoxin-free gelatin was used (type B-based gelMA), it has to be mentioned that most research is currently conducted with gelMA that is based on gelatin with very high endotoxin levels. These endotoxins can cloud the observations by influencing cell behaviour (e.g., stimulation of osteogenesis [94]), or they may elicit other undesired effects. This aspect is generally underestimated in the field.

Other challenges in clinical translation are in batch-to-batch variations and possible disease transfer associated with animal-derived materials. Nonetheless, clinical grade gelatin is nowadays routinely used in the clinics.

The current major bottleneck in the translation of tissue engineered constructs to the clinic is to convert a successful regenerative approach to procedures adhering to good manufacturing practice (GMP) whilst retaining the intended regenerative capacity. The conversion extends from the gelMA synthesis and bioprinting to the cell culturing protocols. The incorporation of cells further complicates translation as tissue engineering products need to be conform the legislation for advanced therapy medicinal products (ATMPs) which is still an underexplored field [95]. In this light, considering its general potency, gelMA might not only be a pioneer for translating semisynthetic biomaterials to ATMPs but it can also act as a “transitional technology” [96]. GelMA can serve as a stepping stone in the field of tissue engineering and accelerate the translation of the technology from bench to bedside.
Concluding remarks and future directions

GelMA has become an attractive biomaterial in recent years for engineering various tissues since the gelatin backbone provides cells with biological cues and the functionalization enables tailored physico-chemical properties. At present, research is either mainly focusing on the generation of viable well-defined 3D constructs or on long-term cell performance in the non-biofabricated construct. The greatest challenge is to scale-up construct dimensions to clinically relevant size. Therefore, future research with gelMA should focus on converging biofabrication and tissue engineering technologies to create large, well-defined and functional tissue equivalents upon maturation. The design of smart geometries, combinations of various materials and tissue types, and maintenance of the complex tissues under ATMP guidelines will be next. In conclusion, gelMA is performing a valuable pioneering role and can accelerate the clinical translation of biofabrication-based tissue repair.

Acknowledgements

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Figure 1, Key Figure. Evolutionary stages from 2D cell culture to the development of 3D tissue analogs

Figure 2. Categories of gelMA-based biofabrication techniques. (A) Preparation of cell-laden microspheres by microfluidics (scale bar= 30µm). Reproduced, with permission, from [97]. (B) Microfabrication of pyramid shape scaffolds with encapsulated cells stained for actin expression (scale bar=100 µm). Adapted and reproduced, with permission, from [56]. (C) Robotic dispensing of cell-laden gelMA and a bioprinted analog of the distal femur from a human knee. Reproduced with permission from [98] and [58].
Outstanding Questions Box

- Is gelMA equally suitable to engineer analogs for all human tissues?
- Will gelMA serve as a stepping stone and be eventually replaced by new synthetic gels with a sufficient level of biofunctionality required for a tissue analog?
- How can multiple cell types be combined, each requiring their specific cues, within one construct to generate multi-tissue type, vascularized, clinical-size, and functional tissue analogs?
- How much pre-defined architectural complexity does a 3D biofabricated tissue analog require and to what extend can this architecture be created and modulated by encapsulated cells?
Figure 2

(A) Microfluidics

(B) Lithography

(C) Bioprinting

Figure 2
**Glossary**

**Biofabrication:** The generation of functional hierarchical 3D living and non-living products with prescribed organisation from raw materials such as living cells, molecules, extracellular matrices, and biomaterials through computer-aided automated additive manufacturing techniques and subsequent (biological) maturation.

**Bioink:** Fluid or gel containing living cells to be used for printing of tissue constructs.

**ECFC:** Endothelial colony forming cells are endothelial progenitors that are able to differentiate into functional endothelial cells. They are present in the adult blood, however, can be obtained with higher yield from umbilical cord blood for engineering endothelial networks or coating the luminal side of vascular structures.

**Habeeb method:** Method to determine the number of free amino groups in proteins.

**Irgacure 2959:** Water-soluble, cytocompatible radical photo-initiator for the UV curing of unsaturated monomers and prepolymers.

**Microfluidics:** Passive or active fluid handling or manipulation within micrometer-sized channels.

**Micromolding:** Production of objects with micrometer-sized features within a mold.

**Micropatterning:** Patterning of (bio)materials to control the fate and geometry of adhering cells.

**Microtissue:** Hydrogels in the millimeter range with encapsulated cells that are used for 3D cultivation.
**MSC:** Mesenchymal stem cells or multipotent stromal cells are adult stem cells that can differentiate towards at least the osteogenic, adipogenic and chondrogenic lineages. MSCs from human bone marrow aspirates are the gold standard human cell source used in tissue engineering and regenerative medicine of bone, fat and cartilage tissue.

**Photo-crosslinking:** Covalent binding of molecules using light as an initiating system.

**Poloxamer:** A triblock copolymer with typical trade names Lutrol or Pluronics. It is used for defined printing processes for creating sacrificial layers or fibers in biofabrication approaches.

**PCL:** Polycaprolactone is a biodegradable and biocompatible polyester that is often used as a scaffolding material in tissue engineering.

**Soft lithography:** combines various fabrication techniques that employ elastomeric materials to fabricate constructs typically on the micrometer or nanometer scale. Photo-patterning is an example of soft lithography that employs molds and/or photomasks.

**Stereolithography:** is an additive manufacturing technique to fabricate scaffolding materials by spatially controlled photo-crosslinking of polymers in a bath of resin.