Gelatin methacryloyl is a slow degrading material allowing vascularization and long-term use in vivo

Stefanie Heltmann-Meyer 1,2, Dominik Steiner 1,2, Claudia Müller, Dominik Schneidereit 3, Oliver Friedrich, Sahar Salehi 3, Felix B Engel, Andreas Arkudas 3,4 and Raymund E Horch 1,2

1 Department of Plastic and Hand Surgery, University Hospital of Erlangen, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, 91054, Germany
2 Laboratory for Tissue Engineering and Regenerative Medicine, University Hospital of Erlangen, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, 91054, Germany
3 Department for Biomaterials, Faculty of Engineering Science, University of Bayreuth, Bayreuth 95447, Germany
4 Institute of Medical Biotechnology, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen 91052, Germany
5 Department of Nephropathology, Experimental Renal and Cardiovascular Research, Institute of Pathology, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen 91054, Germany

E-mail: Stefanie.Heltmann-Meyer@uk-erlangen.de and Raymund.Horch@uk-erlangen.de

Keywords: tissue engineering, hydrogel, GelMA, angiogenesis, proteomics, multiphoton microscopy

Abstract

In situ tissue engineering is an emerging field aiming at the generation of ready-to-use three-dimensional tissues. One solution to supply a proper vascularization of larger tissues to provide oxygen and nutrients is the arteriovenous loop (AVL) model. However, for this model, suitable scaffold materials are needed that are biocompatible/non-immunogenic, slowly degradable, and allow vascularization. Here, we investigate the suitability of the known gelatin methacryloyl (GelMA)-based hydrogel for in-situ tissue engineering utilizing the AVL model. Rat AVLs are embedded by two layers of GelMA hydrogel in an inert PTFE chamber and implanted in the groin. Constructs were explanted after 2 or 4 weeks and analyzed. For this purpose, gross morphological, histological, and multiphoton microscopic analysis were performed. Immune response was analyzed based on anti-CD68 and anti-CD163 staining of immune cells. The occurrence of matrix degradation was assayed by anti-MMP3 staining. Vascularization was analyzed by anti-\(\alpha\)-smooth muscle actin staining, multiphoton microscopy, as well as expression analysis of 53 angiogenesis-related proteins utilizing a proteome profiler angiogenesis array kit. Here we show that GelMA hydrogels are stable for at least 4 weeks in the rat AVL model. Furthermore, our data indicate that GelMA hydrogels are biocompatible. Finally, we provide evidence that GelMA hydrogels in the AVL model allow connective tissue formation, as well as vascularization, introducing multiphoton microscopy as a new methodology to visualize neovessel formation originating from the AVL. GelMA is a suitable material for in situ and in vivo TE in the AVL model.

1. Introduction

Large volume tissue defects or organ failure are treated with autologous tissue or allogenic organs such as the kidney or heart, respectively. Although the technical requirements are well established in reconstructive surgery and transplantation medicine, the required organs or tissues can cause problems due to donor site morbidity, tissue availability, and human leukocyte antigen matching. The generation of bioartificial tissues using the principles of tissue engineering (TE) is a promising approach to solving the aforementioned restrictions. In situ TE is characterized as biomaterial-induced endogenous tissue formation directly at the site of injury or in situ, beginning with readily available resorbable grafts that are progressively transformed into autologous homeostatic replacement tissue with the ability to repair, reshape and grow. Grafts for in situ TE can be natural or synthetic; however, the prerequisite for this approach is first the recruitment of host cells in the transplantation site and its
remodeling in order to achieve adaptive autologous tissue over time and second the biomaterial characteristics such as morphology, biochemical and biophysical properties which must be adapted to the tissue to be replaced [1, 2]. For example, bioartificial bone tissue requires a matrix with solid parts [3–5], while fat tissue requires a rather soft matrix [6]. Furthermore, matrix degradation must also be adapted to the area of application so that the optimal degradation of scaffold materials corresponds to the formation of the required tissue. Another point to consider when choosing a matrix is biocompatibility. Some matrices have already been tested for biocompatibility, such as alginate di-aldehyde-gelatin (ADA-GEL) [7] and polycaprolactone [8], while some materials are already FDA approved and are in medical use, such as fibrin [9] and polymethyl methacrylate [9].

Another new potential field of in situ TE application is the establishment of a drug-producing tissue container. Therefore, an inert container, being used over a longer period (months or years), is implanted subcutaneously. Drug-producing cells are encapsulated into a non-degrading or slowly degrading matrix. The encapsulated cells can produce biological molecules, such as antibodies against tumor necrosis factor receptor II in the case of rheumatoid arthritis in vivo after being directly transferred into the organism. Therefore, the third prerequisite for in situ TE is applying a suitable carrier matrix that is stable for a long time, maintaining the concentration of active substances for years through continuous secretion. Such a system will have a potential application in cancer therapy or the treatment of autoimmune diseases.

Gelatin, a protein-based biopolymer obtained from the hydrolytic degradation of collagen, has shown high biocompatibility and angiogenetic potential in situ as well as in vivo [10–13]. Gelatin is produced from different types of collagen (hydrolyzed collagen), which is easily degradable and does not cause a severe immune reaction, as can be the case with collagen [14, 15]. Structurally, gelatin also contains integrin-binding motif (RGD) sequences improving cell adhesion. RGD motifs are also known to stimulate angiogenesis [16]. Furthermore, morphogenesis, wound healing and angiogenesis is supported by target sequences for matrix metalloproteinases (MMPs) contained in gelatin [17]. MMPs are jointly responsible for the degradation and rearrangement of gelatin [18]. The degradation of the matrix is quite fast with MMPs and proteases. Biodegradation of gelatin can be adjusted via a modification with methyl acrylate resulting in the production of gelatin methacryloyl (GelMA) [19]. The degree of methacrylation can be controlled during the synthesis procedure, and it has been shown that small amounts of MA group to gelatin <2 ml g⁻¹ does not affect the encapsulated cells while preserving the physical and chemical properties show a high cytocompatibility [20]. The amino groups are not affected, and consequently, the RGD sequence remains intact within the gelatin without reacting with MA. Furthermore, GelMA can be optically cross-linked using a photoinitiator system, triggering the formation of free radicals, polymerizing the different methacrylamide, and methacrylate groups within the gelatin, enhancing the stability of the polymer network. Therefore, after preparing the GelMA solution at 37 °C in a liquid state, it can be easily used to fill any cavity before being crosslinked [21].

In order to ensure cell survival in vivo, adequate vascularization is required. Improvement of vascularization can be achieved by, e.g. growth factors such as vascular endothelial growth factor (VEGF) [22], endothelial cells, or surgically induced angiogenesis [23, 24]. The latter is a powerful tool and can be applied in the form of an arteriovenous loop (AVL) model. An AVL between a femoral vein and an artery is micro-surgically anastomosed using a venous interponate. The matrix for in situ TE in the container can thus be tested regarding stability and angiogenic properties. Previous studies with materials like engineered spider silk [25], ADA-GEL and fibrin have already shown that angiogenesis begins after about 14 d [10, 22].

This study targets to prove that GelMA is a biocompatible matrix with angiogenic properties and long-term stability in the rat AVL model.

2. Materials and methods

2.1. GelMA production

GelMA was synthesized according to the protocol provided in Loessner et al [19]. Briefly, 6 g of Gelatin A (Porcine, Sigma-Aldrich, Missouri, USA) was dissolved in 50 ml of Dulbecco’s Phosphate Buffered Saline (DPBS, Sigma-Aldrich) at 50 °C, followed by the addition of 12 ml of Methacrylic anhydride (MA, Sigma-Aldrich) stirred for 1 h. Thereafter, methacrylation process was terminated by adding warm DPBS. Subsequently, the solution was dialyzed at 37 °C for a week and lyophilized for another week. The final product was stored at −20 °C. To prepare a 10 wt% GelMA hydrogel as a matrix for AVL, the lyophilized GelMA was dissolved in sterilized DPBS at 37 °C while being continuously stirred for 30 min. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Sigma-Aldrich) was added to the dissolved GelMA with a concentration of 1 wt%, and the solution was protected from light. The gel was prepared freshly for each operation.

2.2. Degradation assay

A polytetrafluoroethylene (PTFE) chamber with a diameter of 10 mm and a height of 6 mm with 471 μl volume was filled with sterilized warm GelMA 10 wt% solution containing LAP as a photoinitiator and crosslinked for 30 s using a handheld UV-lamp (395–400 nm; 80–150 mcd) (EFL41UV UV, Perel,
Gavere, Belgium). The chambers were then incubated in DPBS and DPBS containing 1.75 µg ml⁻¹ collagenase (Sigma-Aldrich) in a humidified atmosphere (37 °C, 95% relative humidity, 5% CO₂) with a change of media three times a week. The weight of the chambers containing GelMA was taken over 14 d, where the empty chamber weight was subtracted and the degradation calculated via the mass loss.

### 2.3. Arteriovenous loop surgery

Ten male Lewis rats (Charles River Laboratories, Sulzfeld, Germany) with a weight range from 330 to 380 g underwent surgery. The surgeries were approved by the Animal Care Committee of the University of Erlangen and the Government of Mittelfranken (AZ 55.2–2532-2-763). The surgery was carried out under isoflurane general anesthesia (cp-pharma, Burgdorf, Germany) by one surgeon using an operative surgical microscope (Carl Zeiss, Oberkochen, Germany). A 2–3 cm incision in both groins was made, the femoral vessels were dissected, and a vein graft was gathered from the right leg. For the A VL, the vein graft was interposed between the femoral artery and vein on the left side with an 11–0 non-resorbable suture (Ethilon, Ethicon, New Jersey, USA) (figure 1). A PTFE chamber was half-filled with sterilized warm GelMA 10 wt% solution containing LAP and crosslinked as described before. After crosslinking a GelMA layer with a thickness of about 2.5 mm, the AVL was placed on top of it, and another layer of GelMA was cast and crosslinked. In total, approximately one milliliter of GelMA solution was used for each animal. Afterward, the chamber was fixed onto the thigh muscle, closed with a lid, and the skin was closed. Postoperatively, the animals received enoxaparin (10 mg kg⁻¹) for 2 d. Five constructs were explanted 2 weeks after surgery and the other five after 4 weeks.

### 2.4. Explantation procedure

Vascularization of the constructs was visualized after intra-arterial perfusion with India ink. For this, a longitudinal laparotomy was performed, the descending aorta cannulated, and the inferior caval vein cut. The vascular system was flushed with a Ringer–Heparin solution (100 IU ml⁻¹). Then, 20 ml of India ink solution, containing 50% (v/v) India ink (Lefranc-Bourgeois, London, England) in 5% gelatin (Carl Roth, Karlsruhe, Germany) and 4% mannit (Carl Roth) was applied into the aorta. The descending vein and artery were ligated, and the specimens were placed at −20 °C for 2 h before explantation. The constructs were cut in half, one half embedded in Roti®-Histofix 4% (Carl Roth) for histological analysis and the other half frozen at −80 °C for proteome analysis.
2.5. Fluorescein isothiocyanate (FITC) perfusion and explantation
For multiphoton microscopy, 0.8 ml of a 5% fluorescein isothiocyanate (FITC) solution (Sigma-Aldrich) was injected intravenously. After 10 min, the vessels entering the PTFE chamber were ligated, and the chamber was explanted. The animal was euthanized and the construct was fixed in 4% paraformaldehyde (Sigma-Aldrich) for 4 h. Samples were dehydrated and optically cleared according to an established protocol [26] and stored in ethyl cinnamate (Sigma-Aldrich) under the absence of light until multiphoton microscopy.

2.6. Multiphoton microscopy
The samples were imaged while immersed in a clear solution and contained in a polycarbonate chamber that was covered with a glass coverslip. An upright multiphoton microscope system (TriMScope II, LaVision BioTec, Bielefeld, Germany, described in [27]) with a Nikon Plan Fluor 10×/0.3NA objective was used. Images were recorded using an excitation wavelength of 810 nm and detecting the emission wavelengths 525 nm (525/50) and 450 nm (450/70). The images voxel size was 2.2 × 2.2 × 8 µm³ with a 1.1 × 1.1 m² field of view. The images were acquired as 3D mosaic with 10% overlap and stitched using ImageJ to cover the whole sample volume (8.1 × 9.1 × 3.4 mm³) [28].

2.7. Histological staining and analysis
The constructs were embedded in paraffin, and 3 µm cross-sections perpendicular to the longitudinal axis of the AVL were cut with a microtome. Hematoxylin and eosin (H&E) and α smooth muscle actin (α-SMA) staining were carried out according to standard protocols. For macrophage detection, CD68 staining was carried out. Dewaxed sections were first treated with blocking solution (Zytomed Systems GmbH, Berlin, Germany) and then incubated with the primary anti-CD68 (BIO-RAD, Hercules, USA) antibody in a dilution of 1:300. For enzymatic detection, α-SMA staining, counting positive vessels with a clearly visible lumen or lumens filled with India ink. The vessel distribution was calculated using the distance to the closer main loop vessel measured with ImageJ.

2.8. Proteome analysis
Four frozen samples of the 4-week group and four untreated femoral veins were lysed with Triton X-100 (1%) and protease inhibitors (10 µl ml⁻¹ Aprotinin, Sigma-Aldrich, 10 µl ml⁻¹ Leupeptin and 10 µl ml⁻¹ Pepstatin, both Bio-Techne GmbH, Wiesbaden, Germany) and centrifuged at 10 000 × g for 5 min. The proteome assay was performed according to the manufacturer’s protocol (Proteome Profiler Mouse Angiogenesis Array Kit Bio-Techne GmbH). Briefly, the films were incubated with the lysed samples, blocked, and incubated with an antibody cocktail that specifically detects 53 angiogenesis-related proteins. Thereafter, streptavidin and a chemiluminescent detection mix consisting of hydrogen peroxide and luminol were applied, and images were taken with iBright (Thermo Fisher Scientific, Massachusetts, USA). The images were analyzed with the Protein Array Analyzer plugin for ImageJ. Thereby, the measured mean pixel density was normalized to the reference spots on each film. A heatmap of all analyzed and normalized protein values was created with R (RBC, Boston, USA).

2.9. Statistical analysis
Statistical analysis was performed with GraphPad Prism 8.00 (GraphPad Software, California, USA). The normal distribution of the samples was assessed with a Shapiro–Wilk test. An unpaired Student’s t-test was used, and p-values ≤0.05 were considered
statistically significant. Data are shown as mean values ± standard deviation. A Wilcoxon test was done for the proteome analysis, and p-values ≤0.05 were considered statistically significant.

3. Results

3.1. GelMA hydrogels in the AVL model is stable for 4 weeks

In order to determine whether GelMA hydrogels are suitable for long-term in situ TE, the AVL model was utilized by filling the PTFE chamber with GelMA. All animals utilized in this study survived the operation. Furthermore, no surgical side effects or postoperative complications such as wound healing disorder or dislocation of the chamber occurred. Macroscopic analysis of the explanted in situ engineered tissues at 2 and 4 weeks after implantation revealed that all explants were in a stable form (figure 2). The GelMA hydrogel remained translucent, and the loop was clearly visible in the middle of all constructs. No gross-morphological difference was observed between samples at 2 and 4 weeks post-implantation (figures 2(A) and (B)). In accordance with this observation, the weight of the constructs at 2 and 4 weeks post-implantation was not significantly different (0.65 ± 0.04 g vs. 0.63 ± 0.09 g; n = 5 in each group) (figure 2(C)). These data suggest that GelMA hydrogels are stable under in vivo conditions in the AVL model. In the degradation assay, the GelMA was shown to degrade by the collagenase completely after 14 d (figure 2(D)). We measured a mass loss in the first 7 d of 65.50% and 100% until day 14. Whereas the control in PBS remained stable after an initial mass loss. With a weight loss of 19.56% (ns) after 7 d and 18.70% after 14 d (ns). The comparison with the degradation of the construct in vivo shows that the gel loses mass of 3.73% from 2 to 4 weeks.

3.2. GelMA hydrogels are biocompatible

In order to assess whether GelMA is biocompatible or induces an immune response, the presence of CD68-positive multinuclear giant cells as a sign of severe immunoreaction was determined. Anti-CD68 staining revealed only single mononuclear cells (macrophages) in both experimental groups, and no multinuclear giant cells were detected. There was no significant difference between the two groups regarding the number of macrophages per mm² (figure 3(E)). The macrophages tended to be located more peripheral in the interface between the newly formed tissue and the GelMA matrix (figures 3(A)–(D)).

Only a few pro-inflammatory (CD86, M1) and anti-inflammatory (CD163, M2) macrophages were detected in the corresponding staining, without a specific distribution pattern or predominance of one subtype (figures 4 and 3(F)). These data suggest that GelMA hydrogels are biocompatible.

3.3. GelMA hydrogels in the AVL model allow connective tissue formation

In order to assess the suitability of GelMA to promote tissue formation in the AVL model, we analyzed based on H&E staining the formation of connective tissue surrounding the AVL (figure 5). The histological evaluation revealed that all AVL were patent (10/10). In addition, most of the GelMA matrix was visually present after both 2 and 4 weeks post-implantation. Quantifying the cross-sections did not show any statistically significant differences between the two time points (29.53 ± 4.76 mm² vs. 33.01 ± 3.13 mm²; n = 5 in each group; ns) (figure 5(E)). In contrast, the amount of newly formed connective tissue was different. An area of 2.59% (0.76 ± 0.12 mm²) and 3.40% (1.09 ± 0.31 mm²) of the construct area was found at 2 and 4 weeks post-implantation, respectively (figure 5(F)).

Notably, we detected in both experimental groups MMP3 expression, a marker for matrix degradation, predominantly located in the transition zone between the newly formed connective tissue and the GelMA matrix as well as in the proximity of the newly formed vessels (figure 6). Taken together, our data indicate that GelMA hydrogels are suitable for in situ TE as they allow the formation of novel tissues.

3.4. GelMA hydrogels allow the formation of vascularized tissue in the AVL model

One major issue in situ TE is the efficient vascularization of the newly formed tissue. Therefore, the explants were analyzed for new vessel formation surrounding the main AVL vessel (figure 7).

Newly formed vessels originating from the AVL were detected in both groups. Statistically, significant more vessels were identified after 4 weeks compared to the 2 weeks group (16 ± 9 vs. 4 ± 7; p ≤ 0.05). Most new vessels were located in the proximity of the AVL with a mean distance of 0.23 mm (figures 7(A) and (B)). The newly formed vessels were positive for α-SMA in the media layer (figures 7(C) and (D)). In addition to that, India ink was only intravascularly located as a sign for certain vessel patency and maturity, respectively. The evaluation of the lumen area of all vessels in the constructs showed no significant difference between 2 and 4 weeks group (0.06 ± 0.04 mm² vs. 0.08 ± 0.06 mm²).

The formation of new vessels was further underlined by an increased expression of pro-angiogenic factors in the vein graft of the explants 4 weeks post-implantation compared to untreated femoral vein grafts explanted from untreated rats. The analysis of the expression of 53 angiogenesis-related proteins after setting the cutoff at 1.5 revealed that 28 proteins were upregulated, 22 were unchanged, and
three were downregulated compared to the control (table 1).

The data is shown as absolute values plotted in a heatmap. The overview shows that the values of vein and loop are clearly clustered into two groups (figure 8), with the vein values being low and the loop values high. However, the individual values partly show a high variability within the groups of vein and loop. It is also possible to identify a clustered protein group where the vein expression values are at a similar level and upregulated to a similar extent after the implantation. This can be observed especially in the cluster starting with metallopeptidase with thrombospondin type 1 motif 1 (ADAMTS1) to serpin F1 (PEDF) (figure 8).

Overall, of the 31 proteins with a cutoff at 1.5, 13 angiogenesis-relevant proteins were significantly upregulated compared to the control group, and one protein was significantly downregulated (table 2). The proteins with significant changes can be divided into different groups. These comprise paracrine acting molecules such as growth factors (HGF and IGFBPs) and cytokines (MCP-1 and SDF-1). Furthermore, extracellular matrix associated proteins (PAI1, THBS2, SPP1, SERPINEF1 and Endostatin) and proteolytic enzymes (ADAMTS1 and CD26) were upregulated in the loop group (table 2).

To validate the formation of new vessels sprouting from the AVL, FITC was perfused through the constructs, which were then analyzed by multiphoton microscopy (figure 9). This technique allowed the detection of vessels with a diameter between 13 and 265 µm, and the analysis showed that the microvascular network was stained with FITC. This data indicates that at least the majority of newly formed vessels originated from the AVL.

Collectively, GelMA hydrogels allow the formation of mature vessels, further underlining their suitability for in situ TE.

4. Discussion

The in situ TE approach is based on the assumption that a temporary microenvironment is created by the resorbable immunomodulatory scaffold, which acts as an instructive road map for endogenous cells to invade and build fresh, living, and usable tissue. It is hypothesized that the scaffold and biocompatible biomaterial offers help for the development of mature
Figure 3. CD68 macrophages staining and quantification. Macrophages were visualized using an anti-CD68 antibody after 2 weeks (A), (B) and 4 weeks (C), (D) implantation time. No multinucleated giant cells were found. The bold arrows indicate the AVL and the asterisks (*) the GelMA hydrogel. Scale bar = 1 mm (A), (C) and 200 µm (B), (D). Macrophages per mm$^2$ after 2 and 4 weeks (E) and M1 and M2 macrophages per cross section after 2 and 4 weeks (F).

tissue and sufficient mechanical properties to withstand hemodynamic loads upon implantation. The biomaterial should slowly degrade over time, eventually leading to a purely biological structure that has the capacity to repair, remodel and expand [1]. Proof-of-concept has been shown in our previous studies in the formation of small-diameter blood vessels and angiogenesis within the AVL using acellular scaffolds such as ADA-GEL and fibrin [10, 29]. In this regard, hydrogels have thereby gained more and more importance. Hydrogels offer the advantage of a scaffold similar to an extracellular matrix with high porosity, a 3D network, and very high water content of at least 95% [30]. Hydrogels offer the possibility of tunable mechanical properties and a high volume to the surface ratio for safe cell encapsulation in biofabrication and TE applications. In addition, biocompatibility can also be enhanced by bioactive peptides, such as RGD motifs, thus leading to better adhesion, proliferation, and migration of encapsulated cells [31, 32]. However, the matrix should be not only biocompatible but also immunocompatible without causing any severe immune reaction. Materials such as ADA-GEL and fibrin have already been tested in this respect, demonstrating good biocompatibility [10, 29]. Fibrin is an interesting biomaterial already used in clinical practice [9, 33, 34]. Although fibrin and ADA-GEL have shown promising material
characteristics with regard to cell transplantation and angiogenesis, the fast biodegradation within 4 weeks displays a major disadvantage [10, 35]. For a long-term application, such as a drug-producing tissue container or bone TE, biomaterials supporting angiogenesis with slow biodegradation (from months to years) seem to be favorable. With regard to these properties, GelMA was investigated in this study as a hydrogel-based biomaterial that has already been tested in vitro for biocompatibility [36–38]. The GelMA crosslinked hydrogel was well tolerated by the animals without any complications. We were able to show that the constructs were stable even after 4 weeks, as indicated by the construct size and weight. The GelMA incubated in PBS showed a slow degradation rate, which reached 18.70% of mass loss after 14 d of incubation. However, by adding 1.75 µg ml$^{-1}$ collagenase degradation rate increased, but still, it was only fully degraded after 14 d of incubation. This is slightly slower than previous results of degradation assays performed with 5% and 7% (w/v) GelMA, which can be explained by the higher percentage of the present gel [21]. Taking the degradation rate of GelMA in vitro into account the degradation with 3.73% from 2 to 4 weeks is more similar to the degradation rate of GelMA in vivo in PBS. It must be taken into consideration that the implantation of the gel in vivo is a closed system of the chamber with a lid. The only direct interaction between the matrix and the organism is achieved by means of the AVL. The AVL does not seem to deliver degradation products such as collagenase to the gel to the same extent as in the performed in vitro study. Therefore, it can be assumed that the degradation of the matrix is equal to new tissue formation in vivo. Comparing the cross-sectional area as a surrogate parameter for biodegradation, GelMA displayed very slow biodegradation. In former AVL studies using ADA-GEL or fibrin, significantly smaller cross-sectional areas were assessed with 14.6 or 5 mm$^2$, respectively [10, 39]. Especially considering that there was a substantial 75% reduction in construct size with fibrin (20 mm$^2$ after 2 weeks and 5 mm$^2$ after 4) [39]. Besides the well-known MMPs, like MMP1 and MMP2, MMP3 is also involved in the degradation of gelatin [40]. Using immune-histology, we were able to demonstrate MMP3 was involved in the biodegradation of GelMA. With increasing implantation time, we found an accumulation of MMP3 in the layer between GelMA and connective tissue. The slow biodegradation is a positive aspect of this hydrogel, especially with regard to long-term stability, which was demonstrated by the very low weight reduction.

Figure 4. Immuno-histochemical staining of pro- (M1) (A), (C) and anti-inflammatory (M2) (B), (D) macrophages. Pro-inflammatory (M1) macrophages were visualized using an anti-CD86 antibody (A), (C). To stain the anti-inflammatory (M2) macrophages, an anti-CD163 antibody was used (B), (D). Only sporadic M1 (A) and M2 (B) macrophages were found after 2 weeks as well as after 4 weeks (C), (D). Scale bar = 50 µm (A), (C) and 200 µm (B), (D).
of the constructs (0.65 ± 0.04 g after 2 weeks vs. 0.63 ± 0.09 g after 4 weeks).

With regard to angiogenesis, we found newly formed vessels originating from the AVL in both experimental groups. The number of newly formed vessels increased over time from 4 ± 7 per histological cross-section after 2 weeks to 16 ± 9 after 4 weeks. In comparison with other materials, such as recombinant electrospun spider silk or fibrin, with more newly formed blood vessels after 4 weeks (99 ± 52 and 107 ± 25), it is noticeable that the proportion was significantly lower in the GelMA [25, 39]. In this respect, GelMA is more comparable with ADA-GEL with 28 ± 5 vs. 31 ± 24 newly formed blood vessels [10]. Nevertheless, the proangiogenic properties of the material have been shown in the production of proangiogenic signal molecules. Proteins with an influence on early stages of vascularization, such as serpin E1 (21.69-fold), osteopontin (8.03-fold), insulin-like growth factor binding proteins (IGFBP), and fibroblast growth factor (FGF) were significantly upregulated after 4 weeks [41–44]. Consistent with the accumulation of macrophages in the vascularized tissue parts, a significant upregulation of the signal molecule monocyte chemoattractant protein 1 (MCP-1) of 36.74 was detected [45]. In accordance
Figure 6. Matrix degradation. Detailed view at H&E (A), (C) and MMP3 staining (B), (D) images of the same location in the explants, showing an accumulation of MMP3 after 2 (B) and 4 weeks (D), especially at the interface between connective tissue and GelMA matrix. The asterisks (*) indicate the GelMA matrix. Scale bar = 50 µm (A)–(D).

Figure 7. Vascularization and vessel distribution (A), (B) and α-SMA staining (C), (D). The number of vessels showed a significant difference from 2 to 4 weeks (A), whereas the vessel distance was similar after 2 and 4 weeks (B). α-SMA staining revealed newly formed vessels in the connective tissue surrounding the AVL vessels with a defined lumen after 2 weeks (C) and an increased number of newly built vessels after 4 weeks (D). Statistically, significant differences are indicated for *p ≤ 0.05.
Table 1. Proteome analysis of angiogenesis regulated proteins in untreated veins and in the AVL after 4 weeks implantation time.

| Protein                  | Mean vein | Mean loop | Fold induction | P value |
|--------------------------|-----------|-----------|----------------|---------|
| MCP-1 Monocyte chemoattractant protein 1 | 112.13    | 4119.25   | 36.74          | 0.03    |
| PAI1 Serpin E1           | 210.00    | 4554.38   | 21.69          | 0.03    |
| HGF Hepatocyte growth factor | 90.63    | 1557.38   | 17.18          | 0.03    |
| THBS2 Thrombospondin-2   | 997.88    | 16634.84  | 16.67          | 0.03    |
| IGFBP-10 Insulin-like growth factor binding protein 10 | 149.63 | 2160.25   | 14.44          | 0.03    |
| SPP1 Osteopontin         | 232.13    | 1864.25   | 8.03           | 0.03    |
| IGFBP-2 Insulin-like growth factor binding protein 2 | 264.63 | 1560.63   | 5.90           | 0.03    |
| ADAMTS1 Metalloprotease with thrombospondin type 1 motif 1 | 147.63  | 848.88    | 5.75           | 0.03    |
| PTX3 Pentraxin-3         | 64.88     | 298.00    | 4.59           | 0.06    |
| VEGF Vascular endothelial growth factor | 42.38     | 174.25    | 4.11           | 0.06    |
| CD 26 Dipeptidyl peptidase 4 | 100.38   | 385.13    | 3.84           | 0.03    |
| IGFBP-9 Insulin-like growth factor binding protein 9 | 3207.63 | 11515.33  | 3.48           | 0.03    |
| PEDF Serpin F1           | 2157.25   | 5545.63   | 2.57           | 0.03    |
| Endostatin               | 978.38    | 2157.13   | 2.20           | 0.03    |
| MIP-1 alpha Macrophage inflammatory protein 1 alpha | 105.75  | 231.75    | 2.19           | 0.06    |
| PIGF-2 Placental growth factor 2 | 144.13    | 315.75    | 2.19           | 0.06    |
| FGF-7/KGF Fibroblast growth factor 7/keratinocyte growth factor | 177.38 | 383.38    | 2.16           | 0.06    |
| IGFBP-1 Insulin-like growth factor binding protein 1 | 139.00 | 282.50    | 2.03           | 0.03    |
| TIMP-4 Metalloprotease Inhibitor 4 | 81.88 | 157.25    | 1.92           | 0.06    |
| PDGF-AA Platelet-derived growth factor AA | 322.50 | 598.50    | 1.86           | 0.19    |
| EDN1 Endothelin-1        | 1475.38   | 2641.25   | 1.79           | 0.06    |
| DLL4 Delta-like canonical notch ligand 4 | 120.20 | 213.25    | 1.77           | 0.19    |
| LEP Leptin               | 92.00     | 160.63    | 1.75           | 0.11    |
| CXCL16 C-X-C motif chemokine ligand 16 | 104.75 | 176.88    | 1.69           | 0.11    |
| FGF acidic Fibroblast growth factor acidic | 1785.88 | 2979.63 | 1.67           | 0.11    |
| IL-10 Interleukin 10     | 139.88    | 229.88    | 1.64           | 0.11    |
| CD 105 Endoglin          | 63.88     | 99.38     | 1.56           | 0.11    |
| PDGF-AB/BB Platelet-derived growth factor AB/BB | 142.00 | 216.63   | 1.53           | 0.19    |
| Ang Angiogenin           | 3877.13   | 5629.75   | 1.45           | 0.03    |
| MMP3 Matrix metalloproteinase 3 | 49.38 | 71.00    | 1.44           | 0.19    |
| MMP9 (pro and active)    | 189.38    | 252.88    | 1.34           | 0.67    |
| IGFBP-3 Insulin-like growth factor binding protein 3 | 5781.00 | 7665.50 | 1.33          | 0.31    |
| AREG Amphiregulin        | 90.63     | 113.00    | 1.25           | 0.31    |
| CX3CL1 Fractalkine       | 109.63    | 126.50    | 1.15           | 0.47    |
| MMP8 (pro form) Matrix metalloproteinase 8 (pro form) | 389.25 | 445.38    | 1.14           | 0.47    |
| PD-ECGF Platelet-derived endothelial cell growth factor | 199.13 | 227.25    | 1.14           | 0.89    |
| VEGF beta Vascular endothelial growth factor beta | 78.88     | 82.13     | 1.04           | 0.89    |
| FGF basic Fibroblast growth factor basic | 1123.00 | 1162.13  | 1.03           | 0.89    |
| KC Keratinoctyes-derived chemokine | 62.00     | 62.13     | 1.00           | 0.00    |
| ANG3 Angiopoietin-3      | 201.25    | 195.25    | -1.03          | 0.67    |
| PRL Prolactin            | 237.00    | 223.13    | -1.06          | 0.89    |
| IL-1 beta Interleukin 1 beta | 104.63   | 97.63     | -1.07          | 0.89    |
| HBB-EGF Heparin binding epidermal growth factor-like growth factor | 139.63 | 124.88 | -1.12         | 0.89    |
| IL-1 alpha Interleukin 1 alpha | 146.63    | 131.13    | -1.12          | 0.89    |
| PF4 Platelet Factor 4    | 1584.25   | 1416.00   | -1.12          | 0.31    |

(Continued.)
Table 1. (Continued.)

| Protein                      | Mean vein | Mean loop | Fold induction | P value |
|------------------------------|-----------|-----------|----------------|---------|
| ANGPT1 Angiopoietin-1        | 328.75    | 292.25    | -1.12          | 1.00    |
| IP-10 Interferon-gamma induced protein 10 | 134.38    | 116.88    | -1.15          | 0.89    |
| GM-CSF Granulocyte macrophage-colony stimulating factor | 58.13     | 47.38     | -1.23          | 0.31    |
| F3 Coagulation Factor III    | 493.25    | 336.25    | -1.47          | 0.31    |
| PLF Proliferin               | 153.25    | 102.13    | -1.50          | 0.11    |
| SDF-1 Stromal cell-derived factor 1 | 8224.38   | 5015.13   | -1.64          | 0.03    |
| TIMP-1 Tissue inhibitors of metalloproteinase 1 | 446.38    | 230.63    | -1.94          | 0.06    |
| EGF Epidermal growth factor  | 106.25    | 49.50     | -2.15          | 0.11    |

Figure 8. Heatmap of the standardized expression values for the 53 assayed proteins of vein and loop samples. The upper dendrogram shows the hierarchical clustering of the overall values of the vein (blue) and loop (red). The color-coding of the single expression values in the left dendrogram goes from low values in blue via white to red for high values, also hierarchically clustered.

with the hypothesis that angiogenesis is flow-induced in the AVL, proteomic analysis revealed hepatocyte growth factor (HGF) as a flow-dependent molecule being strongly upregulated (17.18-fold) after 4 weeks [46, 47]. Interestingly, no statistical change considering the VEGF expression was found in the AVL after 4 weeks compared to the venous graft. In this context, Henn et al [48] demonstrated a continuous decrease in VEGF expression in the AVL from 4.11 (post-op day 5) to 2.71 (post-op day 15) in a miRNA microarray analysis and from 3.45 to 2.32 in a qPCR analysis. In agreement with previous AVL studies demonstrating that neovascular sprouting occurs between day 10 and day 14, it is likely that VEGF plays
Table 2. Proteome analysis of angiogenesis regulated proteins with statistically significant changes in the AVL after 4 weeks implantation time.

| Protein                           | Vein     | Loop     | Fold induction | P value |
|-----------------------------------|----------|----------|----------------|---------|
| MCP-1 Monocyte chemoattractant protein 1 | 112.125  | 4119.25  | 36.74          | 0.03    |
| PAI1 Serpin E1                    | 210      | 4554.375 | 21.69          | 0.03    |
| HGF Hepatocyte growth factor      | 90.625   | 1557.375 | 17.18          | 0.03    |
| THBS2 Thrombospondin-2            | 997.875  | 16634.875| 16.67          | 0.03    |
| IGFBP-10 Insulin-like growth factor binding protein 10 | 149.625  | 2160.25  | 14.44          | 0.03    |
| SPP1 Osteopontin                  | 232.125  | 1864.25  | 8.03           | 0.03    |
| IGFBP-2 Insulin-like growth factor binding protein 2 | 264.625  | 1560.625 | 5.90           | 0.03    |
| ADAMTS1 Metalloprotease with thrombospondin type 1 motif 1 | 147.625  | 848.875  | 5.75           | 0.03    |
| CD 26 Dipeptidyl peptidase 4      | 100.375  | 385.125  | 3.84           | 0.03    |
| IGFBP-9 Insulin-like growth factor binding protein 9 | 3207.625 | 11550.125| 3.48           | 0.03    |
| PEDF Serpin F1                    | 2157.25  | 5545.625 | 2.57           | 0.03    |
| Endostatin                        | 978.375  | 2157.125 | 2.20           | 0.03    |
| IGFBP-1 Insulin-like growth factor binding protein 1 | 139      | 282.5    | 2.03           | 0.03    |
| Ang Angiogenin                    | 3877.125 | 5629.75  | 1.45           | 0.03    |
| SDF-1 Stromal cell-derived factor 1 | 8224.375 | 5015.125 | −1.50          | 0.03    |

Figure 9. Multiphoton microscopy. A 3D reconstruction of an optically cleared whole construct is shown in (A). The construct was explanted after 4 weeks and stained with FITC perfusion. The reconstruction is created from a stitched 3D mosaic with a voxel size of $2.2 \times 2.2 \times 8 \mu m^3$ and fields of view of 1.1 mm. FITC fluorescence is shown in green (525/50 nm), and sample autofluorescence is shown in blue (450/70 nm). The initial blood vessel (marked by an arrow) sprouts several smaller and microvessels along its path through the construct. The density of sprouted vessels appears higher near the blood entrance and exit points of the construct. The magnified 3D volumes (B) and (C) show that both larger vessels and capillaries are formed in the construct. Scale bar = 1 mm (A) and 400 μm (B), (C).
an important role in the first 2 weeks [29, 39]. Among the downregulated proteins, there is stromal cell-derived factor 1 (SDF-1), tissue inhibitor of metalloproteinase 1 (TIMP-1), and epidermal growth factor (EGF). TIMPs are known for their ability to inhibit MMPs, which in turn are responsible for the degradation of various extracellular matrix proteins [49]. EGF is a protein that, in combination with insulin-like growth factor-II, can contribute to increased angiogenesis in vivo [50]. The significantly downregulated SDF-1 (−1.64-fold) is a chemokine that plays a role in regeneration and angiogenesis in a paracrine manner. In hypoxia, the expression of SDF-1 is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1), which promotes the migration of cells in ischemic tissue [51]. The heatmap shows a good overview of the expression of the proteins, and it can be seen that the cluster with similar low values in the vein group and high values in the loop group are the proteins that have a significant change. For most of the other proteins, however, it can be observed that they show high variability. This variability may be due to the fact that the samples are from different individuals, and it cannot be ruled out that the animals react differently and that therefore different results are obtained in the proteome analysis. In future studies, it may be appropriate to focus on the proteins that are significantly different and investigate them in further detail. Multiphoton microscopy was used to visualize the AVL and neovessel formation originating from the latter one. Vessels with a diameter ranging from 13 µm to 265 µm were recorded with this technique. This methodology is new in the field of AVL studies and in situ TE and represents an advancement to describe tissue formation and vascularization more precisely. Using immune-histology, bioartificial tissues generated in the AVL model can be visualized, and the proximity to the newly formed vessels might be investigated. Also, the migration of previously fluorescence-labeled (e.g. GFP) cells through the scaffold towards the vascularized parts can be described.

In summary, with GelMA, we have found a biocompatible matrix suitable for long-term in vivo use due to its stability and due to its immunomodulatory properties allowing for the recruitment of host cells and angiogenesis within the 3D construct. Another interesting approach might be to modify GelMA to promote angiogenesis further [21]. This can be performed using a lower concentration of GelMA as well as 3D bioprinting microchannels within the construct to enhance the perfusion of the nutrition and oxygen supply for earlier vascularization.

5. Conclusion

GelMA was successfully implanted in the rat AVL model. The constructs were found to be stable for 4 weeks. The degradation of GelMA is suitable for long-term implantation and in situ vascularization. The constructs could be examined by multiphoton microscopy with regard to angiogenesis, and the angiogenic potential could be demonstrated by proteomic analysis. In summary, GelMA offers a good approach for further in vivo implantations and in situ vascularization in the AVL model.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Project Number 326998133–TRR 225 (subprojects B03, B08, C01, and C04). Furthermore, we thank Fulvia Ferrazzi for her help with the heatmap and the Manfred-Roth-Stiftung and the Forschungsförderung Medizin Universitätsklinikum Erlangen for their continuous support of research in regenerative medicine.

Conflict of interest

No competing financial interests exist.

ORCID iDs

Stefanie Heltmann-Meyer  https://orcid.org/0000-0002-3097-854X
Dominik Steiner  https://orcid.org/0000-0002-1904-5925
Dominik Schneidereit  https://orcid.org/0000-0001-7351-6428
Sahar Salehi  https://orcid.org/0000-0002-6740-4195

References

[1] Wissing T B, Bonito V, Bouten C V C and Smits A 2017 Biomaterial-driven in situ cardiovascular tissue engineering-a multi-disciplinary perspective npj Regen Med. 2 18
[2] Gaharwar A K, Singh I and Khademhosseini A 2020 Engineered biomaterials for in situ tissue regeneration Nat. Rev. Mater. 5 686–705
[3] Winkler T, Sass F A, Duda G N and Schmidt-Bleek K 2018 A review of biomaterials in bone defect healing, remaining shortcomings and future opportunities for bone tissue engineering: the unsolved challenge Bone Joint Res. 7 232–43
[4] Mutschall H, Winkler S, Weisbach V, Arkudas A, Horch R E and Steiner D 2020 Bone tissue engineering using adipose-derived stem cells and endothelial cells: effects of the cell ratio J. Cell. Mol. Med. 24 7034–43
[5] Winkler S et al 2020 Human umbilical vein endothelial cell support bone formation of adipose-derived stem cell-loaded and 3D-printed osteogenic matrices in the arteriovenous loop model Tissue Eng. A 27 413–423
vasculogenesis/angiogenesis in human lung development

[44] Murakami M and Simons M 2008 Fibroblast growth factor regulation of neovascularization *Curr. Opin. Hematol.* 15 215–20

[45] Ito W D, Arras M, Winkler B, Scholz D, Schaper J and Schaper W 1997 Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion *Circ. Res.* 80 829–37

[46] Taniyama Y, Morishita R, Hiraoka K, Aoki M, Nakagami H, Yamasaki K, Matsumoto K, Nakamura T, Kaneda Y and Ogihara T 2001 Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat diabetic hind limb ischemia model: molecular mechanisms of delayed angiogenesis in diabetes *Circulation* 104 2344–50

[47] Schmidt V J, Hilgert J G, Covi J M, Leibig N, Wietbrock J O, Arkudas A, Polykandriotis E, De Wit C, Horch R E and Kneser U 2015 Flow increase is decisive to initiate angiogenesis in veins exposed to altered hemodynamics *PLoS One* 10 e0117407–e

[48] Henn D *et al* 2019 MicroRNA-regulated pathways of flow-stimulated angiogenesis and vascular remodeling in *in vivo* *J. Transl. Med.* 17 22

[49] Jiang B, Liu J and Lee M H 2019 Targeting a designer TIMP-1 to the cell surface for effective MT1-MMP inhibition: a potential role for the prion protein in renal carcinoma therapy *Molecules* 24 235

[50] Lee Y M *et al* 2004 Synergistic induction of *in vivo* angiogenesis by the combination of insulin-like growth factor-II and epidermal growth factor *Oncol. Rep.* 12 843–8

[51] Ceradini D J, Kulkarni A R, Callaghan M J, Tepper O M, Bastidas N, Kleinman M E, Capla J M, Galiano R D, Levine J P and Gurtner G C 2004 Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1 *Nat. Med.* 10 858–64