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Research Paper

Bio-inspired green light crosslinked alginate-heparin hydrogels support HUVEC tube formation

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ABSTRACT

Alginate is a polysaccharide which forms hydrogels via ionic and/or covalent crosslinking. The goal was to develop a material with suitable, physiologically relevant mechanical properties and biological impact for use in wound treatment. To determine if the novel material can initiate tube formation on its own, without the dependance on the addition of growth factors, heparin and/or arginyl-glycyl-aspartic acid (RGD) was covalently conjugated onto the alginate backbone. Herein, cell adhesion motifs and bioactive functional groups were incorporated covalently within alginate hydrogels to study the: 1) impact of crosslinked heparin on tubular network formation, 2) impact of RGD conjugation, and the 3) biological effect of vascular endothelial growth factor (VEGF) loading on cellular response. We investigated the structure-properties-function relationship and determined the viscoelastic and burst properties of the hydrogels most applicable for use as a healing cell and tissue adhesive material. Methacrylation of alginate and heparin hydroxyl groups respectively enabled free-radical covalent inter- and intra-molecular photo-crosslinking when exposed to visible green light in the presence of photo-initiators; the shear moduli indicate mechanical properties comparable to clinical standards. RGD was conjugated via carbodiimide chemistry at the alginate carboxyl groups. The adhesive and mechanical properties of alginate and alginate-heparin hydrogels were determined via burst pressure testing and rheology. Higher burst pressure and material failure at rupture imply physical tissue adhesion, advantageous for a tissue sealant healing material. After hydrogel formation, human umbilical vein endothelial cells (HUVECs) were seeded onto the alginate-based hydrogels; cytotoxicity, total protein content, and tubular network formation were assessed. Burst pressure results indicate that the cell responsive hydrogels adhere to collagen substrates and exhibit increased strength under high pressures. Furthermore, the results show that the green light crosslinked alginate-heparin maintained cell adhesion and promoted tubular formation.

1. Introduction

Injury to organs and tissues, either the result of acute trauma or an underlying medical condition, can be fatal if not properly treated. One such example of a traumatic injury is damage to dynamic, vascularized tissue (Meredith and Hoth, 2007; Pawloski and Broaddus, 2010; Trump and Gohar, 2013; Harrison, 2014; Zarogoulidis et al., 2014; Huang et al., 2014). While various natural (Dunn and Goa, 1999; Ono et al., 2001; Fabian et al., 2003; Tansley et al., 2006; Nakajima et al., 2007; Matsutani and Ozeki, 2011) and synthetic (Ranger et al., 1997; Macchiarini et al., 1999; Wallace et al., 2001; Kobayashi et al., 2001; Porte et al., 2001; Campbell et al., 2005; Pedersen et al., 2012) materials have been investigated, many of these fall short in properly addressing the mechanical environment, tissue adhesion, *and* the support of a vascular network. It is imperative in the design of a biomaterial to consider the dynamic mechanical properties of the underlying tissue and recapitulate those properties as closely as possible through an understanding of the structure-properties-function relationship. Indeed, mismatched material and mechanical properties can damage the underlying tissue and increase the chance for failure (Au - Liu and Au - Tschumperlin, 2011). Thus, optimization of material properties to perform in a physiologically relevant manner is often the next step.

Alginate is a hydrophilic polysaccharide derived from brown algae, and forms hydrogels with tunable mechanical and chemical properties *in*

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situ through various crosslinking techniques, providing a myriad of properties for biomedical applications (Alsberg et al., 2001; Lee and Mooney, 2001; Kang et al., 2020; Wagner et al., 2014a, 2014b). Traditional alginate hydrogels are ionically-crosslinked, a structure formation which is reversible in the presence of ionic solutions and affects the material structure and thus the properties *in vivo*. Alginate can also be chemically modified to enable secondary and covalent bonding, creating multi-stimuli responsive materials (Trujillo et al., 2021). In particular, the methacrylation of alginate at the hydroxyl groups imparts a functional group capable of light-activated crosslinking; in this manner, the carboxyl groups remain free to form ionic crosslinks in the presence of divalent cations such as Ca^{2+} (Fenn et al., 2016; Etter et al., 2018; Etter and Oldinski, 2018; Jeon et al., 2009). Photo-crosslinking alginate to form a more structurally sound alginate hydrogel via covalent bonding can employ either ultraviolet (UV) or visible green light activation. UV light is high intensity and can further harm tissue, encapsulated mammalian cells, or biomolecules (Tornaletti and Pfeifer, 1996). A less damaging photo-crosslinking approach is a visible green light photo-initiator system. For additional control over alginate hydrogel mechanical properties, both visible light and UV-activated photo--crosslinking can be used together to create a dual-crosslinked network, as we have shown in our previous publications (Fenn et al., 2016; Etter and Oldinski, 2018).

Despite these beneficial characteristics, alginate inherently lacks cell adhesion motifs and is relatively bioinert. To fill this gap, cell adhesive biomolecules have been conjugated covalently onto the alginate backbone chain resulting in bioactive hydrogels ideal for tissue healing or tissue engineering (Lee and Mooney, 2012). Arginyl-glycyl-aspartic acid (RGD) conjugation, a ligand that promotes cell adhesion, significantly increases cell proliferation and migration (Ruoslahti and Pierschbacher, 1987; Alsberg et al., 2002). The incorporation of growth factors, such as vascular endothelial growth factor (VEGF), into wound dressings has been investigated to promote angiogenesis and vascularization *in situ* to enhance regeneration of damaged vascularized tissues via the development of new three-dimensional (3D) blood vessels (Nagy et al., 2007). Heparin, a glycosaminoglycan found in the extracellular matrix (ECM) of blood vessels, promotes the growth of endothelial cells *in vitro* and binds to several growth factors including VEGF, transforming growth factor (TGF), and fibroblast growth factor (FGF) families (Klagsbrun, 1990; Lyon et al., 1997; Zhang et al., 2020). A polyethylene glycol (PEG)-heparin hydrogel was shown to provide sustained delivery of VEGF release *in vitro* in tubular-shaped human umbilical vein endothelial cells (HUVECs) through affinity bonding and increased angiogenesis *in vivo* in a subcutaneous mouse implant model; however, this material lacked RGD complexes (Tae et al., 2006; Héroult et al., 2004). Heparin-PEG hydrogels have been produced to enhance protein absorption and cell adhesion as well (Benoit and Anseth, 2005; Benoit et al., 2007). Heparin has been conjugated onto alginate to promote growth factor retention or to interact with the ECM (Jeon et al., 2011). Direct conjugation of heparin onto alginate and the subsequent crosslinking of alginate to form heparin-alginate hydrogels has been achieved via methacrylation chemistry (Samorezov et al., 2015) however, heparin itself was not modified in such a way to create a heparin interpenetrating network.

Herein, we investigate the hypothesis that a visible light crosslinked alginate-based hydrogel can support shear and multi-axial loads and *in vitro* tubular network formation via the addition of covalently conjugated RGD and heparin. In the literature, it is unclear how an interpenetrated/co-polymer alginate-heparin hydrogel will perform with HUVECs and what the influence on cellular behavior will be in the presence of RGD and VEGF. Visible light crosslinking was achieved via methacrylation of the alginate and heparin polymers, respectively, imparting a functional group capable of covalent crosslinking by free-radical polymerization in the presence of a photo-initiator *in vitro*. The material properties and burst pressure mechanics were evaluated to determine the structural integrity of the hydrogels for potential applications as dynamic tissue healing biomaterials. The promotion of tube formation was evaluated by directly seeding HUVECs onto photo-crosslinked alginate-RGD-heparin hydrogels with and without encapsulated VEGF. HUVECs are often used to investigate the effect of RGD and heparin biomaterial properties and provide insight into the regulation of subsequent formation of blood vessels, advantageous for tissue and wound healing; tube formation assays are used as a model for studying endothelial tube formation by angiogenic agents/materials.

2. Materials and methods

2.1. Materials

Sodium alginate (Manugel®, $MW_v \approx 170–240$ kDa) was purchased from FMC Biopolymer. Phosphate buffered saline (PBS), methacrylic anhydride, sodium hydroxide (NaOH), N-ethyl-N′(3-dimethylaminopropyl) carbodiimide hydrochloric acid (EDC), N-hydroxysuccinimide (NHS), sodium heparin, glycidyl methacrylate, eosin Y, triethanolamine 1-vinyl-2-pyrrolidinone, and Triton X-100 were purchased from Sigma-Aldrich. Cysteine-L-arginyl–glycyl-L-aspartic acid (cRGD) was purchased from Genscript. VEGF-A was purified and provided by a collaborator (Miao et al., 2014). A WST-8 Cell Proliferation Assay (cataglog #KA1385) was purchased from Abnova. HUVECs and vascular endothelial cell culture medium were purchased from ATCC (ATCC® CRL-1730™, ATCC® PCS-100-030™). A Pierce Protein Assay Kit (catalog #23225), phosphate buffered saline (PBS), and bovine serum albumin (BSA) were purchased from ThermoFisher.

2.2. Synthesis of methacrylated alginate (Alg-MA) and heparin (Hep-GM)

Methacrylated alginate (Alg-MA) was synthesized as described in the literature (Fenn et al., 2016; Charron et al., 2016). Briefly, sodium alginate was dissolved in PBS (pH = 7.4) to create a 1% (w/v) solution at room temperature. A 10-fold molar excess of methacrylic anhydride was added to the alginate solution. The pH of the solution was periodically adjusted to 8.5, using 5N NaOH, and the methacrylation reaction was conducted for 24 h at room temperature. The final pH was adjusted to 7, and the Alg-MA solution was purified via dialysis (MWCO = 6–8 kDa) against deionized (DI) water for three days. The solution was frozen and lyophilized to obtain a dry polymer and stored at −20 °C until use.

Methacrylated heparin (Hep-GM) was synthesized by dissolving sodium heparin, in DI water (2%, w/v) at room temperature. Glycidyl methacrylate (GM) was added to the solution (1g GM:1g heparin), the pH was adjusted to 8.5 using 2N NaOH, and reacted overnight in an oil bath at 50 °C. The product, Hep-GM, was precipitated in cold ethanol and dialyzed for three days. The solution was frozen and lyophilized to obtain a dry polymer and stored at −20 °C until use.

2.3. Alginate-MA-RGD conjugation

Alg-MA was placed in DI water (1%, w/v) at room temperature. EDC was added to the Alg-MA solution while mixing for 30 min at room temperature, followed by the addition of NHS. The COOH:EDC:NHS molar ratio remained consistent (1:8:3.2) during the carbodiimide reactions, where COOH refers to the moles of alginate carboxyl groups (Miao et al., 2014). cRGD was thawed to room temperature and used as is from the supplier; cRGD was added to the functionalized Alg-MA solution. The carbodiimide reaction was conducted for 5 h at room temperature. The product, Alg-MA-RGD, was dialyzed against DI water for three days. The solution was frozen and lyophilized to obtain a dry polymer and stored at −20 °C until use.

2.4. Green light crosslinking and physico-mechanical testing

Covalent crosslinking between Alg-MA and Hep-GM polymer chains

was achieved utilizing visible green light as described in our previous work (Charron et al., 2016; Fenn and Oldinski, 2016). Briefly, polymer precursor solutions (3%, w/v) were prepared in DI water. Control and blended solutions were then mixed with photo-initiator to obtain the following concentrations: 1 mM eosin Y (photo-sensitizer), 125 mM triethanolamine (TEOA, photo-initiator), and 20 mM 1-vinyl-2-pyrrolidinone (1VP, catalyst). Fenn et al. previously published UV–Vis absorbance spectra indicating a maximum absorbance at 530 nm (Fenn and Oldinski, 2016). The polymer solutions were exposed to green light using a custom light set-up (525 nm, SuperBrightLEDs). Photo-crosslinked alginate-based hydrogels were made by injecting 3% (w/v) alginate-based solutions with eosin Y, TEOA, and 1VP in a custom mold and exposing to green light for 5 min. Discs of uniform size were then generated using a 6-mm diameter biopsy punch from the hydrogel sheets for use in cell studies.

Rheometry was performed using an AR2000 rheometer (TA Instruments) equipped with a Peltier plate to determine the shear material properties during and after photo-crosslinking. All tests were performed at 37 °C using a 20-mm diameter 1°59′6″ steel cone geometry with a truncation gap of 57 μm. The solutions were crosslinked utilizing the custom LED ring for 5 min while oscillatory time sweeps were performed at 10% radial strain and 1 Hz. Shear storage (G′) and loss (G″) moduli were calculated after crosslinking, and gelation points were determined using TA Data Analysis software.

ASTM F2392-04 was modified to create a custom-built burst pressure testing device, as described and depicted in the literature, to assess the integrity and performance of the tissue sealant substrates *in vitro* (Charron et al., 2016; Jalalvandi et al., 2019). Collagen-rich substrates (Collagen Casings, The Sausage Maker Inc.), hydrated in PBS at 37 °C for at least 30 min prior to testing, were used as a test membrane. Pressures were recorded digitally using a USB-connected pressure transducer (Omega PX-409-030AUSBH) attached to the burst pressure testing device through a side NPT-port. To verify that each substrate was free from defects and leaks, the substrate was clamped down in the burst pressure device and pressurized with air to a baseline of 3 kPa (normal physiologic lung pressure), at an infusion rate of 75 mL/h, and held briefly ensuring no change in pressure (i.e., no leaking occurred). Next the substrate was removed from the device and a 1.5-mm diameter defect was created in the substrate using a biopsy punch. Over these defects, alginate-based hydrogel precursor solutions were applied to the substrate and then photo-crosslinked for 5 min using a custom LED array. Once cured, the now-sealed collagen-based substrate was returned to the burst-pressure testing device and pressurized with air at an infusion rate of 75 mL/h until delamination or failure of the sealant material was observed. Samples without RGD and VEGF were tested to determine if the mechanical property decreased or increased with heparin modification compared to the bulk alginate materials. Burst pressure was calculated as the ultimate amount of pressure required to cause failure (either delamination or material failure) of the sealant.

2.5. Hydrogel formation and in vitro characterization

Hydrogel precursor solutions were prepared in DI water (3%, w/v). Human VEGF was added to the solutions at a ratio of 10^6:1 (polymer: VEGF). Solutions were blended with photo-initiator (1 mM eosin Y, 125 mM TEOA, 20 mM 1VP), injected into 96-well tissue culture treated plates (0.05 mL/well), and polymerized with visible green light for 5 min at room temperature. Crosslinking was achieved using a custom LED array (*vide supra*). Hydrogels without VEGF were also formed.

HUVECs were thawed and seeded (passage 4) at a density of 1,000 cells/well in 100 μL of complete vascular cell medium (0.2% bovine brain extract, 5 ng/mL recombinant human endothelial growth factor, 10 nM L-glutamine, 0.75 units/mL heparin sulfate, 1 μg/mL hydrocortisone, 50 μg/mL asorbic acid, 2% FBS) directly onto the green light crosslinked hydrogels (Table 1) in a 96-well tissue culture polystyrene plate. The mitochondrial activity of HUVECs was analyzed using a WST-8 Cell Proliferation Assay according to the manufacturers protocol. After 24 h of culture (37 °C, 5% CO_2) the optical density was measured at 450 nm using a plate reader (Biotek Synergy H1). The absorbance values for the cells grown on the hydrogel samples were normalized to hydrogel materials suspended in complete vascular cell medium without cells. HUVEC total protein was measured using a Pierce Protein Assay Kit after 6, 12, 24, and 72 h of incubation on the alginate-based hydrogels. Briefly, in a 96-well plate, BSA solutions with known concentrations ranging from 20 to 2000 μg/mL were used to form a standard curve. Cells were rinsed with PBS then lysed with 100 μL of Triton X-100 lysing solution.; 175 μL of working solution was added to each well containing 25 μL of either the cell lysate sample or BSA standards. The absorbance at 562 nm was measured using a plate reader. The control groups were HUVECs and acellular crosslinked hydrogels in complete vascular cell medium.

Table 1
List of material groups and corresponding group labels. Final polymer hydrogel concentrations were 3% (w/v) for both the single and co-polymer solutions.

Material Group	Abbreviation
Cell Control	Cells
Alg-MA	AM
Alg-MA-RGD	AMR
Alg-MA/Hep-GM	AMH
Ala-MA-RGD/Hep-GM	AMRH

The ability of green light crosslinked alginate-based hydrogels to support tubular network formation and the effects of heparin and RGD conjugation, with and without VEGF, were evaluated qualitatively (Guo et al., 2014). HUVECs were directly seeded onto the green light crosslinked hydrogels in a 96-well tissue culture polystyrene plate. At each time point (6, 12, 24, and 72 h), the 96-well plate was removed from the incubator (37 °C, 5% CO_2) and qualitatively analyzed with phase contrast microscopy to observe and capture the effects on HUVEC tube formation. A total of 12 images were taken at 20x and representative images from four samples were selected; the same materials were imaged at each time point, thereby looking at the effect on the same material over time. Quantitative analysis was not within the scope of the project.

2.6. Statistical analysis

The mean and standard deviation for each sample group in the mechanical analysis and quantitative cell assays were calculated. Shear moduli ($n = 3$) values were selected at a constant time-point ($t = 330s$) and the mean was calculated to allow statistical comparison of moduli values between groups. Burst pressure ($n = 4$) values were determined by averaging the pressure at failure within each group. After the mean absorbance values were calculated for the cellular assays ($n = 4$), the values were normalized to the mean absorbance value of the positive control group which contained untreated cells and culture media. The respective standard deviations associated with this normalization, the propagation of error, was calculated as a combination of the standard deviation of the respective sample and the positive control. ANOVA was performed to determine the statistical significance between all groups for each characterization technique. Additional statistical testing in the form of T-tests (two-tailed distribution assuming unequal variance) was performed to determine the statistical significance ($p < 0.05$) between sample groups.

3. Results and discussion

3.1. Alginate/heparin modification and hydrogel formation

The methacrylation of alginate and heparin, respectively, were

performed to enable covalent photo-crosslinking between adjacent alginate chains to form AM and AMR hydrogels, or between alginate and heparin chains forming crosslinked copolymers AMH and AMRH (Table 1). The methacrylate groups were conjugated at the hydroxyl groups on the alginate backbone, providing an additional functional site capable of covalent crosslinking; thus, the carboxylic acid remains available for ionic crosslinking between alginate backbones as well (Fig.1A,C). As a result of RGD conjugation onto alginate, and the ability to covalently crosslink alginate and heparin together, RGD can be incorporated into a hydrogel formulation. To further illustrate the difference in chemical structure and bioactivity, a schematic detailing the macrostructures of the four different alginate-based hydrogels is shown in Fig. 1G. The creation of a hydrogel that includes crosslinks between alginate chains, heparin chains, or alginate and heparin is unique within the literature, and provides a different structure to the materials (Goldberg et al., 2021). In previous studies of photo-crosslinked alginate-heparin hydrogels, heparin was not modified to enable crosslinking with itself (Jeon et al., 2011). Our novel system provides more flexibility in controlling material properties through the use of inter- and intra-molecular crosslinks.

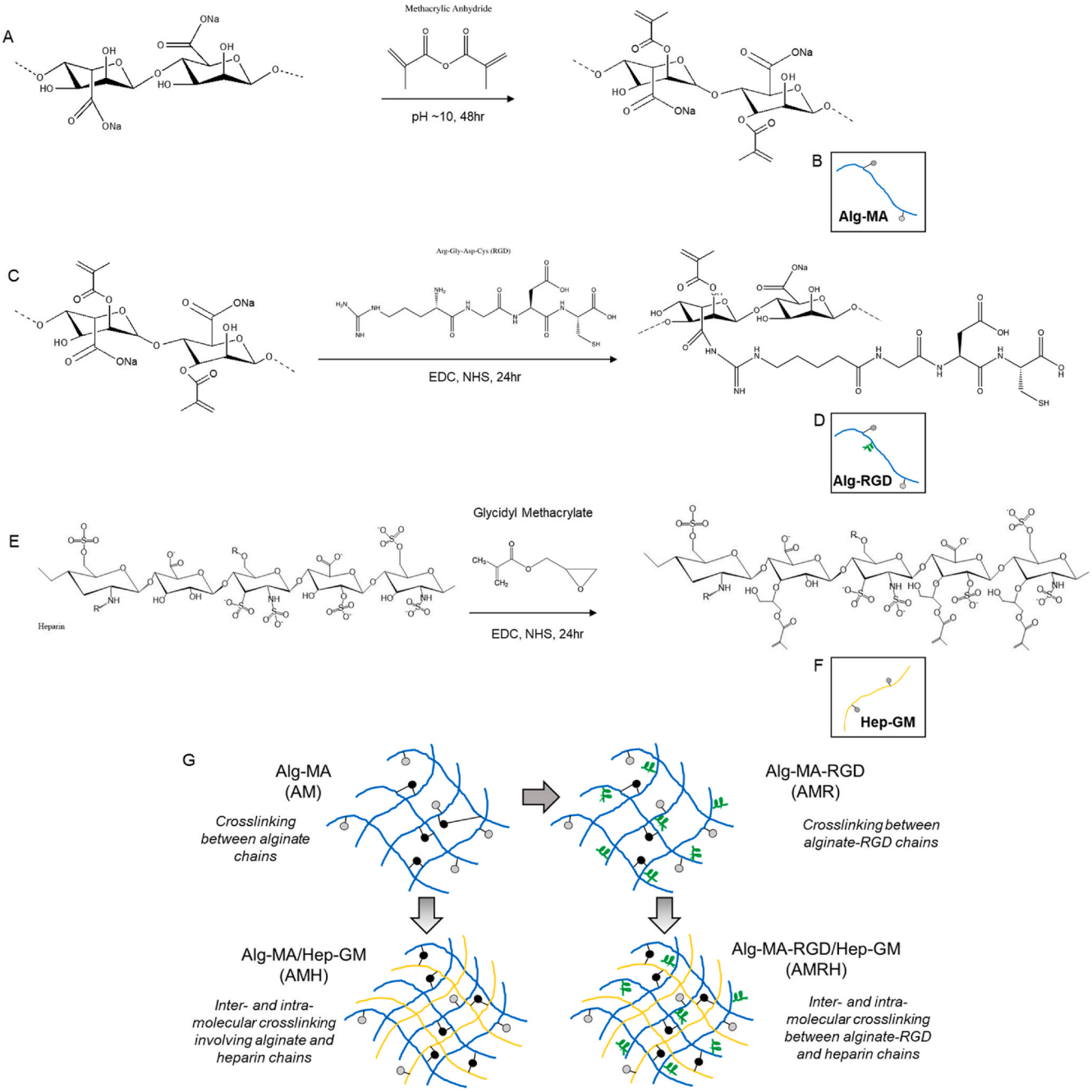


Fig. 1. The chemical synthesis and visual symbols of each polymeric product: **(A,B)** methacrylated alginate (AM); **(C,D)** RGD-conjugated AM (AMR); **(E,F)** methacrylated heparin (AMH). Chemical modification was performed in aqueous solutions and the polymers were filtered via dialysis. **(G)** Schematic diagram of the structural changes between the alginate-based hydrogels. The independent methacrylation of alginate (Alg-MA) and heparin (Hep-GM) enable self and copolymer crosslinking. Grey circles represent methacrylate functional groups; black circles indicate inter- and intra-molecular crosslinks. The inclusion of heparin effected the mechanical performance the most, while RGD displayed less of an effect on the material and mechanical properties.

3.2. Shear mechanical analysis and burst pressure testing

To create an effective tissue healing material, it is important to develop physiologically relevant materials whenever possible, including the physico-mechanical response of the native tissue. Thus, we tested the viscoelastic properties and structural integrity of the materials for healing dynamic tissues. The resistance of the alginate-based hydrogels under shear strain was measured and the storage (G') and loss (G'') moduli were calculated (Fig. 2A and B). The alginate-based material groups, which were fabricated without VEGF for the mechanical study, included AM, AMR, AMH, AMRH and were evaluated under rotational shear conditions. The hydrogel shear moduli for all groups tested ranged from 0.83 to 2.1 kPa for G' (Fig. 2A) and from 10.2 to 23.4 Pa for G'' (Fig. 2B).

Regarding G', the covalent incorporation of heparin with the alginate (AMH) did not significantly change the modulus (nonsignificant), suggesting that the alginate contributes dominantly to material properties. Comparable moduli values in the AMR group indicate that the RGD-conjugation also does not have a significant effect on the modulus. However, the incorporation of RGD-modified alginate with heparin (AMRH), significantly decreased G'compared to all of the other groups (AM, AMR, AMH). Thus, we further hypothesize that the incorporation of all three chemistries may interfere with the degree of crosslinking, and significantly reduce the extent of crosslinking, resulting in a more compliant, weaker material, providing less of a resistance to cell migration and external loading.

To determine if the alginate-based hydrogels could achieve physiological burst pressures and indicate minimum strength of current tissue sealant materials, burst pressure experiments were conducted on the AM and AMH groups. The methods used in the burst pressure testing are shown in Fig. 3A-D which produced the data shown in Fig. 3E. There were no significant differences between AM and AMH ($p = 0.27$), and they both exceeded the published results of current clinical biomaterials Evicel and CoSEAL; however, our materials are improved compared to current biomaterials, in that we obtain strong hydrogels (Annabi et al., 2017). Results demonstrate the potential for the AM and AMH materials to resist not only shear deformation in a viscoelastic manner, but also maintain structural integrity under multi-axial loading. To show that the burst pressures of the alginate-based materials were significantly higher than crosslinked heparin alone, the control hydrogel group was included, and was indeed significantly weaker compared to the alginate-based hydrogels; this is expected, as the molecular weight of the alginate was proposed to be higher and provides more flexible chain ends to physically entangle with an underlying substrate. While the effect of RGD was not investigated in the burst pressure analysis due to preserving use of the biomolecule; RGD is expected to affect the cellular adhesion but will not provide additional tissue adhesive support in regard to the burst pressure testing technique. The main contributor to providing material tissue adhesion and structural integrity is the entanglement of the alginate and heparin chains into the surface structure of the collagen substrate, which has been determined to be the primary source of action for our hydrogel tissue sealants. Also, as shown by the rheology results, there was no significant difference between AM and AMR groups, nor was there a significant difference between AMH and AMR groups.

3.3. Hydrogel cytotoxicity and HUVEC proliferation

The mitochondrial activity of HUVECs seeded on each hydrogel group was quantitatively compared after 24 h of culture; higher values of mitochondrial activity correlate to more active cells and provide information related to the cytotoxicity of the alginate-based hydrogel materials (Fig. 4). Compared to the base AM, modifications with RGD, heparin, and both RGD and heparin, all resulted in significantly higher HUVEC mitochondrial activity after 24 h. Comparing groups with and without RGD modification, groups with RGD had greater degrees of activity compared to the AM and AMH groups, respectively. The presence of conjugated RGD and heparin maintained a significantly greater cell viability as compared to the AM group ($p < 0.05$). An explanation for this effect could be the role that heparin has in binding to receptors on the cell surface (*i.e.,* selectins) to promote cellular adhesion, allowing the cells to adhere to the hydrogel and proliferate by receiving mechanical and chemical cues from the surrounding environment; however, this was after 24 h of culture. Indeed, the significant changes were seen at 72 h in regard to the protein assay (Fig. 5). After comparing mitochondrial activity levels between respective VEGF loaded and unloaded groups, there was little to no observable difference in activity. Despite some groups demonstrating increased amounts of mitochondrial activity over others, all hydrogel groups resulted in lower activity levels compared to the non-modified cell controls. One explanation for this observation could be the presence of the photo-initiator system components.

To further explore what role the chemical modification to alginate-based hydrogels played, cell proliferation was determined using a cell protein assay which quantified total intracellular protein produced over time. As HUVECs proliferate and the popular grows, they produce more total protein. Protein concentration was determined for HUVECs cultured on the various hydrogel groups, with and without encapsulated

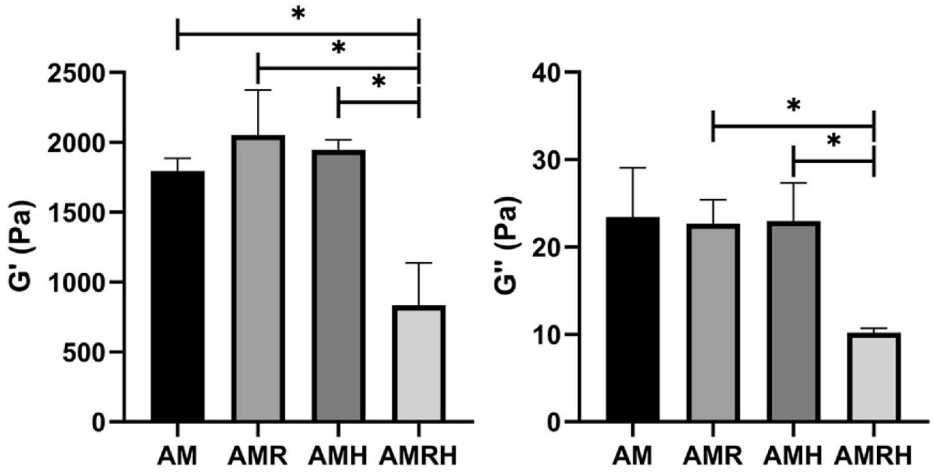
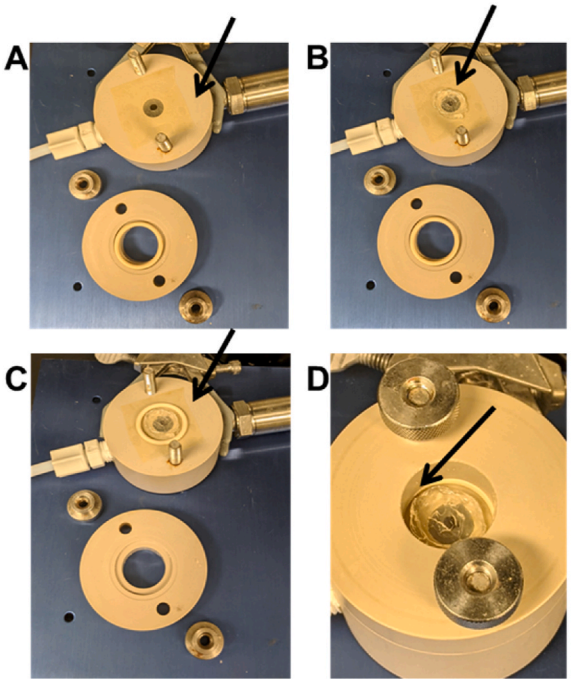


Fig. 2. Shear mechanical properties were calculated for alginate-based hydrogels: methacrylated alginate (AM), methacrylated alginate-RGD (AMR), methacrylated alginate-heparin (AMH), methacrylated alginate-RGD-heparin (AMRH). The testing was conducted at 10% oscillatory strain, 1 Hz. (Left) Shear storage (G') and (Right) loss (G'') moduli were calculated ($n = 3$; mean ± standard deviation; $p < 0.05$).



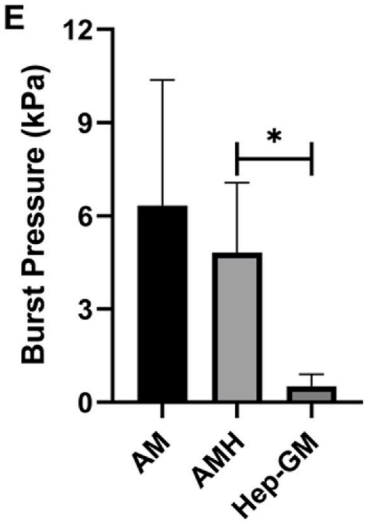


Fig. 3. A custom-made burst pressure testing device was designed and fabricated for testing tissue adhesive hydrogels. (A) A collagen casing is placed on the device, and a small defect is made over the air inlet. (B) The alginate-based materials were crosslinked in place over the defect using visible green light. (C) The crosslinked hydrogel was then tested; a seal was formed on the casing material with the hydrogel covering the defect. (D) Failure, and loss of pressure, was the result of delamination. (E) Burst pressure values were measured for alginate-based hydrogels without VEGF or RGD: methacrylated alginate (AM), methacrylated alginate-heparin (AMH) and Hep-GM were investigated to examine the effect of an interpenetrating heparin network within the alginate-only hydrogels. The adhesion failure analysis and burst pressure testing for the hydrogels were conducted at 37 °C (n = 4; mean ± standard deviation; $p < 0.05$).

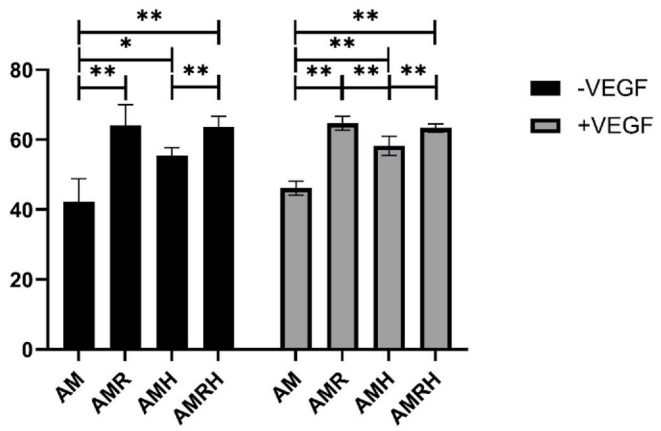


Fig. 4. The mitochondrial activity of human umbilical vein endothelial cells (HUVECs) was quantified using a WST cell viability assay. HUVECs were cultured on alginate-based hydrogels with and without encapsulated VEGF and normalized using non-modified cell controls: methacrylated alginate (AM), methacrylated alginate-RGD (AMR), methacrylated alginate-heparin (AMH), methacrylated alginate-RGD-heparin (AMRH). An absorbance reading was taken after 24 h of culture; (n = 4; mean ± standard deviation; *$p < 0.05$, **$p < 0.005$).

VEGF (Fig. 5). The significant effects of time, material group, and VEGF encapsulation were calculated. Looking at the hydrogels that did not contain VEGF (-VEGF), where hydrogel groups were compared to each other at each time point (Fig. 5A), the AMR, AMH, and AMRH were all significantly higher compared to the AM group at 12, 24 and 72 h. After 6 h of culture, the AMH and AMRH showed increased total protein compared to the AMR group. In the VEGF-encapsulated hydrogels (+VEGF, Fig. 5B), there were no significant differences between material groups at 6 h. At 24 and 72 h of culture, however, the AMH and AMRH hydrogels had significantly higher total protein levels compared the AM group. At 72 h, the AMR group was also significantly higher compared to the AM group.

Looking at the hydrogels that did not contain VEGF (-VEGF), where each time point was compared within a single group type (Fig. 5C), the AM showed significant changes in total protein content over 72 h. The AMR, AMH, and AMRH all showed significant increases in total protein content after 72 h of culture. Indeed, the AMH groups showed a highly significant increase ($p < 0.005$). For the hydrogels encapsulating VEGF (+VEGF, Fig. 5D), only the AMH and the AMRH groups showed significant increases in total protein content after 72 h. Notably, none of the material groups showed significant differences in total protein concentration with and without the encapsulation of VEGF (Fig. 5E-H).

3.4. HUVEC tubular network formation

HUVEC-seeded hydrogels were imaged using phase-contrast microscopy on the surface of hydrogels at 6, 12, 24, and 72 h (Fig. 6). After 6 h, AM hydrogels showed slight to no signs of tube formation or cell adhesion, demonstrated by the round morphology of the HUVECs; in fact, after 6 h all of the cells were dead, and images were not obtained. Similar effects were observed with the methacrylated alginate conjugated with heparin (AMH) in which cells were not seen to effectively adhere; while some cell proliferation did occur, as seen in the VEGF results (Fig. 5), the cells were not able to maintain adhesion to the substrate no produce a tubular network. The incorporation of VEGF into the alginate-based hydrogel formation process resulted in trapped VEGF, with high affinity to the heparin component(Héroult et al., 2004; Jeon et al., 2011), and in our experiments do not show a positive influence on affecting tube formation for the AMH and AMRH groups. Indeed, a more conclusive investigation of the binding of VEGF to the alginate-based hydrogels and the release kinetics was outside the scope of this study.

The latter of these results is not surprising; heparin is a polysaccharide and does not have a cell adhesion motif (RGD). Alginate modified with RGD (AMR), and with RGD and heparin (AMRH), however, demonstrated successful adhesion of the cells to the substrate as evident by the cells elongated morphology and migration. Both materials exhibited angiogenic properties as tubes were formed between cells, but a slightly greater degree of tube formation was seen on the AMRH groups. These same trends were observed when imaging at 12 h but with some structural irregularity of the tubular interactions between cells, most evident on the group modified solely with RGD. At 24 h, peak tube formation was observed for HUVECs cultured on AMR gels. While

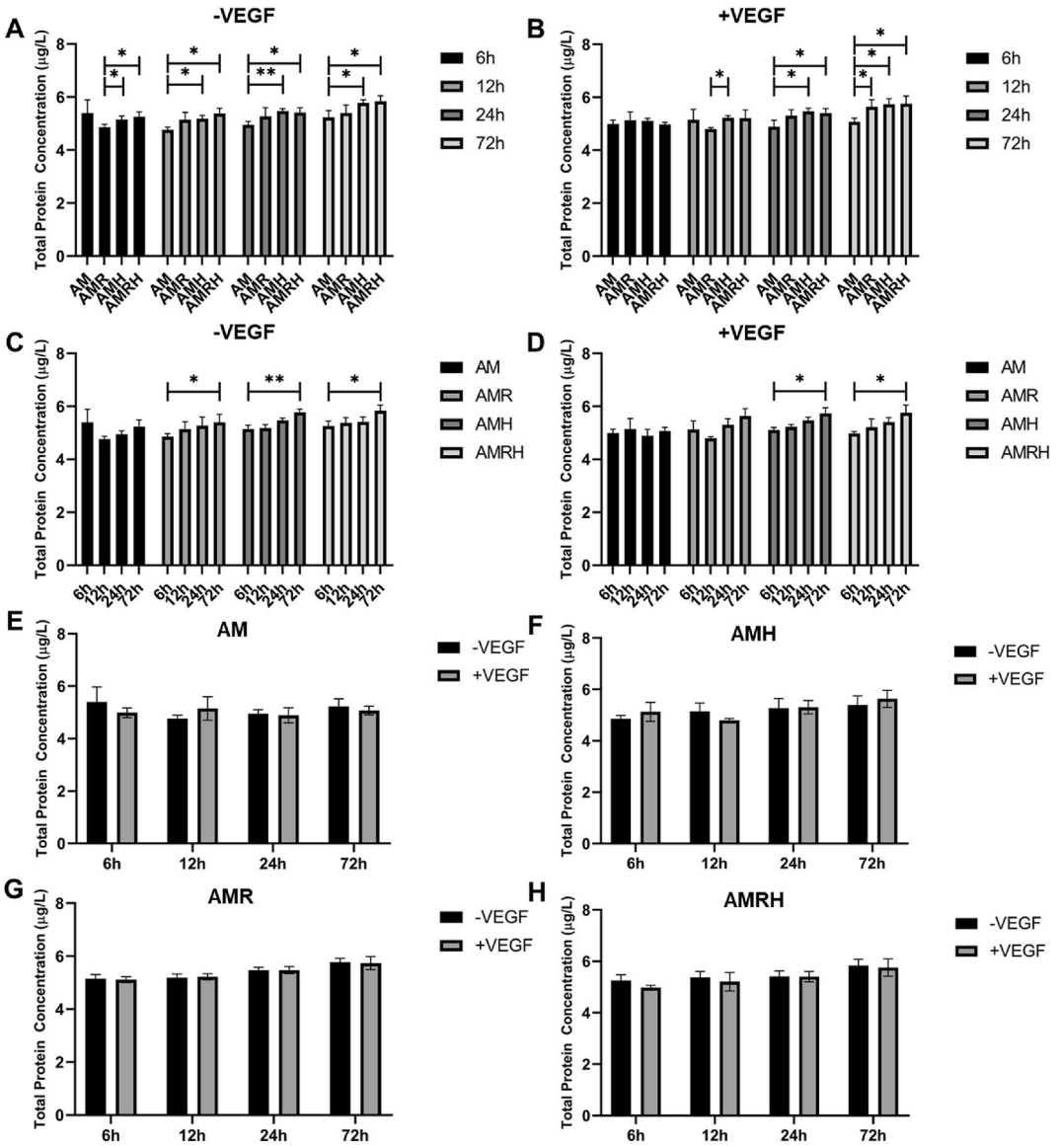


Fig. 5. Total protein content of the population of human umbilical vein endothelial cells (HUVECs) cultured on various hydrogel groups was quantified using a Pierce protein assay. Cells were cultured on alginate-based hydrogels with and without encapsulated VEGF and compared to non-modified cell controls: methacrylated alginate (AM), methacrylated alginate-RGD (AMR), methacrylated alginate-heparin (AMH), methacrylated alginate-RGD-heparin (AMRH). The length of culture time and the material group played a significant role in total protein content **(A–D)**. There were no significant differences between -VEGF and +VEGF hydrogels regarding effect on total protein concentration: **(E)** AM; **(F)** AMH; **(G)** AMR; **(H)** AMRH. Protein absorbance and quantification was performed at 6, 12, 24, and 72 h; ($n = 4$; mean ± standard deviation; *$p < 0.05$, **$p < 0.005$).

tube formation persisted throughout the remainder of the study, by 72 h there were fewer tubular interactions and an increased presence of cells possessing a round morphology. As per the behavior of HUVECs seeded on basement membrane substrates, tubes mature within 6–16 h, and after 24 h, cells undergo apoptosis which cause the tubes to detach from the substrate and break apart (Arnaoutova and Kleinman, 2010). This behavior, however, was not seen in HUVECs cultured on AMRH hydrogels. As opposed to HUVECs cultured on AMR hydrogels, AMRH group continued to show an increase in tube formation from the 24- to 72-h time point, suggesting that the presence of heparin has a role in prolonging the period in which tube formation occurs and in increasing the degree of tubular network formation. These findings were encouraging and suggest an angiogenic potential of our conjugated heparin material, which has also been shown in the literature (Goldberg et al., 2021). Indeed, heparin traditionally plays a role in preventing the formation of a thrombosis due to its anticoagulant activity and is a component of the ECM of blood vessels that has been shown to promote the growth of endothelial cells *in vitro* (Na et al., 2003; Assessing the Angiogenic). These indications provide proof of concept for investigating the influence of mechanical properties, heparin, and RGD concentration.

The effect of VEGF encapsulation was difference between groups with and without heparin conjugation The AMRH group loaded with VEGF exhibited less tube formation compared to their unloaded counterpart at each time point. While VEGF and surface-conjugated heparin have been shown to promote angiogenesis separately, together there seems to be an inhibitory effect; the VEGF-loaded hydrogels resulted in smaller networks (Giraux et al., 1998). Heparin is known to have an affinity for specific growth factors in which, by binding them, can prevent their denaturing and modulate their release into a defect and/or surrounding tissue to promote angiogenesis when necessary. It was originally thought that this behavior of the heparin would increase the benefit of VEGF within the hydrogel as its' incorporation in the hydrogel provides it with more opportunities to interact with and be sequestered

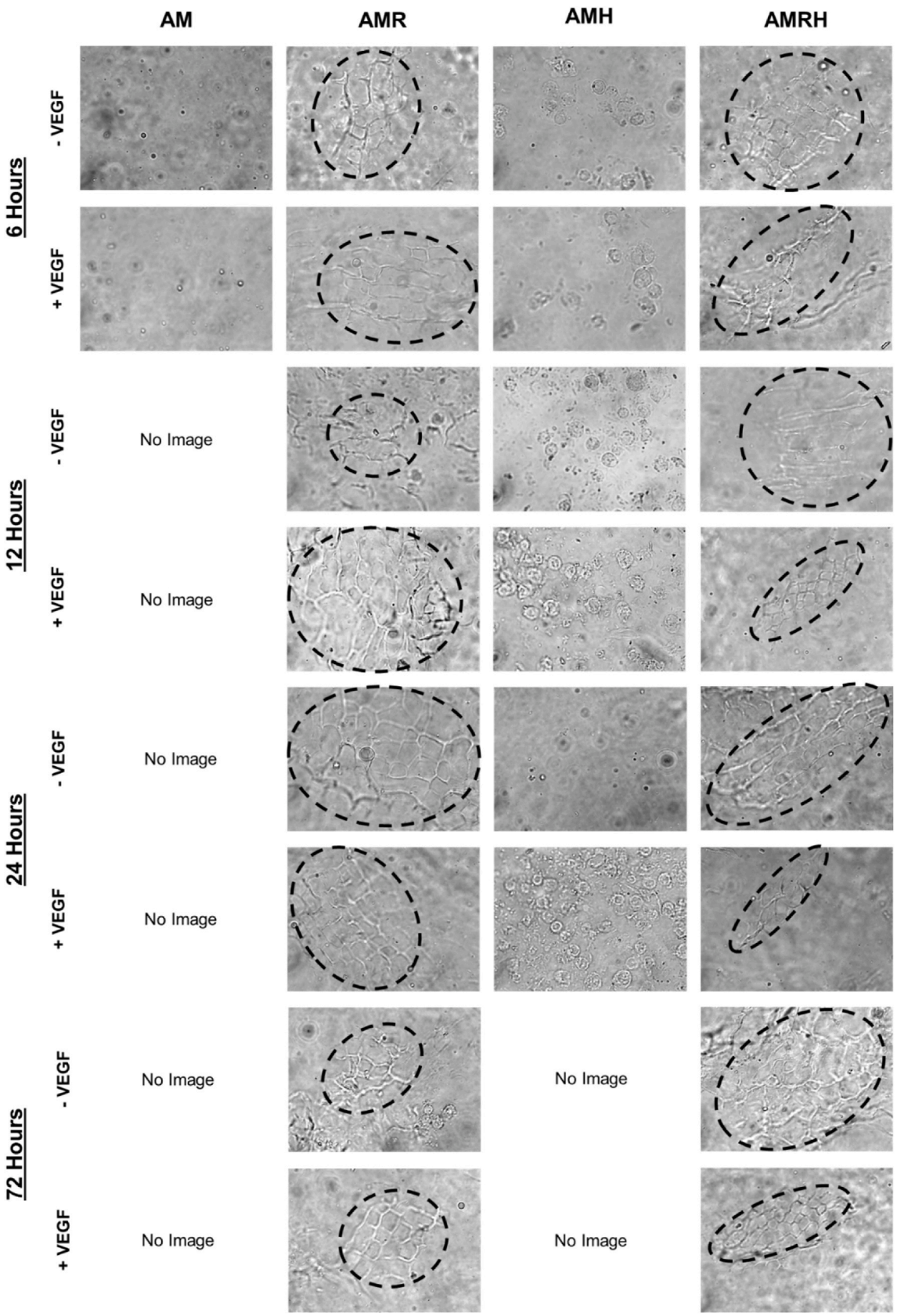


Fig. 6. Phase contrast microscopy images (20× magnification) of HUVECs cultured on alginate-based hydrogels, with and without encapsulated VEGF (AM = methacrylated alginate, AMR = methacrylated alginate-RGD, AMH = methacrylated alginate-heparin, AMRH = methacrylated alginate-RGD-heparin). HUVECs were seeded on top of the hydrogels however appear to migrate into the material indicated by z-axis focus dependence. Images were not obtained for hydrogels void of viable (*i.e.,* adherent) cells. The solid ovals represent average tube network size for hydrogels without VEGF. The dashed ovals represent average tub network size for hydrogels encapsulating VEGF.

by the heparin, but it instead gives rise to an inhibitory effect. The inhibitory effect has been studied before and helps to explain why the samples with heparin and without VEGF behave better than the heparin samples with VEGF – the bioactivity of both factors on angiogenesis is inhibited by the presence of the other (Héroult et al., 2004; Miao et al., 2014). Notably, the AMRH group without VEGF exhibited more uniform and larger tubular networks on average compared to AMRH groups with VEGF (AMRH). Also, the AMRH group without VEGF performed better compared to the AMR group without VEGF and performed just as well on average compared to the AMR group with VEGF. This experiment provides a proof of concept for using conjugated heparin and alginate copolymer hydrogels to aid in angiogenesis without the use of encapsulated growth factors. The AM and AMH groups were not effective at maintaining cell adhesion – no images were collected for materials which contained no viable cells. Also, the heparin conjugation alone, without RGD, did not induce tube formation.

4. Conclusion

Alginate-based hydrogels were developed incorporating RGD and heparin to stimulate tubular network formation for potential use as a tissue healing biomaterial. The purpose of this study was to develop a material incorporating alginate, heparin, and RGD with suitable physico-mechanical properties with potential for use as a therapeutic biomaterial. Methacrylate functional groups were conjugated to the hydroxyl groups of both alginate and heparin, and RGD was conjugated to the carboxyl groups of alginate. All of the hydrogel groups crosslinked under visible green light in the presence of photo-initiators. The rheological properties and burst pressures for the modified alginate-heparin hydrogels indicate advantageous properties for use in loaded environments. HUVECs were seeded onto the alginate-based hydrogels and assessed to investigate the effect of the scaffold components. HUVECs seeded hydrogels were imaged at 6, 12, 24, and 72 h the AMRH group showed the greatest increase and indication of tube formation without VEGF. Interestingly, this is the material with the lowest shear modulus, and it is unknown how this effects the tubular network formation outside of the differences in bioactivity of the hydrogel groups. In the case of a thoracic injury, the therapeutic hydrogels need to form a seal over the wound and expand in a dynamic fashion. Future work will investigate further the potential use of AMRH biomaterials as wound dressings and tissue sealants and a deeper analysis of the angiogenic potential.

Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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