POLYMERIC MATERIALS FOR NEOVASCULARIZATION

BY

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DISSERTATION

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Abstract

Revascularization therapies have emerged as a promising strategy to treat various acute and chronic wounds, cardiovascular diseases, and tissue defects. It is common to either administer proangiogenic growth factors, such as vascular endothelial growth factor (VEGF), or transplant cells that endogenously express multiple proangiogenic factors. Additionally, these strategies utilize a wide variety of polymeric systems, including hydrogels and biodegradable plastics, to deliver proangiogenic factors in a sophisticated manner to maintain a sustained proangiogenic environment. Despite some impressive results in rebuilding vascular networks, it is still a challenging task to engineer mature and functional neovessels in target tissues, because of the increasing complexities involved with neovascularization applications. To resolve these challenges, this work aims to design a wide variety of proangiogenic biomaterial systems with tunable properties used for neovascularization therapies.

This thesis describes the design of several biomaterial systems used for the delivery of proangiogenic factors in neovascularization therapies, including: an electrospun/electrosprayed biodegradable plastic patch used for directional blood vessel growth (Chapter 2), an alginate-g-pyrrole hydrogel system that biochemically stimulates cellular endogenous proangiogenic factor expression (Chapter 3), an enzyme-catalyzed alginate-g-pyrrole hydrogel system for VEGF delivery (Chapter 4), an enzyme-activated alginate-g-pyrrole hydrogel system with systematically controllable electrical and mechanical properties (Chapter 5), and an alginate-g-pyrrole hydrogel that enables the decoupled control of electrical conductivity and mechanical rigidity and is use to electrically stimulate cellular endogenous proangiogenic factor expression (Chapter 6). Overall, the biomaterial systems developed in this thesis will be broadly useful for improving the quality of a wide array of molecular and cellular based revascularization therapies.
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Chapter 1: Introduction*

1.1 Neovascularization

Throughout the body, vessels supply blood to tissues providing nutrients and oxygen, while disposing of cellular wastes and carbon dioxide. Due to diffusion limitations of nutrients and oxygen, tissues can only survive a few micrometers away from blood vessels, making them vital for normal tissue activities. The disruption of normal blood vessel functions, either due to injury or disease, can result in the inadequate supply of oxygen to tissues, a condition known as ischemia [1-3]. For instance, atherosclerosis, a cardiovascular disease that involves the build-up of plaque along blood vessel walls, can reduce the blood supply to tissues [4-5]. Additionally, the plaque build-up from atherosclerosis can break from the vessel walls and completely block blood flow to tissues downstream; this can lead to severe complications including stroke, heart attack, or critical limb ischemia.

Neovascularization, the regeneration of new fully functional blood vessels, has emerged as a therapy to treat various injuries and diseases, including ischemia [3]. These therapies involve the creation of new vascular networks that supply blood to tissues by circumventing injured or malfunctioning vessels. Ultimately, the new blood vessels introduce much needed nutrients and oxygen into deprived tissues, preventing tissue death and necrosis and improving the retention of normal tissue functions.

1.2 Angiogenesis

Neovascularization occurs through the process of angiogenesis, the sprouting of new capillary vessels from existing blood vessels [6]. Angiogenesis plays a role in numerous physiological and pathologic processes, including tissue growth, wound healing and cancer development. The process of angiogenesis is induced through the activation of endothelial cells by exposing them to various angiogenic growth factors [7]. Growth factors that directly or indirectly induce angiogenesis include,
vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factors (PDGF), and transformational growth factor beta-1 (TGFβ1) [8]. Upon activation, endothelial cells produce proteolytic enzymes, including matrix metalloproteinases, which degrade the matrix around the blood vessel wall, allowing the cells to leave the existing vessels. Then, the endothelial cells undergo a proliferation and migration process that ultimately forms sprouting capillary vessels [9]. Finally, capillary vessels become fully functional through a maturation process that involves the formation of a layer of smooth-muscle actin cells that surround the vessels and provide mechanical support.

1.3 Strategies for inducing angiogenesis

The administration of angiogenic growth factors for the induction of angiogenesis remains a significant challenge [3]. In order to induce the sprouting of blood vessels, specific concentrations of angiogenic factors must be present in the surrounding environment. Insufficient levels of growth factors do not result in the induction of angiogenesis; while too high of growth factor concentrations can result in the formation of immature leaky blood vessels. Additionally, many growth factors in physiological environments have extremely short half-life time periods and are readily degraded, making it difficult to sustainably attain desired growth factor levels. Therefore, various polymeric materials in combination with growth factors and/or cells have been traditionally used to sustainably release various growth factors for the induction of angiogenesis [10-11]. These therapies can be categorized into two basic strategies for the sustainable delivery of various growth factors: molecular therapies and cellular therapies.

1.3.1 Molecular Therapies

Molecular based therapies involve the delivery of specify growth factors from polymeric delivery devices for the promotion of angiogenesis. Some common strategies involved the encapsulation of angiogenic growth factors in biodegradable plastic microparticles, such as poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), and poly(ε-caprolactone), which release factors upon the degradation
of the polymers [11-13]. Other strategies use various hydrophilic polymers, in the form of hydrogels that release growth factors via their diffusion through the polymeric matrix [14-15]. A wide variety of synthetic and natural hydrophilic polymers are commonly used for the delivery of growth factors, including poly(ethylene glycol) (PEG), poly(acrylamide), poly(acrylic acid), alginate, collagen, fibrinogen, and hyaluronic acid. Overall, this strategy has the distinct advantage of delivering specific growth factors in a controllable manner for angiogenic therapies. Additionally, many of these polymeric systems are designed to be easily injected locally or systemically, such as PLGA microparticles and in situ forming alginate gels.

1.3.2 Cellular Therapies

Cellular based therapies involve the incorporation of cells with a polymeric matrix for implantations within tissues to promote angiogenesis. The implanted cells endogenously produce growth factors, cytokine, and other bioactive molecules that ultimately activate the angiogenic process, while the polymer matrix provides a protective and stable environment suitable for these cell activities. Cells within the matrices are able to probe their environment for signals through specific bonds, such as those between cellular receptors and soluble factors, cellular integrins and ligands of the extracellular matrix (ECM), and cadherins and neighboring cells [16-18]. These multiple signals orchestrate cell signaling pathways and subsequent diverse cellular activities including the expression of various angiogenic factors.

To further elaborate the role of extracellular microenvironments on inducing specific cellular activities, extensive efforts have been made to develop synthetic ECMs which can recapitulate the natural ECM microstructures and properties for normal and pathologic tissues, using a series of biomaterial systems [19-20]. These synthetic ECMs should thus present a refined control of permeability, mechanical properties, as well as the type and number of cell adhesion molecules and soluble factors. Specifically, a series of hydrogels, commonly formed through chemical or physical cross-linking reactions between
polymers dissolved in aqueous media, are becoming an essential tool for cellular based angiogenic therapies because of their several advantageous features [21-23]. First, hydrogels, by weight, are mostly water, similar to natural ECMs in tissues. Second, a variety of cross-linking reactions—including physical associations, ionic cross-linking reactions, and covalent cross-linking reactions—are available to control the gelation kinetics and the subsequent hydrogel properties. Third, hydrogels can easily be modified to present chemical, mechanical, and electrical properties similar to natural ECMs via chemical modification of gel-forming polymers and cross-linking molecules. Additionally, they have the unique ability to allow three-dimensional (3D) cell cultures, which represents in vivo cellular environments more closely than cells cultured on 2D substrates [24-26].

Recently, it has been shown that the electrical stimulation of cells and tissues can induce the endogenous expression of angiogenic factors, demonstrating the importance of controlling the electrical conductivity of the cell environment for various angiogenic therapies [27-29]. However, cells are typically incorporated in a polymeric matrix, which exhibits an insulative property, ultimately reducing the electrical conductivity of the cell environment. Therefore, hydrogels are being developed containing electrically conductive polymers, such as polyaniline, polypyrrole, and polyacetylene, to improve the conductive properties of various gel systems [30]. Many of these systems have demonstrated significant improvements in their electrical conductive properties; however, it still remains a challenge to process the electrically conductive polymers into hydrogels in a manner suitable for cell based angiogenic therapies.

1.4 Project overview

Despite some impressive work in rebuilding vascular networks, it is still a challenging task to engineer mature and functional neovessels in target tissues, because of the increasing complexities involved with neovascularization applications. To resolve several of these challenges, this work aims to design a variety of proangiogenic biomaterial systems with tunable properties used for neovascularization therapies. This thesis describes the design of several biomaterial systems used for the delivery of
proangiogenic factors in neovascularization therapies, including: an electrospun/electrosprayed biodegradable plastic patch used for directional blood vessel growth (Chapter 2), an alginate-g-pyrrole hydrogel system that biochemically stimulates cellular endogenous proangiogenic factor expression (Chapter 3), an enzyme-catalyzed alginate-g-pyrrole hydrogel system for VEGF delivery (Chapter 4), an enzyme-activated alginate-g-pyrrole hydrogel system with systematically controllable electrical and mechanical properties (Chapter 5), and an alginate-g-pyrrole hydrogel that enables the decoupled control of electrical conductivity and mechanical rigidity and is used to electrically stimulate cellular endogenous proangiogenic factor expression (Chapter 6). Overall, the biomaterial systems developed in this thesis will be broadly useful for improving the quality of a wide array of molecular and cellular based revascularization therapies.
1.5 References


Chapter 2: Directed blood vessel growth using an angiogenic microfiber/microparticle composite patch*

2.1 Abstract

Therapeutic angiogenesis has emerged as a promising strategy to treat various acute and chronic vascular diseases, and to enhance tissue repair and regeneration. Revascularization therapies are commonly conducted by administering angiogenic growth factors, such as vascular endothelial growth factor (VEGF) [1, 2]. Successful angiogenic therapies greatly rely on the ability to engineer mature and functional neovessels, uniformly distributed within target tissues. Immature blood vessels with non-uniform distributions often stimulate an inflammatory response and a limited therapeutic efficacy [3, 4]. This study presents an angiogenic microfiber patch, which releases angiogenic growth factors along aligned fibers and subsequently directs the spacing and orientation of mature and functional neovessels. The angiogenic microfiber patch was prepared by electrostatically binding electrosprayed VEGF-encapsulating poly(lactide-co-glycolide) (PLGA) microparticles with electrospun poly(lactide) (PLA) microfibers. The PLGA microparticles released VEGF in a sustained manner, while the straightly aligned PLA fibers guided cells to adhere along their orientation. Finally, microfiber patches implanted in vivo resulted in the formation of neovessels, with controlled spacings and directionalities, together with a significant increase in the blood vessel density. The angiogenic microfiber patch, developed in this study, will be broadly useful for studying neovessel growth, and will also significantly improve the quality of clinical treatments requiring neovascularization.

2.2 Introduction

Several vascular biological studies have demonstrated that the spatiotemporal organization of multiple angiogenic growth factors and cytokines in tissues control the directional growth and spacing of neovessels during development and self-healing [5]. Therefore, extensive efforts have been made to

engineer the spatial distribution of angiogenic factors within ischemic tissues and tissue defects. For example, polymeric scaffolds assembled by combining growth factor-releasing macro-sized layers with growth factor-free layers have been studied to induce vessel growth towards the bioactive layers [6, 7]. Recently, various micropatterning, microprinting, and microfluidic techniques have been incorporated into the design of scaffolds that aim to control the spatial organization of neovessels by creating micro-sized channels for 3D vessel perfusion [7-10]. However, these approaches have not demonstrated the capacity to control the directional growth and spacing of functionally mature blood vessels at physiologically relevant length scales.

Electrospinning and electrospraying techniques have been extensively studied for the assembly of microfibrous scaffolds and creation of micro-sized particles, respectively. Electrospinning has been used to fabricate fibrous matrices structurally similar to natural extracellular matrices [11, 12]. Prior studies have demonstrated that the spatial organization between the microfibers of the electrospun matrix regulates the spatiotemporal organization of adhered cells [13, 14]. Electrospraying has been used to create drug encapsulating nano- and microparticles with higher drug loading efficiencies compared to methods that use harsh solvents and surfactants [15, 16]. Recently, electrospinning and electrospraying techniques have been coupled with counter electrical charge generators to control the surface charges of the microfibers and microparticles, and ultimately used to create microfiber meshes with bound microparticles. For example, we have recently created a poly(methyl methacrylate) (PMMA) microfiber mesh coated with poly(styrene) (PS) microparticles by electrospraying positively charged PS microparticles on negatively charged PMMA microfibers in situ [17].

We hypothesized that angiogenic microfiber patches, prepared through the electrospraying of angiogenic growth factor releasing microparticles onto electrospun cell adherent microfibers, would allow us to localize the spatial distribution of angiogenic factors around the fibers and stimulate blood vessel growth along the fibers’ orientation (Fig. 2.1). This patch mimics the microstructure and functions of a wound-healing fibrin clot, which presents a fibrous matrix and platelets that release multiple growth
factors, including VEGF [18, 19]. In addition, the use of synthetic polymers allows us to control the mechanical properties of the matrix and control the type and dose of angiogenic factors in a desired manner. The resulting angiogenic microfiber patches stimulates blood vessel growth along the fibers’ orientation because of local increases in angiogenic factors around the microfibers. We examined this hypothesis by electrospraying positively charged VEGF-encapsulating PLGA microparticles on electrospun PLA microfibers with negative surface charges (Fig. 2.2). Cellular adhesion to the bioactive microfiber patches were examined in vitro, and the functionality of the patches towards directing neovessel growth was examined by implanting them on chicken chorioallantoic membranes (CAMs).

2.3 Experimental

2.3.1 Materials

Poly(L-lactide-co-D,L-lactide) (PLA, 70:30, Resomer® LR708, i.v. 5.5-6.5) and poly(D,L-lactide-co-glycolide) (PLGA, 50:50, Resomer® 503H, i.v. 0.32-0.44) were obtained from Boeinger Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany). Other chemicals were from the following vendors: Polyvinylpyrrolidone (PVP, Mw 29000 g mol⁻¹) from Sigma Aldrich (MO, USA), benzaldehyde (BA, ReagentPlus, ≥99 %, carefully protected from light throughout the experimental procedures) from Sigma Aldrich (Steinheim, Germany), vascular endothelial cell growth factor (VEGF, Human) from Koma Biotech (Seoul, Korea), THF from Samchun (Seoul, KOREA), water from J. T. Baker (HPLC grade, NJ, USA), N,N-dimethylformamide (DMF) and Ethyl acetate (EA) from Duksan (Gyeonggi, KOREA) were used without purification. Dubelco’s Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin/Streptomycin, phosphate buffered saline (PBS), Oregon Green ® phalloidin were all obtained from Invitrogen. Bovine Serum Albumin (BSA) was from Sigma and Triton X-100 was from Fisher.

2.3.2 In situ electrospinning and electrospraying of PLA nonwoven microfiber mesh coated with PLGA microparticles:
PLA (4 wt% in DMF/THF (1/1 v/v)) and PLGA (0.1 g ml\(^{-1}\) in EA/BA (1/1 v/v)) solutions were injected by a syringe pump (KDS 100, MA, USA) for electrospinning and electrospraying, respectively. The electrospinning solution was fed at 0.7 ml h\(^{-1}\) into a 19 G stainless nozzle (Norm-Ject, Henke Sass, Germany) of -6.3 kV (DC negative voltage supply, H.V. model, NanoNC, South Korea). The PLGA solution was fed at 0.6 ml h\(^{-1}\) into an 18 G Teflon-coated polypropylene nozzle (Ban Seok Co., South Korea) of 6.0 kV (DC positive high voltage SHV model, Convertech Co., South Korea). A core solution, PVP (0.3 g ml\(^{-1}\)) aqueous solution, was injected into a 26 G stainless nozzle in the middle of the 18 G nozzle at 0.01 ml h\(^{-1}\) with or without VEGF (10 ug ml\(^{-1}\)). Both the nozzles were placed 200 mm apart from the ground and the distance between the nozzles was 165 mm. Composite fibers were taken up by a PTFE ring (\(\phi \) 0.64 or 0.96 cm, Sungjin Co., South Korea), and dried in a freeze dryer (FD-1000, EYELA, Japan, trap chilling temperature -45 °C, 5.6 Pa) for 24 hrs. The process was performed at 23 – 26 °C and 30 – 50 % room humidity.

2.3.3 Microfiber Patch Tensile Testing:

Uniaxial tensile testing was performed on 3×1 cm rectangular microfiber patches (24 hrs vacuum drying after preparation) using Instron 3344 (100 N load cell (model 60275)) at 5 mm/min crosshead speed. An average modulus value was calculated from 6 repeated tests using cross-section areas of fibrils analyzed by SEM.

VEGF release study:

Drug release studies were performed over a two week period using PLA microfiber patches coated with VEGF-encapsulating PLGA particles. The angiogenic patches were incubated in PBS supplemented with FBS (3%). The incubation media was collected at regular intervals and replaced with fresh PBS supplemented with FBS. The samples were immediately stored at -20\(^{\circ}\)F for analysis. After
two weeks, the concentrations of VEGF in all samples were measured using an enzyme-linked immunosorbent assay (DY293B, R&D systems).

Additionally, the local distributions of VEGF on the angiogenic fiber patches were examined, following incubation in FBS-supplemented PBS for 36 hours. The patches were incubated with a mouse monoclonal antibody to VEGF (ABCAM) for 30 min at room temperature. The patch was rinsed three times with PBS, and then incubated for one hour with a goat polyclonal secondary antibody to mouse labeled with fluorescein isothiocyanate (FITC). The patch was again rinsed three times with PBS, and the fluorescence from the fiber surface was imaged using a fluorescence microscope (Leica CTR 6000).

2.3.4 In Vitro Cell Culture on Microfiber Patches:

The mouse endothelial line (C166, passages 7-9) was seeded on the microfiber patches at a concentration of 20,000 cells cm$^{-2}$. The cells were cultured in Dubelco’s Eagle Medium supplemented with FBS (10%) and Penicillin/Streptomycin (1%) at 37 °C and 5 % CO$_2$. After 48 hours, the cells adhered to the microfiber patches were imaged using Scanning Electron Microscopy (SEM, JOEL 6060LV) or fluorescence microscopy (Leica CTR 6000). For the SEM imaging, cell-adhered patches were fixed using neutral buffered formalin in PBS, frozen in liquid nitrogen, lyophilized, and finally coated with palladium. The intracellular actins were imaged at an electron voltage of 20 kV. For the fluorescent imaging, the cells adhered to the patches were also fixed with formalin in PBS, permeabilized with Triton X-100 (0.5%) in PBS, blocked with bovine serum albumin (1%, BSA, Sigma) in PBS, and finally stained with Oregon Green® phalloidin (Invitrogen). The samples were excited at 490 nm and the resulting emissions at 514 nm were captured together with bright field images. The angle of cellular alignments relative to fiber mesh was analyzed using image analysis software (ImageJ).

2.3.5 In vivo angiogenesis assay using Chorioallantoic Membrane (CAM):
Fertilized chicken eggs (Hy-Line W-36) were obtained from the University of Illinois Poultry Farm (Urbana, IL). The eggs were initially incubated for 7 days, while being placed horizontally inside an incubator at 37 °C and 65 % humidity. Next, a hole with a diameter of 2 cm was made on the top of the egg shells, and the microfiber patches were placed on the CAMs. The eggs were incubated for 7 days at 37 °C, and the vascular networks in the CAMs were imaged using a S6E stereomicroscope (Leica) linked with a D-Lux E Camera (Leica). All CAM experiments were performed under sterile conditions. On the 7th day after implantation, the embryos were fixed with a 10% neutral buffered formalin solution (3.7 % formaldehyde in PBS). The area covered with the microfiber patches was excised. The samples were embedded in paraffin, sectioned, and stained with an antibody to α-smooth muscle actin (αSMA). The density and orientation of positively stained blood vessels were quantified using image analysis software (ImageJ). The blood vessel density was determined by measuring the total blood vessel area, and normalizing it to the overall cross-sectional area of the CAM. The blood vessel orientation was determined by measuring the aspect ratio of the cross-sections of the blood vessels. The aspect ratio of the blood vessels was related to the number of corresponding blood vessels to quantify the standard deviation of the resulting Gaussian distribution of aspect ratio values. For each condition, 4 to 6 CAM samples were implanted and evaluated.

2.4 Results and discussion

Angiogenic microfiber patches were prepared by integrating electrospinning and electrospraying processes (Fig. 2.3). PLA microfibers with negative surface charges were extruded through an electrohydrodynamic nozzle, and VEGF encapsulating PLGA microparticles with positive surface charges were simultaneously sprayed over the fiber surfaces. The charge relaxation rates of the PLA microfibers and PLGA microparticles, calculated from $\varepsilon, \varepsilon/K$, where $\varepsilon$ is the dielectric constant and $K$ is the electric conductivity, were tuned to $0.527 \times 10^{-5}$ s and $0.78 \times 10^{-5}$ s, respectively. This process drove PLGA particles, with an average diameter of 4 µm, to adhere to PLA microfibers with an average
diameter of 1.5 µm via electrostatic attraction (Fig. 2.2). The subsequent drying process led to an irreversible fusion between microfibers and microparticles. The density of the PLGA particles on the PLA fibers was kept constant at 14.5 particles per 1000 µm² of fiber area. In contrast, the electrospraying of microparticles onto pre-made PLA microfibers greatly decreased the density of the PLGA microparticles on the fiber mesh (Fig. 2.4). This decreased binding between particles and fibers was attributed to the rapid charge relaxation of PLA microfibers and subsequent absence of electrostatic attraction between microparticles and microfibers.

The PLA microfibers and PLGA particles of the resulting patches imparted two separate functions: the PLA microfibers acted as a sustainable mechanical support while the PLGA microparticles degraded to release VEGF in a sustained manner. This design strategy allows VEGF to be released independent of the patches’ structural integrity. The elastic moduli and ultimate strength of the patches were 697(±55) MPa and 26 (±2) MPa, respectively (Fig. 2.5), which are five orders of magnitude higher than fibrin and collagen matrices—natural fibrillar extracellular matrices [20, 21]. The patch could be extended to 1.3 times its original length under tensile loading (Fig. 2.5). The PLGA microparticles immobilized on the PLA microfibers minimally affected the stiffness of the overall PLA microfiber patches. The orientation and spacing of the PLA microfibers were maintained in physiological media over four weeks, while the PLGA microparticles gradually degraded (Fig. 2.6). The PLGA particles on the PLA microfibers released VEGF over two weeks, which remained bioactive as confirmed by an increase in the proliferation rate of cells exposed to the VEGF (Fig. 2.7). Additionally, the VEGF released from the microparticles locally distributed around the PLA microfibers, as confirmed with a positive fluorescence emission of the antibody to VEGF along the fibers following the incubation of the patch in a cell culture media (Fig. 2.8).

The straight alignment of the PLA microfibers played a significant role in directing cellular spatial organization on the microfiber patches. Endothelial cells adhered to the PLA microfibers immersed in a media supplemented with fetal bovine serum (FBS) (Fig. 2.9). In contrast, culturing
endothelial cells on the microfiber patches, using serum free media, resulted in a limited cell adhesion. These results address that cells adhere to the microfibers by forming specific bonds between cellular integrins and cell adhesion proteins adsorbed on the microfibers. Interestingly, cells adhered to the microfibers stretched anisotropically along the fibers’ orientations, as confirmed through a quantitative analysis. The PLGA microparticles fused to the PLA microfibers minimally influenced the anisotropic alignment of the cells. This alignment greatly contrasts with endothelial cells stretched isotropically on non-fibrous substrates (Fig. 2.9).

PLA microfiber patches, VEGF-encapsulating PLGA microparticles, and PLA microfiber patches coated with VEGF-free or VEGF-encapsulating PLGA microparticles were implanted on CAMs to examine their effects on the orientation and spacing of blood vessels (Fig. 2.10). Implantations of blank PLA patches and VEGF-encapsulating PLGA microparticles resulted in a limited increase of blood vessel density, as compared with control CAMs, which were not exposed to any materials nor soluble factors (Fig. 2.11). Implantation of the PLA microfiber patches coated with VEGF-free PLGA microparticles resulted in randomly oriented blood vessels with small diameters. In contrast, PLA microfiber patch coated with VEGF-encapsulating PLGA microparticles resulted in the formation of a larger number of blood vessels with smaller spacings as compared to the other three conditions and controls. In addition, the blood vessels anisotropically aligned with each other, similar to the anisotropic orientation of the PLA microfibers. The length of the blood vessels along the straightly aligned microfibers reached approximately 7 to 8 mm. However, implantations of patches which present poorly aligned fibers resulted in the formation of blood vessels with a smaller size and irregular spacing (Fig. 2.12).

Histological cross-sections of the CAMs were also imaged to examine the role that the angiogenic fiber patches have on blood vessel density and orientation. Mature blood vessels were marked with an antibody for α-smooth muscle actin (αSMA) (Fig. 2.10 & 2.11). The CAMs covered with blank PLA microfiber patches, VEGF-encapsulating PLGA microparticles, and PLA microfiber patches coated with VEGF-free PLGA microparticles presented a minimal increase in the size and density of mature blood
vessels, as compared to the blank CAMs. In contrast, implantations of PLA microfiber patches coated with VEGF-encapsulating PLGA microparticles resulted in a significant increase in the number and size of mature blood vessels.

Finally, the anisotropic alignment of mature blood vessels was analyzed by quantifying their aspect ratio (Φ), defined as a vessel’s cross-sectional height to width ratio. The blood vessels with a Φ of 1 were classified as vessels aligned perpendicular to the CAM cross-sections, while those with a Φ larger or smaller than 1 were vessels oriented in a direction that deviates from the direction perpendicular to the cross-section (Fig. 2.12). The variation of Φ over the CAM implantation area was fitted to a Gaussian distribution to calculate the standard deviation (σ), an index of isotropic orientation of blood vessels, using Eq (1).

$$\Phi = \frac{1}{\sqrt{2\pi}\sigma^2} e^{-\frac{(N-\Phi_{avg})^2}{2\sigma^2}}$$

(1)

where, N is the number of blood vessels at specific Φ, and Φ_{avg} is the average of all Φs for that specific condition. The CAMs implanted with PLA microfiber patches coated with VEGF-encapsulating PLGA microparticles presented the smallest σ indicating a more anisotropic vessel orientation compared to the other control samples.

Overall, the results of this study demonstrate an advanced method to improve the controllability of neovessels’ orientation and spacing as well as their densities. The integration of electrospinning and electrospraying has enabled us to assemble microfiber patches coated with drug-releasing microparticles through the temporal control of electrostatic interactions between fibers and particles. This in situ assembly technique has the advantage of improving bond strength between fibers and particles compared to previously reported processes such as electrospraying a mixture of microparticles and microfiber-forming polymers and electrospraying microparticles over pre-made microfibers [17, 22]. This allows us to functionalize microfiber surfaces with bioactive growth factor loaded particles without undergoing chemical modification, which can potentially influence fiber strength and stability. Additionally, the use
of PLA as a microfiber material enabled the patch to maintain its original fiber alignment, while the PLGA microparticles degraded and released VEGF.

We interpret that the VEGF released from the microparticles locally distributes around the PLA microfibers, as confirmed with immunofluorescent imaging. The sequestration of VEGF was likely caused by its association with proteins and glycoproteins adsorbed on the fibers’ surfaces, and subsequently limited diffusion from their surfaces [22]. Additionally, the stable fusion of microparticles on microfibers prevents the dislocation of particles at the implantation site, which is often encountered using microparticles as drug carriers [23]. The elastic modulus and ultimate strength of the patches are on a megapascal scale which maintains their structural integrity and fiber alignment against external biomechanical forces exerted at the implant site. The resulting concentration gradients around the microfibers stimulate the migration of endothelial cells towards the PLA microfibers; the cells anchor to the fibers as examined in vitro, and ultimately direct blood vessels’ orientation along the microfibers [22]. This interpretation is supported by a limited in vivo blood vessel growth and alignment from control samples which do not release VEGF or do not have the microfiber structure. It may be suggested that in situ encapsulation of VEGF inside PLA microfibers, using a co-axial spinning technique, will have the same results; however, this approach may encounter difficulties in releasing VEGF at a desired rate.

In addition, current assembly methods of angiogenic patches should further be improved to control the spacing between microparticles and the anisotropic alignment of microfibers at larger length scales in a refined manner—ultimately to enhance the ability to control the orientation and spacing of engineered blood vessels. Finally, the angiogenic microfiber patches developed in this study will be highly useful for a variety of biological applications, including wound healing, tissue regeneration, and in vitro stem cell cultures that require revascularization [24-25]. In addition, the in situ electrospinning-electrospraying process can readily be used to assemble microfiber/microparticle patches with a wide array of biopolymers.
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2.5 Figures and tables

Figure 2.1. Schematic depicting the formation of aligned blood vessels along PLA fibers coated with VEGF-encapsulating PLGA microparticles. The VEGF (green spheres) is locally released from the PLGA microparticles (white spheres), forming concentration gradients that guide vessel growth along the direction of the PLA fibers.
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Figure 2.4. Optical micrographs of PLA fibers coated with PLGA microparticles by (left) simultaneous electrospinning-electrospraying and by (right) sequential electrospraying onto premade PLA fibers. This result demonstrates that simultaneous electrospinning-electrosprinning method is the key to enhance association between PLGA microparticles and PLA fibers.
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Figure 2.6. The PLGA microparticles bound to PLA microfibers gradually degraded over four weeks, while the PLA microfibers remained stable.
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Figure 2.10. (a) Optical images of vascular networks and (b) microscopic images of histological cross-sections of CAMs. In (b), The mature vessels in CAM were indicated through positive staining for α-smooth muscle actin. Samples I through IV represent CAMs implanted with PLA fiber patches (I), VEGF-encapsulating PLGA microparticles (II), PLA fiber patches coated with blank PLGA microparticles (III), and PLA fiber patches coated with VEGF-encapsulating PLGA microparticles, respectively (IV). (b) The blood vessel density, the area of blood vessels normalized to overall tissue area, was the largest for the implantation of PLA fibers coated with VEGF-encapsulating PLGA microparticles (Sample IV). (c) The index of isotropic orientation (σ), quantified using Eq. (1), was minimal for the blood vessels of CAMs implanted with PLA microfiber patches coated with VEGF-encapsulating PLGA microparticles. The difference of the vessel density in (c) and the orientation deviation between the Sample IV and other conditions is statistically significant (* p < 0.05). Four or more different CAM images were used per condition for this image analysis.
Figure 2.11. (a) An optical image of a control CAM’s vascular network and (b) a microscopic image of a CAM cross-section incubated with an antibody for α-smooth muscle actin. The CAM control sample was not exposed to soluble factors or any materials.

Figure 2.12. An optical image of a CAM vascular network implanted with a patch, which presents poorly aligned PLA fibers coated with VEGF-encapsulating PLGA microparticles.

Figure 2.13. Schematic of quantifying the index of isotropic orientation of blood vessels (σ). The σ was determined according to the standard deviations of the aspect ratios’ Gaussian distributions.
2.6 References


Chapter 3: Microfabrication of proangiogenic cell-laden alginate-g-pyrrole hydrogels*

3.1 Abstract

Cells have been extensively studied for their uses in various therapies because of their capacities to produce therapeutic proteins and recreate new tissues. It has often been suggested that the efficacy of cell therapies can greatly be improved through the ability to localize and regulate cellular activities at a transplantation site; however, the technologies for this control are lacking. Therefore, this study reports a cell-laden hydrogel patch engineered to support the proliferation and angiogenic growth factor expression of cells adhered to their surfaces, and to further promote neovascularization. Hydrogels consisting of alginate chemically linked with pyrrole units, termed alginate-g-pyrrole, were prepared through an oxidative cross-linking reaction between pyrrole units. Fibroblasts adhered to the alginate-g-pyrrole hydrogels, and exhibited increased proliferation and overall vascular endothelial growth factor (VEGF) expression, compared to those on pyrrole-free hydrogels. Furthermore, the alginate-g-pyrrole hydrogel surfaces were modified to present microposts, subsequently increasing the amount of pyrrole units on their surfaces. Cells adhered to the microfabricated gel surfaces exhibited increased proliferation and overall VEGF expression proportional to the density of the microposts. The resulting micropatterned alginate-g-pyrrole hydrogels exhibited increases in the size and density of mature blood vessels when implanted on chick chorioallantoic membranes (CAMs). The hydrogel system developed in this study will be broadly useful for improving the efficacy of a wide array of cell-based wound healing and tissue regenerative therapies.

3.2 Introduction

A wide array of cells have been extensively studied for the stimulation of neovascularization in ischemic tissues, wounds, and tissue defects because of their capacity to endogenously produce multiple

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proangiogenic growth factors and recreate new tissues [1-6]. These cells are often transplanted at a target site via an intramuscular injection, but this approach is often plagued by a significant cell loss caused by external mechanical forces, and a limited cellular capacity to sustainably produce angiogenic growth factors [3,7-11]. Therefore, efforts have been made to transplant cells processed in the form of sheets, to prevent cell loss, and to also support cellular therapeutic activities [8,12]. The use of cell sheets in various wound healing and tissue regenerative therapies has reported some impressive results; however, there is still a need to develop methods to increase the mechanical strength of and the endogenous therapeutic protein expression from these cell sheets, at an implant site.

Recently, several studies have reported that certain soluble factors found in inflammatory tissues stimulate angiogenic pathways through the binding of specific cellular receptors [13-14]. As a result, these soluble factors significantly promote revascularization and further enhance wound healing. One of these soluble factors is (carboxyalkyl)pyrrole proteins (CAPs), produced by free radical oxidation of plasma low-density lipoproteins [15]. The pyrrole units of CAPs have been shown to bind with cellular receptors including Toll Like Receptor 2 (TLR2), and subsequently activate oxidative stress pathways involved in cell proliferation and angiogenic factor expression, including vascular endothelial growth factor (VEGF) [13,16-18]. These results imply that the integration of CAP-like pyrrole-based molecules with transplanted cells would improve the cellular activities relevant to neovascularization; however, this strategy has not been carefully explored to date.

Therefore, we hypothesized that a water-soluble polymer chemically linked with pyrrole units can form a hydrogel through the oxidative cross-linking of the pyrrole units, and sustainably modulate the proliferation and angiogenic factor expression of cells adhered to the hydrogel through the pyrrole density (Fig. 3.1). We examined this hypothesis by preparing a hydrogel with alginate chemically substituted with pyrrole units. The amount of pyrrole units on the hydrogel surface was controlled by replacing pyrrole units with cross-linkable methacrylic units, while keeping the total number of cross-linkable units constant. This approach enabled us to control the amount of pyrrole units, without significantly changing
other gel properties, such as the elastic modulus and swelling ratio. The hydrogel surfaces were also modified by introducing micro-sized posts with varied spacings, to further control the amount of pyrrole units available for cell binding. Fibroblasts were seeded on these hydrogel surfaces to examine the role of the pyrrole units on cellular proliferation and VEGF expression. Finally, cell-laden alginate-g-pyrrole hydrogels were implanted on chick chorioallantoic membranes (CAMs) to examine their ability to enhance neovascularization. Overall, this study serves to advance the controllability of cellular therapeutic activities.

3.3 Experimental

3.3.1 Materials

Sodium alginate ($M_w \sim 250,000$, FMC Technologies) was provided by FMC Biopolymer. Ammonium persulfate (APS), 2-aminoethyl methacrylate hydrochloride (AEM), bovine serum albumin (BSA), 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, 1-(2-cyanoethyl)pyrrole (CEP), sodium hydroxide (NaOH), sodium bicarbonate, fluorescein isothiocyanate (FITC), formaldehyde, and lithium aluminum hydride (LAH) were purchased from Sigma-Aldrich Company (St. Louis, MO). Anhydrous ether was purchased from Mallinckrodt Chemicals. 1-hydroxybenzotriazole (HOBt) was purchased from Fluka (St. Louis, MO). SU-8 photoresist was purchased from Microchem Inc. Polydimethylsiloxane (PDMS) silicon elastomer (Sylgard 184 Silicon Elastomer) and curing agent were purchased from Dow Corning. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Thermo Scientific. Celite was purchased from Fisher Chemical. Phosphate buffered saline (PBS), Dulbecco’s modification of Eagle’s medium (DMEM), and Penicillin/Streptomycin (P/S, 10,000U/mL / 10,000mg/mL) was purchased from Cellgro. Fetal Bovine Serum (FBS) and trypsin-EDTA (0.5%) was purchased from Invitrogen. MTT Cell Proliferation Assay was purchased from ATCC. Mouse Duo VEGF enzyme-linked immunosorbent assay (ELISA) and ELISA reagents were purchased from R&D Systems. Anti-integrin beta 3 antibody and anti-integrin $\alpha_v$ antibody were purchased from ABCAM.
Oregon Green® 514 Phalloidin and 4, 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen.

3.3.2 Synthesis of N-(2-aminopropyl)pyrrole (APP)

N-(2-amino propyl)pyrrole (APP) was synthesized according to previously reported procedures [19]. Briefly, a solution of 0.2 M 1-(2-cyanoethyl)pyrrole (CEP) dissolved in anhydrous ether was added dropwise to a suspension of lithium aluminum hydride (LAH) in anhydrous ether, and the resulting mixture was refluxed overnight. After the mixture was cooled, excess LAH was quenched by the addition of water and a 15% NaOH solution. The mixture was then filtered through Celite and the water in the mixture was evaporated to collect APP.

3.3.3 Conjugation of pyrrole or methacrylate units to alginate

Sodium alginate was irradiated with γ-rays from a 60Co source at a dose of 2 Mrad for 4 hours, in order to decrease the M_w to 100,000 g/mol, as determined through gel permeation chromatography (GPC), as previously reported [20,21]. Irradiated alginate was dissolved in 0.1 M MES buffer (pH 6.4) at a concentration of 1% (w/v). Next, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and APP and/or 2-aminoethyl methacrylate hydrochloride (AEM) were dissolved in the alginate solutions and stirred for 18 hours. The molar ratio of HOBt, EDC, and APP/AEM was kept constant at 2:2:1. The molar ratio of APP to uronic acids of the alginate was varied from 0 to 0.2, while the molar ratio of AEM to uronic acids was varied from 0 to 0.1. The mixture was then dialyzed with deionized (DI) water for three days, while replacing it with fresh water every 12 hours. The dialyzed polymer solutions were lyophilized and reconstituted to a 7 wt% stock solution with PBS.

The degree of pyrrole substitution to alginate was evaluated by measuring the UV absorbance of the resulting polymers. Alginate substituted solely with pyrrole units (alginate-g-pyrrole), alginate substituted with both pyrrole and methacrylic groups (alginate methacrylate-g-pyrrole), and alginate
substituted only with methacrylic groups (alginate methacrylate) were dissolved in DI water at a 0.01\%(w/v) concentration, and the UV absorbance values of the solutions at a wavelength of 210 nm were measured using a CARY 500 Scan UV-Vis NIR Spectrometer. A standard curve, created by measuring the absorbance values of 0.01\%(w/v) alginate solutions containing known quantities of APP, was used to determine the degree of pyrrole substitution. Separately, the degree of methacrylate substitution to alginate was evaluated with the ratio of acrylate to pyrrole peaks of $^1$H NMR (500 MHz, D$_2$O).

3.3.4 Viscosity measurements of pre-gelled solutions

The viscosities of pre-gelled polymer solutions were measured using a rheometer. Polymer solutions with a concentration of 5wt\% were loaded between the cone and plate of a rheometer (Bohlin, CS-50). The solution was subject to a steady shear stress of 1.13 Pa over 20 seconds, and the viscosity was calculated from the shear rate measured by the rheometer. The temperature was kept constant at 25$^\circ$C.

3.3.5 Preparation of hydrogels

Alginate-g-pyrrole, alginate methacrylate-g-pyrrole, and alginate methacrylate hydrogels were prepared through the cross-linking of the pyrrole and/or methacrylic groups conjugated to alginate. Pre-gel polymer solutions were first mixed with a solution of ammonium persulfate (APS). The final polymer and APS concentrations were 5 \text{ wt}\% and 0.1 M, respectively. Next, the mixtures were poured between two glass plates separated by 1 mm spacers, and incubated at 70 $^\circ$C for one hour. The hydrogels formed between the glass plates were punched into disks with a 0.5 or 1 cm diameter for further characterizations.

For experiments introducing controlled micropost densities on the hydrogel surfaces, the pre-gelled mixtures containing APS were poured onto PDMS substrates with controlled densities of micro-sized wells (Fig. 3.2). The mixtures were incubated at 70 $^\circ$C for one hour to prepare hydrogel disks with varied densities of micro-posts: 0, 25, 100, and 225 posts/cm$^2$. The diameter of hydrogel disks was kept
constant at 8 mm. All of the hydrogels were incubated in PBS (pH 7.4) at 37°C for 24 hours, exchanging it with fresh media every 4 to 8 hours.

3.3.6 Characterization of hydrogel properties

The stiffness of the hydrogels was evaluated by measuring a compressive elastic modulus. Following the incubation in PBS for 24 hours, the gel disks with a 1 cm diameter and 1 mm thickness were compressed at a rate of 1 mm/min using a mechanical testing system (MTS Insight). The elastic moduli \( (E) \) of the gels were calculated from the linear slope of the stress \((\sigma)\) versus strain \((\epsilon)\) curve for the first 10% strain. The shear moduli \( (G) \) were calculated from the linear slope of the stress versus \( -(v-v^2) \) curve, where \( v = 1 - \epsilon \), for the first 10% strain. In parallel, the degree of swelling \((Q)\) of the gels were calculated following:

\[
Q = \rho_p \left[ \frac{Q_m}{\rho_s} + \frac{1}{\rho_p} \right]
\]

where \( \rho_p \) is the polymer density \((1.6 \text{ g/cm})\), \( \rho_s \) is the density of water, and \( Q_m \) is the swelling ratio, which is defined as the mass ratio of hydrated gels to dried gels. The number of cross-links \((N)\) were then calculated based on rubber elasticity theory as follows [22]:

\[
N = \frac{GQ^{1/3}}{RT}
\]

where \( R \) represents the gas constant \((8.314 \text{ J mol}^{-1} \text{ K}^{-1})\) and \( T \) represents the temperature at which the modulus was measured, 25°C.

3.3.7 In vitro cell culture on hydrogels

Fibroblasts (NIH 3T3) were seeded on hydrogels with varied pyrrole concentrations and micropost densities to examine their proliferation and VEGF expression. Fibroblasts between passage numbers 10 and 15 were seeded on hydrogels disks at a density of 1,000 cells per disk, and were cultured in DMEM supplemented with 10% FBS and 1% P/S at 37°C. After 4, 24, 48, and 96 hours, media was
collected and exchanged with fresh media. The VEGF concentrations in the media were measured using a VEGF ELISA kit, following the manufacturer’s protocol. A calibration curve, prepared by measuring the absorbance values of standards with known concentrations of VEGF, was used to quantify the concentrations of VEGF in the collected cell culture media.

In parallel, the number of viable cells on the hydrogel surfaces was measured using an MTT Cell Proliferation Assay, following the manufacturer’s protocol. Briefly, cells on the hydrogels were incubated in fresh media containing the MTT reagent for four hours. Metabolically active, viable cells reduce the MTT reagent to a measurable colorimetric formazan dye, and the UV absorbance values of the formazan dye are therefore proportional to the cell density. Next, an MTT detergent was added to the cell culture media, followed by a four-hour incubation at room temperature. The absorbance values of the extracted formazan dye, at 570 nm, were measured using a spectrophotometer (Synergy HT, BioTek).

Cell-hydrogel interactions were also evaluated by examining cell morphologies and intracellular actin polymerizations. Again, cells were seeded on hydrogels at a density of 1,000 cells per gel, and were further incubated in DMEM supplemented with 0.1% or 10% FBS and 1% P/S. Cells were imaged throughout a 24-hour incubation period using a DIML inverted microscope (Leica) linked with a D-Lux E camera (Leica). In parallel, after 24 hours, cells were fixed using 10% neutral buffered formalin—3.7% formaldehyde in PBS—and were subsequently incubated with Oregon Green® phalloidin and DAPI using standard fluorescent staining procedures. Fluorescence images of the cells were captured using a multi-photon microscope (Zeiss 710, Mai Tai eHP Ti:sapphire laser), following standard imaging procedures.

Additionally, cellular integrin blocking experiments were performed to examine specific cell-hydrogel interactions. Fibroblasts were separately exposed to anti-β3 and anti-αV antibodies, for 30 minutes, prior to seeding on hydrogels. Then, the cells were seeded on the hydrogels, and were incubated in DMEM supplemented with 0.1 % FBS and 1% P/S. After 24 hours, cellular morphology and intracellular actin polymerizations were examined, following the procedures described above.
3.3.8 In vivo chorioallantoic membrane assay for neovascularization

Fertilized chicken eggs (Hy-Line W-36) were obtained from the University of Illinois Poultry Farm (Urbana, IL). The eggs were incubated for 7 days horizontally inside an incubator at 37°C and 65% humidity. Next, a hole with a diameter of ~ 2 cm was made in the side of the egg shells by carefully removing shell fragments. The hole on the egg shells were covered with tape (Scotch) and incubated at 37°C overnight to ensure survival after initial shell opening. Next, hydrogel samples were placed on the individual CAMs of the embryos. After implantation, the eggs were incubated at 37°C for 7 days. Images of the vasculature were captured at the initial implantation and 7 days after the initial implantation using a S6E stereomicroscope (Leica) linked with D-Lux E Camera (Leica). All CAM assays were performed under sterile conditions.

On the 7th day after the initial implantation, embryos were fixed with a 10% neutral buffered formalin solution. After 4 hours, a 1 cm x 1 cm CAM tissue sample at the implantation site, was excised using suture scissors. The samples were then processed for paraffin embedding, sectioned, and stained for α-smooth muscle actin (αSMA), using standard immunohistological techniques, in order to examine and quantify mature blood vessels. CAM cross-sections stained for αSMA were used to quantify blood diameters and vessel densities using image processing software (ImageJ).

3.3.9 Statistical Analysis

Four to six samples were analyzed per condition unless otherwise specified for all experiments. One-way analysis of variance (ANOVA) was used to determine the statistical significance of data and Scheffe Post Hoc tests were applied to all pair-wise differences between means. Data was considered significant for \( p \) values < 0.05.
3.4 Results

3.4.1 Chemical modification of alginate with pyrrole and/or methacrylate units

Pyrrole and methacrylate units were chemically conjugated to alginate using carbodiimide chemistry. For the pyrrole conjugation, 1-(2-cyanoethyl)pyrrole (CEP) was first reduced to N-(2-amino propyl)pyrrole (APP) using LiAlH₄ in ether (Fig. 3.3). The chemical structure of APP was confirmed with ¹H NMR and matched that of the reference compound (Fig. 3.4). Next, APP was attached to alginate by coupling the amine of APP with the carboxylate group of a uronic acid of alginate using 1-hydroxybenzotriazole (HOBT) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Fig. 3.5). The conjugation of pyrrole to alginate was confirmed by the loss of the peak α to the amine at 2.4 ppm in the ¹H NMR of alginate obtained from the reaction. The degree of pyrrole substitution to alginate (DS\textsubscript{py}) was determined to be 0, 8, and 14 %, as evaluated using UV absorbance measurements at 210 nm.

The alginate-g-pyrrole with a DS\textsubscript{py} of 8% was further reacted with 2-aminoethyl methacrylate hydrochloride (AEM) to substitute alginate with both pyrrole units and methacrylate units, termed alginate methacrylate-g-pyrrole (Fig. 3.5). Separately, AEM was reacted with unmodified alginate to prepare alginate methacrylate, free of pyrrole units. The methacrylates linked to the alginate were also confirmed with ¹H NMR. According to NMR and UV-Vis analysis, the DS of methacrylic units (DS\textsubscript{MA}) to the alginate-g-pyrrole was 10%. Overall, three different polymers were synthesized for the proceeding studies: (1) alginate methacrylate with a DS\textsubscript{MA} of 10 %, (2) alginate methacrylate-g-pyrrole with a DS\textsubscript{py} of 8 % and DS\textsubscript{MA} of 10 %, and (3) alginate-g-pyrrole with a DS\textsubscript{py} of 14 % (Table 3.1).

3.4.2 Hydrogel preparation

All polymers prepared above were soluble in aqueous media. At a polymer concentration of 5wt%, the viscosities of the pre-gel solutions, measured at a low shear stress of 1.13 Pa, are in order from highest to lowest as follows: alginate-g-pyrrole > alginate methacrylate > alginate methacrylate-g-pyrrole. The incorporation of ammonium persulfate in these pre-gel polymer solutions, and a subsequent
application of heat, activated cross-linking reactions forming hydrogels. A control mixture of unmodified alginate and ammonium persulfate did not activate the formation of a hydrogel (not shown here). Independent of the viscosity of pre-gelled solution, there were no significant differences in the elastic moduli and swelling ratios between the conditions. Accordingly, there were no significant differences in the cross-linking density between the hydrogel conditions, calculated using Eq. (2) (Table 3.1). Overall, the hydrogels prepared in this study present controlled quantities of pyrrole units, with limited differences in gel stiffness and water content.

3.4.3 Cell adhesion to hydrogels with varied pyrrole densities

3T3 mouse fibroblasts were seeded on hydrogels with controlled amounts of pyrrole units to examine the effects of the pyrrole units on cell adhesion. In media supplemented with 10 % FBS, cells adhered to alginate-g-pyrrole hydrogels with a DS$_{py}$ of 14 % and presented a larger surface area and contour length compared to those on alginate methacrylate hydrogels with a DS$_{py}$ of 0% (Fig. 3.6). Decreasing the FBS concentration to 0.1 % resulted in a more significant difference in the cell surface area and contour length between the two conditions (Fig. 3.7). Specifically, cells adhered to the alginate methacrylate hydrogels, free of pyrrole units, exhibited minimal cell spreading. Additionally, at the low FBS concentration, there were more active actin polymerizations in the cells adhered to the alginate-g-pyrrole hydrogels compared to those adhered to the alginate methacrylate gels.

Additionally, cells were exposed to antibodies for α$_v$ and β$_3$ integrins before seeding them on alginate-g-pyrrole hydrogels, and were cultured in media supplemented with 0.1 % FBS. This experiment was conducted to examine whether the role of pyrrole units in inducing cell adhesion to the gel is mediated by the blocking of cellular integrins, similar to previous studies using (carboxy ethyl) pyrrole. These integrin blocking experiments were conducted to examine whether the cells adhere to the alginate-g-pyrrole hydrogels through specific interactions between cellular integrins and the pyrrole units.
Interestingly, cells exposed to either an antibody for the $\alpha_V$ integrin or an antibody for the $\beta_3$ integrin did not exhibit cell spreading on the hydrogels.

3.4.4 Cellular proliferation and vascular endothelial growth factor expression on hydrogels with controlled amounts of pyrrole

The effect of the amount of pyrrole on cellular proliferation was evaluated by measuring increases in the number of metabolically active, viable cells over time, using the MTT cell proliferation assay—the number of viable cells is proportional to the absorbance of a reduced MTT reagent. Interestingly, cells proliferated more actively on hydrogels modified with the largest quantity of pyrrole units, exhibited by a significant increase in the absorbance of reduced MTT reagent over time. Additionally, the cellular expression of vascular endothelial growth factor (VEGF) was evaluated for cells on hydrogels with controlled pyrrole amounts, by measuring the amount of VEGF released into the cell culture media. Cells cultured on the hydrogels exhibited a linear increase in the levels of overall VEGF expression over three days, independent of the pyrrole amount; however, after 4 days, the overall cellular VEGF expression increased for hydrogels with a larger amount of pyrrole (Fig 3.8).

3.4.5 Micropatterned alginate-g-pyrrole hydrogel surfaces for tuning cellular growth and VEGF expression

Alginate-g-pyrrole hydrogels having a $D_{SPy}$ of 14% were further modified to present controlled densities of micro-sized posts on their surfaces, and subsequently increase exposure of pyrrole units. These hydrogels were formed by pouring pre-gel polymer solutions on PDMS molds with controlled densities of microwells and activating the cross-linking reaction between pyrrole units. The resulting hydrogels presented the same micropost densities as the microwell densities of the PDMS molds. The diameter and height of the microposts were kept constant at 500 and 50 $\mu$m, respectively, so that the cell
would not recognize differences in surface roughness. The densities of the microposts were varied from 0 to 225 posts/cm² (Fig. 3.9).

Once more, fibroblasts were seeded on the alginate-g-pyrrole hydrogels with controlled micropost densities. Cells were fully spread over the micropost surface, similar to those adhered to the flat alginate-g-pyrrole hydrogels (Fig. 3.10). Increasing the micropost density from 25 to 225 posts/cm² resulted in a larger increase in the number of metabolically active cells over time, as characterized through an increase in the absorbance of the reduced MTT reagent over time. In addition, increasing the micropost density from 0 to 25 posts/cm² resulted in a five-fold increase in overall VEGF expression levels over four days. Additionally, increasing the micropost density from 25 to 250 posts/cm² led to an even larger increase in the overall VEGF expression.

3.4.6 Neovascularization stimulated by cell-laden alginate-g-pyrrole hydrogels

The capacity of the alginate-g-pyrrole hydrogels to stimulate neovascularization was examined by implanting fibroblast-laden gels on chicken chorioallantoic membranes (CAMs). Fibroblasts were seeded on flat alginate methacrylate hydrogels, flat alginate-g-pyrrole hydrogels, and alginate-g-pyrrole hydrogels with microposts at a density of 225 posts/cm², and then were implanted on CAMs. After seven days, the fibroblast-laden alginate-g-pyrrole hydrogels stimulated the formation of blood vessels in larger densities and with larger sizes at the implantation site, as compared to hydrogels containing no pyrrole. There were no significant inflammatory responses at the implantation site for all conditions, as confirmed with minimal number of inflammatory cells recruited into the area. According to histological images stained with an antibody for α-smooth muscle actin, cell-laden alginate-g-pyrrole hydrogels induced a 1.5-fold increase in the blood vessel density, from 0.15 to 0.2 mm²/mm², and exhibited blood vessels with significantly larger diameters (Fig. 3.11). There was a minimal increase in the blood vessel density for CAM samples implanted with cell-laden alginate methacrylate hydrogels compared to samples without any implants.
Furthermore, cell-laden alginate-g-pyrrole hydrogels modified to present microposts increased the blood vessel density from 0.2 to 0.4 mm$^2$/mm$^2$ compared to flat cell-laden alginate-g-pyrrole gels, and exhibited the largest blood vessels compared to all other conditions (Fig. 3.11). Overall, fibroblast-laden hydrogels modified with pyrrole units and microposts led to an over three-fold increase in the blood vessel density, with significantly larger blood vessels, compared to cell-laden alginate methacrylate hydrogels and control conditions with no implants.

3.5 Discussion

The results of this study demonstrate that cells adhered to hydrogels, designed to sustainably present CAP-mimicking pyrrole units, exhibit an increased proliferation and an overall larger VEGF expression, and an enhanced neovessel formation when implanted in vivo. Hydrogels were designed to present controllable amounts of cross-linkable pyrrole units, by chemically conjugating varied amounts of pyrrole units to alginate and fabricating the gel surfaces with controlled micropost densities. Fibroblasts adhered to these gels exhibited increased proliferation rates, which resulted in high levels of overall VEGF expression proportional to the degree of pyrrole substitution to alginate and the micropost densities on the hydrogel surfaces. Therefore, fibroblasts adhered to the hydrogels presenting the largest quantity of pyrrole units significantly increased the density and size of the blood vessels formed at and around implantation sites.

One of the interesting findings of this study is that the pyrrole units conjugated to alginate can act as a cross-linkable unit to form hydrogels. The alginate-g-pyrrole has been previously synthesized and used in various biosensor applications; however, there has not been any study that prepares hydrogel matrices via the cross-linking reaction between pyrrole units [23-25]. We interpret that the pyrrole units were activated chemically through the addition of APS, and covalently linked with one another to form a hydrogel, according to the mechanism proposed by Diaz and colleagues (Fig. 3.12) [26,27]. Furthermore, the addition of methacrylate units to form alginate methacrylate-g-pyrrole can make additional...
interconnected cross-linking junctions, through an independent radical polymerization mechanism. These interpretations are supported by an insignificant difference in the number of cross-links calculated from elastic moduli and swelling ratios of the hydrogels.

The resulting cross-linked pyrrole units of the hydrogels were advantageous in sustainably presenting insoluble biochemical signals for the stimulation of cell growth and VEGF expression. According to the immuno-blocking experiments, fibroblasts could anchor to the alginate-g-pyrrole hydrogels by forming specific bonds between pyrrole units and integrins, including αVβ3 heterodimers. We therefore suggest that the integrins expressed on the cellular membranes activate intracellular actin polymerizations, and further lead to more extensive cell spreading than cells seeded on pyrrole-free alginate methacrylate hydrogels. This pyrrole-induced cell adhesion might have stimulated cellular signaling related to proliferation, as displayed with increases in the cellular growth rates and overall VEGF expression levels, proportional to the degree of pyrrole substitution to the alginate. However, it would be important to examine which specific integrins modulate cellular VEGF expression via fluorescence-activated cell sorting (FACS) analysis in future studies [28,29]. Additionally, there is also a possibility that the pyrrole units facilitate the adsorption of cell adhesion proteins to the hydrogel surface and subsequently activate cellular integrin expression, specifically in media supplemented with sufficient levels of serum proteins; however, this possibility needs to be systematically investigated in future studies.

Furthermore, this study has demonstrated that the surface topology of alginate-g-pyrrole hydrogels is also an important factor for regulating cellular activities, because it significantly increases the amount of pyrrole units that can bind with cellular integrins. With an assumption that the pyrrole units are uniformly distributed in the hydrogel matrices, we interpret that increasing the density of microposts on the hydrogel surfaces from 0 to 225 posts/cm² lead to nearly an 18% increase in the number of pyrrole units, because of an additional vertical dimension. This increase in the amount of pyrrole units on the hydrogel surfaces should provide cells with a larger number of sites to anchor and
further spread. It is not likely that the micropost structures such as the surface roughness stimulates cellular proliferation and VEGF expression, independent of the pyrrole units, because the micropost diameters are significantly larger than the diameter of fully spread cells. The enhanced cellular growth and VEGF expression on hydrogels modified with microposts are attributed solely to larger cell adhesion areas.

Based on the in vitro studies, we propose that cells adhered to alginate-g-pyrrole hydrogels modified with microposts, sustainably secrete proangiogenic growth factors, including VEGF, at the implantation sites. These subsequent angiogenic growth factors actively stimulate the sprouting of mature blood vessels from pre-existing ones. The limited sprouting of blood vessels around the alginate methacrylate hydrogels also demonstrates the critical role pyrrole units have on stimulating neovascularization. Another interesting finding was that the cells that adhered to alginate-g-pyrrole hydrogels had also significantly increased the diameter of blood vessels around the implant. Several of these blood vessels had diameters equivalent to those of arterioles with an average diameter of 30 μm. We envisage that the hydrogels prepared in this study stimulated fibroblasts to express additional growth factors and cytokines crucial to the expansion of blood vessels; however, this aspect needs to be examined more systematically in future studies [30].

As a follow-up study, the alginate-g-pyrrole hydrogel may further be modified to have controllable biodegradation, in order to effectively remove the gel from the implantation site following treatment. Previous studies have demonstrated that oxidation of alginate with sodium periodate can create hydrolytically labile acetal-like linkage in the backbone of alginate and the gel prepared with the oxidized alginate gradually degrades under physiological conditions [20,21]. Furthermore, the gel degradation can be tuned according to the mass fraction of the oxidized alginate and also the number of acetal-like linkage in the polymer. Additionally, it would be essential to study whether both alginate-g-pyrrole and degraded polymeric fragments present any toxicity and also stimulate any inflammation over a desired treatment period.
3.6 Conclusion

This study presents an advanced hydrogel system that can significantly enhance the efficacy of transplanted cells for stimulating neovascularization. This hydrogel was assembled by activating the chemical cross-linking reaction between pyrrole units linked to alginate and further introducing micro-sized posts on the gel surfaces. The resulting hydrogels modulated the proliferation and overall VEGF expression of fibroblasts through controlling the amount of pyrrole units. Additionally, the alginate-g-pyrrole hydrogels fabricated to present microposts on their surfaces further improved cellular proangiogenic activities for vascularization, because of the larger amount of pyrrole units on the hydrogel surface than the flat hydrogels. We believe that this material design strategy can be readily extended to a wide array of hydrogels used as cell carriers. In addition, the hydrogel developed in this study will be broadly useful to regulate a wide range of stem and progenitor cells that can potentially be used for wound healing and tissue regeneration.
3.7 Figures and tables

**Figure 3.1.** Schematic of cell-laden alginate-g-pyrrole hydrogels with microposts on the surface. The hydrogel was designed to stimulate cellular endogenous expression of proangiogenic growth factors. These hydrogels sustainably present oxidized pyrrolic units (b) similar to CAPs (a), and biochemically stimulate cellular production of proangiogenic growth factors (c).

**Figure 3.2.** Preparation of PDMS micromolds used to make hydrogels with microposts. PDMS molds with varied densities of micro-wells were created using standard microfabrication techniques. First, silicon master molds were made by spin coating SU-8 photoresist resin over the surface of 4 inch silicon wafers. The photoresist layer thickness was kept constant at 50 μm. Then, the wafers were exposed to a 365nm UV light through a mask prepared to present controlled densities of 500 μm diameter circles. The unpolymerized photoresist resin was removed, resulting in a master mold with controlled micropost densities. Second, PDMS molds were created by pouring a 1:10 mixture of curing agent to silicon elastomer on the master molds, and curing them at 90°C for two hours. The resulting PDMS molds exhibited circular microwells with a 50 μm depth and a 500 μm diameter. The microwell densities were varied from 0, to 25, 100, 225 wells/cm².
Figure 3.3. Chemical reaction schemes. (a) APP synthesized through the reduction of the cyano group of CEP using LiAlH₄. The conjugation of APP (b) and AEM (c) to alginate using HOBT and EDC.

Figure 3.4. ¹H NMR (500Hz, D₂O) analysis. (a) NMR spectra that confirms the synthesis of APP and its conjugation to alginate. (b) NMR spectra of alginate methacrylate, alginate methacrylate-g-pyrrole, and alginate-g-pyrrole.
Figure 3.5. Chemical reaction schemes of (a) alginate methacrylate, (b) alginate methacrylate-g-pyrrole, and (c) alginate-g-pyrrole. These polymers were prepared via the chemical conjugation of methacrylic and/or pyrrole units to alginate.

Figure 3.6. Adhesion of fibroblast onto hydrogels with controlled pyrrole densities. (a) Cells adhered on the alginate methacrylate hydrogels with a DS$_{py}$ of 0%, termed 0% PY, and on the alginate-g-pyrrole hydrogels with a DS$_{py}$ of 14%, termed 14% PY. Cells adhered to the hydrogels were cultured in media supplemented with 0.1% and 10% FBS. Intracellular actin and nuclei were stained with phalloidin (green color) and DAPI (blue color), respectively. (b) Immuno-blocking experiments for testing cellular adhesion to the hydrogels. Prior to seeding cells on alginate-g-pyrrole hydrogels, cells were incubated with antibodies for either α$_{V}$ or β$_{3}$ integrins. In (b), two top images show the bright field image of cells and the lower two images exhibit intracellular actin and nuclei stained with
phalloidin (green color) and DAPI (blue color). Cell images displayed in (a) and (b) were captured 24 hours after seeding the cells on the hydrogels.

Figure 3.7. Bright field images of fibroblasts seeded on alginate methacrylate hydrogels with a DS_{py} of 0%, termed 0% PY, and alginate-g-pyrrole hydrogels with a DS_{py} of 14%, termed 14% PY. Cells were cultured in DMEM supplemented with 0.1% or 10% FBS.

Figure 3.8. Proliferation and VEGF expression of fibroblasts adhered to hydrogels with controlled amounts of pyrrole units. (a) Increases in the absorbance of reduced MTT reagents over four days, which represents the increased number of metabolically active cells. (b) Increases in the VEGF expression over four days. Data points and error bars represent the averages and standard deviations of cells adhered to alginate methacrylate (0% PY), alginate methacrylate-g-pyrrole (8% PY), and alginate-g-pyrrole (14% PY). * indicates the statistical significance between 14% PY and 0% PY for differences in MTT absorbance and VEGF expression (*p < 0.01). Separately, #
indicates statistical significance between 8 % PY and 0 % PY for differences in MTT absorbance and VEGF expression (*p < 0.04).

**Figure 3.9.** Cellular activities on alginate-g-pyrrole hydrogels with controlled micropost densities. (a) Hydrogels with controlled micropost densities on their surfaces were formed by pouring pre-gel solutions on PDMS molds with multiple microwells, and subsequently activating the cross-linking reaction. (b) Bright field images of hydrogels with controlled micropost densities ranging from 0 to 225 post/cm². (c) A bright field (left) and fluorescent image (right) of the cells adhered to the micropost surfaces. In the fluorescent image, cellular actin and nuclei were stained with phalloidin (green) and DAPI (blue), respectively. (d) Cell proliferation on the hydrogels with controlled micropost densities, quantified through increases in the absorbance of reduced MTT reagents at 570 nm. (e) Cellular VEGF expression normalized to hydrogels containing no microposts on their surfaces. In (d), * separately indicates the statistical significance for 225 posts/cm², as compared to the values for 0 and 25 posts/cm² (*p < 0.01). In (e), * indicates the statistical significance of the difference between 225 posts/cm² and 0 post/cm² (*p from <0.0001 to 0.03), and # indicates the statistical significance between 25 posts/cm² and 0 post/cm² (*p from 0.0002 to 0.031).
Figure 3.10. Neovascularization controlled by the cell-laden alginate hydrogels with varied pyrrole amounts and micropost densities. Conditions include fibroblasts adhered to flat alginate methacrylate hydrogels with a DS$_{Py}$ of 0%, termed Flat 0 % PY (top row), those adhered to flat alginate-g-pyrrole hydrogels with a DS$_{Py}$ of 14%, termed Flat 14 % PY (second row), and those adhered to alginate-g-pyrrole hydrogels with microposts, termed Microposts 14 % PY (third row). Images (a) and (b) exhibit vascular networks around the cell-laden hydrogels implanted on the CAMs, which were captured at the initial implantation (a) and 7 days after implantation (b), respectively. Additionally, images (c) show the CAM excised after 7 days. Images (d) exhibit cross-sections stained with an antibody to α-smooth muscle actin.
Figure 3.11. Quantitative analysis of the vascular networks controlled by the cell-laden hydrogels with varied pyrrole amounts and micropost densities. (a) The blood vessel densities of the CAM samples were quantified by the area of blood vessels identified with α-smooth muscle actin layers normalized to the overall tissue area. Differences in the vascular density between conditions were statistically significant (*p < 0.05). (b) Percentage of blood vessels with diameters larger than 100μm. The percentages of blood vessels were calculated from more than 30 vessels per condition. ■ represents percentages of blood vessels with diameters larger than 100μm, ● those larger than 200μm, ▲ and those larger than 600μm. The control condition was CAM tissues with no implantations.

Figure 3.12. Schematics of the hydrogel microstructures for (a) alginate methacrylate, (b) alginate methacrylate-g-pyrrole, and (c) alginate-g-pyrrole.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>DS$_{py}$ (%)</th>
<th>Viscosity (Pa·s)</th>
<th>Elastic Modulus (kPa)</th>
<th>Swelling Ratio (g/g)</th>
<th>Cross-Linking Density (x 10$^{-6}$ mol/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate methacrylate</td>
<td>0</td>
<td>0.17 ± 0.01</td>
<td>14 ± 3</td>
<td>24 ± 1</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>Alginate methacrylate-g-pyrrole</td>
<td>7.7</td>
<td>0.03 ± 0.00</td>
<td>18 ± 6</td>
<td>25 ± 3</td>
<td>6.2 ± 1.7</td>
</tr>
<tr>
<td>Alginate-g-pyrrole</td>
<td>13.7</td>
<td>0.49 ± 0.09</td>
<td>12 ± 2</td>
<td>23 ± 1</td>
<td>5.9 ± 0.7</td>
</tr>
</tbody>
</table>

*Table 3.1.* Analysis of the degree of substitution of pyrrole units to alginate (DS$_{py}$), viscosity of the pre-gel solutions, and properties of alginate methacrylate, alginate methacrylate-g-pyrrole, and alginate-g-pyrrole hydrogels.
3.8 References


Chapter 4: Enzymatically cross-linked injectable alginate-g-pyrrole hydrogels for neovascularization

4.1 Abstract

Microparticles capable of releasing protein drugs are often incorporated into injectable hydrogels to minimize their displacement at an implantation site, reduce initial drug burst, and further control drug release rates over a broader range. However, there is still a need to develop methods for releasing drug molecules over extended periods of time, in order to sustain the bioactivity of drug molecules at an implantation site. In this study, we hypothesized that a hydrogel formed through the cross-linking of pyrrole units linked to a hydrophilic polymer would release protein drugs in a more sustained manner, because of an enhanced association between cross-linked pyrrole groups and the drug molecules. To examine this hypothesis, we prepared hydrogels consisting of alginate substituted with pyrrole groups through a horse-radish peroxidase (HRP)-activated cross-linking of the pyrrole groups. The hydrogels were encapsulated with poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with vascular endothelial growth factor (VEGF). The resulting hydrogel system released VEGF in a more sustained manner, compared to an unmodified alginate hydrogel system. Finally, the implantation of the VEGF-releasing alginate-g-pyrrole hydrogel system on a chicken chorioallantoic membrane resulted in the formation of blood vessels with higher densities and with larger diameters, compared to other control conditions. Overall, the drug releasing system developed in this study will be broadly useful for regulating release rates of a wide array of protein drugs, and further enhance the quality of protein drug-based therapies.

4.2 Introduction

Over the past several decades, various proteins, including growth factors and antibodies, have been extensively studied to treat various wounds, tissue defects, and chronic diseases [1-6]. These
proteins have often been incorporated into various biodegradable microparticles, in order to administer them in a non-invasive manner and further attain a local and sustained drug delivery; however, microparticles often exhibit an undesirable initial drug burst, and also tend to dislocate from a delivery site due to mechanical forces [7-8]. These factors ultimately result in an unsatisfactory therapeutic treatment.

To address these challenges while retaining the capability of administering drugs in a non-invasive manner, protein-encapsulating microparticles are often incorporated in injectable hydrogels [9-13]. Hydrogels can be injected at a local delivery site during clinical procedures and can also localize microparticles at the target tissue. Furthermore, gels can modulate the release profile of drug molecules eluted from the microparticles [14-18]. Despite some impressive results, there remains a need to improve the controllability of the drug release rates from microparticle-filled hydrogel composites by modulating the interaction between protein drugs and the hydrogel matrices.

Therefore, in this study, we hypothesized that loading drug-encapsulating microparticles into a hydrogel formed from the cross-linking of alginate modified to present pyrrole groups, termed alginate-g-pyrrole, would allow us to release protein drugs in a more sustained manner, because of an enhanced association between pyrrole groups and the drug molecules. Specifically, hydrogels formed from the cross-linking reaction between pyrrole units would release protein drugs more sustainably than hydrogels containing non-cross-linked pyrrole pendants, because of a stronger association between cross-linked pyrrole units and protein molecules. To examine this hypothesis, pyrrole groups conjugated to alginate were cross-linked via a mild, enzymatic reaction, activated by a mixture of horse-radish peroxidase (HRP) and H₂O₂. Control hydrogels were also prepared by inducing ionic cross-linking for both unmodified alginate and alginate-g-pyrrole. These hydrogels were loaded with poly(lactic-co-glycolic acid) (PLGA) microparticles encapsulating vascular endothelial growth factor (VEGF), a model protein drug. The drug release from the microparticle-laden hydrogels was examined in vitro. Finally, this hydrogel system was implanted on chick chorioallantoic membranes (CAMs) to examine its capability to stimulate the
development of mature blood vessels. Overall, the hydrogel system developed in this study will serve to improve the therapeutic efficacy of a wide array of protein drug delivery systems.

4.3 Experimental

4.3.1 Materials

Sodium alginate ($M_w \sim 250,000$ Da, FMC Technologies) was provided by FMC Biopolymer. Calcium sulfate, poly(lactic-co-glycolic acid) ($M_w \sim 40$ kDa, 50:50; PLGA), 2-(N-morpholino)ethane sulfonic acid (MES) hydrate, 1-(2-cyanoethyl)pyrrole (CEP), sodium hydroxide (NaOH), poly(vinyl alcohol), lithium aluminum hydride (LiAlH$_4$), horse radish peroxidase (250-330 U/mg; HRP) and paraformaldehyde, were purchased from Sigma-Aldrich Company (St. Louis, MO). Anhydrous ether and hydrogen peroxide ($H_2O_2$) were purchased from Mallinckrodt Chemicals. 1-hydroxybenzotriazole (HOBt) was purchased from Fluka (St. Louis, MO). Dichloromethane (DCM) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Thermo Scientific. Celite was purchased from Fisher Chemical. Phosphate buffered saline (PBS) was purchased from Cellgro. VEGF, Mouse Duo VEGF enzyme-linked immunosorbent assay (ELISA), and ELISA reagents were purchased from R&D Systems.

4.3.2 Synthesis of $N$-(3-aminopropyl)pyrrole (APP)

$N$-(3-amino propyl)pyrrole (APP) was synthesized according to previously reported procedures (Fig. 4.1) [19]. Briefly, a solution of 0.2 M 1-(2-cyanoethyl)pyrrole (CEP) dissolved in anhydrous ether was added drop-wise to a suspension of lithium aluminum hydride (LiAlH$_4$) in anhydrous ether, and the resulting mixture was refluxed overnight. After the mixture was cooled, excess LiAlH$_4$ was quenched by the addition of water and a 15% NaOH solution. The mixture was then filtered through Celite, and the water in the mixture was evaporated to collect APP.
4.3.3 Conjugation of pyrrole units to alginate

Sodium alginate was irradiated with γ-rays from a $^{60}$Co source at a dose of 2 Mrad for 4 hours, in order to decrease the $M_w$ to 100,000 g/mol, as determined through gel permeation chromatography and as previously reported [20]. Irradiated alginate was dissolved in 0.1 M MES buffer (pH 6.4) at a concentration of 1% (w/v). Next, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and APP were dissolved in the alginate solutions and stirred for 18 hours. The molar ratio of HOBt, EDC, and APP was kept constant at 2:2:1. The molar ratio of APP to uronic acids of the alginate was 0.2. The mixture was then dialyzed with deionized (DI) water for three days, while replacing it with fresh water every 12 hours. The dialyzed polymer solutions were lyophilized and reconstituted to a 7 wt% stock solution with PBS. The conjugation of pyrrole to alginate was confirmed using $^1$H NMR (500 MHz, D$_2$O).

The degree of pyrrole substitution to alginate was evaluated by measuring the UV absorbance of the resulting polymer. Alginate-g-pyrrole molecules were dissolved in DI water at a 0.01% (w/v) concentration, and the UV absorbance values of the solutions at a wavelength of 210 nm were measured using a CARY 500 Scan UV-Vis NIR Spectrometer. A standard curve, created by measuring the absorbance values of 0.01% (w/v) alginate solutions containing known quantities of APP, was used to determine the degree of pyrrole substitution.

4.3.4 Preparation of VEGF-releasing PLGA microparticles

PLGA microparticles were synthesized using a double emulsion/evaporation process [21]. First, a water-in-oil emulsion was created by adding a 50 μL of a 50 mg/mL VEGF solution to 1 mL DCM containing 100 mg/mL PLGA, followed by sonication for 20 seconds (Sonic Dismembrator; Fisher Scientific). The mixture was immediately vortexed for 20 seconds in a 1% PVA aqueous solution, creating an oil-in-water emulsion. Next, the mixture was added to a 0.1% PVA aqueous solution and vigorously stirred for 3 hours, allowing the DCM to evaporate. The resulting microparticles were
repeatedly centrifuged and re-suspended in deionized water to remove residual PVA. The microparticles were then sterilized by re-suspending them in a mixture of ethanol and water with a volumetric ratio of 70 to 30, respectively. Finally, the microparticle solution was freeze dried (FreeZone6; Labconco) to attain a powder.

4.3.5 Hydrogel preparation

Alginate-g-pyrrole hydrogels were prepared through the enzymatic cross-linking of pyrrole units linked to alginate. Pre-gel polymer solutions were mixed with a solution of HRP, a solution of its substrate H$_2$O$_2$, and 0 or 10 mg/mL VEGF-releasing PLGA microparticles. The final concentrations of alginate-g-pyrrole, HRP, and H$_2$O$_2$ concentrations were 5 wt%, 2 mg/mmol pyrrole, and 12 mol/mol pyrrole, respectively. Additionally, control hydrogels including calcium cross-linked alginate gel and calcium cross-linked alginate-g-pyrrole gel were prepared by mixing a 1 mL pre-gel polymer solution with 40 μL of a 1 M calcium sulfate slurry; the final polymer concentration was kept constant at 5 wt%.

4.3.6 Characterization of hydrogel properties

The stiffness of the hydrogels was evaluated by measuring the compressive elastic modulus. Hydrogels with and without PLGA microparticles were formed between two glass plates separated by 1 mm spacers. The hydrogels were punched into 1 cm disks and incubated in PBS at 37⁰C for 24 hours. Next, the gel disks were compressed at a rate of 1 mm/min using a mechanical testing system (MTS Insight). The elastic moduli of the gels were calculated from the linear slope of the stress versus strain ($\varepsilon$) curve for the first 10% strain. The shear moduli were calculated from the linear slope of the stress versus $-(\nu - \nu^{-2})$ curve, where $\nu = 1 - \varepsilon$. In parallel, the water content, termed swelling ratio ($Q_m$), of the hydrogel disks was determined by measuring the mass ratio of hydrated gels to freeze dried polymers. The degree of swelling ($Q$) of the gels was calculated following:

$$Q = \rho_p \left[ \frac{Q_m}{\rho_s} + \frac{1}{\rho_p} \right]$$  

(1)
where $\rho_p$ is the polymer density (1.6 g/cm$^3$) and $\rho_s$ is the density of water. The number of cross-links ($N$) was then calculated based on rubber elasticity theory as follows:

$$N = \frac{GQ^{1/3}}{RT}$$  \hspace{1cm} (2)

where $R$ represents the ideal gas constant (8.314 J mol$^{-1}$ K$^{-1}$) and $T$ represents the temperature at which the modulus was measured (25°C).

### 4.3.7 Analysis of PLGA microparticle-laden hydrogels using scanning electron microscopy

Cross-section of the enzymatically cross-linked alginate-g-pyrrole hydrogels encapsulating PLGA microparticles were examined using scanning electron microscopy. The alginate-g-pyrrole hydrogels were flash frozen using liquid nitrogen, fractured, and lyophilized for imaging. Cross-sections of samples were coated with palladium and imaged at an electron intensity of 25kV (JOEL 6060LV SEM). In addition, freeze dried PLGA microparticles were imaged to quantify an average diameter and size distribution.

### 4.3.8 Characterization of VEGF release rates

Drug release profiles for hydrogels containing VEGF-encapsulated PLGA microparticles were characterized by measuring the amount of VEGF released from the hydrogels over time. Hydrogel samples containing 10 mg/ml VEGF-releasing PLGA microparticles were prepared in 15 mL conical tubes. A 2 mL release buffer, consisting of PBS, was added to the hydrogels. The samples were then incubated at 37°C over a 25 day period. The PBS was collected and replaced with fresh release buffer throughout the 25 day period. The collected buffers were immediately stored at -20°C until further analysis. After the 25 day period, the concentration of VEGF in the collected samples was measured using a VEGF ELISA kit (R&D Systems) according to the manufacture’s protocol. The release profiles for the 25 day time period were fit to a first-order drug release kinetics approximation as follows:

$$\frac{c}{c_0} = e^{-kt}$$  \hspace{1cm} (3)
where $C$ is the concentration of VEGF remaining in the hydrogel and $C_o$ is the initial amount of VEGF encapsulated, $t$ is the incubation time period, and $k$ is the VEGF release rate constant (day$^{-1}$).

4.3.9 Analysis of neovascularization using a chicken choriollantoic membrane (CAM) assay

Fertilized chicken eggs (Hy-line W-36) were obtained from the University of Illinois Poultry Farms. The eggs were initially incubated horizontally at 37°C with 65% humidity for a 7 day period. Next, a hole with a diameter of 2 cm was made in the top of the egg shells by carefully removing shell fragments. The hole was covered with tape and then incubated at 37°C overnight. Next, 100 μL hydrogel samples were injected onto the CAMs. The samples contained 10 mg/mL VEGF-encapsulating PLGA microparticles and were formed in situ, as described above. Sample conditions consisted of calcium cross-linked alginate hydrogels, enzymatically cross-linked alginate-g-pyrrole hydrogels, and VEGF encapsulating PLGA particles alone. After implantation, the holes were covered with scotch tape and then samples were incubated at 37°C for 7 days. Images of the vasculature were taken at the initial implantation and 7 days after implantation using a S6E stereomicroscope (Leica) linked with a D-Lux E Camera (Leica). All CAM assays were performed under sterile conditions.

On the 7th day after the initial implantation, embryos were fixed with a 10% neutral buffered formalin solution: a 3.7% paraformaldehyde solution in PBS. After 4 hours, 1 cm x 1 cm CAM tissue samples at the implantation sites were excised using suture scissors. The samples were then processed, paraffin embedded, sectioned, and stained for α-smooth muscle actin (αSMA), using standard immunohistological techniques, in order to examine and quantify mature blood vessels. CAM cross-sections stained for αSMA were used to quantify blood vessel densities using image processing software (Image J).

4.3.10 Statistical analysis
Four to six samples were analyzed per condition unless otherwise specified for all experiments. One-way analysis of variance (ANOVA) was used to determine the statistical significance of data, and Scheffe Post Hoc tests were applied to all pairwise difference between means. Data was considered significant for p values < 0.05.

4.4 Results

4.4.1 Preparation of alginate-g-pyrrole

Alginate chemically conjugated with pyrrole groups, termed alginate-g-pyrrole, was prepared through a chemical reaction between amine groups of N-(3-amino propyl) pyrrole (APP) and carboxylate groups of alginate. (Fig. 4.2) The reaction was activated by converting carboxylate groups of alginate to succinimidyl groups using 1-hydroxybenzotriazole and 1-ethyle-3-(3-dimethylaminopropyl) carbodiimide. The degree of pyrrole substitution to alginate, determined by measuring the UV absorbance of the pyrrole groups at 210 nm, was approximately 14%. The resulting alginate-g-pyrrole remained soluble in water with no phase separation observed over four weeks.

4.4.2 Encapsulation of VEGF-releasing PLGA microparticles in the alginate-g-pyrrole hydrogels via an enzyme-activated cross-linking reaction

Alginate-g-pyrrole hydrogels encapsulating VEGF-releasing PLGA microparticles were prepared through the enzymatic cross-linking reaction of pyrrole groups (Fig. 4.2). VEGF was first encapsulated in PLGA microparticles through an in situ double emulsification (Fig. 4.3). The subsequent addition of HRP and H$_2$O$_2$ to aqueous alginate-g-pyrrole solutions containing VEGF-releasing PLGA microparticles activated the formation of brown-colored hydrogels consisting of 5 wt% polymer and 10 mg/mL PLGA microparticles within 10 minutes. Throughout this in situ cross-linking reaction, the PLGA microparticles were immobilized in the gel matrix without any structural damage, as confirmed with scanning electron microscopic (SEM) images of the cross-sections of freeze fractured hydrogels.
In addition, two control hydrogels containing VEGF-releasing PLGA microparticles were prepared: 1) a calcium cross-linked hydrogel of unmodified alginate and 2) a calcium cross-linked hydrogel of alginate-g-pyrrole (Fig. 4.2). The calcium cross-linked hydrogels of alginate-g-pyrrole allow the pyrrole groups to remain uncross-linked pendants. Both gels also immobilized PLGA microparticles without any structural breakage of the particles, as also confirmed with SEM images of the fractured gel’s cross-section (Fig. 4.4).

4.4.3 Characterization of hydrogel elastic modulus, swelling ratio, and cross-linking density

First, the rigidity of the hydrogels was examined by measuring the compressive elastic moduli for gels encapsulating and not encapsulating PLGA particles. The elastic modulus of the hydrogels formed from an enzymatic cross-linking reaction of alginate-g-pyrrole, termed as HRP cross-linked alginate-g-pyrrole hydrogels, was 25 kPa (Fig. 4.5). The incorporation of 10 mg/mL PLGA microparticles slightly decreased the elastic moduli of the hydrogels to 17 kPa. In contrast, the calcium cross-linking reaction of alginate-g-pyrrole resulted in a softer hydrogel compared to the enzymatically cross-linked gel. The incorporation of the PLGA microparticles in this calcium cross-linked gel had a more significant decrease in the elastic modulus compared to the HRP cross-linked gel. The calcium induced cross-linking reaction between unmodified alginate resulted in the most rigid hydrogel with an elastic modulus of 50 kPa. Similar to the calcium cross-linked alginate-g-pyrrole hydrogel, inclusion of 10 mg/mL PLGA microparticles in the calcium cross-linked alginate hydrogel also resulted in a significant decrease of the elastic modulus to 30 kPa.

In parallel, the degrees of swelling of the hydrogels were quantified by measuring the mass of water up-taken by the gels; Eq. (1) (Fig. 4.5). The incorporation of the PLGA microparticles in the HRP cross-linked alginate-g-pyrrole gels led to a significant increase in the swelling ratio compared to the calcium cross-linked gel systems. The degree of swelling of the microparticle-filled hydrogels in order
from largest to smallest was: HRP cross-linked alginate-g-pyrrole gel > calcium cross-linked alginate-g-pyrrole gel > calcium cross-linked alginate gel.

Furthermore, the number of cross-links in the hydrogels were calculated through the elastic modulus and degree of swelling, using a rubber based elasticity theory; Eq. (2). The inclusion of PLGA microparticles decreased the number of cross-links, more significantly for the calcium cross-linked hydrogels. Additionally, the number of cross-links of the microparticle-filled hydrogels in order from largest to smallest was as follows: calcium cross-linked alginate gel > HRP cross-linked alginate-g-pyrrole gel > calcium cross-linked alginate gel.

4.4.4 Drug release rates controlled via hydrogel formulation

The release kinetics of VEGF from PLGA microparticles or microparticle-filled hydrogels was evaluated over a 25-day period. For all conditions, the VEGF release profiles followed a first order release kinetics; Eq. (3) (Fig. 4.6). The PLGA microparticles not incorporated into hydrogels displayed the largest initial burst and fastest VEGF release rate (k) of 0.15 day\(^{-1}\). Loading PLGA microparticles into hydrogels significantly decreased the initial burst rate of VEGF from the microparticles by 85%. The subsequent VEGF release rates from the hydrogels, characterized by k values, decreased by one order of magnitude (Table 4.1).

The VEGF release profiles from the microparticle-filled hydrogels were further modulated by the chemical structure of the gel-forming polymer and cross-linked structure of the gel. The total VEGF released from the HRP cross-linked alginate-g-pyrrole gels during the first five days was smaller than that from the calcium cross-linked alginate gels by 45%. The difference in the cumulative VEGF mass became more significant during the subsequent 20 days. Therefore, the k value for the HRP cross-linked alginate-g-pyrrole gels was smaller than that of the calcium cross-linked alginate gels by 30%. In contrast, the VEGF release profile for the calcium cross-linked alginate-g-pyrrole hydrogels was similar to the calcium cross-linked alginate gels. For instance, after 25 days 53% of the VEGF was released from
both the calcium cross-linked alginate-g-pyrrole hydrogels and the calcium cross-linked alginate gels, while 44% of the VEGF was released from the HRP cross-linked alginate-g-pyrrole. Overall, the encapsulation of VEGF-releasing PLGA particles within a hydrogel effectively reduced the initial burst release of VEGF from the PLGA microparticles. Additionally, the enzyme-activated alginate-g-pyrrole hydrogels exhibited a more sustained VEGF release than the calcium cross-linked alginate gel.

The VEGF release rates (k) were not correlated to the number of cross-links of the hydrogels (Fig. 4.6). Interestingly, the calcium cross-linked alginate hydrogels with the largest number of cross-links exhibited the highest k value. In contrast, the HRP cross-linked alginate-g-pyrrole gels with an intermediate cross-linking density displayed the lowest k value.

4.4.5 Neovascularization controlled by PLGA microparticle-filled hydrogels

The capacity of the HRP cross-linked alginate-g-pyrrole hydrogels to stimulate neovascularization was examined by implanting gels on chicken chorioallantoic membranes (CAMs). Hydrogels encapsulating VEGF-releasing PLGA microparticles were prepared on CAMs by injecting mixtures of microparticles and pre-gel solutions, and then activating the subsequent cross-linking reaction with calcium or HRP/H$_2$O$_2$. Similar to in vitro studies, the hydrogels formed on the CAMs within 10 minutes. The subsequent gels remained at the implantation site throughout the 7 days, while the VEGF-releasing PLGA microparticles were displaced from the initially implanted site (Fig. 4.7).

After seven days, control CAMs without any implants exhibited an increase in the number, but not the diameter of the blood vessels (Fig. 4.8). CAMs implanted with VEGF-releasing PLGA microparticles alone exhibited vascular networks with a similar size and density to control samples. In contrast, implantations of calcium cross-linked alginate gels encapsulating VEGF-releasing PLGA microparticles increased the vascular densities at the implantation sites. Furthermore, there was a noticeable increase in the number and size of blood vessels developed around the HRP cross-link
alginate-g-pyrrole hydrogels encapsulating VEGF-releasing PLGA microparticles compared to all other conditions.

According to quantitative analyses of the density and diameter of mature blood vessels that were stained positive for \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), the blood vessel densities of the CAMs implanted with the HRP cross-linked alginate-g-pyrrole gels were the largest among all four conditions tested in this study (Fig. 4.9). Specifically, the vascular densities of CAMs implanted with HRP cross-linked gels were four times larger than all other conditions. CAMs implanted with PLGA particles alone and calcium cross-linked alginate hydrogels had similar blood vessel densities compared to controls. Additionally, the average blood vessel diameters were the largest for implantations of HRP cross-linked alginate-g-pyrrole hydrogels, as compared to all three other conditions.

4.5 Discussion

The results of this study demonstrate the effectiveness of using an enzymatically cross-linked alginate-g-pyrrole hydrogel system to control the release rate of a proangiogenic growth factor, and ultimately improve neovascularization when implanted \textit{in vivo}. The alginate chemically linked with pyrrole units formed a hydrogel via an enzyme-activated cross-linking reaction between the pyrrole groups. The incorporation of VEGF-releasing PLGA microparticles in the enzymatically cross-linked alginate-g-pyrrole hydrogels resulted in a composite which presents a more sustained VEGF release and promotes an increased neovascularization compared to calcium cross-linked alginate and alginate-g-pyrrole gels, also loaded with PLGA microparticles.

The alginate-g-pyrrole synthesized in this study could form hydrogels via a HRP-activated cross-linking reaction or ionic cross-linking reaction. This enzymatic cross-linking reaction was activated by the addition of HRP and its substrate \( \text{H}_2\text{O}_2 \); the HRP catalyzes the transfer of electrons from the pyrrole units to \( \text{H}_2\text{O}_2 \) using a ferric group, ultimately forming cross-linked pyrrole groups and water. As a result, the elastic properties and swelling ratios of the gels should be controlled solely through the amount of
cross-linking between pyrrole units. Therefore, it is likely that the gel properties could further be controlled over a broader range by controlling the number of pyrrole units linked to the alginate and concentration of alginate-g-pyrrole. These pyrrole units linked to alginate, however, significantly reduced the number of ionic cross-links, as exhibited by the lower elastic modulus of the calcium cross-linked alginate-pyrrole gels compared to the unmodified alginate gels. This result implies that the pyrrole units are mostly linked to blocks of the guluronic acid residues; because calcium cross-linked alginate gels are formed exclusively from the cross-linking reaction between guluronic acid blocks and calcium [22].

Loading VEGF-releasing PLGA microparticles in the HRP cross-linked alginate-g-pyrrole hydrogels significantly reduced the initial burst release and also released VEGF in a more sustainable manner than the stiffer calcium cross-linked alginate gels. Typically, the release rate of macromolecules from a hydrogel is dictated by the cross-linking density of the system, which is inversely related to the mesh size of the hydrogel; however, the hydrogels formed from the pyrrole cross-linking reaction released VEGF more sustainably than the calcium cross-linked alginate gels with a larger cross-linking density. Additionally, the calcium cross-linked alginate-g-pyrrole gels do not exhibit a VEGF release profile as sustainable as the enzymatically cross-linked alginate-g-pyrrole gels. We therefore suggest that cross-linked pyrrole units have a higher affinity to VEGF than uncross-linked pyrrole units or carboxylic acid groups of alginate. Therefore, it is likely that VEGF released from PLGA microparticles is better sequestered by the pyrrole units cross-linked to each other in the gel matrix. We expect that the VEGF release rate can be further decreased by increasing the number of cross-linked pyrrole units through controlling the degree of substitution of pyrrole units to alginate, as well as the concentration of alginate-g-pyrrole. This approach will not only increase the affinity of VEGF to the gels but also decrease the pore size of the gels.

The importance of controlling the VEGF release rate from a gel matrix was further highlighted through the neovascularization study, using a CAM assay. The implantation of enzymatically cross-linked alginate-g-pyrrole hydrogels significantly increased both the number and size of blood vessels at
the implantation site, as compared to all other conditions. We propose that this improved vascular development attained with HRP-cross-linked alginate-g-pyrrole hydrogels should be attributed to several factors. First, the encapsulated drug-releasing microparticles inside the hydrogel minimized particle dislocation, thus leading to a localized drug delivery. Second, the enzymatically cross-linked gel resulted in a more sustained VEGF release over an extended period of time, as compared to the other control and hydrogels samples. Finally, as we previously demonstrated, it is plausible that pyrrole units of the hydrogel biochemically stimulate activities of host cells that contribute to vasculature formation [19].

Additionally, the enzymatically cross-linked alginate-g-pyrrole gel system will allow us to circumvent challenges encountered with calcium cross-linked gel systems, which undergo changes in their properties and microstructures by calcium leaching under physiological conditions. Therefore, this system will allow one to improve the long-term structural stability of the gel. Conversely, the degradation rate of the gel and subsequent drug release rate can be tuned in a more elaborate manner, for example through the oxidation of alginate molecules. Overall, the microparticle-filled alginate-g-pyrrole hydrogel system developed in this study will be broadly useful for delivering a wide array of macromolecular drugs at a controlled rate.

4.6 Conclusion

This study presents an advanced drug delivery system that promotes the sustained release of protein drugs and subsequently enhances local vascular development. This drug delivery system was assembled through the encapsulation of VEGF-releasing PLGA particles into a hydrogel formed from an enzymatic cross-linking reaction between pyrrole groups that are conjugated to an alginate backbone. The cross-linked pyrrole groups contributed to a more sustained VEGF release, by enhancing the affinity between the growth factor and the gel matrix. Subsequently, this microparticle-hydrogel construct was advantageous for improving neovascularization in vivo. Overall, the drug releasing system developed in
this study will be broadly useful for controlling release rates of various protein drugs and ultimately improving their therapeutic efficacies.

Acknowledgements

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4.7 Figures and tables

**Figure 4.1.** The synthesis of 1-(3-aminopropyl)pyrrole through the reduction reaction of 1-(2-cyanoethyl)pyrrole using LiAlH₄.

**Figure 4.2.** Preparation of PLGA microparticle-encapsulating alginate-g-pyrrole hydrogels via enzyme-activated cross-linking reaction. (a) Synthesis of alginate-g-pyrrole by conjugation of 1-(3-aminopropyl)pyrrole to alginate through the activation of carboxylatic acid using EDC and HOBr. (b) A schematic depicting the formation of a hydrogel network encapsulating PLGA microparticles through the HRP/H₂O₂-catalyzed cross-linking reaction of

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pyrrole groups of alginate-g-pyrrole. (c) Photos of enzyme-catalyzed alginate-g-pyrrole hydrogels with and without PLGA particles. (d) An SEM micrograph that exhibits the cross-section of HRP/H2O2-activated alginate-g-pyrrole hydrogels encapsulating PLGA microparticles. (e) Images of Ca\(^{2+}\) cross-linked alginate, Ca\(^{2+}\) cross-linked alginate-g-pyrrole, and HRP/H2O2-catalyzed alginate-g-pyrrole hydrogels encapsulating 10 mg/mL PLGA microparticles.

Figure 4.3. SEM images of VEGF-releasing PLGA microparticles formed through a double emulsion process.

| Ca Alginate | Ca Alginate-g-pyrrole |

Figure 4.4. SEM images of cryo-fractured Ca\(^{2+}\) cross-linked alginate and alginate-g-pyrrole hydrogels encapsulating VEGF-releasing PLGA microparticles.
Figure 4.5. Analysis of hydrogel properties. The compressive elastic moduli (a), swelling ratios (b), and crosslinking densities (c) of calcium (Ca$^{2+}$) cross-linked alginate, Ca$^{2+}$ cross-linked alginate-g-pyrrole, and HRP/H$_2$O$_2$-catalyzed alginate-g-pyrrole hydrogels with and without PLGA microparticles. * indicates statistical significance between conditions for hydrogels with and without PLGA microparticles ($p < 0.05$).
Figure 4.6. Analysis of the hydrogel's capability to control protein release rate (a & b) The VEGF release profiles over a 25 day period from PLGA microparticles alone (■) and PLGA microparticles encapsulated in Ca$^{2+}$ cross-linked alginate (●), Ca$^{2+}$ cross-linked alginate-g-pyrrole (▲), and HRP/H$_2$O$_2$-catalyzed alginate-g-pyrrole (▼) hydrogels. Plot (b) displays VEGF release profiles only from the PLGA microparticle-encapsulating hydrogels, in order to show the significant difference between conditions. (c) The relationship between the VEGF release rate constant ($k$) and the cross-linking density of the hydrogels ($N$); the dotted line indicates the relationship between $k$ and $N$ for hydrogel systems releasing drugs solely controlled by mesh size.
Figure 4.7. Images of neovasculature tuned by VEGF-releasing hydrogels. Images of CAM blood vessels captured 0 days (1st column) and 7 days (2nd column) after implantation, excised CAM tissues (3rd column), and histological cross-sections of CAMs stained with an antibody for α-smooth muscle actin (4th column). Conditions tested in this study include implantations of VEGF-releasing PLGA microparticles alone, implantations of Ca\(^{2+}\) alginate hydrogels encapsulating VEGF-releasing PLGA microparticles, and HRP/H\(_2\)O\(_2\)-catalyzed alginate-g-pyrrole hydrogels encapsulating VEGF-releasing PLGA microparticles.

Figure 4.8. Images of CAM blood vessels captured after 0 days and 7 days, an excised CAM tissue, and a CAM histological cross-section stained with an antibody for α-smooth muscle actin for control samples with no implantations.
Figure 4.9. Quantitative analysis of CAM neovasculature. (a) The average blood vessel densities and (b) vessel diameters in CAMs were quantified using histological cross-sections stained positive for α-smooth muscle actin. * indicates statistical significance between the conditions compared to the implant of VEGF-releasing HRP/H₂O₂-catalyzed alginate-g-pyrrole hydrogels and the three other conditions (p < 0.05).

<table>
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<th>Delivery System</th>
<th>k (day⁻¹)</th>
<th>R²</th>
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<td>0.87</td>
</tr>
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<td>Ca Alginate-g-pyrrole</td>
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<td>0.94</td>
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<tr>
<td>HRP Alginate-g-pyrrole</td>
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<td>0.92</td>
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</table>

Table 4.1. The VEGF release rate constants (k) and R² values for the first-order release fitting.
4.8 References


Chapter 5: Enzymatic modulation of hydrogel rigidity and electrical conductivity

5.1 Introduction

Over the past several decades, electrically conductive hydrogels have been studied for various applications, including biosensors, actuators and various biomedical devices [1-4]. Many of these hydrogels are prepared by inducing the polymerization of electrically conductive monomers such as pyrrole and aniline within a hydrogel, forming an interpenetrating network system [5-7]. The success of these applications greatly relies on the ability to interconnect the electrically conductive polymers in a hydrogel matrix, while retaining their uniform distributions within the gel [8]. However, the polymerization process often results in the precipitation of polypyrrole or polyaniline due to hydrophobic aggregations, and the resulting hydrogels present limited electrical conductivity [9-12]. The polymerization of electrically conductive polymers requires oxidizing agents such as iron chloride, which may not be advantageous for use of the gels in biological applications [13-14]. Additionally, the gel-forming polymers also inadvertently act as insulators in proportion to its number of cross-links, and subsequently reduce the electrical conductivity of the gel with increasing stiffness.

Therefore, this study presents a new method for increasing both the electrical conductivity and rigidity of a hydrogel system, while circumventing the use of toxic oxidizing agents for the polymerization. We hypothesized that pyrrole units conjugated to a water-soluble polymer can cross-link with each other to form a hydrogel via a one-step enzymatic cross-linking reaction. Increasing the number interconnected pyrrole units of the resulting hydrogels, would produce a more rigid and electrically conductive hydrogel system. We examined this hypothesis using alginate conjugated with pyrrole groups, termed alginate-g-pyrrole. The hydrogels were formed using two different peroxidase enzymes: horse radish peroxidase (HRP) and laccase, which utilize hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$) as substrates, respectively [15-18]. We also examined the properties of the alginate-g-pyrrole
hydrogels, formed through a chemical cross-linking reaction between carboxylic acid groups of the polymers, in parallel. Overall, the results of this study serve to improve the performance of electrically conductive hydrogels in various applications.

5.2 Experimental

5.2.1 Materials

Sodium alginate ($M_w \sim 250,000$, FMC Technologies) was provided by FMC Biopolymer. Calcium Sulfate, 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, 1-(2-cyanoethyl)pyrrole (CEP), sodium hydroxide (NaOH), lithium aluminum hydride (LiAlH$_4$), laccase (>10 U/mg) and horse radish peroxidase (250-330 U/mg; HRP) were purchased from Sigma-Aldrich Company (St. Louis, MO). Anhydrous ether and hydrogen peroxide ($H_2O_2$) were purchased from Mallinckrodt Chemicals. 1-hydroxybenzotriazole (HOBt) was purchased from Fluka (St. Louis, MO). Dichloromethane (DCM) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Thermo Scientific. Celite was purchased from Fisher Chemical. Phosphate buffered saline (PBS) was purchased from Cellgro.

5.2.2 Synthesis of N-(2-aminopropyl)pyrrole

N-(2-amino propyl)pyrrole (APP) was synthesized as previously reported [19]. Briefly, a solution of 0.2 M 1-(2-cyanoethyl)pyrrole (CEP) dissolved in anhydrous ether was added drop-wise to a suspension of lithium aluminum hydride (LiAlH$_4$) in anhydrous ether, and the resulting mixture was refluxed overnight. After the mixture was cooled, excess LiAlH$_4$ was quenched by the addition of water and a 15% NaOH solution. The mixture was then filtered through Celite and the water in the mixture was evaporated.

5.2.3 Pyrrole conjugation to alginate
Sodium alginate was irradiated with γ-rays from a $^{60}$Co source at a dose of 2 Mrad for 4 hours, in order to decrease the $M_n$ to 100,000 g/mol, as determined through gel permeation chromatography and as previously reported [20]. Irradiated alginate was dissolved in 0.1 M MES buffer (pH 6.4) at a concentration of 1% (w/v). Next, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1-hydroxybenzotriazole (HOBr), and APP were dissolved in the alginate solutions and stirred for 18 hours. The molar ratio of HOBr, EDC, and APP was kept constant at 2:2:1. The molar ratio of APP to uronic acids of the alginate was 0.2. The mixture was then dialyzed with deionized (DI) water for three days, while replacing it with fresh water every 12 hours. The polymer solutions were dialyzed, lyophilized, and reconstituted to a 7 wt% stock solution with PBS. The conjugation of pyrrole to alginate was confirmed using $^1$H NMR (500 MHz, D$_2$O).

The degree of pyrrole substitution to alginate was evaluated by measuring the UV absorbance of the resulting polymer. Alginate-g-pyrrole were dissolved in DI water at a 0.01% (w/v) concentration, and the UV absorbance values of the solutions at a wavelength of 210 nm were measured using a CARY 500 Scan UV-Vis NIR Spectrometer. A standard curve, created by measuring the absorbance values of 0.01% (w/v) alginate solutions containing known quantities of APP, was used to determine the degree of pyrrole substitution.

5.2.4 Enzyme-catalyzed alginate-g-pyrrole hydrogel formation

Alginate-g-pyrrole hydrogels were prepared through the enzymatic cross-linking of pyrrole units attached to alginate. Pre-gel polymer solutions were mixed with a solution of HRP and a solution of its substrate H$_2$O$_2$. The final polymer concentration was kept constant at 5 wt%. The molar ratio of HRP to pyrrole groups of alginate-g-pyrrole was varied from 2 to 12 mg/mmol, and the molar ratio of H$_2$O$_2$ to pyrrole groups of alginate-g-pyrrole was varied from 1 to 12 mg/mmol. The mixtures were placed between two glass plates separated by a 1 mm spacer. The resulting hydrogel was punched into disks with a diameter of 10 mm. Additionally, control hydrogels were prepared by adding AAD, HOBr, and
EDC into pre-gel solutions of unmodified alginate and alginate-g-pyrrole with a final concentration of 5 wt%. The molar ratio between ADD, HOBt, and EDC was kept constant at 1:2:2. Again, the hydrogel formed between two glass plates were punched into disks with a 10 mm diameter and 1 mm thickness.

5.2.5 Characterization of gelation time

The time required to form a hydrogel through the enzyme-catalyzed cross-linking of alginate-g-pyrrole was examined. Pre-gel polymer solutions of alginate-g-pyrrole were mixed with varied concentrations of HRP and H₂O₂ as described above, in small vials with final polymer concentrations 5 wt%. The vials were turned upside-down every five minutes to examine whether the mixtures flow to the opposite side of the vial under the force of gravity. The time required before the mixture did not flow, was determined as the gelation time.

5.2.6 Characterization of hydrogel mechanical properties

The stiffness of the hydrogels was evaluated by measuring the compressive elastic modulus. Hydrogels were formed between two glass plates separated by 1 mm spacers following the same procedures described above. The hydrogels were punched into 1 cm disks and incubated in PBS at 37°C for 24 hours. Next, the gel disks were compressed at a rate of 1 mm/min using a mechanical testing system (MTS Insight). The elastic moduli of the gels were calculated from the linear slope of the stress versus strain (ε) curve for the first 10% strain as previously described. The shear moduli were calculated from the linear slope of the stress versus \(-(\nu - \nu^{-2})\) curve, where \(\nu = 1 - \varepsilon\). In parallel, the water content, termed the swelling ratio \(Q_m\), of the hydrogel disks was determined by measuring the ratio of saturated to freeze dried hydrogel weights. The degree of swelling (Q) of the gels was calculated following:

\[
Q = \rho_p \left[ \frac{Q_m}{\rho_s} + \frac{1}{\rho_p} \right]
\]  

(1)
where \( \rho_p \) is the polymer density (1.6 g/cm\(^3\)) and \( \rho_w \) is the density of water. The number of cross-links (N) was then calculated based on rubber elasticity theory as follows:

\[
N = \frac{6Q^{1/3}}{RT}
\]

(2)

where \( R \) represents the ideal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)) and \( T \) represents the temperature at which the modulus was measured (25°C) [21].

5.2.7 Characterization of electrical conductivity

The electrical conductivity of the hydrogels was evaluated by measuring current through the gels. Hydrogels were formed between two glass plates as described above and punched into 1.5 cm x 0.5 cm x 1 mm rectangles. One end of the hydrogels were exposed to a 100 mV alternating potential difference at 1 Hz using an Agilent 33220A Waveform Generator, while the subsequent current through the gels were measured using an Agilent 34411A Digital Multimeter at the other end. The electrical conductivity (\( \sigma \)) of the hydrogels was determined as follows:

\[
\sigma = \frac{l \cdot I}{V \cdot A}
\]

(3)

where \( I \) is the measured current, \( l \) is the length of the hydrogels (1.5 cm), \( V \) is the exposed potential (100 mV), and \( A \) is the cross-sectional area of the hydrogels (0.05 cm).

5.2.8 Statistical analysis

Four to six samples were analyzed per condition unless otherwise specified for all experiments. One-way analysis of variance (ANOVA) was used to determine the statistical significance of data and Scheffe Post Hoc tests were applied to all pair wise difference between means. Data was considered significant for \( p \) values < 0.05.
5.3 Results and discussion

Alginate-g-pyrrole was synthesized through the conjugation of pyrrole units to alginate using carbodiimide chemistry. First, N-aminopropyl pyrrole (AAP) was synthesized through a reduction reaction of N-cyanoethyl pyrrole (CEP), and was subsequently conjugated to the carboxylic acid groups of alginate through amide linkages (Fig. 5.1). The conjugation of pyrrole units to alginate was confirmed by the loss of the peak α to the amine at 2.4 ppm in the $^1$H-NMR for the reaction. The degree of pyrrole unit conjugation to the carboxylic acid groups of alginate was 14%, determined through UV-Vis absorbance measurements at 210 nm. The resulting alginate-g-pyrrole remained soluble in an aqueous media for over two months.

Hydrogels were formed through the addition of peroxidase enzymes and their respective substrates to pre-gel alginate-g-pyrrole polymer solutions (Fig. 5.1). The addition of horseradish peroxidase (HRP) and H$_2$O$_2$ to a 7 wt% solution of alginate-g-pyrrole resulted in the formation of a gel. In contrast, polymer solutions mixed only with HRP or H$_2$O$_2$ did not form a hydrogel. These results demonstrate that the cross-linking of pyrrole groups occurs through enzymatic activities utilizing substrates. The time required to form a hydrogel, termed the gelation time, was decreased by increasing the concentration of horseradish peroxidase (HRP) or that of H$_2$O$_2$, fitted to a linear regression. Additionally, the resulting hydrogel did not present toxicity to cells cultured in vitro, as confirmed with active adhesion and spreading of fibroblasts on the gel after formation (Fig. 5.2).

Separately, both unmodified alginate and alginate-g-pyrrole hydrogels were prepared through the cross-linking reaction between carboxylate groups of alginate and adipic acid dihydrazide (AAD) (Fig. 5.3). This cross-linking reaction was activated by incorporating a mixture of sulfo-N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide into the pre-gel solution. The hydrogels were formed more quickly than those formed through the enzymatic cross-linking reaction.

The physical properties of the post-hydrogels formed through the enzymatic cross-linking reaction activated by HRP/H$_2$O$_2$ were predominantly influenced by the amount of H$_2$O$_2$ incorporated into
the pre-gel solutions, not by the amount of HRP. Increasing the molar ratio between H$_2$O$_2$ and pyrrole units of alginate-g-pyrrole (M$_{H2O2}$) from 3 to 12 resulted in more than a two-fold increase in the elastic modulus from 10 to 24 kPa, and a decrease in the swelling ratio from 80 to 59 (Table 5.1). Note that gels did not form at an M$_{H2O2}$ of 1. Accordingly, the number of cross-links calculated using a rubber elasticity theory (Eq. (2)) was increased with M$_{H2O2}$. In contrast, increasing the molar ratio between HRP and pyrrole units of alginate-g-pyrrole (M$_{HRP}$) from 2 to 12 minimally changed the elastic moduli, swelling ratio, and subsequent number of cross-links of the gels.

The alginate-g-pyrrole hydrogel formed from the enzymatic cross-linking reaction displayed increases in the electrical conductivity with M$_{H2O2}$ (Fig. 5.4). Specifically, increasing M$_{H2O2}$ from 3 to 12 resulted in a three-fold increase in the electrical conductivity, from 4 to 12 mS/cm, which is equivalent to the conductivity of cerebrospinal fluid. In contrast, increasing M$_{HRP}$ minimally altered the electrical conductivity of the hydrogels. Overall, the cross-linking density of the gels formed from the enzymatic reaction was proportionally related to the electrical conductivity.

Additionally, alginate-g-pyrrole hydrogels formed using HRP and H$_2$O$_2$ exhibited a higher electrical conductivity than unmodified alginate and alginate-g-pyrrole hydrogels cross-linked by AAD, for gels with similar cross-linking densities (Fig. 5.5). The electrical conductivity of the enzymatically cross-linked alginate-g-pyrrole hydrogels incubated in neutral phosphate buffer saline (PBS, ~0.015 S/cm) was rather comparable to that of PBS. In contrast, the electrical conductivity of the AAD cross-linked alginate-g-pyrrole hydrogels was similar to that of AAD cross-linked unmodified alginate gels, indicating that non-cross-linked pyrrole groups have little contribution to the overall conductivity of the gels. The difference in the electrical conductivity between the gels became more significant by increasing the ionic strength of the media. This higher electrical conductivity of the enzymatically cross-linked gel is attributed to increases in the number of motile charged species within the gel system as well as the number of interlinked pyrrole units through which electrons move.
5.4 Conclusion

Overall, the results of this study demonstrate an advanced method to prepare a rigid, electrically conductive hydrogel via a one-step, biocompatible enzymatic cross-linking reaction between electrically conductive epitopes. The hydrogels were formed through the enzymatic cross-linking of pyrrole groups conjugated to alginate and were controlled via the concentrations of HRP and H₂O₂ added to a pre-polymer solution. The exclusive cross-linking of pyrrole groups linked to alginate not only improves the rigidity of the system, but also improves the overall electrical conductivity of the material. The simple, one step polymerization makes this system advantageous for a broad range of applications with improved processability compared to interpenetrating network systems prepared with multi-steps. We believe that this material design strategy can be extended to a wide array of electrically conductive hydrogels systems, and will be broadly useful for numerous applications including biosensors, actuation, and biomedical applications.
5.5 Figures and tables

Figure 5.1. (a) Synthesis of N-aminopropyl pyrrole (APP) via a reaction between N-cyanoethyl pyrrole (CEP) and lithium aluminum hydride. (b) The synthesis of alginate-g-pyrrole through the conjugation of APP to the carboxylate groups of alginate. (c) Schematic depicting the cross-linked structure of an alginate-g-pyrrole hydrogel formed through the enzymatic cross-linking of pyrrole groups using an enzyme, HRP, and its substrate, H2O2. (d) Alginate-g-pyrrole hydrogels formed only in the presence of both HRP and H2O2. (e) Control of the gelation time by varying M_{HRP} at a constant M_{H2O2} (3 mol/mol-Py) or M_{H2O2} at a constant M_{HRP} (2 mg/mmol-Py), respectively.
Figure 5.2. Cells (NIH 3T3 Mouse Fibroblasts) viably adhered and spread on alginate-g-pyrrole hydrogels formed via the HRP-catalyzed cross-linking reaction of pyrrole groups. The cell images were captured 24hrs after seeding.

Figure 5.3. A schematic depicting the structures of (a) alginate and (b) alginate-g-pyrrole hydrogel formed by the cross-linking with adipic acid dihydrazide (AAD).
Figure 5.4. (a) The electrical conductivity for alginate-g-pyrrole hydrogels formed with varied $M_{\text{HRP}}$ or $M_{\text{H}_2\text{O}_2}$. $M_{\text{HRP}}$ was varied at a constant $M_{\text{H}_2\text{O}_2}$ of 3 mol per mol of pyrrole, or $M_{\text{H}_2\text{O}_2}$ was varied at a constant $M_{\text{HRP}}$ of 2 mg per mmol of pyrrole. (b) A plot of electrical conductivity versus the cross-linking density. (c) The electrical conductivity normalized to the cross-linking density for alginate gels cross-linked with AAD (■), alginate-g-pyrrole gels cross-linked with AAD (▲), and alginate-g-pyrrole gels cross-linked with HRP and $\text{H}_2\text{O}_2$ (●).
Figure 5.5. Alginate hydrogel cross-linked with AAD, alginate-g-pyrrole hydrogel cross-linked with AAD, and alginate-g-pyrrole hydrogel with HRP and H₂O₂ were engineered to exhibit similar cross-linking structures. In this study, the molar ratio between AAD and uronic acids of alginate or alginate-g-pyrrole was tuned to 1:25. The molar ratio between HRP and uronic acids of alginate-g-pyrrole (M_HRP) and the molar ratio between H₂O₂ and uronic acids of alginate-g-pyrrole (M_H₂O₂) were tuned to 2 mg per mmol of pyrrole and 12 mol per mol of pyrrole, respectively.

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<th>H₂O₂ (mol/mol Py)</th>
<th>Elastic Modulus (kPa)</th>
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</tr>
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<td>11.41 ± 1.70</td>
<td>82.22 ± 2.47</td>
<td>5.06 ± 0.81</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>10.04 ± 0.55</td>
<td>80.21 ± 4.54</td>
<td>4.39 ± 0.288</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
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<tr>
<td>2</td>
<td>12</td>
<td>23.97 ± 3.68</td>
<td>59.25 ± 2.59</td>
<td>6.38 ± 0.39</td>
</tr>
</tbody>
</table>

Table 5.1. The compressive elastic moduli, swelling ratio, and cross-linking density for alginate-g-pyrrole hydrogels formed with varied concentrations of HRP and H₂O₂.
5.6 References


Chapter 6: Decoupled control of mechanical rigidity and electrical conductivity of a proangiogenic alginate-g-pyrrole hydrogels

6.1 Introduction

Over the past several decades, hydrogels have been increasingly used for various biomedical applications including, drug delivery, cell culture, and tissue engineering [1-6]. The successful utilization of hydrogel systems greatly relies on the ability to control their inherent properties, including the mechanical and electrically conductive properties; however, controlling these aspects still remains a significant challenge [7-9]. For example, the electrical conductivity of typical hydrogel systems is based on the transport of ions through a polymeric cross-linked mesh. Increasing the mechanical rigidity of these systems requires increases the cross-linking, which inhibits ion transport, subsequently reducing the electrical conductivity of the system [10-12].

Recently, the incorporation of conductive polymers in hydrogel systems has been used to improve the electrical conductive properties of gels [13-16]. These strategies typically include the diffusion of monomers, such as pyrrole or aniline, within a pre-formed hydrogel network, followed by the subsequent oxidative polymerization [17-18]. This process forms an inter-penetrating (IPN) network consisting of electrically conductive polymers (e.g., polypyrrole or polyaniline) within a polymer cross-linked network. These IPNs have demonstrated some improved conductive properties; however, systematically controlling the mechanical and electrical properties still remains a challenge. Additionally, the multi-step processing procedures of these co-networks are inefficient compared to single step hydrogel formation strategies.

Therefore, we hypothesized that an advanced electrically conductive hydrogel system can be formed in a single polymerization step, through the cross-linking of conductive monomer-units conjugated to a hydrophilic polymeric backbone. This strategy was examined through the cross-linking of pyrrole groups that were conjugated to an alginate backbone, termed alginate-g-pyrrole. Additionally, we hypothesized that this system could be used to eliminate the inverse dependence between the
mechanical and conductive properties of hydrogels. Furthermore, the enhanced conductivity of the gel would stimulate cells adhered to the gel to produce proangiogenic factors more actively, in response to electrical stimuli. This was examined by forming gels using alginate-g-pyrrole with controlled degrees of pyrrole conjugation, which simultaneously controls the cross-linking density and the quantity of conductive polymer within the hydrogel system. Finally, these hydrogels were used in electrical cell stimulation experiments, for controlling various cellular activities.

6.2 Experimental

6.2.1 Materials

Sodium alginate ($M_w \sim 250,000$ Da, FMC Technologies) was provided by FMC Biopolymer. Poly(ethylene glycol) diacrylate (PEGDA, MW 400 g/mol) was purchased from Polysciences. Ammonium persulfate (APS), 2-(N-morpholino)ethane sulfonic acid (MES) hydrate, 1-(2-cyanoethyl)pyrrole (CEP), 2-aminoethyl methacrylate (MA), sodium hydroxide (NaOH), poly(vinyl alcohol), and lithium aluminum hydride (LiAlH₄) were purchased from Sigma-Aldrich Company (St. Louis, MO). Anhydrous ether was purchased from Mallinckrodt Chemicals. 1-hydroxybenzotriazole (HOBt) was purchased from Fluka (St. Louis, MO). Polydimethylsiloxiane (PDMS), curing agents, and silicone glue were purchased from Dow Corning. 3-aminopropyl(diethoxy)methyilsilane and N-(trimethoxysilylpropyl)ethylene diamine triacetic acid were purchased from Gelest Industries. Dichloromethane (DCM) and 1-ethyl-3-(3-dimethyaminopropyl)carbodiimide (EDC) were purchased from Thermo Scientific. Celite was purchased from Fisher Chemical. Phosphate buffered saline (PBS) was purchased from Cellgro. VEGF, Mouse Duo VEGF enzyme-linked immunosorbent assay (ELISA), and ELISA reagents were purchased from R&D Systems. Phosphate buffered saline (PBS), Dulbecco’s modification of Eagle’s medium (DMEM), and Penicillin/Streptomycin (P/S, 10,000U/mL / 10,000mg/mL) was purchased from Cellgro. Fetal Bovine Serum (FBS) and trypsin-EDTA (0.5%) was purchased from Invitrogen. MTT Cell Proliferation Assay was purchased from ATCC. Type 1 Collagen,
Mouse Duo VEGF enzyme-linked immunosorbent assay (ELISA) and ELISA reagents were purchased from R&D Systems.

6.2.2 Synthesis of N-(3-aminopropyl)pyrrole (APP)

N-(3-amino propyl)pyrrole (APP) was synthesized according to previously reported procedures [19]. Briefly, a solution of 0.2 M 1-(2-cyanoethyl)pyrrole (CEP) dissolved in anhydrous ether was added drop-wise to a suspension of lithium aluminum hydride (LiAlH₄) in anhydrous ether, and the resulting mixture was refluxed overnight. After the mixture was cooled, excess LiAlH₄ was quenched by the addition of water and a 15% NaOH solution. The mixture was then filtered through Celite, and the water in the mixture was evaporated to collect APP and was confirmed through ¹H NMR (500 MHz, D₂O).

6.2.3 Conjugation of pyrrole or methacrylate units to alginate

Sodium alginate was irradiated with γ-rays from a ⁶⁰Co source at a dose of 2 Mrad for 4 hours, in order to decrease the Mₚ to 100,000 g/mol, as determined through gel permeation chromatography (GPC), as previously reported [20]. Irradiated alginate was dissolved in 0.1 M MES buffer (pH 6.4) at a concentration of 1% (w/v). Next, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and APP was dissolved in the alginate solutions and stirred for 18 hours. The molar ratio of HOBt, EDC, and APP/AEM was kept constant at 2:2:1. The molar ratio of APP to uronic acids of the alginate was varied from 0 to 0.35. The mixture was then dialyzed with deionized (DI) water for three days, while replacing it with fresh water every 12 hours. The dialyzed polymer solutions were lyophilized and reconstituted to a 7 wt% stock solution with PBS.

The degree of pyrrole substitution to alginate was evaluated by measuring the UV absorbance of the resulting polymers. The alginate polymers with varied substitutions of pyrrole units (alginate-g-pyrrole) were dissolved in DI water at a 0.01% (w/v) concentration, and the UV absorbance values of the solutions at a wavelength of 210 nm were measured using a CARY 500 Scan UV-Vis NIR Spectrometer.
A standard curve, created by measuring the absorbance values of 0.01% (w/v) alginate solutions containing known quantities of APP, was used to determine the degree of pyrrole substitution. Separately, control samples of alginate were conjugated with methacrylic groups following the same procedures as described above and previously [21]. 2-aminoethyl methacrylate hydrochloride (MA) was conjugated to alginate with the molar ratio of MA to uronic acid groups varied from 0.05 to 0.2, and was confirmed using $^1$H NMR (500 MHz, D$_2$O).

6.2.4 Hydrogel Preparation

Alginate-g-pyrrole, polyethylene glycol (PEG), and alginate methacrylate hydrogels were prepared through the cross-linking of the pyrrole, acrylate, or methacrylic groups, respectively. Pre-gel polymer solutions were first mixed with a solution of ammonium persulfate (APS) to induce cross-linking. The final polymer concentrations of the alginate-g-pyrrole and alginate methacrylate pre-gel solutions with varied substitutions of pyrrole and methacrylic were 5 wt%, while the concentration of polyethylene glycol diacrylate was varied from 8 to 12 wt%; the final APS concentrations was 0.1 M. Additionally, control hydrogels of adipic acid (AAD) cross-linked alginate hydrogels were formed by mixing solutions of ADD with NHS and EDC in a 1:2:2 ratio respectively with the final polymer concentration of 5 wt%. All of the hydrogels were incubated in deionized water (DI), PBS (pH 7.4) or 10x PBS (pH 7.4) at 37°C for 24 hours, exchanging it with fresh media every 4 to 8 hours.

6.2.5 Characterization of hydrogel mechanical properties

The stiffness of the hydrogels was evaluated by measuring a compressive elastic modulus. Following the incubation in PBS for 24 hours, gels formed in the shape of disks with a 1 cm diameter and 1 mm thickness were compressed at a rate of 1 mm/min using a mechanical testing system (MTS Insight). The elastic moduli ($E$) of the gels were calculated from the linear slope of the stress ($\sigma$) versus strain ($\epsilon$) curve for the first 10% strain. The shear moduli ($G$) were calculated from the linear slope of the stress
versus \(-(v-v^2)\) curve, where \(v = 1 - \epsilon\), for the first 10% strain. In parallel, the degree of swelling \((Q)\) of the gels were calculated following:

\[
Q = \rho_p \left[ \frac{Q_m}{\rho_w} + \frac{1}{\rho_p} \right]
\]

where \(\rho_p\) is the polymer density (1.6 g/cm), \(\rho_w\) is the density of water, and \(Q_m\) is the swelling ratio, which is defined as the mass ratio of hydrated gels to dried gels. The cross-linking densities \((N)\) were then calculated based on rubber elasticity theory as follows [22]:

\[
N = \frac{GQ^{1/3}}{RT}
\]

where \(R\) represents the gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)) and \(T\) represents the temperature at which the modulus was measured, 25\(^\circ\)C.

6.2.6 Characterization of hydrogel electrical conductivity

The electrical conductivity of the hydrogels was evaluated by applying a voltage potential and subsequently measuring the current through the gels. Following the incubation in DI water, PBS, or 10x PBS for 24 hours, rectangular hydrogels with dimensions of 1.5 cm x 0.5 cm x 1 mm were exposed to a 100 mV alternating potential difference at 1 kHz using an Agilent 33220A Waveform Generator. Simultaneously, the current through the gels was measured using an Agilent 34411A Digital Multimeter. The electrical conductivity \((\sigma)\) of the hydrogels was determined through the relation:

\[
\sigma = \frac{I}{V \cdot A}
\]

where \(I\) is the measured current, \(l\) is the length of the hydrogels (1.5 cm), \(V\) is the exposed potential (100 mV) and \(A\) is the cross-sectional area of the hydrogels (0.05 cm).

6.2.7 Preparation of hydrogels for electrical stimulation

Hydrogels were formed within an electrical stimulation platform for future cellular electrical stimulations experiments. The platforms were constructed using PDMS molds adhered to electrically
conductive indium tin oxide (ITO) coated glass slides (Sigma Aldrich) (Fig. 6.1). First, PDMS was poured around 8 mm glass tubes and cured to form a mold with 8 mm wells. Next, the molds were adhered to ITO slides using silicone glue, resulting in a multiple array of wells with electrically conductive ITO as well bottoms. A 0.5% solution of either N-(trimethoxysilylpropyl)ethylene diamine triacetic acid or 3-aminopropyl(diethoxyl)methylsilane were added to the wells and incubated for one hour in order to present reactive carboxylic acid or amine groups on the surfaces of the ITO. The carboxylic acid functionalized surfaces were subsequently reacted with APP in the presence of EDC and HOBT, in excess, resulting in pyrrole presenting surfaces (Fig. 6.2)). Finally, AAD cross-linked alginate and alginate-g-pyrrole hydrogels were formed, as described above, on the amino and pyrrole functionalized surfaces, respectively, forming gels linked to the ITO substrates of the electrically conductive platforms.

6.2.8 Examination of cellular activities electrically stimulated on alginate-g-pyrrole hydrogels

Fibroblasts (NIH 3T3) were seeded on hydrogels of alginate-g-pyrrole and AAD linked alginate with similar cross-linking structures in the electrical stimulation platform, exposed to electrical stimulation, and examined for subsequent cellular activities. The hydrogels formed in the stimulation platform were soaked in Type 1 Collage at a concentration of 0.5 mg/mL for two hours before cell seeding. Fibroblasts between passage numbers 10 and 15 were seeded on hydrogels at a density of 1,000 or 15,000 cells per well, and were cultured in DMEM supplemented with 10% FBS and 1% P/S at 37°C. After 24 hours, gels with cells seeded at 1,000 cells per well were electrically stimulated with a direct potential of 1 V for 2 hours, by connecting electrodes to the ends of the ITO glass slide of the stimulation platform. Throughout the stimulation period, images were taken at intervals of 1 minute and were examined using analytical software (Image J). In parallel, gels with cells at 15,000 cells per well were stimulated with an alternating potential of 1 V at a frequency of 10 Hz for 20 minutes per day for 2 days. Cell media was collected before stimulation (day 0) and after the stimulation period (day 2). The VEGF
concentrations in the media were measured using a VEGF ELISA kit, following the manufacturer’s protocol. A calibration curve, prepared by measuring the absorbance values of standards with known concentrations of VEGF, was used to quantify the concentrations of VEGF in the collected cell culture media.

6.2.9 Statistical Analysis

Four to six samples were analyzed per condition unless otherwise specified for all experiments. One-way analysis of variance (ANOVA) was used to determine the statistical significance of data and Scheffe Post Hoc tests were applied to all pair-wise differences between means. Data was considered significant for p values < 0.05.

6.3 Results

6.3.1 Chemical modification of alginate with varied degrees of pyrrole substitution

Pyrrole units were chemically conjugated to alginate using carbodiimide chemistry. First, 1-(2-cyanoethyl)pyrrole was reduced to N-(3-amino propyl) pyrrole (AAP) using LiAlH₄ in ether. Next varied amounts of APP were attached to alginate by coupling the amine of APP with the carboxylic acid groups of alginate’s uronic acid groups using 1-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Fig. 6.3). By controlling the ratio of APP to uronic acid groups, alginate was conjugated with controlled quantities of pyrrole, and termed alginate-g-pyrrole. The degree of pyrrole substitution to alginate was determined to be 12, 17, 22, 26, and 32%, as evaluated using UV absorbance measurement at 210 nm. In parallel, alginate was chemically linked with varied quantities of 2-aminoethyl methacrylate forming alginate methacrylate with varied degrees of methacrylate substitution: 5, 10, and 15% substitution.

6.3.2 Hydrogel preparation
Hydrogels were formed through inducing the cross-linking of pyrrole of alginate-g-pyrrole (Fig. 6.3). The addition of ammonium persulfate (APS) to pre-gel polymer solutions of alginate-g-pyrrole with varied degrees of pyrrole substitution, and a subsequent application of heat activated the cross-linking reactions of pyrrole groups, forming gels with controlled pyrrole content. All alginate-g-pyrrole hydrogels were formed with final polymer concentrations of 5 wt%. The activation of alginate-g-pyrrole with a 12% pyrrole substitution did not result in the formation of a rigid hydrogel, because of an insufficient quantity of cross-linked pyrrole necessary to form a gel; while alginate-g-pyrrole with higher degrees of substitution formed rigid hydrogel networks. Additionally, control hydrogels of polyethylene glycol (PEG), alginate methacrylate, and adipic acid hydrazide (AAD-linked alginate were also prepared. PEG gels were formed by cross-linking varied concentrations of PEG diacrylate (PEGDA) through the addition of APS. Similarly, alginate methacrylate gels with varied substitutions of methacrylate units were formed by cross-linking methacrylate using APS. AAD cross-linked alginate hydrogels were formed through the chemical linking of AAD with the carboxylic acid groups of alginate through the activated coupling reaction using HOBt and EDC. The final polymer concentration of both alginate methacrylate and AAD linked alginate gels was 5 wt%.

The mechanical properties of the hydrogels were characterized through compressive elastic modulus measurements, and the water content of the gels, termed as the swelling ratio, was characterized by measuring the masses of hydrated and lyophilized gels. The elastic moduli of the alginate-g-pyrrole, PEG, and alginate-methacrylate hydrogels were controlled through the degree of pyrrole substitution, the concentration of PEGDA, and the degree of methacrylate substitution, respectively (Fig. 6.4 & 6.5). The elastic moduli of the alginate-g-pyrrole and PEG gels could be controlled from 0 to 200 kPa. On the other hand, alginate-methacrylate could only be formed with moduli ranging from 0 to 50 kPa, due to the flexible methacrylate linker and solubility limitations of methacrylate substitutions higher than 24%. The swelling ratios of hydrogels decreased with increasing pyrrole substitution, PEGDA concentrations, or methacrylic substitutions. The alginate hydrogels exhibited higher swelling ratios compared to PEG.
hydrogels due to the charged carboxylic acid groups of alginate backbone. Finally, the overall quantity of cross-links for the hydrogel networks, termed as the cross-linking density, was determined using Eqn. (2). The cross-linking density for the hydrogels was controlled through varying the degree of pyrrole substitution, the PEGDA concentration, and the degree of methacrylate substitution for the respective hydrogels.

6.3.3 Characterization of hydrogel electrical conductivity

The electrical conductivity of the hydrogels was quantified by measuring the current through the hydrogels under a voltage potential. The alginate-g-pyrrole hydrogels exhibited increases in electrical conductivity by increasing the number of cross-links within the hydrogel network in DI water, PBS, and 10x PBS (Fig. 6.6 & 6.7). On the other hand, PEG and alginate methacrylate gels exhibited decreases in electrical conductivity by increasing the number of cross-links within the hydrogel networks. Additionally, PEG gels in solutions without any ions exhibited a constant conductivity, independent of the cross-linking density.

6.3.4 Examining the activities of cells adhered to hydrogels under direct potential stimulation

The effects of direct potential stimulation on the activities of cells adhered to alginate-g-pyrrole hydrogels were examined using an electrical stimulation platform (Fig. 6.8). Alginate-g-pyrrole hydrogels with a 32% degree of pyrrole substitution were prepared in the wells of the electrical stimulation platform. Fibroblast cells were seeded to the gels and then stimulated with potential of 1 V over a 2 hour period. Additionally, control hydrogels of ADD linked alginate with a similar cross-linking density, but with a 2-fold lower electrical conductivity were used for cellular stimulation experiments (Fig.6.9). The cells adhered to the ADD cross-linked alginate gels remained adhered in spread morphology throughout the entire stimulation period, while the cells on the alginate-g-pyrrole retracted
their filopodia. The stimulated cells on the alginate-g-pyrrole gels remained viable, as demonstrated by a subsequent re-spreading of the cells after the stimulation period (Fig. 6.10).

6.3.5 Examining the VEGF expression of cells adhered to hydrogels under alternating potential stimulation

The effects of alternative potential stimulation on the endogenous VEGF expression of cells adhered to alginate-g-pyrrole hydrogels was examined using an electrical stimulation platform (Fig. 6.11). Alginate-g-pyrrole hydrogels with a 32% degree of pyrrole substitution were prepared in the wells of the electrical stimulation platform. Fibroblast cells were seeded to the gels and then stimulated with an alternating potential of 100 mV at 10 Hz for 20 minutes a day for two days. Again, control hydrogels of ADD linked alginate with a similar cross-linking density, but with a 2-fold lower electrical conductivity were used. The stimulated cells on the AAD cross-linked alginate gels exhibited similar VEGF expression levels to that of non-stimulated cells on both AAD-alginate and alginate-g-pyrrole gels. The cell stimulated on the alginate-g-pyrrole gels exhibited a significant increase in the endogenous VEGF expression compared to all other control conditions.

6.4. Discussion

The results of this study demonstrate a strategy to eliminate the inverse dependency between the mechanical rigidity and electrical conductivity of hydrogels by controlling the quantity of electrically conductive cross-linking pyrrole units bound to an alginate polymer, and demonstrate its capacity to manipulate cellular activities including endogenous VEGF expression. The simultaneous increase in the mechanical and electrical properties of the alginate-g-pyrrole hydrogels is controlled through the quantity of pyrrole groups conjugated to alginate, which subsequently determines the quantity of electrically conductive cross-linked pyrrole in the system. Furthermore, the improved electrical properties of the system was demonstrated to have significant impacts on cell adhesion morphology and cellular
expression of VEGF for adhered cells, compared to gels with similar mechanical properties, but lower electrical conductivities.

The cross-linking structure of hydrogels significantly impacts their electrical properties by inhibiting ionic transport. Similarly, alginate-g-pyrrole hydrogels formed through the cross-linking of pyrrole groups inhibits ionic transport; however, the cross-linked pyrrole groups provide an additional electron based conductivity, in which electrons transport through the backbone of the polymerized pyrrole. The added electron transport of our system overcomes reductions in conductivity associated with inhibited ion transport, and ultimately increases the conductivity of the system. This is demonstrated by a significant enhancement in electrical conductivity associated with electron transport for the alginate-g-pyrrole gels in DI which contains no ions when compared to PEG gels. Additionally, the similar conductivities of alginate-g-pyrrole and PEG gels with low cross-linking densities in PBS can be attributed to the dominant ionic transport and due to the lack of electron transporting pyrrole groups.

Additionally, the composition of a hydrogel can have a significant impact on the overall conductivity of the system. Hydrogels containing ionic groups have been demonstrated to be more absorbent compared to hydrogels lacking charged groups, which subsequently improves the electrical conductivity of the system via ionic transport. For instance, the alginate-methacrylate and AAD cross-linked alginate that contain negatively charged carboxylate groups have higher conductivities compared to PEG gels without charged groups at similar cross-linking densities. Therefore, increasing the substitution of linkers to alginate to improve the mechanical properties of the system can reduce the overall electrical conductivity by reducing or eliminating charged groups. The addition of a conductive linker such as pyrrole is a strategy that can be used to overcome these decreases in electrical conductivity.

The addition of electrically conductive polymers within a hydrogel system is a common strategy to improve the electrical properties of hydrogels. These strategies tend to require a two-step polymerization process forming a co-network system. On the other hand, the alginate-g-pyrrole system has the advantage of using an electrically conductive cross-linker to form a gel, which ultimately allows
the formation of a single network system, all with a one-step cross-linking process. Additionally, this strategy uniformly distributes the cross-linked pyrrole throughout the gel; whereas other strategies struggle to evenly distribute the electrical conductive polymer throughout the system, due to its tendencies to aggregate from hydrophobic associations. The even distribution of pyrrole throughout the alginate-g-pyrrole system, contributes to the overall systematic control of the hydrogel properties.

Finally, the electrical stimulation of fibroblasts adhered to alginate-g-pyrrole hydrogels exhibited enhanced activities compared to cells adhered to control gels. Cells stimulated on the alginate-g-pyrrole gels were exposed to an environment with a significantly improved transport of charge through the system by the addition of electron transport through the backbone of polymerized pyrrole groups. Under a direct potential cells began to reduce their surface area most likely due to the forced desorption of charged binding proteins in this environment with improved charge transport. Additionally, cells adhered to alginate-g-pyrrole gels stimulated with an alternating potential exhibited a higher expression of endogenous VEGF, which can be attributed to the improved electrical stimulatory environment of the cells.

6.5 Conclusion

This study presents an advanced electrically conductive hydrogel system designed with systematically controllable mechanical and electrical properties. The hydrogels were formed through a single polymerization step by cross-linking conductive pyrrole groups conjugated to alginate with controlled degrees of pyrrole substitution. Hydrogels with increased degrees of pyrrole substitution exhibited a simultaneous increase in the gels mechanical rigidity and electrical conductivity. The alginate-g-pyrrole hydrogels were used to control the adhesion and proangiogenic growth factor expression of cells adhered to the gels via applied electrical stimulation. We believe that this material design can be extended to a wide array of hydrogel systems, and will be broadly useful for various actuation, cell culture, and biomedical applications.
Figure 6.1. Schematic depicting the design of the electrical stimulation platforms. PDMS molds with wells were created and bound to the surface of indium tin oxide slides (ITO), subsequently forming wells with ITO bottoms.
Figure 6.2. A schematic of the surface modification of indium tin oxide (ITO) to present pyrrole (left) and amine groups (right). Pyrrole groups were conjugated to silane coupling agents presenting carboxylic acid groups using carbodiimide chemistry, and silane coupling agents with amine groups were used to for amine surface modification.
Figure 6.3. (a and b) A schematic of the pyrrole group conjugation to the carboxylic acid groups of alginate with varied degrees of pyrrole substitution using carbodiimide chemistry. (c) Alginate-g-pyrrole hydrogels formed containing a high and low degree of conjugated pyrrole to alginate. (d) Images of gels formed with varied concentrations of pyrrole substitution to alginate.
Figure 6.4. The compressive elastic modulus (a), swelling ratio (b), and cross-linking density (c) of alginate-g-pyrrole gels with varied degrees of pyrrole substitution and PEG gels formed with varied concentrations of PEGDA.
Figure 6.5. The compressive elastic modulus (a), swelling ratio (b), and cross-linking density (c) of alginate methacrylate gels with varied degrees of methacrylate (MA) substitution.
Figure 6.6. The electrical conductivity of alginate-g-pyrrole (●) and PEG (■) gels with varied cross-linking densities in DI water (a) and in PBS (b).
Figure 6.7. (a) The electrical conductivity of alginate-\textit{g}-pyrrole (●) and PEG (■) gels with varied cross-linking densities in 10x PBS. (b) The normalized electrical conductivity of alginate methacrylate gels with varied cross-linking densities in PBS.
Figure 6.8. (a) Image of the electrical stimulation platform constructed of PDMS molds bound to ITO slides. (b) Direct potential cell stimulation images of fibroblasts adhered to AAD cross-linked alginate and alginate-g-pyrrole hydrogels after 0 and 2 hours of stimulation. The scale bars equal 40 μm. (c) Ratio of cell areas before and after 2 hours of direct potential stimulation, termed the cell spreading ratio, for fibroblasts adhered to AAD cross-linked alginate and alginate-g-pyrrole gels. * indicates significant difference between conditions ($p < 0.05$).
Figure 6.9. The cross-linking density (a) and electrical conductivity (b) of the AAD cross-linked alginate and alginate-g-pyrrole hydrogels used for the cellular electrical stimulation experiments.

Figure 6.10. An image of fibroblasts adhered to alginate-g-pyrrole gels 24 hours after direct potential stimulation experiments. The scale bar equals 40 μm.
Figure 6.11. (a) A schematic of cellular endogenous proangiogenic factor expression under alternating current potential stimulation for cells adhered to alginate-g-pyrrole hydrogels in the stimulation platform. (b) The VEGF expression normalized to the overall cellular protein content for fibroblast adhered to AAD cross-linked alginate and alginate-g-pyrrole hydrogels before and after 2 days of alternating potential stimulation.
6.7 References


