Creating a Three-Dimensional, Perfusable Capillary *In Vitro* to model Cerebral Cavernous Malformation

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Bachelor of Science, School of Engineering

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Cerebral Cavernous Malformation (CCM) is a vascular disease that affects the brain and spinal cord and causes the formation of lesions. CCM affects approximately 1 in every 100-200 individuals and approximately 75% of patients with CCM experience symptoms, including inflammation, seizures, headache, hemorrhage, and focal neurological deficits including stroke.[1] CCM affects the function of endothelial cells in cerebral capillaries by increasing their permeability and disrupting their ability to form cell-to-cell junctions.[2] I hypothesize that CCM formation is related to endothelial cell exposure to abnormal shear stress due to blood flow. Studying cell response to shear stress due to flow through a tubular vessel requires a model that recapitulates the anatomical structure of vasculature. CCM has previously been studied in mouse models, but it is very difficult to study cellular mechanisms and cell response to shear stress in vivo.[3] Hydrogel scaffolds have been widely researched as a basis for manufacturing engineered tissues, organs, and blood vessels in three dimensions (3D).[4] This capstone project aims to design a 3D, perfusable, artificial cerebral capillary in a hydrogel that can be used to study CCM formation in vitro.

Keywords: Cerebral cavernous malformations, hydrogel, hyaluronic acid, capillary

Introduction

Cerebral Cavernous Malformation (CCM) is a vascular disease that affects the brain and spinal cord and causes the formation of lesions. CCM affects the function of endothelial cells in cerebral capillaries by increasing their permeability and disrupting their ability to form cell-to-cell junctions.[2] CCM affects approximately 1 in every 100-200 individuals and approximately 75% of patients with CCM experience symptoms.[5] CCM lesions are characterized by their irregular, mulberry-like appearance and their thin, leaky walls. Though it is believed that CCM formation occurs before or shortly after birth, the median age of symptom onset is 37 years. Symptoms of CCM can include inflammation, seizures, headache, hemorrhage, and focal neurological deficits including stroke.[1]

There are two forms of CCM: familial CCM, which is genetically inherited, and sporadic CCM, which occurs due to a spontaneous genetic mutation. There are three genes that have been confidently implicated in the development of CCM and they are labeled: KRIT-1, Malcavernin, and PDCD10.[6] Loss or mutation of any one of these three genes can cause CCM, yet the exact mechanisms involved remain unknown. Because the majority of CCM cases are due to a loss-of-function mutation in the KRIT-1 gene, that was chosen as the primary gene of focus for this project.

It has been hypothesized that CCM functions with Knudson's two-hit mechanism: the first “hit” is a germline, loss-of-function mutation of one of the three implicated genes mentioned previously, and the second “hit” is unknown.[7] I hypothesize that the second “hit” is related to endothelial cell exposure to shear stress due to blood flow. It has been proven that hemodynamic shear stress significantly impacts endothelial cell morphology and function, specifically decreased cell permeability.[8] However, there have been no published findings linking shear stress to the initiation of CCM. One proposed mechanism for CCM initiation is the up-regulation of β1 integrin activation due to decreased ICAP-1 inhibition from a loss of function of the KRIT-1 gene. β1 integrin activation destabilizes cell-to-cell tight junctions in cerebral capillaries, which leads to the formation of lesions.[9] These findings suggest that endothelial cell shear stress exposure could play a role in CCM initiation.

Studying cell response to shear stress due to flow through a tubular vessel requires a model that recapitulates the anatomical structure of vasculature. CCM has previously been studied in mouse models, but it is very difficult to study cellular mechanisms and cell response to shear stress in vivo.[3] One of the main limitations of mouse models is the short time span that a mouse with induced CCM can be studied before it dies.[10] In addition, in vitro models are reproducible, cost effective, and more accessible than animal models.[11]

Hydrogel scaffolds have been widely researched as a basis for manufacturing engineered tissues, organs, and blood vessels in three dimensions (3D).[4] A hydrogel scaffold can mimic the extracellular matrix (ECM) by providing support for cells, and with the addition of biochemical ligands, such as arginine-glycine-aspartate (RGD), cells can adhere directly to a hydrogel.[12] Hydrogel models have been used for a variety of tissue engineering and biofabrication applications, including the development of in vitro blood vessels, but challenges remain. This capstone project aimed to elucidate the role of endothelial shear stress in CCM causation by designing an artificial capillary in a hydrogel.

The specific aim of this capstone was to develop an in vitro artificial capillary to model cerebral blood flow within a hydrogel. The developed model was three-

dimensional, perfusable, and reproducible and was characterized in terms of rheological properties as well as permeability.

Results

Hydrogel Composition

To create a model capillary, several polymer options were considered for the hydrogel: hyaluronic acid crosslinked with added norbornene groups (NorHA), polyethylene glycol crosslinked with added norbornene and thiol groups (PEG-NB/SH), polyethylene glycol crosslinked with added acrylate groups (PEG-AC), and gelatin. Pugh analysis was used to compare the various polymers (Figure S1 in Appendix) and NorHA was selected as the polymer for hydrogel fabrication, primarily due to its highly tunable mechanical properties. Literature has shown that modifying the weight percentage of base hydrogel solution as well as the degree of crosslinking can adjust the stiffness of the NorHA hydrogel.[13]

The norHA was synthesized using an aqueous esterification route via reaction with carbic anhydride.[14] Following synthesis, the norHA was dialyzed in deionized (DI) water for three days and then lyophilized for storage. The hydrogel precursor solution was formed with the lyophilized norHA polymer, deionized water, lithium phenyl-2, 4, 6- Trimethylbenzoylphosphinate (LAP) as a photoinitiator, thiolated arginyl-glycyl-aspartic acid (RGD) as a cell adhesive peptide, and dithiothreitol (DTT) as a crosslinker.

Hydrogel Fabrication

To create a space for a bulk hydrogel with a channel running through it, a custom negative mold was 3D printed out of resin using a stereolithography (STL) FormLabs printer. The design of the mold went through several iterations before developing a mold that allowed for easy removal. The resin was washed three times in 100% isopropanol and then heat treated at 80 °C. Supports were removed from the resin print using tweezers and a razor blade. Then, two blunt-tipped 22G needles were inserted into the slots in the resin mold.Following needle insertion, the mold was filled with polydimethylsiloxane (PDMS). The resulting PDMS device then punctured with a 8 mm biopsy punch on either side of the center well to create media wells and was plasma bonded to a glass slide (Figure 1). Early iterations of the PDMS device used a 5 mm biopsy punch to create media wells, but this was sized up to allow for a greater volume of media to be used.

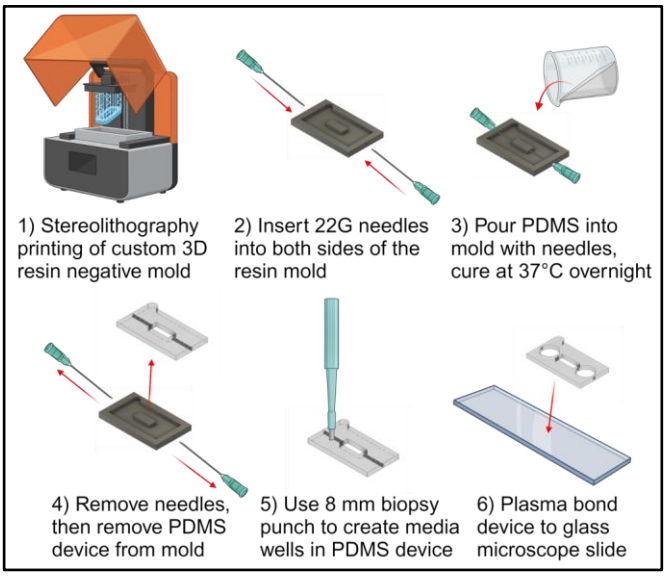

Figure 1. Schematic of PDMS device production. Image created in BioRender.

After the PDMS device was bonded to the glass slide, the 22G needle was inserted back into the device. The hydrogel precursor solution was pipetted into the center well of the device and crosslinked with ultraviolet (UV) light exposure at 10 mW/cm² for 60 seconds. The dimensions of the final resulting hydrogel were 2 mm by 4 mm by 8 mm. A 22 gauge needle has an outer diameter of 0.718 mm, giving the resulting channel an approximate diameter of 0.718 mm and a length of 8 mm.[15] This value is larger than a typical cerebral capillary, but is in the <1 mm range while still being large enough to be feasibly fabricated in the lab. The needle was then removed from the hydrogel, forming the channel (Figure 2, Figure 3)

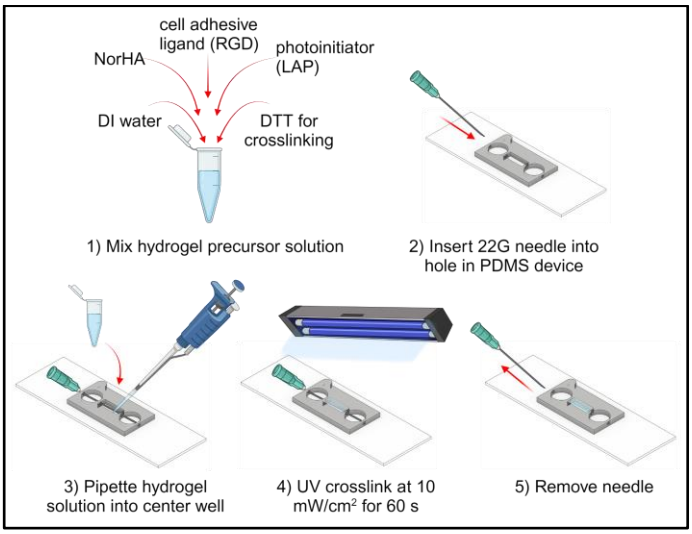

Figure 2. Schematic of hydrogel fabrication. Image created in Biorender.

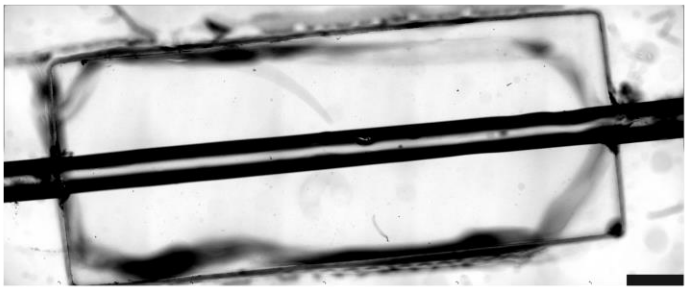

Figure 3. Brightfield image of finished 1.5 wt% NorHA hydrogel within PDMS device with channel running through the center. Scale bar = 1 mm

Rheological Testing

The storage modulus of the native brain extracellular matrix has been observed to be roughly 1 kPa, which can be mimicked by a norHA bulk hydrogel.[16]

Hydrogels with 1, 1.5, 1.75, and 2 weight percent NorHA, all with a 0.6 ratio of thiol (-SH) to norbornene groups, were tested using a TI 83 DH-3 Rheometer to determine the ideal concentration to mimic the stiffness of brain tissue. The hydrogel precursor solution was pipetted onto the rheometer stage where it was exposed to UV light at 10 mW/cm² for 60 seconds before performing a frequency sweep at 1% strain from 0.1 rad/s to 100 rad/s (Figure S2).

Data was recorded by the rheometer and exported into Microsoft Excel (Figure S3). By modifying the weight percentage of NorHA in the overall hydrogel formulation, the mechanical properties of the bulk gel changed (**Figure 4**). Each weight percentage was tested three times and the average storage modulus across the entire frequency was calculated.

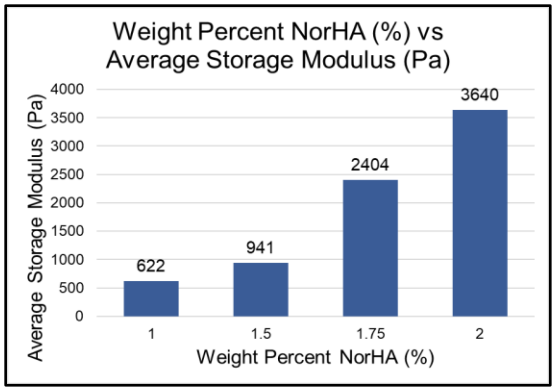

Figure 4. Modifying the weight percentage of NorHA in the hydrogel impacted the storage modulus.

1.5 wt% was chosen as the ideal concentration for the NorHA hydrogels to mimic the mechanical properties of native human brain tissue, with a storage modulus of approximately 1 kPa.[16]

Permeability Assay

FITC-dextran permeability assays are a widely used method to quantify permeability. FITC-dextran is easily dissolvable in water and excites fluorescently with light of wavelength 495 nm.[17] By injecting a FITC-dextran solution into the hydrogel channel and measuring the fluorescence of the surrounding hydrogel over time via fluorescent microscopy, the diffusion of FITC-dextran into the hydrogel over time can be calculated.

Hydrogels were perfused with 200 µg/mL of 70 kDa FITC-conjugated dextran in DI water at a rate of 40 µL/min using a syringe pump (Figure S4). Images were taken of the hydrogel every 20 seconds for 4 minutes (Figure S5) and then analyzed in ImageJ. The measured total fluorescence intensity was normalized with respect to the baseline images and then was plotted versus time (Figure S6). The data was used to solve for the function of intensity over time (Equation 1), where intensity increases exponentially until reaching an asymptote. However, because the fluorescence level on the microscope was saturated, only the four data points in the interval from t=60 s to t=120 s (where fluorescence was increasing before reaching 100% saturation) were viable. The derivative of each side of the equation was solved and then further simplified (Equation 2). Equation 2 was plotted with the data in Microsoft Excel and a linear regression was performed to determine the fit of the data to the equation (Figure S7). However, the calculated value for the best fit slope, -*k*, gave a negative value for *k* and statistical analysis gave a *p* value of 0.18, which is a non-significant value.

$$I(t) = A(1 - e^{-kt}) \quad [1]$$

$$I(t) = \text{function of intensity over time}$$
$$A = \text{constant, asymptote value}$$
$$k = \text{time constant of dye transport into hydrogel}$$
$$t = \text{time}$$

Equation 1. Change in intensity over time

$$\frac{dI}{dt} = Ake^{(-kt)} \quad [2]$$
$$ln(\frac{dI}{dt}) = ln(Ak) - kt$$

Equation 2. Taking the derivative and then natural log of Equation 1 to

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in the Highley lab space. HUVECs were cultured in 2D on culture-treated plastic in a T-75 flask until they reached confluence. HUVECs are considered to be

confluent in 2D culture at values of approximately 10^5 cells per square cm.[18] The HUVECs were then passaged, resuspended in medium, and manually counted using a hemocytometer.

The hydrogel precursor solution was sterilized under UV light for 2 hours before incorporating the photoinitiator (LAP) to prevent any contamination that would harm the cells. After sterilization, incorporating LAP, and UV crosslinking the hydrogels within the PDMS devices, a count of 2.5 million cells in 20 µL of medium was injected into each hydrogel using a blunt tip, 22G needle. The hydrogels were inverted and left upside down for 30 minutes to allow for cell adhesion on both sides of the channel. Following this, the devices were turned right side up and 200 µL of cell medium was added to the media wells. The devices were placed on a rocker to induce flow through the channel and incubated at 37 ℃ for 48 hours. The cell media was changed every 24 hours before applying a 4% paraformaldehyde solution for 15 minutes and then completing live/dead staining (Figure 6).

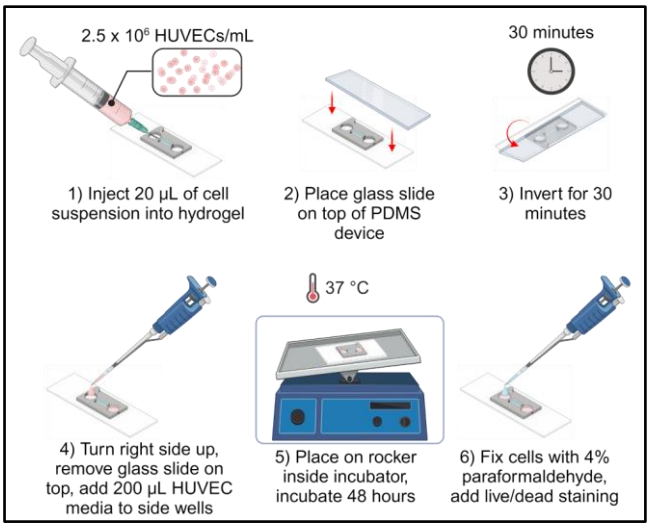

Figure 7. Schematic of cell experiment. Image created in Biorender.

Confocal imaging of the resulting devices did not show any live or dead cells in the channel or surrounding hydrogel. Multiple trials were completed to eliminate the possibility of error in staining or imaging protocols. I hypothesize that although the cells were injected as gently as possible, because the volume of the channel was so small, the cell suspension traveled through the capillary and into the media well, where they were aspirated up at the 24 hour media change. The cells did not adhere to the channel and create an endothelialized vessel despite the inclusion of a cell-adhesive ligand in the hydrogel precursor solution–thiolated RGD.[12]

Discussion

This capstone succeeded in creating a reproducible 3D, perfusable, artificial channel in vitro, that will allow for the modeling of CCM and other vascular diseases in a controlled environment in the future.

This capstone elucidated that a hydrogel model made of 1.5 wt% norHA recapitulated the storage modulus of native human brain tissue. In addition, the permeability of70 kDa within the 5 wt% norHA hydrogel was characterized. In addition, this research showed that despite including thiolated RGD in the hydrogel, carefully injecting cells, and rotating the hydrogel construct 180° for 30 minutes, cells did not adhere to the channel to create an artificial capillary.

This novel hydrogel model is advantageous over existing models because of its unique rheological properties. Existing models of cerebral capillaries are formed within gels with stiffness that far exceeds the storage modulus of native tissue, limiting their physiological relevance.[19]

Future Work

The next step in this research is to repeat the permeability assay experiments with the exposure time of the microscope calibrated to prevent reaching saturation. This will allow more data points to be collected to fit the data to an equation.

The next step in this research is to successfully seed endothelial cells into the channel. One method that was not used for this capstone (due to time constraints) that could be helpful in creating a fully endothelialized vessel would be to coat the needle used to create the channel in thiolated RGD solution, ensuring that thiolated RGD is present on the walls of the vessel and not just throughout the hydrogel. Another method that could be utilized to seed cells into the channel would be by using a syringe pump at a very low feed rate to prevent the cells from flowing out of the channel when injected by hand.

After a successfully endothelialized vessel is created, the permeability of the channel with cells in it should be determined via a FITC-dextran permeability assay and compared to the hydrogel with no cells. It is expected that the permeability of a fully endothelialized channel would be significantly lower than a channel with no cells. Then, the cells in the channel can be exposed to fluid flow via perfusion with a peristaltic pump, and the permeability of HUVECs not exposed to flow and HUVECs exposed to flow can be compared. This will provide quantitative data to show the effect of exposure to shear stress due to fluid flow on endothelial cell permeability.

In addition, endothelial cells could be transfected with silencing RNA to genetically model CCM. Loss of function in proteins encoded by the KRIT-1 gene have been confidently linked to the development of CCM.[20] KRIT-1 expression can be silenced using siRNA to genetically model CCM, then the concentrations of KRIT-1 proteins in transfected and wild type endothelial cells can be compared via Western blotting.

Finally, the permeability of wild type HUVECs not exposed to flow, wild type HUVECs exposed to flow, transfected HUVECs not exposed to flow, and transfected HUVECs exposed to flow can be compared. This will model the initiation of CCM via two "hits" according to the hypothesized Knudson's two-hit mechanism: a genetic factor (modeled by siRNA transfection) and an environmental factor (modeled by endothelial cell exposure to shear stress).[7]

Broader Impacts

This capstone project is just one step towards understanding the overall pathways involved in CCM formation. Establishing a clear understanding of the role of endothelial cell exposure to shear stress in CCM caused by loss-of-function in the KRIT-1 gene *in vitro* will then allow for the investigation of the role of shear stress in CCM caused by Malcavernin and PDCD10 mutations. In addition, the development of a 3D, perfusable, *in vitro* capillary model has applications in studying a wide variety of vascular diseases beyond just CCM. Cardiovascular disease remains the leading cause of death in the United States.[21] Developing a functional *in vitro* model for studying the cause of vascular diseases can also lead to innovations in diagnostic modeling and treatment experimentation while reducing reliance on animal models.

Roadblocks and Challenges

This capstone originally sought to genetically model CCM by silencing KRIT-1 gene expression in endothelial cells and confirm this with Western blotting. This aim was not successfully achieved due to several different factors. Though transfection of endothelial cells was attempted multiple times, no successful Western blots were completed. In addition, the Helmke lab liquid nitrogen tank containing all of the cells malfunctioned, causing all of the cells to thaw. This left us without a cell source for several weeks, halting progress on this aim.

This capstone also aimed to compare the permeability of transfected and wild-type endothelial cells with and without exposure to shear. This aim was also not fully achieved due to the challenges in transfecting cells. Because the artificial capillary was never successfully seeded with cells, their permeability within the model was not able to be studied. However, the permeability of the hydrogel with no cells was characterized to provide a baseline of comparison for future study.

Limitations

This capstone research has several limitations. First, the channel generated by the 22 G needle has a diameter of approximately 700 µm, and the average diameter of a capillary is between 5 and 10 µm, making this artificial capillary larger than biological capillaries by a factor of nearly 100. In addition, the channel generated by the needle is straight, which is only able to model linear flow. This is not representative of how blood flows through vasculature *in vivo*, which is characterized by branching structures in 3D.

The cell types used for this research were HUVECs, but the primary cell type affected by CCM is human brain microvascular endothelial cells (HBMECs). Studying HBMECs would be the most accurate cell type to model CCM *in vitro*, but I opted to study BAECs and HUVECs because they were available to me in the Helmke and Highley labs.

In addition, the data collected from the permeability assay was not sufficient to be able to fit the data to Equation 2. This data collection was limited because the fluorescence intensity level was 100% saturated, giving only four viable data points. Repeating the experiment with decreased exposure time on the microscope and calibrating the saturation point will allow for more accurate data that will be able to be fitted to the transport equation (Equation 1). This will allow for the time constant of 70 kDa FITC-dextran's transport into the hydrogel to be calculated.

Materials and Methods

Resin Negative Mold and PDMS Device Fabrication

Resin negative molds were designed in AutoDesk Fusion and printed on the Highley lab's FormLabs Form 2 stereolithography printer. The resin mold went through ten iterations before settling on a 36 mm by 22 mm by 3 mm size with a 4 mm half-circle tab designed for easy removal of PDMS.

PDMS was made with 90 percent by volume liquid PDMS and 10 percent Sylgard 184 Silicone Elastomer Curing Agent and cured at 37 °C for 45 minutes. PDMS was removed from the mold using tweezers and excess material was trimmed off using a razor blade.

Hydrogel Fabrication

Hydrogels were made in 0.5 mL batches with 7.5 mg NorHA, 100 µL of 25 mM LAP (giving a final concentration of 5 mM), 3.57 µL of 50 mg/mL DTT (giving a final ratio of [-SH]:[nor] of 0.6), 10.25 µL of 50 mg/mL thiolated RGD (giving a final concentration of 1 mM), and 386.18 µL of deionized water. Hydrogels were crosslinked with UV light at 10 mW/cm² for 60 seconds.

Cell Culture

HUVECs were cultured on culture-treated plastic in a T-75 flask until they reached confluence using American Type Culture Collection (ATCC) Vascular Cell Basal Medium with added Endothelial Cell Growth Kit (ATCC) and additional 10 volume % anti-anti and 25 volume % fetal bovine serum. For passaging, cells were rinsed with Dulbecco's phosphate-buffered saline, separated using trypsin-EDTA (Gibco) and centrifuged for 5 minutes at 250*g* before resuspending in Vascular Cell Basal Medium.

Live/dead staining was completed by first fixing cells with 4% paraformaldehyde solution in phosphate buffered saline (PBS) for fifteen minutes, then washing three times with PBS, then applying 1 mL of calcein blue solution (1 µL per 1 mL PBS) and e. Homodimer solution (2 µL per 1 mL PBS).

End Matter

Author Contributions and Notes

L.G.P. and B.P.H. designed research, L.G.P. performed research, L.G.P. and B.P.H. analyzed data; and L.G.P. and wrote the paper. The authors declare no conflict of interest.

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Appendix. Supplemental Information

	Criteria	NorHA	PEG-NB/SH	PEG-AC	Gelatin
Primary Criteria (2 points)	Biocompatible polymer	2	2	2	2
	Customizable stiffness	2	0	0	0
	Cells form a monolayer on it	2	2	2	2
	Easy to image (transparent)	2	2	2	2
Secondary Criteria (1 point)	Easy to crosslink	1	1	1	0
	Easy to synthesize	1	1	1	1
	Affordable	0	1	1	1
	Total:	**10**	**9**	**9**	**9**

Figure S1. Pugh analysis of hydrogel polymers

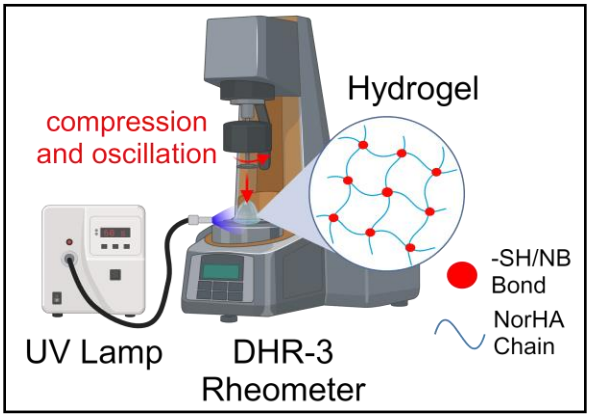

Figure S2. Schematic of rheometer set up with UV light. Image created in Biorender.

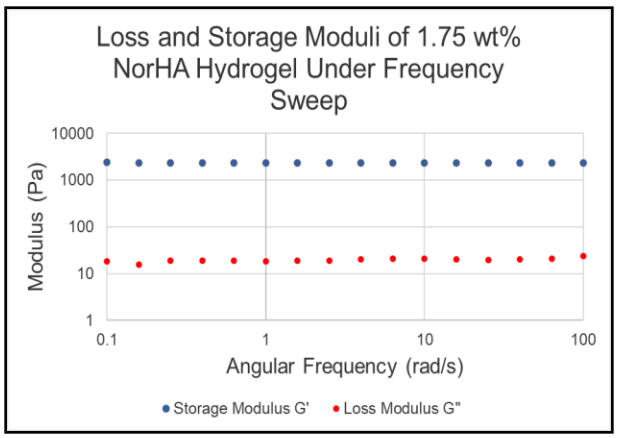

Figure S3. Rheological properties of 1.75 weight % NorHA under frequency sweep.

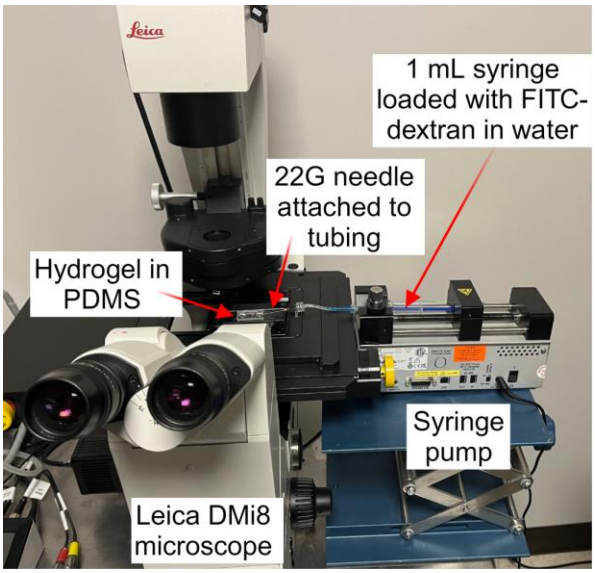

Figure S4. Permeability assay set up

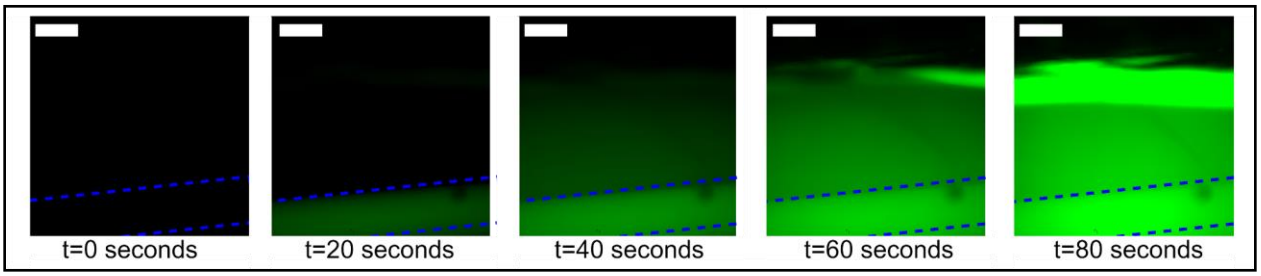

Figure S5. Fluorescent images of FITC dextran (green) permeability assay through NorHA hydrogel at representative time points. Channel outlined in blue dashed line. Images processed in ImageJ. Scale bars= 500 µm

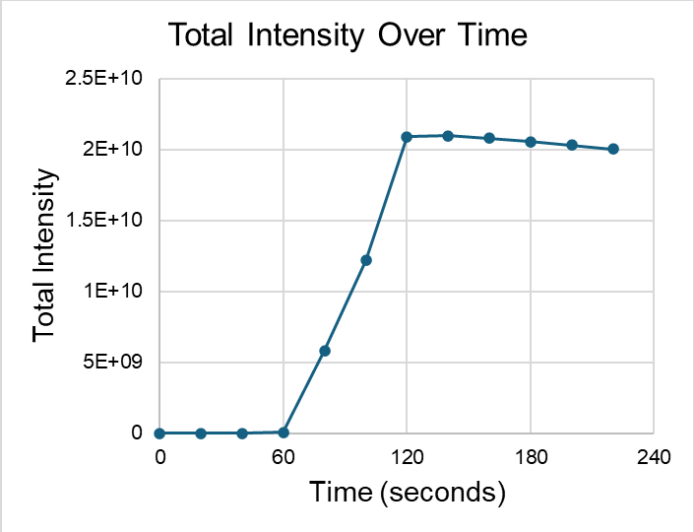

Figure S6. Total fluorescence intensity measured over time at 20 second intervals. This data can be used to solve for the permeability constant of the NorHA hydrogel using Equation 1

Time (seconds)	Time (normalized)	Intensity	dI/dt	ln(dI/dt)	fit	A(1-e(-kt))
60	0	67134528			19.1184	0
80	20	5805268648	2.87E+08	19.4747	19.3837	4601715220
100	40	12158383270	3.18E+08	19.5765	19.649	10601617806
120	60	20888183530	4.36E+08	19.8943	19.9143	18424533680

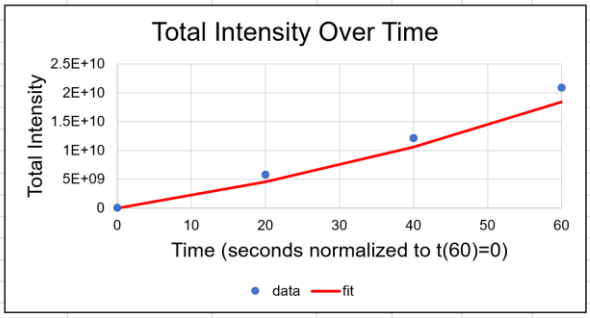

Figure S7. Total intensity over time from t=60 to t=120 plotted (blue circles) with Equation 2 (red line)