Analysis of Hydrogel Degradation with Blood Suspension

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Abstract

The cold chain is common practice to stabilize biologics for storage and transport, but it is costly and challenging to support in underdeveloped countries. This work addressed the potential of using hydrogels to induce reversible biostasis as an alternative to the cold chain, using blood as a model. Polyethylene glycol-based, stimuli-responsive hydrogel networks were used to stabilize biologics. Encapsulating biologics within the network slows cellular processes, protecting the biologic from the external environment. This study used two hydrogel networks: polyethylene glycol phthalaldehyde methacrylate (PEG-p-MA) and polyethylene glycol phthalaldehyde norbornene (PEG-p-Norb). Rheology assessed network stiffness, and a hemolysis assay evaluated the membrane integrity of red blood cells. The hydrogels were crosslinked with a photopolymerization and degraded mechanically with sonication. However, the hemolysis assay did not provide any conclusive results up to this point regarding the efficacy of stabilization with PEG-p-Norb. These novel stimuli-responsive hydrogel networks can encapsulate and release red blood cells, which can be used to explore the stabilization of biologics.

Analysis of Hydrogel Degradation with Blood Suspension

The Cold Chain

The cold chain is the current standard to preserve biologics, which slows metabolic processes. It maintains the viability of biologics, from donor/manufacturer to recipient, by storing materials at freezing temperatures (Feyisa et al., 2022; Rooney et al., 2015). The viability of tissue and blood for a transplant requires temperatures between -20°C and -40°C to ensure viability for up to 6 months (Rooney et al., 2015). Vaccines are another biologic that must be preserved at low temperatures to prevent denaturation (Feyisa et al., 2022). Maintaining these cold conditions requires meticulous monitoring and is not always possible, especially in developing countries. In addition, the cold chain requires a significant amount of money and human resources. Efforts towards eliminating the cold chain are challenging, and generally, they aim to put cells into a state of stasis—an ametabolic state.

Stasis in Nature

The technologies that induce stasis are inspired by organisms found in nature. For example, Tardigrades are microscopic invertebrates known to be resilient to extreme conditions (Boothby et al., 2017; Hashimoto et al., 2016). Tardigrades enter a state of reversible stasis in response to extreme dehydration; the organism is able to withstand extreme environmental conditions in this state (Boothby et al., 2017; Hashimoto et al., 2016). Tardigrade Disordered Proteins are made, which vitrify the cytosol and maintain cellular integrity under these harsh conditions (Boothby et al., 2017; Hashimoto et al., 2016). These proteins also increase the level of crowding intracellularly. The ability of molecules to flow through a cell depends on the macromolecular crowding level, which thereby affects the rates at which reactions occur (Minton, 2001; Zimmerman & Minton, 1993). These rates can be adjusted by increasing or

decreasing the level of crowding within a cell. An increase in crowding is achieved by intracellular gelation. Under physiologically stressful conditions, eukaryotic cells are known to form hydrogels by synthesizing ribonucleoproteins, which form stress granules (Riback et al., 2017). Poly(A)-binding protein and its orthologs are common among eukaryotes in the formation of stress granules to increase the tolerance to stressful conditions (Riback et al., 2017). Therefore, hydrogels have a precedent of being an appropriate model to mitigate harmful environmental conditions of eukaryotic tissues. Furthermore, the cross-linked structure of hydrogels resembles the extracellular matrix found in all connective tissues, which makes hydrogels an excellent candidate for biological applications (Kharkar et al., 2013; Saroia et al., 2018).

Hydrogels

Broadly, hydrogels encompass a wide variety of biological and synthetic materials that absorb significant amounts of water (Kharkar et al., 2013; París & Quijada-Garrido, 2009). Synthetic hydrogels have been formed intracellularly to maintain the integrity of the cell membrane of dead dendritic cells, and the integrity has been maintained even in hypoosmotic conditions (Lin et al., 2021). Dead dendritic cells can be stored to serve as antigen-presenting cells, which shows promise in immunotherapy applications (Lin et al., 2021). A specific subset of hydrogels is stimuli-responsive hydrogels, encompassing materials that polymerize and respond to various external stimuli (Saroia et al., 2018). These stimuli can include but are not limited to light, heat, pH, chemical, and mechanical such as ultrasound (Kharkar et al., 2013; Zardad et al., 2016). Some stimuli, such as light and ultrasound, provide spatiotemporal control, which allows the targeting of a specific area and control over the duration of exposure to the stimulus (Chatani et al., 2014; Zardad et al., 2016). Stimuli-responsive hydrogels provide a

variety of advantages to drug delivery. For example, a drug can be encapsulated within the network during cross-linking or diffused into the network (Kharkar et al., 2013). Subsequently, the drug could be released when the network is broken down in response to a stimulus found within the target environment (Kharkar et al., 2013). Most importantly, synthetic hydrogels have been shown to induce a stasis-like state by slowing the cell cycle and slowing down processes such as translation (Macdougall et al., 2022). The material has been transfected into cells, and ultraviolet (UV) light triggers the network to form through photolabile cross-links (Macdougall et al., 2022). Through macromolecular crowding, this network induces a stasis-like state (Macdougall et al., 2022).

Hydrogels and Reversible Biostasis

While hydrogels have a wide range of capabilities, reversible biostasis has not been achieved (Macdougall et al., 2022). This thesis aims to explore the applications of stimuli-responsive hydrogels in reversible stasis with the use of blood as a model. While red blood cells do not participate in many metabolic events due to a lack of organelles and nuclei, the stabilization of red blood cells through macromolecular crowding demonstrates the potential to be used in reversible stasis in the future. The first phase of this study was aimed at characterizing the physical properties of the hydrogels. The second phase involved the encapsulation and release of blood from these networks. It is hypothesized that stimuli-responsive polyethylene glycol (PEG) based hydrogels will be able to stabilize red blood cells through the formation of a network with photopolymerization, and sonication degradation will release red blood cells (RBCs) unharmed. The two hydrogel networks herein include polyethylene glycol phthalaldehyde methacrylate (PEG-p-MA) and polyethylene glycol phthalaldehyde norbornene (PEG-p-Norb). PEG-p-MA homopolymerizes to form the hydrogel network. PEG-p-Norb

polymerizes with polyethylene glycol thiol (PEG-SH). After characterizing these networks, this study aims to model the reversibility of this process by mechanical degradation of the hydrogel with sonication. Sonication serves the current model to show that these networks degrade mechanically, with the ultimate goal of using ultrasound. Ultrasound is a milder version of sonication. The phthalaldehyde units in both networks have been shown to degrade networks mechanically (Soars et al., 2021). Importantly, ultrasound is biologically safe, and UV light is biologically safe under the conditions used here (Ruskowitz & DeForest, 2019). PEG is known to be inert in a cellular environment; therefore, it is an ideal material for use in this context (Sung & Nesbitt, 2021). Short-chain PEGs can diffuse through the cell membrane (Dupuis et al., 2014). Thus, the hydrogel network could possibly be formed intracellularly and extracellularly. By forming the hydrogel network surrounding cells, macromolecular crowding should stabilize red blood cells to model a stasis-like state. The degradation products are expected to be non-toxic to cells and can easily be removed from an organism. Sonication provides an added advantage because it's able to penetrate tissues (compared to a photo-trigger). Thus, this scheme can be scaled up to target hydrogels placed in the human body. Placement of the hydrogel by injection, surgical, or oral means the network could be degraded with ease from outside the organism.

Reversible biostasis will create a new application for hydrogels by storing biologics at room temperature or allowing the biocomponents to undergo swings in environmental conditions. These specific hydrogels provide the advantage of external stimulation of release because sonication can penetrate tissues to degrade the network at a distance. With that, these networks could also be used for drug delivery through distant stimulation of release. Once the drug is encapsulated, it could be placed in the body through a variety of means, and the network could be broken down from outside the body.

Materials and Methods

Materials

All materials were obtained from Sigma Aldrich. The materials are 4-arm 10K PEG, 4-arm 10K PEG-SH, phthalaldehyde, butylated hydroxytoluene (BHT), pyridine, methacryloyl chloride, sodium hydride, and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP).

PEG Macromer Synthesis

PEG macromers were synthesized to serve as the units that crosslink to form one hydrogel network. With the use of a schlenk line, 4-arm PEG (10K, 1 equiv., 0.2 mmol) was dissolved in dichloromethane (DCM) (30 mL) and added to a 250 mL round bottom flask with a magnetic stir bar under inert conditions (N_2 gas). It was cooled to 0°C. Sodium hydride (60% dispersion in mineral oil, 20 equiv., 4 mmol) was added and stirred for 30 minutes at 0°C. Phthalaldehyde (16 equiv., 3.2 mmol) dissolved in DCM (4 mL) was added and stirred for 1 hour at 0°C. Pyridine (24 equiv., 4.8 mmol) and BHT (1 mg) were dissolved in DCM (3 mL). BHT serves as a radical scavenger. Pyridine, BHT, and methacryloyl chloride (20 equiv., 4 mmol) were added to make PEG-p-MA. To make PEG-p-Norb, methacryloyl chloride was substituted for norbornene acyl chloride (20 equiv., 4 mmol). The reaction was allowed to warm to 25°C. The mixture was quenched in a dropwise fashion with methanol (5 mL). A rotary evaporator was used until the mixture was approximately a 5 mL slurry. Methanol (140 mL) was added to the mixture. The mixture was cooled to -78°C, centrifuged, and the solid was collected.

Figure 1

Chemical Structure of PEG-p-MA

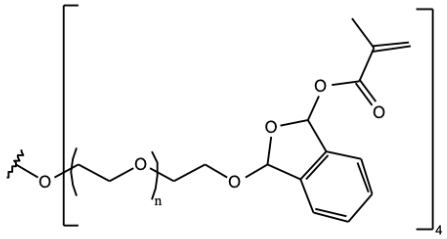
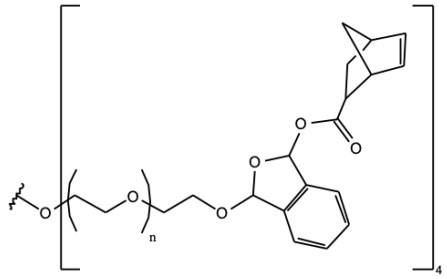


Figure 2

Chemical Structure of PEG-p-Norb



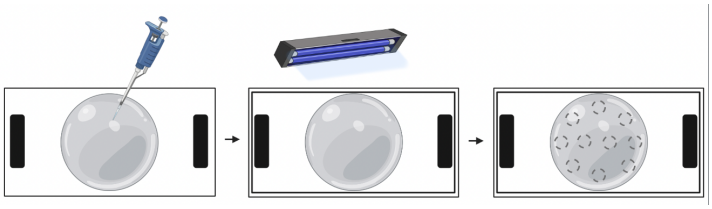
Hydrogel Formation

The two different hydrogel networks were made to be, thereafter, used in degradation. One network was formed with PEG-p-Norb and PEG-SH (15 or 20 weight percent (wt%) macromer). The macromers were added to LAP stock (0.1 wt%) with deionized water. The other network was formed with PEG-p-MA (15 or 20 wt% macromer) and added to LAP stock (0.1 wt%). LAP was the photoinitiator used to initiate crosslink formation in the network through radical polymerization. The solution was pipetted between two glass slides sprayed with RainX to be able to remove the hydrogels from the slides post-cure. The entire solution was pipetted between the two slides (Figure 3), or equivalent amounts of solution were pipetted equidistant from each other onto the glass slide (Figure 4). While both are commonly accepted methods, a switch was made to the ladder method to avoid unnecessary loss of materials (Figure 4). 500 μm or 2 mm spacers were inserted between the glass slides to create a uniform thickness. The solution was

exposed to 365 nm UV light with an intensity of 20 mW/cm^2 for 5-20 minutes to cure the hydrogels. The resultant hydrogels were placed into individual glass vials (20 mL).

Figure 3

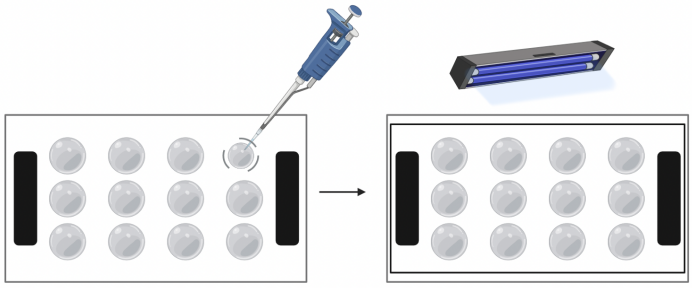
Hydrogel Formation Procedure



Note. The entire solution was pipetted between two glass slides and exposed to light. Uniform hydrogels (8 cm diameter) were cut out from the large hydrogel.

Figure 4

Updated Hydrogel Formation Procedure



Note. Aliquots of the solution were pipetted onto the glass slide and exposed to UV light.

Rheometry

Rheological measurements were obtained with Ares-G2 TA Instruments DH3 rheometer. Experiments were carried out at 25°C with an 8 mm plate. Measurements were collected with 10% strain, a frequency of 10 rad/s, and 0.105 N axial force.

Sonication Degradation

Fisher Scientific Sonic Dismembrator, Model 100 was used to degrade the hydrogels mechanically. Hydrogels were allowed to swell completely in DI water, and initial rheometry was run. Hydrogels were placed in 5-10 mL of DI water for sonication. The vials were placed on ice to prevent heating of the solution, and the sonication probe was inserted into the water with the hydrogels. Hydrogels were sonicated at 7 W for predetermined lengths of time. Final rheometry was run. The time frame was chosen, knowing the time scale at which the hydrogel networks degrade completely.

Lyophilization

Lyophilization was used to obtain the dry weight of the hydrogels for mass loss studies with sonication degradation. Hydrogels were placed in ~2 mL of deionized (DI) water and frozen by submersion in liquid nitrogen. Hydrogels were lyophilized using LABCONCO FreeZone FreezeDry System at -53°C and 0.2 mbar for ~24 hours.

The following equation was used to calculate mass loss of the hydrogels:

$$M_{loss} = \frac{M_o - M_t}{M_o} \times 100 \quad (1)$$

where M_{loss}= percent mass loss, M_0= initial mass, M_t= mass at a pre-established time. After initial lyophilization, the hydrogels were heated in the oven at 80°C for one hour because it was observed the hydrogels did not degrade otherwise. Hydrogels were then swollen completely and degraded with the sonication probe. The supernatant was removed to get rid of soluble degradation products. The hydrogels were lyophilized again to obtain a final dry weight.

Heat Degradation

Hydrogels were placed in glass vials with 5 mL of DI water, and the caps were loosely twisted on. The oven was allowed to come to temperature (60°C, 80°C, or 100°C), and the vials were

placed in the oven for predetermined lengths of time to demonstrate the networks' ability to withstand extreme temperature conditions.

Blood Assay

The following procedure was adapted from the Haeussler Group (Locock et al., 2013). Fresh human whole blood was obtained from Vitalant with ethylenediaminetetraacetic acid (EDTA) added as the anti-coagulant (1.8 mg/mL). Whole blood was diluted in phosphate-buffered saline (PBS, 20 mL). The whole blood was washed four times by centrifugation (10 mins, 2000 rpm, 15°C) to obtain washed RBCs. As found in the human body, PBS was added to achieve 40% RBCs (vol/vol). The hydrogels created were 100 μm thick and 12 wt% macromer. Each solution (Table 1) was prepared in microcentrifuge tubes and diluted to a final concentration of 2% RBCs (vol/vol) before being added to the plate. Three samples (100 μL) of each solution were pipetted into individual wells of a 96-well round-bottom plate. The assay plate was tapped to adequately mix all solutions of polymer and RBCs. The plate was put in a plate reader to assess hemolysis, and the absorbance of hemoglobin was measured at 570 nm. PBS and RBCs were used as a negative control for hemolysis and hemagglutination. Sonication of RBCs and PBS (12W, level 5, 90 s) was used as a positive control for hemolysis. Blood exposed to oxygen for an extended period of time was used as a positive control for hemagglutination. Percent hemolysis was calculated according to the absorbance of the positive control (extended sonication of RBCs) and negative control (PBS and RBCs).

$$\%\ hemolysis\ = \frac{Abs\ of\ sample - Abs\ of\ PBS\ negative\ control}{Abs\ of\ positive\ control - Abs\ of\ PBS\ negative\ control} * 100 \quad (2)$$

Table 1

Blood Assay Samples

Sample	PBS	RBCs (40% v/v)	PEG-p-Norb (6 wt%)	PEG-SH (6 wt%)	LAP (0.1 wt%)	UV Light (365 nm, 30 s)	Sonication N (5 W, Level 2, 30 s)	Sonication E (12 W, Level 5, 90 s)
1	X	X						
2	X	X	X					
3	X	X		X				
4	X	X			X			
5	X	X				X		
6	X	X			X	X		
7	X	X	X	X	X	X	X	
8	X	X	X	X	X	X		X
9	X	X (O_2)						
10	X	X					X	
11	X	X						X

Note. Sonication N indicates sonication normal at level 2, 5 W, and 30 seconds. Sonication E indicates sonication extreme at level 5, 12 W, and 90 seconds. O_2 indicates blood exposed to oxygen for a period of time and coagulated. Weight percentages indicate the macromer or LAP. Abbreviations: phosphate buffered saline (PBS), red blood cells (RBCs), polyethylene glycol phthalaldehyde norbornene (PEG-p-Norb), polyethylene glycol thiol (PEG-SH), weight percent (wt%), volume/volume (v/v), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), ultraviolet (UV).

Results

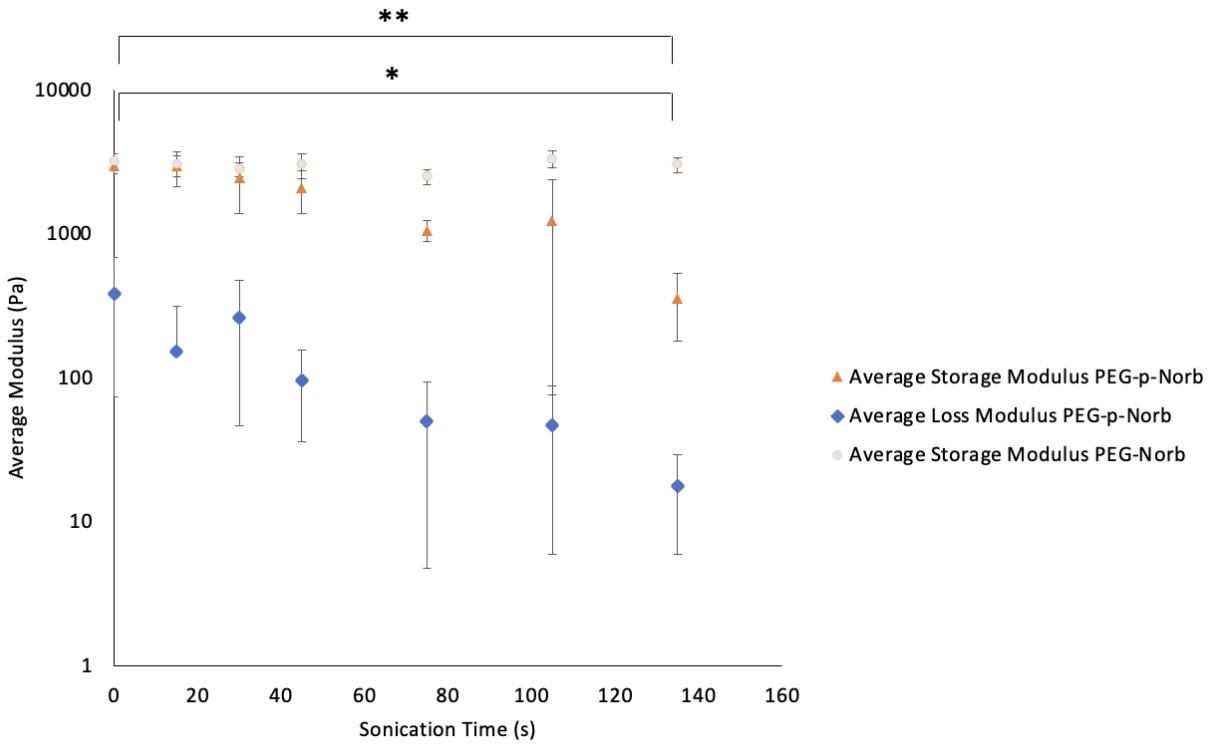
Here, the aim was first to characterize the physical properties of the hydrogels, namely mechanical degradability, in order to use the hydrogels in blood encapsulation and release. Secondly, the network stiffness during heat exposure was examined to determine how these hydrogel networks respond to physiological conditions or swings in environmental conditions.

Rheological measurements were taken for the PEG-p-Norb hydrogel network to determine whether this network degrades mechanically. Three 15 wt% macromer hydrogels were made using 0.1 wt% LAP. The solution was put between two glass slides and exposed to 365 nm UV light with an intensity of 20 mW/cm^2 for 8 minutes on each side. The average storage

modulus of the network declines significantly from 3000 Pa to 400 Pa (Figure 5). These results show that PEG-p-Norb degrades mechanically.

Figure 5

PEG-p-Norb Hydrogel Degrades Mechanically with Sonication

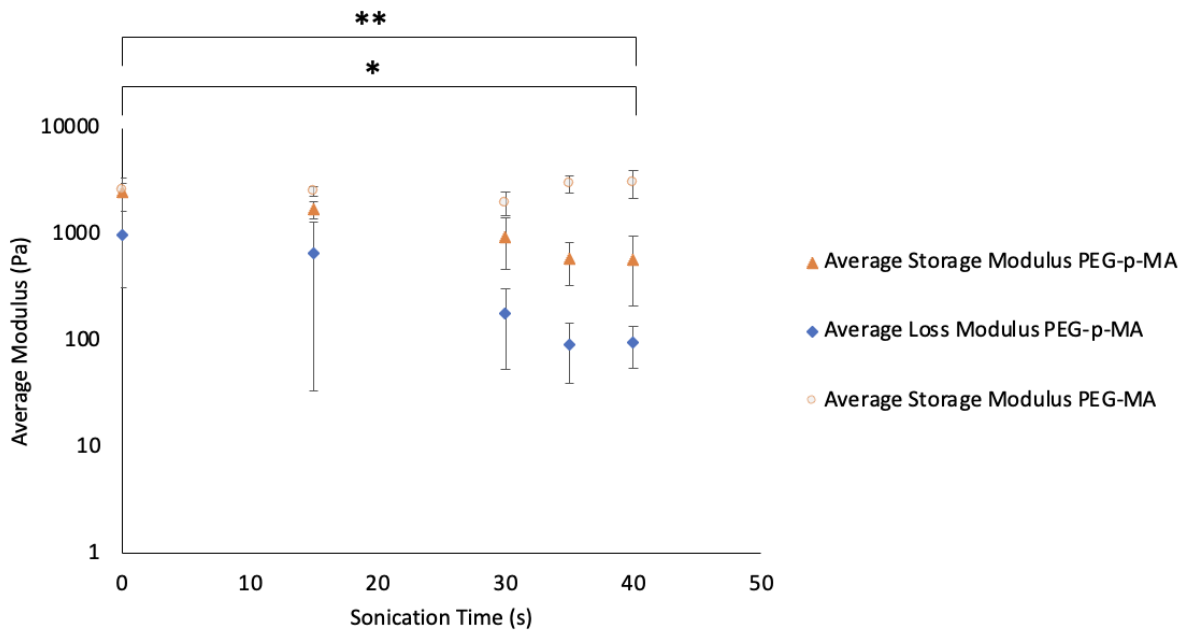


Note. The 2 mm gels were swollen in water, sonicated at 7 W, and rheometry was run. Average Storage and Loss Modulus indicate hydrogel stiffness after the indicated period of sonication. *indicates statistical significance at p<alpha of 0.05 for Average Storage Modulus PEG-p-Norb. **indicates statistical significance at p<alpha of 0.05 for Average Loss Modulus of PEG-p-Norb. Abbreviations: polyethylene glycol phthalaldehyde norbornene (PEG-p-Norb), polyethylene glycol norbornene (PEG-Norb).

Rheological measurements were taken during sonication to determine whether the PEG-p-MA network degrades mechanically. Three 15 wt% macromer hydrogels were made using 0.1 wt% LAP. The solution was put between two glass slides and exposed to 365 nm UV light with an intensity of 20 mW/cm^2 for 8 minutes on each side. The average storage modulus decreases significantly from 3000 Pa to 500 Pa (Figure 6). These results demonstrate that the PEG-p-MA network degrades mechanically.

Figure 6

PEG-p-MA Hydrogel Degrades Mechanically with Sonication

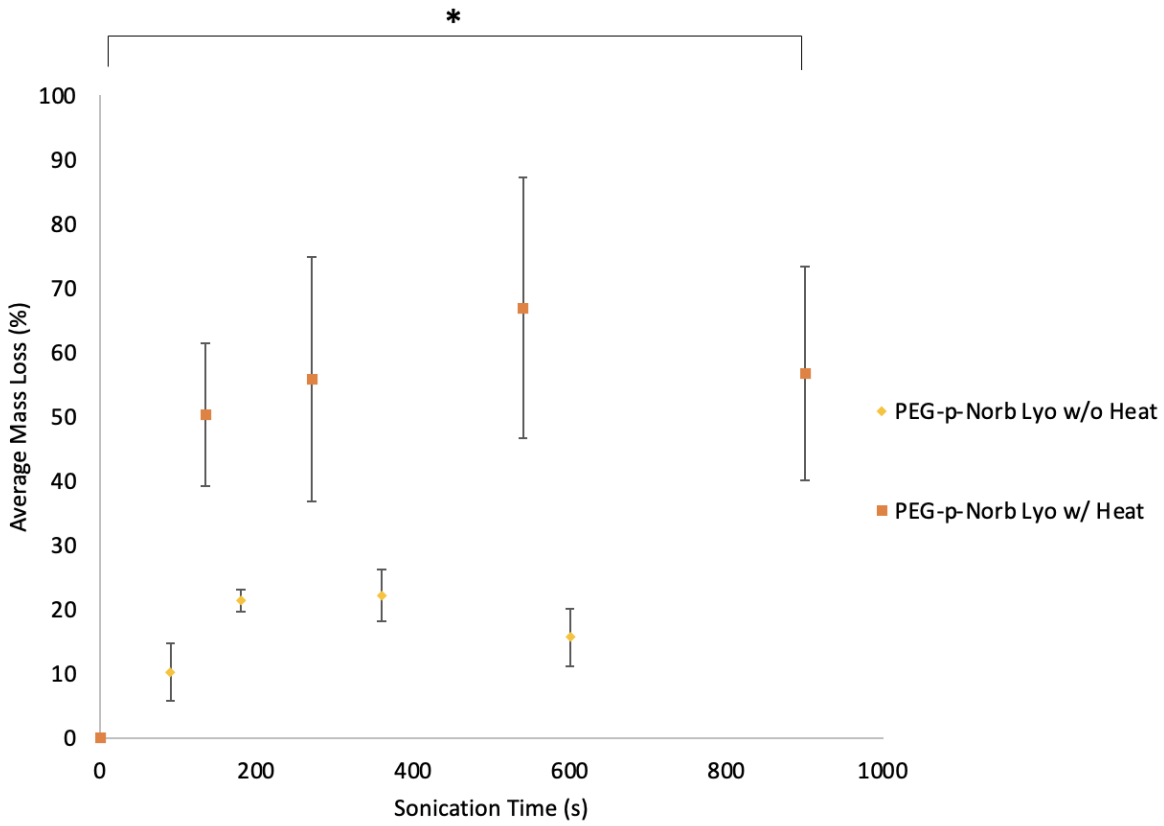


Note. The gels were allowed to swell completely, sonicated at 7 W, and rheometry was run. Average Storage and Loss Modulus indicate hydrogel stiffness after the indicated period of sonication. *indicates significance at p<alpha of 0.05 for Average Storage Modulus PEG-p-MA. **indicates significance at p<alpha of 0.05 for Average Loss Modulus PEG-p-MA. Abbreviations: polyethylene glycol phthalaldehyde methacrylate (PEG-p-MA), polyethylene glycol methacrylate (PEG-MA).

Mass loss of the two networks with sonication was looked at to further confirm the networks were breaking down mechanically. The gels were 20 wt% macromer and 500 µm thick. The gels were formed by exposure to 365nm UV light at an intensity of 20mW/cm^2 for 5 minutes. They were then lyophilized, heated or not, allowed to swell, and sonicated. There was a 57% mass loss of the PEG-p-Norb network with heat exposure at 900 seconds of sonication (Figure 7). There was a 100% mass loss of the PEG-p-MA network with heat exposure at 600 seconds of sonication (Figure 8). This further confirms that both networks broke down mechanically with sonication.

Figure 7

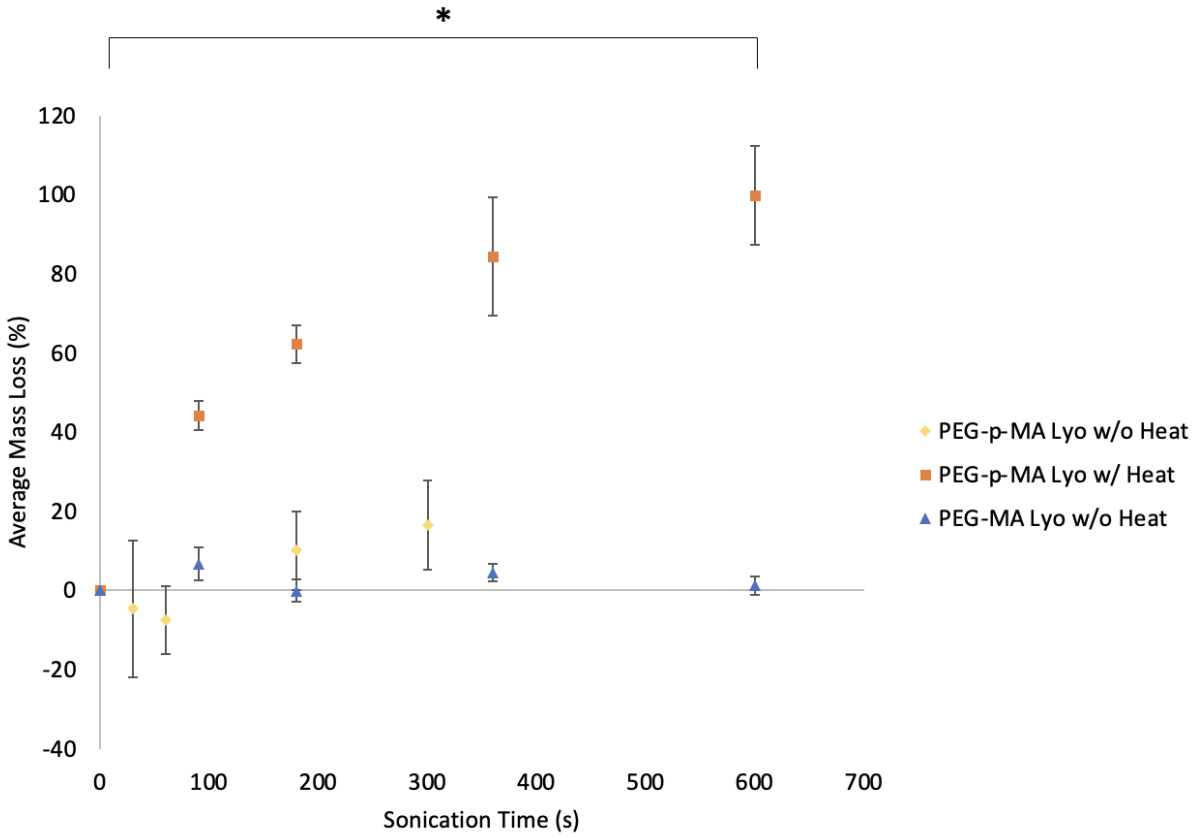
Lyophilization of PEG-p-Norb Showed Greater Percent Mass Loss



Note. PEG-p-Norb Lyo w/o Heat indicates mass loss of the network with lyophilization and without heating prior to sonication. PEG-p-Norb Lyo w/ Heat indicates mass loss of the network with lyophilization and heating prior to sonication. *indicates significance at p<alpha of 0.05 for PEG-p-Norb Lyo w/ Heat. Abbreviations: polyethylene glycol phthalaldehyde norbornene (PEG-p-Norb), polyethylene glycol norbornene (PEG-Norb), lyophilization (lyo).

Figure 8

Lyophilization of PEG-p-MA with Heat Showed Greatest Percent Mass Loss



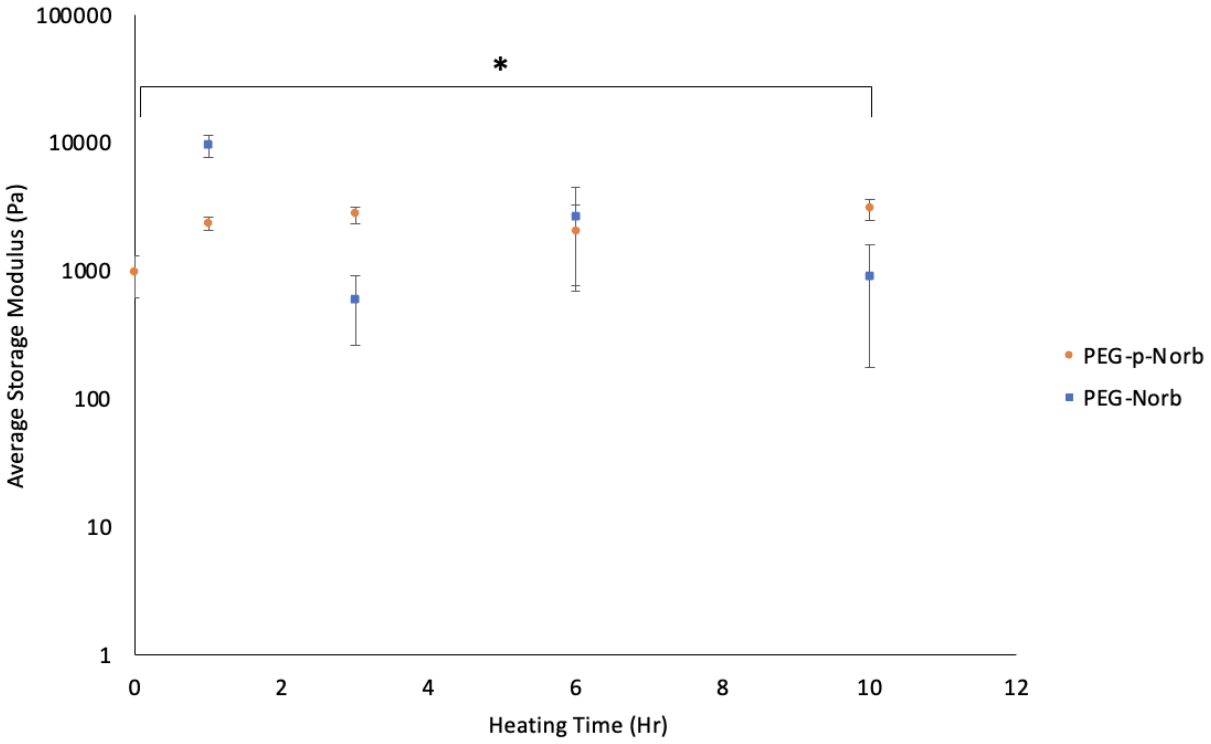
Note. PEG-p-MA and PEG-MA Lyo w/o heat indicates mass loss of the network with lyophilization and without heating prior to sonication. PEG-p-MA Lyo w/ Heat indicates mass loss of the network with lyophilization and heating prior to sonication. *indicates significance at

p<alpha of 0.05 for PEG-p-MA Lyo w/ Heat. Abbreviations: polyethylene glycol phthalaldehyde methacrylate (PEG-p-MA), polyethylene glycol methacrylate (PEG-MA), lyophilization (lyo).

Rheological measurements were taken to determine how the PEG-p-Norb network stiffness responds to heat. 15 wt% macromer, 500 µm gels were made using 0.1 wt% LAP. The solution was placed between two glass slides and exposed to 365 nm, 20 mW/cm^2 UV light for 5 minutes. The average storage modulus increases significantly from 1000 Pa to 6000 Pa (Figure 9). These results show that PEG-p-Norb does not degrade with heat.

Figure 9

PEG-p-Norb Hydrogel Does Not Significantly Degrade with Heat



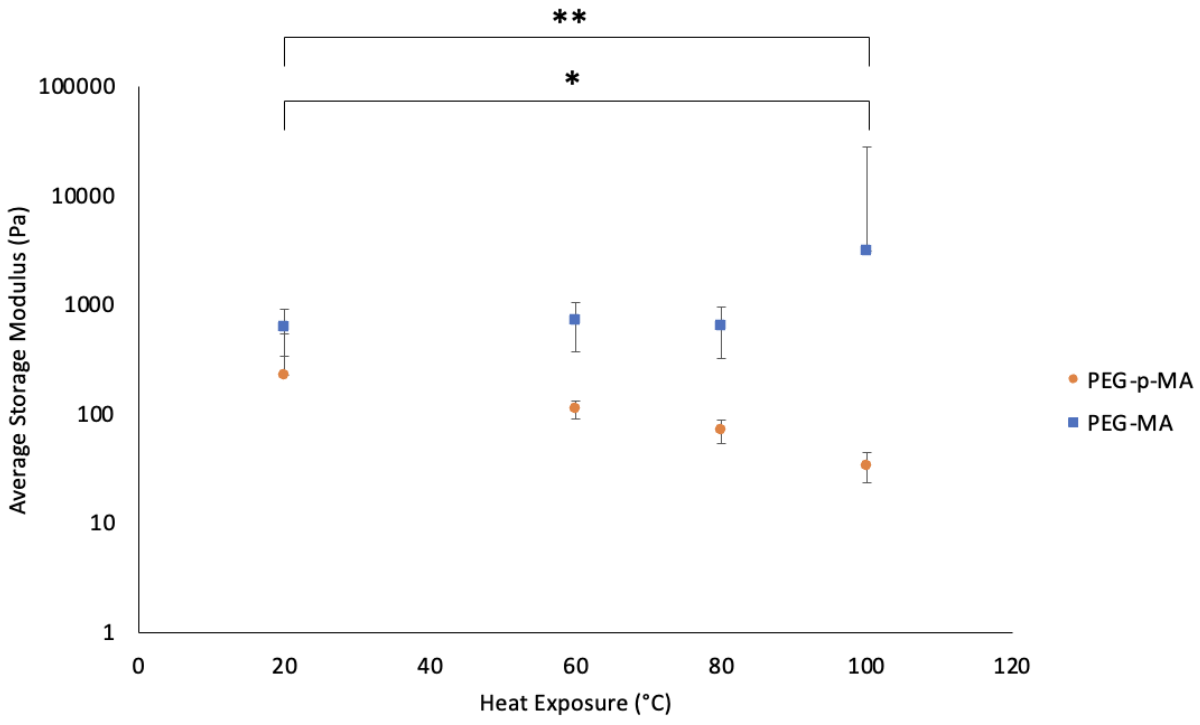
Note. The 500 µm gel was swollen in 5 mL of water, heated in the oven, and rheometry was run. The oven temperature was set to 100°C. PEG-p-Norb indicates polyethylene glycol

phthalaldehyde norbornene average storage modulus after a period of heating. PEG-Norb indicates polyethylene glycol norbornene average storage modulus after a period of heating. *indicates significance at p<alpha of 0.05 for PEG-p-Norb.

Rheological measurements were taken to determine how the PEG-p-MA network stiffness responds to heat. 15 wt% macromer, 500 μm gels were made with 0.1 wt% LAP. The solution was placed between two glass slides and exposed to 365 nm UV light with an intensity of 20 mW/cm^2 for 5 minutes. The average storage modulus decreases significantly from 200 Pa to 30 Pa (Figure 10). These results demonstrate that PEG-p-MA does degrade with heat.

Figure 10

PEG-p-MA Degraded with Heat Stimulus



Note. The gels were allowed to swell completely, heated in the oven at varying temperatures for 15 minutes, and rheometry was run. PEG-p-MA indicates polyethylene glycol phthalaldehyde methacrylate average storage modulus after a period of heating. PEG-MA indicates polyethylene glycol methacrylate average storage modulus after a period of heating. *indicates significance at p<alpha of 0.05 for PEG-p-MA. **indicates significance at p<alpha of 0.05 for PEG-MA.

The degradation of these networks with acid was explored to determine if they respond to other stimuli for possible drug delivery applications. The PEG-based macromers were expected to be acid labile because of the ester groups. Three PEG-p-MA gels at 15 wt% macromer were placed in a 5 mL solution at pH 1 for three days. After three days, the gels were visually still intact and did not appear to have degraded to a significant extent. Following this, three PEG-p-MA gels at 15 wt% macromer were placed in a 5 mL solution at pH -1. It took 30 minutes for the gels to degrade completely, creating a clear solution. Acid degradation was not further explored due to the extreme conditions required to degrade the gel for relevant physiological applications.

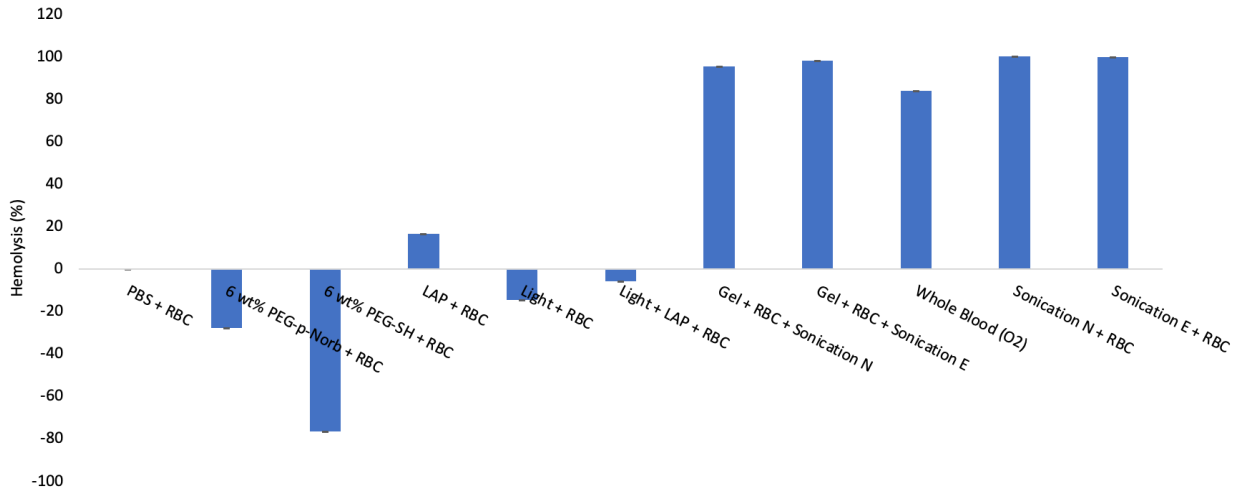
The same procedure was followed to examine the PEG-Norb network's acid degradation. Three PEG-Norb gels with 15 wt% macromer were placed in a 5 mL solution at pH 1 for three days. At the end, the gels were visually intact and did not appear to have degraded to a significant extent. Acid degradation was not further explored because the time scale was not relevant for physiological applications.

Percent hemolysis was measured to determine if red blood cells could be successfully encapsulated and released with sonication. 12 wt% macromer, 100 μm gels with 40% (vol/vol) RBCs, and PBS were exposed to 365 nm UV light for 30 seconds at 20 mW/cm^2. The gels were

sonicated under normal and extreme conditions. Samples of the solution were taken and pipetted into a 96-well round-bottom plate, and the absorbance of hemoglobin was measured at 570 nm. Percent hemolysis of normal sonication was 95%, and extreme sonication was 98% for RBCs encapsulated in the gels (Figure 11). Conclusions cannot be drawn from this data because some controls had a negative percent hemolysis, which is not possible.

Figure 11

RBC Assay Proved Inconclusive



Note. Each label on the x-axis indicates a sample that was assessed for percent hemolysis of RBCs (Table 1). PBS + RBCs served as a negative control for hemagglutination and hemolysis. Individual components of the hydrogel network formation were added as controls for toxicity to RBCs. 6 wt% PEG-p-Norb and 6 wt% PEG-SH were the macromers used to make the hydrogels. LAP was the photoinitiator used to make the hydrogels. Light (365 nm) was used to cross-link the network with LAP. Light + LAP + RBCs served as an indicator of free radical harm to RBCs. Sonication N indicates sonication normal of RBCs at level 2, 5 W, and 30 seconds. Sonication E indicates sonication extreme of RBCs at level 5, 12 W, and 90 seconds. O_2 indicates blood exposed to oxygen for a period of time and coagulated, which served as a positive control for

hemagglutination. Sonication N and Sonication E + RBCs served as the positive controls for hemolysis. Abbreviations: phosphate buffered saline (PBS), red blood cells (RBCs), polyethylene glycol phthalaldehyde norbornene (PEG-p-Norb), polyethylene glycol thiol (PEG-SH), weight percent (wt%), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP).

Discussion

Broadly, the aim of this work was to create a stimuli-responsive hydrogel network that can be modeled for applications in reversible stasis using blood. The first phase involved the characterization of the physical properties of the hydrogel networks. The second phase involved the stabilization of blood within these networks to subsequently be released. The duration and intensity of sonication to completely break down the network were elucidated to use the PEG-based hydrogel networks for stabilization. Knowing the minimum stimulus intensity required to release the material is essential because biologics can only withstand stimuli in a narrow range.

Experiments with PEG-p-Norb and PEG-p-MA were run to determine whether the network degraded mechanically with sonication. Non-phthalaldehyde materials are used as controls and should not degrade mechanically. The PEG-p-Norb network demonstrated a significant decline in the storage modulus after 130 seconds of sonication (Figure 5). Therefore, the network stiffness decreased, and the network broke down mechanically with sonication. The PEG-p-MA network showed a significant decrease in the storage modulus after 40 seconds of sonication (Figure 6). These results demonstrated that the network stiffness decreased, and the PEG-p-MA network broke down mechanically. Mass loss studies of the two networks with sonication degradation were also done to support the rheological data. The PEG-p-Norb network demonstrated significant mass loss after 900 seconds of sonication (Figure 7), and the PEG-p-MA network showed a significant mass loss after 600 seconds of sonication (Figure 8).

This further supported the rheological data, showing both networks broke down mechanically with sonication. It was observed that mass loss of both networks increased with heating prior to sonication, which was unexplained by this data (Figures 7 & 8).

Although sonication is the intended degradation method for these networks, it is important to characterize how the hydrogel networks respond to heat. For these networks to be applicable to humans, it is necessary to understand how they respond to physiological conditions. Furthermore, these networks are intended to eliminate the cold chain; thus, the networks have to remain unharmed during swings in environmental conditions. To explore these issues, the network stiffness was measured after exposure to heat. Network stiffness should decrease if there is degradation of the network. The average storage modulus of the PEG-p-Norb increased significantly from 1000 Pa to 6000 Pa (Figure 9). The increase in the average storage modulus is significant, and this could be explained by the formation of more cross-links or a loss of water within the system. A post-cure process is more plausible because the gels were submerged in water throughout the heating process. These results further confirm that the degradation observed during sonication resulted from mechanical degradation, as opposed to any heat generated during sonication (Figure 5). Following the same reasoning as the PEG-p-Norb system, the PEG-p-MA was tested to determine whether a thermal stimulus degrades the network. Initially, it was proposed that the PEG-p-MA system would behave like the PEG-p-Norb system. Therefore, the hydrogels were heated in the oven for an hour at 100°C. The gels were no longer visible after one hour of heating at 100°C, and this showed that the heat caused the gels to degrade.

The following experiment looked at the thermal degradation of the PEG-p-MA system under lower temperature conditions and shorter time periods to determine the rate of degradation. The average storage modulus of PEG-p-MA decreased significantly from 200 Pa to 30 Pa

(Figure 10). The decrease in storage modulus demonstrates that the gels degraded when exposed to heat. It is essential to consider how these results lend to sonication degradation. Hydrogels are kept on ice during sonication to minimize heat generation impacting degradation. It is unlikely that a significant amount of heat was generated to cause the PEG-p-MA network to degrade during sonication.

By demonstrating that these PEG-based hydrogels form a network with UV light and degrade mechanically with sonication, the hydrogels could then be used to explore the stabilization of biologics with blood as a model. In the second phase of this project, red blood cells were suspended within the network. Red blood cells were then released through mechanical degradation of the network with sonication. Cellular integrity was evaluated with a hemolysis assay created by the Haeussler Group (Locock et al., 2013). The individual components of the network were also evaluated for toxicity to red blood cells. The PEG-p-Norb network was used because it was previously shown that the PEG-p-MA network only achieved gelation with 25% blood and 75% PBS. Furthermore, the PEG-p-MA network degraded with heat, making it a poor candidate for the stabilization of biologics in extreme environments. Percent hemolysis of normal sonication was 95%, and extreme sonication was 98% for RBCs encapsulated in the gels (Figure 11). It is difficult to make conclusions based on the controls in this assay. A negative percent hemolysis was indicated for the controls, which is not possible (Figure 11). More importantly, the procedure taken from the Haeussler Group was not carried out in its entirety. Once the samples were pipetted into the plate, they should have been centrifuged to leave any hemoglobin released from lysed cells in the supernatant. The supernatant then should have been replated and absorbance measured. The resuspension was not done, and the absorbance measurement was taken from the entire sample. Hemolysis cannot be measured this way, as the

absorbance of intact RBCs was also being measured. The hemolysis equation normalizes the negative control (PBS + RBCs) to zero percent hemolysis and the positive control (Sonication E + RBCs) to one hundred percent hemolysis. By measuring the absorbance of the entire sample, minor differences in the quantity of RBCs between samples could generate negative percent hemolysis. In all, the data shown here is not an accurate measure of hemolysis of the samples, and more trials need to be run with resuspension. The pitfalls of this assay include limited quantifiable data. Hemagglutination was only assessed visually, and it can be subjective. Other methods to measure hemagglutination should be explored.

It has been demonstrated that these PEG-based networks can form with the photoinitiator LAP and 365 nm UV light. The networks broke down mechanically with sonication. In the future, ultrasound should be explored to replace sonication, as ultrasound is biologically safe. The PEG-p-Norb network was shown to withstand temperatures up to 100°C for 10 hours (Figure 9). Thus, PEG-p-Norb has the potential to stabilize biologics that may undergo temperature swings. Uncontrolled environments would not impact the integrity of the gel, allowing it to be used in transport without cooling or in underdeveloped countries. The PEG-p-MA network degraded in response to conditions of 100°C after 15 minutes of exposure (Figure 10). This network is not a viable candidate for stabilizing biologics that may undergo temperature swings. Using RBCs as a model for stabilizing biologics did not produce conclusive data. In the future, the assay should be rerun appropriately to evaluate percent hemolysis. When a reasonable percentage of red blood cells are intact after encapsulation and release, the RBCs can be tested to determine how long the RBCs can maintain cellular integrity within the network.

If this can be accomplished, the applications of hydrogels will be opened even beyond their wide range of current applications. Furthermore, it will allow the cold chain to be left in the

past. Biologics— vaccines and transplants— could be stored at room temperature and preserve cellular integrity through swings in environmental conditions. When the two networks are sensitive to ultrasound, they could be used in drug delivery applications. The network could be broken down from outside the body through distant stimulation. It would allow the targeted delivery of drugs in a non-invasive manner. In all, these novel stimuli-responsive hydrogel networks can encapsulate and release red blood cells, which can be used to explore the stabilization of biologics.

Abbreviations

BHT Butylated Hydroxytoluene

DI Deionized

LAP Lithium Phenyl-2,4,6-trimethylbenzoylphosphinate

PEG Polyethylene Glycol

PEG-p-MA Polyethylene Glycol Phthalaldehyde Methacrylate

PEG-p-Norb Polyethylene Glycol Phthalaldehyde Norbornene

PEG-SH Polyethylene Glycol Thiol

UV Ultraviolet

wt% Weight percent

RBCs Red Blood Cells

EDTA Ethylenediaminetetraacetic Acid

PBS Phosphate buffered saline

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