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A hydrogel sealant for the treatment of severe hepatic and aortic trauma with a dissolution feature for post-emergent care

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Non-compressible hemorrhage is an important cause of pre-hospital death following trauma, and immediate control of blood loss is critical. An ideal material for hemorrhage management does not require manual pressure to control bleeding, does not rely on the natural clotting cascade, is suitable for intracavitary hemorrhage, and is removed without debridement. A dissolvable dendritic thioester hydrogel sealant is described for intracavitary wounds. The hydrogel is composed of a lysine-based dendron and a PEG-based crosslinker, which are synthesized in high yields and subsequently characterized by ^{1}H, ^{13}C NMR spectroscopy, and MALDI. The hydrogel dissolution relies on a thiol-thioester exchange mechanism. When compared to untreated controls, the application of the hydrogel sealant reduces blood loss by 33% in a rat model of severe hepatic hemorrhage (23.57 ± 8.27 mL/kg v. 35.21 ± 7.47 mL/kg; p = 0.02) and by 22% in a rat model of aortic injury (17.95 ± 3.84 mL/kg v. 23.09 ± 3.80 mL/kg; p = 0.03). A unique feature of the hydrogel is its dissolution with a biocompatible solution following initial application – thus the treated wound area can be re-exposed for definitive surgical care in an operative setting.

Hemorrhagic shock is the first cause of preventable death after traumatic injury in military personnel[1, 2] and the second in civilian populations.[3] In actively bleeding patients, prompt arrest of hemorrhage is the most important intervention to prevent death and reduce the adverse systemic consequences of the inflammatory cascade after initial resuscitation.[4] In some scenarios, such as trauma sustained in military operations or in rural/wilderness settings, surgical care may not be available for several hours. Immediate compression of external wounds by a first responder substantially reduces volume loss, and limb tourniquets can control hemorrhage and reduce mortality.[5, 6] However, the majority of uncontrolled hemorrhages leading to death are either non-compressible or not amenable to treatment with a tourniquet.[2, 7] These wounds are often junctional, as in incompressible inguinal bleeding, or intracavitary such as intra-abdominal or intrapelvic bleeding. Therefore, easily applied

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sealants, hemostatic dressings, or adhesives are critically needed to stop severe hemorrhage and prevent death after traumatic injury in emergent scenarios.[8–13]

The US Army Institute of Surgical Research and the Naval Medical Research Center recently evaluated the performance of several advanced sealants, adhesives and hemostatic dressings. The results consistently show that three agents (WoundStat® [WS], Combat Gauze® [CG], and Celox®) are effective in reducing blood loss and improving survival in large animal models of junctional hemorrhage.[14–17] The reaction of water with some materials affords an exothermic reaction resulting in burn injuries, a significant adverse event.[18] Furthermore, residues of these agents remain in the lumen of the majority of treated vessels even after extensive irrigation and debridement.[19] Given the clotting activity of these agents, their residues promote local or systemic thrombosis,[20] and are also associated with significant endothelial injury and transmural damage to the vessels that render them nonviable for primary surgical repair.[19, 21] Finally, the need for extensive surgical or mechanical debridement before definitive repair contributes to additional blood loss and damage to adjacent uninjured tissues.

Although hemostatic agents, sealants, and tissue adhesives were originally designed for external use, a natural extension of their applications is for control of intracavitary bleeding in the chest, abdomen, or pelvis (non-compressible by external means). These injuries represent the greatest need as up to one third of potentially preventable deaths in both civilian and military trauma are due to non-compressible hemorrhage.[22] However, with currently clinically available materials, hemostasis is only achieved when extrinsic pressure is applied on the wound. The reported clinical research for intra-abdominal, intrathoracic or intrapelvic use of dressings is limited to date,[23] as only uncontrolled, off-label experiences for refractory bleeding are reported.[24–26] An alternative to these topical agents is the use of systemically administered hemostatic agents, however, selective clotting at only the intended site is a critical and required design feature.[27, 28]

Consequently, there is a critical unmet need for a topically applied material that: 1) is easily applied and forms *in situ*, 2) is of sufficient mechanical flexibility to accommodate complex wound contours and volumes, 3) can be removed atraumatically under controlled conditions for definitive surgical care, 4) is biocompatible, and 5) stops severe arterial and/or venous bleeding without the need for external compression. Recently, we introduced a dissolvable hydrogel sealant whose dissolution is based on a thiol-thioester exchange reaction, and evaluated its performance in *ex vivo* models.[29] Although the reaction between the thiol-terminated dendron and an NHS-activated PEG-based crosslinker afforded a thioester crosslinked sealant, a faster rate of hydrogel formation is desired for securing *in vivo* hemorrhage. Herein, we report the synthesis and characterization of a new hydrogel-based, dissolvable sealant which is an advancement over our previous system. Its dissolution, based on a thiol-thioester exchange reaction, will allow for staged surgical care of injured tissues without the need for debridement of the hydrogel. Specifically, a thiol-terminated dendron is reacted with a maleimide end-capped PEG crosslinker containing two internal thioester linkages to form the sealant. Additionally, we evaluate its efficacy in reducing intracavitary blood loss in small animal models of severe hepatic and aortic injuries in the absence of direct pressure.

The hydrogel sealant is composed of two components: a dendron **1** and a crosslinker **2** (Scheme 1A and B, also see ESI). A dendritic macromonomer is chosen as it enables fine control of the composition, structure and molecular weight and provides a species with multiple reactive sites to ensure rapid gelation. As documented in the literature, such materials are finding utility in various biomedical applications.[29–40] The tri-lysine dendron **1** possesses four reactive thiols for rapid gelation with the bifunctional *N*-(2-aminoethyl)maleimide-capped (MAL) crosslinker. Poly(ethylene glycol) (PEG, Mw = 2 kDa) is attached to the focal point of the dendritic structure to increase aqueous solubility. The dendron **1** is synthesized following a previously reported procedure.[29] Briefly (Scheme 1A), the tri-lysine dendron is coupled to MPEG-amine via a standard peptide coupling reaction with 1-hydroxybenzotriazole (HOBt) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDCI) and, subsequently, the carboxybenzyl (Cbz) protecting groups are removed via hydrogenolysis with Pd/C and H_2. Next, the thiols are introduced by means of a coupling with pentafluorophenol-activated (PFP) and trityl-protected (Tr) mercaptopropionic acid. Finally, the Tr protecting groups are removed with trifluoroacetic acid (TFA) and triethylsilane (Et_3SiH) to give free thiols as the nucleophilic moieties on the dendron. The two lysine-based peptide dendrons, possessing four terminal thiols (**1**) or amines (**3**), were synthesized in good yields and reproducibility, *i.e.* 46% over seven steps and 64% over five steps on average, respectively. The crosslinker **2** is based on PEG (Mw = 3.4 kDa) with two internal thioester linkages and two MAL end-caps (Scheme 1B). It is synthesized by reacting SVA-PEG-SVA (Mw = 3.4 kDa) with thioglycolic acid in the presence of *N,N*-diisopropylethylamine (DIPEA) to introduce two thioester moieties. Next, the macromolecule is capped with MAL moieties using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) as the coupling agent in the presence of DIPEA. Due to its fast reaction kinetics and high specificity for thiols at physiological pH, the MAL reactive group is extensively used in peptide bioconjugate chemistry.[41]

To prepare the hydrogel sealant, a solution of the dendron **1** in borate buffer at pH 9.0 is mixed with a solution of the crosslinker **2** in PBS at pH 6.5 (see ESI). The ratio of thiol to MAL is 1:1, and the total concentration of the polymer in solution is 30 wt%. A hydrophilic hydrogel sealant forms spontaneously within one second at room temperature upon mixing the two aqueous solutions. The thiols of the tri-lysine dendron react with the MAL end-caps of the crosslinker via Michael-type addition reaction, resulting in the formation of thioester bonds, giving a crosslinked network (Figure 1). Due to its instant *in situ* gelation, the hydrogel does not flow from the administration site, and is not diluted in the presence of other fluids. Instead, the sealant fills in the cavities of the tissue surface and mechanically interlocks with it. This feature also allows for application on areas difficult to access or junctional wounds not amenable to tourniquet control. In addition, the hydrogel dressing is stable to hydrolysis for several days in PBS at pH 7.4.

Rheological studies reveal the mechanical strength and viscoelastic properties of the hydrogel sealant (see ESI). An oscillatory stress sweep, performed at a frequency of 1 Hz, establishes the linear viscoelastic region (Figure 2, top). Then, the frequency sweep is run at a constant oscillatory stress of 50 Pa (value chosen from the linear viscoelastic region) and

between 0.1 and 5 Hz frequencies, G' > G". At a frequency of 1 Hz, the G' and G" values for the hydrogel are ~9,000 and 500 Pa, respectively (Figure 2, bottom). Since G' is one order of magnitude higher than G", the hydrogel is more elastic than viscous at the investigated frequency range. The hydrogel sealant is transparent and exhibits viscoelastic properties that enable flexibility to fit complex wound contours and areas.

To investigate the dissolution feature of the hydrogel sealant, it is first applied to a 2.5 mm full thickness incision made on the otherwise intact tissue (see ESI). The sealant prevents leaks when the pressure within the system is increased and held at approximately 120 mmHg. This pressure is similar to arterial pressure (70–100 mmHg) and significantly greater than venous pressure (8–12 mmHg). As shown in Figure 3, the thioester-containing hydrogel is dissolved only upon exposure to cysteine methyl ester solution (CME, 0.3 M, pH 8.5) after an average of 9.6 ± 0.59 min. On exposure to lysine methyl ester (LME, 0.3 M, pH 8.5) or air, the hydrogel remains intact and no hydrogel dissolution is observed. In addition, a control non-dissolvable hydrogel that does not possess thioester bonds, and is prepared with a commercially available crosslinker SVA-PEG-SVA (Mw = 3.4 kDa) and **3** (Scheme 1A), does not dissolve after exposure to CME (0.3 M, pH 8.6). These results confirm that dissolution occurs via thiol-thioester exchange reaction between the thioester present in the hydrogel network and an exogenous thiolate solution (Scheme 2).[32,42] This reaction yields a new thioester intermediate which undergoes a spontaneous sulfur to nitrogen acyl shift through a 5-member cyclic intermediate to form an amide bond. This amide bond is thermodynamically stable and prevents hydrogel reformation.

Cleavage of thioether bonds formed between maleimides and thiols via a retro Michael reaction in the presence of glutathione has been reported in the literature.[43] The rate of this reaction depends on the pKa of the thiol that was used to form the thioether bond; thiols with higher pKa decrease the reaction rate significantly. Therefore, a Michael donor with a sufficiently high pKa can be used to suppress thioether cleavage via retro Michael reaction. In our system, the dendron is capped with 3-mercaptopropionic acid with pKa of 10.3. Based on published studies with glutathione at various concentrations, the retro Michael reaction does not take place within 250 h between the Michael adduct of 3-mercaptopropionic acid and *N*-ethylmaleimide.[43] Based on all of the data, the dissolution mechanism of the hydrogel sealant proceeds via the thiol-thioester exchange reaction.

The viability of the NIH3T3 murine fibroblasts cultured with the hydrogel sealant via transwells for six hours is assessed via MTS assay in triplicate (see ESI). NIH3T3 fibroblasts were chosen due to their major physiological role in wound healing,[44] and there use in standard FDA biocompatibility testing. The cells exposed to the hydrogel sealant exhibit 100.4 ± 6.2% viability as a percentage of the positive control. The viability of NIH3T3 fibroblasts was reduced with treatment with CME and CME with hydrogel dissolution products to 73.5 ± 8.3% ($p = 0.033$) and 89.3 ± 7.7% ($p = 0.018$), respectively. The observed increase in *in vitro* cytotoxicity may be attributed to extensive metal ion chelation by CME. Furthermore. These cytotoxic effects are difficult to remediate *in vitro*; however, the oral and intraperitoneal LD50 (mouse) of CME are 2300 mg/kg and 1340 mg/kg, respectively. Additionally, in the United Kingdom, 100 mg CME tablets are sold under the name Visclair or Mecysteine Hydrochloride.

As a measure of macrophage activation, IL-6 concentration is measured using an ELISA assay (see ESI). The colorimetric ELISA signal is translated into IL-6 concentration through a generated standard curve of known concentrations of murine IL-6. RAW 264.7 macrophages exposed to LPS, the hydrogel sealant, and media only secrete IL-6 to a concentration of 1345 ± 162 pg/mL, 114 ± 46 pg/mL, and 128 ± 4.1 pg/mL, respectively. The significant difference ($p = 0.0003$) in IL-6 levels in the growth media and the insignificant difference ($p = 0.58$) between the hydrogel and media only samples indicate that the hydrogel sealant does not elicit an immune response involving IL-6.

To demonstrate the efficacy of the hydrogel sealant, it is necessary to establish that it reduces bleeding in the absence of direct pressure, thus decreasing blood loss when compared with no treatment (see ESI). There are no differences in the severity of the wounds inflicted (as determined by the mass of excised liver normalized by total body weight) or the volume of pre-injury blood loss within either the hepatic or aortic injury models (Table 1). Out of the 30 rats used, only three are excluded from the analyses due to inadequate pre-operative heparinization (large blood clots are found in the peritoneal cavity). All of the excluded animals belong to the hydrogel sealant arm of the hepatic injury model. The above quantitative assurances of comparable injuries allow the differences observed in blood loss to reflect the efficacy of the hydrogel sealant. Upon application, the hydrogel sealant reduces the post-injury blood loss by 33% in severe hepatic hemorrhage and by 22% in aortic hemorrhage when compared to untreated controls (Figure 4; $p = 0.02$ and $p = 0.03$, respectively) at 20 minutes (Table 1).

Other groups have evaluated the efficacy of different sealants, without direct pressure, in intracavitary hemorrhage using comparable small animal models of hepatic injury.[46–48] Similar to our study design, they also use untreated animals as control groups. The use of thrombin + collagen, fibrin, or chitosan sealants results in blood loss reduction of 24, 53 and 67%, when compared to untreated controls, with the blood loss volume quantified at 90, 30 or 60 min after injury induction and sealant application, respectively. These prolonged time points complicate result interpretation as all groups report mortality in their intervention groups. The reduction in blood loss volume is attributed to both the effect of the sealant and cardiac arrest, as the latter also decreases total blood loss. Additionally, these time points artificially magnify the effective blood loss volume difference between the treatment and control groups, since controls will continue to bleed actively until total exsanguination occurs. In contrast, by quantifying blood loss at 20 min, we attribute the blood loss reduction to the hydrogel sealant because all of the animals in our study are alive at the time of volume quantification. In the severe aortic trauma model, previous efforts focused on the time necessary to achieve hemostasis and not on the effects on blood volume loss, and thus, comparable data are unavailable.[49]

When interpreting these results, several caveats must be considered. First, in both *in vivo* models, the injuries are designed to be readily exposed, allowing the hydrogel sealant easy access to the bleeding organ. This is not an accurate portrayal of an emergency scenario of intracavitary hemorrhage. In such settings, the source of bleeding is not easily identified and oftentimes is difficult to access. However, an initial assessment of efficacy is warranted before testing the hydrogel sealant under more strenuous conditions. Additionally, we do not

evaluate the effect of the hydrogel sealant on other important outcomes such as mortality, due to the short follow-up period (20 min). However, it is essential to avoid the effect that confounding factors such as cardiac arrest can have on blood loss quantifications. In addition, translation of any solid organ hemorrhage model from an animal to human is challenging due to anatomical and physiologic differences. Future investigations will focus on large animal models of hemorrhage and biocompatibility studies required by regulatory agencies (e.g., FDA, ISO 10993) in anticipation of translation activities.

Conclusions

The currently used methods for external hemorrhage control are not applicable to non-compressible or intracavitary hemorrhage. In order to decrease the mortality of severely injured patients, efforts must be directed at the development of biomaterials that allow for efficient hemorrhage control in emergent scenarios and subsequent treatment in the operating room. The hydrogel sealant described herein significantly decreases blood loss in hepatic and aortic *in vivo* animal models of hemorrhage and is of comparable efficacy to other materials described in the literature. Moreover, it introduces the novel capability of on-demand dissolution, via thiol-thioester exchange reaction, thus permitting atraumatic wound re-exposure during definitive surgical care. This feature is not present in any currently available wound sealant systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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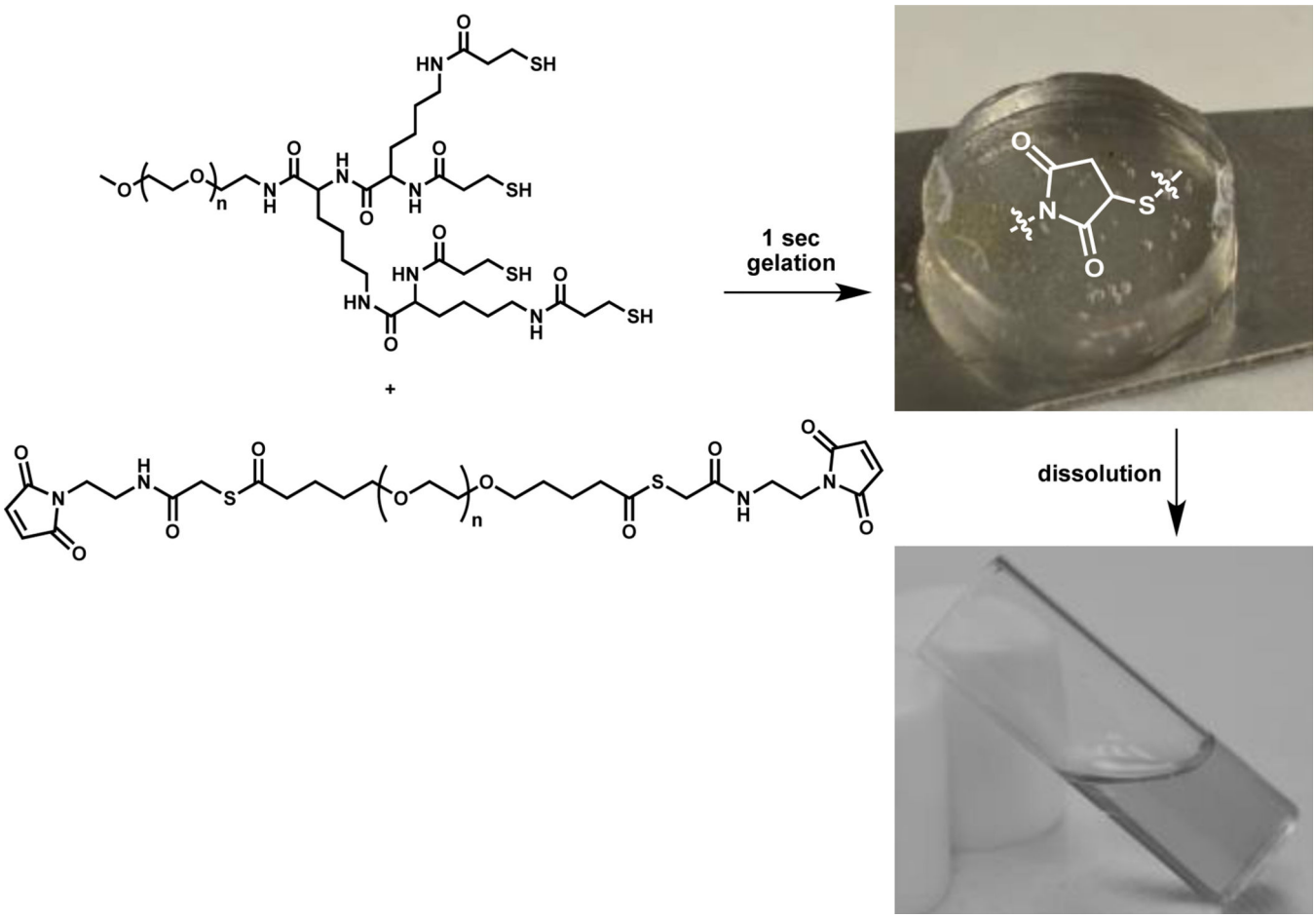


Figure 1.

A transparent hydrogel is formed upon mixing the dendron with the crosslinker, and subsequently dissolved.

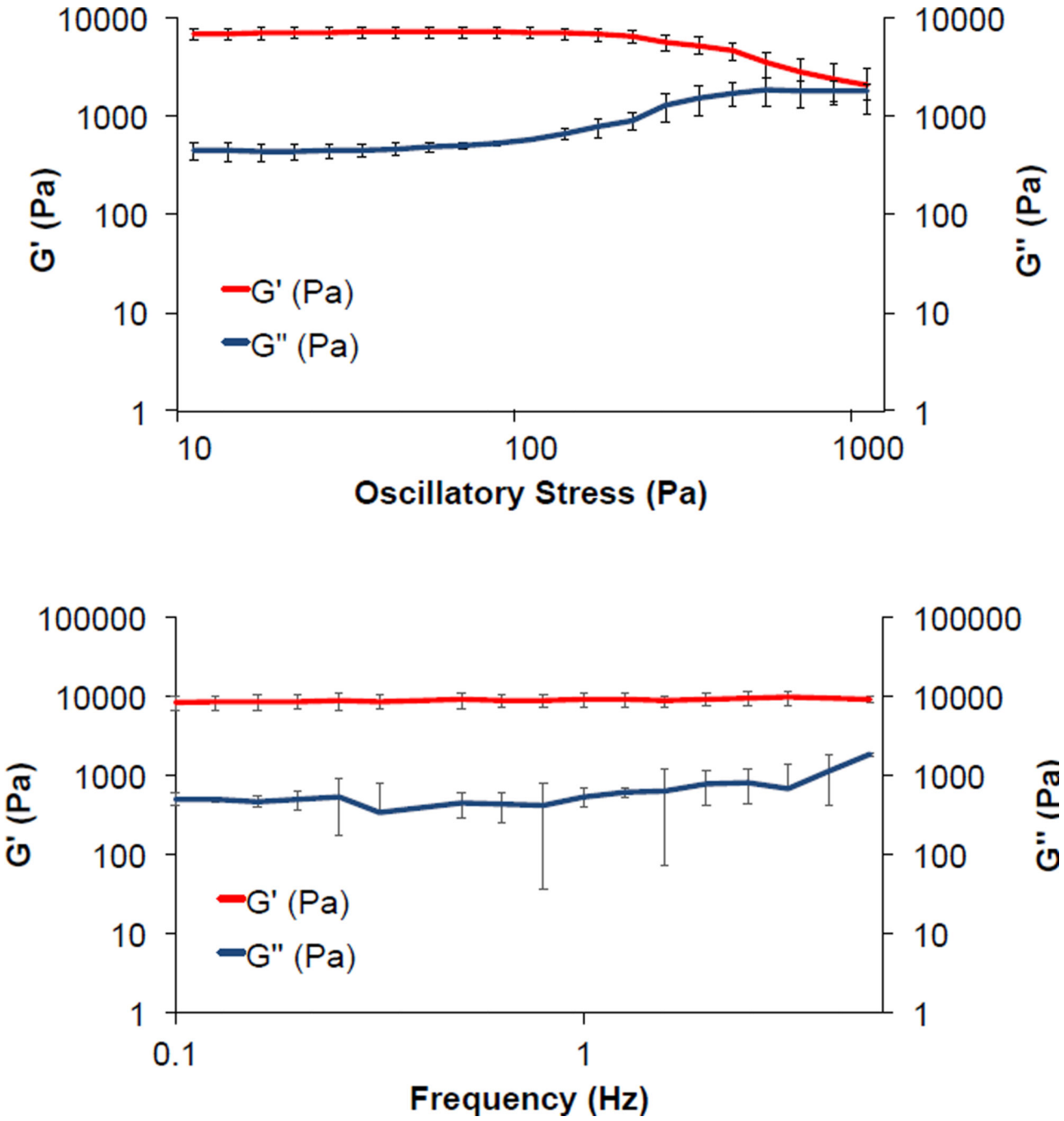


Figure 2.
Oscillatory stress (top) and frequency (bottom) sweeps of the hydrogel sealant. Data are expressed as mean ± SD (n = 3).

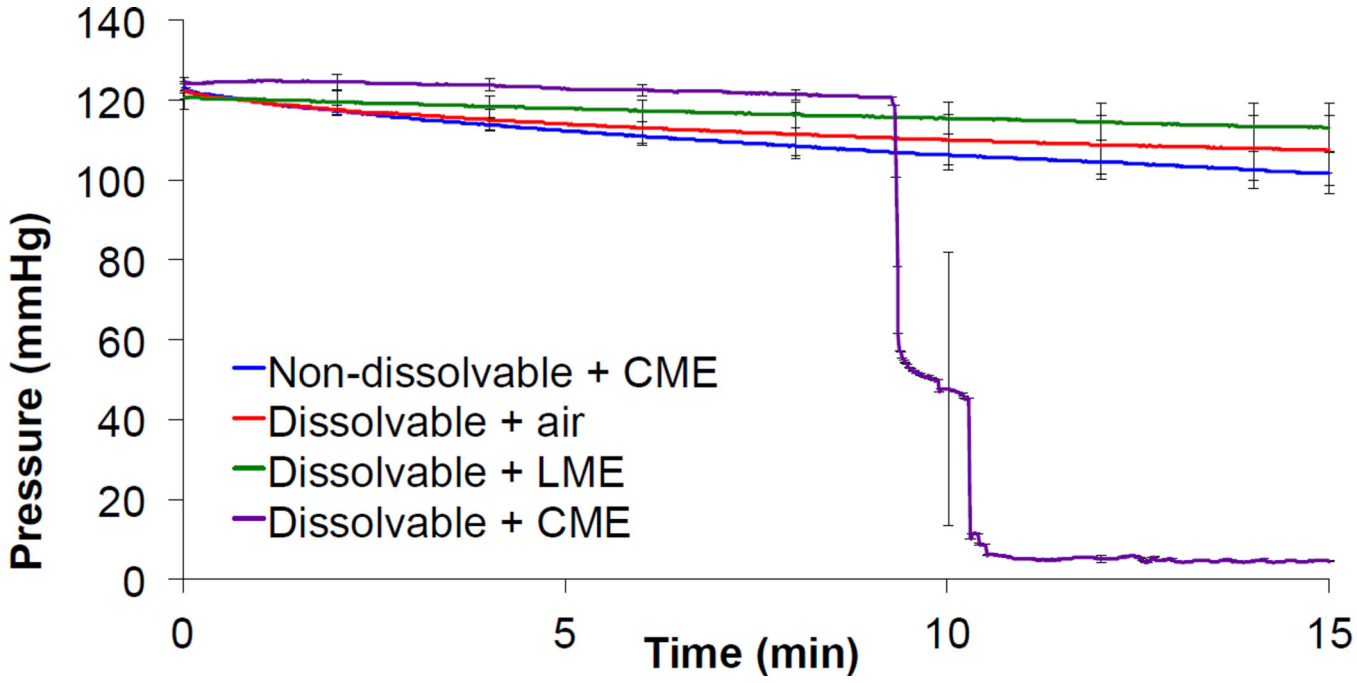


Figure 3.
Hydrogel dissolution is noted by a sudden drop in pressure recordings.

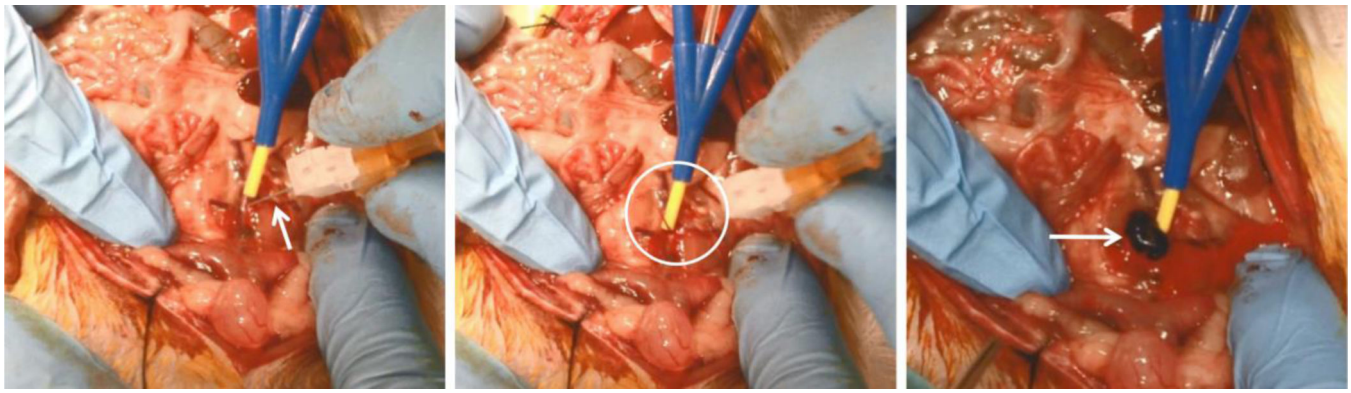
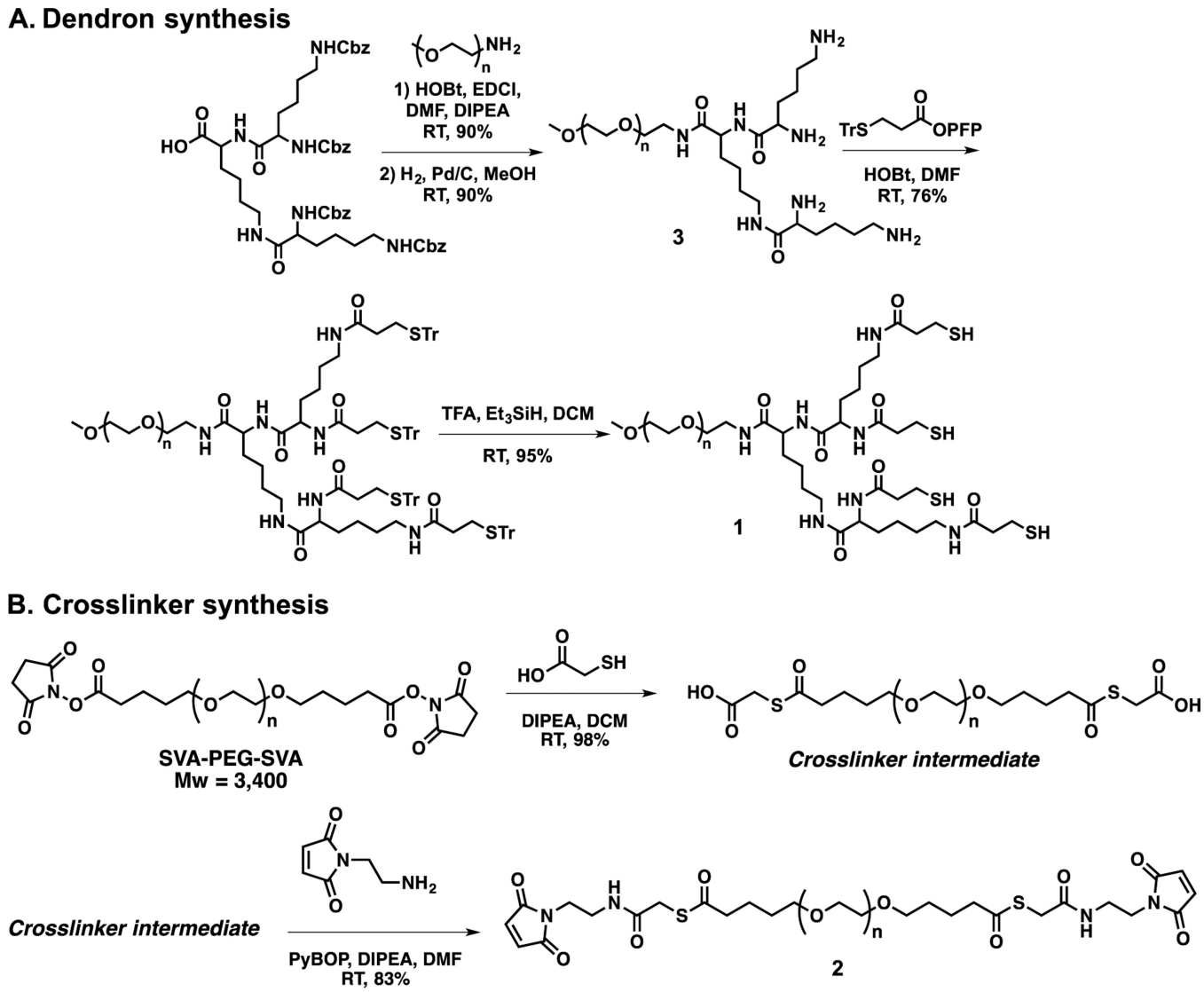


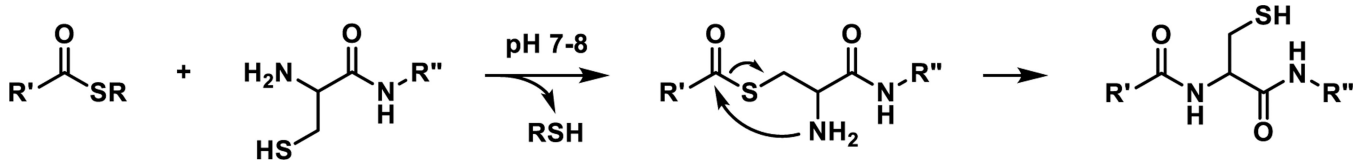
Figure 4.

Consecutive photographs of the hydrogel sealant to secure an aortic injury. (Left) Image of the aorta prior to inflecting hemorrhage with a needle (arrow). (Middle) Image of the aortic hemorrhage (circle). (Right) Image of the secured aortic injury using the hydrogel sealant (arrow).



Scheme 1.

A. Synthesis of the dendron **1**; **B.** Synthesis of the crosslinker **2**.



Scheme 2.

Hydrogel dissolution proceeds via thiol-thioester exchange reaction.

Table 1

Results of *in vivo* animal model studies.

	Hepatic model			Aortic model		
	Hydrogel	**Control**	**p value**	**Hydrogel**	**Control**	**p value**
n	7	5	–	10	5	–
BW%	0.25 ± 0.02	0.22 ± 0.04	0.12	–	–	–
Pre-injury blood loss (mL/kg)	3.61 ± 4.38	4.66 ± 3.08	0.66	1.80 ± 0.56	1.81 ± 0.60	0.98
Post-injury blood loss (mL/kg)	23.57 ± 8.27	35.21 ± 7.47	**0.02**	17.95 ± 3.84	23.09 ± 3.80	**0.03**

n = number of animals; BW% = excised liver as a fraction of total body weight.