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

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I. General information

Chemicals were either used as received or purified according to *Purification of Common Laboratory Chemicals*. All reactions were carried out under nitrogen using standard techniques, unless otherwise noted. SVA-PEG-SVA (Mw = 3.4 kDa) was purchased from Laysan Bio and all other reagents were purchased from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 500 MHz spectrometer. Chemical shifts for ^{1}H NMR were reported as δ, parts per million (ppm), relative to the signal of residual $CHCl_3$ in $CDCl_3$ at 7.26 ppm. Chemical shifts for ^{13}C NMR were reported as δ, ppm, relative to the centerline signal of the $CDCl_3$ triplet at 77.0 ppm. Proton and carbon assignments were established using spectral data of similar compounds. The abbreviations s, t, and tt stand for the resonance multiplicity singlet, triplet and triplet of triplets, respectively. Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired using a Voyager MALDI-TOF spectrometer from Applied Biosystems. Positive ion mass spectra were acquired in linear mode. 2,5-dihydroxy benzoic acid solution in acetonitrile (1 mg/mL) was used as a matrix. 0.3-0.5 mg of the crosslinker was mixed to 10 μL of the matrix solution and 1 μL of the solution was then deposited on a MALDI plate.

II. Synthesis and characterization of hydrogel precursors

The dendron was synthesized following a previously reported procedure.[1] 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. Reactions were performed three times. The *N*-(2-aminoethyl)maleimide (MAL) was synthesized following a previously reported procedure.[2]

*Crosslinker intermediate (**Scheme 1 in the manuscript**):* A flame-dried round bottom flask was equipped with a rubber septum and magnetic stir bar and was charged with SVA-PEG-SVA ($2.94x10^{-4}$ mol, 1.0 equiv), thioglycolic acid ($8.82x10^{-4}$ mol, 3.0 equiv), and *N*,*N*-diisopropylethylamine (DIPEA, $1.18x10^{-3}$ mol, 4.0 equiv). The flask was purged with a stream of nitrogen and dry dichloromethane (DCM) [0.15 M] was added with a syringe. The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 16 h. Then, the mixture was poured into a separatory funnel containing 25 mL of DCM and 50 mL of a saturated citric acid solution. The layers were separated, and the organic layer was washed with water, brine and then dried over Na_2SO_4. The solvent was evaporated under reduced pressure, and the residue was purified by precipitation in diethyl ether to afford the desired *crosslinker intermediate* as a white solid (0.98 g, 98% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.80-3.44 (overlap, 210H), 2.65 (t, J = 7.4 Hz, 4H), 1.77 (tt, J = 7.4, 7.4 Hz, 4H), 1.62 (tt, J = 7.6, 6.2 Hz, 4H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz): δ 197.3, 169.9, 70.5, 70.1, 43.2, 31.0, 28.6, 22.2 ppm; MALDI-TOF (pos) ($M+Na^+$): 3607.

*MAL-PEG-MAL crosslinker **2** (**Scheme 1 in the manuscript**):* A flame-dried round bottom flask was equipped with a rubber septum and magnetic stir bar and was charged with the *crosslinker intermediate* ($2.88x10^{-4}$ mol, 1.0 equiv), *N*-(2-aminoethyl)maleimide ($1.15x10^{-3}$ mol, 4.0 equiv), (benzotriazol-1-

yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (1.15×10^{-3} mol, 4.0 equiv), and DIPEA (1.15×10^{-3} mol, 4.0 equiv). The flask was purged with a stream of nitrogen and dry DCM [0.05 M] was added with a syringe. The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 16 h. The mixture was then added to excess of cold diethyl ether and filtered. The resulting residue was dissolved in DCM and poured into a separatory funnel containing 25 mL of DCM and 50 mL of a saturated citric acid solution. The layers were separated, and the organic layer was washed with water, brine and then dried over Na_2SO_4. Solvent was evaporated under reduced pressure, and the residue was dissolved in water, filtered, and lyophilized to afford the desired MAL-PEG-MAL crosslinker **2** as a white solid (0.81 g, 83% yield). ^{1}H NMR (D_2O, 500 MHz): δ 6.88 (s, 4H), 3.90-3.38 (overlap, 210 H), 2.75 (t, J = 7.2 Hz, 4H), 1.74 (tt, J = 9.6, 6.7 Hz, 4H), 1.64 (tt, J = 8.21, 6.18 Hz, 4H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz): δ 198.8, 170.6, 168.4, 134.1, 69.9, 43.2, 38.6, 37.0, 32.3, 28.6, 21.9 ppm; MALDI-TOF (pos) ($M+Na^+$): 3896.

III. Hydrogel formation

To prepare the hydrogel, the dendron was dissolved in borate buffer at pH 9.0, and was mixed with the crosslinker dissolved in phosphate buffered saline (PBS) buffer at pH 6.5. The ratio of thiol (dendron) to the MAL (crosslinker) was 1:1, and the total concentration of the polymer in solution was 30 wt%. Hydrophilic gels formed spontaneously within one second upon mixing the two aqueous solutions at room temperature as judged by the "inverted tube method."[3]

IV. Rheological studies

The rheological measurements were obtained on a TA Instruments RA 1000 rheometer. Cylindrical hydrogel samples of 9 mm diameter and 3 mm thickness were prepared in a precast Teflon mold and analyzed after sitting in a moisture chamber at 23 °C for 15 min. All rheological measurements were performed at 23 °C to avoid evaporation. The oscillatory stress sweeps of the hydrogel samples were recorded at a frequency of 1 Hz. The frequency sweeps were measured at frequencies from 0.1 to 5 Hz with a controlled oscillatory stress of 50 Pa. A normal force of 0.5 N was applied to the hydrogel using 8 mm steel plate geometry. Data are expressed as mean ± standard deviation (SD) (n = 3). The hydrogel exhibited viscoelastic properties.

V. *Ex vivo* hydrogel dissolution

The dissolution of the hydrogel was defined as failure to hold pressure in a closed system with mean time to failure reported in minutes ± SD.

The testing device consisted of a sensor assembly connected to a cylindrical reservoir.[4] The sensor assembly contained a flow sensor (FLR-1007, Omega Engineering, Stamford, CT, USA) and a pressure sensor (PX-309, Omega Engineering, Stamford, CT, USA), which acquired data at a per-second rate and sent it to a data logger (DAQPRO-5300, Omega Engineering, Stamford, CT, USA). The sensor assembly was connected to the reservoir through polyvinyl chloride (PVC) pressure monitoring lines (MX561,

Smiths Medical, Dublin, OH, USA), resulting in a closed system. The reservoir was lined with *ex vivo* murine skin, and 0.9% sodium chloride solution was fed into the system using a pressure infuser (Infusable, Vital Signs Inc., Totowa, NJ, USA).

A 2.5 mm incision was made on the otherwise intact tissues and then sealed with the hydrogels (n = 3 per group). A thioester-containing hydrogel ("dissolvable hydrogel") was exposed to aqueous cysteine methyl ester solution (CME, 0.3 M, pH 8.5). Additionally, three groups were included in the tests as controls: 1) dissolvable hydrogel exposed to aqueous lysine methyl ester solution (LME, 0.3 M, pH 8.5), 2) dissolvable hydrogel exposed to air only, and 3) hydrogel with no thioester bonds ("non-dissolvable") exposed to CME (0.3 M, pH 8.5). Fifteen minutes after application, pressure within the system was increased to a maximum of 120 mmHg. Hydrogel dissolution was noted by a sudden drop in pressure recordings and visible leakage of saline through the puncture site.

VI. *In vitro* cytotoxicity studies with the hydrogel sealant

VI.1. Cell maintenance

NIH3T3 murine fibroblast cells were maintained as recommended by ATCC in Dulbecco's Modified Eagle Media supplemented with 10% bovine calf serum. Cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. Subconfluent cells were harvested, and 200,000 cells were plated in each well on 12-well plates for use in *in vitro* cytotoxicity studies.

VI.2. Cytotoxicity studies

Hydrogel sealant samples (N = 3) were prepared in polycarbonate transwells with pore size of 0.4 μm. Each hydrogel sealant sample was submerged in PBS at pH 7.4 and allowed to swell overnight at room temperature. Transwells containing pre-swelled hydrogels were inserted into wells with pre-seeded NIH3T3 and the Dulbecco's Modified Eagle Media was aspirated and replaced with Opti-MEM Reduced Serum Media to ensure sufficient contact with the transwell and hydrogel. The NIH3T3 were exposed to the hydrogel sealant for six hours before cell viability was assayed using a colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell proliferation assay. Absorbance was read at 490 nm, and cell viability in each well was calculated as the percentage of the positive control absorbance.

In vitro cytotoxicity of the dissolution agent and dissolved hydrogel products was assessed by culturing NIH3T3 murine fibroblast cells in DMEM supplemented with 10% fetal calf serum at 37 °C with 5% CO_2. Hydrogel samples (N = 4) were prepared and left to gel for 60 min at room temperature. Each gel was then submerged in sterile phosphate buffered saline for 2 hours at room temperature and allowed to swell. Each gel was subsequently dissolved by addition of 50 mM CME over the course of 10 minutes in DMEM media supplemented with 10% fetal calf serum. The NIH3T3 fibroblasts were treated with media containing 50 mM CME with or without dissolved hydrogel sample at 37 °C for 10 minutes, the time it took for the hydrogel samples to fully dissolve in media. Cell viability and death were both measured via a multiplexed, protease-based, fluorescence assay (Promega, Madison, WI). Treatment with CME only, and CME + hydrogel dissolution products resulted in 73.5 ± 8.3 (P = 0.033) and 89.3 ± 7.7 (P =

0.018) percent viability compared to a media-only control. The decrease in cell viability may reflect the limitations of this in vitro assay where cysteine and its analogs, including CME, act as metal chelators,[5-7] leading to cytotoxic effects.[8] CME may further afford a hypertonic shock due to its high osmolarity[9] as the commercially available hydrochloride salt. In the clinic, the CME solution and the dissolution products would be irrigated with saline and these contributions would be minimized. Additionally, CME is generally considered safe. The oral LD50 (mouse) of the compound is 2,300 mg/kg and intraperitoneal LD50 (mouse) is 1,340 mg/kg, as reported in the MSDS. In the United Kingdom, tablets containing CME are sold under the name Visclair or Mecysteine Hydrochloride 100 mg Gastro-resistant Tablets. This medication is indicated as an adjunct in the management of conditions such as chronic obstructive pulmonary disease, when characterized by thick viscid or glutinous mucus, including the symptomatic relief of cough with sputum. Moreover, CME has been used in shaving compositions (Patent 5,902,574, The Gillette Company) and products for hair waving (Patent 4,218,435, Yamanouchi Pharmaceutical Co.). In the literature, CME has been reported to protect cultured rodent lung tissue from sulfur mustard. It was assessed that CME is non-toxic to cultured rat lung slices at 5 mM after 30 min of treatment.[10]

VII. Macrophage activation with the hydrogel sealant

VII.1. Cell maintenance

RAW 264.7 murine macrophages were maintained as recommended by ATCC in Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and were grown at 37 °C with 5% CO_2 in a humidified incubator. Subconfluent cells were harvested, and 50,000 cells were plated in each well on 24-well plates for use in macrophage activation studies.

VII.2. Macrophage activation studies

Hydrogel sealant samples (N = 4) were prepared in polycarbonate transwells with pore size of 3.0 μm. Each hydrogel sealant sample was swelled in PBS at pH 7.4, overnight at room temperature. RAW 264.7 macrophages were exposed to either hydrogel sealant via transwell or to lipopolysaccharide (1 μg/mL), a component of gram-negative bacteria that elicits a strong immune response. The Dulbecco's Modified Eagle Media was aspirated and replaced with additional Opti-MEM Reduced Serum Media to ensure sufficient contact with the transwell. Media samples were collected after six hours and tested for IL-6, a cytokine secreted by macrophages as a marker of macrophage activation. IL-6 concentration was determined by a commercially available ELISA assay (abcam, Cambridge, MA).

VIII. *In vivo* experiments

Thirty adult female Sprague Dawley rats (220-290 g) were used in this study. All animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Beth Israel Deaconess Medical Center and Boston University (Boston, MA, USA).

Two experiments were designed and conducted sequentially. In the first experiment, 15 rats were divided into two groups and the efficacy of the hydrogel sealant was tested in a *hepatic injury model*[11, 12] (n = 10) and compared with no treatment (n = 5). In the second experiment, 15 rats were divided into two groups and the efficacy of the hydrogel sealant was tested in an *aortic injury model*[13] (n = 10) and compared with no treatment (n = 5).

VIII.1. Hepatic injury model

Anesthesia was induced with 5% isoflurane in an induction chamber and then maintained with 2% isoflurane administered through a nose cone. Body temperature was maintained throughout the procedure with a water-circulating heating pad. The animals were anticoagulated with 3000 IU/kg of unfractionated heparin administered intravenously (IV) through the lateral tail vein 5 min before the start of the procedure (our pilot studies demonstrated that a dose of 1000 UI/kg was insufficient to induce uncontrolled hemorrhage as the bleeding stopped spontaneously). After confirming the anesthetic plane, a ventral midline laparotomy was made, and bleeding was controlled with a handheld electrocautery. Any blood present at this point was removed with a pre-weighed 2 x 2" gauze and its blood-soaked weight recorded as pre-injury blood loss. The capsule of the median lobe was scored in three spots (lateral, medial, and in the midline), 1 cm from the hepatic border, with the handheld cautery. The portion of the median lobe distal to the marks was sharply excised with scissors. The weight of the excised median lobe divided by the total body weight of the rat was used as a measure of the injury reproducibility.

In the intervention group, the hydrogel sealant (1 mL) was applied directly on the wound surface 20 sec after the excision, whereas in the control group no treatment was administered. The liver was actively bleeding as the hydrogel was applied. The abdominal cavity was left open and, after 20 min elapsed, shed blood was collected with pre-weighed, 2 x 2" gauzes (post-injury blood loss).

VIII.2. Aortic injury model

In the aortic injury model, the animals were anticoagulated with 1000 IU/kg of unfractionated heparin and underwent midline laparotomy under general anesthesia, as previously described. The abdominal aorta was exposed and the peritoneal cavity was dried with 2 x 2" gauzes in order to quantify the pre-injury blood loss. A 25-gauge needle was inserted into the artery causing severe arterial bleeding. In the intervention group, the hydrogel (1 mL) was immediately applied on the actively bleeding wound surface, whereas in the control group no treatment was administered. The abdominal cavity was left open and, after 20 min elapsed, shed blood was collected with pre-weighed, 2 x 2" gauzes for the evaluation of post-injury blood loss.

VIII.3. Data collection and analysis

The primary endpoint of the *in vivo* studies was post-injury blood loss volume. After each procedure, the blood loss was calculated as the difference between blood-soaked sponges minus the weight of pre-weighed dry sponges. Any blood loss was corrected for body weight (mL/kg) and all measures are presented as mean ± SD. Differences in group means were determined with the Student's t-test (Stata Ver 13.0,

StataCorp, College Station, TX). Statistical significance was assigned at a greater than 95% confidence level ($\alpha = 0.05$).

Procedural criteria were developed to include only animals that represented a consistent challenge to the hydrogel sealant. Animals were eliminated from the study if any of the following criteria applied: 1) copious uncontrolled bleeding from a site other than the site of injury, 2) inadequate anticoagulation (spontaneous hemorrhage arrest in the 20 sec prior to hydrogel application in the hepatic injury model or presence of blood clots in the peritoneal cavity after the injury was inflicted), 3) death due to some identifiable reason other than hepatic/aortic blood loss, and 4) accidental disruption of the hydrogel sealant during the test period or other irreparable procedural error.

IX. References

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