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A Dendritic Thioester Hydrogel Based on Thiol-Thioester Exchange as a Dissolvable Sealant System for Wound Closure

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Abstract

A dissolvable dendritic thioester hydrogel based on thiol-thioester exchange for wound closure is reported. The hydrogel sealant adheres strongly to tissues, closes an *ex vivo* vein puncture, and withstands high pressures placed on a wound. The hydrogel sealant can be completely washed off upon exposure to thiolates based on thiol-thioester exchange and allow gradual wound re-exposure during definitive surgical care.

Keywords

Dendrimer; Reversibility; Thiol/thioester exchange; Hemostasis/Sealant; Wound dressing; Hydrogel

Reactions readily occurring in water are particularly appealing for their potential use in biological and biomedical applications. Thiol-thioester exchange, the reaction between a thioester and a thiolate anion to produce a new thioester and thiolate as products, proceeds in high yield in water, in solutions of pH relevant to biological processes, and at room temperature.^[1] While common in biological processes and in native chemical ligation, thiol-thioester exchange is less well understood and much less used in organic synthesis or in the

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construction of reversible molecular assemblies.^[2] As a reversible covalent reaction, thiol-thioester exchange has the potential to be useful in the design of functional biomaterials. Hydrogels are one class of biomaterials widely used in medical applications,^[3] including the sealing of wounds with several formulations in clinical use. However, there are no hydrogels reported for emergency care where the sealant is applied to the wound and then subsequently dissolved to allow for definitive surgical care at a later time. As a first step towards this goal, we report the synthesis of a covalently crosslinked dendritic thioester hydrogel, its use to close an *ex vivo* jugular vein puncture, and its controlled dissolution for gradual wound re-exposure, based on thiol-thioester exchange.

An ideal sealant system for trauma scenarios sustained in military injuries or in rural or wilderness settings should: 1) stop the bleeding for several hours; 2) adhere to the tissue; 3) be easily applied; and 4) enable controlled dissolution of the sealant in the surgical theatre setting to allow for gradual wound re-exposure during definitive surgical care.^[4] This capability is not present in any available wound closure system, as removal of the clotting agent or dressing is performed via mechanical debridement and/or surgical excision. Synthetic hydrogel based sealants offer a number of advantages as the chemical composition as well as tissue adhesion, mechanical properties, degradation, swelling, etc can be tuned. To that end, we are investigating a strategy based on thiol-thioester exchange and dendritic macromers. While hydrogels based on a thiol-disulfide interchange or native chemical ligation (NCL) are known,^[5] this is the first example of hydrogel disassembly based on thiol-thioester exchange (Figure 1). A dendritic^[6] macromer was selected since the composition, structure and molecular weight can be precisely controlled to afford a macromer with multiple reactive sites to ensure rapid formation of a hydrogel, and such materials have been used successfully for wound closure.^[5a, 7]

As the mechanism behind hydrogel dissolution relies on thiol-thioester exchange, we prepared a thioester-linked hydrogel as well as a control amide-linked hydrogel. Specifically, a lysine-based peptide dendron **1** or **2** possessing four terminal thiols or amines was synthesized in high yield (Scheme 1). First, the Cbz-protected G1 lysine **4** was synthesized following a previously reported procedure.^[8] A poly(ethylene glycol) amine of 2000 *M_w* was then introduced on the peptide dendron by a classic peptide coupling reaction to enhance aqueous solubility, followed by the catalytic hydrogenolysis of the Cbz groups to afford **2**. Dendron **1** was prepared by coupling the activated OPFP-3-(tritylthio)propionic acid **6** to dendron **2**, followed by removal of the trityl groups (tr) using TFA and triethylsilane in DCM. The dendrons were characterized by ¹H NMR, ¹³C NMR, IR, MALDI and TGA (see SI).

To prepare the hydrogels, a solution of dendron **1** or **2** in borate buffer pH 9 was reacted with a solution of poly(ethylene glycol) disuccinimidyl valerate) of 3400 *M_w* (**3**, SVA-PEG-SVA) in PBS buffer at pH 6.5. The ratio of amine or thiol to SVA was 1:1, and the total concentration of polymer in solution was either 10 or 30 wt%. A hydrophilic gel formed spontaneously within seconds upon mixing the two aqueous solutions at either concentration. The gels exhibited viscoelastic properties and were transparent. Cylindrical hydrogel samples of 9-mm diameter and 3-mm thickness were prepared and analyzed after sitting at 25 °C for 2 h (See SI for the kinetic of gelation). The mechanical strength and

viscoelastic properties of the hydrogels were investigated using rheological measurements. First, the strain sweep test was performed on both hydrogels, at a frequency of 1 Hz, in order to establish the range of linear viscoelasticity (LVE) (See SI). Then, the frequency sweep at a constant oscillatory stress of 50 Pa was determined for all hydrogels before and after swelling (See SI). PEG-LysSH and PEG-LysNH₂ hydrogels at either concentration showed strong elastic properties with low $\tan \delta$ ($<5^\circ$) and exhibited a G' greater than G'' at frequencies between 0.1 and 10 Hz (See SI). Before swelling, G' values for 30 wt% PEG-LysSH (**1:3**; reversible) and PEG-LysNH₂ (**2:3**; non-reversible) hydrogels were 37×10^3 and 14×10^3 Pa, respectively, at 1 Hz frequency (Figure 2). The increase in modulus was also consistent with the increase in the wt% of the polymer, 37×10^3 Pa (30 wt%) vs. 6×10^3 Pa (10 wt%) for PEG-LysSH hydrogel, at 1 Hz frequency. The dendron was required for formation of a crosslinked hydrogel as replacement of dendron **1**, containing four thiol groups, with HS-PEG-SH ($M_w = 3400$) gave a **HS-PEG-SH:3** composition with weak mechanical properties ($G' \approx 20$ Pa at 30 wt%), unsuitable for sealing a wound.

After exposure to 4 mL PBS buffer at pH 7.4, 30 wt% PEG-LysSH and PEG-LysNH₂ hydrogels swelled up to 400 and 600%, respectively, and reached equilibrium after 48 hrs (See SI). The G' values, at swelling equilibrium, decreased by approximately half, for both hydrogels (Figure 2). For the 10 wt% hydrogels, the G' also decreased in a similar manner after 48 hrs of exposure to PBS buffer, with the PEG-LysSH hydrogel possessing the lowest G' value (~ 200 Pa) at 1 Hz frequency. Overall the rheological data show that both the reversible and non-reversible hydrogels, at high wt%, exhibited suitable mechanical properties even after swelling for 48 hrs. These results are promising as the hydrogel can maintain its integrity while absorbing fluid from the wound prolonging its contact time on the tissue. It is worth noting that thioesters spontaneously hydrolyze in water to form carboxylic acids in a competing process, which could prevent the formation of the gel. Under our conditions, the PEG-LysSH hydrogels were formed within seconds and were stable to hydrolysis for several days.

Next, the dissolution capability of PEG-LysSH and PEG-LysNH₂ based hydrogels at 30 wt % was evaluated to test the hypothesis that thiol-thioester exchange between the thioester bonds in the hydrogel and a thiolate in aqueous solution (e.g., cysteine) would dissolve the hydrogel and form an amide linkage to prevent hydrogel re-formation. Three conditions were examined: i) a solution of (*L*)-cysteine methyl ester (CME) was used as nucleophile based on the NCL based mechanism; ii) a water soluble thiolate solution, 2-mercaptoethanesulfonate (MES); and iii) a (*L*)-lysine methyl ester (LME) solution where the amine acts as the nucleophile. In all three conditions, the dissolution of the hydrogel was evaluated at equilibrium after swelling in PBS buffer at pH 7.4. The PEG-LysNH₂ hydrogel containing the amide bonds was used as a control. It was observed that the pH of the buffer solution as well as the concentration of the thiolate solution played a significant role in the rate of exchange, thus in the dissolution time of the thioester hydrogel. As observed in Figure 3, increasing the concentration of CME solution up to 0.5 M, at constant pH of 7.4, decreased the dissolution time of the hydrogel from $t_{1/2} = 30$ min to $t_{1/2} = 18$ min. Similarly, when increasing the pH to 8.5, at a constant concentration of CME solution (0.3 M), the thioester bridges in the gel were rapidly cleaved and the hydrogel completely dissolved in

$t_{1/2}$ = 12 min as compared to 25 min at pH 7.4 (Figure 3). Upon exposure of PEG-LysSH hydrogel to an excess solution of MES (0.3M) in PBS at pH 8.5, the dissolution time of the gel was comparable to that with CME, with $t_{1/2}$ = 10 min. Interestingly, 0.3 M LME solution in PBS at pH 8.5 did not cleave the thioester bridges in the PEG-LysSH hydrogel even after 60 min, demonstrating that a thiol-thioester exchange is responsible for the dissolution of the hydrogel with CME. As expected, reacting the PEG-LysNH₂ hydrogel with 0.1 M CME solution at pH 7.4 did not dissolve the gel even after 1 hr exposure.

In order to evaluate the PEG-LysSH hydrogel for closure of a wound, we first investigated its adherence to *ex vivo* human skin tissues. A solution of 30 wt% PEG-LysSH hydrogel (or 30 wt% PEG-LysNH₂ hydrogel as a control) in borate buffer was mixed with a solution of **3** in PBS and quickly applied to the skin. The gel formed within seconds. As shown in Figure 4, torsion stress was applied on both hydrogels to test their adherence strength and flexibility on the skin. Despite the stress applied, the gels remained intact. Next, we evaluated the dissolution of the thioester hydrogel upon exposure to 0.3 M CME in PBS buffer, at pH 8.5. After 30 min, PEG-LysSH hydrogel was completely dissolved and washed off while PEG-LysNH₂ swelled and remained adhered to the skin even after several hrs.

An *in vitro* cytotoxicity study with the 30 wt% PEG-LysSH hydrogel was performed with NIH3T3 murine fibroblast cells (See SI). The viability of the cells was 97±3% after exposure to the hydrogel for 24 hrs and similar to the untreated control ($p>0.05$). The cytotoxicity of 0.1 M and 0.3 M CME buffer solution at pH 7.4 and 8.5 in the presence of the thioester hydrogel was also assessed (See SI). The cells were completely viable after 1 hr exposure to the cysteine buffer solutions at either pH or concentration. The degradation products of the hydrogels are (L)-lysine, mercaptopropionic acid and poly(ethylene glycol) chains.

Next, an *in vitro* macrophage activation study was performed with PEG-LysSH to determine if the hydrogel induces an immune response (See SI). Briefly, macrophages were exposed to the 30 wt% PEG-LysSH hydrogel for 24 hours ($n=3$), or 1 µg/mL Lipopolysaccharide (LPS), a component of Gram-Negative bacteria that elicits an immune response as the positive control. Media samples were then tested for IL-6, a marker of macrophage activation. LPS exposure afforded a statistically higher IL6-response than the hydrogel ($p<0.01$), whereas the hydrogel's response was statistically indistinguishable ($p>0.05$) to that of a media only control. The 30 wt% PEG-LysSH hydrogel does not activate macrophages with production of IL-6.

Finally, the PEG-LysSH hydrogel was tested as a sealant on an *ex vivo* bovine jugular vein, in order to simulate a trauma puncture. The vein was first linked to a syringe pump and filled with PBS solution. Prior to the application of the gel, the pressure was increased in the vein to ensure a leak proof system and that it could withstand pressures of ~250 mmHg (the upper limit of detection; $n=3$), significantly greater than normal arterial blood pressure of 120 mmHg. Next a 2.5 mm hole was made on the vein surface, and the pressure dropped to 0. Dendron **1** and SVA-PEG-SVA **3** were mixed quickly at room temperature, and then a 100 µL solution of the hydrogel at 30 wt% was applied to the puncture site (Figure 5). Within 5 min of closing the incision, the hydrogel sealant secured the wound without

leakage, as the syringe pump continuously increased the pressure to ≈ 250 mmHg ($n=3$). Application of CME afforded dissolution of the sealant and the wound leaked again. The procedure with the hydrogel sealant was facile and did not inflict additional tissue trauma.

In summary, the synthesis of a dendritic thioester hydrogel is reported that gels within seconds from the multiple thioester linkages formed between the thiol residues of dendron **1** and the poly(ethylene glycol) macromer **3**. The crosslinked hydrogel sealant is transparent, adhesive, elastic, hydrophilic, and acts as a physical barrier on the vein surface. The hydrogel sealant also exhibits strong mechanical properties even after swelling in PBS buffer, and completely adheres to human skin tissue even when torsional stress is applied. The hydrogel sealant can be completely washed off of the skin upon exposure to a thiolate solution based on the thiol-thioester exchange mechanism. The use of a thioester hydrogel based on the thiol-thioester exchange for wound repair as opposed to commercially available wound dressings introduces the novel capability of gradual dissolution in the surgical theatre setting to allow for controlled wound re-exposure during definitive surgical care.

Experimental Section

Synthesis of dendron 2

HOBt (0.59 g, 4.4 mmol) and EDCI (0.84 g, 4.4 mmol) were added at room temperature and under nitrogen to a solution of compound **4** (3.75 g, 4 mmol) in DMF (40 mL). Next, a solution of MPEG2000-NH₂ (7.7 g, 4 mmol) and DIPEA (0.85 mL, 4.8 mmol) in DMF (20 mL) was added dropwise. The reaction mixture was stirred at room temperature over night. The solvent was concentrated under vacuum and the crude was redissolved in DCM. The organic phase was extracted with sodium bicarbonate, water and brine to yield the Cbz-protected dendron. The compound was then dissolved in methanol (200 mL) and 10% of Pd/C was added. Next, the reaction was stirred under hydrogen for 48 hours. The solution was then filtered over celite, washed several times with methanol and concentrated under vacuum. The transparent oil was triturated with ether until a precipitate was formed. The solid was filtered and dried under vacuum to afford dendron **2** as a white solid (8.3 g, 90%) which was used in the next step without further purification. ¹H NMR (500 MHz, D₂O) δ 1.36-1.99 (m, 18H), 2.92 (m, 4H), 3.20 (m, 2H), 3.36-3.85 (m, $\sim$ 180H), 3.38 (s, 3H), 4.15 (m, 1H), 4.24 (m, 1H) ppm; MALDI-TOF-MS (pos) (M+Na⁺): 2334; IR (neat): 3280, 2883, 1634, 1466, 1343, 1105, 963, 842 cm⁻¹

Synthesis of dendron 1

Et₃SiH (0.6 mL, 3.75 mmol) and TFA (2 mL) were added to a solution of compound **5** (0.9 g, 0.25 mmol) in DCM (5 mL). The solution was stirred at room temperature for 3 hours. The solvent and TFA were removed under vacuum and the product was triturated in ether until a precipitate is formed. The solid was filtered, washed several times with ether and dried under vacuum. A solution of HCl 1N was added and the aqueous phase filtered and lyophilized. Water was then added and the pH adjusted to 7. The aqueous phase was lyophilized again to afford dendron **1** as a white solid (0.6 g, 95%). The last step was quickly conducted to avoid oxidation of the thiols in water. ¹H NMR (500 MHz, D₂O) δ 1.37-1.77 (m, 12H), 1.82 (m, 6H), 2.57-2.70 (m, 8H), 2.80 (m, 8H), 3.24 (m, 6H), 3.42 (s,

3H), 3.50-3.74 (m, ~180H), 4.28-4.33 (m, 3H) ppm; ^{13}C NMR (100 MHz, D_2O) δ 174.2, 173.8, 173.7, 71.1-66.6 (OCH_2CH_2), 58.5, 58.2, 54.4, 54.1, 40.2, 39.8, 39.5, 39.1, 30.8, 28.2, 22.8, 20.9, 20.4, 19.9 ppm; MALDI-TOF (pos) ($\text{M}+\text{Na}^+$): 2687; IR (neat): 3300, 3056, 2869, 2553, 1653, 1558, 1457, 1348, 1096, 949, 843 cm^{-1}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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a) Chemistry of Native Chemical Ligation (NCL)

b) Dissolution of PEG-LysSH hydrogel based on NCL

Figure 1.

a) Chemistry of Native Chemical Ligation (NCL). b) Schematic representation of an idealized cross-linked PEG-LysSH hydrogel formed between **1** and **3** and its dissolution based on NCL.

Figure 2.

G' of 10 and 30 wt% PEG-LysSH and 30 wt% PEG-LysNH₂ hydrogels, at 50 Pa oscillatory stress, 1 Hz frequency, and 20°C.

Figure 3.

Dissolution of PEG-LysSH and PEG-LysNH₂ hydrogels at 30 wt% upon exposure to different concentrations of CME in PBS at pH 7.4 and 8, 0.3M LME and 0.3M MES in PBS at pH 8.5. G' values were normalized to the highest G' value for each experiment.

Figure 4.

(a & b) Photographs of hydrogels PEG-LysSH (green) and PEG-LysNH₂ (pink) adhered to human skin tissue, under torsion. (c) Dissolution of PEG-LysSH hydrogel in 0.3 M CME solution in PBS at pH 8.5, at different time intervals (0, 10, 20 and 30 min).

PEG-LysNH₂ used as control, swelled and did not dissolve. The PEG-LysSH and PEG-LysNH₂ hydrogel sealants were dyed with green food coloring and Nile red dye, respectively.

Figure 5.

a) beef jugular vein linked to a syringe pump and filled with PBS at pH 7.4; b) 2.5 mm puncture on the vein surface; c) PEG-LysSH 30 wt% hydrogel applied on the puncture. The hydrogel was dyed in green; d) Vein placed in 0.3 M CME solution in PBS pH 7.4; e) PEG-LysSH hydrogel completely dissolved.

Scheme 1.

Synthetic route to PEG-peptide dendrons **1** and **2**.

- a) MPEG2000-NH₂, DIPEA, HOBt, EDCI, DMF, rt, 16 hrs, 90%; b) Pd/C, H₂ (1 atm), MeOH, rt, 16 hrs, 90%; c) OPFP-3(tritylthio)propionic acid (**6**), HOBt, DMF, rt, 24 hrs, 76%; d) Et₃SiH, TFA, DCM, rt, 3 hrs, 95%.