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DOI: 10.1002/ange.201308007

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

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Reactions that readily occur 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 a new thiolate, proceeds in high yield in water, in solutions with pH values relevant to biological processes, and at room temperature.[1] Although thiol–thioester exchange commonly occurs in biological processes and in native chemical ligation (NCL), it is poorly understood and hardly used in organic synthesis or in the construction of reversible molecular assemblies.[2] As a reversible reaction that forms and breaks covalent bonds, 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, no hydrogels have been reported for emergency care where a sealant is applied to the wound and subsequently dissolved to allow for surgical care at a later time. As a first step towards the development of such a sealant, we report the synthesis of a covalently cross-linked 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 for surgery to allow for gradual wound re-exposure during definitive surgical care.[4] None of the currently available wound-closure systems feature these characteristics, as removal of the clotting agent or dressing is performed by mechanical debridement and/or surgical excision. Sealants that are based on synthetic hydrogels offer a number of advantages, as the chemical composition and other properties, including tissue adhesion, mechanical properties, degradation, and swelling, can be tuned. To that end, we are investigating a strategy that is based on thiol–thioester exchange and dendritic macromers. Although hydrogels that are based on thiol–disulfide interchange or NCL have been developed,[5] this is the first example of a hydrogel disassembly that is based on thiol–thioester exchange (Figure 1). A dendritic[6] macromer was selected as its composition, structure, and molecular weight can be precisely controlled to afford a macromer with multiple reactive sites to ensure rapid formation of a hydrogel; such materials have been used successfully for wound closure.[5a,7]

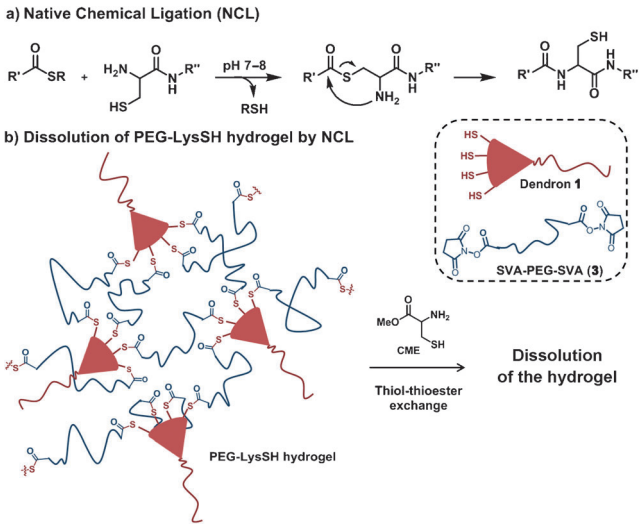

Figure 1. a) Native chemical ligation (NCL). b) An idealized cross-linked PEG-LysSH hydrogel formed by the reaction of **1** and **3** and dissolution of the hydrogel by thiol–thioester exchange.

As the mechanism behind hydrogel dissolution relies on thiol–thioester exchange, we prepared a thioester-linked hydrogel and an amide-linked hydrogel as the control material. Specifically, the lysine-based peptide dendrons **1** and **2**, which possess four terminal thiols or amines, respectively, were synthesized in high yields (Scheme 1). First, the Cbz-protected G1 lysine **4** was synthesized following a previously reported procedure (G1 = first generation).[8] A PEG-substituted amine ($M_w = 2000$; PEG = poly(ethylene glycol)) was then introduced on the peptide dendron by a classic peptide coupling reaction to enhance aqueous solubility, which was followed by catalytic hydrogenolysis of the Cbz groups to afford **2**. Dendron **1** was prepared by the coupling of

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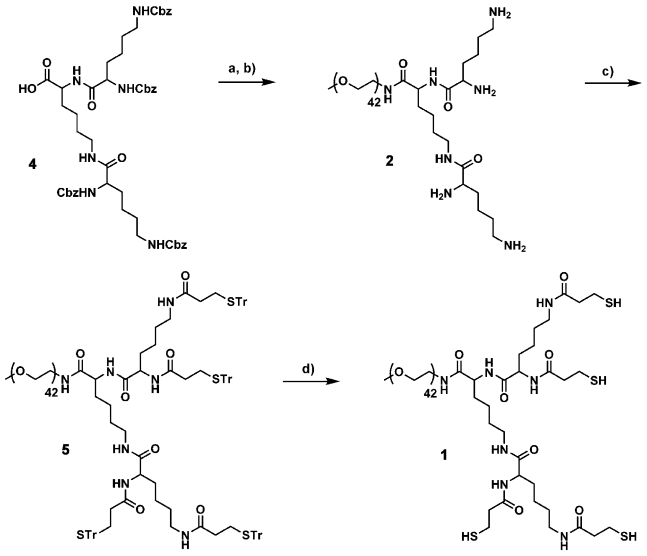
[**] This work was supported in part by Boston University and the NIH (R21 EB013721). We thank Creekstone Farms Premium Beef LLC for samples of bovine jugular vein. We also thank Dr. Michel Wathier for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201308007.



Scheme 1. Synthetic route to PEG–peptide dendrons **1** and **2**. a) MPEG2000-NH_2, DIPEA, HOBt, EDCI, DMF, RT, 16 h, 90%; b) Pd/C, H_2 (1 atm), MeOH, RT, 16 h, 90%; c) PFP-3-(tritylthio)propionic acid (**6**), HOBt, DMF, RT, 24 h, 76%; d) Et_3SiH, TFA, CH_2Cl_2, RT, 3 h, 95%. Cbz = benzyloxycarbonyl, DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, EDCI = 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide, HOBt = 1-hydroxybenzotriazole, PFP = pentafluorophenol, Tr = trityl, TFA = trifluoroacetic acid.

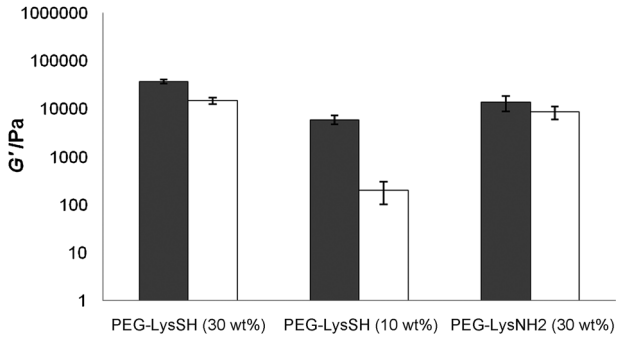


Figure 2. G′ of PEG-LysSH (10 and 30 wt%) and PEG-$LysNH_2$ (30 wt%) hydrogels before swelling (■) and after swelling for 48 hours (□), at an oscillatory stress of 50 Pa, a frequency of 1 Hz, and 20°C.

activated PFP-3-(tritylthio)propionic acid **6** to dendron **2**, followed by removal of the trityl groups using TFA and triethylsilane in CH_2Cl_2. The dendrons were characterized by ^{1}H NMR, ^{13}C NMR, IR, MALDI, and thermal gravimetric analysis (TGA; see the Supporting Information).

To prepare the hydrogels, a solution of dendron **1** or **2** in borate buffer (pH 9) was mixed with a solution of poly(ethylene glycol disuccinimidyl valerate) (**3**, SVA-PEG-SVA; $M_w = 3400$) in phosphate-buffered saline (PBS) at pH 6.5. The ratio of amine or thiol to SVA was 1:1, and the total concentration of the 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 with a diameter of 9 mm and a thickness of 3 mm were prepared and analyzed after sitting at 25°C for two hours (for the gelation kinetics, see the Supporting Information). 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 to establish the range of linear viscoelasticity (LVE; see the Supporting Information). Then, the frequency sweep at a constant oscillatory stress of 50 Pa was determined for all hydrogels before and after swelling (see the Supporting Information). PEG-LysSH and PEG-$LysNH_2$ hydrogels at either concentration showed strong elastic properties with low tan δ values ($< 5°$) and exhibited storage moduli (G′) higher than loss moduli (G″) at frequencies between 0.1 and 10 Hz (see the Supporting Information). Before swelling, the G′ values for PEG-LysSH (30 wt%, **1**:**3**; reversible) and PEG-$LysNH_2$ (**2**:**3**; non-reversible) hydrogels were 37×10^3 and 14×10^3 Pa, respectively, at a frequency of 1 Hz (Figure 2). For the PEG-LysSH hydrogel and at a frequency of 1 Hz, the increase in modulus was consistent with the increase in the relative amount (wt%) of the polymer (30 wt%: 37×10^3 Pa vs. 10 wt%: 6×10^3 Pa). The dendron was required for the formation of a cross-linked hydrogel. For example, replacement of dendron **1**, which contains four thiol groups, with HS-PEG-SH ($M_w = 3400$) gave a viscous solution upon reaction with **3**, which was unsuitable for sealing a wound because of its weak mechanical properties (G′ $\approx$ 20 Pa at 30 wt%, 1 Hz).

After exposure to PBS buffer (4 mL) at pH 7.4, PEG-LysSH and PEG-$LysNH_2$ hydrogels (30 wt%) swelled up to 400 and 600%, respectively, and reached equilibrium after 48 hours (see the Supporting Information). For both hydrogels, the G′ values decreased by approximately half at the swelling equilibrium (Figure 2). For hydrogels at a concentration of 10 wt%, G′ also decreased in a similar manner after 48 hours of exposure to PBS buffer, with the PEG-LysSH hydrogel possessing the lowest G′ value (ca. 200 Pa) at a frequency of 1 Hz. Overall, the rheological data show that at high wt%, both the reversible and non-reversible hydrogels exhibited suitable mechanical properties, even after swelling for 48 hours. These results are promising as the hydrogel can maintain its integrity while absorbing fluid from the wound, which prolongs its contact time with the tissue. Thioesters spontaneously hydrolyze in water to form carboxylic acids in a competing process that 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 capabilities of hydrogels based on PEG-LysSH and PEG-$LysNH_2$ (30 wt%) were evaluated to determine whether 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, thus preventing hydrogel re-formation. Three solutions that contained different nucleophiles were tested; these contained 1) L-cysteine methyl ester (CME; reacts by an NCL-based mechanism); 2) the water-soluble thiolate 2-mercaptoethanesulfonate (MES); and 3) L-lysine methyl ester (LME; the amine acts as the nucleophile). Under all three conditions, the dissolution of the hydrogel was

evaluated at equilibrium after swelling in PBS buffer at pH 7.4. The PEG-LysNH$_2$ hydrogel that contains the amide bonds was used as a control system. It was observed that the pH of the buffer solution and the concentration of the thiolate solution had a significant impact on the rate of exchange, and thus on the dissolution time of the thioester hydrogel. Increasing the concentration of the CME solution to 0.5 M at a constant pH of 7.4 led to a decrease in the dissolution time of the hydrogel from $t_{1/2} = 30$ min to $t_{1/2} = 18$ min (Figure 3). Similarly, when the pH was increased to 8.5 at a constant concentration of the CME solution (0.3 M), the thioester bridges in the gel were rapidly cleaved, and the hydrogel completely dissolved with $t_{1/2} = 12$ min ($t_{1/2} = 25$ min at pH 7.4; Figure 3). Upon exposure of the PEG-LysSH hydrogel to an excess of MES solution (0.3 M) in PBS at pH 8.5, the dissolution time of the gel ($t_{1/2} = 10$ min) was comparable to that in CME solution. Interestingly, an LME solution (0.3 M) in PBS at pH 8.5 did not cleave the thioester bridges of the PEG-LysSH hydrogel even after 60 min, which demonstrates

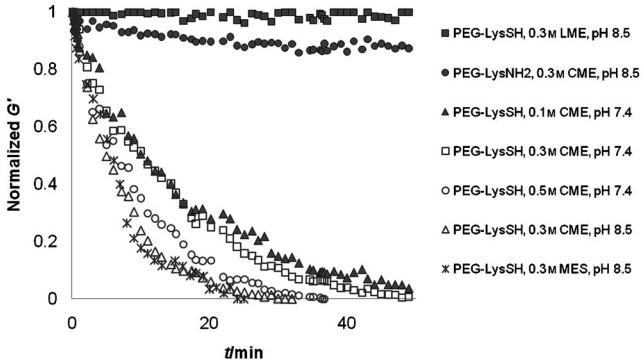


Figure 3. Dissolution of PEG-LysSH and PEG-LysNH$_2$ hydrogels (30 wt%) upon exposure to different concentrations of CME, LME, or MES in PBS. G′ values were normalized to the highest G′ value for each experiment.

that a thiol–thioester exchange is responsible for the dissolution of the hydrogel in the presence of CME. As expected, when the PEG-LysNH$_2$ hydrogel was exposed to CME solution (0.1 M) at pH 7.4, the gel did not dissolve, even after one hour of exposure.

To evaluate the potential of the PEG-LysSH hydrogel for closure of a wound, we first investigated its adherence to ex vivo tissues of human skin. A solution of PEG-LysSH hydrogel (30 wt%; or 30 wt% PEG-LysNH$_2$ 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. Torsion stress was applied on both hydrogels to test their adherence strength and flexibility on the skin (Figure 4). Despite the stress applied, the gels remained intact. Next, we evaluated the dissolution of the thioester hydrogel upon exposure to CME (0.3 M) in PBS buffer at pH 8.5. After 30 min, the PEG-LysSH hydrogel had completely dissolved and washed off, whereas PEG-LysNH$_2$ swelled and remained adhered to the skin even after several hours.

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

Next, an in vitro macrophage activation study was performed with PEG-LysSH to determine whether the hydrogel induces an immune response (see the Supporting Information). Macrophages were exposed to the PEG-LysSH hydrogel (30 wt%) for 24 hours ($n = 3$), or lipopolysaccharide

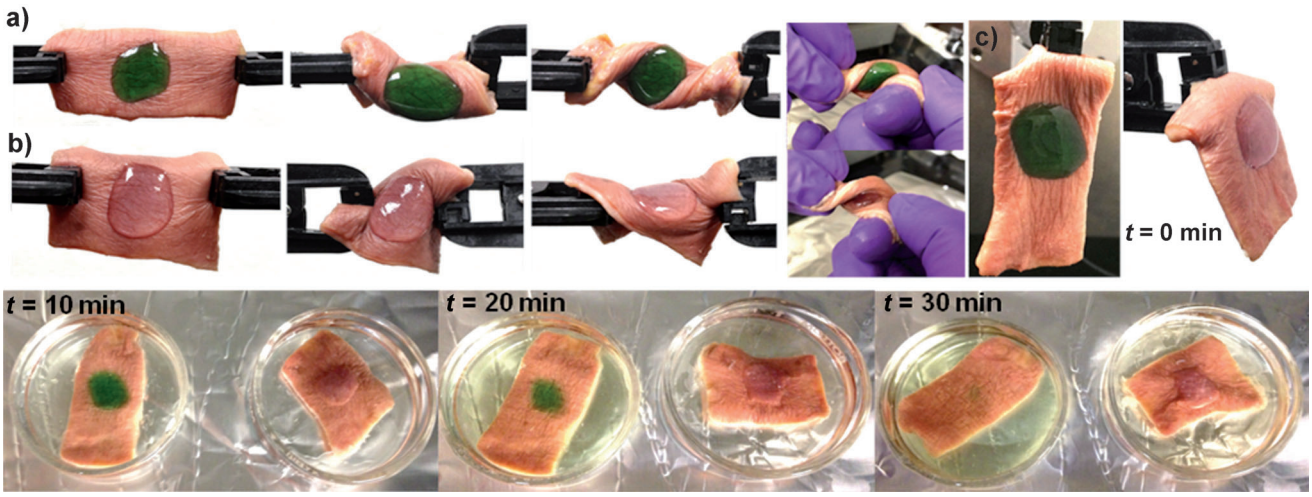


Figure 4. a, b) Photographs of hydrogels PEG-LysSH (green) and PEG-LysNH$_2$ (pink) adhered to human skin tissue, under torsion. c) Dissolution of PEG-LysSH hydrogel in CME solution (0.3 M) in PBS at pH 8.5 at different time intervals (0, 10, 20, and 30 min). PEG-LysNH$_2$ was used as a control, it swelled and did not dissolve. The PEG-LysSH and PEG-LysNH$_2$ hydrogel sealants were dyed with green food coloring and Nile Red, respectively.

(LPS; 1 μg mL^{-1}), 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 ($p < 0.01$); the response to the hydrogel was statistically indistinguishable ($p > 0.05$) to that of a media only control. The PEG-LysSH hydrogel (30 wt %) does not activate macrophages with concomitant production of IL-6.

Finally, the PEG-LysSH hydrogel was tested as a sealant on an ex vivo bovine jugular vein to simulate a trauma puncture (Figure 5). 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 that the system is leak-proof and that it could withstand pressures of ca. 250 mmHg (the upper limit of detection; $n = 3$), which is significantly greater than normal arterial blood pressure (120 mmHg). Next, a 2.5 mm hole was made on the vein surface, and the pressure dropped to zero. Dendron **1** and SVA-PEG-SVA **3** were quickly mixed at room temperature, and a solution (100 μL) of the hydrogel (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 approximately 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 to carry out and did not inflict additional tissue trauma.

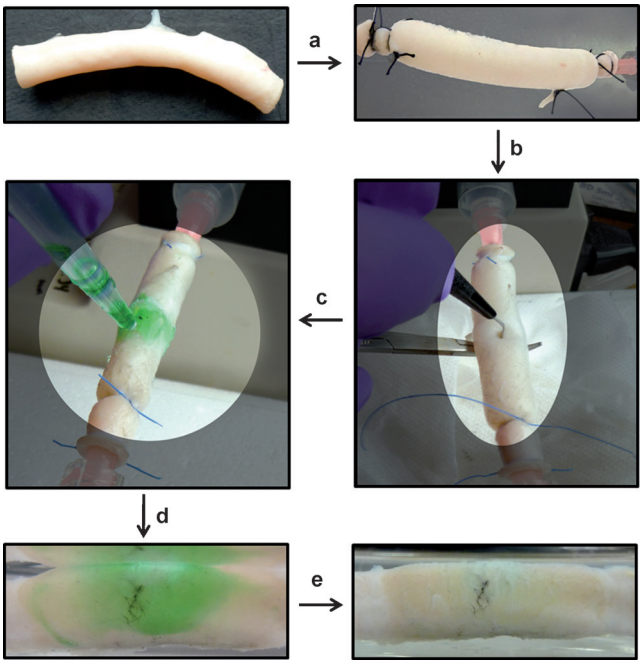


Figure 5. a) Bovine jugular vein linked to a syringe pump and filled with PBS at pH 7.4; b) 2.5 mm puncture on the vein surface; c) PEG-LysSH hydrogel (30 wt %) applied on the puncture; the hydrogel was dyed in green; d) Vein placed in a CME solution (0.3 M) in PBS at pH 7.4; e) PEG-LysSH hydrogel completely dissolved.

In summary, the synthesis of a dendritic thioester hydrogel that gels within seconds because of the formation of multiple thioester linkages between the thiol residues of dendron **1** and the poly(ethylene glycol) macromer **3** has been reported. The cross-linked 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 from the skin upon exposure to a thiolate solution, because a thiol–thioester exchange takes place. The use of a thioester hydrogel based on thiol–thioester exchange for wound repair as opposed to commercially available wound dressings enables gradual dissolution 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 to a solution of **4** (3.75 g, 4 mmol) in DMF (40 mL) at room temperature and under nitrogen. Next, a solution of MPEG2000-NH_2 (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 overnight. The solvent was removed under vacuum, and the crude product mixture was redissolved in CH_2Cl_2. The organic phase was extracted with an aqueous sodium bicarbonate solution, water, and brine to yield the Cbz-protected dendron. This compound was then dissolved in methanol (200 mL), and Pd/C (10 %) was added. Next, the reaction was stirred under hydrogen for 48 h. The solution was then filtered through celite, washed several times with methanol, and concentrated under vacuum. The transparent oil was triturated with ether until a precipitate 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. ^{1}H NMR (500 MHz, D_2O): $\delta = 1.36$–1.99 (m, 18 H), 2.92 (m, 4 H), 3.20 (m, 2 H), 3.36–3.85 (m, ca. 180 H), 3.38 (s, 3 H), 4.15 (m, 1 H), 4.24 ppm (m, 1 H); MALDI-TOF-MS (positive ion) [M+Na^+]: 2334; IR (neat): $\tilde{\nu} = 3280, 2883, 1634, 1466, 1343, 1105, 963, 842$ cm^{-1}.

Synthesis of dendron **1**: Et_3SiH (0.6 mL, 3.75 mmol) and TFA (2 mL) were added to a solution of **5** (0.9 g, 0.25 mmol) in CH_2Cl_2 (5 mL). The solution was stirred at room temperature for 3 h. The solvent and TFA were removed under vacuum, and the product was triturated in ether until a precipitate 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 was 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 conducted quickly to avoid oxidation of the thiols in water. ^{1}H NMR (500 MHz, D_2O): $\delta = 1.37$–1.77 (m, 12 H), 1.82 (m, 6 H), 2.57–2.70 (m, 8 H), 2.80 (m, 8 H), 3.24 (m, 6 H), 3.42 (s, 3 H), 3.50–3.74 (m, ca. 180 H), 4.28–4.33 ppm (m, 3 H); ^{13}C NMR (100 MHz, D_2O): $\delta = 174.2$, 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 (positive ion) [M+Na^+]: 2687; IR (neat): $\tilde{\nu} = 3300, 3056, 2869, 2553, 1653, 1558, 1457, 1348, 1096, 949, 843$ cm^{-1}.

Received: September 11, 2013
Published online: November 26, 2013

Keywords: dendrimers · hemostasis · hydrogels · supramolecular chemistry · wound dressing

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