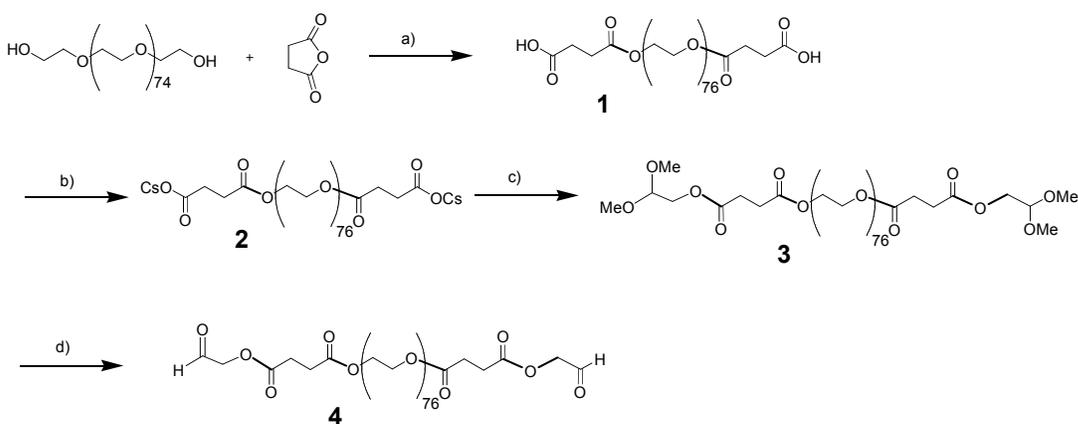


# Hydrogels Formed by Multiple Peptide Ligation Reactions to Fasten Corneal Transplants

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## Experimental section

**Material.** All solvents were dried and freshly distilled prior to use (CH<sub>2</sub>Cl<sub>2</sub> with CaH<sub>2</sub> and MeOH with Na) or were purchased from Acros (DMF). All chemicals were purchased from Aldrich, Acros as highest purity grade and used without further purification. All reactions were performed under nitrogen atmosphere. NMR spectra were recorded on a Varian INOVA spectrometer (for <sup>1</sup>H and <sup>13</sup>C at 400 and 100.6 MHz, respectively). Elemental analysis was obtained from Atlantic Microlab, Inc. A TA Instruments RA 1000 was used for the rheological measurements. DCM = dichloromethane, DMF = *N,N*-dimethylformamide, TFA = trifluoroacetic acid, PEG = polyethylene glycol, Py = pyridine. The buffer pH=7.4 was prepared from HEPES (100 mM) with NaOH 1M.



a) Py, 2 days, 25 °C, 95%, b) Cs<sub>2</sub>CO<sub>3</sub> 1M, 99%, c) DMF, bromoacetaldehyde dimethyl acetal, 2 days, 80 °C, 90%, d) DCM/TFA (90/10), 2h, 25 °C, 90%.

Figure 1. Synthesis of the polyethylene glycol diester-aldehyde.

### Synthesis of Bis-succinic acid-poly(ethylene glycol), 1

Polyethylene glycol Mw=3400 g/mol (10 g, 3 mmol) was dissolved in pyridine (50 mL). After 5 minutes, the succinic anhydride (1.17 g, 12 mmol) was added at 0 °C. The

reaction mixture was stirred for 24 h at 25 °C under N<sub>2</sub>. After concentration under vacuum the mixture was dissolved in DCM and purified by precipitation in cool ether to afford a white powder 10 g (95 %). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.61 (m, 8, CH<sub>2</sub>-CO); 3.45-3.80 (m, 340, PEG); 4.22 (m, 4, CH<sub>2</sub>O). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.13 and 29.60 (CH<sub>2</sub>CO); 64.02 (CH<sub>2</sub>O); 69.18-70.75 (PEG), 172.73 (CO), 182.43 (CO<sub>2</sub>H). MALDI MS: 3652 *m/z* (M<sup>+</sup>) (theory: 3600 *m/z* (M<sup>+</sup>)).

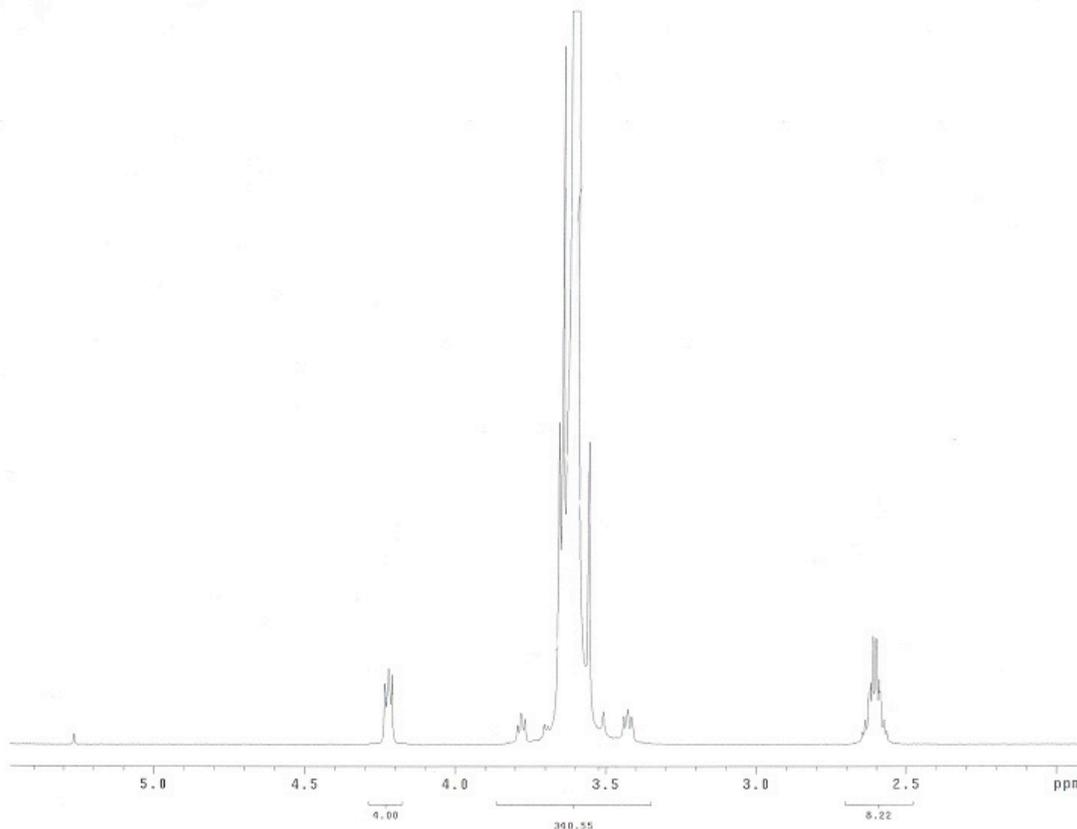


Figure 2. NMR spectrum of compound 1.

### Synthesis of Bis-succinic cesium salt-poly(ethylene glycol), 2

The Cs salt of the compound was prepared to increase the nucleophilicity of the acid for the subsequent reaction. CsCO<sub>3</sub> 1M was added to a solution of bis-succinic acid-poly(ethylene glycol) (10 g, 3 mmol) in water (100 mL) until pH=8. The sample was freeze-dried to give 10.8 g of the white compound (99%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.53 (m, 8, CH<sub>2</sub>-CO); 3.42-3.80 (m, 340, PEG); 4.25 (m, 4, CH<sub>2</sub>O). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.70 and 31.21 (CH<sub>2</sub>CO); 63.16 (CH<sub>2</sub>O); 70.19-70.35 (PEG); 173.14 and 174.82 (CO). MALDI MS: 3901 *m/z* (M<sup>+</sup>) (theory: 3866 *m/z* (M<sup>+</sup>)).

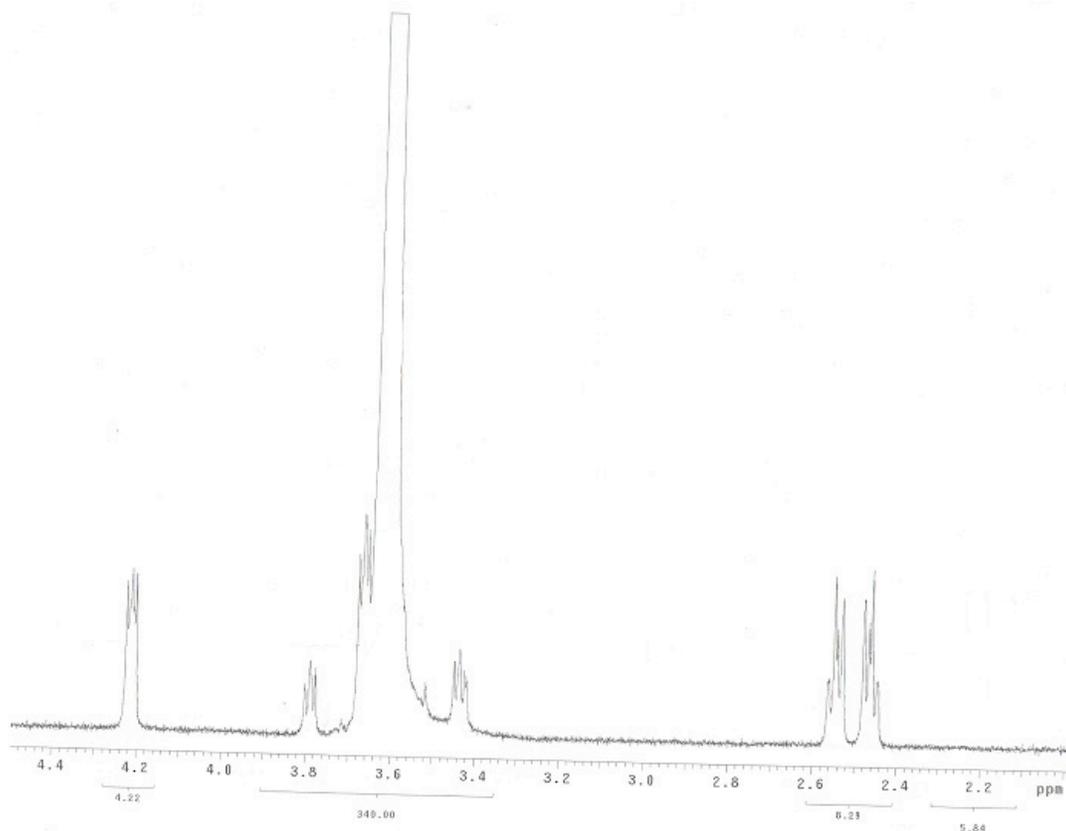


Figure 3. NMR spectrum of compound **2**.

### Synthesis of Bis-2,2-dimethoxyethyl succinate-poly(ethylene glycol), **3**

Bis-succinic cesium salt-poly(ethylene glycol) (3 g, 0.7 mmol) was dissolved in DMF (40 mL). After 5 min the 2-bromo-1,1-dimethoxyethane (520 mg, 3 mmol) was added at 80 °C and the reaction mixture was stirred for 24 h under N<sub>2</sub>. After concentration under vacuum the mixture was dissolved in DCM (200 mL) and washed with NaHCO<sub>3</sub> (2x100 mL), water (2x100 mL), and then dried over Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the organic solvent gave an oil that was purified by precipitation in cool ether to give a white powder 2.62 g (90 %). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.64 (s, 8, CH<sub>2</sub>-CO); 3.36 (s, 12, CH<sub>3</sub>); 3.44-3.79 (m, 340, PEG); 4.10 (d, 4, CH<sub>2</sub>); 4.21 (m, 4, CH<sub>2</sub>OCO); 4.53 (t, 2, CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.18 (CH<sub>2</sub>CO); 54.15 (CH<sub>3</sub>); 63.36 (CH<sub>2</sub>O); 64.12 (CH<sub>2</sub>CH); 69.27-70.78 (PEG); 105.25 (CH); 173.09 and 173.12 (CO). MALDI MS: 3787 *m/z* (M<sup>+</sup>) (theory: 3772 *m/z* (M<sup>+</sup>)).

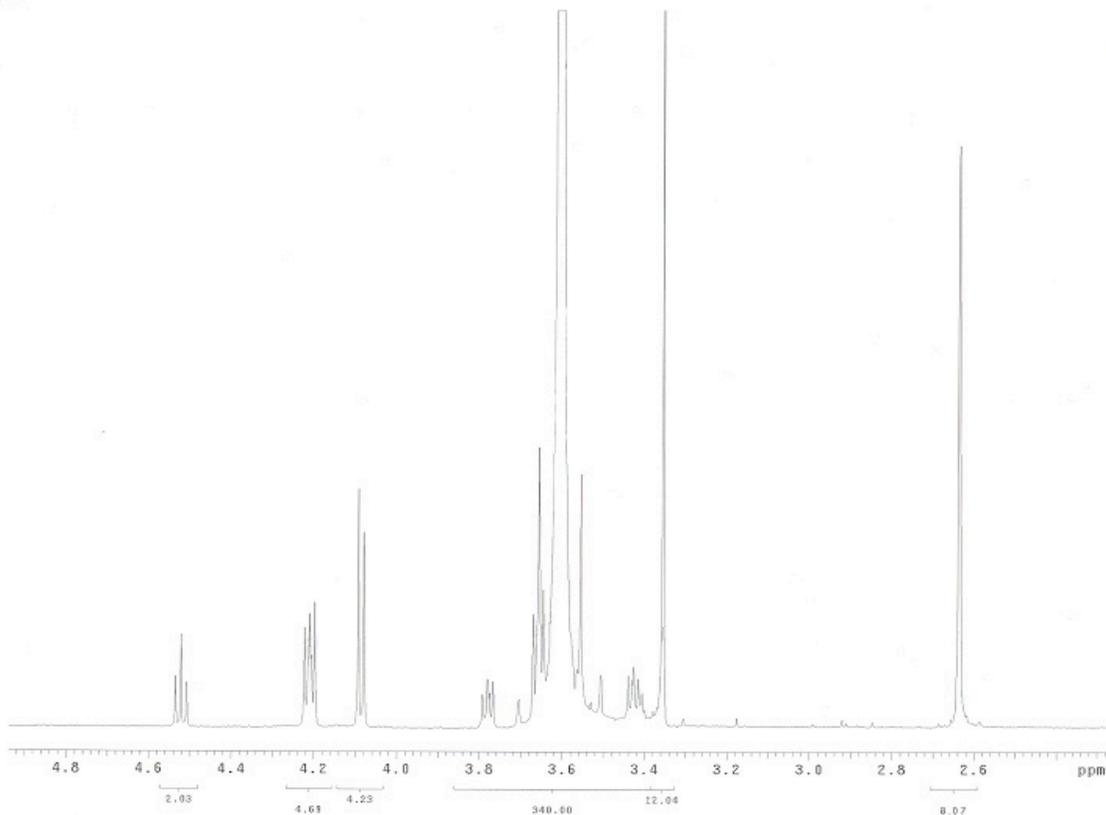


Figure 4. NMR spectrum of compound **3**.

#### Synthesis of Bis-2-oxoethyl succinate-poly(ethylene glycol), **4**

TFA (2.5 mL) was added in 10 portions over 10 minutes to a solution of bis-2,2-dimethoxyethyl succinate-poly(ethylene glycol) (1 g, 0.26 mmol) in freshly distilled  $\text{CH}_2\text{Cl}_2$  (25 mL) at 0 °C. The reaction mixture was stirred under  $\text{N}_2$  at 25 °C for 2 h. The product was isolated after evaporation of the solvent and precipitation in cool ether to give a pure white compound 877 mg (90 %).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  2.71 (m, 8,  $\text{CH}_2\text{CO}$ ); 3.44-3.81 (m, 340, PEG); 4.24 (m, 4,  $\text{CH}_2\text{OCO}$ ); 4.67 (s, 4,  $\text{CH}_2\text{CHO}$ ); 9.58 (s, 2,  $\text{CHO}$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  29.72 ( $\text{CH}_2\text{CO}$ ); 69.20 ( $\text{CH}_2\text{CHO}$ ); 69.40-70.74 (PEG); 173.20 (CO); 169.27 (CO), 196.12 (CHO). MALDI MS: 3694 ( $\text{M}^+$ ) (theory: 3680 ( $\text{M}^+$ )).

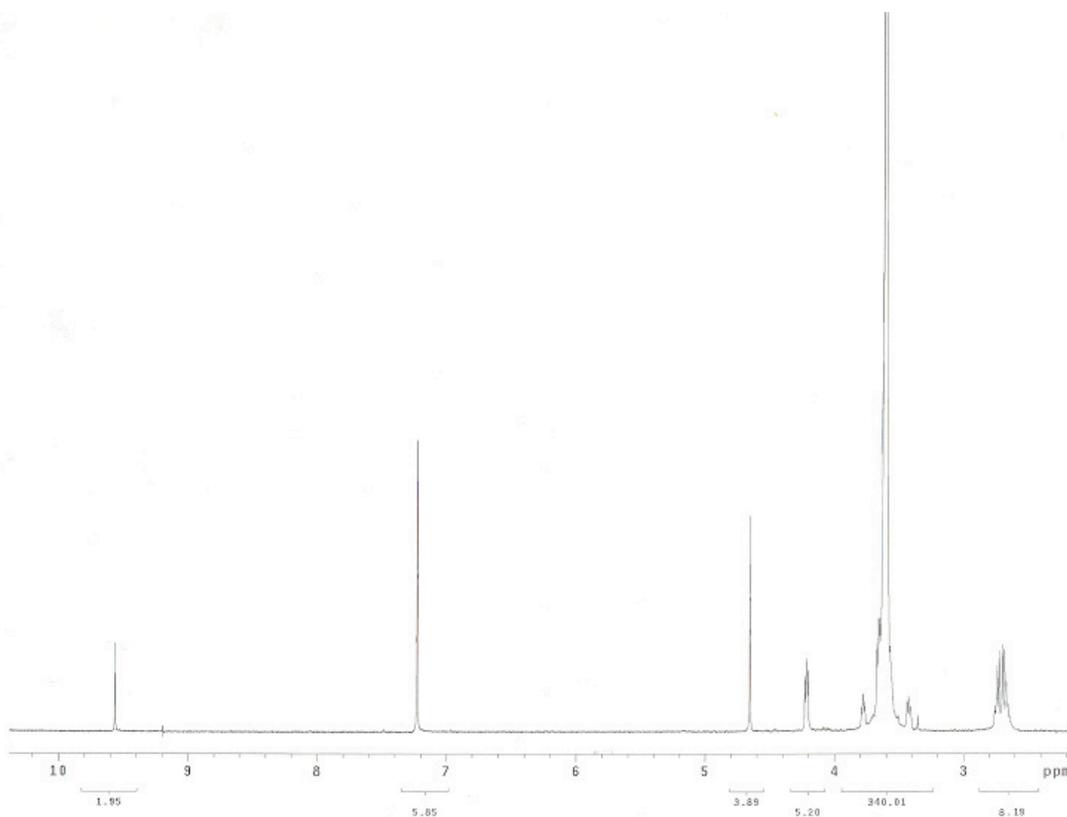
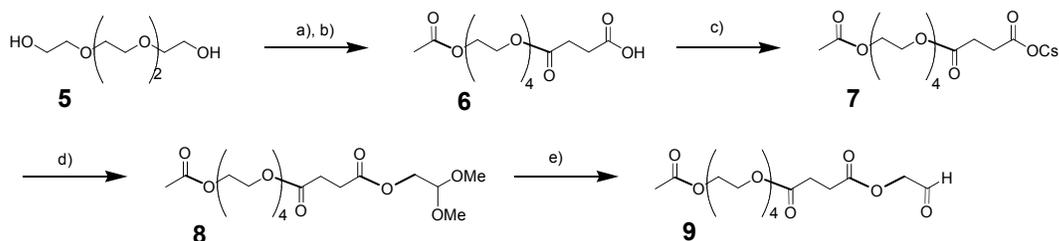


Figure 5. NMR spectrum of compound **4**.

The *N*-terminated cysteine lysine-based dendron was prepared accordingly to Wathier, M.; Jung, P. J.; Carnahan, M. A.; Kim, T.; Grinstaff, M. W., *J. Am. Chem. Soc.* **2004**, *126*, 12744-12745, and characterized by NMR and elemental analysis.

### Synthesis of the model compound



a) Acetic anhydride, Py, 1 day, 25 °C, 35%, b) Succinic anhydride, Py, 2 days, 25 °C, 92%, c) CsCO<sub>3</sub> 1M, 99%, d) DMF, bromoacetaldehyde dimethyl acetal, 2 days, 80 °C, 98%, e) DCM/TFA (90/10), 2h, 25 °C, 99%.

Figure 6. Synthesis of the tetraethylene glycol diester-aldehyde.

**Acetic acid 2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester, 5**

Tetraethylene glycol (25 g, 129 mmol) was dissolved in pyridine (100 mL). After 5 min the acetic anhydride (13.1 g, 129 mmol) was added at 0 °C. The reaction mixture was stirred for 24 h at 25 °C under N<sub>2</sub>. The reaction mixture was poured in water (200 mL) then extracted with DCM (3\*100 mL). The organic layers were washed with HCl 1M (100 ml), brine (100 mL), and dry over sodium sulfate. After concentration under vacuum the compound was purified by silica gel column with DCM/MeOH (95/5) to give an colorless oil 10.6 g (35 %). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.00 (s, 3, CH<sub>3</sub>); 2.83 (bt, 1, OH); 3.51-3.65 (m, 14, CH<sub>2</sub>O), 4.15 (m, 2, CH<sub>2</sub>OCO). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.42 (CH<sub>3</sub>); 62.19 (CH<sub>2</sub>OH); 64.15 (CH<sub>2</sub>OCOCH<sub>3</sub>); 69.98-71.56 (CH<sub>2</sub>-O). FAB<sup>+</sup> MS: 237 *m/z* (MH<sup>+</sup>) (theory: 236 *m/z* (M<sup>+</sup>)).

**Succinic acid mono-(2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl) ester, 6**

Acetic acid 2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester (10 g, 4.2 mmol) was dissolved in pyridine (50 mL). After 5 min the succinic anhydride (840 mg, 8.4 mmol) was added at 0 °C. The reaction mixture was stirred for 24 h at 25 °C under N<sub>2</sub>. After concentration under vacuum the mixture was dissolved in NaHCO<sub>3</sub> 10% (100 mL) then extracted with DCM (3\*100 mL). The organic layers was acidified with HCl 1M, dry over sodium sulfate and concentrated under vacuum to afford a colorless oil 12.47 g (92 %). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.99 (s, 3, CH<sub>3</sub>); 2.57 (s, 4, CH<sub>2</sub>-CO); 3.56-3.62 (m, 12, CH<sub>2</sub>CH<sub>2</sub>OCO), 4.12-4.18 (m, 4, CH<sub>2</sub>OCO). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.44 (CH<sub>3</sub>); 64.11 and 64.39 (CH<sub>2</sub>-OCO); 69.53 and 69.61 (CH<sub>2</sub>CO); 71.00-71.17 (CH<sub>2</sub>-O); 171.77 and 172.69 (CO); 177.11 (CO<sub>2</sub>H). FAB<sup>+</sup> MS: 337 *m/z* (MH<sup>+</sup>) (theory: 336 *m/z* (M<sup>+</sup>)).

**Succinic cesium salt mono-(2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl) ester,**

**7**

CsCO<sub>3</sub> 1M was added to a solution of succinic acid mono-(2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl) ester (10 g, 21 mmol) in water (100 mL) until pH=8. The flask was freeze-dried to give 13.8 g of the white compound (99%). The compound was used without characterization.

**Succinic acid 2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester 2,2-dimethoxy-ethyl ester, 8**

Succinic cesium salt mono-(2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl) ester (1 g, 2.1 mmol) was dissolved in DMF (40 mL). After 5 min, the 2-bromo-1,1-dimethoxyethane (540 mg, 3.2 mmol) was added at 65 °C. The reaction mixture was stirred for 24 h at 65 °C under N<sub>2</sub>. After concentration under vacuum the mixture was dissolved in DCM (200 mL) and washed with NaHCO<sub>3</sub> (2x100 mL), water (2x100 mL), and then dried over Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the organic solvent gave an oil that was purified by silica gel column in DCM/MeOH (95/5) to lead a colorless oil 0.887 g (98 %). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.08 (s, 3, CH<sub>3</sub>); 2.67 (s, 4, CH<sub>2</sub>-CO); 3.39 (s, 6, CH<sub>3</sub>); 3.65-3.67 (m, 8, CH<sub>2</sub>-O); 3.70 (m, 4, CH<sub>2</sub>CH<sub>2</sub>OCO); 4.12 (d, 2, CH<sub>2</sub>CH), 4.21-4.26 (m, 4, CH<sub>2</sub>OCO), 4.56 (t, 1, CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.40 (CH<sub>3</sub>); 54.42 (OCH<sub>3</sub>); 63.64-64.38 (CH<sub>2</sub>-OCO and CH<sub>2</sub>CH); 69.56 (CH<sub>2</sub>CO); 71.08-71.11 (CH<sub>2</sub>-O); 101.63 (CH); 171.57, 172.44, and 172.69 (CO). FAB<sup>+</sup> MS: 225 *m/z* (MH<sup>+</sup>) (theory: 424 *m/z* (M<sup>+</sup>)).

**Succinic acid 2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester 2-oxo-ethyl ester), 9**

TFA (3 mL) was added in 10 portions over 10 minutes to a solution of succinic acid 2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester 2,2-dimethoxy-ethyl ester (0.8 g, 1.88 mmol) in freshly distilled CH<sub>2</sub>Cl<sub>2</sub> (20 mL) at 0 °C. The reaction mixture was stirred under N<sub>2</sub> at 25 °C for 3 h. The product was isolated after evaporation of the solvent to give a pure white compound 700 mg (99 %). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.11 (s, 3, CH<sub>3</sub>); 2.72-2.79 (m, 4, CH<sub>2</sub>CO); 3.72-3.77 (m, 12, CH<sub>2</sub>O and CH<sub>2</sub>CH<sub>2</sub>OCO); 4.27 (m, 4, CH<sub>2</sub>OCO); 4.72 (s, 2, CH<sub>2</sub>CHO), 9.60 (s, 1, CHO). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 29.45 (CH<sub>3</sub>); 64.16 (CH<sub>2</sub>-OCO); 69.40-71.12 (CH<sub>2</sub>CO, CH<sub>2</sub>-O, and CH<sub>2</sub>CHO); 171.82, 172.33, and 172.66 (CO, and CHO). FAB<sup>+</sup> MS: 379 (MH<sup>+</sup>) (theory: 378 (M<sup>+</sup>)).

### General sample procedure preparation:

To prepare a sufficient amount of the crosslinked hydrogel to secure a corneal transplant, dendron (4.68 mg ( $1.92 \times 10^{-5}$  mol of Cysteine)) in 37.5  $\mu\text{L}$  of HEPES 100 mM, pH 7.4) was mixed with polyethylene glycol diester-aldehyde (32.8 mg ( $1.92 \times 10^{-5}$  mol of aldehyde)) in 37.5  $\mu\text{L}$  of HEPES 100 mM, pH 7.4) to give 75  $\mu\text{L}$  of hydrogel adhesive. The ratio of cysteine to PEG functionality was 1:1, and the total concentration of polymer in solution was 33 % wt. Upon mixing the two aqueous solutions, a network formed spontaneously within 30 seconds. When the ratio of cysteine to polyethylene glycol diester-aldehyde was changed from 1:1 to 1:2 or 2:1 the gelation time slowed and the crosslinked network was weaker. For example E decrease from  $7 \times 10^5$  Pa to  $4.6 \times 10^5$  Pa and  $2.8 \times 10^5$  Pa for the 33 % gel at the 1:1, 1:2 or 2:1 ratio, respectively (see Figure 7).

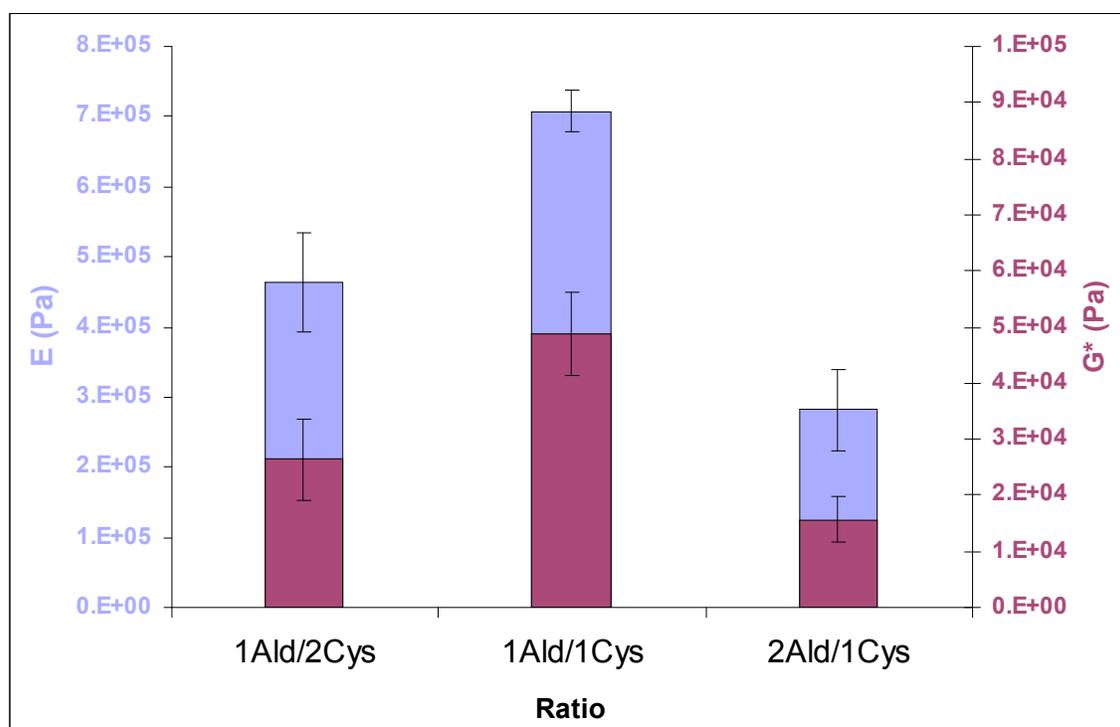


Figure 7. Compress modulus E (blue) and  $G^*$  (red) of 33 wt% gel with different ratios of aldehyde to cysteine reactive functionality.

### General procedure for rheological measurement:

Cylindrical samples for dynamic mechanical testing were prepared by crosslinking biodendrimer solutions at three different % w/t by mixing the dendron (75  $\mu\text{L}$ ) with the

polyethylene glycol diester-aldehyde (75  $\mu$ L) in HEPES buffer at pH = 7.4. A cylindrical mold (dia. = 8 mm, h = 3 mm) was used to ensure inter-sample consistency and each preparation was made in triplicate. The crosslinked samples were removed from the molds and subsequently analyzed. The samples were investigated with a TA 1000 rheometer equipped with a peltier temperature control at 0.1 to 10 Hz (maximum strain amplitude of 0.5%) with a parallel-plate geometry. Before the first measurement, the samples were placed at 25  $^{\circ}$ C for 24 hours. This ensured that the crosslinking reaction was complete as mechanical measurements as a function of time show no change in E after 1 hour of formation. An 8 mm steel plate diameter geometry was used to measure the rheological properties. The choice of a small geometry less stress-sensitive plate was based on the amount of material available. Next the samples were placed in buffer for 48 hours at 25  $^{\circ}$ C. Swelling is complete after 24 hours as determined by following the mass increase of the hydrogel. After swelling, either 12 mm diameter steel plate or 20 mm aluminum plate geometry were used to measure the rheological properties of the hydrogel. Care was taken to use always a bigger geometry than sample. All rheological measurements were performed with a cover and at 25  $^{\circ}$ C to avoid evaporation. The mechanical measurements were insensitive to temperature and this was confirmed at two different temperature (25  $^{\circ}$ C and 37  $^{\circ}$ C) on one sample of each group. Each rheological measurement was performed at 25  $^{\circ}$ C as follow. First to determine the Pseudo-Linear Viscoelastic Region (LVR) an oscillatory strain sweep (strain amplitude from 0.01 to 10%) at fixed frequency (1Hz) was perform. Secondly, a normal force (enough to obtain a flat surface) was applied to the gel using either geometry and a strain-rate compression test (maximum compression as 10% of the height) was realized to determine the compress modulus, E. After equilibrium (15 min at zero strain) an oscillatory frequency sweep (from 0.1 to 10 Hz) with a controlled strain for a linear response (as determine in the LVR test) was performed at 25  $^{\circ}$ C. This measures the storage modulus  $G'$ , the lost modulus  $G''$  and the complex modulus  $G^*$ . All data are reported at a frequency of 1 Hz. No significant frequency dependence was detected in the observed frequency range. The frequency independent complex shear modulus  $|G^*|$ , storage modulus  $G'$ , loss modulus  $G''$ , and the loss angle  $\delta$  were determined. The dynamic mechanical properties ( $G'$ ,  $G''$ ,  $|G^*|$ , and  $\delta$ ) showed no appreciable frequency dependence from 0.1 to 10 Hz.

## NMR procedure for following the O,N acyl migration in the model compounds

The succinic acid 2-{2-[2-(2-acetoxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester 2-oxo-ethyl ester), **9** (30 mg, 0.08 mmol) was dissolved in D<sub>2</sub>O (300 μL). The resulting solution has acidic because of remaining TFA from the aldehyde deprotection step. The first spectrum acquired was of the starting material to assign the protons. Next, a second solution of cysteine methyl ester, HCl (13.7 mg, 0.08 mmol) and dry NaHCO<sub>3</sub> (6.7 mg, 0.08 mmol) was prepared in D<sub>2</sub>O (100 μL) and added to the previous solution. A second spectrum was acquired as soon as possible and noted as t=0. Every 15 min a new spectrum was acquired and noted as t=15 min, t=30 min... up to 3h to obtain the fully thiazolidine formation (no more change observed on the integrations). After 3h, the pH was adjusted to 8 with dry NaHCO<sub>3</sub> and a new spectrum was acquired as soon as possible and noted as t=0. Again, every 15 min a new spectrum was acquired and noted as t=15 min, 30 min... up to 3h to obtain the fully O,N acyl rearrangement (no more change observed on the integrations).

With this model, we can follow the methylene protons alpha to the aldehyde and monitor the change in chemical shift as the reaction progress. For this study we controlled the pH from acid to block the *O,N* acyl rearrangement and then changed the pH to basic to allow for the final step to occur. The first step of the reaction (before rearrangement) can be followed by the disappearance of the starting α methylene aldehyde at 4.05 ppm and appearance of the α methylene ester at 3.88 ppm. By changing the pH of the solution to basic the *O,N* acyl rearrangement occurs and the α methylene ester peak at 3.88 ppm disappears and a new peak corresponding to the α methylene alcohol at 3.80 ppm appears (see figure 8).

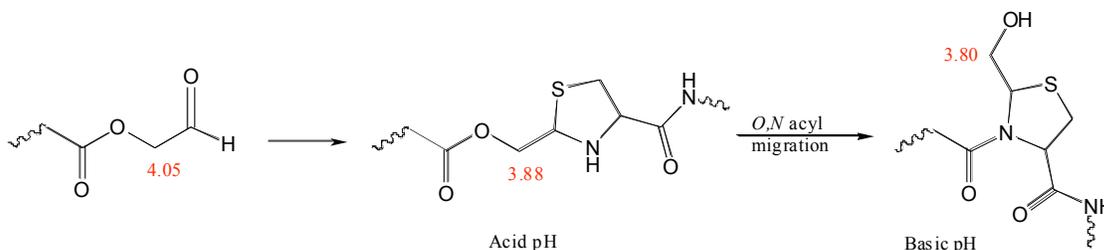


Figure 8. Sequential steps of the O,N acyl migration in the model compounds

## PKP:

Human donor eyes were obtained from the North Carolina Eye Bank. A full-thickness circumferential excision of the posterior sclera was made just anterior to the vortex veins using scissors. The vitreous, retina, uvea and lens were removed. The remaining corneal-scleral shell was mounted on a two-port artificial anterior chamber and secured in place. The chamber was constructed of rigid plastic and stainless steel by the Ophthalmic Biophysics Department at Duke University Eye Center. One port was attached via rigid tubing to a micro-infusion pump (KD Scientific, Model 100, Holliston, Massachusetts). The other port was attached via rigid tubing to a pressure monitor (Hewlett Packard, model CMS 24 Omnicare, Palo Alto, California). Air was flushed from the system and the transducer was zeroed with the system open to atmospheric pressure with the eye soft. The system was closed and the pressure remained at zero. The pressure was increased to 20 mm Hg and an 8 mm central corneal trephination was made. This newly formed button was then autografted back to the original eye. The host-graft tissue interface was secured using sutures (16 or 8), sutures plus the hydrogel sealant, or the hydrogel alone. For the hydrogel sealant, 60  $\mu$ L of the sealant was applied to the wound to secure the autograft. Next, a balanced salt solution was infused into the chamber at a rate of 10 mL/hr. The eye was viewed under an operating microscope and as soon as a leak was visualized, the pressure was recorded (leaking pressure). Evidence of major wound leakage or wound dehiscence was used as endpoints for our leaking pressure studies. Figure 9

summarizes the result obtained during these experiments. The polymer solutions can be sterilized by filtration through 0.2  $\mu$ m filter (aseptic fill). This is one of the approved FDA methods for sterilization

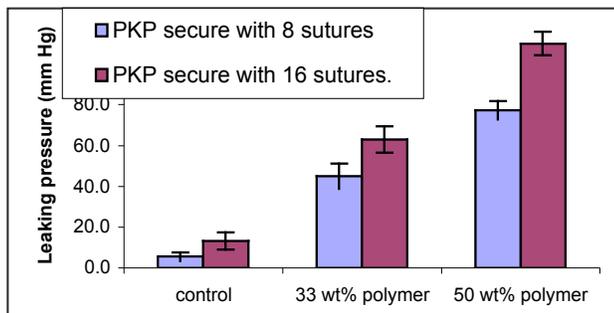


Figure 9. Leaking pressure for autographed eyes.