Supporting Information

An antithrombotic hydrogel with thrombin-responsive fibrinolytic activity: breaking down the clot as it forms

Hui Du,[a] Cong Li,[a] Yafei Luan,[a] Qi Liu,[a] Weikang Yang,[a] Qian Yu,[a] Dan Li,*[a] John L. Brash[ab] and Hong Chen[a]*

[a] State and Local Joint Engineering Laboratory for Novel Functional Polymeric Materials, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, Jiangsu, 215123, P.R. China. E-mail: yuqian@suda.edu.cn; lidan@suda.edu.cn.

[b] School of Biomedical Engineering and Department of Chemical Engineering, McMaster University , Hamilton, Ontario, L8S4L7, Canada.

1. Cleavage of crosslinker by thrombin

Thrombin-induced degradation of the crosslinker peptide was verified by high performance liquid chromatography (HPLC). 10 µL peptide crosslinker in phosphate-buffered saline (PBS) (50 mg mL^{-1}) was mixed with 20 µL thrombin (100 U mL^{-1}) and 70 µL PBS. The mixture was incubated at 37 °C for 2 h. As a control, 10 µL peptide (50 mg mL^{-1}) was incubated with 90 µL PBS in 37 °C for the same time. The chromatograms are shown in **Fig. S1**. The peptide sample without thrombin showed a doublet peak at 2.5 min. Peaks at longer elution times of about 3.5 min appeared in the sample incubated with thrombin, indicating degradation of the peptide.

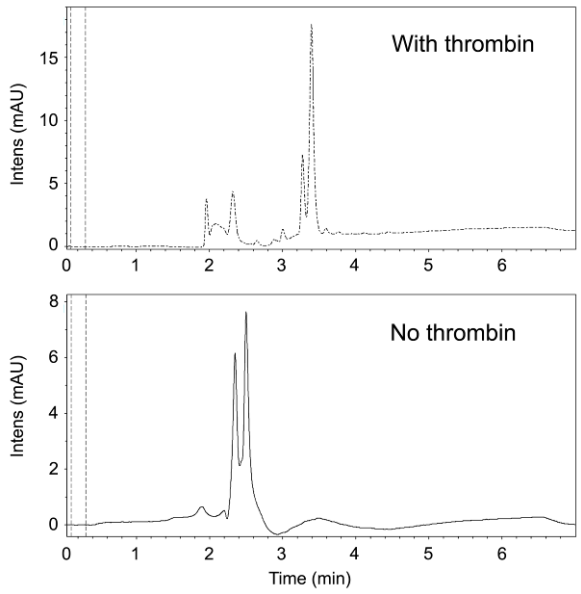

Fig. S1 HPLC of peptide incubated in presence and absence of thrombin.

2. Cyto- and blood-compatibility assessment

2.1 Cytotoxicity test

The cytotoxicity of the hydrogel degradation products was investigated using the cells counting kits-8 (CCK-8) (from Beyotime Biotechnology Co., Ltd. (Shanghai, China)) assay with human umbilical vein endothelial cells (HUVECs) and human umbilical artery smooth muscle cells (HUASMCs).

For the control, cells were seeded at a density of 1×10^4 /well (96-well plates) in the wells. The cells were incubated at 37 °C in RPMI-1640 medium (Hyclone) in a humidified chamber with 5% CO_2; the medium was changed every second day. Media were supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U mL^{-1} penicillin (Genview) and 100 μg mL^{-1} streptomycin (Solarbio). After incubation for 24 or 72 h, the cells attached to the well surfaces were washed twice with sterilized PBS (pH 7.4), followed by the addition of 200 μL fresh RPMI-1640 medium (Hyclone) and 20 μL CCK-8. The cells were then incubated for 1 h at 37 ℃ in a humidified incubator with 5% CO_2 (v/v). 200 μL of the resulting solutions were drawn and the absorbance at 450 nm was determined using a microplate reader (Thermo Fisher Scientific Inc.)

For solutions of the hydrogel degradation products, 180 μL medium mixed with 20 μL hydrogel degradation products, diluted four–fold, was added to the wells containing the cells. The cells were incubated at 37℃ in a humidified chamber with 5% CO_2 for 24 or 72 h. The CCK-8 assay was then carried out as described above.

As shown in **Fig. S2**, the adhesive ability and multiplication capacity of the HUVEC and HUASMC samples were not significantly different from those of the control, demonstrating the compatibility of the degradation products of the hydrogel with respect to these vascular cells.

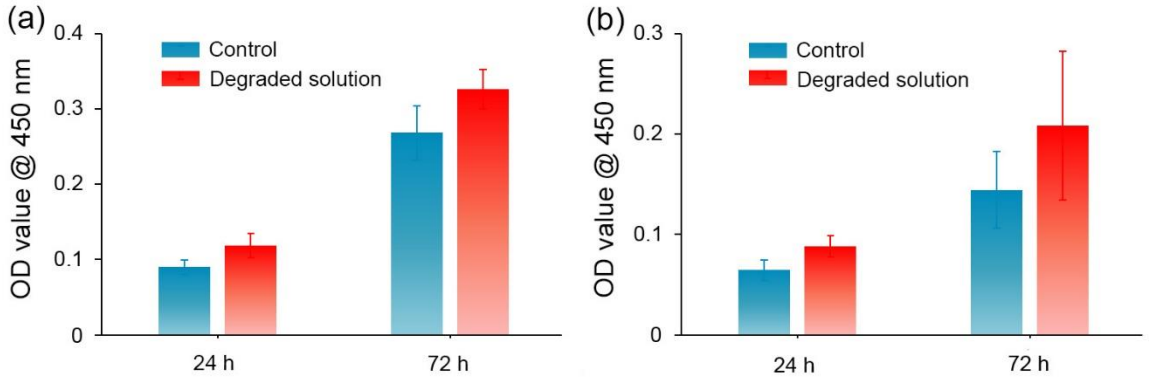

Fig. S2 Viability of (a) human umbilical vein endothelial cells and (b) human umbilical artery smooth muscle cells in contact with solution containing thrombin-induced hydrogel degradation products determined by CCK-8 assay. Error bars represent standard deviation (n = 3*).

2.2. Hemolysis test

Hemolysis provoked by the hydrogels was investigated as described elsewhere.[1] Fresh blood was collected from human volunteers into citrate anticoagulant and diluted with normal saline (4:5 ratio by volume). The hydrogels and the solutions of hydrogel degradation products were placed in tubes containing 10 mL normal saline; 0.2 mL diluted blood was then added. The mixtures were incubated in a 37 ℃ water bath for 1 h and then centrifuged at 239 g for 10 min. The absorbance of the supernatant was measured at 545 nm using a microplate reader (Thermo Fisher Scientific, Inc.). The mean value of three measurements was calculated. Positive controls consisted of 0.2 mL diluted blood in 10 mL deionized water, while negative controls consisted of 0.2 mL diluted blood in 10 mL normal saline. The degree of hemolysis was calculated from:

$$\text{Hemolysis (\%)} = (D_s - D_{nc})/(D_{pc} - D_{nc})*100$$

Where D_s is the absorbance of the sample, D_{nc} is the absorbance of the negative control, D_{pc} is the absorbance of the positive control. The observed hemolysis of the samples was normalized to that of the positive control.

As shown in **Fig. S3**, hemolytic activity was less than 4% for all samples. According to American Society for Testing and Materials (ASTM) guidelines, materials showing hemolysis lower than 5% can be considered nonhemolytic.

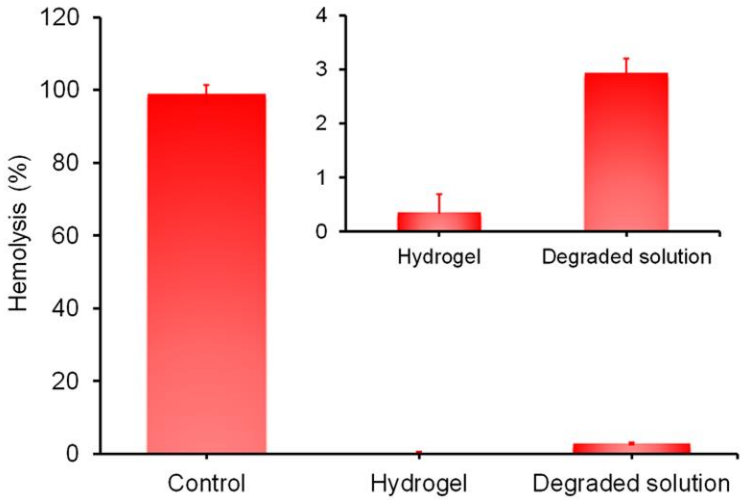

Fig. S3 Normalized degree of hemolysis of blood in the absence/presence of hydrogel and solutions of hydrogel degradation products. Error bars represent standard deviation (n = 3).

3. Average degradation rates of hydrogels

The average degradation rates of hydrogels with different D_{CL} in thrombin solution (20 U mL^{-1}) were estimated by the linear part of the initial rising portion of the curves in **Fig. 2b** and shown in **Fig. S4a**. The average degradation rates of hydrogels (medium D_{CL}) in thrombin solution with different concentrations calculated from the slopes of the curves in **Fig. 2c** were shown in **Fig. S4b**.

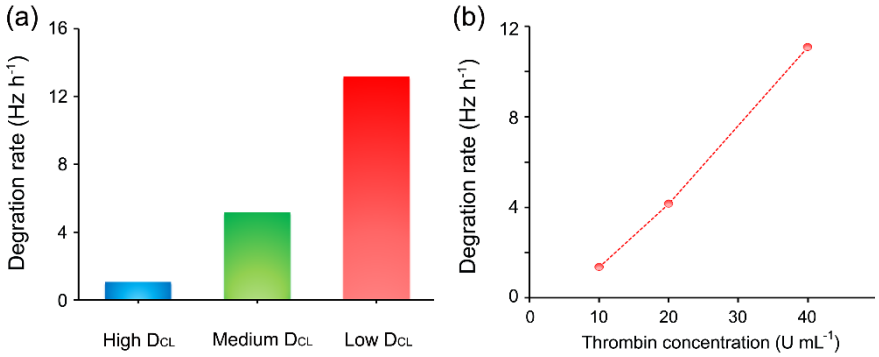

Fig. S4 (a) Degradation rates of hydrogels with varying D_{CL} on exposure to thrombin solution (20 U mL^{-1}). (b) Degradation rates of the hydrogel with medium D_{CL} on exposure to thrombin solutions of different concentration.

4. t-PA loading and release into buffer

t-PA was loaded into the gels during gel formation. The loading and release of t-PA were quantified using radiolabeled t-PA. Hydrogels loaded with radiolabeled t-PA (prepared as described in the experimental section) were placed in 100 µL PBS containing 0, 10, 20 and 40 U mL^{-1} thrombin, respectively, at 37 °C. At pre-determined time intervals, the radioactivity of the hydrogels was measured and converted to quantity of retained t-PA.

The loading data are shown in **Fig. S5**. It is seen that t-PA loading increased with increasing D_{CL}. Loading also increased markedly with increasing concentration of t-PA in hydrogel preparation, and in this case loading increased more strongly with increasing D_{CL}. The phenomena above were attributed to that the hydrogels of high crosslinking degree have smaller pores and a tighter network and thus may retain more t-PA following washing.

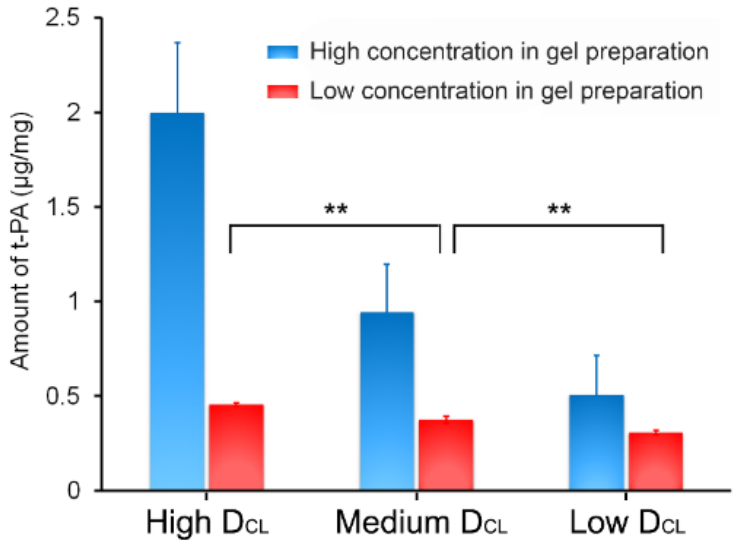

Fig. S5 t-PA loading into hydrogels of different D_{CL} measured by radiolabeling method. The t-PA concentrations in gel preparation were 3.75 µg mg^{-1} (high) and 0.88 µg mg^{-1} (low). Error bars represent the standard deviation of the mean (n = 3, ** $p < 0.01$).

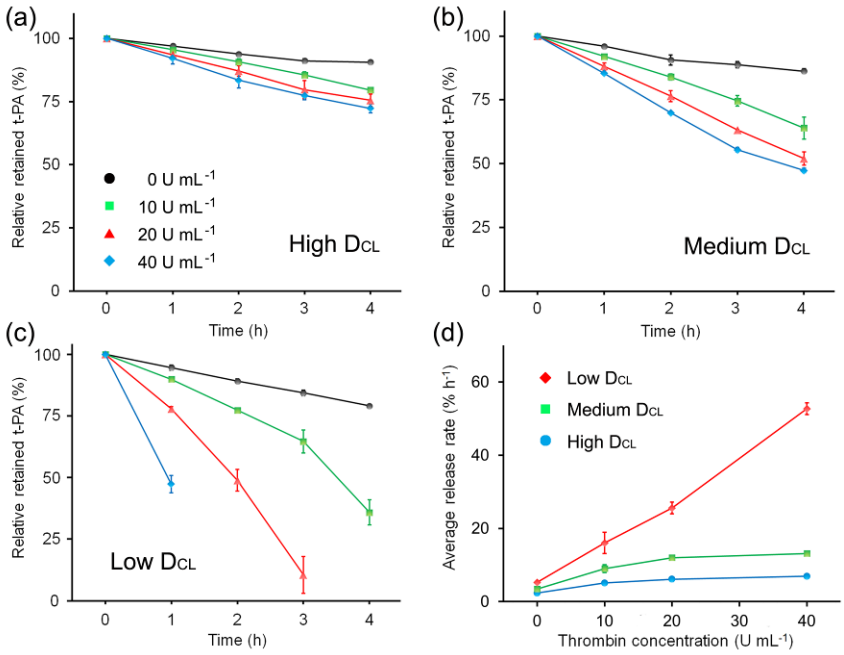

Fig. S6 Release of t-PA from hydrogels of: (a) high D_{CL}, (b) medium D_{CL}, and (c) low D_{CL} in thrombin-containing PBS with varying thrombin concentration. (d) Average release rates calculated by linear fitting of the release curves in (a-c). Error bars represent standard deviation ($n = 3$).

5. *Calculation of t-PA concentration achieved by release from hydrogel if applied as a surface coating on a vascular graft*

The formula for calculating the t-PA concentration (*C*) achieved by release is as follows:

$$c = \frac{r \times t \times \pi dl}{l \times \pi \left(\frac{d}{2}\right)^2} = \frac{4rt}{d}$$

where r is the rate of t-PA release (μg cm^{-2} h^{-1}), d and l are the diameter and the length of the vascular graft (cm), respectively and t is the releasing time (h).

If t-PA is released from the hydrogels prepared using a concentration of 0.875 μg mg^{-1} in response to 10 U mL^{-1} thrombin, r is ranging from 0.032 to 0.077 μg cm^{-2} h^{-1} according to the curves in **Fig. 4**. Assuming t and d are 1 h and 0.3 cm, respectively, then C is ranging from 0.42 - 1.03 μg mL^{-1}.

6. Thrombogenicity in Whole Blood

The thrombogenicity of the thrombin-responsive hydrogel was also studied using a whole blood experiment. Fresh human blood anticoagulated with acid citrate dextrose was drawn from healthy, medication-free donors. t-PA-loaded hydrogel (medium D_{CL}) was placed in the whole blood and pure whole blood (no hydrogel) was used as control. Then thrombin was added to give a final concentration of 2.5 U mL^{-1}. After incubation at 37 °C for 3 h, photographs of the blood were taken.

As seen in **Fig. S7**, after incubation for 3 h, blood in contact with the thrombin-responsive hydrogel remained fluid, whereas the control sample (whole blood, no hydrogel) coagulated. Clearly t-PA was released from the hydrogel in response to thrombin and promoted the generation of plasmin which lysed the nascent clot. This result indicates that the thrombin-responsive hydrogel can prevent thrombus formation by clearing initially formed fibrin in a whole blood environment.

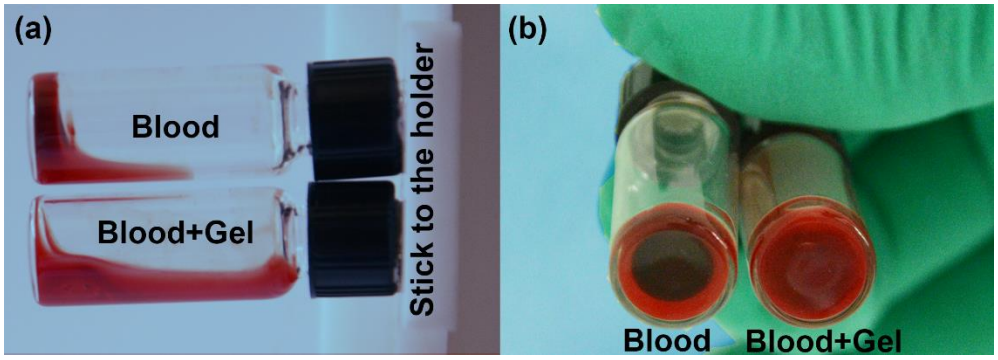

Fig. S7 Typical photographs of pure and hydrogel-containing whole blood after incubation with thrombin (2.5 U mL^{-1}) for 3 h: (a) side view when the bottles were lying down; (b) bottom view by looking up to the bottles. The hydrogel is of medium D_{CL} with 5 μg t-PA loaded. The dark area in the pure blood sample indicates blood clotting, whereas the gel-containing blood remains bright red fluid indicating non-thrombogenicity of the gel.

References

1. X. Wang, N. Zhou, J. Yuan, W. Wang, Y. Tang, C. Lu, J. Zhang and J. Shen, *J. Mater. Chem.*, 2012, **22**, 1673-1678.