Supporting Information

Self-Healing Hydrogel with a Double Dynamic Network Comprising Imine and Borate Ester Linkages

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MATERIALS AND METHODS

1. Materials

Poly(ethylene glycol) (PEG_{2000}) (Sinopharm Chemical Reagent), poly(vinyl alcohol) (PVA-1788) (Sinopharm Chemical Reagemt), triethylamine (TEA, 99%, Aladdin Industry), *o*-phathalic anhydride (99%, J&K Chemicals), 4-(diethoxymethyl) benzaldehyde (98%, Shuya Chemical Science and Technology), 4-aminophenylboronic acid hydrochloride (95%, ARKpharm), cyclohexyl isocyanide (98%, 3A Chemicals), glycol chitosan (GC, 90% degree of deacetylation, Wako Pure Chemical Industries) were used as purchased. PEG derivative with terminal benzaldehydes (DA-PEG) was prepared according to previous literatures.[1] All solvents were purchased from Beijing Chemical Works and used without further purification.

RPMI-1640 culture media, trypsin-EDTA, PBS solution, penicillin, and streptomycin were purchased from Life Technologies, Inc. Fetal calf serum was purchased from Thermo Fisher Scientific. Fluorescein diacetate (FDA), propidium iodide (PI), and the CCK-8 cell

counting kit were purchased from Sigma. Nude mice (Balb/c-Nu) were purchased from the Beijing Vital River Laboratory Animal Technology.

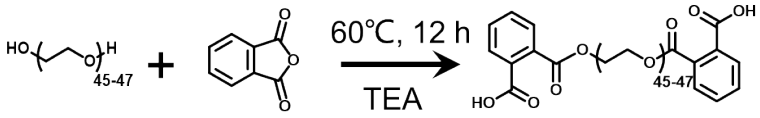
2. Measurements

^{1}H NMR and ^{11}B NMR spectra were obtained using a JEOL JNM-ECA400 (400 MHz) spectrometer. The rheology analysis was carried out in an AR-2 rheometer with a parallel plate geometry (diameter: 20 mm). The mucoadhesive tests were carried out using a DCAT 21 tensiometer, followed by a modified standard test method for the strength properties of the tissue adhesives in the tension method (ASTM F2258-05). The hematoxylin and eosin stain (H&E) histological pictures were captured on a Leica DM3000 B microscope. The ESI-MS data were collected using a Micro TOF-QII Bruker. The UV-vis spectra were measured on a Shimazu UV-2450 UV-vis spectrometer. Cell viability was determined using a VICTOR X3 PerkinElmer 2030 Multilabel Plate Reader.

All *in vivo* tests were performed under the technical guidelines for non-clinical studies issued by the China Food and Drug Administration (CFDA) and authorized by the ethics committee of the Cancer Hospital, Chinese Academy of Medical Science.

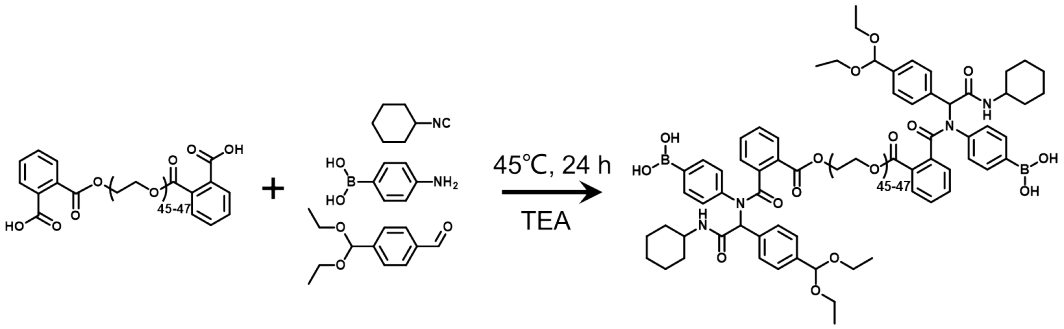
3. Methods

3.1 PEG-COOH



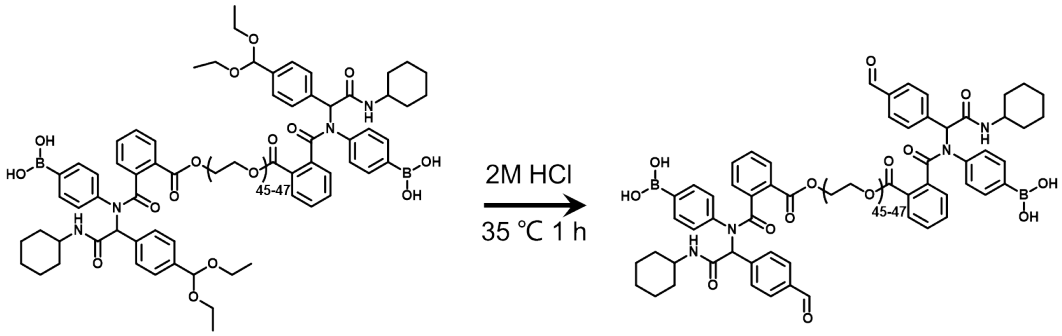
PEG_{2000} (6 g, 3.0 mmol), *o*-phathalic anhydride (1.8 g, 12.0 mmol), and TEA (1.69 mL, 12.0 mmol) were dissolved in THF (50 mL). The mixture was stirred at 60℃ for 12 h. The PEG-COOH was purified as a white powder (6.38 g, yield: 92.5%) by double precipitation in diethyl ether.

3.2 PEG intermediate



PEG-COOH (5.75 g, 2.5 mmol), 4-(diethoxymethyl) benzaldehyde (2.0 mL, 10.0 mmol), 4-aminophenylboronic acid hydrochloride (1.74 g, 10.0 mmol), cyclohexyl-isocyanide (1.25 mL, 10.0 mmol), and TEA (1.4 mL, 10.0 mmol) were dissolved in methanol (5.0 mL). The mixture was kept at 45℃ for 24 h. Then, the polymer intermediate was purified as a white powder (6.91 g, yield: 85.2%) by precipitation in diethyl ether thrice.

3.3 MF-PEG



The polymer intermediate (6.91 g, 2.13 mmol) was dehydrated in a HCl/THF solution (1/6 (v/v), 25 mL, 2 M HCl). The mixture was kept at 35℃ for 1 h, then the MF-PEG was obtained as a white powder (5.55 g, yield: 83.9 %) by triple precipitation in diethyl ether.

3.4 DDN hydrogels

A GC aqueous solution (5.0 wt%) was prepared by dissolving 0.5 g of glycol chitosan in 8.0 g of water; this solution was neutralized using a HCl aqueous solution (37%) followed by adding water to 10 g. A PVA solution (5.0 wt%) was prepared by dissolving PVA (0.5 g) into water (9.5 g). A MF-PEG aqueous solution (5.0 wt%) was prepared by adding MF-PEG (0.5 g) into water (9.0 g), then neutralized by NaOH and added water to 10 g. All DDN hydrogels were prepared through the same procedure using different GC/PVA ratios. For example, to

prepare Gel 2, a GC solution (0.1 g) was first mixed with a PVA solution (0.4 g), then a MF-PEG solution (0.5 g) was added. The mixture was stirred vigorously to generate a hydrogel in a few seconds.

3.5 Gelation time of different hydrogels

The gelation time were tested by the tube-inversion method. For example, Gel 4 was made by mixing chitosan and PVA solutions (0.1 g, 60:40) in a centrifuge tube and then by adding an MF-PEG solution (0.1 g) with vortex. The gelation time of Gel 4 hydrogel was determined when the mixture did no longer flow from the inverted tube. The gelation times of other hydrogels was tested similarly.

3.6 Gelation mechanism study

Small molecular model reactions were carried out to study the gelation mechanism. Phenyl boronic acid and glycerol were used to study the formation of borate ester. Typically, phenyl boronic acid (122 mg, 1 mmol) and glycerol (92 mg, 1 mmol) were dissolved in a mixed solution of CD_3OD and D_2O (2 mL, CD_3OD/D_2O = 1/2 (v/v)). Mesitylene (6 μL) was added as the internal standard. NaOH was used to adjust the pH from 6 to 8. ^1H NMR was used to monitor the borate ester.

The formation of imine under different pH was similarly studied using benzaldehyde and glucosamine for the model reaction.

3.7 Storage modulus (G') and loss modulus (G") of the hydrogels

All hydrogels were tested through the same process. Typically, 400 μL of hydrogel was prepared, then applied to the measuring plate of a rheometer. The rheological analysis at 0.1% strain recorded the storage modulus (G') and loss modulus (G") versus frequency to monitor the mechanical property of the hydrogel.

3.8 Shear-thinning evaluation of the hydrogels

All hydrogels were tested through the same process. Typically, 400 μL of a hydrogel was prepared and applied to the measuring plate of the rheometer. Rheological analyses (6.3 rad s-

[1]) of the storage modulus (G') and the loss modulus (G") at different strain values (stain% 0.01~2000) were recorded to monitor the shear-thinning property of the hydrogels.

3.9 Step-changing experiments on the hydrogels

All hydrogels were tested through the same process. Typically, 400 μL of a hydrogel was prepared and applied to the measuring plate of the rheometer. Rheological analyses at, alternatively, 1% and 200% strains were carried out to record the storage modulus (G') and the loss modulus (G") over time. For Gel 1, the high strain was set as 1000% according to its shear-thinning property.

3.10 Mucoadhesive tests

The tissue mucoadhesive strength of the hydrogel was assessed according to the standard test method for strength properties of tissue adhesives in tension (ASTM F2258-05). A tensiometer (DCAT 21) was used to record mass−distance curves using porcine intestine as the mucous tissue.

The intestine was cleaned and cut before being fixed to the substrates and maintained at 37°C in a humid environment. The hydrogels were applied on the intestinal tissue surface (bonding area: 300 mm^2); then, the motor was moved up to touch the upper mucous membrane. After 10 min, the motor was moved down for the detaching step (motor speed ~ 1.0 mm/s). The bonded mucous tissues were loaded until they reached complete separation (~ 3.0 mm). The attaching/detaching cycles were recorded thrice and the maximum mass in the mass−distance curve corresponded to the dissociation force of the mucoadhesive strength.

The mucoadhesive ability of PVA, GC, PVA/GC solutions was similarly tested.

3.11 Cell culture

SMMC-7721 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin at 37 °C in 5% CO_2. The medium was changed every day. The cells were harvested with PBS containing trypsin (0.025 (w/v) %) and EDTA (0.01 (w/v)%), centrifuged, and re-suspended in RPMI-1640 medium. L929 cells were cultured with the same method.

3.12 Cytotoxicity of the gelators (GC, PVA, and MF-PEG)

The cytotoxicity of the gelators were tested by a CCK-8 assay. First, 100 μL of cell suspension (5×10^4 cells/mL) were seeded in a 96-well plate and cultured for 24 h in the media containing different polymers (0.5 to 16 mg/mL). After removing the culture media, cells were washed three times with a PBS solution, then cultured in the culture media (100 μL) containing 10% of CCK-8 dye for 4 h before analyses with a microplate reader (VICTOR X3 PerkinElmer 2030 Multilabel Plate Reader, 450 nm).

3.13 2D cell culture

Cells (3.3×10^5 cells mL^{-1}, 1 mL) were seeded into a glass petri-dish (diameter: 3.5 mm). Gel 4 (1 mL) was deposited onto the cells. The hydrogel-covered cells were incubated at 37 °C in 5% CO_2 for 24 h followed by staining with FDA/PI and observation in a confocal microscopy (488 nm and 543 nm, respectively) to visualize live and dead cells.

3.14 3D cell culture

Typically, cells in a RP 1640 medium (3.3×10^6 cells mL^{-1}, 300 μL) were mixed with a culture medium containing glycol chitosan and PVA (5 wt%, GC: PVA = 60:40, 500 μL). Then, the cell suspension was mixed with a culture medium containing MF-PEG (12.5 wt%, 200 μL) to form a cell-embedded Gel 4 hydrogel. The cell-laden hydrogel was incubated at 37 °C in 5% CO_2 for 24 h before observation. The FDA/PI double staining was used to evaluate the cell viability and the proliferation conditions (488 nm and 543 nm, respectively). Z-stacks were recorded throughout the depth of the gels to verify the even distribution of the cells.

3.15 In vitro release of DOX

Dox•HCl (0.4 mg, Dox: 0.38 mg) was dissolved in 1 mL of MF-PEG solution (5.0 wt%, NaOH was added to adjust the pH value around 7), then mixed with 1 mL of PVA and the GC solution (PVA 1.0 wt%, GC 4.0 wt%). The prepared Dox was loaded in Gel 4 (~2 mL) was put in a syringe and injected into a dialysis bag (MWCO: ~1.0 kDa). The dialysis bag was sealed and submerged in a centrifuge tube with a 40 mL PBS solution. The whole release

system was placed in an isothermal shaker (37 ℃). Samples were taken out to assess the fluorescence at different times (Excitation: 485 nm, Emission: 585 nm).

3.16 Ethical declaration

All *in vivo* tests were performed under the technical guidelines for non-clinical studies of cytotoxic anti-tumor drugs issued by the CFDA and authorized by the ethics committee of the Cancer Hospital, Chinese Academy of Medical Science.

3.17 Bio-safety assessment of the hydrogels

Female Bala/c mice (10, 5-week old, 18-21 g) were shaved before injection with Gel 4 hydrogel (200 μL) on the back (6 points/mouse) for biosafety assessment. Animals were sacrificed on the 4th day after photographs of the skin at the implant sites were recorded. The dissected tissues were taken out for H&E staining.

3.18 Biodegradability of the hydrogel

Gel 4 hydrogel (200 μL) was injected subcutaneously to female Bala/c mice (10, 5-week old, 18-21 g, 6 points/each animal). Animals were sacrificed at different times (4, 6, 8, 11, 13, 16, and 18 days) to take out the remaining hydrogels. The gel weight versus time was recorded to monitor the biodegradation of the Gel 4 hydrogel.

3.19 Post-operation treatment of the tumor

A GFP-transfected human hepatocarcinoma tumor (MHCC97H, 50 μL) was injected under the armpits of 24 female nude mice (Balb/c-Nu, 8 weeks old, ~15 g) to mimic a residual tumor after surgery. All animals were randomly divided into 4 groups and treated separately. Different treatments were carried out, including a negative control (saline solution), a positive control (Dox solution through tail vein injection and *in situ* injection, respectively), and a Dox-loaded hydrogel group. The survival rate and the tumor volume (including the gel volume) were recorded over 24-days. Fluorescent images of the tumor were taken on the 1st and the 24th day. All animals were sacrificed and dissected on the 24th day. The tumors were peeled off and weighed prior to H&E staining.

3.20 Statistical analyses.

All data were expressed as the mean ± SD of, at least, triplicate determinations. Statistical difference between the values was examined by Student's t-test. The P values less than 0.05 were considered significant.

Supporting data:

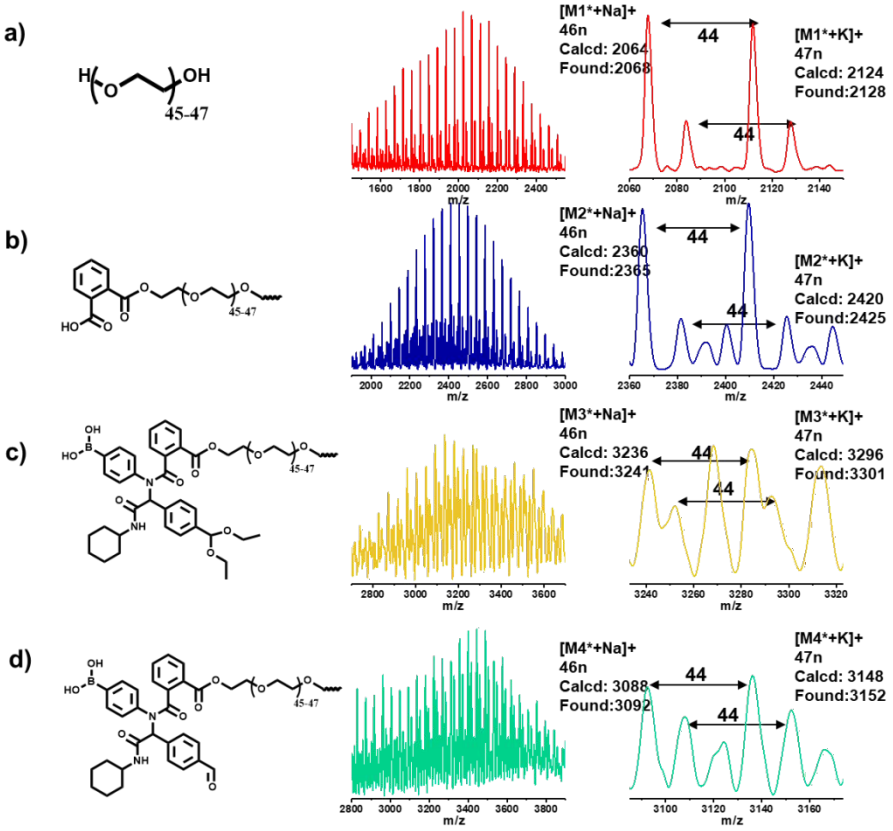


Figure S1. MALDI-TOF analyses of the different PEG derivatives: a) PEG, b) PEG-COOH, c) PEG intermediate, and d) MF-PEG.

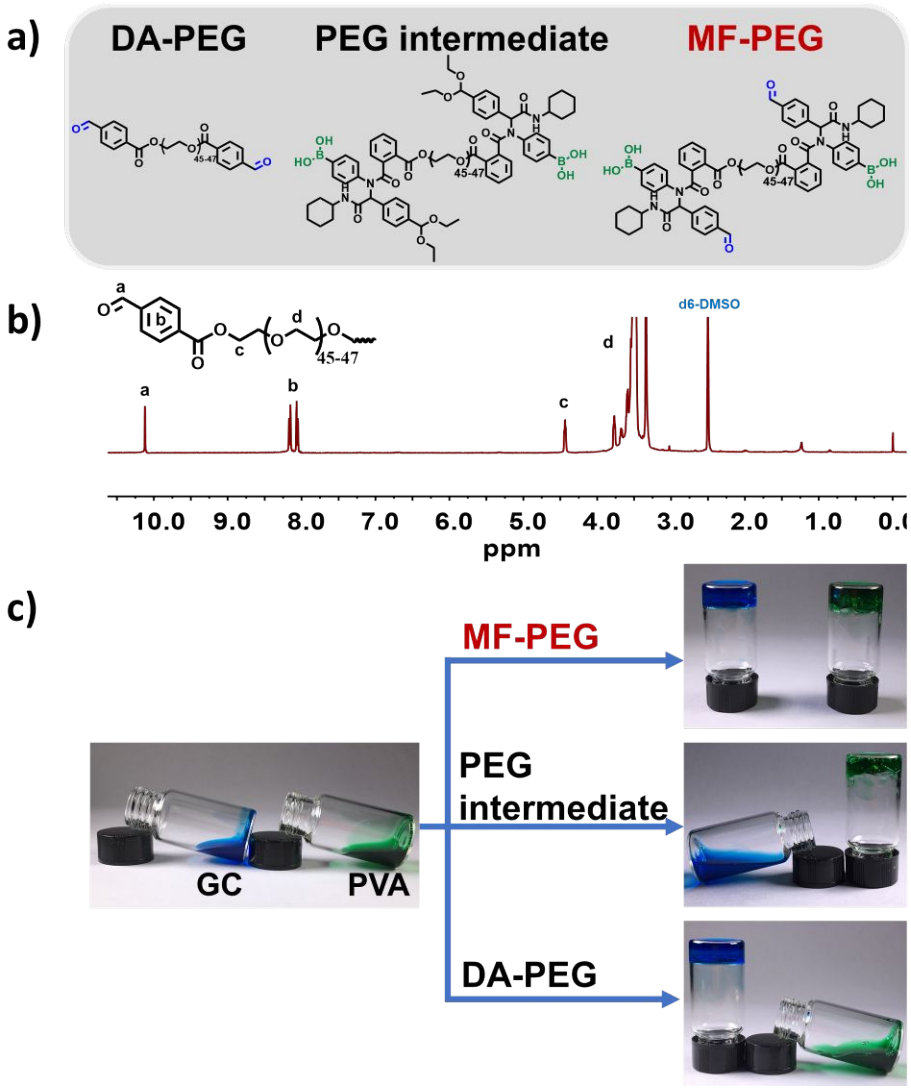


Figure S2. Illustration of the gelation mechanism of the DDN hydrogel. a) Three tested polymers; b) ^{1}H NMR spectra (DMSO-d_6, 400 M) of DA-PEG; c) photos of GC and PVA solutions mixed with different polymers.

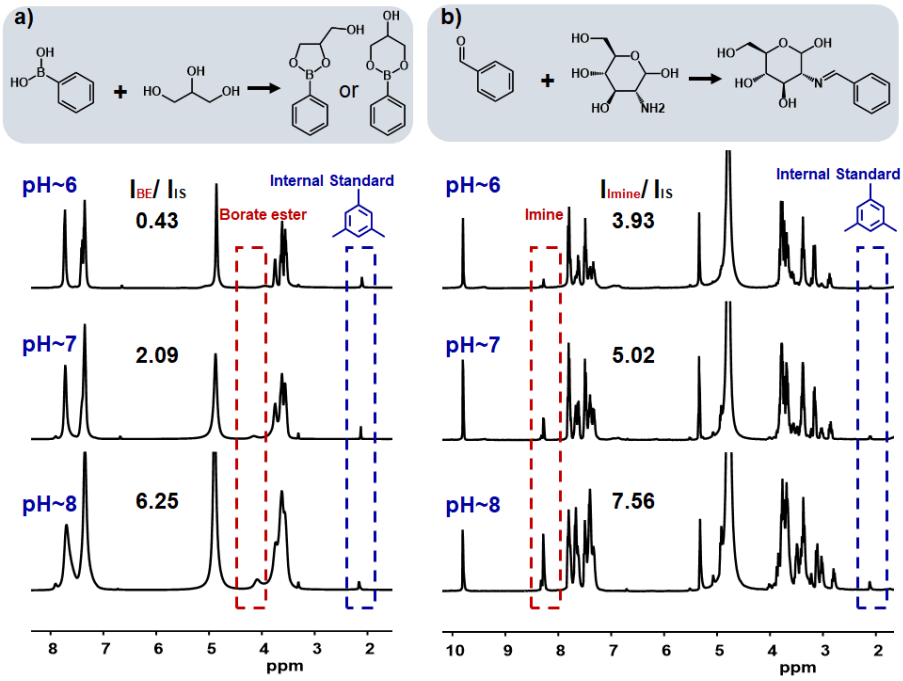


Figure S3. a) Small molecule model reaction of borate ester formation and ^{1}H NMR spectra ($CD_3OD/D2O$ = 1/2 (v/v), 400 M) of borate ester at different pH; b) small molecule model reaction of imine formation and ^{1}H NMR spectra ($CD_3OD/D2O$ = 1/2 (v/v), 400 M) of imine at different pH.

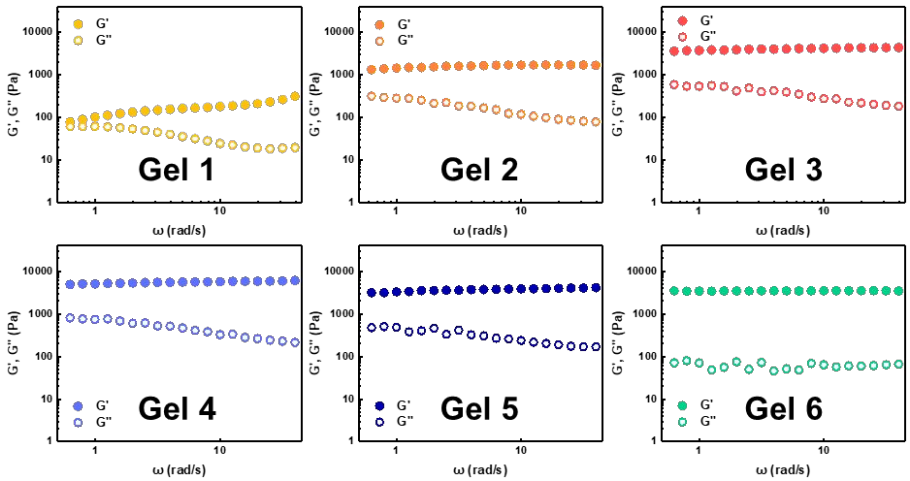


Figure S4. Storage moduli (G') and the loss moduli (G'') of the different hydrogels.

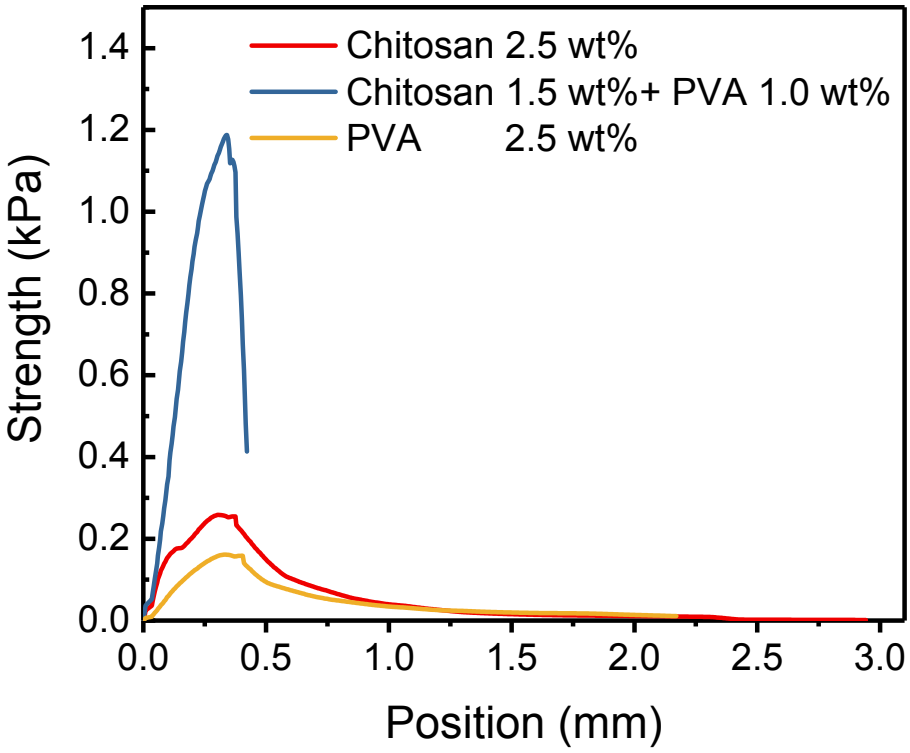


Figure S5. Mucoadhesive ability of chitosan, PVA, and chitosan/PVA solutions.

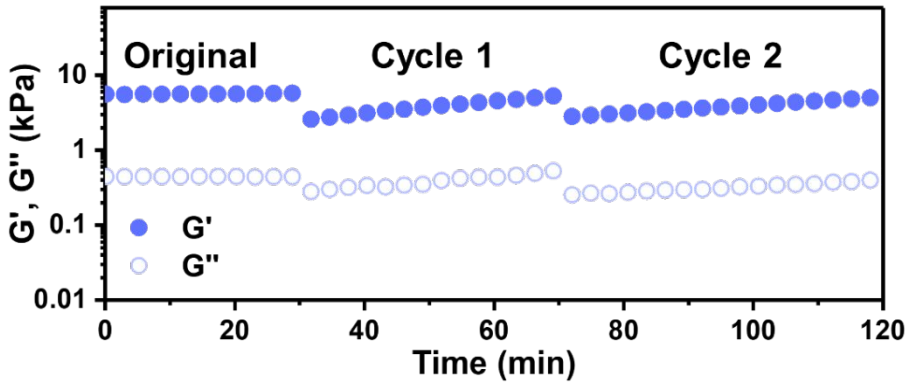


Figure S6. Rheology analyses of the hydrogel during the transformation experiment.

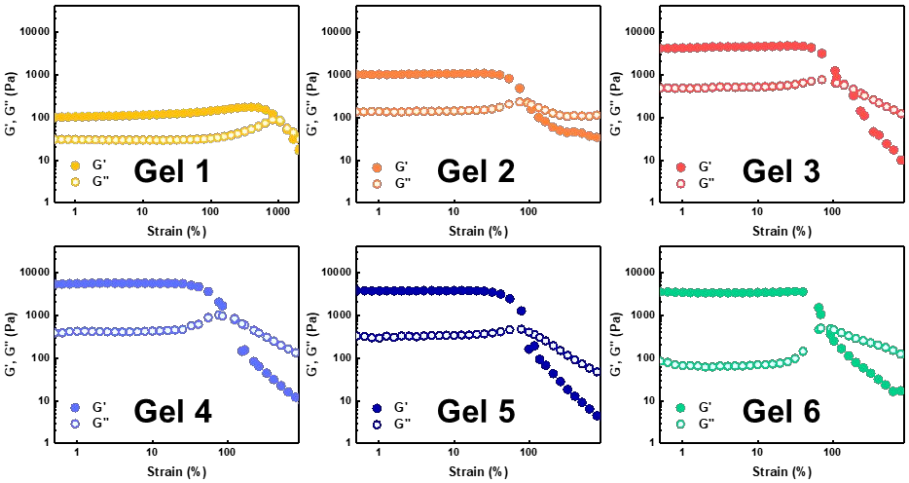


Figure S7. Shear-thinning tests of different hydrogels.

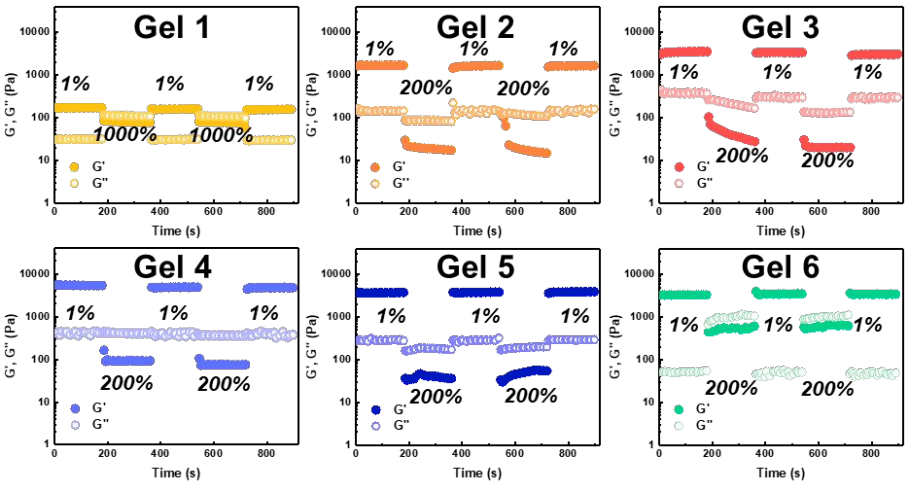


Figure S8. Step-changing tests of different hydrogels.

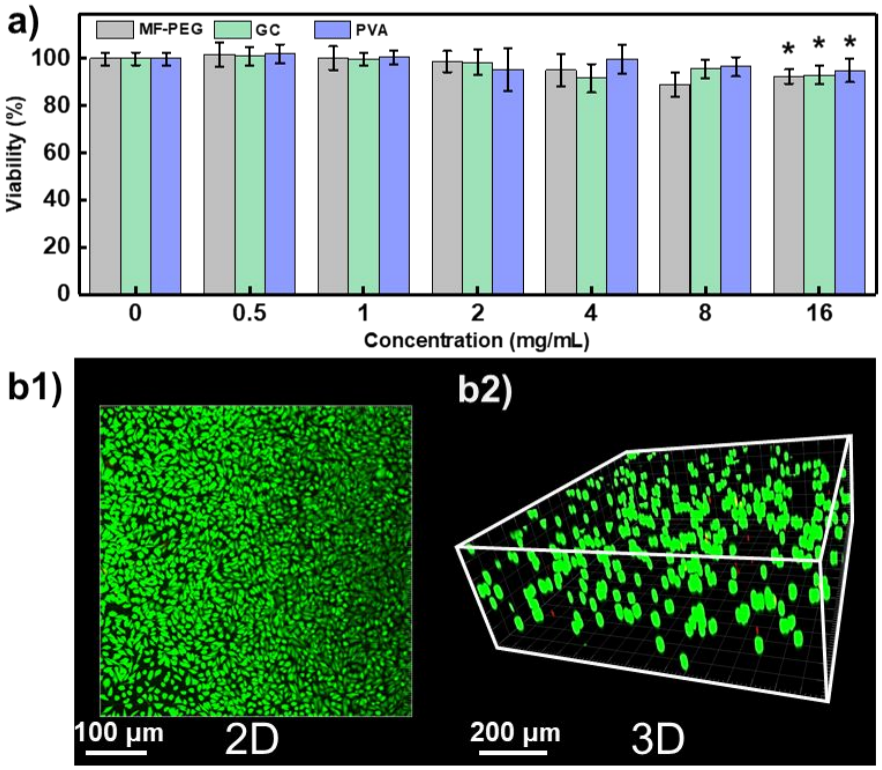


Figure S9. a) Cytotoxicity of the three components in the DDN hydrogels using a CCK-8 assay with a L929 mouse fibroblast cell line. Data are represented as Mean ± SD, n = 5, **p* < 0.05, compared with the blank. b1, b2) Cell viability after a 24 h culture with Gel 4 on the top (b1), and inside Gel 4 (b2).

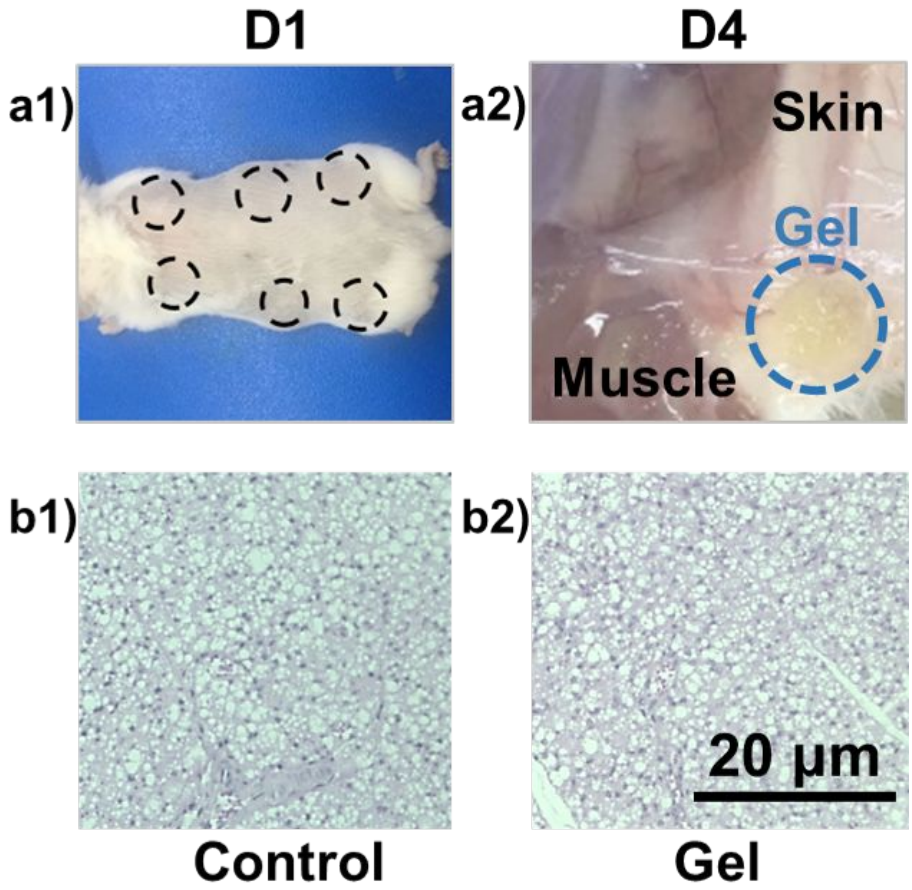


Figure S10. Bio-safety evaluation of the Gel 4 hydrogel. a1) Photo of a mouse after injection of Gel 4 hydrogel at 1st day. a2) Gel implantation site in the mouse on the 4th day. b1) H&E staining of normal subcutaneous tissue. b2) The subcutaneous tissue of the implanting site on the 4th day (right).

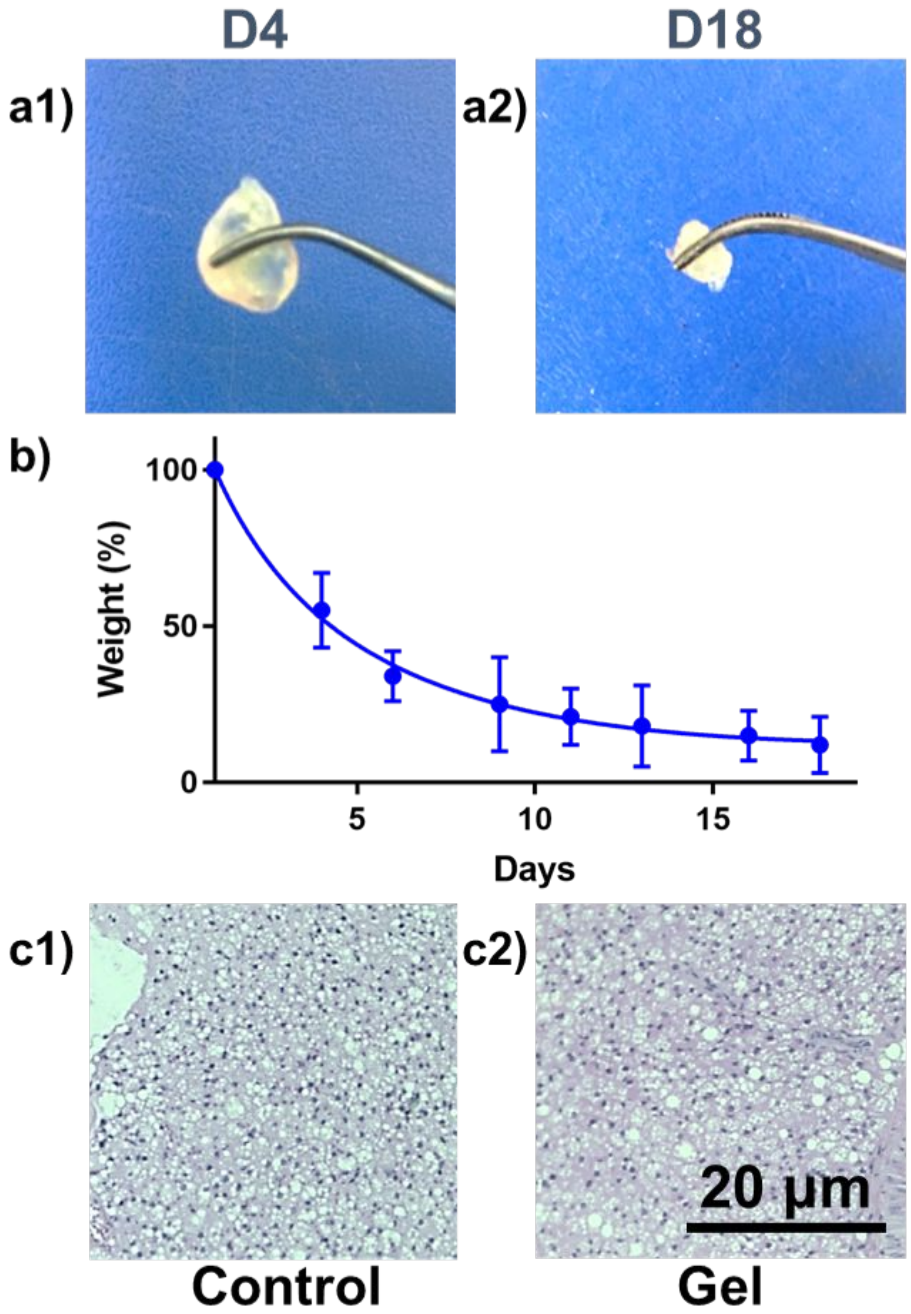


Figure S11. Bio-degradation of the Gel 4 hydrogel under the sink of mice. a1) Photographs of Gel 4 after 4 and a2) 18 days; b) Weight versus time of Gel 4, mean ± SD (n = 6). c1) H&E staining of normal subcutaneous tissue c2) the subcutaneous tissue of the implanting site on the 18th day (right).

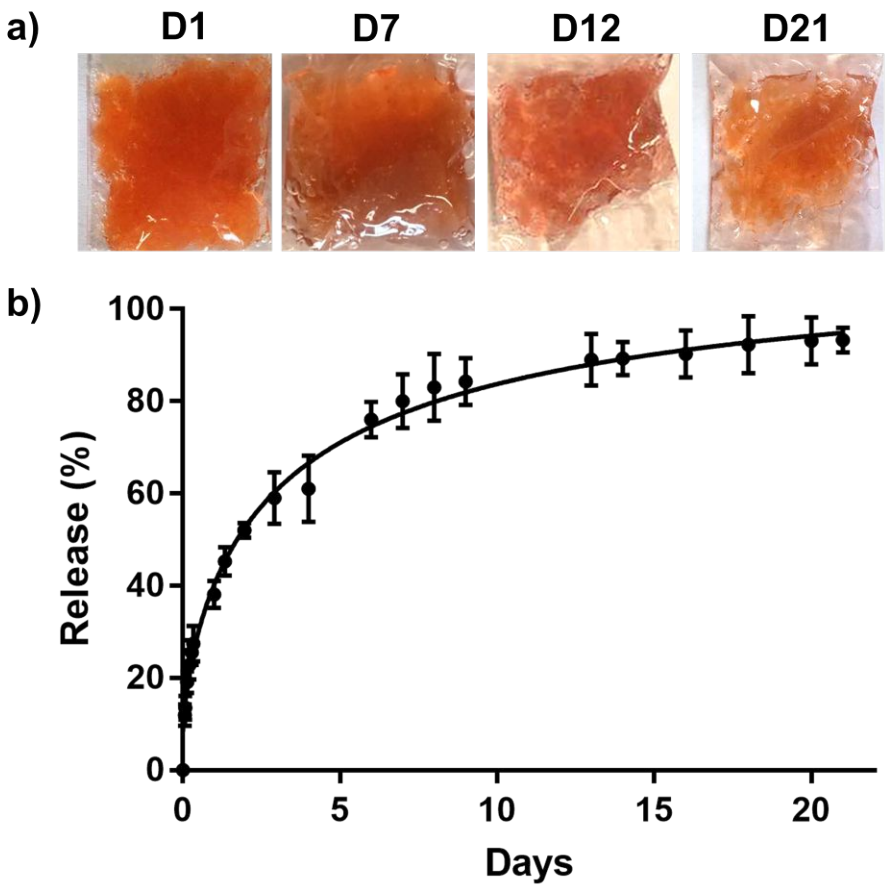


Figure S12. Controlled release of Dox from Gel 4, mean ± SD (n = 5).

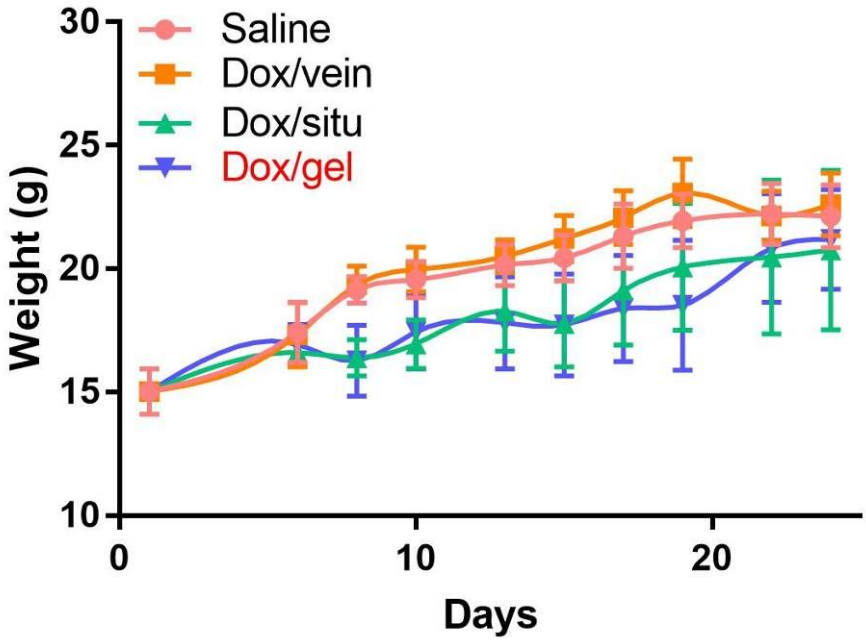


Figure S13. Weight changes versus time in different groups. Mean ± SD (n = 5).

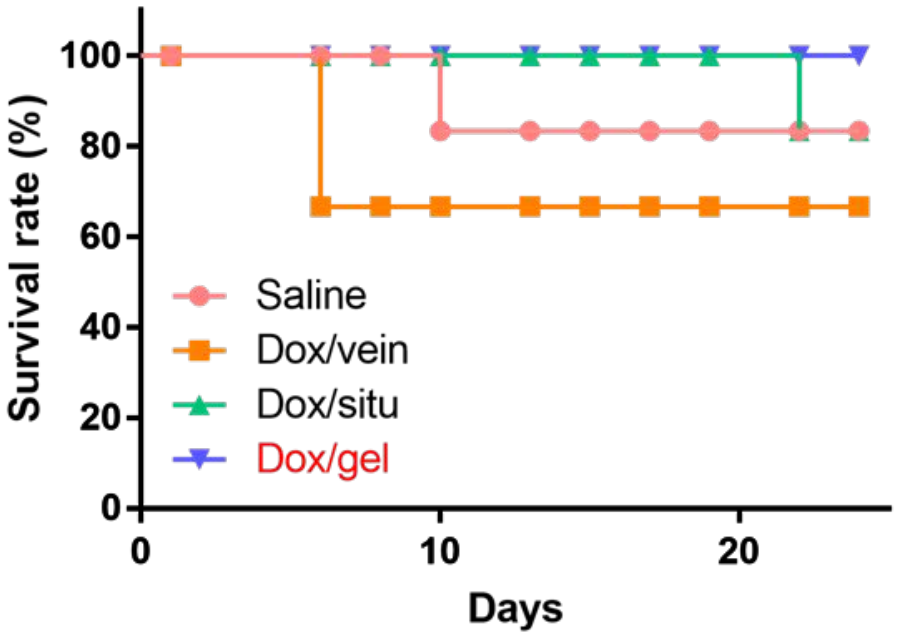


Figure S14. Mice survival rate depending on the injected treatment drug.

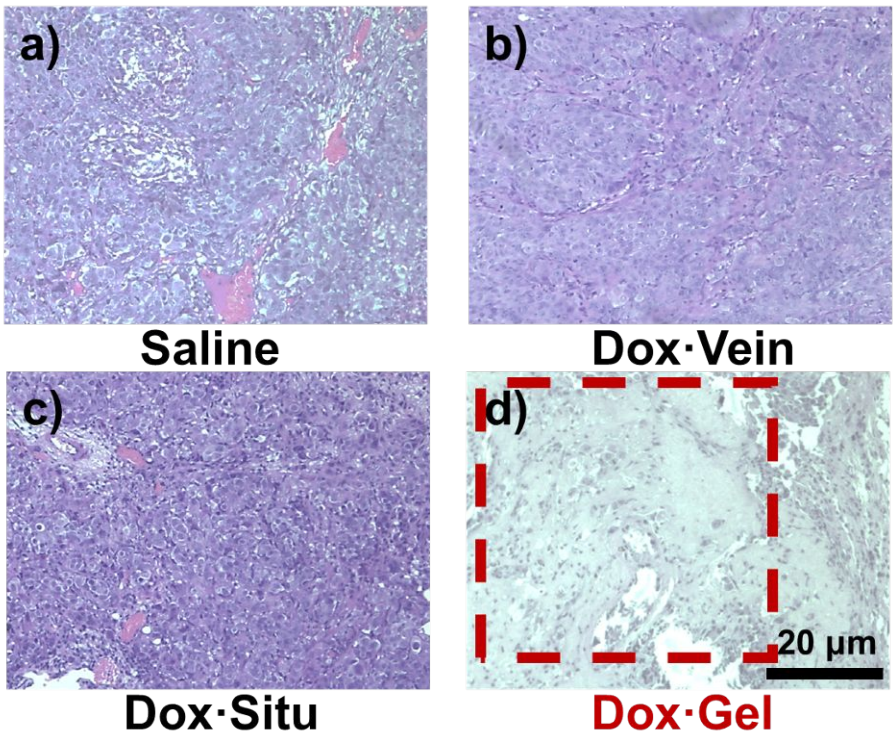


Figure S15. H&E staining of the tumors from a) the saline group, b) the Dox by intravenous injection group, c) the Dox injected *in situ*, and d) the Dox injected in the gel group.

References

1. Zhang, Y.; Tao, L.; Li, S.; Wei, Y., Synthesis of multiresponsive and dynamic chitosan-based hydrogels for controlled release of bioactive molecules. *Biomacromolecules* **2011,** *12* (8), 2894-2901.