Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

Long-term Antibacterial, Antioxidative, Bioadhesive Hydrogel Dressing for Infected Wound Healing

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Materials

Gelatin (Gel, type A) from porcine skin was supplied by Sigma-Aldrich (USA). Methacrylic Anhydride (MA, >94%) and 2,3-Epoxypropyltrimethylammonium chloride (EPTAC, >95%) were purchased from Adamas-beta (Shanghai, China). Alkali lignin (wn= 1000~10,000) was purchased from Taotaole chemical industry Co., (Zhejiang, China). Poly (hexamethylene biguanide) hydrochloride (PHMB, 95%) was bought from Meryer Chemical Technology Co., Ltd (Shanghai, China). Acrylamide (AM, AR), Ammonium Persulfate (APS, AR) and N, N, N', N'-Tetramethylethylenediamine (TMEDA, AR) were purchased from Aladdin industrial corporation (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH, ＞ 97%) was purchased from Tokyo Chemical Industry Co., Ltd (Japan). Hydrogen peroxide (H_2O_2, 30%, AR) and ethyl alcohol (AR) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Deuterium oxide (D_2O, 99.9%) was purchased from Energy Chemical (Shanghai, China). Staphylococcus aureus strain (*S. aureus*), Escherichia coli strain (*E. coli*), Methicillin-resistant staphylococcus aureus strain (*MRSA*) and Mouse fibroblasts (L929) were provided by the Jiangsu Center for Disease Prevention and Control. Phosphate-buffered saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM, high glucose) were purchased from Adamas-life (Shanghai, China). Fetal bovine serum (FBS) was bought from Boster (Wuhan, China). Dichlorodihydro-fluorescein diacetate (DCFH-DA), Cell Counting Kit-8 (CCK-8), Calcein/PI Cell Viability/Cytotoxicity Assay Kit, Triton X-100 and 4% Paraformaldehyde Fix Solution were obtained from Beyotime (Shanghai, China). Haematoxylin and eosin (H&E) dye were supplied from Nanjing Jiancheng Technology co., LTD (Nanjing, China).

Synthesis of gelatin methacryloyl (GelMA)

GelMA was prepared according to the previous study[1]. Briefly, Gel (5 g) was dissolved in PBS solution (50 mL) at 50 °C until Gel was dissolved completely. 4 mL of MA was added dropwise into the Gel solution with stirring for 3 h at 50 °C. Then, the obtained solution was diluted with

PBS solution to stop the reaction. And the diluted solution was purified by dialyzing with deionized water (DI) water for 6 days (0.8-1.4 KDa molecular weight cutoff). Finally, the dialyzed solution was lyophilized and stored at 4 °C. The graft ratio of GelMA was detected by ^{1}H NMR (AVANCE III HD, Bruker, German) at 45 ℃ using D_2O as deuterated reagent.

Synthesis of quaternized lignin (QL)

The QL was prepared by grafting EPTAC onto lignin[2]. Firstly, lignin was dissolved in 120 mL NaOH solution (pH > 11). Next, EPTAC (6 g) was added to the above mixture in an oil bath (85 °C) for 4 h. Then, the reaction solution was cooled to room temperature. Subsequently, the mixture was purified by dialyzing through a dialysis tube (3500 Da) for 2 days until the pH of the solution changed to neutral. Finally, the dialyzed solution was freeze-dried, and the QL was obtained. The Fourier-Transform Infrared Spectroscopy (FT-IR spectra, ALPHA II, Bruker, German) was used to characterize the chemical structure of lignin and QL.

Preparation of the hydrogel

The hydrogels were fabricated via the following steps. First, the QL and PHMB were dissolved in DI water with stirring for 30 min. Second, the GelMA, AM, APS, and TMEDA were added into the above solution under 45 °C. The solution was homogeneous by a vortex mixer for 5 s. Then, the mixture was moved into poly(tetrafluoroethylene) (PTFE) molds immediately for the polymerization of the hydrogel. QL-PAM hydrogel with different GelMA, PAM hydrogel with different content of QL, and QL-PAM hydrogel with different content of PHMB was prepared. The content of various hydrogels was listed in Table S1~S3.

Table S1. The compositions of the hydrogel pre-precursor in detail.

Sample	QL (g)	GelMA (g)	AM (g)	PHMB (g)	APS (g)	TMEDA (μL)	H_2O (mL)
$GelMA_{0.6\%}$-$QL_{1\%}$-PAM	0.1	0. 06	2.6	0	0. 045	30	10
$GelMA_{1.2\%}$-$QL_{1\%}$-PAM	0.1	0.12	2.6	0	0. 045	30	10

Table S2. The compositions of the hydrogel pre-precursor in detail.

Sample	QL (g)	GelMA(g)	AM (g)	PHMB (g)	APS (g)	TMEDA (μL)	H_2O (mL)
PAM	0	0. 06	2.6	0	0. 01	30	10
$QL_{0.2\%}$-PAM	0. 02	0. 06	2.6	0	0. 03	30	10
$QL_{0.6\%}$-PAM	0. 06	0. 06	2.6	0	0. 04	30	10
$QL_{1\%}$-PAM	0.1	0. 06	2.6	0	0. 045	30	10

Table S3. The compositions of the hydrogel pre-precursor in detail.

Sample	QL (g)	GelMA(g)	AM (g)	PHMB (g)	APS (g)	TMEDA (μL)	H_2O (mL)
PAM	0	0. 06	2.6	0	0. 01	30	10
$QL_{0.6\%}$-PAM	0. 06	0. 06	2.6	0	0. 04	30	10
$PHMB_{0.5\%}$-PAM	0	0. 06	2.6	0. 05	0. 01	30	10
$QL_{0.6\%}$-$PHMB_{0.5\%}$-PAM	0. 06	0. 06	2.6	0. 05	0. 04	30	10
$QL_{0.6\%}$-$PHMB_{2\%}$-PAM	0. 06	0. 06	2.6	0.2	0. 04	30	10

Characterization of hydrogels

Morphologies of the hydrogels

The cross sections of the lyophilized hydrogels were sprayed with gold. And their microstructures were observed by scanning electron microscope (JSM-7600F, JEOL, Japan).

The swelling ratio (SR) of the hydrogels

Various fresh hydrogels (8 mm × 2 mm) were completely immersed in 2 mL of PBS at 37 °C with a shaking speed of 100 rpm. The samples were weighed at different time points until the swelling equilibrium of the hydrogels was reached. The SR was calculated according to the

following equation:

$$\text{SR (\%)} = \frac{W_s - W_i}{W_i} \times 100\% \quad \text{(Equation S1)}$$

Where W_i and W_s represented the initial weight and the swollen weight of the hydrogels respectively. Six parallel samples were tested for each group.

Tensile tests

The tensile property of the hydrogels (25 mm in length, 25 mm in width, and 2 mm in thickness) was tested by a universal testing machine (MTS20210217, Meitesi, Jinan, China) equipped with a 100 N load cell. The uniaxial tensile test was carried out at room temperature with a constant peed of 20 mm/min. At least three parallel specimens were tested for each group.

The strength and ductility product (SDP) is an indicator of the comprehensive mechanical performance to characterize the levels of strength and toughness at a static state.

SDP (MPa %) = Maximum tensile strength (MPa) × Maximum tensile strain (%) (Equation S2).

Adhesion tests

The adhesive strength of the various hydrogels was evaluated via the tensile adhesive test according to the reported methods[3]. The specimens were attached to the surface of substrates with an adhesive area of 2 cm × 2 cm. The adhesiveness of the hydrogels on various substrates, such as glass, titanium, silicone rubber, and porcine skin was evaluated. The experiment was tested by five parallel samples for each group. The adhesive strength was calculated by the maximum load divided by the initial bonded area. The adhesion-peeling cyclic test was used to measure the repeatable adhesive ability of the hydrogels.

PHMB release behavior of the hydrogels

The hydrogel slices (8 mm × 2 mm) were immersed in 1 mL of PBS at 37 ℃ to evaluate the PHMB release behavior. Besides, the released PBS was collected completely at the predetermined interval and measured by a UV-Vis spectrophotometer (lambda 650s, PE, America) at 235 nm. And the cumulative release was calculated from the standard curve of PHMB. Four parallel samples were measured for each group.

Antibacterial activity

To evaluate the antibacterial efficiency of the hydrogels, *S. aureus* (G+), *MRSA,* and *E. coli* (G-) were chosen for the experiment according to the previously reported methods[4]. And, five kinds of hydrogels, including PAM, QL-PAM, PHMB-PAM, and QL-PHMB-PAM with different content of PHMB, were measured with the three kinds of bacteria, respectively. Briefly, 1 mL of bacterial suspension (1×10^4 CFU/mL) was co-cultured with purified hydrogel samples (n = 3, 10 mm × 1 mm) at 37 ℃ for 12 h. Then, 200 μL of the diluent bacterial suspension (50 times by PBS) was spread onto an agar plate. After incubation at 37 °C for 24 h, agar plates were taken photos. And the number of bacterial colonies was counted. The bacterial viability (%) was acquired as the following equation:

$$\text{Bacterial viability (\%)} = \frac{CFU\ of\ experimental\ group}{CFU\ of\ control\ group} \times 100\%. \quad \text{(Equation S3)}$$

To evaluate the long-term antibacterial ability of hydrogels against *S. aureus*, the release solution at 12th day from the various hydrogels was used for antibacterial experiment. And 900 μL release solution was mixed with 100 μL *S. aureus* suspension (10^5 CFU/mL) co-cultured for 12 h at 37 ℃. The bacterial suspensions were treated according to the above methods.

In vitro antioxidant ability

The antioxidant efficiency of the hydrogels was analyzed through DPPH free radical scavenging experiment according to the published methods[5]. First, the freezing-dried hydrogel was grinded and dispersed in methanol for using. Next, the different amounts of the hydrogel powders were incubated with 50 μM DPPH-methanolic solution at 37 ℃ in dark. The absorbance of the supernatant from each experimental group was recorded at 517 nm (A_H) with an UV-Vis spectrophotometer (lambda 650s, PE, America). The DPPH scavenging ratio (%) was obtained from the following equation:

$$\text{DPPH scavenging ratio (\%)} = \frac{AC - AH}{AC} \times 100\%. \quad \text{(Equation S4)}$$

Where A_C was the absorption of control group and A_H was the absorption of the various hydrogels.

Intracellular ROS scavenging ability

The intracellular ROS scavenging ability in L929 cell was measured by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, the L929 cell was seeded in 48-well plate with the density of 3×10^4 cells/well and cultured in a humid atmosphere containing 5% CO_2 at 37 °C for 24 h. After L929 cell cultured for 24 h, the purified hydrogel slices (10 mm × 1 mm) were co-cultured with cells in DMEM complete medium. After 1 day's culture, 200 μL H_2O_2 (50 μM) was added to the well to stimulate the cells for 1.5 h. Subsequently, the cells were washed carefully with sterile PBS 2 times after removing the medium and hydrogels. The cells were incubated with 200 μL DCFH-DA solution (50 μM) to assess the intracellular ROS at 37 °C for 20 min in dark. Cells without treatment were served as negative control. Finally, the intracellular ROS was measured by inverted phase contrast fluorescence microscope (Nikon, Ti-S). And the fluorescence intensity of each group determined b

quantitatively by image J.

Hemolytic activity assay of hydrogels

The hemolysis activity assay was conducted to evaluate the blood compatibility of hydrogels according to the previous methods[6]. Briefly, the erythrocytes were collected by centrifuging (1500 rpm) from the fresh whole blood of rabbit for 10 min. Next, the obtained erythrocytes were purified with PBS for three times and diluted to a final concentration of 2% (v/v) by PBS. The purified hydrogel specimens (8 mm × 1 mm) mixed with 3 mL of the erythrocytes suspension and then shook with a shaking speed of 100 rpm at 37 ℃ for 1 h. After that, the whole solution was centrifuged (1500 rpm) for 10 min. The absorbance of supernatants was recorded at 540 nm using an ultraviolet spectrophotometer (Metash, V-5000). 2% Triton X-100 and PBS served as positive and negative control group, respectively. The hemolysis ratio was calculated by the following formula:

$$\text{Hemolysis ratio (\%)} = \frac{A_H - A_P}{A_T - A_P} \times 100\%. \quad \text{(Equation S5)}$$

where A_H represented the absorbance value for hydrogels. A_T and A_P meant the absorbance value for the Triton X-100 positive control and PBS negative control. Three parallel samples were used for the hemolysis experiment.

In vitro cytotoxicity assay

The cell adhesion and proliferation ability of hydrogels was evaluated by CCK-8 assay and Live/Dead staining of the L929 cell. The L929 cell were cultured on PAM, PHMB-PAM, QL-PAM, and QL-PHMB-PAM hydrogels for cell biocompatibility evaluation. And the pure PAM hydrogel served as a control group. First, the hydrogels (10 mm × 1 mm) were purified via repeatedly soaking in PBS and pure ethanol. Furthermore, the hydrogels were sterilized by 75% ethanol overnight. The sterilized samples were placed in 48-well cell culture plate and swelled

in DMEM. The L929 cell (4×10^4 cells/well) were seeded on the surface of hydrogel and incubated for 2 h at 37 °C in a 5% CO_2 humidified incubator. Then, DMEM with 10% FBS was added into the well slowly. Cell proliferation was measured via CCK-8 assay at 1, 3, 5 days. The cell viability adhered on the hydrogels was evaluated by Calcein-AM/Propidium iodide staining after 3 days` culture. The staining cells were observed under a confocal laser scanning microscope (CLSM) (Nikon A1, Nikon, Japan).

In vivo wound healing experiment

All the following animal experiments were carried out according to protocols approved by the animal ethical committee of Nanjing Normal University and laboratory animal administration rules of China. The animal experiments were performed according to the previous reported methods[7]. Four Sprague-Dawley (SD) rats (6 weeks, male, Zhejiang Academy of Medical Sciences) with full-thickness skin defect model under *S. aureus* infection were used for evaluating the wound healing ability of various hydrogels. Briefly, the rats were anesthetized by intraperitoneal injection of chloral hydrate (10%, dissolved in PBS) with a dose of 3 mL/kg. After that, the dorsal region of rats was depilated. Four circular full-thickness skin wounds with diameters of 8 mm were created on the dorsal back of the rats using a skin biopsy punch. Then, 10 μL *S. aureus* suspension (1×10^6 CFU/mL) was injected into each defect wound site. Afterward, the PAM, QL-PAM, and QL-PHMB-PAM hydrogels (8 mm × 2 mm) were implanted on corresponding infected wound sites. And blank wound without hydrogel treatment was severed as control. Four parallel samples were tested in each group. Finally, Tegaderm™ Film (3 M, St. Paul, MN, USA) was used to cover the wounds to make the hydrogel in place. The wound areas were monitored on days 0, 3rd, 7th, and 14th using a digital camera. The wound closure ratio (%) was quantified by Image J software and calculated according to the following equation:

$$\text{Wound closure ratio (\%)} = \frac{area\ of\ day\ 0 - area\ of\ day\ n}{area\ of\ day\ 0} \times 100\% \quad \text{. (Equation S6)}$$

After 14 days, all rats were euthanized by injecting excessive chloral hydrate. The surrounding tissue of the wound sites was harvested and stained with hematoxylin and eosin (H&E) for histological analysis. Images of the stained slices were obtained by inverted phase contrast fluorescence microscope.

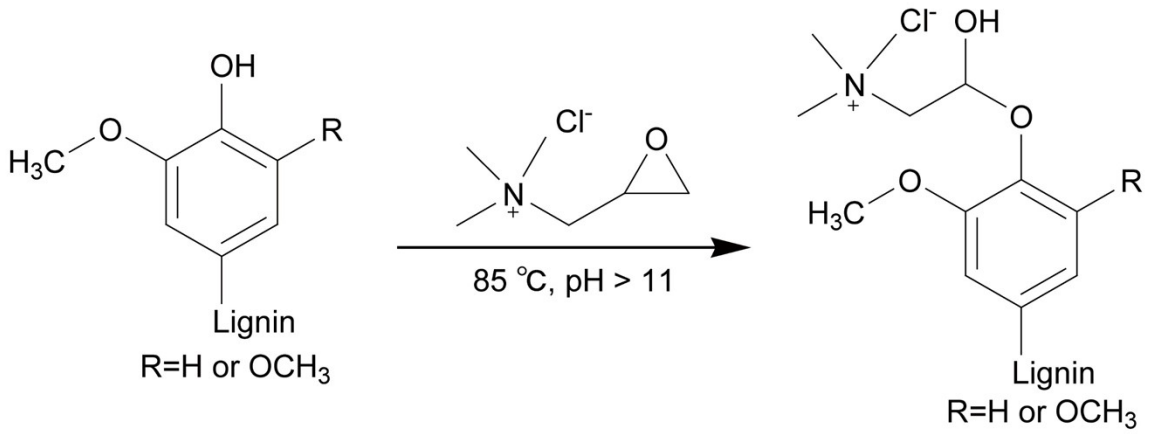

Fig. S1. Synthesis of the quaternized lignin (QL).

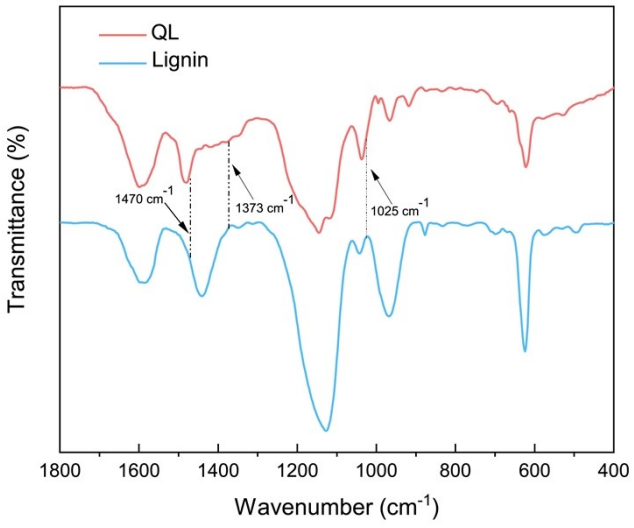

Fig. S2. FT-IR spectra of Lignin and QL.

To verify the successful grafting of quaternary ammonium group on lignin, FT-IR analysis of lignin (L) and quaternized lignin (QL) samples were displayed in Fig. S2. The peak at 1373 cm^{-1} were associated with the phenolic hydroxyl group in lignin and the prominent decrease of

intensity of this peak confirmed the enormous phenolic hydroxyl group was reacted after modification. Meanwhile, a new peak at approximately 1470 cm^{-1} was attributed to the bending vibration of the quaternary ammonium group, which indicated the phenolic hydroxyl group in lignin had been successfully quaternized by EPTAC. These results indicated the QL was synthesized successfully.

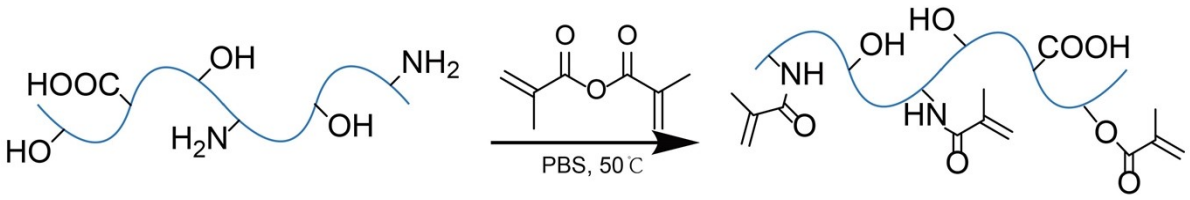

Fig. S3. Synthesis of gelatin methacryloyl (GelMA).

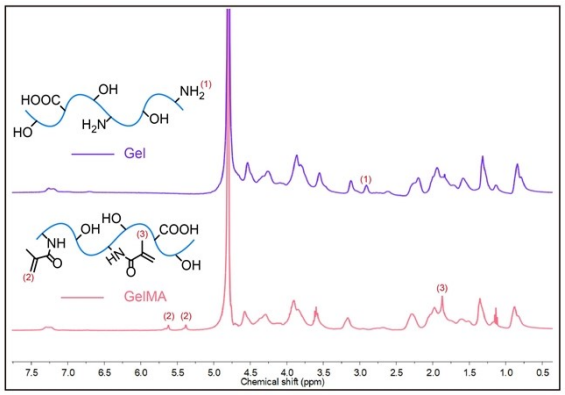

Fig. S4. ^{1}H NMR spectra of gelatin (Gel) and GelMA.

The synthesis of GelMA was presented in Fig. S3. As depicted in the spectrum (Fig. S4), the

methacrylate degree of gelatin is 52%, which is confirmed from the disappearance of the peak of the amino group (δ = 2.9 ppm) in the spectrum of GelMA. In addition, new signals appeared at 5.4 ppm and 5.6 ppm in the spectrum of GelMA, which corresponded to the double bonds of the methacrylate groups. The peaks at 7.3 ppm and 1.87 ppm were characteristic of the aromatic amino acid residues of gelatin and the methyl group of methacrylic acid, respectively.

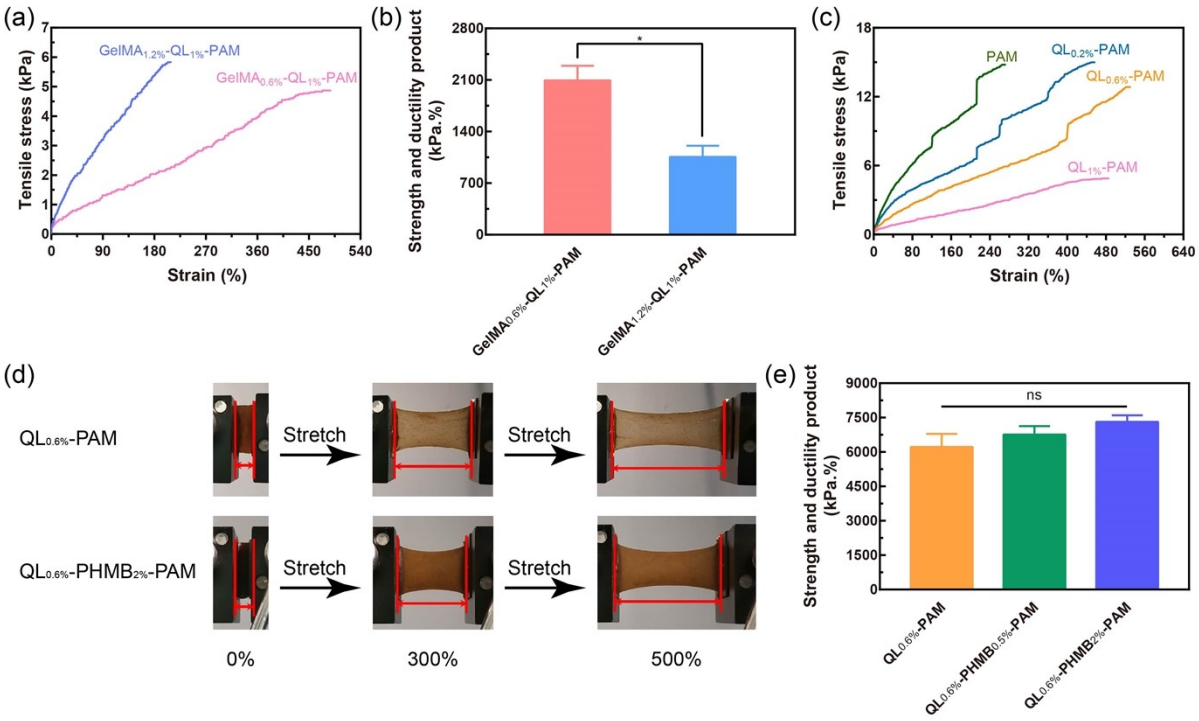

Fig. S5. The (a) typical tensile stress-strain curve and (b) strength and ductility product of GelMA-$QL_{1\%}$-PAM hydrogel with different GelMA content (n = 3, mean ± SEM). Pairwise comparisons have significant differences as denoted as "*" ($p < 0.05$, determined by unpaired t-test). (c) The typical tensile stress-strain curve of QL-PAM hydrogel with different QL content. (d) The digital photographs of $QL_{0.6\%}$-PAM and $QL_{0.6\%}$-$PHMB_{2\%}$-PAM hydrogel during the tensile process (the red line is the tensile elongation of the hydrogel). (e) The strength and ductility product of $QL_{0.6\%}$-PHMB-PAM hydrogel with different PHMB content (n = 3,

mean ± SEM). Pairwise comparisons have no significant difference as denoted as "ns" ($p > 0.05$, determined by one-way ANOVA multiple comparisons).

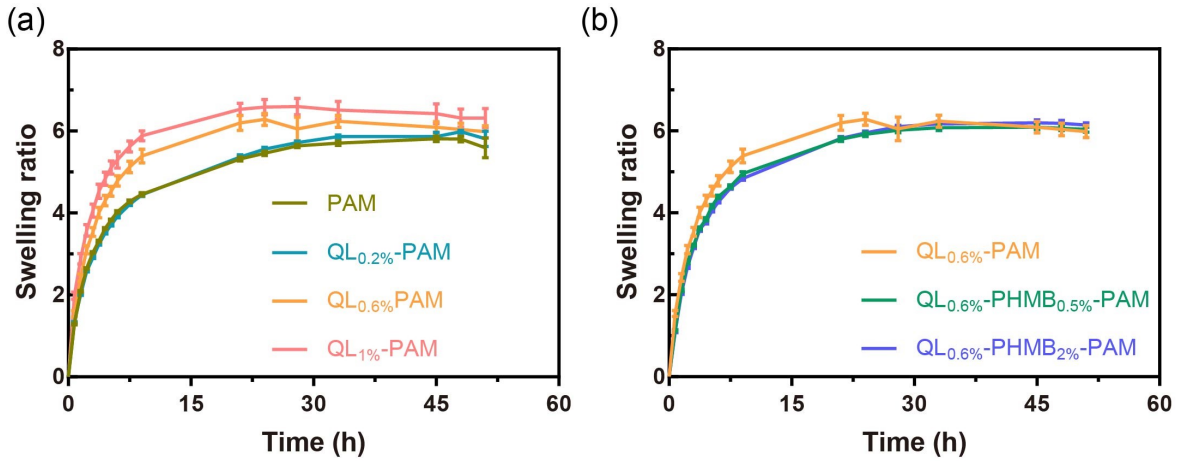

Fig. S6. (a) The swelling ratio of QL-PAM hydrogel with different QL content (n =6, mean ± SEM). (b) The swelling ratio of $QL_{0.6\%}$-PHMB-PAM hydrogel with different PHMB content (n =6, mean ± SEM).

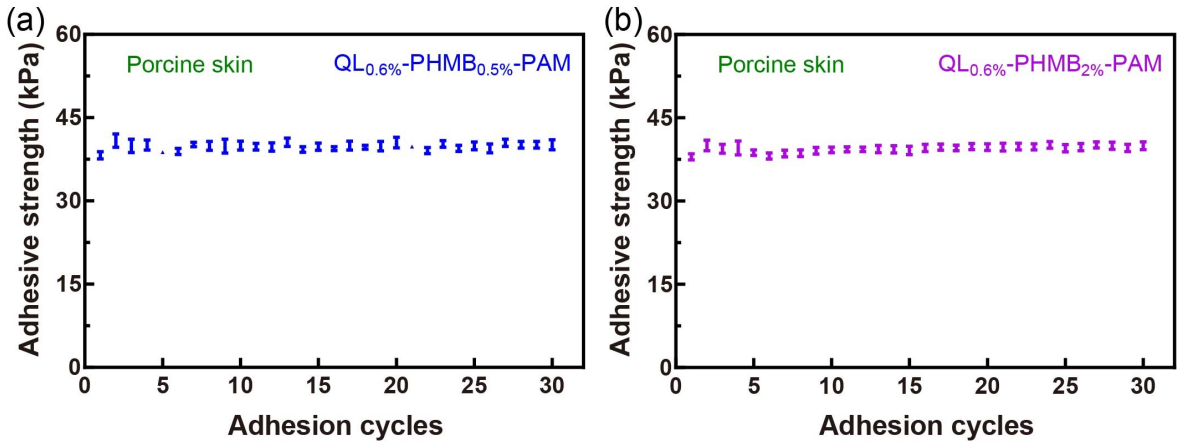

Fig. S7. The repeated adhesion of $QL_{0.6\%}$-$PHMB_{0.5\%}$-PAM and $QL_{0.6\%}$-$PHMB_{2\%}$-PAM hydrogel to porcine skin and titanium after 30 cycles of adhering-stripping (n =5, mean ± SEM).

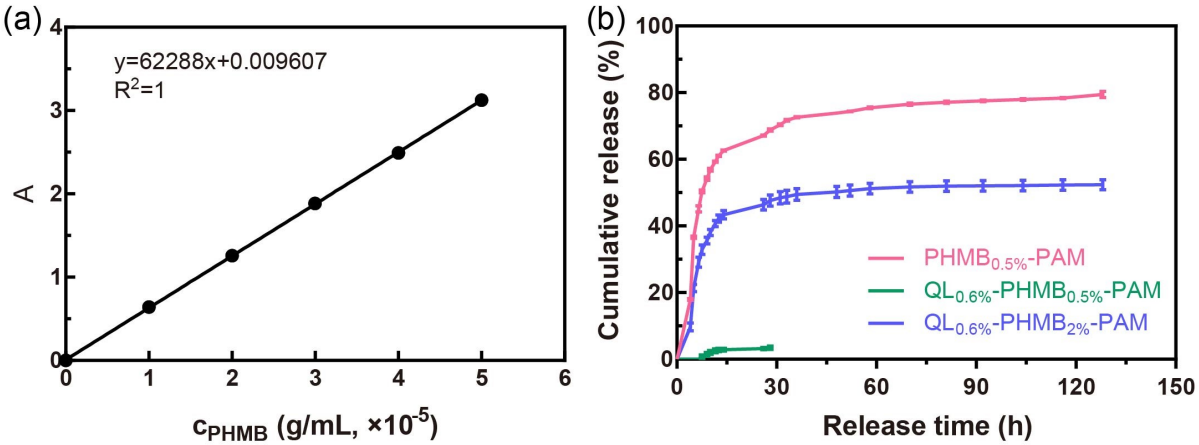

Fig. S8. (a) The standard curve of A-c_{PHMB}. (b) The release profiles of PHMB from $PHMB_{0.5\%}$-PAM, $QL_{0.6\%}$-$PHMB_{0.5\%}$-PAM, and $QL_{0.6\%}$-$PHMB_{2\%}$-PAM hydrogel over 5 d into PBS (n =4, mean ± SEM).

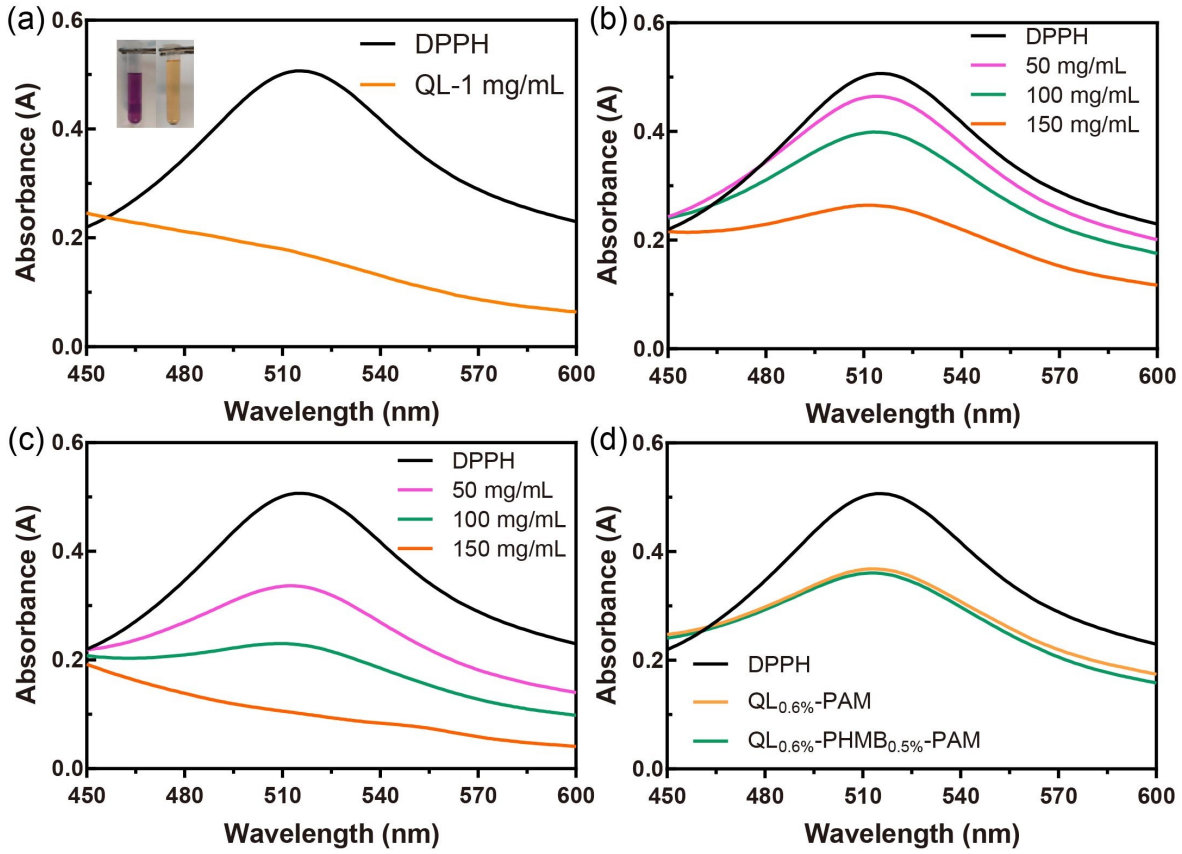

Fig. S9. (a) The DPPH UV−vis spectra of the blank and QL of 1 mg/mL after reaction for 5 min. The DPPH UV-vis spectra of the blank and $QL_{0.6\%}$-PAM hydrogel with different content varying from 50 mg/mL to 150 mg/mL after reaction for (b) 2 h and (c) 24 h. (d) The DPPH UV−vis spectra of the blank and $QL_{0.6\%}$-PHMB-PAM hydrogel of 150 mg/mL with different PHMB content after reaction for 1 h.

Reference:

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