

Supporting information

Engineering bioactive self-healing antibacterial exosomes hydrogel for promoting chronic diabetic wound healing and complete skin regeneration

Chenggui Wang^{a#}, Min Wang^{b#}, Tianzhen Xu^a, Xingxing Zhang^c, Cai Lin^c, Weiyang Gao^a, Huazi Xu^a, Bo

Lei^{b,d,e*}, Cong Mao^{a*}

^a *Key Laboratory of Orthopedics of Zhejiang Province, Department of Orthopedics, the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325027, China*

^b *Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi'an Jiaotong University, Xi'an 710000, China*

^c *Center of Diabetic Foot, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, China*

^d *Frontier Institute of Science and Technology, Xi'an Jiaotong University, Xi'an 710054, China*

^e *Instrument Analysis Center, Xi'an Jiaotong University, Xi'an 710054, China*

*Corresponding author: maocong@wmu.edu.cn (Cong Mao) and rayboo@xjtu.edu.cn (Bo Lei).

These authors contributed equally to this work.

2. Materials and methods

2.1 Synthesis of oxidized hyaluronic acid (OHA) and FHE hydrogel

Hyaluronic acid (HA, 1.2×10^6 g/mol, Sigma) was dissolved in double-distilled water and oxidized with sodium periodate (NaIO_4) (Sigma) in DDW as previously described [1]. FHE hydrogel was prepared using Pluronic F127 (F127) (Sigma), oxidized hyaluronic acid (OHA) and poly- ϵ -lysine (EPL) (Mw 3.5kDa from Nanjing Shineking Biotechnology) by the Schiff base reaction and thermal-responsive sol-gel process at different blending ratio. Briefly, 20% (wt/vol) of F127 and EPL with different concentration (5% and 10%) were dissolved in phosphate buffered solution (PBS, 0.01 M, pH 7.4), and 5% (wt/vol) of oxidized hyaluronic acid was then added in the solution to make homogenous mixture. Subsequently, the mixture was kept at 37 °C for complete gel formation. FH, FHE5, and FHE10 represent F127/OHA hydrogel, F127/OHA/EPL hydrogel with 5 % (wt/vol) of EPL and F127/OHA/EPL hydrogel with 10 % (wt/vol) of EPL, respectively.

2.2 Physicochemical and multifunctional properties characterizations of the FHE hydrogel

The chemical compounds were characterized by the FT-IR spectroscopy (NICOLET 6700, Thermo), which were obtained from 4000 to 400 cm^{-1} at a scan resolution of 4 cm^{-1} . The porous morphology of FHE hydrogels was observed by a scanning electron microscopy (SEM) (Quanta 250 FEG, FEI). All of these samples were placed on double-sided tape and sprayed with a thin gold layer before observation. The rheological properties of hydrogels including the storage modulus (G') and loss modulus (G'') were performed by employing a rheometer (DHR-2, TA Instruments). The hydrogels were placed between the parallel plates of 20 mm diameter and with a gap of 1000 μm . The temperature effect of various hydrogels in the range of 10-37.5 °C was evaluated by keeping the strain and frequency constant as 1% and 1 Hz, respectively.

The injectability of the FHE-5 hydrogels was evaluated as follows. 20% (wt/vol) of F127 and 5% (wt/vol) of EPL were dissolved in PBS (pH 7.4) with rhodamine B (0.002%, wt/vol, Sigma), and 5% (wt/vol) of oxidized hyaluronic acid was then added in the solution to make homogenous mixture. Subsequently, when the FHE-5 turned into a gel state, it was put into a syringe with a medical plastic catheter (0.8 mm of inner diameter, 10 cm of length) to evaluate whether the hydrogel can be extruded through the catheter without clogging.

The self-healing performance of hydrogels was testified by macroscopic experiments and quantitative method, respectively. In the macroscopic self-healing experiments, the FHE-5 hydrogel was made into a columnar (15 mm diameter, 5 mm thickness) with a cavity (5 mm diameter, 5 mm thickness), and stained with rhodamine B. Then, the new FHE-5 hydrogel was injected into the cavity and placed in a humidior at 25°C to allow them self-heal into a complete columnar hydrogel. The self-healing process of the FHE-5 hydrogel was recorded by photographs. The quantitative analysis of hydrogels during the self-healing process was tested by employing the rheometer. The strain amplitude effect of various hydrogels from 1% (100 s for each interval) to 1000% (100 s for each interval) were evaluated by keeping the temperature and frequency constant at 25°C and 1 Hz, respectively, and three cycles were carried out. On the other hand, the hydrogel was performed using the time sweep test with 1% strain and frequency constant of 1 Hz at 25°C. It was then snapped into two pieces and were put together to obtain the completely self-healed hydrogel. Then, the same test was performed for the self-healed hydrogels.

2.3 *In vitro* and *in vivo* degradation of the FHE hydrogel

The degradation properties of FHE hydrogels was evaluated *in vitro* and *in vivo*. Briefly, 200 µL FHE-5 hydrogel was placed in the upper transwell chamber placed in a 24-well plate, and 1 mL PBS was then added in the plate. The weight of hydrogel was recorded at day 0, 0.5, 1, 2, 3, 5, 7, 9, 11 and 13 day

and the weight residual was calculated. In addition, 150 μL FHE-5 hydrogel was injected into the mouse skin by subcutaneous injection, and photographed to record the degradation of hydrogel at 1, 3, 7 and 13 day.

2.4 Antibacterial activity evaluation

The *E. coli* and *S. aureus* were used to assess the antibacterial ability of FHE hydrogel. Briefly, the 500 μL hydrogel were added into a 24-well microplate. Then, 10 μL bacterial suspensions (10^6 CFU mL^{-1}) were added onto hydrogel surface. After 2 h incubation, the bacterial survivor on the hydrogel surface was re-suspended using 1 mL sterilized PBS buffer solution and was added on the bacterial culture medium to incubate for 24 h. The bacterial suspension suspended in PBS was used as a negative control (NC). The bacterial colony plaque was captured and the killing ratio was calculated according to the equation: kill % = (cell count of control-survivor count on hydrogel) \times 100% / (cell count of control).

2.5 Isolation and identification of exosomes, and exosomes release profile of FHE@exo hydrogel

Firstly, AMSCs were isolated from the adipose tissue surrounding the epididymis of the four-week-old ICR mice according to previous reports [2, 3]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose medium containing 10% FBS at a 37°C incubator. Flow cytometric analysis was performed for identification of cellular characteristics after cell-labeling with appropriate antibodies: anti-CD90 (ab226, 1:1000, Abcam), anti-CD45 (ab25670, 1:1000, Abcam), anti-CD44 (ab25579, 1:1000, Abcam) and anti-CD34 (RM3604, 1:2000, ThermoFish) on day 14.

Exosomes were extracted and purified from AMSCs as previously described [18]. Briefly, AMSCs culture medium was collected and centrifuged at 800 g for 5 min and then for an additional 10 min at 2000 g to remove lifted cells. Then the supernatant was subjected to filtration on a 0.2- μm pore membrane filter, followed by concentration using a 100,000-kDa molecular mass cut-off membrane (Millipore) to remove

large membrane vesicles. The supernatant was ultra-centrifuged at 100,000 g for 90 min at 4 °C using a 100Ti rotor (Beckman Coulter). Final exosomes were obtained and stored at -80 °C. The protein content, as the quantification of exosomes, was determined using a micro BCA protein assay kit (Beyotime, Shanghai, China). The morphology of the extracted exosomes was observed using a transmission electron microscopy (Hitachi, Tokyo, Japan). Size distribution within exosome samples was analyzed using NanoSight (Malvern, UK). The Alix, CD9, CD63, and CD81 molecules which frequently located on the surface of exosomes were analyzed using Western blot with primary antibodies as follows: anti-Alix (ab186429, 1:1000, Abcam), anti-CD63 (sc-5275, 1:500, Santa), anti-CD9 (ab92726, 1:1000, Abcam), anti-CD81 (sc-7637, 1:500, Santa) according to previous study [18]. The bands were detected by electrochemiluminescence reagent (Invitrogen) and the signals were visualized by a ChemiDocXRS+ Imaging System (Bio-Rad).

The FHE@exo hydrogel was obtained by mixing 1 µg exosomes with 100 µL FHE hydrogel with stirring at 4 °C. The exosome release profile was tested by a micro BCA protein assay kit (Beyotime, China). Briefly, the above prepared 100 µL FHE@exo hydrogel containing 1 µg exosomes were placed in the upper transwell chamber placed in a 24-well plate, while 100 µL PBS was added in the lower chamber. Then 10 µL PBS (pH=5.5 or 7.5) was collected and replaced by 10 µL fresh PBS at day 0, 3, 6, 9, 12, 15, 18 and 21. The content of released exosomes was detected and the exosomes released percentage was calculated.

2.6 Cell cytotoxicity and proliferation assessment

Human umbilical vein endothelial cells (HUVECs, from ATCC) were cultured in 1640 complete medium with 10% FBS and 1% penicillin-streptomycin in a cell incubator. FHE@exo hydrogel was prepared as described in 2.5. The cytotoxicity of HUVECs on different time point after co-culture with FHE or FHE@exo was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo) using transwell (Corning, 8 µm, US). Briefly, suspended HUVECs (1×10^5 cells/cm²) were transferred on the lower chamber and 100

μl FHE or FHE@exo was added to the upper chamber. After culturing for 1 to 4 days, samples were then incubated with medium containing the CCK-8 reagent for 1 h, and the absorbance of the medium was read at 450nm by a microplate reader.

2.7 *In vitro* HUVECs migration and tube formation assay

The migration and tube formation ability of HUVECs was tested through a Transwell (Corning, 8 μm) co-culture system. In brief, HUVECs (1×10^5) were cultured on the lower chamber and 100 μl FHE or FHE@exo was placed on the upper chamber. Cells were then harvested after incubation for 48h. For migration assay, HUVECs (1×10^5) were cultured on the upper chamber, and culture medium with 1% FBS was added into the lower chamber. After 12 h, the upper chamber membrane was fixed and stained with crystal violet to visualize the migrated cells, which were then observed under a microscope (Nikon, FHEIPSE Ti, Japan). To assess exosomes on HUVECs tube formation, HUVECs were cultured on growth factor-reduced Matrigel (BD Biosciences, US). Firstly, thawed Matrigel was added to a μ -Slide (10 μl /well, IBIDI, Germany) and then incubated for 30 min to solidify. After cultured with FHE or FHE@exo hydrogel for 48 h, 5000 harvested cells were transferred into the Matrigel-coated μ -Slide and incubated for 4 h. Formed tubes were observed by five independent fields under the above microscope.

2.8 Diabetic wound model

Male ICR mice weighing about 30g were provided by SLAC laboratory animal company (China), and all animal treatments were licensed by Wenzhou Medical University Animal Care and Use Committee. After a 12 h fast, mice were intraperitoneally injected with streptozotocin (STZ, 100 mg/kg) for 2 days, and after 2 weeks, the blood glucose was measured by a SureStep Complete Blood Glucose monitor (Johnson & Johnson). Diabetic mice were successfully induced when the blood glucose was above 16.7 mM. Before surgery, 48 diabetic mice were anesthetized with 4% chloral hydrate (0.1mL/10g). After shaving and

sterilization, two 8-mm full-thickness wounds were made on each mouse's back. Saline, FHE hydrogel alone, 10 µg free exosomes or FHE@exo hydrogel (containing 10 µg exo) were used to cover the wounds (n = 12). All treated mice were kept in separate cages and closely watched during the whole time. At day 0, 3, 7, 14, and 21, wound areas were measured and the wound closure rate was calculated by Image-Pro plus.

2.9 Histological analysis, immunohistochemically and immunofluorescence staining

Wound samples were harvest after mice were sacrificed, fixed and embedded in paraffin. Tissue sections (5µm) were mounted on slides for histological analysis. Hematoxylin and Eosin (H&E) staining and Masson's trichrome staining were performed to visualize the pathological changes of formed tissue and collagen formation at different healing time. The stained slides were assessed using a Nikon microscope.

For immunohistochemically and immunofluorescence staining, sections were deparaffinized, rehydrated, and treated with H₂O₂ and carbinol for blocking the endogenous peroxidase activity. Samples were heated in a microwave oven twice to recover the antigen, and treated with 5% bovine serum albumin to block the non-specific reactions. Then, sections were treated with the primary antibodies of rabbit anti-Cytokeratin (ab9377, 1:75, Abcam), anti-Ki67 (ab15580, 1:200, Abcam), rabbit anti-collagen III (ab7778, 1:300, Abcam), or rabbit anti-collagen I (ab21286, 1:300, Abcam) overnight, 1 h treatment with HRP goat anti-rabbit (1:400, Abcam), and visualized by a DAB kit (ZSGB-BIO, China). After counterstained with hematoxylin, slides were assessed with a fluorescent microscope (40FL Axioskop, Zeiss). For α-SMA staining, samples were firstly cultured with rabbit anti-α-SMA, then treated with goat anti-rabbit IgG Alexa Fluor® 647-conjugated secondary antibody (ab150083, 1:1500, Abcam) and DAPI. The stained slides were observed under a confocal laser microscope (Nikon, A1 PLUS, Japan).

2.10 Statistical analysis

Data from at least three individual experiments are listed as mean ± standard deviation (mean ± SD).

Statistical differences were evaluated by one-way ANOVA with Tukey's test (Graph Pad Prism 7.0). The value of $*P < 0.05$ was considered as significant difference.

References

- [1] Y. Cai, E. López-Ruiz, J. Wengel, L.B. Creemers, K.A. Howard, A hyaluronic acid-based hydrogel enabling CD44-mediated chondrocyte binding and gapmer oligonucleotide release for modulation of gene expression in osteoarthritis, *J. Control. Release* 253 (2017) 153-159.
- [2] Y.T. Chen, C.C. Yang, Y.Y. Zhen, C.G. Wallace, J.L. Yang, C.K. Sun, T.H. Tsai, J.J. Sheu, S. Chua, C.L. Chang, C.L. Cho, S. Leu, H.K. Yip, Cyclosporine-assisted adipose-derived mesenchymal stem cell therapy to mitigate acute kidney ischemia-reperfusion injury, *Stem Cell Res. Ther.* 4(3) (2013) 62.
- [3] M. Maumus, D. Guerit, K. Toupet, C. Jorgensen, D. Noel, Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology, *Stem Cell Res. Ther.* 2(2) (2011) 14.

Table S1 Characterization of aldehyde hyaluronic acid (OHA)

Sample	Theoretical oxidation (%)	Actual oxidation degree (%)
OHA	40	32.34 ± 1.75

Figures

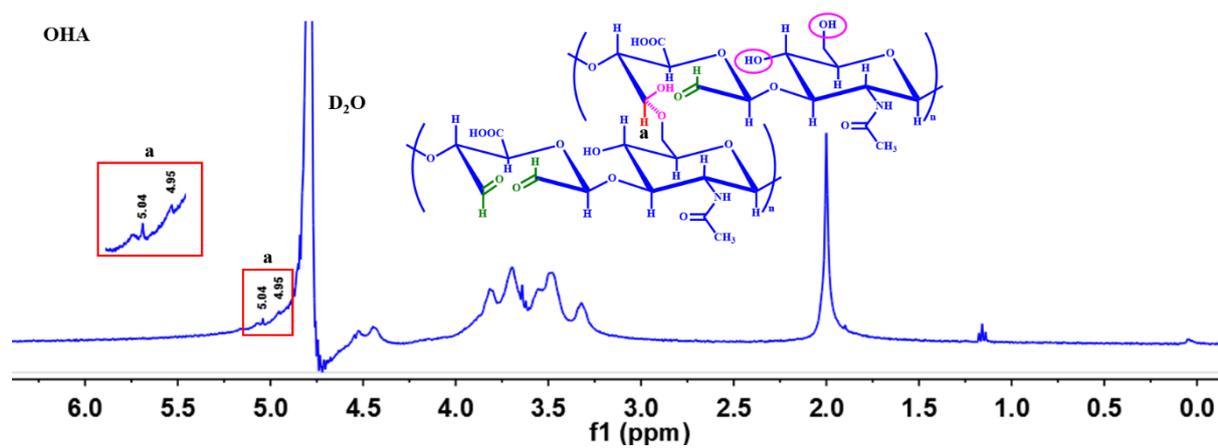


Figure S1 ¹H NMR spectra of OHA

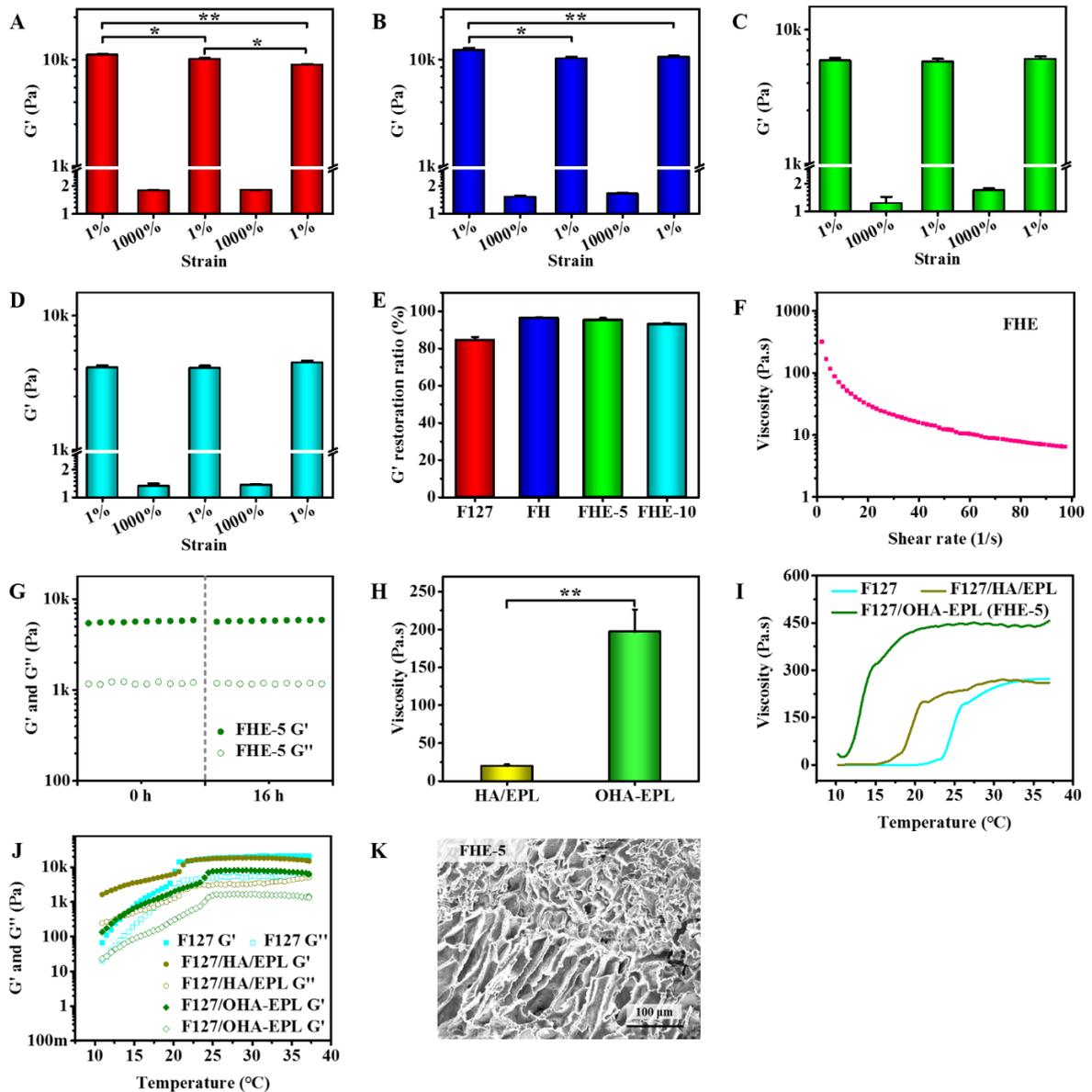


Figure S2 Rheological properties of FHE hydrogel. (A-D) The G' of F127 (A), FH (B), FHE-5 (C) and FHE-10 (D) hydrogel during cycling three times between 1 % and 1000 %; (E) The G' restoration ratio of the hydrogel after healing; (F) The viscosity of FHE-5 hydrogel with the shear rate from 1 to 100 1/s. (G) The G' and G'' of the initial FHE-5 hydrogel and after 16 h hydrogel repair; (H) The viscosity of HA/EPL and OHA-EPL hydrogel at the shear rate of 1 1/s; (I) The viscosity of F127, F127/HA/EPL and F127/OHA-EPL hydrogel from 10 to 37 °C at the shear rate of 1 1/s; (J) The G' and G'' of F127, F127/HA/EPL and F127/OHA-EPL hydrogel from 10 to 37 °C at the strain of 1 %; (K) The SEM after 16 h FHE-5 hydrogel repair.

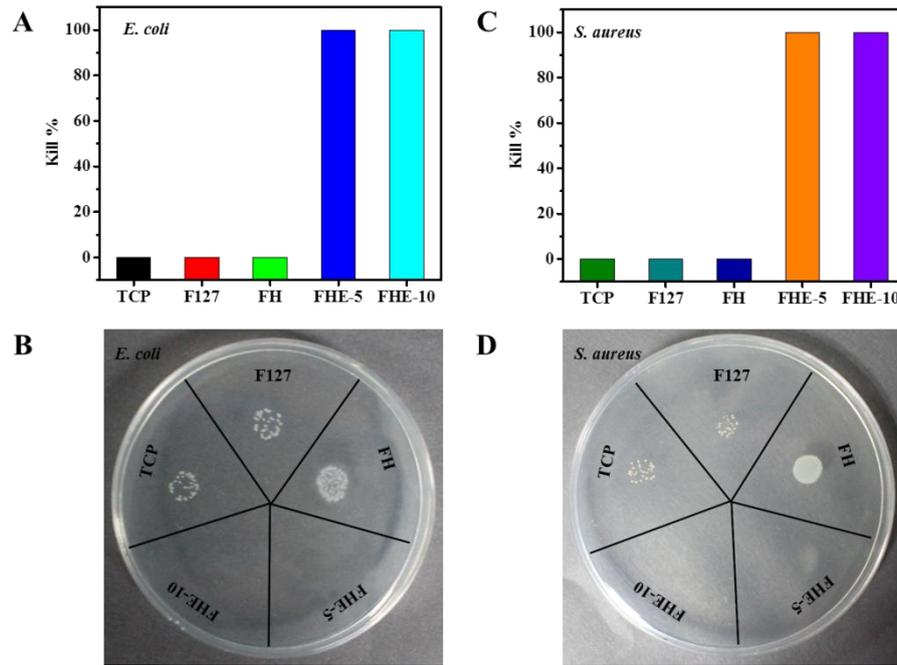


Figure S3 Robust antibacterial activity of FHE hydrogel. (A-B) Antibacterial efficiency (A) and bacteria clones (B) against *E. coli* after incubation for 2 h at 37°C; (C-D) Antibacterial efficiency (C) and bacteria clones (D) against *S. aureus* after incubation for 2 h at 37°C. The TCP represent the blank control. Almost above 99% antibacterial efficiency could be observed for FHE-5 and FHE-10 hydrogel.

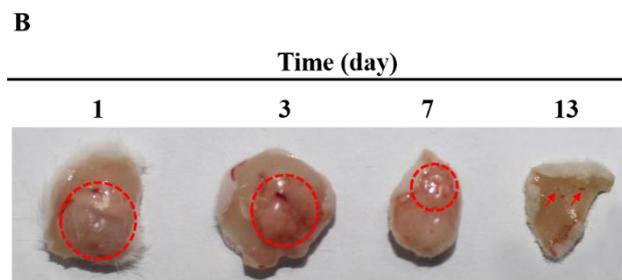
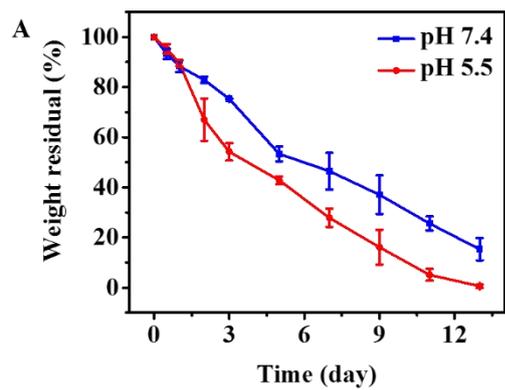


Figure S4 The degradation properties of the hydrogels *in vitro* (A) and *in vivo* (B).

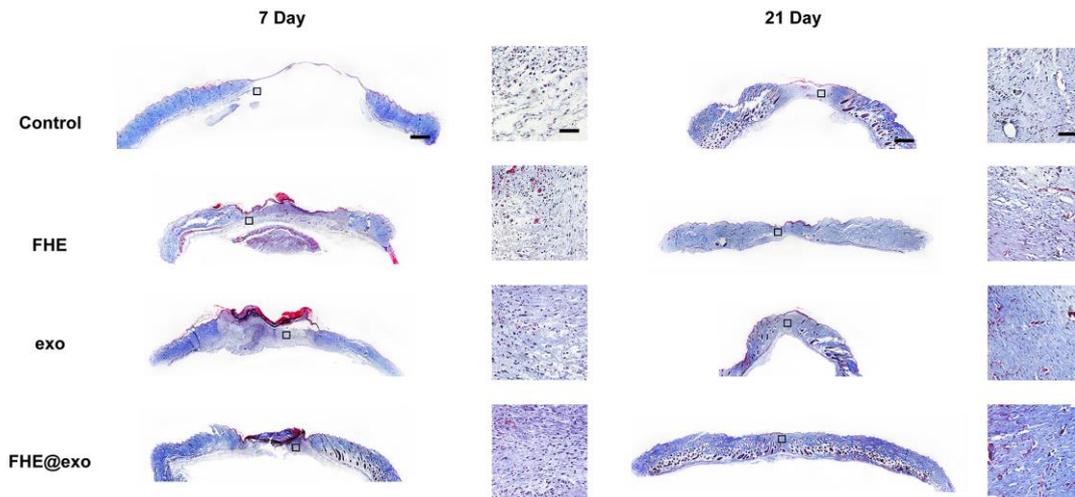


Figure S5 Masson staining of collagens in wounds treated by FHE@exo hydrogel. Rectangles refer to the close-up areas. Original scale bar = 1000 μm and close-up scale bar = 50 μm , n=3 per group.

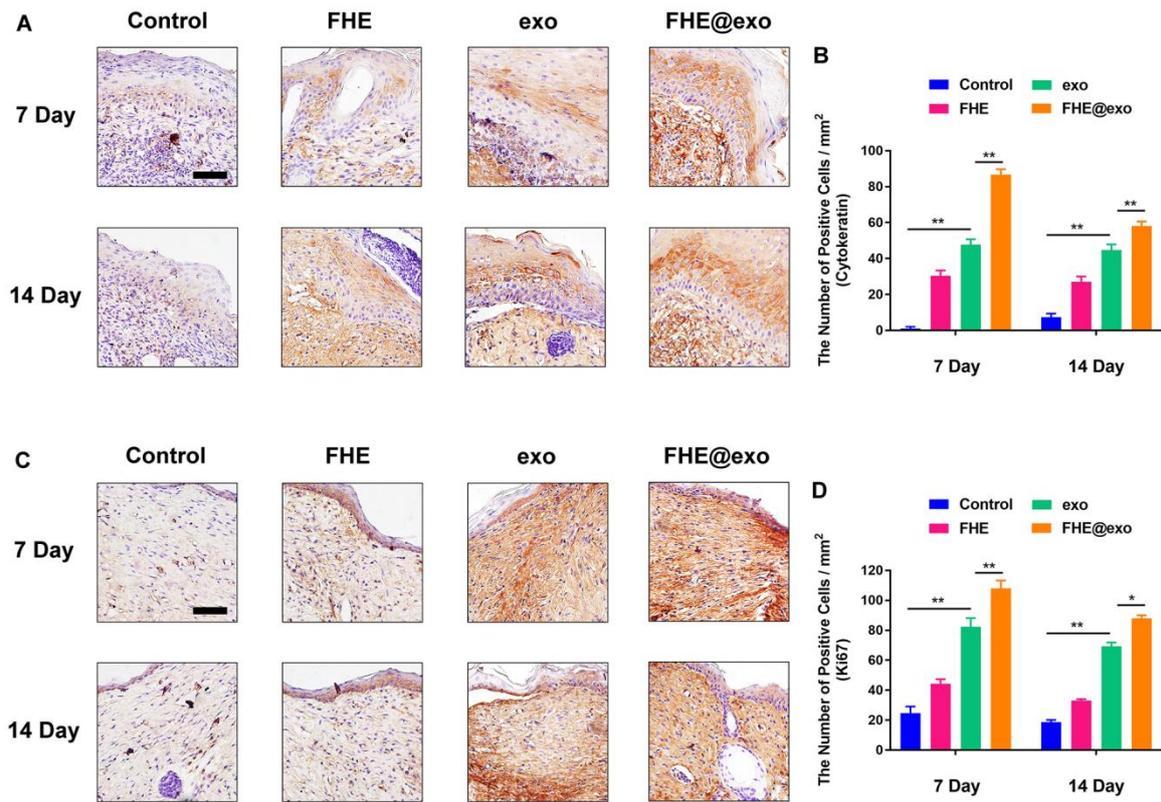


Figure S6 Immunofluorescence staining of cytokeratin and Ki67 expression in wounds treated by FHE@exo hydrogel. (A) Immunohistochemical images of wound sections stained with cytokeratin (a marker of epithelial cells) at day 7 and 14, scale bars: 50 μm ; (B) Quantification of the number of cytokeratin positive cells in the wound area on days 7 and 14; (C) Representative images of Ki67 immunohistochemistry staining on days 7 and 14, showing enhanced cellular proliferation in FHE@exo group as evidenced by increased expression of Ki67; (D) Quantification of the number of Ki67 positive cells in the wound area on days 7 and 14.