Supplementary information

Chitosan-based multifunctional hydrogel containing in-situ rapidly bioreduced silver nanoparticles for accelerating infected wound healing

Fengxin Zhao [a], Yifan Liu [a], Tao Song [a], Bin Zhang [a], Dongxiao Li [b], Yumei Xiao [a, *], Xingdong Zhang [a]

[a] National Engineering Research Center for Biomaterials, Sichuan University, Chengdu 610065, Sichuan, China

[b] Sichuan Academy of Chinese Medicine Science, Chengdu 610041, Sichuan, China

*Corresponding authors. E-mail address: xymzl2000@126.com (Yumei Xiao)

1. Synthesis and characterization of QCS, CMA, and ODex

Synthesis of quaternized chitosan (QCS)

The QCS was prepared according to the previous method with modification [1]. Briefly, 1 g of chitosan was dissolved in 72 mL 0.175 M acetic acid solution at 55 °C. Then 0.94 g of GTMAC was added into the chitosan solution. After reacting 24 hours, the solution was purified through dialysis (MWCO: 8000-14,000 Da) against deionized water for 3 days and then lyophilized to obtain QCS. The chemical structure of QCS was detected by ^{1}H NMR spectrum. From Fig. S2, a new signal at 3.1 ppm attributing to trimethylammonium groups was detected in the ^{1}H NMR spectrum of QCS, demonstrating that the GTMAC was successfully grafted onto the chitosan backbone. The substitution degree of QCS was about 15.5 ± 1.5% according to the ^{1}H NMR spectrum.

Synthesis of methacrylate anhydride-modified collagen (CMA)

Furthermore, the CMA was successfully synthesized following our previous literature [2]. Briefly, 0.2g of type I collagen was dissolved in 100 mL 0.2 M acetic acid solution overnight at 4 ℃ and the pH was then adjusted to 7.4 by 5 M NaOH. Subsequently, 9.8 μl MA was added into the overhead solution. After 4 h, dialysis (MWCO: 8000-14,000 Da) against three times with pre-cooled 0.5 M acetic acid /ethyl alcohol mixture (4:1, v/v) and several times 0.5M acetic acid solution for 3 days at 4 ℃. The lyophilization was performed to give the product CMA. In the 1H NMR spectrum of CMA (Fig. S3), there have two new peaks at 5.3 ppm and 5.6 ppm, indicating that CMA

was synthesized by specifically conjugating methacryl groups to amino groups of collagen molecules [2]. The substitution degree according to the 1H NMR spectrum was approximately 50.6 %.

Synthesis of oxidized dextran (ODex)

ODex was prepared as previous literature [3]. Briefly, 1 g of dextran (Concentration:10 mg/ml) was dissolved in deionized water, and the equal quality of sodium periodate was added into the dextran solution. After continuously stirring at room temperature for 2 h in the dark, 2 mL of diethylene glycol was added into the mixture solution for stopping the reaction. Finally, the ODex was freeze-dried after dialysis (MWCO: 3500 Da) against deionized water for 3 days. The oxidation degree of the ODex samples was determined by measuring aldehyde content using the hydroxylamine hydrochloride titration method, the actual oxidation degree of ODex was about 76.2 ± 3.1% according to the titration experiments. Furthermore, the new peaks at 5.7-4.8 ppm in the 1H NMR spectrum of ODex further indicated that there were aldehyde groups and hemiacetal [4] (Fig. S4). It may be that after the most hydroxyl group on the dextran is oxidized to an aldehyde group, the unoxidized hydroxyl group reacts with the adjacent aldehyde group to form a hemiacetal structure, which is more stable than the free aldehyde group [5, 6].

2. *In vitro* degradation, swelling, and mechanical behaviors of hydrogels

Hydrogel was weighed (W1) and immersed in 2 mL of PBS (pH = 7.4) at 37 °C. At the designated time point, hydrogel was taken out from the solution and its weight was recorded as W_2. The remaining weight was calculated as follows:

$$\text{Remaining weight (\%)} = W_2/W_1 \times 100\%.$$

The swelling capacity of hydrogels was determined. Briefly, the freeze-dried hydrogel samples were immersed in 2 mL PBS (pH = 7.4) at 37 °C until swelling equilibrium, and then hydrogel samples were weighed again after removing the superficial water of samples. The swelling ratio was calculated as follow:

$$\text{Swelling ratio (\%)} = (W_s - W_o)/W_o \times 100\%.$$

where W_s refers to the weight of hydrogel sample after swelling equilibrium, W_o refers to the weight of the original freeze-dried hydrogel sample.

The compression mode of dynamic mechanical analyzer (TA-Q800, USA) was used to analyze the mechanical behaviors of hydrogels samples. The ramp force was 0.1 N/min to 5 N. After fitting the linear portion of the stress−strain curve, the slope at 10% strain was taken as the elastic modulus.

3. *In vitro* hemocompatibility and cytocompatibility evaluations of hydrogels

The hemocompatibility of hydrogels was evaluated by a hemolysis experiment. The 4% w/v erythrocyte suspension was prepared from Sprague-Dawley (SD) rat's blood (1500 rpm, 10 mins) and resuspending the erythrocyte solution with saline solution [7]. And then, COC and COCAg hydrogels were weighted at 0.1 g with 750 μL saline solution in 2ml tube [8]. The positive control and negative control respectively were established by another 750 μL deionized water and saline. Next, 750 μL of the 4% erythrocyte suspension was added to each tube. After 2 h incubating at 37 °C, 100 μL of the solution was collected from each tube and the hemolysis ratio was calculated by optical density value at 540 nm by a microplate reader (BioTek).

$$\text{Hemolysis ratio (\%)} = (A_s - A_n)/ (A_p - A_n) \times 100\%.$$

where A_s, A_n, and A_p refer to the optical density value of experimental groups, negative control groups, and positive control groups, respectively.

The cytotoxicity of the hydrogels was estimated by L929 cells. Sterilized hydrogels were completely immersed in DMEM media supplemented for 24 h to acquire the hydrogel extract. And the used hydrogel extract concentration was 0.2 g/mL as the previous studies reported [9, 10]. Meanwhile, L929 cells (1×10^4 cells/well) were seeded in a 96-well plate. After 1 day, the culture medium was replaced by the extraction of different hydrogels, and then the cell viability was detected by CCK-8 kit after cultured 24 h. The cell viability was calculated by OD value at 450 nm.

$$\text{Cell viability} = (OD_e - OD_b) / (OD_c - OD_b) \times 100\ \%$$

where OD_e, OD_c, and OD_b refer to the optical density value of experimental groups, control groups, and blank groups, respectively.

Furthermore, the extraction of different hydrogel samples was also used to evaluate the cell proliferation and morphology of L929 cells and HUVECs. Briefly, L929 cells and HUVECs (5×10^3 cells/well) were seeded in 96-well plates. And then the culture medium was replaced with the extraction after 24 h. On days 1, 2, and 3, the cell proliferation was detected by the CCK-8 kit and the optical density value at 450 nm was recorded. Also, the cells were stained with fluorescein diacetate (FDA, Sigma)/propidium iodide (PI, Sigma) and the cell morphology was imaged by fluorescence microscope (LSM880, Germany).

4. *In vitro* antibacterial performance of hydrogels

The bactericidal ability of the COCAg hydrogels was verified using gram-positive organism (S. aureus, ATCC6538) and gram-negative organism (E. coli, ATCC25922) by the bacterial inhibition zones. The 10 mm in diameter and 2 mm in thickness of hydrogel samples were putted on the surface of LB solid media, which was coated with 10^5 CFU/mL bacterial liquid. After 12h, the diameter of inhibition zone was measured. Additionally, the antibacterial properties of the hydrogels co-culturing with a large number of bacteria was further conducted by turbidimetry measurements. Briefly, 600 μL of S. aureus and E. coli (10^6 CFU/mL) suspensions were respectively co-culture with hydrogels in 48-well plate for 24 h at 37 °C by LB media. Afterward, the optical density (OD) value at 600 nm from co-culture medium of different hydrogels were recorded. Meanwhile, a 10 μL LB co-culture medium from different hydrogels was diluted 1000 times and coated onto the LB solid media. After being cultured for 12 h, the pictures of living bacterial from co-cultured medium were captured.

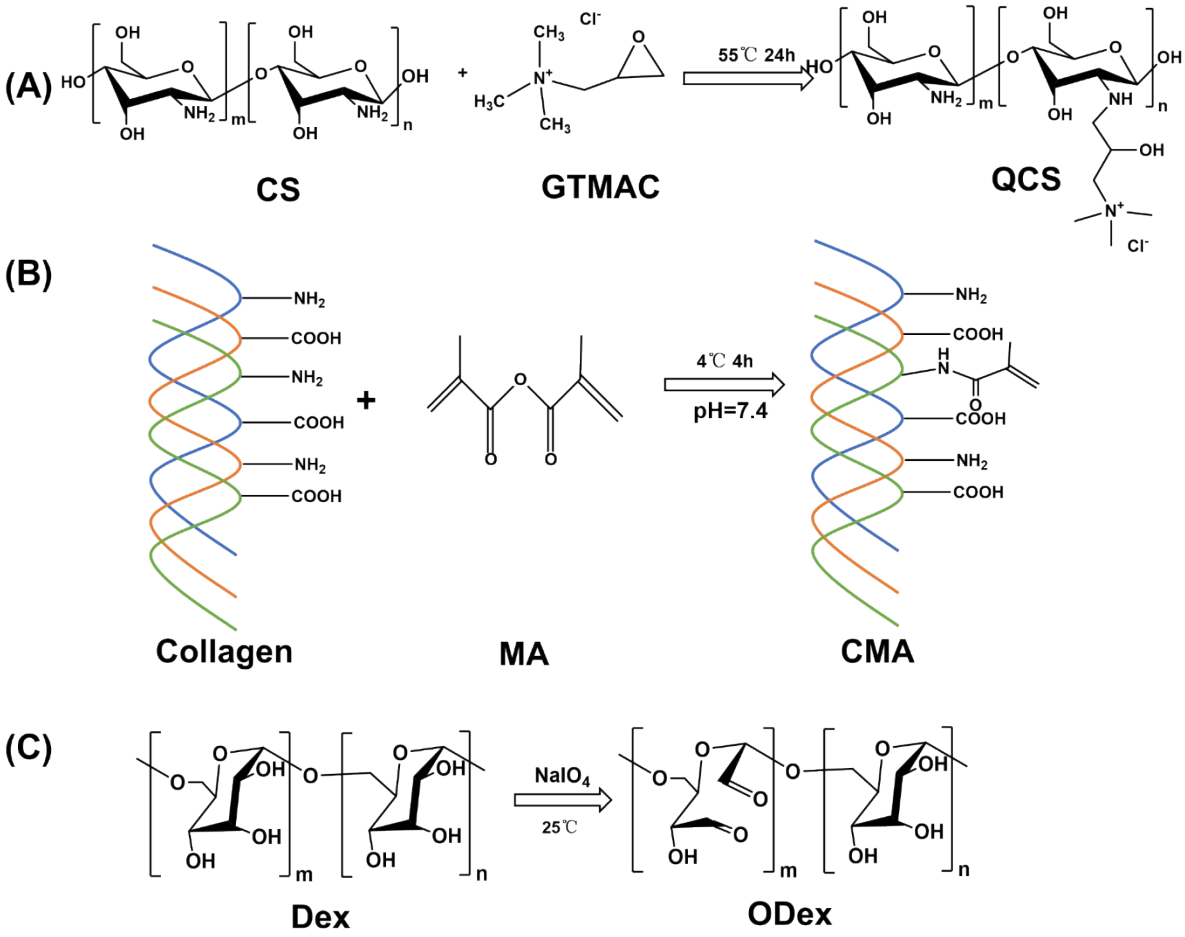

Figure S1 The schematic diagram of the synthesis of (A) QCS, (B) CMA and (C) ODex.

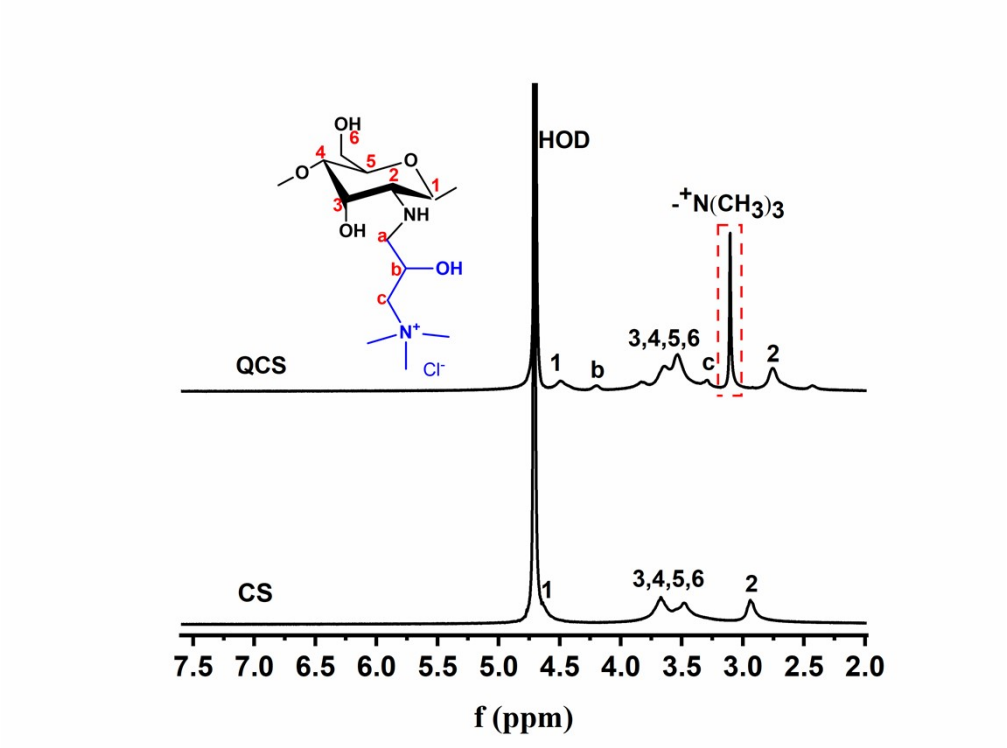

Figure S2 ^{1}H NMR spectra of QCS and CS.

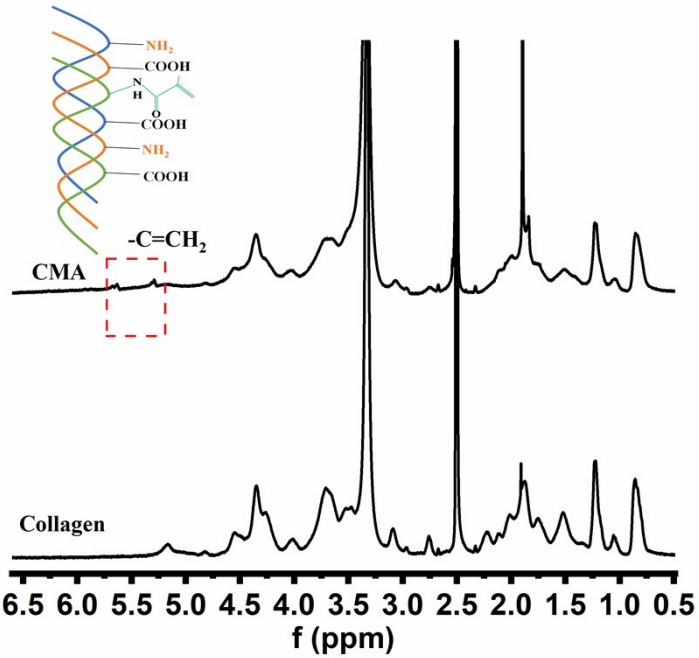

Figure S3 ^{1}H NMR spectra of CMA and Collagen.

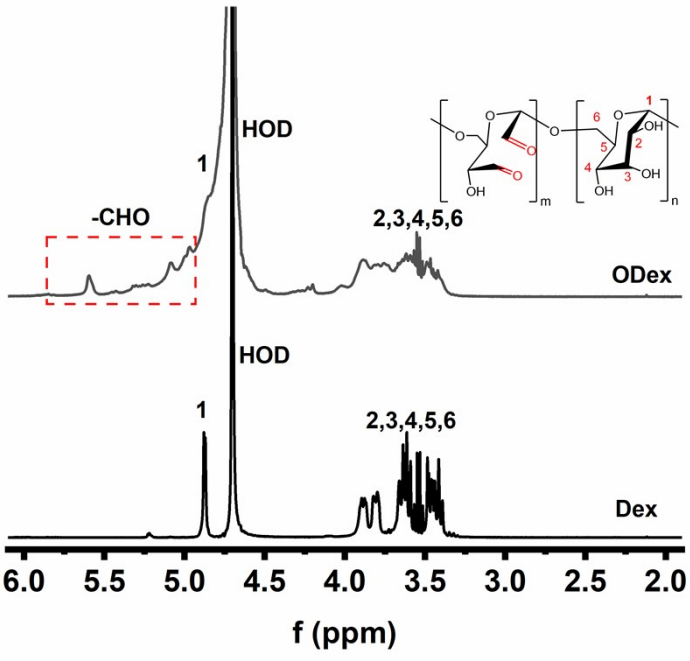

Figure S4 ^{1}H NMR spectra of ODex and Dex.

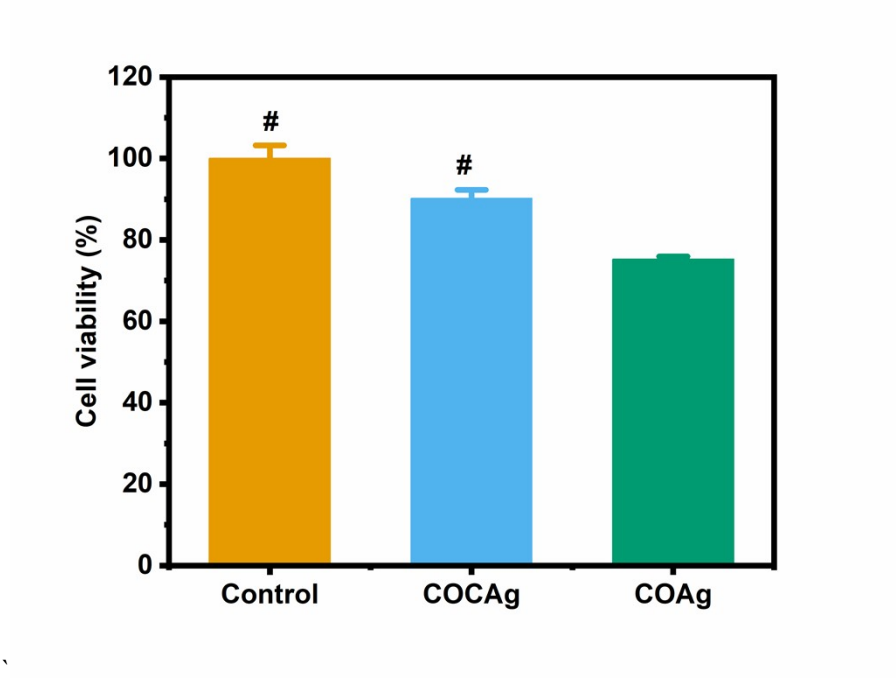

Figure S5 The cell viability of COCAg and COAg hydrogels. ($^{\#}p < 0.05$ versus COAg)

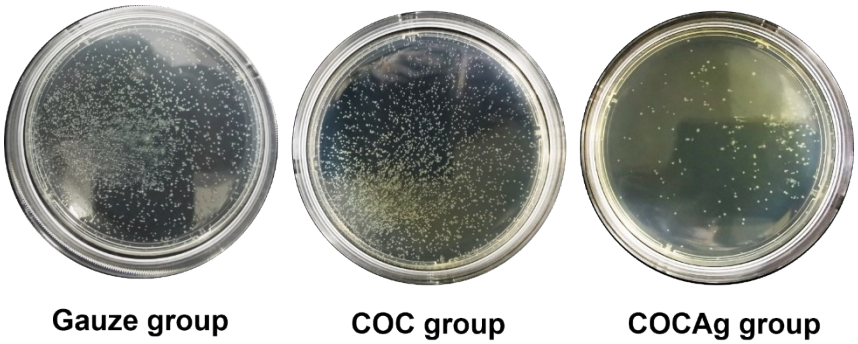

Figure S6 The digital photos of bacterial colonies collected from the wounds of different groups at day 7 incubated on an LB agar plate at 37 ℃ for 12 h.

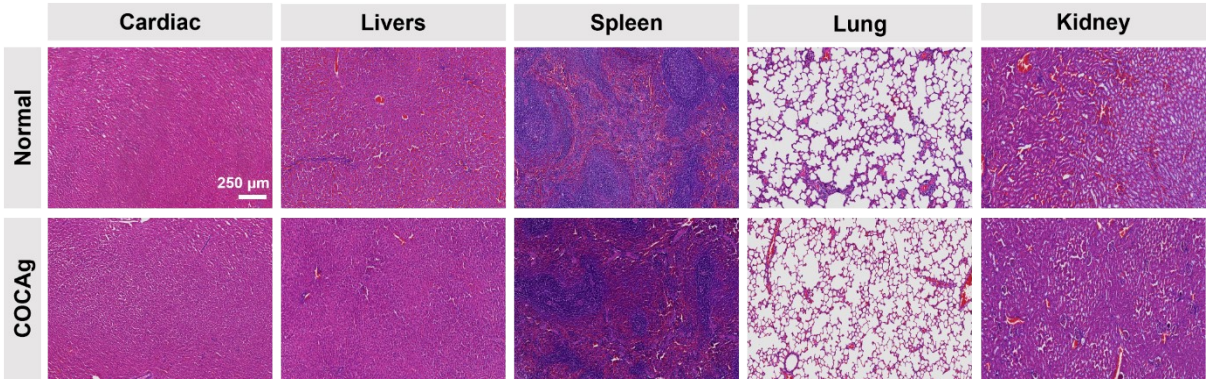

Figure S7 H&E stained histological images of cardiac, liver, spleen, lung, and kidney from different groups after 14days. All scale bars: 250 μm**.**

References

1. J. Cho, J. Grant, M. Piquette-Miller and C. Allen, *Biomacromolecules*, 2006, **7**, 2845-2855.
2. J. Yang, Y. Li, Y. Liu, D. Li, L. Zhang, Q. Wang, Y. Xiao and X. Zhang, *Acta Biomater*, 2019, **91**, 159-172.
3. F. Jiang, Z. Tang, Y. Zhang, Y. Ju, H. Gao, N. Sun, F. Liu, P. Gu and W. Zhang, *Biomater Sci*, 2019, **7**, 2335-2347.
4. X. Zhang, Y. Yang, J. Yao, Z. Shao and X. Chen, *Acs Sustain Chem Eng*, 2014, **2**, 1318-1324.
5. J. Maia, L. Ferreira, R. Carvalho, M. A. Ramos and M. H. Gil, *Polymer*, 2005, **46**, 9604-9614.
6. Z. Bao, A. Yu, H. Shi, Y. Hu, B. Jin, D. Lin, M. Dai, L. Lei, X. Li and Y. Wang, *Int J Biol Macromol*, 2021, **167**, 659-666.
7. S. Li, L. Wang, W. Zheng, G. Yang and X. Jiang, *Adv Funct Mater*, 2020, **30**, 2002370.
8. C. Hu, L. Long, J. Cao, S. Zhang and Y. Wang, *Chemical Engineering Journal*, 2021, **411**, 128564.
9. Y. Wu, Y. Wang, L. Long, C. Hu, Q. Kong and Y. Wang, *J Control Release*, 2021, **341**, 147-165.
10. C. Hu, F. Zhang, L. Long, Q. Kong, R. Luo and Y. Wang, *J Control Release*, 2020, **324**, 204-217.