Supplementary file

Facile and green approach towards biomass-derived hydrogel powders with hierarchical micro-nanostructures for ultrafast hemostasis

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1 Experimental Section

1.1 Characterization of PCSCPs

1.1.1 Morphology

Scanning electron microscopy (SEM) was used to observe the internal microstructure of PCSCPs (JMS-7500F SEM JEOL, Japan) at an acceleration voltage of 20 kV. The surface element content was analyzed by energy dispersive spectroscopy for elemental mapping (EDS mapping) (JMS-7500F SEM JEOL, Japan).

1.1.2 Chemical analysis

The chemical structure of PCSCPs was characterized by Fourier transform infrared spectroscopy (FTIR, Nicolet 560, USA) with the wavelength range of 600-2000 cm^{-1}. Thermal gravimetric analysis (TGA) of PCSCPs was conducted on a thermos gravimetric analyzer (METTLLE TOLEDO, TGA/DSC 3+, Switzerland) from 25 to 800 °C with a heating rate of 10 °C/min in the nitrogen flow. Surface elements of the PCSCPs were analyzed by an X-ray photoelectron spectroscopy (XPS) analyzer (Kratos Analytical, UK). X-ray diffraction (XRD) was characterized at the diffraction angle (2θ) ranging from 2° to 80° (Rigaku Ultima IV).

1.2 Water absorption ability tests

1.2.1 Water absorption ratio

The water absorption ability of PCSCPs was determined using normal saline (NS, 0.9% NaCl), phosphate-buffered saline (PBS), and rabbits' whole blood and performed as previously reported.[1] First, pre-weighted freeze-dried samples were immersed in different solutions at 37 °C. At time intervals, extra solutions were removed by

centrifugation at 700 rpm and residual wet PCSCPs were weighed. The weights of dry samples and wet samples were recorded as W_{dry} and W_{wet}, respectively. The water absorption ratio of PCSCPs was determined using the following formula:

$$Water\ absorption\ ratio\ (\%) = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100\%$$

Six parallel measurements were conducted and the results were presented as mean ± standard deviation (SD).

1.2.2 Water absorption rate

The water absorption rate was measured by a simple device with slight adjustment as previously reported.[2, 3] The device included a conical funnel, a rubber tube and a graduated pipette as shown in **Fig. S11**. During measurement, water was added to the funnel until the liquid level in the graduated pipette was stable, and the liquid level position was recorded as V_0. Then previously weighted dry PCSCPs were soaked in the conical funnel and the liquid level in the graduated pipette began to move. Then the time from the beginning of the level movement to the stability of the level was recorded as t and the final level position was recorded as V_1. The inside diameter of the conical funnel was measured as d. The fluid absorption rate was calculated by the following equation:

$$Fluid\ absorption\ rate\ (mL/(s \cdot cm^2)) = \frac{4(V_0 - V_1)}{t\pi d^2}$$

Six parallel measurements were conducted and the results were presented as mean ± standard deviation (SD).

1.3 Porosity, density and water contact angles

The porosity of PCSCPs was investigated using a liquid displacement method with ethanol as the displacement liquid.[4] Preweighted dry PCSCPs were immersed into a glass bottle which filled with absolute ethanol. Then the bottle was vibrated by ultrasonic machine for 15 min and replenished with absolute ethanol. Finally, PCSCPs soaked with ethanol were collected by discarding the ethanol and wiped by filter paper to suck the remaining ethanol. The weight of dried PCSCPs was recorded as M_S. The weight of a glass bottle only filled with absolute ethanol was recorded as M_1. The weight of glass bottle filled with PCSCPs and ethanol was recorded as M_2. The weight of wet PCSCPs which absorbed with ethanol was recorded as M_3. The volume of PCSCPs was $Vs = (M_1 - M_2 + M_3 - M_S)/\rho^e$, where the ρ^e was the density of ethanol. The density (ρ) and porosity (P) of PCSCPs were calculated by the following two equations:

$$\rho = \frac{M_S}{(M_1 - M_2 + M_3 - M_S)/\rho^e}$$

$$P = \frac{(M_3 - M_S)/\rho^e}{(M_1 - M_2 + M_3 - M_S)/\rho^e}$$

The surface water contact angles (WCAs) of PCSCPs were determined using a video capture system with 2 μL of water droplet (SL200KS, Surface-science, China).

1.4 Hemostasis assays

1.4.1 Blood-related component preparation

The preparation of blood-related components was referred to a previous report with a slight adjustment.[5] Citrated whole blood (CWB) was prepared by extracting whole blood from healthy New Zealand White Rabbit heart at a ratio of 9:1 for 3.8% sodium

citrate. Red blood cells (RBCs) were obtained by centrifugation of CWB under 2000 rpm for 15 min and rinsed five times with PBS. After that, 5% RBCs suspension was prepared with 100 μL of RBCs per 2 mL of PBS. Platelet poor plasma (PPP) was obtained as the supernatant by centrifugation of CWB under 4000 rpm for 15 min. Platelet-rich plasma (PRP) was obtained as the supernatant by centrifugation of CWB under 1000 rpm for 10 min.

1.4.2 Whole blood clotting kinetics

Whole blood clotting kinetics of PCSCPs was evaluated following the reported procedure.[6] 100 μL of CWB was immediately added to 10 mg PCSCPs. At time intervals of 1 min, 2 min, 4 min and 6 min, 15 mL of deionized water was added to wash the RBCs that not trapped by the clot and shaken at 30 rpm for 10 min. The absorbance measurement procedure at 540 nm was the same as the BCI measurement described.

1.4.3 RBCs attachment

RBCs attachment was assessed according to a previous report with slight modifications.[4, 7] 200 μL of 2% RBCs suspension was dropped on 20 mg of samples and incubated in a test tube at 37 °C. After 15 min, 800 μL of PBS was added into each tube to wash the uncombined RBCs from the sample surface and the RBCs suspension was collected. 200 μL of the RBCs suspension diluted with 2 mL DI was measured at 540 nm by an UV-vis spectrometer (UV-1750, Shimadzu Co., Ltd, Japan) and recorded as OD_{sample}. 200 μL of 2% RBCs directly diluted with 800 μL PBS and 2 mL DI was

used as reference and the absorbance was recorded as $OD_{reference\ value}$. The percentage of adhered RBCs on the sample surface was quantified by the following equation:

$$Percent\ of\ adhered\ RBCs\ (\%) = \frac{OD_{reference\ value} - OD_{sample}}{OD_{reference\ value}} \times 100\%$$

Six parallel measurements were conducted and the results were presented as mean ± standard deviation (SD).

1.4.4 Coagulation pathway analysis

Activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were tested by an automatic coagulation analyzer (CA-50 semiautomatic blood coagulation analyzer, Sysmex Corporation, Kobe, Japan). Fresh anticoagulation blood from human donors was centrifuged at 4000 rpm for 15 min (37 °C) and supernatant liquor was collected as platelet poor plasma (PPP). Before measurement, PPP was incubated with 10 mg samples for 5 min, respectively. 200 μL PPP was extracted and added into the test tube. APTT, PT and TT values were obtained finally. The blank control group was the untreated PPP and the other control groups included CMC, SA, CMC/SA and Baxin.

1.5 Evaluation of biocompatibility *in vitro*

1.5.1 Hemolysis ratio

Hemolysis ratios of samples were investigated according to a previous report.[8] Briefly, samples were immersed in NS and cultured for 24 h at 37 °C. Then, the samples were collected by centrifugation for hemolysis test. 5 mL of anticoagulant blood was firstly added to 10 mL of NS, and then RBCs were isolated from plasma by centrifuging at 1000 rpm for 15 min. The centrifugation procedure was repeated at least 5 times to

gain 10% RBCs suspension in PBS for further use. 0.2 mL of 10% RBCs suspension and 0.8 mL NS was added to the pretreated samples and incubated at 37 °C for 3 h. 0.2 mL of 10% RBCs suspension treated with 0.8 mL NS and 0.8 mL deionized water were used as negative control and positive control, respectively. Then the suspension was centrifuged at 8000 rpm for 3 min and the absorbance of the released hemoglobin in the suspension was measured at 540 nm. The hemolysis percentage of PCSCPs was calculated by the following equation:

$$Hemolysis\ ratio = \frac{A_s - A_n}{A_p - A_n} \times 100\%$$

where A_s is the absorbance of the supernatant isolated from samples test group, A_P and A_n are the absorbance of deionized water and NS treated control, respectively.

1.5.2 Cytotoxicity *in vitro*

1.5.2.1 Cell culture

L929 fibroblast cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in an incubator (5% CO_2, 37 °C). When reaching the logarithmic phase, L929 cells were collected and centrifuged at 1000 g for 5 min. The cytocompatibility of PCSCPs was tested by CCK-8 assays, F-actin and nuclear staining, and live/dead assays. Prior to culture with L929 cells, PCSCPs were sterilized. In brief, PCSCPs were placed in a 1.5 mL ep tubes, sterilized with 75% alcohol for 24 h and washed with PBS. At time intervals, PBS was taken away to remove residual alcohol. Then, pretreated samples were collected by centrifugation and transferred to 96-well TCPS.

1.5.2.2 CCK-8 assays

100 μL of L929 cells (1000 cells per well) were added into each well and the plate was incubated at 37 °C under 5% CO_2 atmosphere. The medium was changed every day. At 24, 48 and 96 h of culture, 100 μL of CCK-8 (final dilution=1:10), which could react with dehydrogenase in mitochondria to form a water soluble formazan, was added to the sample well. After incubating at 37 °C for 0.5-2 h, the absorbance was read at 450 nm. Relative cell viability was calculated as the following:

$$Cell\ viability = \frac{OD_{sample}}{OD_{control}} \times 100\%$$

where OD_{sample} is the absorbance of the supernatant isolated from sample groups and $OD_{control}$ is the absorbance of the supernatant isolated from control groups where L929 cells were cultured with normal DMEM.

1.5.2.3 Live/Dead staining assays

L929 cells (10000 cells per well) were seeded in 24-well TCPS and cultured with normal DMEM in a 5% CO_2 atmosphere under 37 °C. After cell culture for 4 h, pretreated samples were added in each well to co-culture with cells for 48 h. L929 cells were stained by Live/Dead Staining Kit (Solarbio life sciences) and observed with a fluorescent microscope (LEICA DMi8).

1.5.2.4 F-actin and Nuclear staining of living cells

L929 cells (10000 cells per well) were seeded in 24-well TCPS and cultured with normal DMEM in a 5% CO_2 atmosphere under 37 °C. After cell culture for 4 h, pretreated samples were added in each well to co-culture with cells for 48 h. Then L929 cells were stained with F-actin and DAPI for cell cytoskeleton staining. Cells without

treatment were used as the negative control. Finally, cells were observed with a fluorescent microscope.

1.5.3 Antibacterial performance

The antibacterial property of PCSCPs for both *E. coli* (ATCC 8739) and *S. aureus* (ATCC 29213) was evaluated according to a previous report.[9] Luria−Bertani agar (LB agar) and Luria−Bertani broth (LB broth) were used as culture media. Before measurement, all samples were sterilized for 2 h under UV radiation. Briefly, preweighted samples were incubated with the LB broth suspension of *E. coli* and *S. aureus* 1×10^4 CFU/mL at 37 ℃ for 18 h. After incubation, OD values of the solutions were measured at 600 nm. The OD values of the bacterial suspension without samples were chosen as control. The antibacterial ratio was calculated following the following equation:

$$Antibacterial\ ratio\ (\%) = \frac{OD_{control} - OD_{samples}}{OD_{control}} \times 100\%$$

Different amounts of samples were added until the inhibition of bacterial growth reached 90% and the weight was chosen for bacteria counting experiment.

In bacterial counting assay, weighted samples were incubated with 4 mL of PBS suspension of bacteria (1×10^4 CFU/mL) for 4 h. 100 μL of the bacterial suspension was spread on the surface of LB agar and incubated at 37 ℃ for 24 h. The bacterial solutions without any treatment were used as control. Bacterial colonies were counted to represent survival bacteria and recorded as N_s for the sample groups and N_c for the control group. The antibacterial ratio was obtained according to the following formula:

$$Antibacterial\ ratio\ (\%) = \frac{N_c - N_s}{N_c} \times 100\%$$

Three parallel measurements were conducted and the results were presented as mean ± standard deviation (SD).

1.6 Hemostatic assays *in vivo*

1.6.1 Rodent bleeding models-animal hemostasis for rats

For rat-tail amputation, a third of the tail (10 cm) was cut by surgical scissors and exposed in air for 15 s to allow an average blood loss. Afterward, the wound was covered with PCSCPs. For rat-liver injury, the chest of the rat was opened, and a cut of 1.0 cm × 0.5 cm (length and depth) was created on the liver. Free bleeding was allowed for 20 s, and the bleeding site was covered with PCSCPs. Once hemostasis was achieved, the hemostatic time and blood loss were recorded immediately. Bleeding sites treated with medical gauze and Baxin were served as the negative control and the positive control, respectively.

1.6.2 H&E staining of the liver tissues

The liver tissues in the rat-liver bleeding model were collected and fixed with 4% paraformaldehyde. After embedding in paraffin, fixed liver tissues were cut into sections and heated in a vacuum oven at 60 °C for 20 min. Then, samples were deparaffinized and hydrated. The sections were stained with hematoxylin and eosin (H&E), observed and photographed using an inverted microscope.

1.6.3 Rodent bleeding models-animal hemostasis for rabbits

All the rabbits were fixed onto a wooden corkboard for operation and were anesthetized with 1.5% sodium pentobarbital by intravenous injection into the ear (0.3 mL per 100 g). For rabbit-ear artery bleeding, the rabbit's ear artery was cut off, and

blood flow was immediately observed until samples were placed over the wound site. For rabbit-liver bleeding model, the rabbit underwent an abdominal incision to expose the liver, and then a liver defect with 2.0 cm × 0.5 cm (length and depth) was made using surgical scissors. Free bleeding was allowed for 20 s, and then preweighted PCSCPs were immediately applied onto the defect. The subsequent operation followed the method of hemostasis on the rat′s liver. After being observed for 2 h, the rabbits were finally euthanized with an overdose of sodium pentobarbital.

1.7 Hemostatic mechanism

1.7.1 Adherence of RBCs and platelets

PCSCPs were added into 24-well tissue culture polystyrene plates (TCPS) followed by the addition of 200 μL PRP or 5% RBCs. After incubating at 37 °C for 1 h, 1 mL PBS was added into each well to remove no-adhered erythrocytes and the procedure was repeated for three times. Then samples were fixed with freshly prepared 2.5% (v/v, in PBS) glutaraldehyde for 1 h, washed three times with PBS and gradually dehydrated by 30%, 50%, 70%, 80%, 90%, 95%, 100% ethanol with a time interval of 15 min. Finally, they were dried for SEM observation.

1.7.2 Comparison of whole blood clots morphology

The whole blood used in this experiment was fresh blood without any anticoagulants donated by a 24 years-old adult. Whole blood clots treated with PCSCPs and the naturally coagulation clots were collected and transferred to 24-well TCPS. The treatment of clots for SEM observation was the same as the description above.

The whole blood used in this experiment was fresh blood without any anticoagulants donated by a 24 years-old adult. The experiments were approved and performed by West China Hospital, Sichuan University, and all the experiments were performed in compliance with the relevant laws and national guidelines (GB/T 16886.4-2003/ISO 10993-4:2002, General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, Standardization Administration of the People's Republic of China). Informed consent was obtained for any experimentation with human subjects, and all regulations (e.g. IRB) were fulfilled for using human blood.

1.7.3 PF4 expression of platelets

Commercial enzyme-linked immunosorbent assays (ELISA) were carried out to evaluate platelet activation (Human Platelet Factor4 (PF4), Cusabio Biotech, China). The samples were added in 24-well TCPS, and 250 μL of human whole blood was introduced. After being incubated at 37 °C for 1 h, the whole blood was centrifuged for 10 min at 2500 g centrifugal force (2-8 °C) to obtain plasma. 40 μL of the obtained plasma was diluted for 10 times with PF4-Sample Diluent; then 200 μL of the diluted plasma was added into another Antibody Coated Well (provided by the PF4 kit). Finally, the detection was conducted according to the respective instruction manuals. Whole blood was used as control sample. For ELISA tests, At least 3 parallel sample groups were applied to get a reliable value, and the results were expressed as mean ± SD.

1.7.4 Ca^{2+} release from PCSCPs

The release of Ca^{2+} from PCSCPs in PBS solution was evaluated by an atomic absorption spectrometer (AAS, SpectrAA 220FS/220Z). Briefly, 200 mg of PCSCPs were soaked in 25 mL of PBS buffer solution with different pH values under gently shaking at 37 °C. At time interval of 10 s, 20 s, and 30 s, 60 s, 90 s, 120 s, 2 mL of supernatant solution was taken as the test solution to measure the Ca^{2+} concentration.

1.8 Degradability

1.8.1 Degradation *in vivo*

Preweighted freeze-dried PCSCPs were immersed in the PBS (pH = 7.4) containing lysozyme (10 KU/mL) and incubated in a shaker at 37 °C. The mixture was centrifuged to isolate the residue at appropriate intervals. Then, the residue was lyophilized and weighed. The degradability was expressed by weight loss:

$$Weight\ loss = \frac{W_0 - W_t}{W_0} \times 100\%$$

where W_t is dry weight of the residue after degradation, and W_0 is the initial weight of the sample.

1.8.2 Degradation and histocompatibility *in vivo*

The degradation experiment *in vivo* was implemented with a subcutaneous implantation model on the back of SD rats. Briefly, the back area of the SD rat was shaved and cleaned with 75% alcohol before incision. Then, a length of 10 mm and down to the dermis incision was created and PCSCPs were subcutaneously implanted. The incised skin tissue without implantations was served as control. The incision was opened and recorded at different time intervals (3^{th}, 7^{th}, 14^{th}). Finally, tissues contacted with PCSCPs were collected and fixed with 4% paraformaldehyde at room temperature.

Five internal organs of rats including heart, liver, spleen, lung and kidney were also collected and fixed. Following the same steps for H&E staining above, the sections were observed and photographed using an inverted microscope.

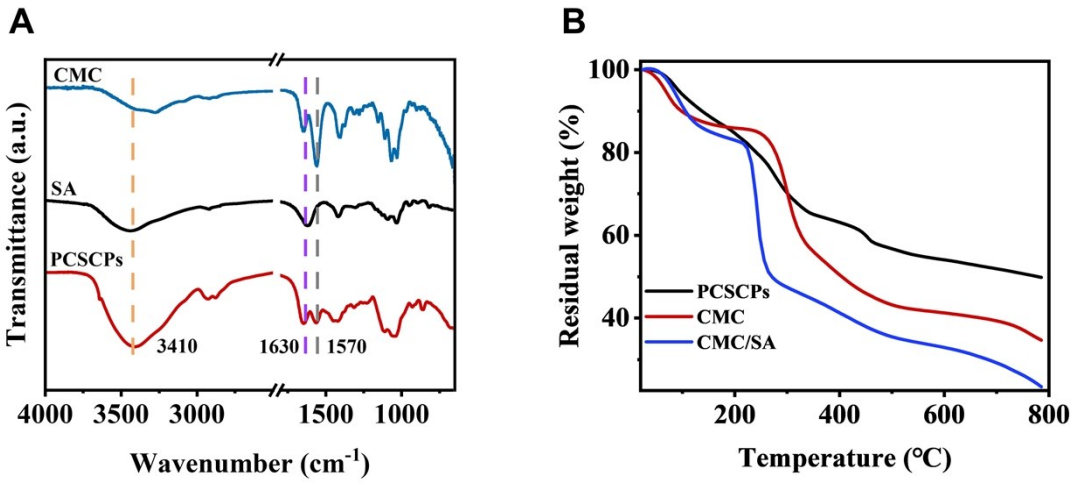


Fig. S1. FTIR (A) and TGA curves (B) of CMC, CMC/SA and PCSCPs**.**

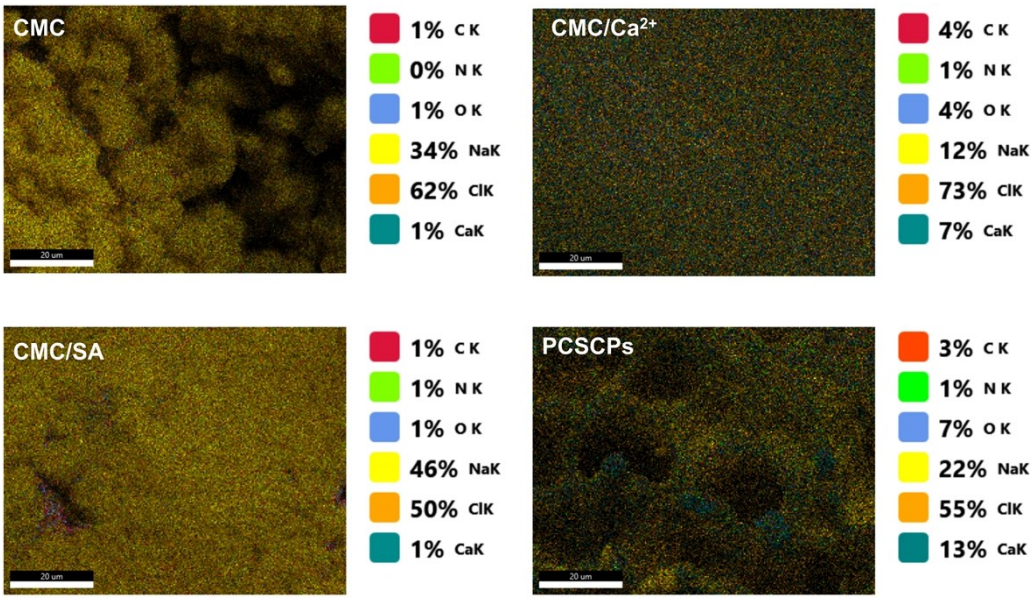


Fig. S2. EDS mapping of CMC, CMC/Ca^{2+}, CMC/SA and PCSCPs.

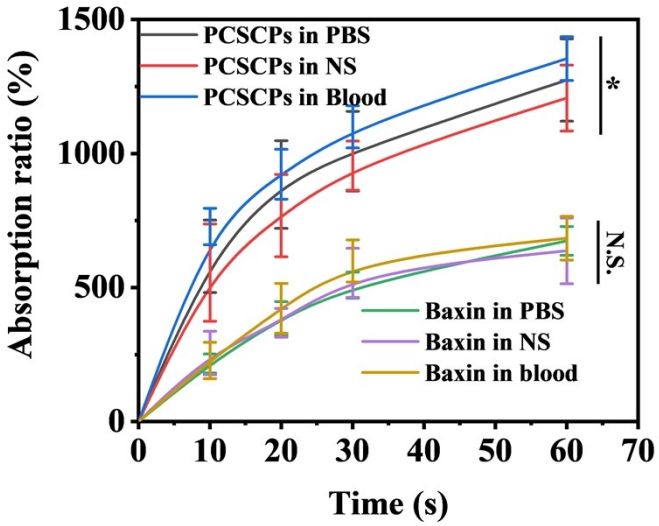
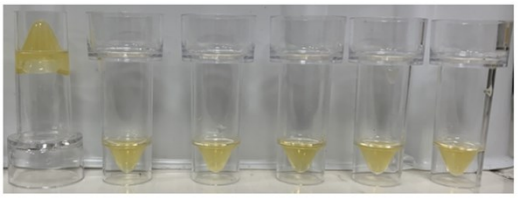


Fig. S3. The water absorption ratio of PCSCPs and Baxin in one minute in three different liquids.

A

Sample	APTT(s)	PT(s)	TT(s)
Control	40±1.3	10.2±0.3	25.1±1.1
CMC	40.4±0.9	10.2±0.1	24.4±0.9
SA	38.7±1.1	10.5±0.4	24.6±0.6
CMC/SA	44.7±0.6	11.7±0.2	27.4±0.8
PCSCPs	-	-	-
Baxin	44.7±0.6	9.6±0.1	31.3±0.5

B



C

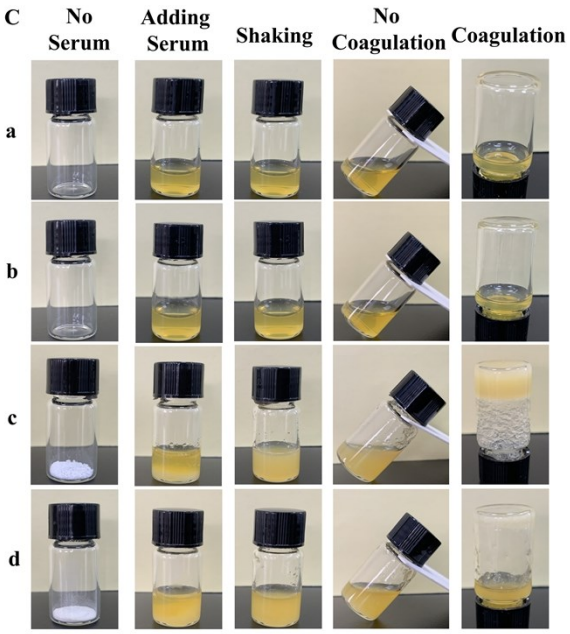


Fig. S4. A) APTT, PT and TT time of different samples. CMC, SA, CMC/SA and Baxin had no significant influence on APTT, PT and TT. B) Images of the status of PPP treated with different samples during the plasma gelation time measurement. The plasma coagulation time of PCSCPs could not be determined because the PPP had already coagulated before analyzing due to the great procoagulant activity. C) Images of PPP coagulation treated with different samples including the negative control (a), treated with $CaCl_2$ (b), treated with PCSCPs (c) and Baxin (d). The PPP treated with PCSCPs coagulated rapidly to form a yellowish gel, while other groups remained flowable.

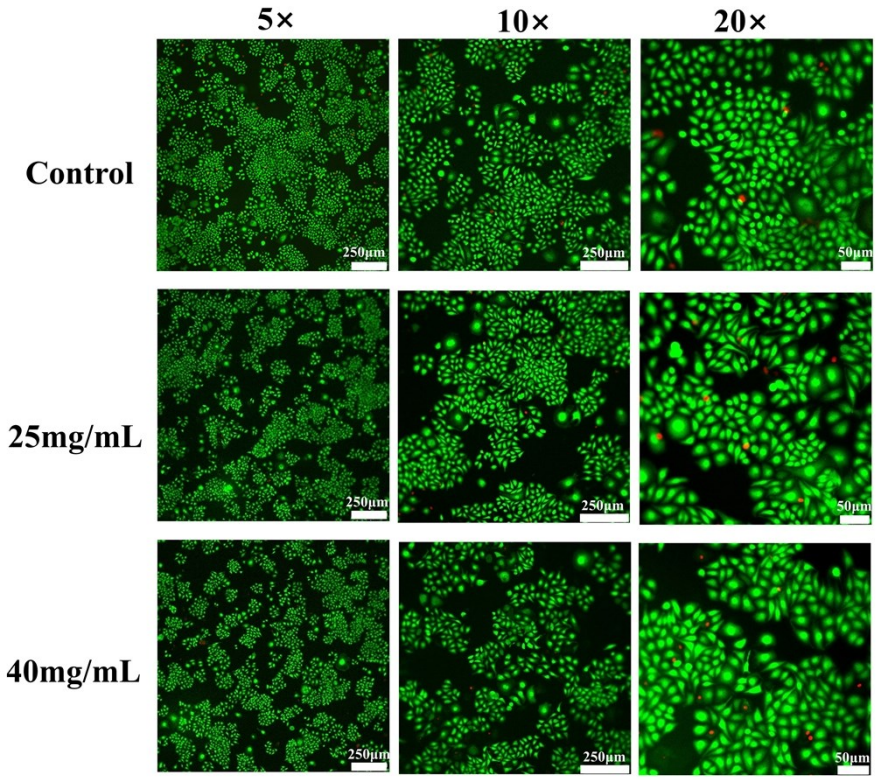


Fig. S5. The live/dead assay of L929 cells treated with different concentration of PCSCPs.

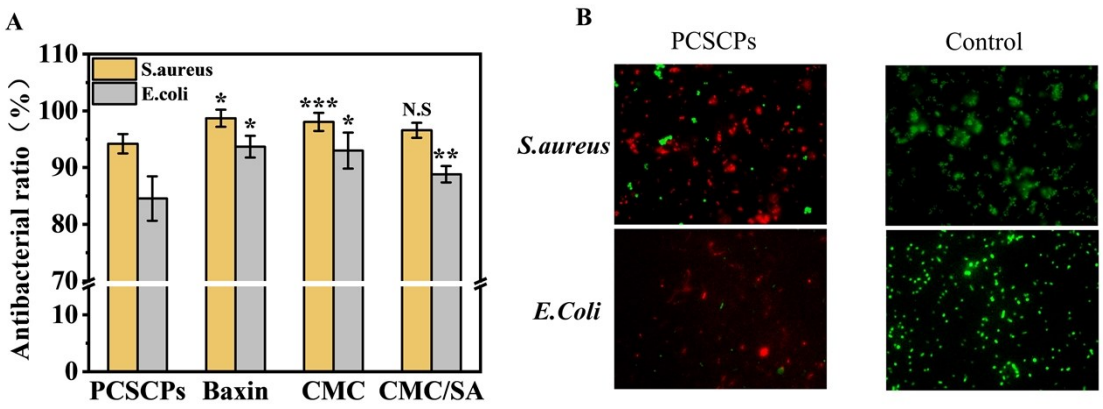


Fig. S6. A) The antibacterial ratios of different samples calculated by OD values (OD 600 nm). B) The corresponding bacterial fluorescence of *S.aureus* and *E.coli* after co-culturing with PCSCPs for 18 h.

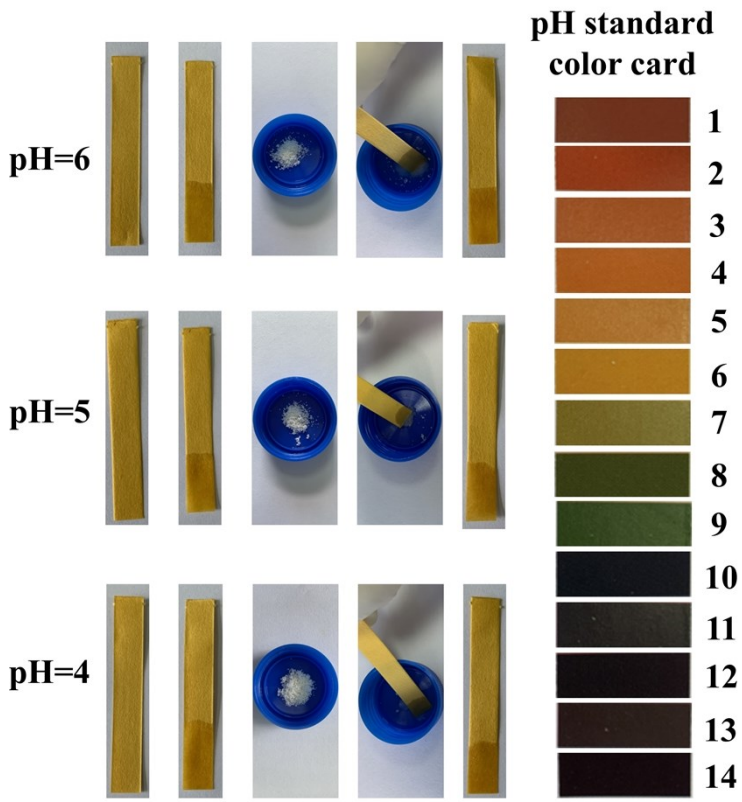


Fig. S7. Images of pH variation of PCSCPs contacting with surroundings by pH test paper.

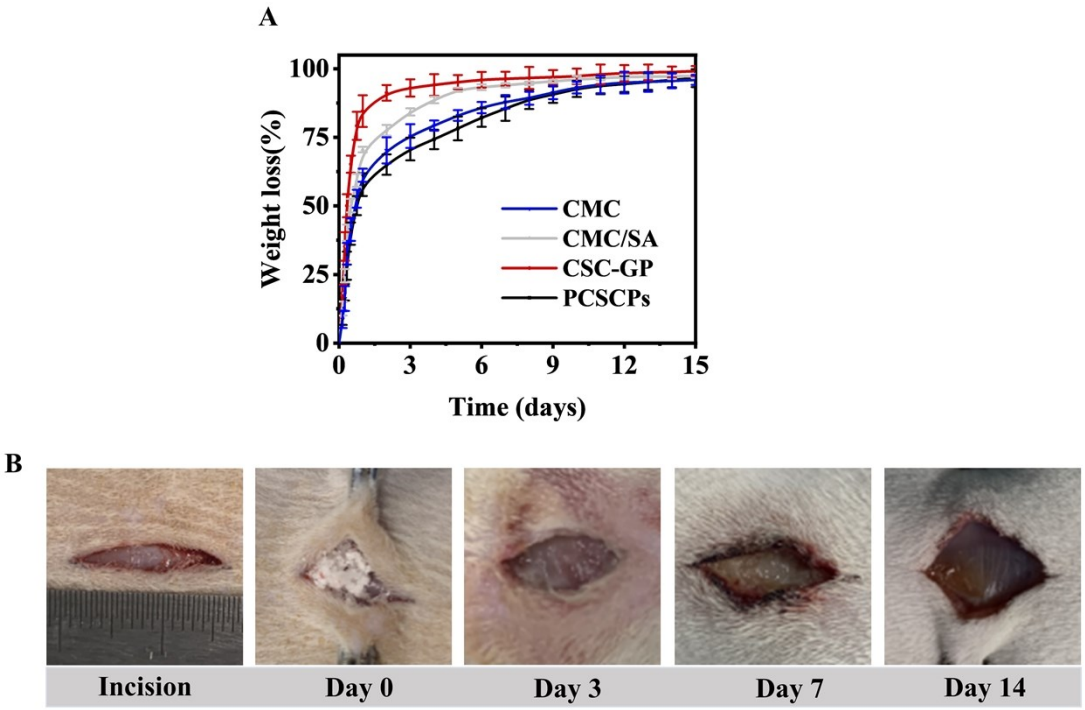


Fig. S8. A) The degradability of different samples *in vitro* by enzyme degradation. The weight loss ratio of PCSCPs reached 75% after 5 days , which proved an efficient degradation process of PCSCPs in vitro. CSC-GP showed a faster weight loss tendency, demonstrating that the ECH crosslinking was more stable than the ionic crosslinking. (B) Photographs of PCSCPs on the implantation sites on the 3rd, 7th, 14th day.

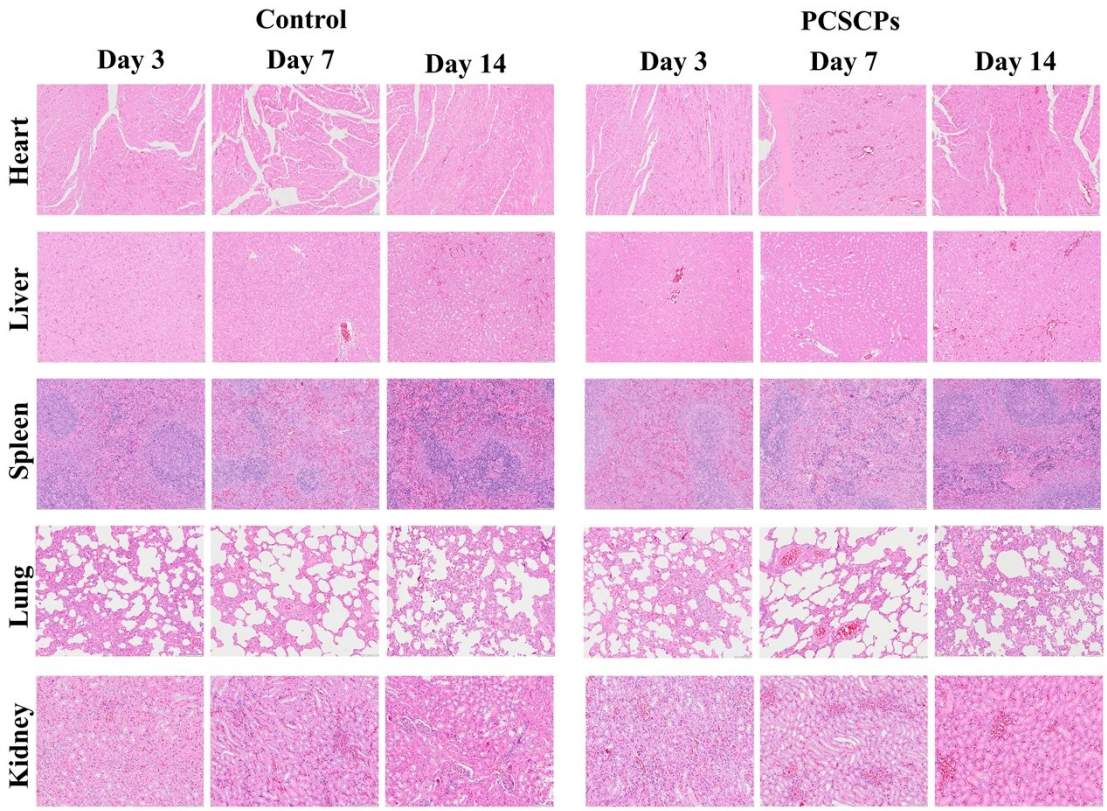


Fig. S9. H&E staining of visceral organs including heart, liver, spleen, lung and kidney of rats on the 3rd, 7th, 14th day.

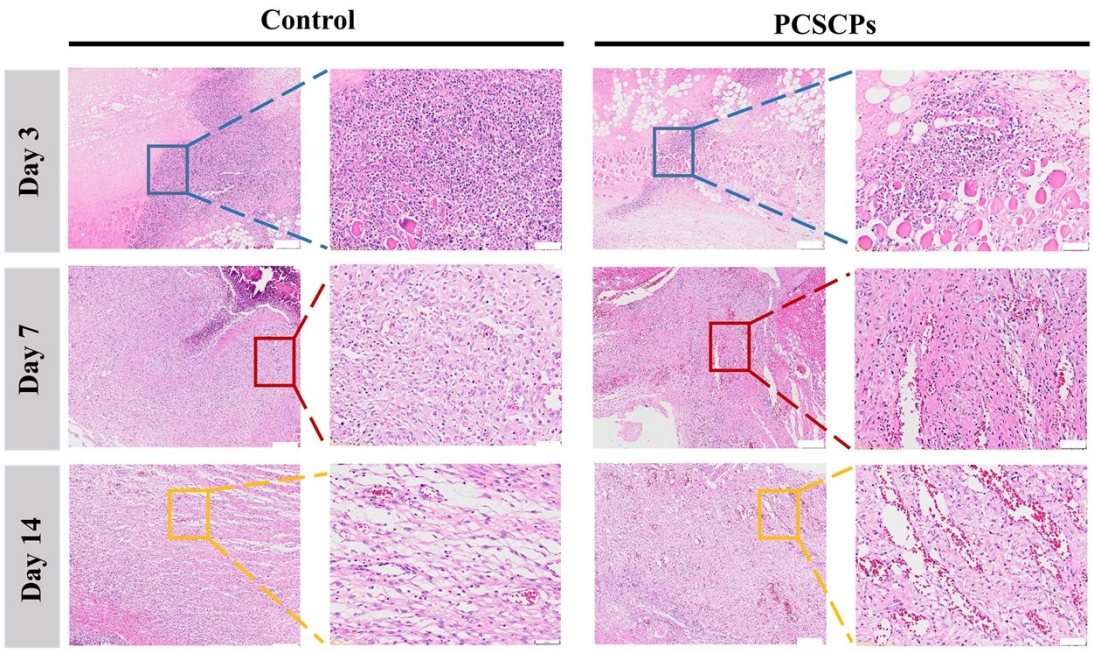


Fig. S10. H&E staining analysis of implantation sites on the 3rd, 7th, 14th day. Less inflammatory cells (circled and amplified by the blue box) were found around the implantation sites of PCSCPs group contrary with the control group on the 3rd day. The mild acute inflammation responses occurred in both groups in a normal wound condition. A slighter inflammation of PCSCPs was possibly due to their reasonable antibacterial property. With tissue repair and regeneration, the inflammation was reduced in both groups and the early angiogenesis (circled and amplified by the red box) was observed in PCSCPs group on the 7th day. Amounts of newly formed blood vessels (circled and amplified by the orange box) were occurred in PCSCPs group on the 14th day and indicated an excellent tissue repair and regeneration process.

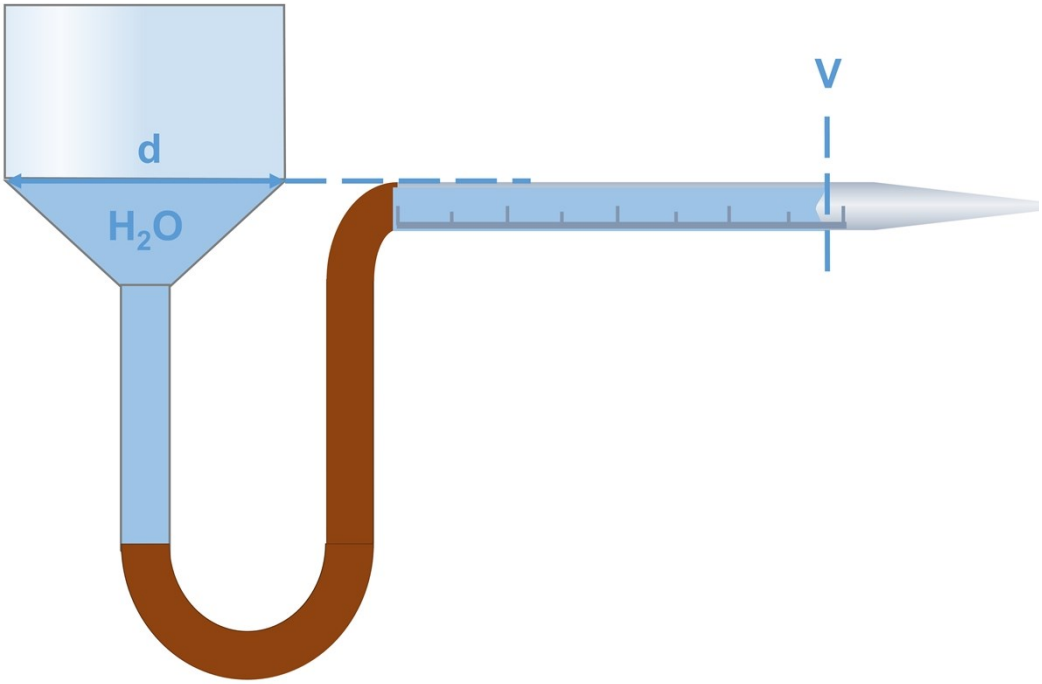


Fig. S11. Schematic diagram of water absorption rate testing device including a Conical funnel, a rubber tube and a graduated pipette

References

1. X. Yang, W. Liu, G. Xi, M. Wang, B. Liang, Y. Shi, Y. Feng, X. Ren and C. Shi, *Carbohydr Polym*, 2019, **222**, 115012.
2. G. H. Xi, W. Liu, M. Chen, Q. Li, X. Hao, M. S. Wang, X. Yang, Y. K. Feng, H. C. He, C. C. Shi and W. Z. Li, *Acs Appl Mater Inter*, 2019, **11**, 46558-46571.
3. D. Markl and J. A. Zeitler, *Pharm Res-Dordr*, 2017, **34**, 890-917.
4. J. J. Guan, K. L. Fujimoto, M. S. Sacks and W. R. Wagner, *Biomaterials*, 2005, **26**, 3961-3971.
5. C. Wang, H. Niu, X. Ma, H. Hong, Y. Yuan and C. Liu, *ACS Appl Mater Interfaces*, 2019, **11**, 34595-34608.
6. M. F. Shih, M. D. Shau, M. Y. Chang, S. K. Chiou, J. K. Chang and J. Y. Cherng, *Int J Pharmaceut*, 2006, **327**, 117-125.
7. S. S. Biranje, P. V. Madiwale, K. C. Patankar, R. Chhabra, P. Bangde, P. Dandekar and R. V. Adivarekar, *Carbohyd Polym*, 2020, **239**.
8. Z. Shi, G. Lan, E. Hu, F. Lu, P. Qian, J. Liu, F. Dai and R. Xie, *Carbohydr Polym*, 2020, **232**, 115814.
9. X. Zhao, B. L. Guo, H. Wu, Y. P. Liang and P. X. Ma, *Nat Commun*, 2018, **9**.