**Supporting Information**

# A Well-Designed Two-Fold Crosslinked Biological Valve Leaflets with Heparin-Loaded Hydrogel Coating for Enhancing Anticoagulation, Endothelialization, and Anticalcification

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# 1. Experimental section

## 1.1. Decellularization of PP

Fresh PP obtained from the Hongye slaughterhouse factory (Chengdu) was used as the basis material. The decellularization of PP was carried out according to the method described by Tedder et al with a little modification [1]. In brief, fresh PP was incubated with 0.1 wt% tyrisin and 0.02 wt% EDTA and gently, continuously oscillated for an hour at 37°C, rinsed thoroughly, and then transferred to the PBS solution containing 1.0 wt% Triton X-100 under the same conditions. Next, the treated PP was placed into the mixed solution of 0.02 mg/ml RNaseA enzyme and 0.2 mg/ml DNaseI enzyme after 24 hours, continually shaking for four hours. Finally, the decellularized PP (D-PP) was harvested after cleaning with sterile PBS solution three times.

### 1.2. Characterization of D-PP

To validate the cell nuclei removal and microstructure integrity of D-PP, Hematoxylin, and Eosin (H&E) and Masson staining was carried out. And, to quantify the DNA content of D-PP, the Quant-iT PicoGreen dsDNA assay kit (Invitrogen) was carried on, following the manufacturer's instructions. To determine the sulfated GAG remaining in the D-PP by the way of colorimetric method, which was served as an indicator of the alteration in the structural composition of decellularized tissues [2].

### 1.3. Hemocompatibility Assessment

*2.10.1 Hemolysis.* 5% red blood cells (RBCs) suspension was first obtained from fresh whole rabbit blood centrifuged at 1500 rpm for 15 min and diluted with PBS buffer. Then, PP samples were incubated at 37°C for two hours in the above suspension. After centrifugation at 3000 rpm for 5 min, a 100 μL supernatant was transferred to a 96-well plate and read the OD value at 540 nm by a microplate reader. $H_2O$ and PBS buffer were set as positive and negative control samples within the same environments, respectively. The hemolysis rate ($R_H$) of samples was computed using the following Eq. (4):

$$R_{\mathrm{H}} = \frac{A_{\mathrm{sample}} - A_{\mathrm{negative}}}{A_{\mathrm{positive}} - A_{\mathrm{negative}}} \times 100\% \quad (1)$$

*2.10.2 Static platelet adhesion.* Platelet-rich plasma (PRP) was first collected from supernatant obtained after centrifuging fresh whole blood at 1500 rpm for 20 min. PP samples were immersed into the PRP and incubated for an hour in the 37°C oscillators. Then, the samples were softly washed with PBS three times and fixed with 2.5% glutaraldehyde. SEM was surveyed to characterize the morphology of PP samples treated by the critical point drying method. Moreover, platelets adhesion to the surface of PP samples was detected by the lactate dehydrogenase (LDH) test as instructed. Moreover, platelet activation counts on the surface of PP samples were studied by measuring β-thromboglobulin (β-TG) expression levels of platelets with a rabbit β-TG ELISA (USCN Life Science Inc., Wuhan, China) according to instructions. Six parallel samples per group were collected.

*2.10.3 Whole blood clotting assay:* Whole blood clotting time (WBCT) of PP samples was measured as formerly described [3, 4]. Coagulation of 5 mL fresh whole blood occurred by adding 500 µL, 0.1 M $CaCl_2$. Then, 100 µL above activated whole blood was rapidly transferred into each well where PP samples were pre-stored and incubated for 5, 15, 25, and 35 min at room temperature, respectively. Next, 2 mL ultrapure water was added to each well and subsequently incubated for 5 min after reaching the predefined time point. OD value of the hemoglobin released by the broken free RBCs was measured at 540 nm. The reduction in OD value was positively correlated with clotting. Here, five parallel samples were established for each group.

*2.10.4 Plasma recalcification assay:* Plasma recalcification time (PRT) of PP samples was determined in the polystyrene plate wells as previously described [3]. Each sample immersed in a 500 µL PPP was incubated for one hour at 37°C in a 110 rpm thermostatic oscillator. Empty polystyrene plate wells with $CaCl_2$ and without $CaCl_2$ were regulated separately as positive and negative controls. Then 100 µL PPP extracted from each sample well was transferred to a 96-well plate and added 100 µL 0.025 M $CaCl_2$ except for the negative control. The 96-well plate was rapidly placed into the microplate reader and incubated at 37°C. The absorption recorded at 405 nm was measured every 30 s in 45 min to investigate the clotting process's kinetics. Half-maximum absorption time (W50) was an essential indicator of thrombogenicity. Five parallel samples were taken from each group.

*2.10.5 Blood clotting time.* PP samples were incubated with 500 µL PPP under continuous slight shaking at 37°C for one hour. Next, the supernatant was used to be detected by an automatic coagulation analyzer (ACL 200, Beckman-Coulter, Inc., USA). The activated partial thromboplastin time (APTT) and the prothrombin time (PT) were obtained, and six parallel samples from each group were collected.

# 2. Supporting figure

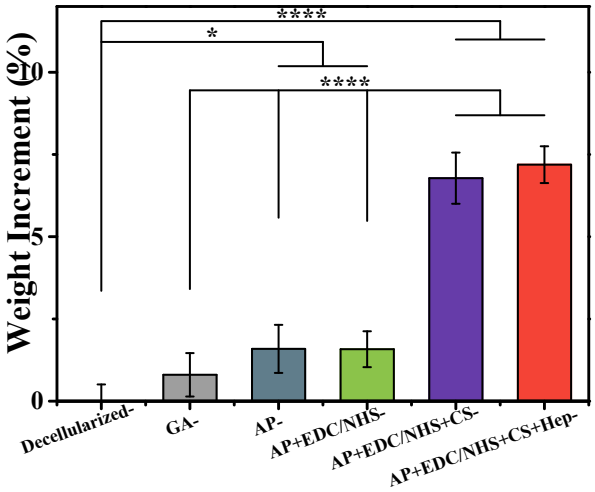


**Figure S1.** Weight increment of modified PP samples, compared with the D-PPs.

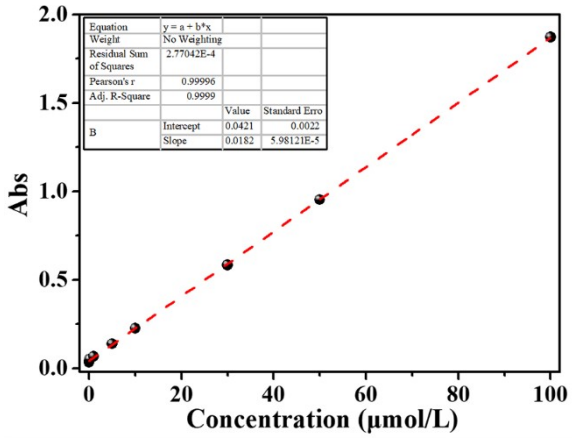


**Figure S2.** The calibration curve of heparin in the PBS buffer.

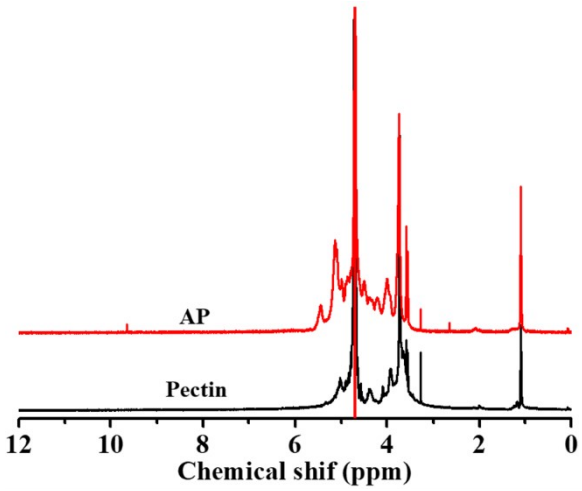


**Figure S3.** $^{1}$H-NMR spectra of pectin and AP in $D_2O$, respectively.

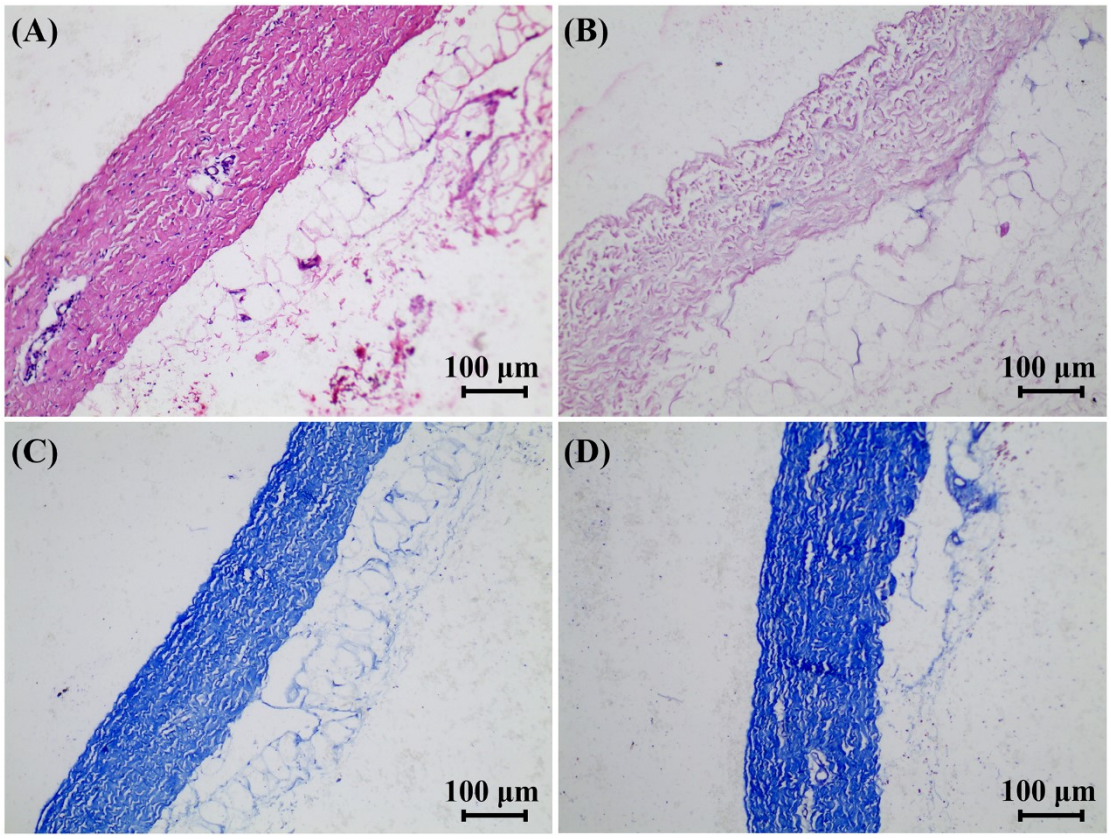


**Figure S4.** H&E staining of (A) PP and (B) D-PP and Masson staining of (C) PP and (D) D-PP, respectively.

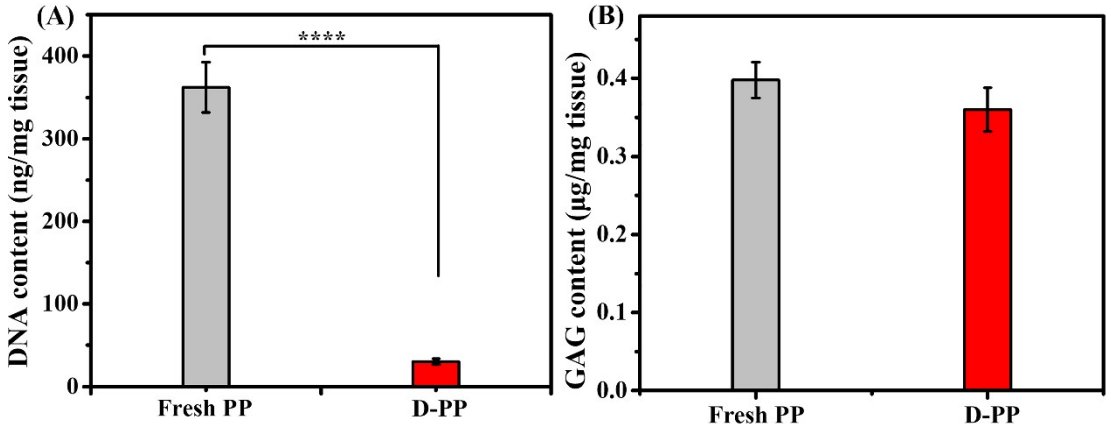


**Figure S5.** Quantitative determination of (A) DNA and (B) GAG in the PP and D-PP.

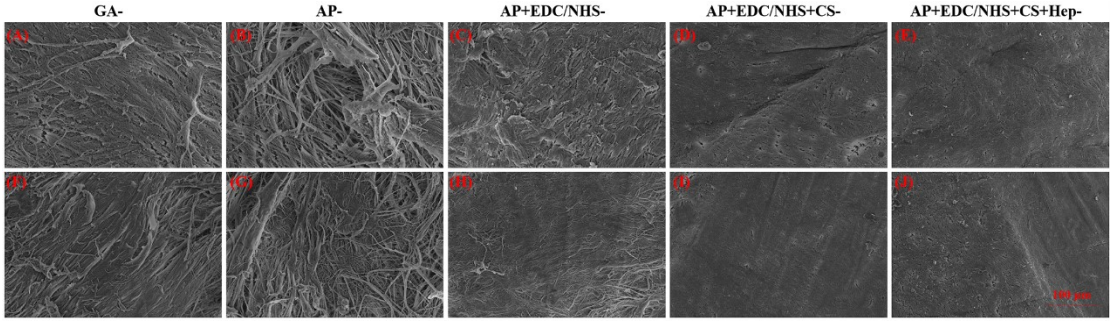


**Figure S6.** The surface morphology of (A-E) the modified PP samples after

collagenase enzymolysis and (F-J) the modified PP samples after elastase enzymolysis.

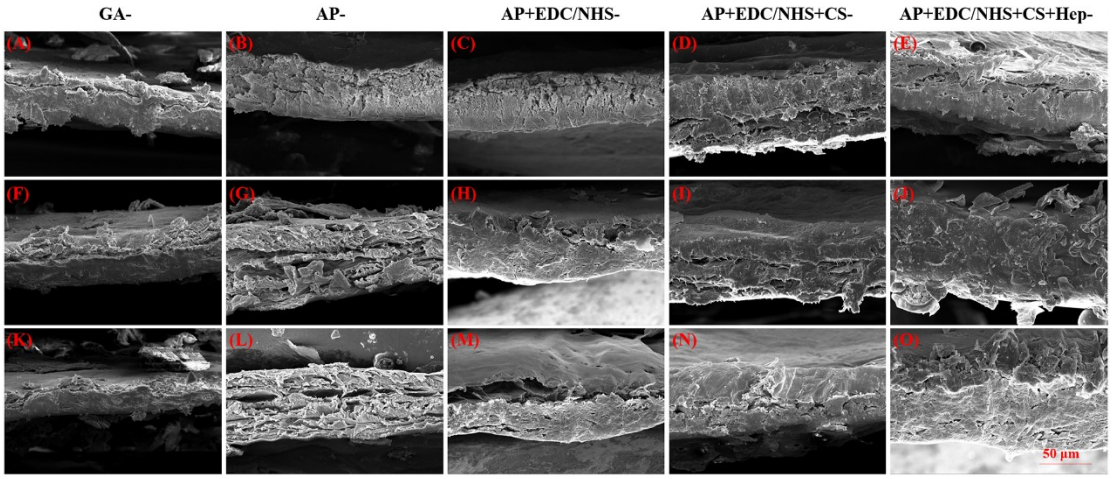


**Figure S7.** The cross-sectional morphology of (A-E) the modified PP samples before the enzymolysis test, (F-J) the modified PP samples after collagenase enzymolysis, and (K-O) the modified PP samples after elastase enzymolysis.

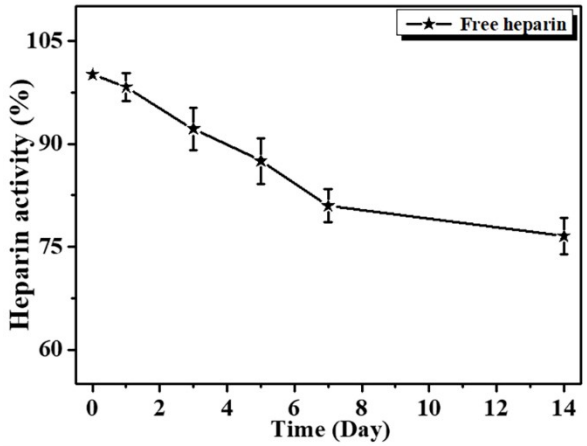


**Figure S8.** The bioactivity of free heparin in the PBS buffer.

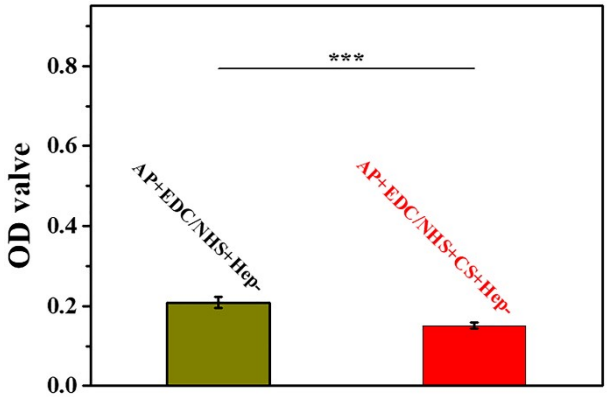


**Figure S9.** LDH quantification of adhered platelet on AP+EDC/NHS+Hep-PP and AP+EDC/NHS+CS+Hep-PP (n=6).

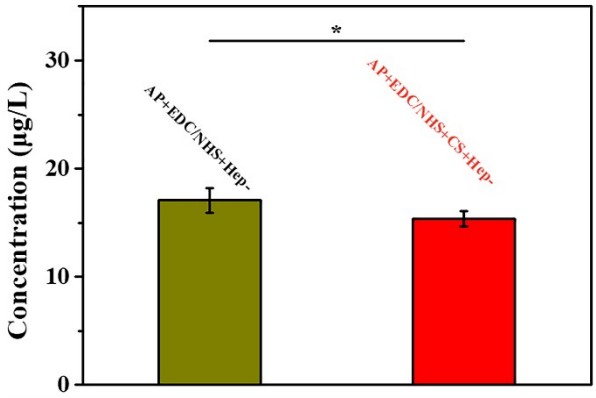


**Figure S10.** β-thromboglobulin (β-TG) levels of adhered platelet on AP+EDC/NHS+Hep-PP and AP+EDC/NHS+CS+Hep-PP (n=6).

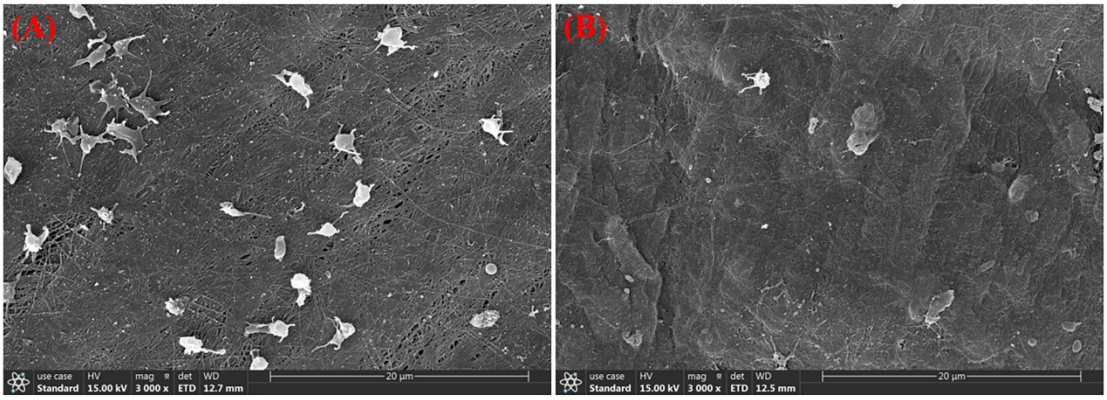


**Figure S11.** SEM images of adhered platelet on the surface of (A) AP+EDC/NHS+Hep-PP and (B) AP+EDC/NHS+CS+Hep-PP (n=6).

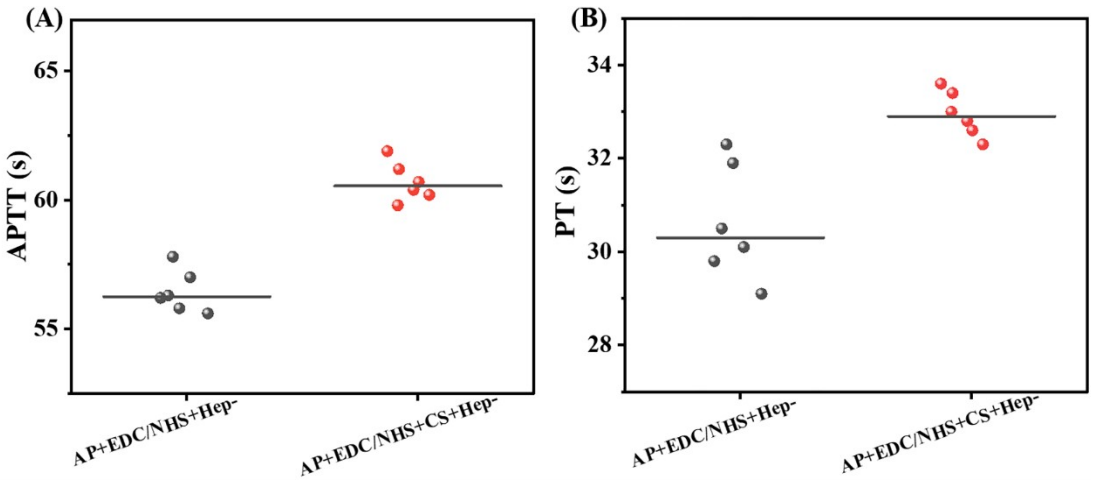


**Figure S12.** (A) APTT and (B) TT tests.

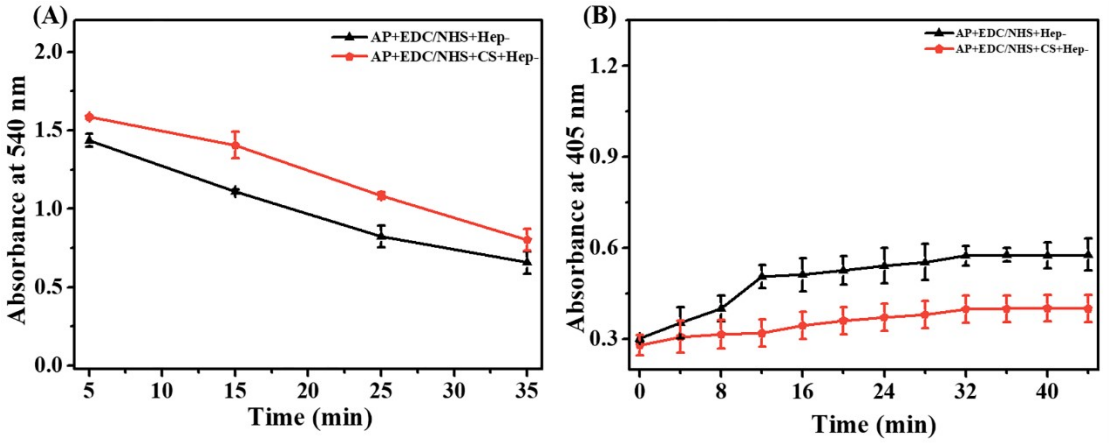


**Figure S13.** (A) Whole blood clotting assay for heparin-loaded PP and (B) plasma recalcification profiles of the PPP in the presence of heparin-loaded PP.

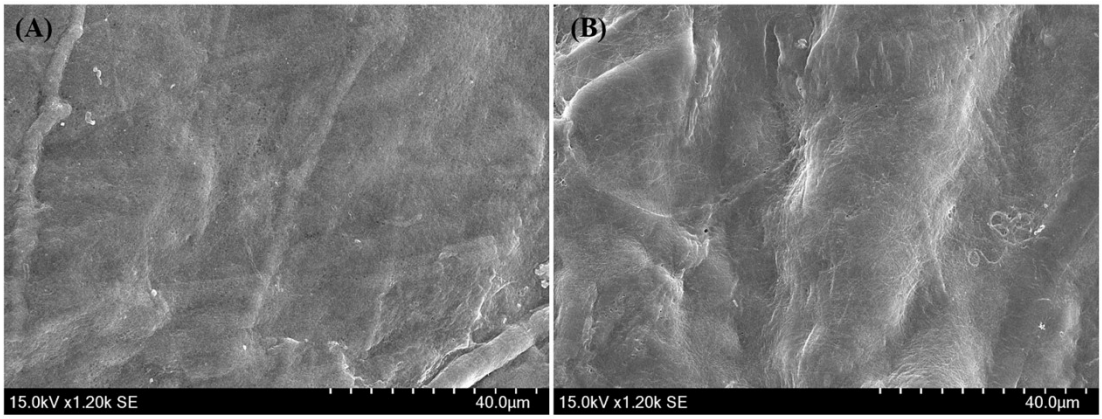


**Figure S14.** SEM images of AP+EDC/NHS+CS+Hep-PP immersed in simulated body fluid for (A) 15 and (B) 30 days. Scale bars = 40 µm.

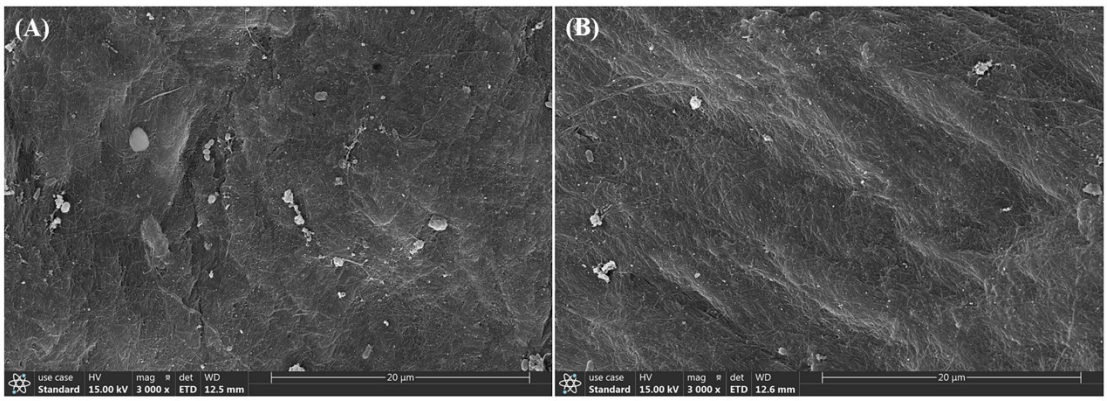


**Figure S15.** SEM images of adhered platelet on the surface of AP+EDC/NHS+CS+Hep-PP immersed in simulated body fluid for (A)15 and (B) 30 days. Scale bars = 20 µm.

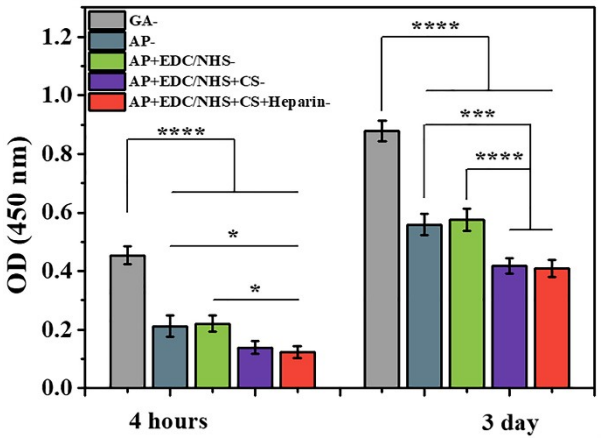


**Figure S16.** Cell viability of Raws 264.7 cells on PP samples at different time.

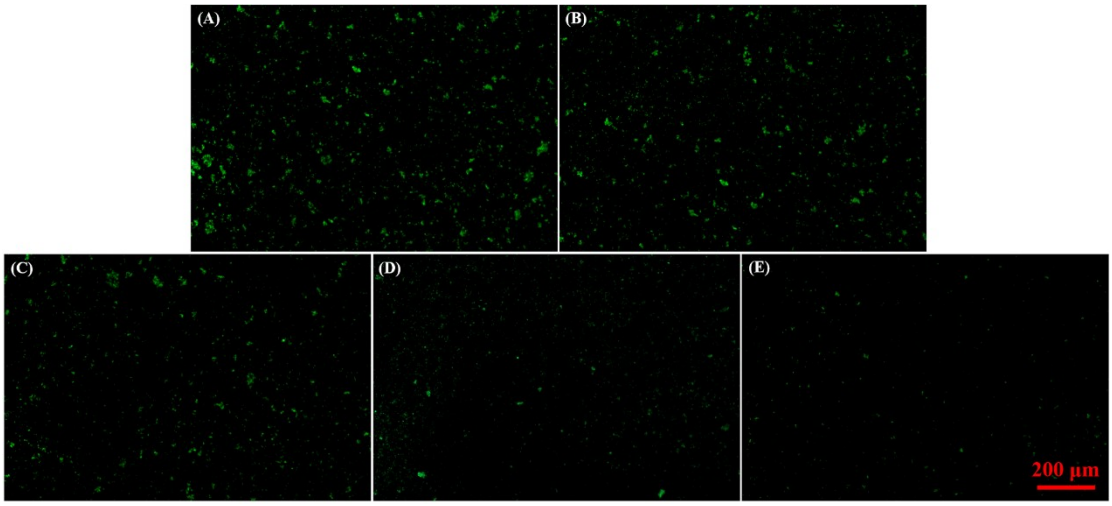


**Figure S17.** FDA staining of RAW264.7 adhered on (A) GA-PP, (B) AP-PP, (C) AP+EDC/NHS-PP, (D) AP+EDC/NHS+CS-PP, and (E) AP+EDC/NHS+CS+Hep-PP after culturing for 3 days.

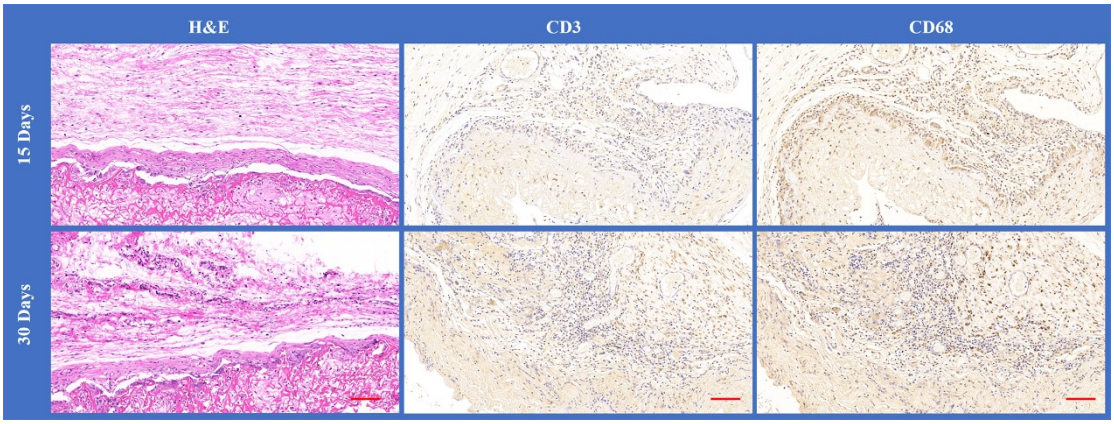


**Figure S18.** H&E staining and Immunohistochemistry staining with CD3 and CD68 markers in sections of AP+EDC/NHS+Hep-PP at the 15 days and 30 days post-implantation into the rats; the scale bar = 100 μm.

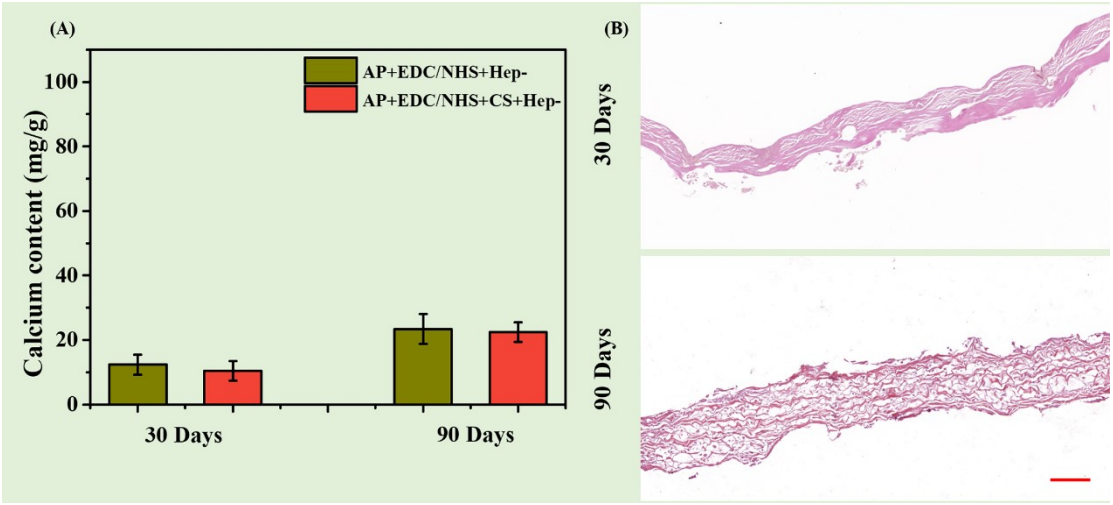


**Figure S19.** (A) Calcium contents of the PP after 30 and 90 days of implantation (n=6); (B) staining of calcium depositions by Alizarin red in the sections of AP+EDC/NHS+Hep-PP and AP+EDC/NHS+CS+Hep-PP after subcutaneous implantation in the rats for 30 days and 90 days (n=6); the scale bar = 100 μm.

# 3. Reference

1. M. E. Tedder, J. Liao, B. Weed, C. Stabler, H. Zhang, A. Simionescu and D. T. Simionescu, *Tissue Engineering Part A*, 2009, **15**, 1257-1268.
2. P. Akhyari, H. Aubin, P. Gwanmesia, M. Barth, S. Hoffmann, J. Huelsmann, K. Preuss and A. Lichtenberg, *Tissue Engineering Part C: Methods*, 2011, **17**, 915-926.
3. D. Motlagh, J. Yang, K. Y. Lui, A. R. Webb and G. A. Ameer, *Biomaterials*, 2006, **27**, 4315-4324.

4. X. Li, J. Tang, L. Bao, L. Chen and F. F. Hong, *Carbohydrate polymers*, 2017, **178**, 394-405.