Supplementary Materials

Histology analysis of explanted bioprosthetic valves

The characteristics of endothelialization coverage and fibroblastic proliferation were further observed by immunofluorescence of α-SMA (Fig S2A), vWF (Fig S2B), and CD31 (Fig S2C). The positive staining of CD31 in swim bladder group was consistent with its results of SEM, indicating that the valve fabricated by swim bladder was covered with endothelium 6 months after implantation. However, the negative staining of CD31 and vWF in bovine pericardium group showed insufficient endothelium migration. In addition, the strong positive staining of α-SMA in both swim bladder and bovine pericardium may be related to fibroblastic proliferation.

Considering the existence of abnormal tissue hyperplasia on the surface of valve fabricated by bovine pericardium, we tested the markers of macrophage by immunofluorescence staining. Figure S3 showed the results of immunofluorescence staining of CD68 (Fig S3A), CD206 (Fig S3B), and CCR7 (Fig S3C). The positive staining of CD68, CD206, and CCR7 hinted high level of immunogenicity of bovine pericardium tissue and subsequent induced macrophage recruitment and infiltration, which may explain the existence of aberrant foreign body response. The negative staining of CD68 and CD206 in swim bladder indicated a lower immune response level compared to bovine pericardium.

Materials and methods

1. Tissue fixation and cross-linking

Fresh swim bladder of Carp (Yangcheng Lake, Jiangsu, China) and bovine pericardium (Shanghai Beef and Mutton Corp. Ltd., Shanghai, China) were stored in icebox, and immediately transported to the laboratory. The attached adipose tissue and connective tissue were removed carefully by surgical scissors. The tissues were rinsed in sterile phosphate buffer (PBS) solution, and then immersed in 0.625% glutaraldehyde (GA) solution for 48 h for fixation and cross-linking. At last, the fixed tissues were stored in 0.2% GA solution after rinsing by PBS solution.

2. Histological analysis

A slicing machine was used to cut specimens into 5-μm-thick paraffin sections. Then, a routine protocol was conducted, including sectioning, dewaxing, and hydrating. Paraffin sections were stained by hematoxylin and eosin solution consecutively for 5 min and 1 min. Victoria blue (VB) staining was used for fiber analysis. In brief, the rehydrated sections were stained with VB solution and Ponceau S Stainning Kit consecutively for 1 h and 1min. Further, scanning electron micrograph (SEM) was performed to observe the endothelialization of valve samples. In brief, samples were fixed in cold 2.5% (v/v) GA before sequential dehydrations. Images were captured by scanning electron microscope (Hitachi SU-8010, Japan) after air-drying and gold sputter coating procedure. Transmission electron micrograph (TEM, Hitachi HT7700, Japan) was performed to observe the fiber morphology of samples.

3. Mechanical property analysis

Human valves open and close 3.2 billion times regularly with the heartbeat in their lifespan. Therefore, excellent mechanical properties were the basic requirements for swim bladder to be acted as a biomaterial for bioprosthetic valve. It was reported that the native valve was anisotropic. Therefore, we studied the mechanical properties of swim bladder and bovine pericardium in two directions by a tensile testing machine. A tensile test platform (Shore western 306, USA) was used for mechanical property analysis. The structures of swim bladder and bovine pericardium were anisotropic. Strips were cut into 2 directions (10mm×7mm), the direction of the fiber bundle was defined as "Circumferential" and the direction perpendicular to the fiber was defined as "Axial". The parameter of test platform was set as 5 mm/min. The ultimate tensile strength, elongation at break, and elastic modulus were calculated from the stress-strain curve.

4. Differential scanning calorimetry (DSC)

In order to evaluate the thermodynamic stability of harvested samples, 5 mg of dry sample was sealed in a DSC (DSC 204F1, Germany) pot, and then heated to 100 ℃ at a rate of 10 ℃/min in a nitrogen gas environment. The denaturation temperature (DT) was calculated from the DSC curve.

5. Cytotoxicity analysis

Mouse L929 cells and MTT assay were used for cytotoxicity analysis. Tissues were immersed in DMEM (6 cm^2 tissue: 1ml DMEM) for 24 hours at 37 ℃. The L929 cells were incubated with the immersing supernatant for another 48 h. 0.5% phenol and polyethylene were used as positive and negative controls, respectively. After each

treatment, the cytocompatibility was assess by MTT kit under the instruction.

6. Hemocompatibility analysis of platelet deposition/activation assay

The experiment was approved by the ethics committee of Changhai Hospital, Second Military Medical University. Healthy volunteers signed informed consent before experiment. Tissues were immersed in human whole blood (6 cm^2 tissue: 1ml blood) for 60 min at 37 ℃. Zymosan A and human blood gamma globulin (0.01g: 1ml blood) were set as positive control and negative control, respectively. CD62p-PE (Immunotech, France) and CD61-FITC (Immunotech, France) were added and incubated in the dark for 1 hour. The reaction was stopped by adding 1 ml of cold paraformaldehyde solution. At last, flow cytometer was used for calculating the ration of activated platelets.

7. Hemolytic test *in vitro*

The fixed tissue were immersed into saline (6cm^2 tissue: 1ml saline) at 37 ℃ for 60 min, distilled water and saline were set as positive control and negative control, respectively. 60 μL diluted human whole blood was added into each tube and incubated at 37 ℃ for 60 min. The supernatant was harvested for measurement of the absorbance at 545 nm after centrifuge (2500 rpm, 5min).

8. Rat subcutaneous implantation model

The animal studies were approved by the ethics committee institution of Second Military Medical University, all experimental protocols complied with NIH Guide. 24 male Sprague Dawley rats were anesthetized by isoflurane inhalation, and 4 subdermal pockets were made at dorsal area of each rat. 2 swim bladder strips (1×1cm) were

inserted into the pockets close to forelimbs, and 2 bovine pericardium strip (1×1cm) were inserted into the pockets close to posterior limbs. At the indicated time points, 8 rats of each group were randomly scarified, and the implanted tissues were taken out for histological analysis.

9. Alizarin red staining and Von Kossa staining

Paraffin sections were stained by Alizarin red solution (Servicebio, Wuhan, China) for 5 min after the consecutive procedures of sectioning, dewaxing, and hydrating. The slices were washed three times with 95% ethanol to remove non-specific staining. Matrix calcification in alizarin red staining was manifested with red deposition. In addition, the hydrated slices were incubated with 1% silver nitrate solution under UV light for 40 min for Von Kossa staining (Solarbio, China). Then, the slices were incubated with 5% sodium thiosulfate for 1 min. At last, neutral red solution or hematoxylin solution was used for counterstaining. The calcium content of valves were calculated quantitatively by inductively coupled plasma atomic emission spectrometry.

10. Bioprosthetic valve replacement model

The long-term durability of the bioprosthetic valve *in vivo* could be assessed by sheep pulmonary bioprosthetic valve replacement *in situ* model. The fixed swim bladder or bovine pericardium were sutured on a cobalt-chromium alloy stent to construct a bioprosthetic valve with an outer diameter of 24 mm (inner diameter: 23mm). Fatigue test was used to verify the function and durability of the fabricated valve before animal experiment (Movie 1). 40-45 kg male adult sheep were selected as experimental animal. After anesthesia procedure by 2% isoflurane inhalation, fourth

intercostal incision at left side was taken as surgical approach into the pericardial cavity. 1.5 mg/kg heparin was used intravenously for perioperative anticoagulation. Double circular suture was performed at right ventricular outflow tract. The bioprosthetic valve was immersed in 0.2% (v/v) heparin saline and then loaded into a customized delivery system. The pulmonary valve was replaced by bioprosthetic valve *in situ* through the path of right ventricular outflow tract. Echocardiography was performed before and after implantation procedure to evaluate the existence of valve regurgitation, the position of bioprosthetic valve, and the existence of perivalvular leakage. All echocardiographic imaging and analyses were collected by the same experienced investigator blinded to the assignments. Warfarin was given orally for 3 months post-operation. The bioprosthetic valve was removed 6 months after implantation for histological analysis.

11. Immunofluorescence

Briefly, hydrated slices were blocked for 0.5 h at room temperature using BSA (Maixin Biotech, Fujian, China). Primary antibodies against CD68 (ab125212, abcam, 1:200), CD206 (ab64693, abcam, 1:500), CCR7 (BS9847M, Bioworld, 1:200), α-SMA (AF1032, Affinity, 1:200), CD31 (ab28364, abcam, 1:50), and vWF (AF3000, Affinity, 1:200) were incubated overnight at 4℃. Subsequently, slices were incubated with corresponding secondary antibodies labeled with Cy3 (Servicebio, Wuhan, China) for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (Servicebio, Wuhan, China) for 5 min. Fluorescent images were acquired using an inverted fluorescent microscopy.

12. Statistics analysis

Continuous data are expressed as mean ± standard deviation, the normality of distribution of all continuous variables was confirmed by the Kolmogorov-Smirnov test. Statistical analysis was performed by GraphPad Prism 5 software and SPSS version 21. Normally distributed variables were analyzed using Student t test. Student's t-test and nonparametric test were applied for comparison between 2 groups appropriately. Bonferroni's test was performed for multiple comparative analysis between two independent groups after ANOVA. The echocardiography results during follow-up were test by Two-way Repeated Measures ANOVA. P value < 0.05 was considered statistically significant for all tests.

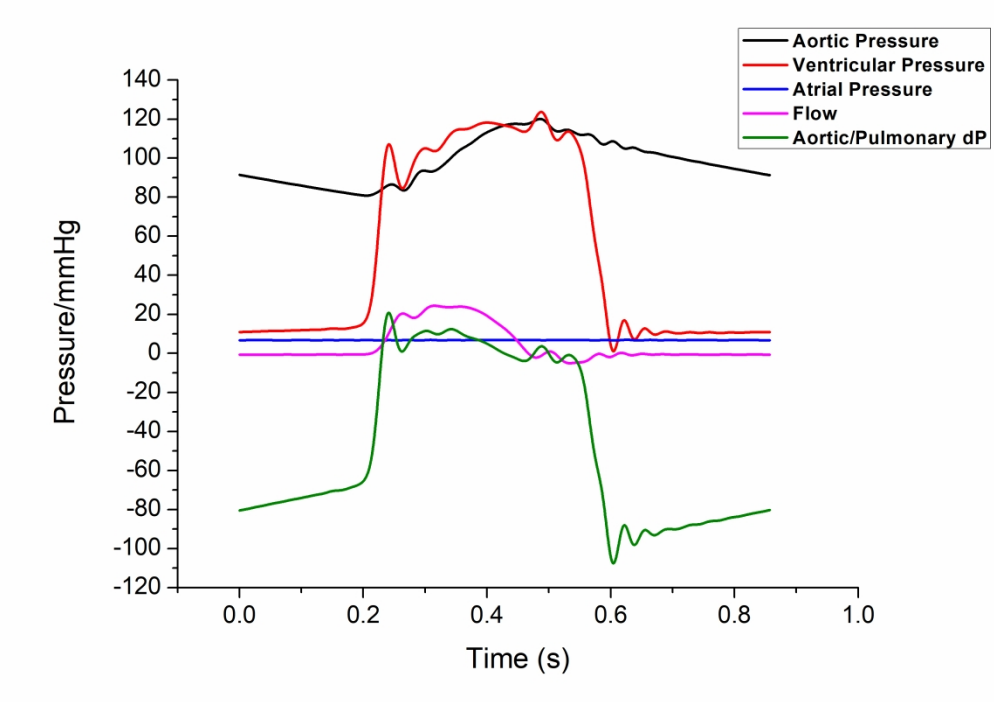
Fig S1. Pulse data of pulsating flow test

Fig S2. Pulmonary bioprosthetic valve replacement *in situ* model. A, the bioprosthetic valve was fabricated by suturing BP-GA or SB-GA on a cobalt-chromium alloy stent. B, the bioprosthetic valve was loaded into a customized delivery system, the yellow triangles represent the uncompressed bioprosthetic valve, and the yellow star represents the customized delivery sheath. C, the customized delivery system loading with bioprosthetic valve. D, the native pulmonary valve was replaced by bioprosthetic valve *in situ*, the white dotted lines represent the pulmonary artery, and the white arrow represents the direction of blood flow and surgical path. E, the short axis of pulmonary valve in echocardiography postoperatively, the yellow triangles represent cobalt-chromium alloy stent. F, the long axis of pulmonary valve in echocardiography postoperatively, the yellow triangles represent cobalt-chromium alloy stent. BP, bovine pericardium; SB, swim bladder; GA, glutaraldehyde.

Fig S3. Endothelium coverage and fibroblastic hyperplasia evaluation by immunofluorescence staining. A, fibroblastic hyperplasia was detected by α-SMA (red) and DAPI (blue). B, endothelium coverage was detected by vWF (red) and DAPI (blue). C, endothelium formation was detected by CD31 (red) and DAPI (blue). Scale bar was 100 μm.

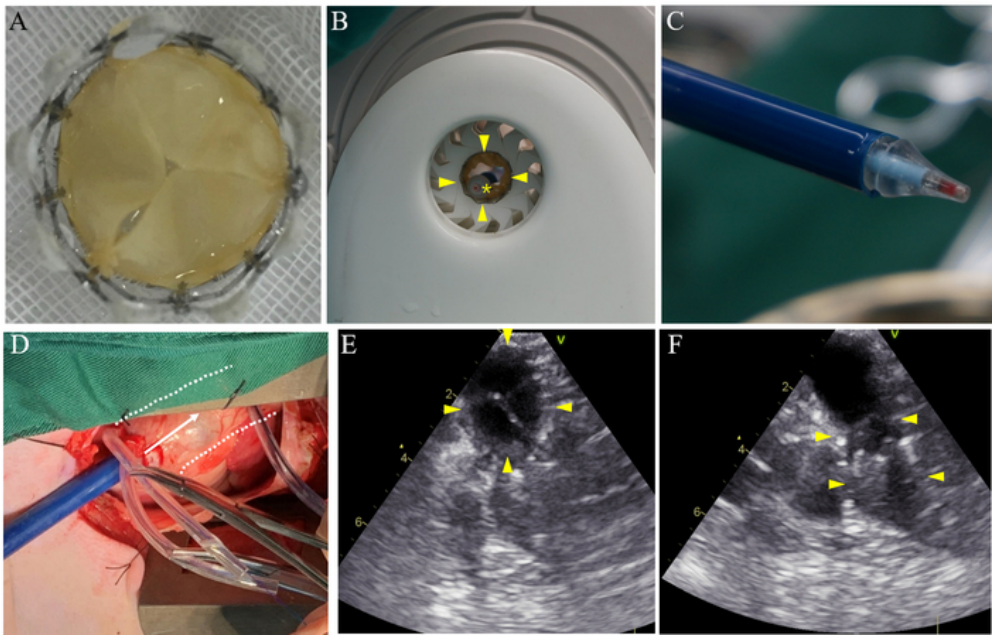
Fig S4. Macrophage infiltration evaluation by immunofluorescence staining. A, Macrophage infiltration was detected by CD68 (red) and DAPI (blue). B, M2 macrophage phenotype was detected by CD206 (red) and DAPI (blue). C, M1 macrophage phenotype was detected by CCR7 (red) and DAPI (blue). Scale bar was 100 μm.

Video 1. Pulsating flow test of bioprosthetic valve fabricated by carp swim bladder



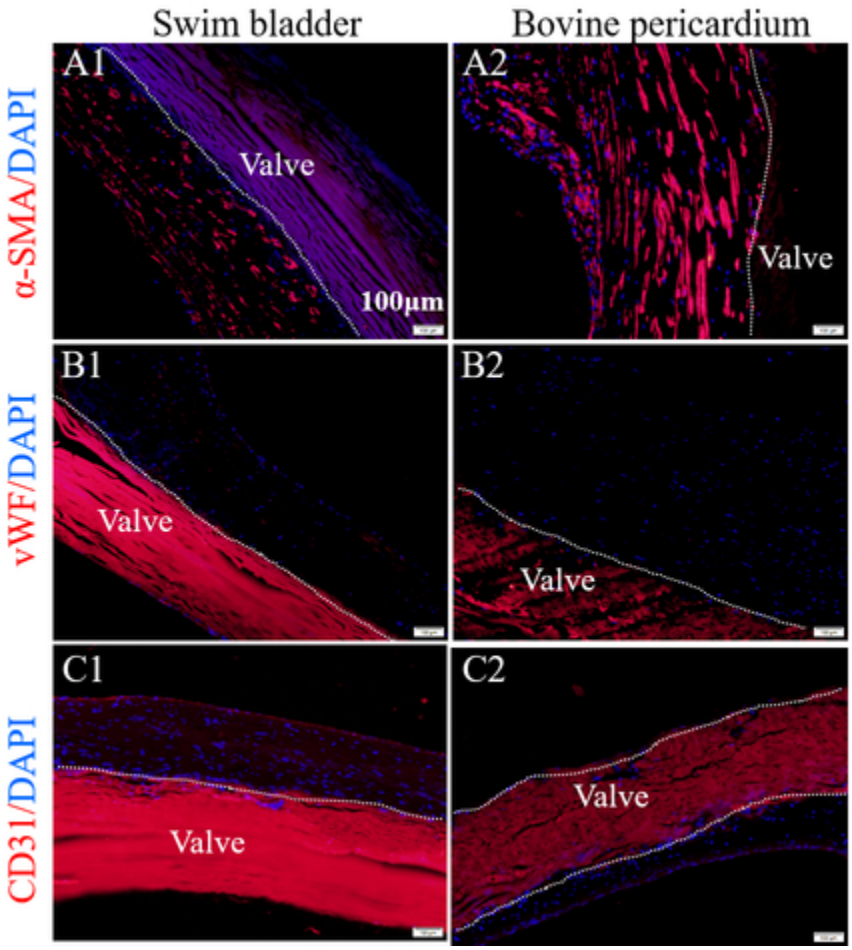
Pulse data of pulsating flow test

288x203mm (300 x 300 DPI)



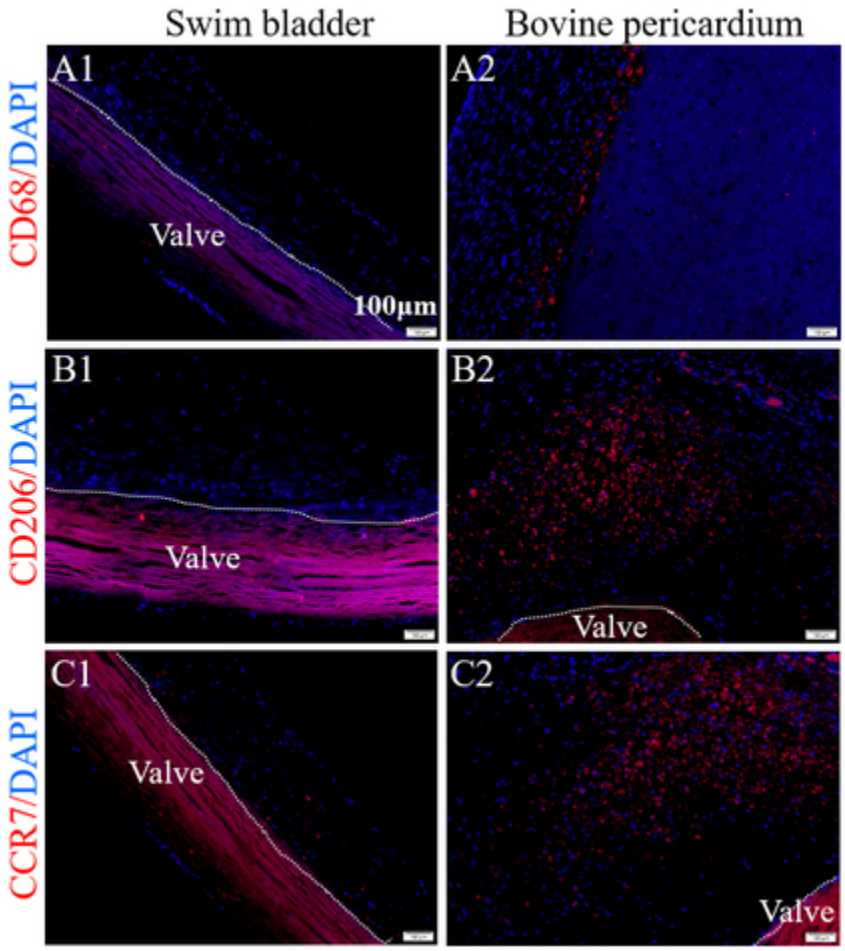
Pulmonary bioprosthetic valve replacement in situ model. A, the bioprosthetic valve was fabricated by suturing BP-GA or SB-GA on a cobalt-chromium alloy stent. B, the bioprosthetic valve was loaded into a customized delivery system, the yellow triangles represent the uncompressed bioprosthetic valve, and the yellow star represents the customized delivery sheath. C, the customized delivery system loading with bioprosthetic valve. D, the native pulmonary valve was replaced by bioprosthetic valve in situ, the white dotted lines represent the pulmonary artery, and the white arrow represents the direction of blood flow and surgical path. E, the short axis of pulmonary valve in echocardiography postoperatively, the yellow triangles represent cobalt-chromium alloy stent. F, the long axis of pulmonary valve in echocardiography postoperatively, the yellow triangles represent cobalt-chromium alloy stent. BP, bovine pericardium; SB, swim bladder; GA, glutaraldehyde.

59x38mm (300 x 300 DPI)



Endothelium coverage and fibroblastic hyperplasia evaluation by immunofluorescence staining. A, fibroblastic hyperplasia was detected by α-SMA (red) and DAPI (blue). B, endothelium coverage was detected by vWF (red) and DAPI (blue). C, endothelium formation was detected by CD31 (red) and DAPI (blue). Scale bar was 100 μm.

36x40mm (300 x 300 DPI)



Macrophage infiltration evaluation by immunofluorescence staining. A, Macrophage infiltration was detected by CD68 (red) and DAPI (blue). B, M2 macrophage phenotype was detected by CD206 (red) and DAPI (blue). C, M1 macrophage phenotype was detected by CCR7 (red) and DAPI (blue). Scale bar was 100 μm.

36x40mm (300 x 300 DPI)

Table S1. Follow-up of pulmonary bioprosthetic valve replacement in sheep

	bioprosthetic valve	Post-operation		15 days after operation			180 days after operation		
		PV velocity (max, m/s)	PV gradient (max, mmHg)	PV velocity (max, m/s)	PV gradient (max, mmHg)	PV regurgitation	PV velocity (max, m/s)	PV gradient (max, mmHg)	PV regurgitation
1	Swim Bladder	DOT	-	-	-	-	-	-	-
2	Swim Bladder	-	-	0.95	4.00	None	1.01	4.10	None
3	Swim Bladder	1.00	4.69	1.40	4.00	None	1.00	4.06	None
4	Swim Bladder	1.29	6.69	0.84	2.80	None	1.84	13.60	None
5	Swim Bladder	1.02	4.13	0.95	3.60	Mild	1.62	10.50	Mild
6	Swim Bladder	1.18	5.54	0.96	3.70	Mild	1.19	5.73	None
7	BP	1.40	7.82	1.19	5.60	Mild	1.02	4.19	None
8	BP	1.37	7.49	1.27	6.40	None	1.39	7.82	None
9	BP	1.19	5.69	0.94	3.50	Mild	1.54	9.55	Mild

DOT, die on table; BP, bovine pericardium; PV, pulmonary valve.