A chitosan modified small-diameter vascular graft with dual functions of anti-thrombotic and anti-bacterial for vascular tissue engineering

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1. Blood compatibility experiments

1. 1. Hemolysis ratio

The hemolysis potentials of the PCL and bilayered SDVGs were measured as reported.[1] 5 mL of whole blood was firstly added to 10 mL of calcium- and magnesium-free PBS solution, and then the red blood cells (RBCs) were isolated from plasma by centrifuging at 2000 rpm for 10 min, the centrifugation procedure was repeated at least 5 times. For the hemolysis test, 1 mL of the diluted RBC suspension (approx. 10^8 cells per mL) was added to the sample (the SDVGs was previously immersed in PBS overnight) and incubated at 37 °C for 3 h. PBS (pH = 7.4) was selected as negative control while deionized water was used as positive control. Then the suspensions were centrifuged at 8000 rpm for 3 min, and the absorbance of the released hemoglobin in the suspensions was measured at 540 nm using a UV-vis spectrometer (UV-1750, Shimadzu Co., Ltd, Japan), and then the hemolysis ratio was calculated by the following equation:

$$Hemolysis\ ratio\ (\%) = \left(\frac{A_s - A_{nc}}{A_{pc} - A_{nc}}\right) \times 100$$

where A_s, A_{nc} and A_{pc} are absorbance of sample, negative control and positive control,

respectively.

1. 2. Anticoagulant activity

To evaluate the antithrombogenicity of the SDVGs, activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), were measured by a semi-automatic blood coagulation analyzer (CA-530, Sysmex Corporation, Kobe, Japan). Healthy human fresh blood (healthy man, 25 years old) was collected using vacuum tubes (5 mL, Terumo Co.) containing sodium citrate as anticoagulant (anticoagulant to blood ratio, v/v, 1:9). The blood samples used for all the blood-related tests came from the same donor. Platelet-poor plasma (PPP) was obtained by centrifuging at 4000 rpm for 15 min. The test samples were pre-immersed in normal saline overnight and then incubated at 37 °C for 1 h. After that, the normal saline was removed, and 200 μL of fresh PPP was introduced. After incubating at 37 °C for 30 min, 50 μL of the incubated PPP was moved to a test cup and mixed with 50 μL of APTT agent (Dade Actin Activated Cephaloplastin Reagent, Siemens; incubated 10 min at 37 °C before use), followed with adding 50 μL of a 0.025 M $CaCl_2$ solution, and then the APTT was measured. For the TT test, 50 μL of the incubated PPP was added in a test cup and mixed with 100 μL of thrombin agent (Sysmex; incubated 10 min at 37 °C before use), and then the TT was measured. For the PT test, 50 μL of the incubated PPP was added in a test cup, followed by the addition of 100 mL Thromborel S (Siemens; incubated 10 min before use), and incubated at 37 °C for 2 min, and then the PT was measured. At least 3 parallel samples were applied to get a reliable value, then the values were averaged and the results were expressed as mean ± SD (n = 3).

1. 3. Enzyme-linked immunosorbent assay (ELISA)

For the enzyme-linked immunosorbent assays (ELISA), commercial enzyme-linked immunosorbent assays were used to evaluate coagulation activation (Thrombinantithrombin III complex (TAT), Enzygnost TAT micro, Assay Pro, USA) and complement activation (Human Complement Fragment 3a (C3a) and Human Complement Fragment 5a (C5a), Cusabio Biotech, China).

The samples were pre-immersed in normal saline in a 24-well cell culture plate overnight. Then, the normal saline was removed and 250 μL of human whole blood was introduced. After being incubated at 37 °C for 1 h, the whole blood was withdrawn and then centrifuged for 10 min at 2500 g centrifugal force (2-8 °C) to obtain plasma.

For the TAT test, 50 μL of the obtained plasma was added into an Antibody Coated Well (provided by the TAT kit). For the C3a test, 5 μL of the obtained plasma was diluted for 500 times with C3a-Sample Diluent, and 100 μL of the diluted plasma was added into an Antibody Coated Well (provided by C3a kit). For the C5a test, 10 μL of the obtained plasma was diluted for 10 times with C5a-Sample Diluent, and the diluted plasma was added into another Antibody Coated Well (provided by C5a kit).

Finally, the detections were conducted according to the respective instruction manuals. Whole blood was used as control sample. For the blood tests (ELISA) of each sample, three replicates were used to reduce error. Then, the obtained values were averaged and the results were expressed as mean ± SD (n = 3).

1. 4. Platelet activation

In order to eliminate the interference of other components in blood, such as erythrocyte and leucocyte, PRP was used for studying platelet adhesion. To study platelet adhesion, the PCL, PCL/CMC and PCL/CS nanofibrous membranes were prepared. Then the nanofibrous membranes were immersed in PBS and equilibrated at 37 ^{o}C for 1 h. Then, the PBS solution (pH=7.4) was removed and 200 μL of fresh PRP was introduced. The nanofibrous membranes were incubated with PRP at 37 ^{o}C for 2 h. Then, the PRP was decanted off and the slices were rinsed three times with PBS solution. Finally, the nanofibrous membranes were treated with 2.5 wt% glutaraldehyde in PBS solution at 4 ^{o}C for 1 day. The nanofibrous membranes were washed with PBS solution, subjected to a drying process by passing them through a series of graded alcohol-PBS solutions (30, 50, 70, 80, 90, 95 and 100%). Platelet adhesion was observed using a FE-SEM (JSM-7500F, JEOL, Japan).

1. 5. Whole blood circulation

To explore the anti-thrombotic ability of the vascular graft. We simulated the whole blood circulation in vitro. The vascular grafts were pre-immersed in PBS overnight. Healthy human fresh blood (healthy man, 25 years old) was collected using vacuum tubes (5 mL, Terumo Co.) containing sodium citrate as anticoagulant (anticoagulant to blood ratio, v/v, 1:9). 0.025 mol/L $CaCl_2$ solution was added in the whole blood before use. The added volume of $CaCl_2$ solution was 10% of the total volume of blood. Add the whole blood to a 5 mL syringe, then use the syringe to pump the whole blood into the vascular graft. After being fixed by fresh prepared glutaraldehyde (2.5 wt. %), the lumen of the vascular graft were observe by naked eyes. The blood cells adhered to the vascular graft were visualized using Field Emission Scanning Electron Microscope (Apreo S Hi Vac, ThermoFisherScientific (FEI)) after being fixed by fresh prepared glutaraldehyde (2.5 wt. %) and dehydrated by ethanol solutions with series of concentrations.

2. Results and discussion

2.1 Whole blood circulation

As shown in **Fig. S1. A and B**, the simple whole blood circulation tests were performed. The surface of the vascular grafts was shown in **Fig. S1. C and D.** After fixed by glutaraldehyde, the surface of the PCL SDVGs formed a lot of thrombus while the surface of the bilayered SDVGs was non-adhesive. The results demonstrated that the pure PCL SDVGs have poor blood compatibility, but the bilayered SDVGs exhibited good blood compatibility after modified with CMC and CS. Therefore, the bilayered SDVGs can effectively inhibit thrombus formation.

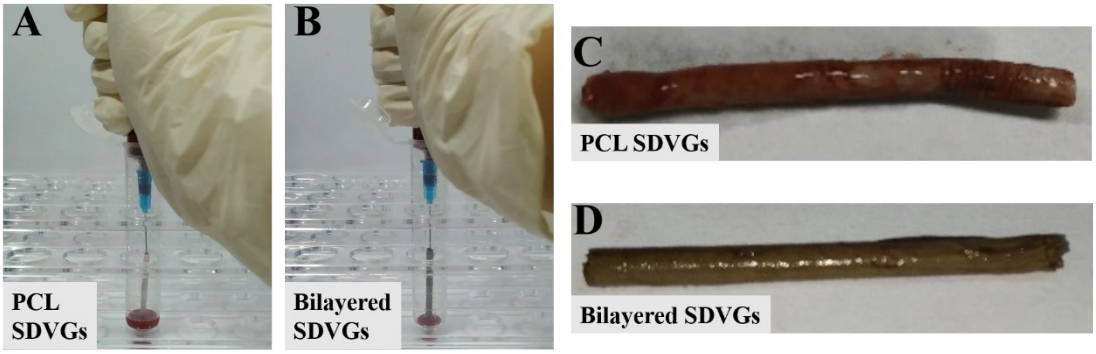

Fig. S1 (A, B) Picture of the whole blood circulation tests. (C, D) Picture of the vascular grafts after fixed by glutaraldehyde.

2.2 Thermogravimetric analysis

The thermogravimetric curves of the nanofibrous scaffolds are shown in **Fig. S2**. The decomposition temperature of the crosslinking SDVG was higher than that of uncrosslinking SDVG, and the weight loss of crosslinking SDVG was less than that of uncrosslinking SDVG, which indicated that the thermal stability of the SDVG was improved after crosslinking.

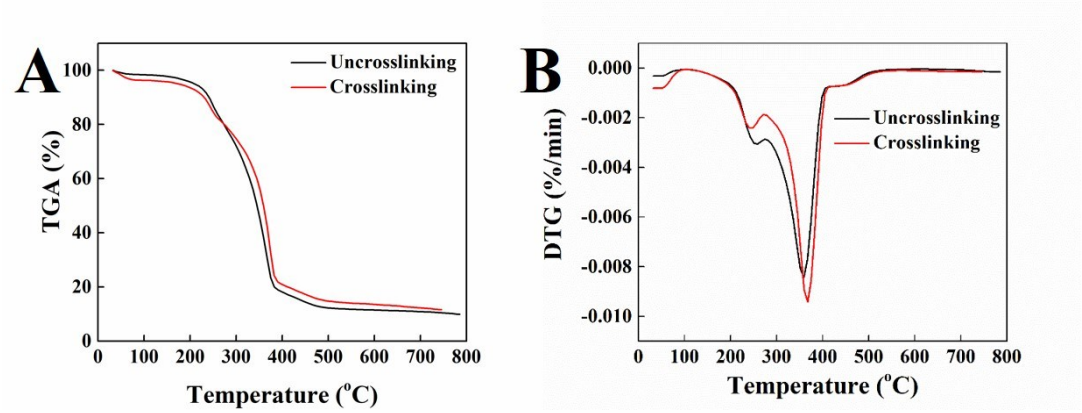

Fig. S2 (A) TGA and (B) DTG curves for uncrosslinking and crosslinking SDVGs.

References

1. D. Ma, K. Tu and L.-M. Zhang, *Biomacromolecules*, 2010, **11**, 2204-2212.