ESI:

Facile fabrication of copper incorporated poly(ε-caprolactone)/keratin mats for tissue-engineered vascular grafts with the potential of catalytic nitric oxide generation

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1. Experiments

1.1 Hemolysis

Prior to the hemolysis experiment, the pristine PCL and PCL/Ker-Cu mats were immersed into normal saline for 24 h. Then, whole rabbit blood samples were centrifuged and resuspended in normal saline to get the red blood cells (RBCs). 2.5 mL RBCs suspension mixed with 2.5 mL normal saline and 2.5 mL distilled water were served as negative control (producing no hemolysis) and positive control (producing 100% hemolysis), respectively. The samples were placed in 2.5 mL RBCs suspension mixed with 2.5 mL normal saline. These mats were incubated for 60 min at 37 °C. After incubation, all mats were centrifuged at 2500 rpm for 5 min, the supernatant was taken and its absorbance was recorded on a microplate reader (Bio-Tek ELX 800, USA) at 540 nm. The final data of each group were an average of five samples. The formula for calculating the hemolysis rate was as follows:

$$\text{Hemolysis}(\%) = [(\text{OD}_{test} - \text{OD}_{neg})/(\text{OD}_{pos} - \text{OD}_{neg})] \times 100$$

where OD_{test}, OD_{neg} and OD_{pos} were the absorbance values of the test sample, the negative control (normal saline) and the positive control (water), respectively.

1.2 Platelet adhesion

PCL and PCL/Ker-Cu films were cut into 1.5 cm × 1.5 cm pieces and placed in a 48 well plate. Fresh PRP was dropped onto the center of a film and incubated for 3h at 37 °C. After the films were washed with phosphate buffered saline (PBS, pH 7.4) solution, the platelet adhering on the film was fixed by 2.5 wt% glutaraldehyde in PBS for 2h. Finally, the film was washed several times with PBS, and dehydrated with a series of ethanol/water mixtures (25, 50, 75, 90, 100 vol% ethanol; 20 min in each mixture). The surface of the film was observed with field emitted scanning electron microscopy (FESEM, XL 30 ESEM FEG, FEI Company) [1]. The number of adherent platelets was determined by detecting the amount of lactate dehydrogenase (LDH)[2]. Briefly, the adherent platelets were rinsed with PBS three times and the mats were permeated with 0.05% TritonX-100. An LDH Cytotoxicity Assay Kit was used to measure the LDH activity in the lysed platelet suspensions. The absorbance was measured at 490 nm using a microplate reader.

1.3 Cytotoxicity of PCL/Ker-Cu mats

The cytotoxicity of PCL/Ker-Cu mats in vitro was carried out against L929 and HUVEC cells for three days, respectively. PCL/Ker and PCL/Ker-Cu mats (Φ 1.5 cm) were sterilized under UV

irradiation for 1 h, and then put into a 24-well plate with a glass ring fixed. The cell suspension (3×10^4 cells/mL, 1 mL) was added to each well and cultured. After incubation for three days, MTT solution (0.5 mg/mL, 100 μL) was added to each well and cultured for another 4 h. The medium was discarded and washed with PBS, then 500 μL DMSO was added in each well to dissolve the formed crystals absolutely for 30 min in the dark. Finally, the OD value of the solution was measured by a microplate reader (BioTek ELx800, USA) at 490 nm.

1.4 Detailed procedure of grafts implantation

All procedures were conducted respecting the ARRIVE guidelines and were approved by the Animal Ethics Committees of Shanghai Children's Medical Center (SCMC), Shanghai Jiaotong University (Shanghai, CN). The experiments strictly followed the National Institutes of Health guide for the care and the use of laboratory animals (NIH Publications No.8023, revised 1978). A total of 6 rabbits were used in the experiment and 3 parallel samples were obtained at each time point (1 m, 3 m) for each group. All scaffolds were sterilized via ethylene oxide and rehydrated with sterile PBS before implantation. The implantation surgeries were conducted at the Shanghai Children's Medical Center (Shanghai, CN). Preoperative anesthesia was performed by slowly injecting 3% pentobarbital sodium into the rabbit ear vein at a dose of 1 mL/kg. After routinely preparing the skin on the neck, a 2 cm incision was made. Then, we separated the superficial fascia and muscle layer by layer, and dissociated the carotid artery, and blocked it with two vascular clamps after systemic half heparinization (100 U/kg). We resected the carotid artery to establish a defect and inserted a scaffold with 1.5 cm in length and 2 mm in diameter by end-to-end anastomosis and reopened the vascular clamp after anastomosis. We then compressed the anastomosis for several seconds until no active bleeding occurred and sutured the incision layer by layer, after confirming the transplanted scaffolds were beating well. After surgery, antimicrobial antibiotics were administered intramuscularly for 1 week and aspirin with 10 mg/kg was administered orally daily.

2. Results

2.1 EDS spectrum of PCL/Ker-Cu mats

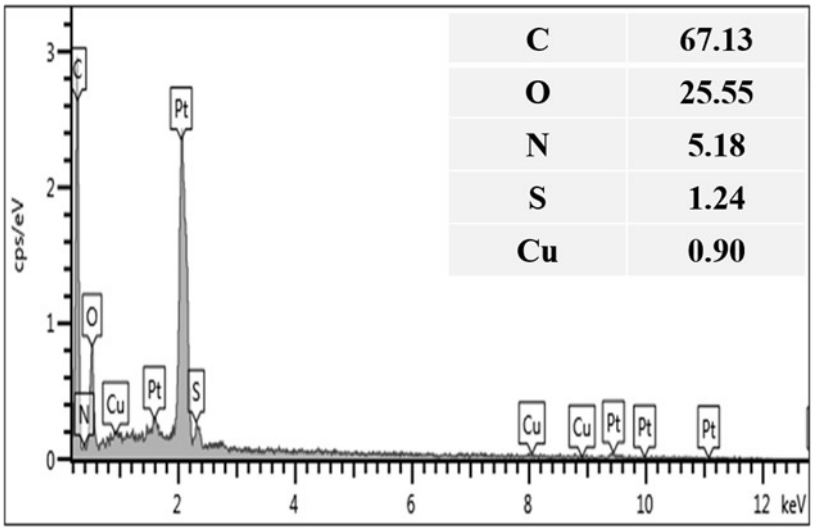
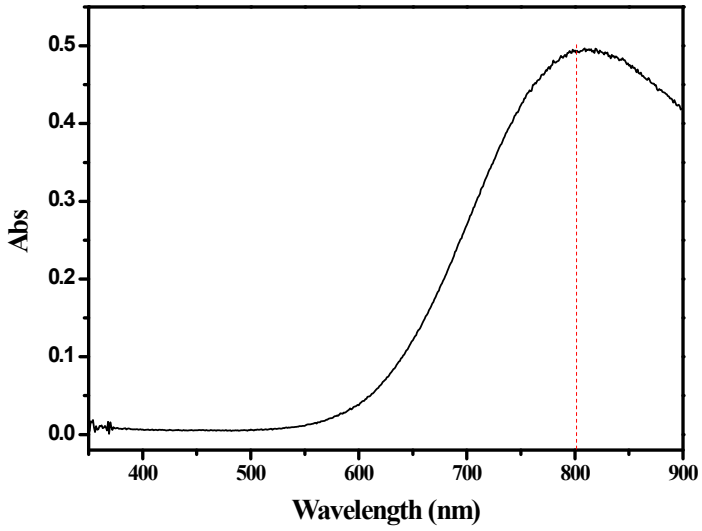


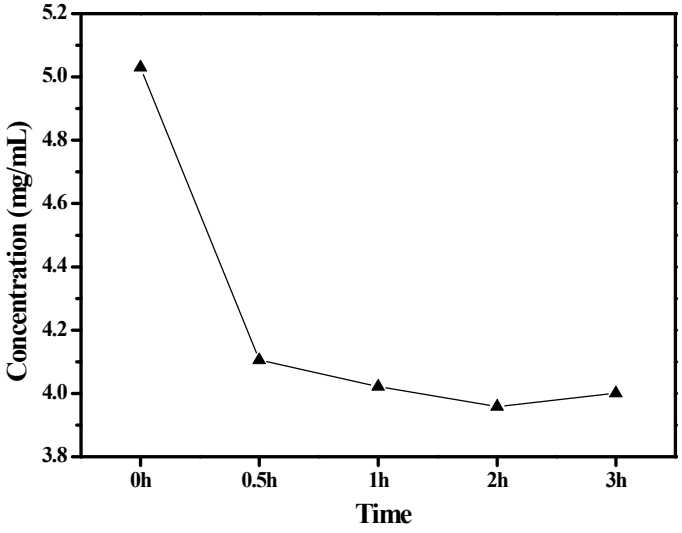
Fig.S1 EDS spectrum of PCL/Ker-Cu mats.

2.2 Detection of the adsorbed Cu(II) content

It has been reported that Cu(II) has a strong absorption peak at 800 nm, as shown in Fig. S1A. The optimal reaction time was determined by the different reaction time between the fiber membrane and Cu(II). The Cu(II) content within the mats was calculated by the concentration of Cu(II) in the $CuSO_4$ solution before and after the reaction, according to the standard curve line. Fig.S1B shows that the adsorption amount of Cu(II) on the PCL/Ker film reached a stable level after 2 h. Therefore, 2 h was set as the optimal reaction time, and the adsorption amount was close to 98 μg/cm^2. The highly reactive of PCL/Ker nanofibers to metal ions was mainly depend on the main functional groups of keratin including the side chains of peptide bonds and amino acid residues, not the porosity of the nanofibrous membrane. The amide, carboxyl, and hydroxyl groups could electrostatically complex with Cu(II) to form the polar bond. Overall, the electrostatic complexation between the nanofibrous vascular stent and Cu(II) is the main reason why the composite film had the adsorption of Cu(II)[3,4].



A



B

Fig.S2 (A) UV spectrum of Cu(II) ions as a function of concentrations; (B) Curve of copper ion content and reaction time.

2.3 ATR-FTIR

ATR-FTIR was also conducted to chemically characterize the PCL/Ker and PCL/Ker-Cu mats(Fig.2B). There are no substantial differences between them. The peaks at 1650 cm^{-1} and 1545 cm^{-1} were ascribed to the amide I and amide II of keratin. The peak at 1721 cm^{-1} related to the C=O stretching vibrations of free carboxyl groups became weak due to the formation of copper (II) ion/carboxyl complexes[3].

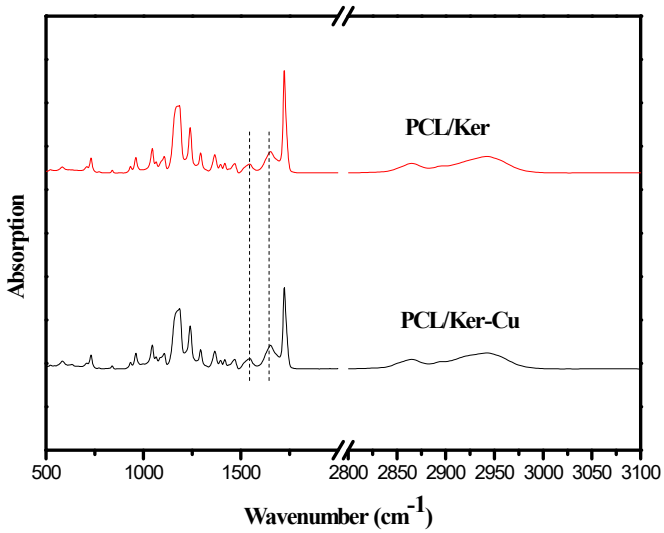


Fig.S3 ATR-FTIR spectra of PCL/Ker and PCL/Ker-Cu mats.

2.4 Water contact angles

The water contact angle was performed by the sessile drop method using the DSA30S standard contact angle meter (KRUSS, Germany) at room temperature with 2 μL DIW droplets. The contact angle of each sample was measured at three various positions of one sample. It can be seen from Fig.S3 that the surface water contact angles of the PCL/Ker and PCL/Ker-Cu samples were 24.8 ± 5.2° and 44.6 ± 5.4°, respectively, illustrating the excellent hydrophilicity of mats.

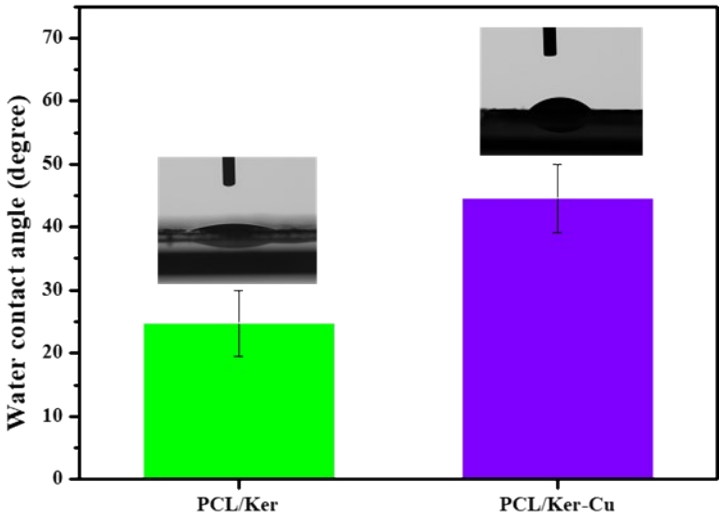


Fig.S4 Water contact angles of the PCL/Ker and PCL/Ker-Cu mats.

2.5 Hemolysis

The rate of hemolysis is an important indicator for evaluating the blood toxicity of biological materials, and is used to assess the degree of damage to RBCs. According to ISO 10993-4, biological materials with a hemolysis rate of less than 5% are considered safe, in line with

biological safety standards and can be used as blood contact materials. As shown in Fig.S5, the hemolysis rates for the pure PCL and the PCL-Ker/Cu were 1.114% and 0.164% respectively. This suggested that PCL and PCL-Ker/Cu both had excellent blood compatibility.

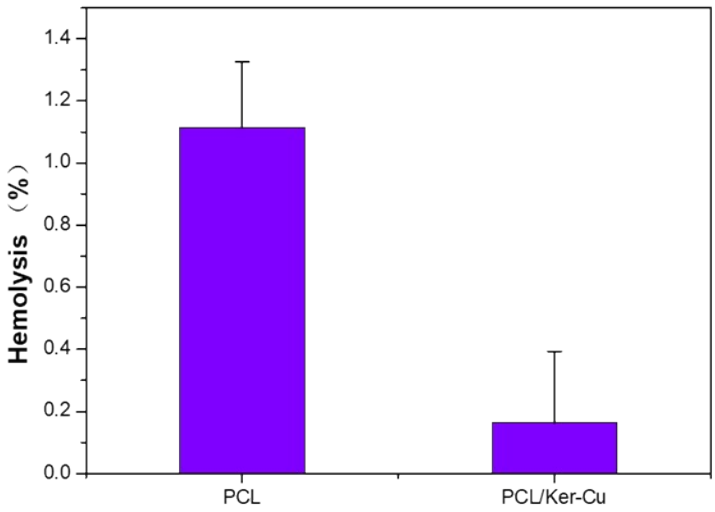


Fig.S5 Hemolysis rate of PCL and PCL/Ker-Cu, respectively.

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