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Synthesis and Antimicrobial Activity of Copper nanoparticles Loaded Regenerated Bacterial Cellulose Membranes

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Experimental

CuNPs content determination

RC membranes were cut into round shapes with 10 mm diameter and weighed (W_0). Then the fabricated RC-Cu membranes were weighed (W_1). CuNPs contents were determined with the following equation:

CuNPs content=(W_1- W_0)/A (1)

where A is the area of the dry membranes.

Swelling behavior

Swelling studies were carried out by direct immersion in 0.01M PBS buffer (pH=7.4) at room temperature. The RC and RC-Cu membranes were maintained for 72 h. The swollen membranes were periodically (5 min, 10 min，20 min，30 min，1 h，2 h，3 h，5 h, 8 h, 12 h, 24 h, 48 h and 72 h) removed from the solution, and the wet weight of the swollen membranes (W_t) was measured after the removal of excess surface water by gently blotting with a filter paper. The degree of swelling (S) was calculated according to the equation:

$S(\%) = (W_t - W_0)/W_0 \times 100\%$ (2)

where W_t is the weight of the swelled membranes at time t; W_0 is the weight of dry membrane.

Antibacterial growth activity

Single colony of *S. aureus* and *E. coli* grown on TSA were transferred into 100mL TSB, respectively. After agitated cultivation at 37°C for 6 h, 10 μL of bacterial suspension was introduced into a 100 mL flask containing 29.99 mL of TSB, and then 30 mg samples was added into the flask. The culture was kept at 37°C and 0.4 mL was drawn from the systems every 1 h. Then they were analyzed using a SHIMADZU UV 2450 spectrophotometer at the monitoring wavelength of 600 nm. Triplicate experiments were carried out.

Hemolysis assay

The procedure for hemolysis was modified from Choudhury et al.[1] Sodium citrate (3.8%) stabilized blood (4 mL) was collected from two healthy rabbits. RC and RC-Cu membranes (3 mm×3 mm) were separately incubated for 30 min at 37°C in siliconized tubes each containing 1 mL of blood (diluted with sterilized PBS in 1:9 ratio). Positive and negative controls were prepared by the same procedure but adding the blood into 0.9 mL water and PBS, respectively. Each tube was gently inverted twice to ensure the blood contact with the tested membranes. The absorbance of the supernatant is measured at 545 nm in a Shimadzu UV-2450 spectrophotometer. The percentage of hemolysis was calculated as follows:

$\text{Hemolysis}\% = [(Abs_{sample} - Abs_{negative\ control})/(Abs_{positive\ control} - Abs_{negative\ control})] \times 100\%$

Cytotoxicity tests

The HUVEC cell lines were cultured in 1640 medium supplemented with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin. The RC-Cu membranes were placed in transwell chambers in 24-well plate and the cytotoxicity was measured using the MTT assay. 200 μL of HUVEC cells, at a density of 1×10^5, were placed in each well of a 24-well plate. Then the cells were incubated over night at 37°C in a humidified 5% CO_2-containing atmosphere. RC-Cu membranes with same size (3 mm×3 mm) were placed slightly in the transwell chambers and

then fresh media was added. Wells containing only the cells were used as control. The cells were treated for another 24 h. Then the transwell chambers with samples were removed. The media in plate was changed with fresh media and 20 μL of dimethyl thiazolyldiphenyl (MTT) was added and the incubation continued for 6 h. Medium was removed, and 200 μL DMSO was added to each well to dissolve the formazan. The absorbance was measured with a test wavelength of 570 nm and a reference wavelength of 630 nm. Empty wells (DMSO alone) were used as blanks. The relative cell viability was measured by comparison with the control well containing only the cells.

Table S1 Cu content before and after 30 min ultrasonication

	Cu content (wt%) before ultrasonication	Cu content (wt%) after ultrasonication
RC_5	36.74±2.03	36.16±2.8

Cu contents of RC_5 membrane before and after 30 min ultrasonication were calculated based on EDS result. The tests were replicated on three places of sample surface, the average values were calculated and listed in Table S1. It can be seen that the Cu content of RC_5 membrane didn't change after 30 min ultrasonication, indicating the synthetic CuNPs is not easy to fall off from RC surface. Thus, the prepared RC-Cu membranes showed good stability. This is because RC is a linear polyhydroxy polymer, so it can be used as templates for metal nanoparticles. Thus, CuNPs can't fall off from RC due to the coordination and charge effects between RC and CuNPs.

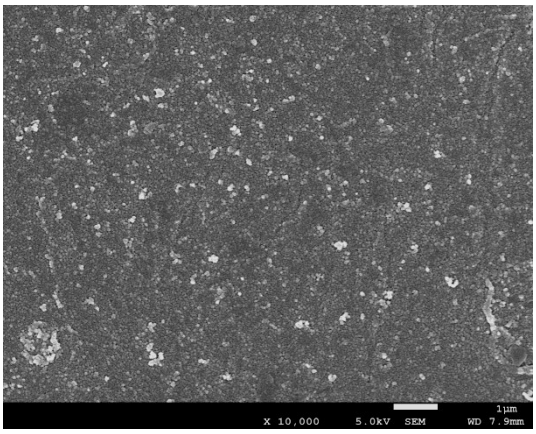

Fig. S1 SEM image of RC_5 after 30 min ultrasonication

RC_5 membrane was put into an ultrasonic bath (KH3200B with 40kHz, Hechuang Ultrasonic, China) and treated for 30 min. The morphology was shown in Fig. S1. There is no change of the morphology after 30 min ultrasonication compared to that of RC_5 membrane without any ultrasonication, which further proves good stability of the fabricated RC-Cu membranes.

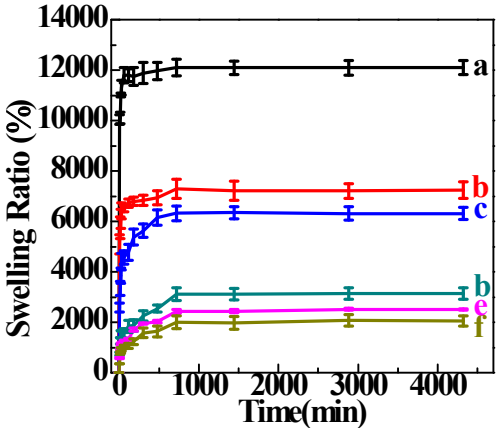

Fig. S2 Swelling behavior of RC and RC-Cu membranes. a–f are RC, RC_1, RC_2, RC_3, RC_4 and RC_5.

The swelling behaviors were tested for 3 days and the result was shown in Fig. S2. It can be seen that the swelling ratio decreased with increasing CuNPs loadings in the membranes, which was due to the denser structure with increased CuNPs loadings. However, the swelling ratio is still high that the prepared RC-Cu membranes can absorb the exudates, which can protect the wound bed from the accumulation of exudates and reduce the frequency of replacement.

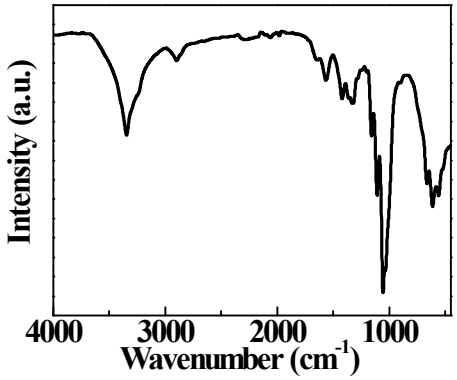

Fig. S3 FTIR spectrum of BC membrane

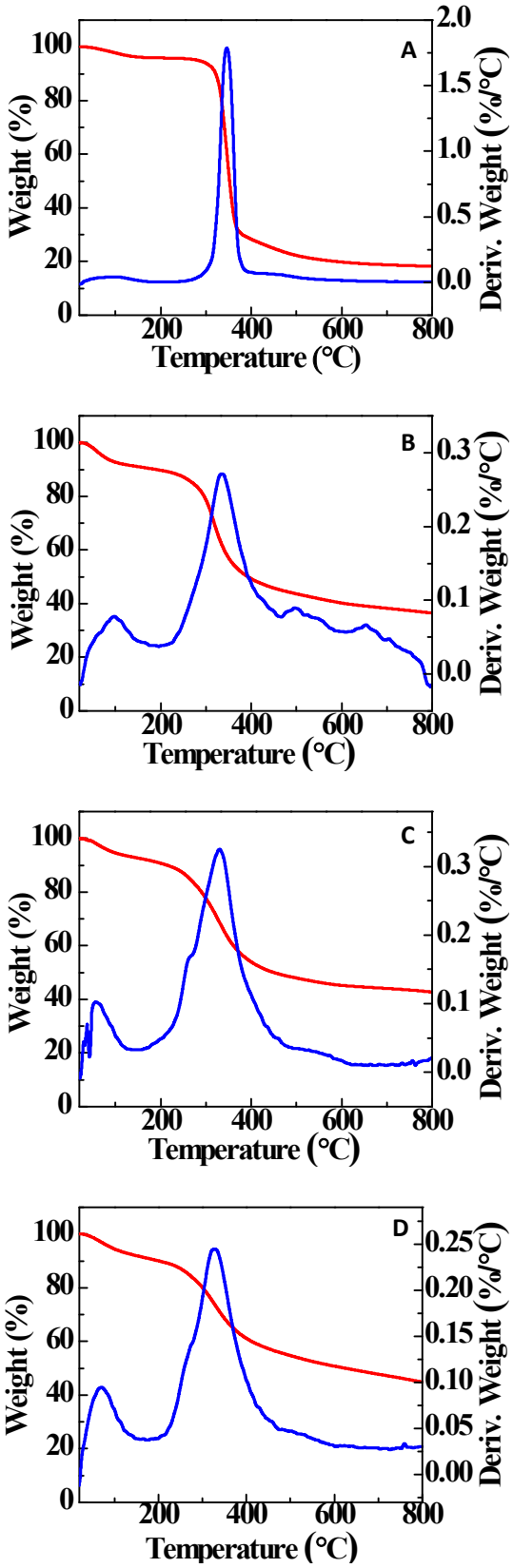

Fig. S4 TG and DTG analysis of RC_1 (A), RC_2 (B), RC_3 (C) and RC_4 (D) membranes

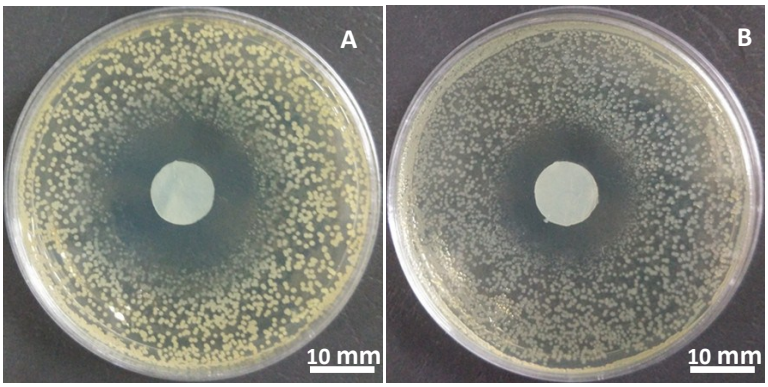

Fig. S5 Optical images of inhibition zones of RC_5 membranes: (A) *S. aureus* and (B) *E. coli*.

The inhibition activity of RC_5 membrane against both *S. aureus* and *E. coli* for 3 days was shown in Fig. S5. There are still clear inhibition zones after 3 days incubation, exhibiting excellent long-lasting antibacterial performance.

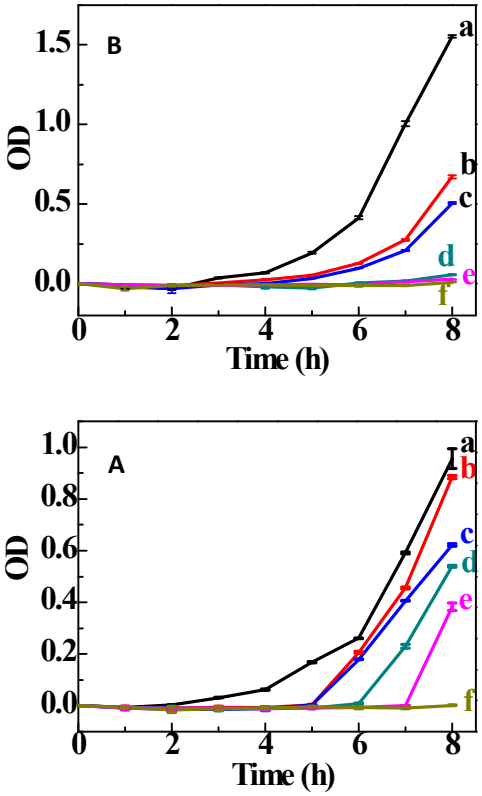

Fig. S6 Inhibitory effect of RC and RC-Cu membranes on bacterial growth, a–f are RC, RC_1, RC_2, RC_3, RC_4 and RC_5. (A) represents the growth of *S.aureus* and (B) of *E.coli*.

We did the antibacterial curve incubated with our prepared samples for 8 h. The antibacterial activity of the RC-Cu membranes was tested at different time points (1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h) and the result was shown in Fig. S6. It can be found that RC_5 membrane inhibited bacterial growth efficiently , exhibiting good antibacterial activity against *S. aureus* and *E. coli*.

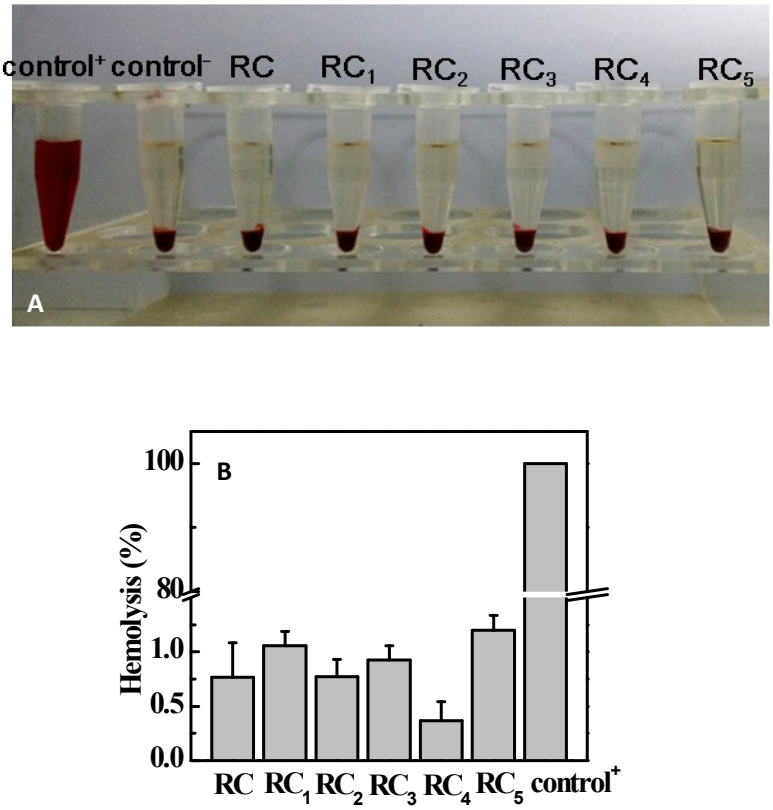

Fig. S7 Hemocompatibility studies of RC and RC-Cu membranes against mouse erythrocytes. (A) Photographs of diluted blood after exposure to tested membranes for 30 min. (B) Hemolysis percentage incubated with different membranes.

Hemocompatibility is an essential requirement for blood-contacting biomaterials with less than 5% hemolysis.[2] Fig. S7A and B show the photographs of diluted blood after exposure to tested membranes for 30 min and their hemolytic activities against mouse erythrocytes. It can be seen that the percentages of hemolysis for RC_5 is 1.5%, which proves the fabricated RC-Cu membranes are hemocompatible biomaterials.

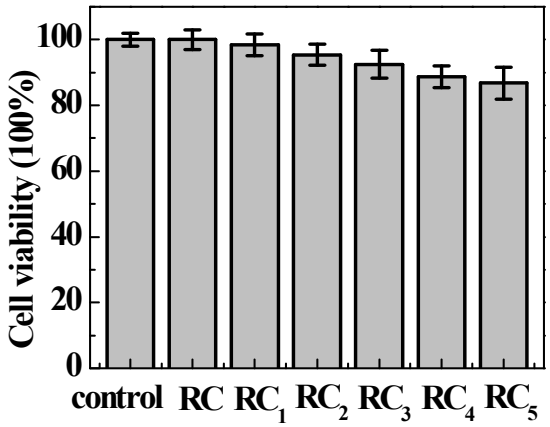

Fig. S8 In vitro cytotoxicity analysis of RC and RC-Cu membranes towards HUVEC cells

Cytotoxicity studies were performed on HUVEC cells to investigate the effect of CuNPs in the RC membrane on proliferation of HUVEC cells. The MTT result was shown in Fig. S8. All the materials showed negligible toxicity although the cell viability slightly decreased with increasing CuNPs loadings. Cell viability of all fabricated RC-Cu membranes was higher than 86%, which indicated that the fabricated membranes had no toxicity. It is considered to be good wound dressing candidate when samples with cell viability larger than 75%[3].

References

1. Choudhury A J, Gogoi D, Kandimalla R, Kalita S, Chaudhari Y B, Khan M R., Kotoky J, Chutia J. Penicillin impregnation on oxygen plasma surface functionalized chitosan/Antheraea assama silk fibroin: Studies of antibacterial activity and antithrombogenic property, Materials Science and Engineering C, 2016, 60: 475-484.
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