HEMOCOMPATIBILITY OF CELLULOSE-BASED PAPER COATED WITH HEPARIN

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Heparin was coated onto the surface of cellulose-based paper either directly (Paper-Hep) or through polyurethane drug loaded nanoparticles (Paper-PU-Hep), and the hemocompatibility of these two resulting materials was examined through *in vitro* anticoagulation tests, hemolysis tests and blood adhesion tests. It was found that loading with heparin significantly improved the hemocompatibility of the paper. Loading the heparin with polyurethane nanoparticles to the paper matrix rendered milder and more persistent anticoagulation efficacy in Paper-PU-Hep, compared with Paper-Hep, for which heparin was loaded plainly through physical adsorption.

Keywords: cellulose-based paper, heparin, hemocompatibility, surface coating, drug loaded nanoparticles

INTRODUCTION

Natural and manufactured biomaterials can diagnose, repair, replace and enhance organs and living tissues in humans and animals, and have thus become indispensible in contemporary clinical practices.[1-4] Novel biomaterials are constantly needed to conquer the medical challenges that traditional metals, ceramics and polymers fall short to resolve.[5-7] Paper has been widely used in life science as a conventional material. However, its use in medicinal research and practices has been limited because fresh blood will clot upon direct and prolonged contact with paper to cause thrombosis and foreign body reactions. Therefore, research on improving the hemocompatibility of cellulose-based materials, such as paper, may broaden their scope in medicinal practices.

Heparin is an effective and regularly prescribed glycosaminoglycan polysulfate anticoagulant in treating cardiovascular and cerebrovascular diseases and in blood dialysis.[8-10] Numerous studies have indicated that heparin can effectively activate antithrombin and accelerate its inhibition to serine proteases in the coagulation cascade.[11-13] The immobilization of heparin on biomaterials has been a continuous pursuit in medicinal research even since Gott *et al.* first reported the anticoagulation of heparinized polymer.[14-17] In general, heparin can be coated on the material surface either physically or chemically, where the former gives better anticoagulation performance, but is less stable regarding heparin attachment.[18,19] Consequently, novel methods are needed to resolve this dilemma, *i.e.*, produce stably immobilized heparin-coated materials without sacrificing the anticoagulation properties. In this paper, we coated cellulose-based paper with heparin in two ways, *i.e.*, directly and *via* drug loaded nanoparticles, and we then evaluated the properties of the resulting materials.

EXPERIMENTAL

Materials

Glutaraldehyde (25% solution), tetrahydrofuran (AR) and acetone (AR) were supplied by Sinopharm Chemical Reagent Co., Ltd. Absolute ethanol (AR) and

n-hexane (AR) were obtained from Nanjing Chemical Reagent Co. Ltd. and Beijing Chemical Works, respectively. Heparin (98%) and toluidine blue (GR, 99%) were both purchased from Sigma-Aldrich. Natural saline (0.9%) and PBS buffer solution (pH = 7.4) were both prepared in the lab.

The APTT (activated partial thromboplastin time)/PT(prothrombin time)/TT(thrombin time) reagents (solution) and polyurethane (R180A, one polymer from isocyanate and Polyhydroxy) were supplied by Shanghai Sun Biotech Co., Ltd. and Suzhou Kesong Polymers Co., Ltd., respectively. Quantitative filter paper (Φ = 9 cm, slow speed) was acquired from Hangzhou Xinhua Paper Industry Co., Ltd. Fresh rabbit whole blood used in anticoagulation studies was supplied by Jiangsu Key Laboratory of Biofunctional Materials. Platelet-rich plasma (PRP) was prepared by adding the prescribed amount of citrate anticoagulant to the rabbit whole blood. Platelet-poor plasma (PPP) was obtained by centrifuging the PRP at 3000 rpm for 15 min and collecting the supernatant.

Surface coating of heparin

A solution of heparin (0.2% wt, 10 mL) was freshly prepared and sprayed onto a piece of quantitative filter paper (Φ = 9 cm) until the surface appeared saturated. The paper was then allowed to dry naturally and its flip side was further sprayed with heparin until saturation. This procedure was repeated until the heparin solution was exhausted. The heparin-coated paper (Paper-Hep) was obtained after drying the sprayed filter paper naturally.

Polyurethane (0.02 g) was dispersed in a mixture of tetrahydrofuran (1 mL) and acetone (4 mL). Heparin (0.02 g) was dissolved in distilled water (5 mL) and rapidly added into the polyurethane dispersion. The mixture was thoroughly stirred to form a microemulsion of the polyurethane-heparin nanoparticles (PU-Hep) and then dialyzed with water to remove the organic solvents. The resulting solution of PU-Hep was sprayed similarly onto a piece of quantitative filter paper to give polyurethane–heparin coated paper (Paper-PU-Hep).

Characterization of PU-Hep

The size and morphology of the PU-Hep nanoparticles were examined under a JEOL-2000F transmission electron microscope (TEM) operated at 80 kV. Some PU-Hep solution was dried naturally at room temperature, and the residue was scrubbed, ground with KBr, and examined on a Nicolet 170SX infrared spectrometer (4000-650 cm^{-1}, 32 scans at 4 cm^{-1} resolution).

The calibration curve of heparin (Hep) was determined as follows. A certain amount of toluidine blue (TB) was added to the aqueous solution of heparin, and the resulting stable TB–Hep complex was extracted with *n*-hexane. The absorbance of the remaining aqueous TB solution at 631 nm was measured on an Agilent 8453 diode array UV-Vis spectrophotometer (Agilent, USA) and plotted against the known heparin concentration to generate the standardized calibration curve of TB absorbance *versus* heparin concentration, which had good linearity over the range of 0-40 mg/L heparin.

The sustained release of heparin from PU-Hep was evaluated as follows. A series of PU-Hep nanoparticles (0.1 mg) were kept at 37 °C in PBS (1 mL) for a certain time (2, 4, 8, 12, 20, 24, 48, 72 h respectively) and then each was centrifuged to give the supernatant to measure absorbance and calculate the amount of heparin released. The heparin release curve was then plotted accordingly.

Dynamic coagulation time *in vitro*

Heparin-free filter paper and the two heparin-coated filter papers were cut to identical size and tested on an RT-2204C semi-automatic coagulation analyzer (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China) according to the procedures defined by the manufacturer and reproduced as follows. Each sample was measured in triplicate and the results were averaged. PBS was used as the negative control.

Activated partial thromboplastin time (APTT)

The three paper samples (heparin-free filter paper, Paper-Hep and Paper-PU-Hep) were mixed with PPP (0.1 mL), added into the blood coagulation cups and cultured for 3 min at 37 °C. The APTT reagent (0.1 mL, maintained at 37 °C) was then added and the mixture was incubated for 5 min at 37 °C before the addition of $CaCl_2$ (0.1 mL, 0.025 mol/L, maintained at 37 °C). The PPP then started to coagulate in the presence of Ca^{2+} and the time elapsed for coagulation was recorded as the APTT.

Prothrombin time (PT)

The three paper samples were each mixed with PPP (0.1 mL), added into the blood coagulation cups and cultured for 3 min at 37 °C. The PT reagent (0.2 mL, maintained at 37 °C) was then added and the time taken for the PPP to coagulate was recorded as the PT.

Thrombin time (TT)

The three paper samples were each mixed with PPP (0.2 mL), added into the blood coagulation cups and the TT reagent was then added. The time taken for the PPP to coagulate was recorded as the TT.

Hemolysis

The three paper samples were cut into disks (Φ = 1 cm) and immersed in natural saline for 12 h. Then, to each sample, rabbit whole blood (0.2 mL) and a suitable amount of anticoagulant were added. The mixture was incubated for 1 h at 37 °C and the hemolysis was terminated by addition of natural saline

(4 mL). The positive and negative controls were measured using double distilled water (4 mL) and natural saline (4 mL), respectively, with rabbit whole blood (0.2 mL). The incubated samples were centrifuged and the absorbance of the supernatant layer at 541 nm was measured. The hemolysis ratio was calculated as follows:

$$\text{Hemolysis ratio} = (OD_{test} - OD_{neg}) / (OD_{pos} - OD_{neg}) \times 100\% \quad (1)$$

where OD_{test}, OD_{neg} and OD_{pos} are the absorbance of the tested sample, the negative control and the positive control, respectively. The sample was considered biomedically suitable if the hemolysis ratio was no greater than 5%.

Adhesion tests

The three paper samples were placed into a 24-well cell culture plate (TPP Techno Plastic Products AG, Switzerland) and freshly prepared PRP (1 mL) was added into the cells. The mixtures were cultured for 90 min at 37 °C, PRP was then removed with a suction tube and the paper samples were thoroughly rinsed with PBS (3 × 1 mL). Afterwards, aqueous glutaraldehyde (2.5% w/w, 1 mL) was added to each well and the mixtures were kept at room temperature for 30 min to immobilize the platelets adhered to the paper surface. Glutaraldehyde was removed once the reaction finished, and the samples were again rinsed with PBS and dehydrated stepwise using ethanol of escalating concentration (50%, 60% 70%, 80%, 90%, 95% and 100% v/v). Each dehydration step took 30 min. The paper samples were dried naturally at room temperature after the series of ethanol treatments. The dried paper samples were sputter coated with gold and examined under a JSM-5410LV scanning electron microscope (JOEL Ltd., Japan).

The adhesion test of rabbit whole blood was run according to an identical procedure, except for changing the incubation time of papers in blood from 90 min to 30 min.

Anticoagulation performance of heparin-coated paper after extended use

The durability of Paper-Hep and Paper-PU-Hep was tested by first immersing the paper samples in PBS for a total of 24 h inside the 24-well cell culture plate, during which time PBS was removed from the cells and new PBS was added every 6 h. Adhesion tests were then run with the aged paper samples for both PRP and rabbit whole blood.

RESULTS AND DISCUSSION

Characterization of PU-Hep

The PU-Hep nanoparticles appear as well-dispersed microspheres in the TEM image (Fig. 1). The FT-IR spectrum (Fig. 2) shows that PU-Hep has strong absorbance at 1000-1100 cm^{-1}, which resulted from the overlaid signals of the S=O and C–O–C bonds in the heparin molecule.[20] Besides, the characteristic peak of the sulfonyl group in heparin at 1230 cm^{-1} is also clearly visible in the spectrum of PU-Hep, thus confirming that heparin was successfully loaded onto the PU nanoparticles. For PU-Hep, the peaks at 2960 and 2860 cm^{-1} result from the symmetric and asymmetric stretch of the $–CH_2–$ units, the signal at 1731 cm^{-1} is characteristic of the –NHCOO– urethane linkage, and the absorbance of the benzene rings appears at 1595 cm^{-1}.[21]

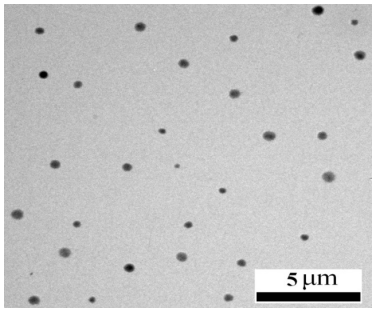

Figure 1: TEM image of PU-Hep nanoparticles

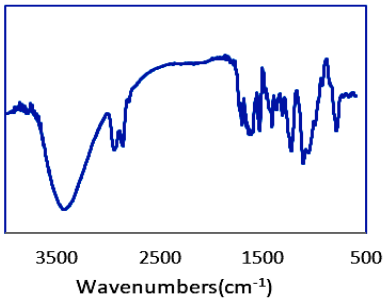

Figure 2: FT-IR spectrum of PU-Hep

It was also found that only about 10% heparin was liberated from PU-Hep after 24 h in the sustained release experiment (Fig. 3). Heparin has a high molecular weight of ~12,000 and can entangle with the polymer chain in the PU nanoparticle, thus slowing its release rate. Further monitoring shows that the release of heparin reached only 21% after 72 h. This again demonstrates the sustained release of heparin from PU-Hep.

Coagulation tests

Figure 4 shows that, compared with the negative control of PBS, the APTT and PT of the PPP decreased significantly after crude filter paper was added, indicating its relatively poor

hemocompatibility. In contrast, when Paper-Hep was added into the PPP, the APTT and TT went beyond the measurable range (>50 s) and the PT also increased significantly. Therefore, the heparin adsorbed onto the paper surface rapidly and evidently inhibited coagulation. In addition, Paper-PU-Hep gave a milder anticoagulation performance, where the observed APTT, TT and PT were also longer than for the PBS control, but shorter than for Paper-Hep.

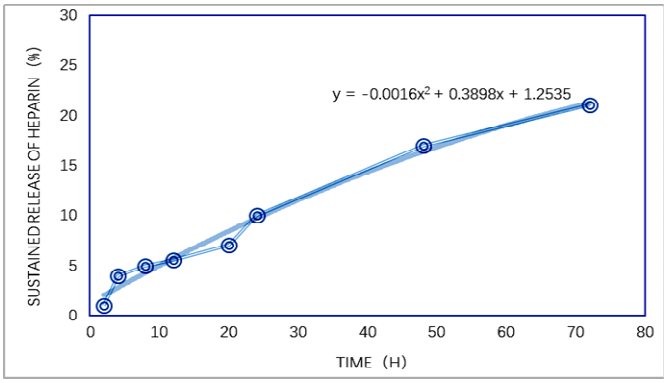

Figure 3: Sustained release of heparin from PU-Hep nanoparticles

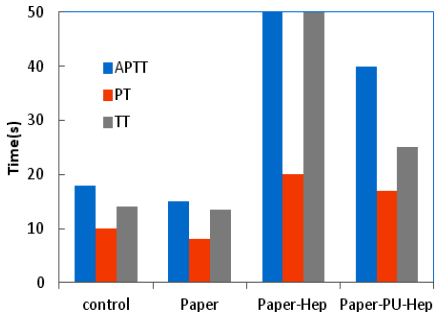

Figure 4: APTT, PT and TT results of crude filter paper, Paper-Hep and Paper-PU-Hep. PBS was used as negative control; the APTT and TT of Paper-Hep were beyond measurable

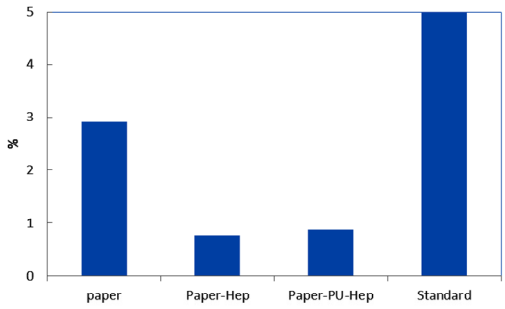

Figure 5: Hemolysis results of crude filter paper, Paper-Hep and Paper-PU-Hep. Double distilled water was used as positive control and natural saline (0.9%) was used as negative control

Hemolysis

Although the hemolysis ratios of crude filter paper, Paper-Hep and Paper-PU-Hep were all less than 5% (Fig. 5), that of the paper coated with heparin is obviously lower. The hemolysis ratio is not only an important reference for evaluating the hemocompatibility of biomaterials, but also a supplementary indicator of the material's cytotoxicity.[22] According to the benchmark specified by ISO, materials should have hemolysis ratios no greater than 5% to be safe for medical use. Therefore, all the three samples passed the hemolysis test, while the two heparinized samples had a much lower hemolysis ratio than the crude filter paper (Fig. 5).

Adhesion tests

Because platelets tend to adhere to the surface of foreign materials, the hemocompatibility of materials can be evaluated by the adhesion of platelets on their surface upon prolonged contact.[23] The SEM images in Figure 6 show that after exposure in PRP for 90 min, many platelets adhered to the surface of the crude filter paper with their pseudopods extended (Fig. 6 a-1). In contrast, Paper-Hep and Paper-PU-Hep had a clean surface, showing barely any adhesion of platelets, thus suggesting their excellent anticoagulation properties after coating with heparin.

The adhesion of whole blood undergoes a more complicated coagulation mechanism and is

a straightforward method to determine the material's hemocompatibility. It can better mimic the physiological condition than using only PRP due to the presence of fibrinogen, red blood cells *etc*. The SEM images in Figure 7 show that fibrinogen and blood cells are abundant on the surface of the crude filter paper, but basically non-existent on the surface of Paper-Hep and Paper-PU-Hep, thus indicating the excellent anticoagulation effect of the latter two for the introduction of heparin.

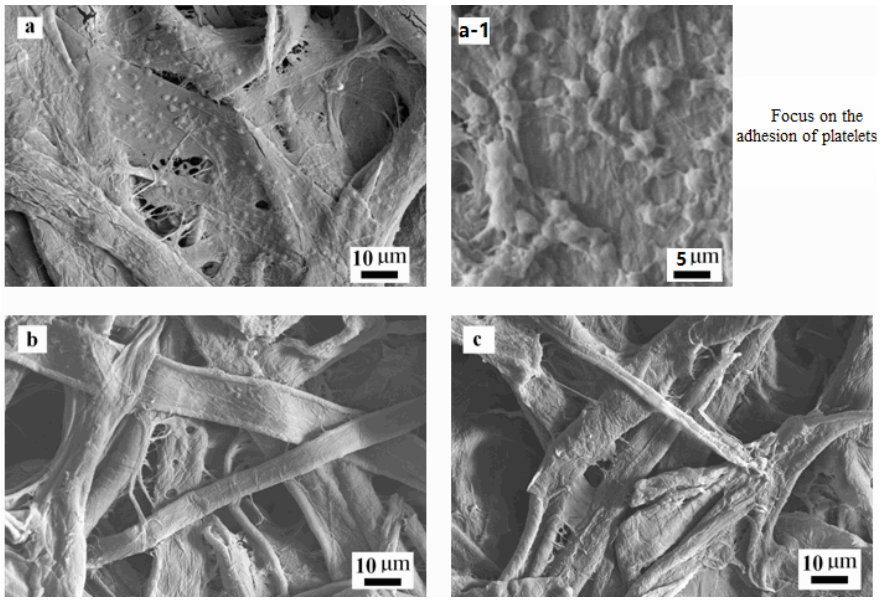

Figure 6: SEM images of the samples after incubation with PRP for 90 min; (a) Crude filter paper, (b) Paper-Hep, (c) Paper-PU-Hep

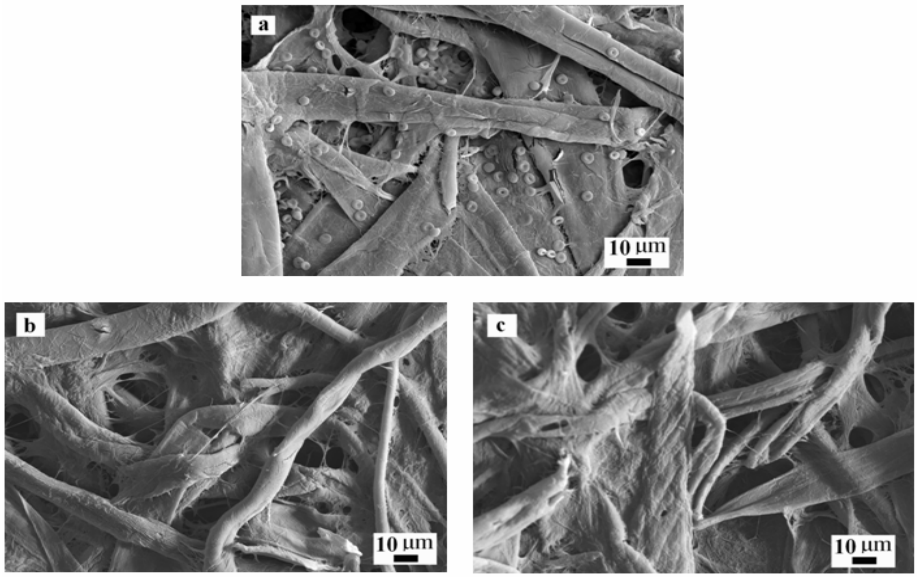

Figure 7: SEM images of the samples after incubation with rabbit whole blood for 30 min; (a) Crude filter paper, (b) Paper-Hep, (c) Paper-PU-Hep

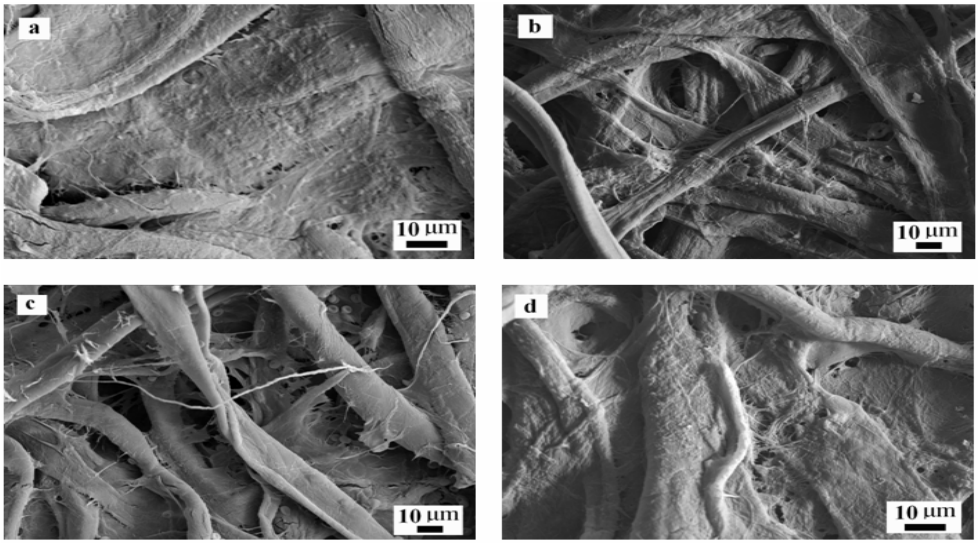

Figure 8: SEM images of aged Paper-Hep and Paper-PU-Hep samples after incubation; (a) Paper-Hep with PRP, (b) Paper-PU-Hep with PRP, (c) Paper-Hep with rabbit whole blood, (d) Paper-PU-Hep with rabbit whole blood

Durability tests

Platelets and blood cells were found to adhere to the surface of the aged Paper-Hep samples (Fig. 8 a, c), whereas the Paper-PU-Hep samples were resistant to adhesion and maintained a clean surface (Fig. 8 b, d). Prolonged immersion in PBS displaced the weakly adsorbed heparin on the Paper-Hep and erased its anticoagulation property. On the contrary, for Paper-PU-Hep, heparin was loaded onto the PU nanoparticles and was bound more strongly to the paper matrix, maintaining superior anticoagulation performance due to the sustained release of heparin under extended PBS treatment.

CONCLUSION

Heparin was coated onto cellulose-based paper and the hemocompatibility of the resulting materials was examined. Coagulation tests (APTT, PT and TT), hemolysis tests and adhesion tests all demonstrated that the heparin-coated paper samples exhibited notably enhanced anticoagulation performance. In addition, compared with direct adsorption of heparin onto the paper matrix, loading the heparin through polyurethane nanoparticles enabled a milder and more persistent anticoagulation effect. The results herein may assist the development of novel anticoagulation biomaterials.

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