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Preparation and Characterization of Controlled Heparin Release Waterborne Polyurethane Coating Systems*

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Abstract In this study, to improve hemocompatibility of biomedical materials, a waterborne polyurethane (WPU)/heparin release coating system (WPU/heparin) is fabricated *via* simply blending biodegradable WPU emulsions with heparin aqueous solutions. The surface compositions and hydrophilicity of these WPU/heparin blend coatings are characterized by attenuated total reflectance infrared spectroscopy (ATR-FTIR) and water contact angle measurements. These WPU/heparin blend coatings show effectively controlled release of heparin, as determined by the toluidine blue method. Furthermore, the biocompatibility and anticoagulant activity of these blend coatings are evaluated based on the protein adsorption, platelet adhesion, activated partial thromboplastin time (APTT), thrombin time (TT), hemolysis, and cytotoxicity. The results indicate that better hemocompatibility and cytocompatilibity are obtained due to blending heparin into this waterborne polyurethane. Thus, the WPU/heparin blend coating system is expected to be valuable for various biomedical applications.

Keywords: Waterborne polyurethane; Heparin; Blend coating; Hemocompatibility.

INTRODUCTION

In the past three decades, numerous synthetic polymers have been widely applied in biomedical fields, such as artificial hearts, vascular grafts, catheters, inter-aortic balloons, and extracorporeal circuits[1–3]. Polyurethanes (PUs) have attracted more and more attention for their excellent mechanical and biological properties among them[4, 5]. However, like most materials, once PUs make contact with blood, protein adsorption immediately occurs at the interface, resulting in platelet adhesion and other cellular interactions[6]. Various approaches have been developed in subsequent researches, such as grafting polyethylene oxide (PEO) or PEO-sulfonate to the surface and incorporation of bioactive agents on the surface to enhance the hemocompatibility of PUs[7–10]. However, these approaches could hardly overcome the anticoagulation of blood-contact materials.

As an important anticoagulant, heparin is a glycosaminoglycan composed of chains of alternating uronic

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acid and residues of D-glucosamine[11], of which anticoagulant activity is determined from a unique pentasaccharide that contains a high-affinity binding sequence to antithrombin III[12]. Thus, heparin is extensively applied to minimize thrombus formation on various biomaterials. Currently, there are two main methods used to modify biomaterials with heparin. One is the chemical immobilization of heparin. Young *et al*. introduced a carboxylic acid group on the poly(ethylene terephthalate) (PET) surface by grafting with acrylic acids (AA)[13], and then the grafting product (PET-AA) was bonded with heparin by PEO as a spacer. This method can provide long-term antithrombogenicity[14–16], but it may induce the conformational changes of heparin because of bounding to the surface by the functional group or spacer arms, resulting in a significant decrease in its anticoagulant activity. The other method is the employment of a heparin-release system based on a polymer. In the heparin-release system, heparin can be slowly released over a defined period while maintaining high bioactivity[17]. Except for its anticoagulant properties, the released heparin can also regulate some biological pathways involved in signal transduction, cell-cell recognition, and growth processes[18]. Therefore, most researchers have shown interest in heparin-release systems to improve hemocompatibility of biomaterials. Marconi *et al*. synthetized a novel PU consisting of quaternary ammonium to adsorb a high amount of heparin through ionic bonding[19]. The heparin-deoxycholic acid conjugate was homogeneously dispersed as nanoparticles in PU through the co-solvent of dioxane, water, and propanol[15]. To obtain a novel heparin-functional PU microsphere, the phase separation method was developed by Tong *et al*. Heparin can be simply dissolved in water or formamide, however, they are not suitable solvents for most polymers[20].

In our previous study, a series of nontoxic biodegradable waterborne polyurethanes (WPUs) have been developed using isophorone diisocyanate (IPDI), 1,4-butandiol (BDO), L-lysine, poly(ethylene glycol) (PEG), and poly(ε-caprolactone) (PCL)[21]. Due to their nontoxic degradation products and good mechanical properties, the WPUs have exhibited great potential for biomedical applications, such as drug delivery, biodegradable WPU elastomer, and porous tissue engineering scaffolds[21–23].

Herein, to overcome the blending issues between heparin and polymers, the WPU emulsions are utilized to blend with heparin because water serves as a good solvent for heparin. Nontoxic components: IPDI, PEG, PCL, L-Lysine are the main structure compositions of the synthetic polyurethanes, thus this blend system is simple, efficient, and nontoxicity. These blend coating surfaces are characterized by attenuated total reflectance infrared spectroscopy (ATR-FTIR) and water contact angle (WCA) measurements. The hemocompatibility aspects are evaluated by the protein adsorption, platelet adhesion and hemolysis. Activated partial thromboplastin time (APTT), thrombin time (TT) and cytocompatibility are investigated as well.

EXPERIMENTAL

Materials

Porcine heparin (molecular weight, 6000–20000), toluidine blue and bovine serum albumin (BSA; fraction V) were purchased from Aladdin, and used without purification. Fibrinogen (FG; fraction I human) was purchased from Sigma and used without purification. PEG (molecular weight, 1450) and PCL (molecular weight, 2000; Dow Chemical, USA) were dehydrated at 70–80 °C under vacuum for 2 h before use. IPDI (BASF, Germany) was redistilled under vacuum before use. Micro BCA™ Protein Assay Reagent kits were obtained from PIERCE. L-Lysine and other chemical reagents and solvents of reagent grade were obtained from commercial suppliers and used without additional purification.

Synthesis of Waterborne Polyurethane

A two-step method was utilized for the synthesis of WPU. The feed ratios are shown in Table 1. First, a prepolymer was synthesized by PEG, PCL, and IPDI in the presence of a small amount of organic bismuth. Second, the prepolymer was added to lysine solution for emulsification under high-speed stirring (1000 r/min). Concurrently, the dilute sodium hydroxide solution was slowly added to the polymer solution to neutralize the carboxyl group of the chain extender lysine[21, 22].

Table 1. Contents of synthetic WPU and WPU/heparin blend coatings

Samples [a]	Feed molar ratio				Heparin (mg/mL)
	IPDI	PCL	PEG	Lysine	
WPU	3.00	0.75	0.25	1.70	0.0
WPU-1h	3.00	0.75	0.25	1.70	1.0
WPU-3h	3.00	0.75	0.25	1.70	3.0
WPU-9h	3.00	0.75	0.25	1.70	9.0

[a] WPU/heparin blend coatings were denoted as WPU-*X*h, where h is for heparin, and *X* is for the concentration of heparin solution.

Preparation of WPU/Heparin Blend Coatings

WPU was added into isochoric heparin at different concentrations (1 mg/mL, 3 mg/mL, 9 mg/mL) as listed in Table 1 under ultrasonic vibration and stirring at room temperature to obtain various concentrations of WPU/heparin blend coatings (denoted as WPU-1h, WPU-3h, WPU-9h). Then, the solution was spread on a cover glass and the water was evaporated at 37 °C. Finally, the coatings were dried in a vacuum oven at 37 °C for 24 h for testing.

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy of WPU/Heparin Blend Coatings

The surface chemistry of WPU and WPU/heparin coatings was characterized by ATR-FTIR using a Tensor-27 spectrophotometer at 4000–600 cm^{-1} with a resolution of 4 cm^{-1} (Tensor-27; Bruker, Germany).

Water Contact Angle

The hydrophilicity of WPU and WPU/heparin coating surfaces was characterized on the basis of WCA measurements using a contact angle goniometer (Kruss; Hamburg, Germany) equipped with a video camera. The measurement was carried out by putting a drop of deionized water onto the air-facing side of the coating at room temperature, and the WCAs as a function of the contact time were performed and recorded. Each specimen was tested for three replicates.

Release Test of Heparin from WPU/Heparin Blend Coatings

The heparin release from blend coatings was determined using the toluidine blue method[24]. 2 mL of heparin release aqueous solution was added to 3 mL of the toluidine blue solution for 2 h. Then, 3 mL of hexane was added and shaken well. The absorbance values of the aqueous phase were measured by a UV-1800PC spectrophotometer (Shanghai Mapada Instrument Co. Ltd., China) at 631 nm, and the amount of released heparin in the aqueous phase was calculated based on the standard curve of heparin.

Protein Adsorption

The protein adsorption tests were executed with 1 mg/mL BSA or 0.1 mg/mL FG dissolved in isotonic PBS. The coating samples (1 cm × 1 cm) were incubated in the solution for 24 h at 4 °C and then immersed in the protein solution for 2 h at 37 °C. After incubation, the samples were gently rinsed with PBS and deionized water and then transferred to a 2 wt% sodium dodecyl sulfate (SDS; 0.05 mol/L NaOH) solution at 37 °C for 30 min under mechanical shock to remove the adsorbing proteins. Finally, the proteins eluted in the SDS solution were quantified using a Micro BCA protein assay reagent kit, and the amount of protein adsorption was calculated with reference to the standard curve of BSA.

Blood Collection

Blood samples were obtained from a healthy experimental rabbit provided by the Huaxi Animal Center. The blood was centrifuged to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP).

Platelet Adhesion

To evaluate the platelet adhesion and activation, the coatings were immersed in phosphate buffered saline (PBS) at 37 °C for 1 h. After removing the PBS solution, 1 mL PRP was added. After incubating for 1 h, the PRP was removed and the samples were washed in PBS for 5 times. Then, 2.5 wt% glutaraldehyde was added at 25 °C for

2 h. Finally, the coatings were washed in PBS solution and dehydrated with alcohol. Platelet adhesion and activation were observed under a scanning electron microscope (SEM; Inspect F; FEI Company). Each sample was observed 3 times by using three individual specimens.

Hemolysis Ratio

First, after washing with physiological saline solution, the samples (1 cm × 1 cm) were immersed in 10 mL of the same solution at 37 °C for 30 min. Then, 200 μL of blood samples, consisting of fresh rabbit blood (4 mL) and physiological saline solution (5 mL), was added to the mixture at 37 °C for another 60 min. Finally, the mixture was centrifuged at 2500 r/min for 10 min, and 2 mL supernatant sample was analyzed using ultraviolet spectroscopy at 540 nm. The percent hemolysis was evaluated by using the following formula:

$$\text{Hemolysis rate}=\frac{(\text{sample absorbance}-\text{negative control absorbance})}{(\text{positive control absorbance}-\text{negative control absorbance})}\times 100\%$$

Activated Partial Thromboplastin Time (APTT) and Thrombin Time (TT)

The APTT and TT values were determined using a CA-50 automatic blood coagulation analyzer (Sysmex Corporation; Kobe, Japan) and used to assess the antithrombogenicity of the prepared coatings. The coating samples (0.5 cm × 0.5 cm) were incubated in 0.2 mL PPP at 37 °C for 30 min, and then APTT and TT were measured. The experiment was performed three times for each sample.

Cell Viability Assay

Cytotoxicity of the coatings was evaluated by the 3-[4,5-dimethylthiazoylyl-2]-2,5-diphenyltetrazolium bromide (MTT) assay. Firstly, fibroblasts (L929 cells) were seeded in 96-well plates and incubated for 24 h. Then, the cells were incubated on the samples for 72 h, and 20 μL of MTT solution was added into each well. After incubating for 4 h, the MTT solution was removed and the insoluble formazan crystals were dissolved in 100 μL of dimethyl sulfoxide. Finally, the absorbance was measured at a wavelength of 490 nm. The cell viability was evaluated and normalized using untreated cells as a control[25].

RESULTS AND DISCUSSION

Surface Structures of Waterborne Polyurethane/Heparin Blend Coatings

A series of waterborne polyurethane/heparin blend coatings containing various heparin contents were obtained *via* direct blending the polyurethane emulsions and heparin aqueous solutions under ultrasonic vibration. Heparin contents in these blends are listed in Table 1. To investigate the fact that heparin was successfully dispersed in WPU, the water contact angles of WPU/heparin blend coatings were measured (Fig. 1), since heparin can generally improve the hydrophilicity of polymer surfaces[26]. Unexpectedly, although all the WCAs of blend coatings are smaller than that of WPU, the values increase with the increasing amount of heparin in the blend coating systems. That is to say, the hydrophilicity of WPU-1h is better than the other samples with higher heparin contents. The microstructure changes of blend coatings induced by the incorporation of heparin might be taken into account to explain this result. The heparin would immigrate and aggregate on the surface of the coating, which constructs a new microstructure. The new microstructures of the coating surfaces make the hydrophilicity decrease with increasing the content of heparin. This hypothesis will later be discussed on account of the SEM results. Besides, the surface compositions of blend coatings are further characterized by ATR-FTIR. As shown in Fig. 2, the absorption peak at 3360 cm^{-1} corresponds to the N—H stretching vibration, and the carbonyl stretching vibration is observed at approximately 1730 cm^{-1}. These characteristic absorption peaks are attributed to WPU. However, the peaks at 1066 cm^{-1}, which appear for WPU-1h, WPU-3h, and WPU-9h, correspond to the S=O of heparin, indicating that some heparin molecules have dispersed on the surfaces of these blend coatings.

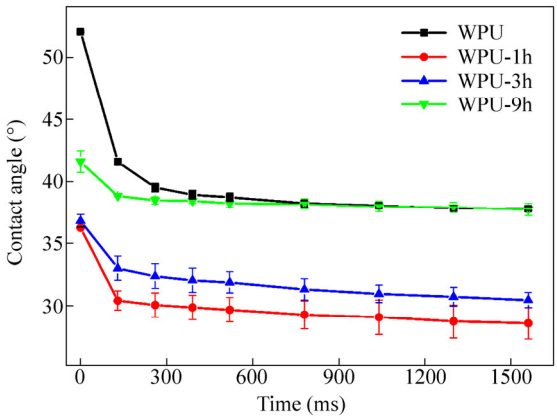

Fig. 1 Contact angle dynamics of WPU and WPU/heparin coatings (Error bars represent means ± standard deviation for $n = 3$.)

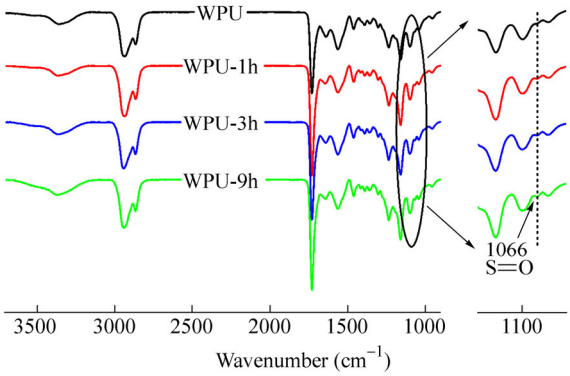

Fig. 2 ATR-FTIR spectra of WPU and WPU/heparin coatings

Biocompatible Evaluation of WPU/Heparin Blend Coatings

Nonspecific protein adsorption is an essential feature for blood-contacting biomaterials. When materials first contact blood, plasma proteins become adsorbed onto the materials surface in just a few seconds. Then, this adsorption induces aggregation and activation of platelets, and blood coagulation. As a result, hemocompatibility of biomaterials should be evaluated on the amount of protein adsorption. Albumin (BSA) is the most abundant protein in the plasma and has many functions, such as transporting small molecules in blood and maintaining the osmotic pressure of blood. Meanwhile, fibrinogen (Fg), one of the most important proteins in the plasma, plays a leading role in platelet adhesion on biomaterials and blood coagulation process. Herein, we evaluated hemocompatibility based on the amount of BSA and Fg adsorbed on the coating surfaces, using traditional biomedical polyether urethane (PEU) as a control. The BSA and Fg adsorption results are shown in Fig. 3. All of the experimental groups show lower amount of BSA adsorption and Fg adsorption compared with PEU. Interestingly, the amount of BSA adsorption and Fg adsorption on WPU-1h is the lowest among these samples, which is around 3.50 and 0.30 μg/cm^2, respectively. Since many factors can influence the protein adsorption on biomaterial surfaces, such as surface topography, surface roughness, surface charge, surface free energy and surface hydrophilicity. Among them, surface hydrophilicity is regarded as the most important factor. In this research, the results of BSA and Fg adsorption are consistent with those of water contact angles, suggesting that high hydrophilicity of the surface brings about less protein adsorption again[8, 27].

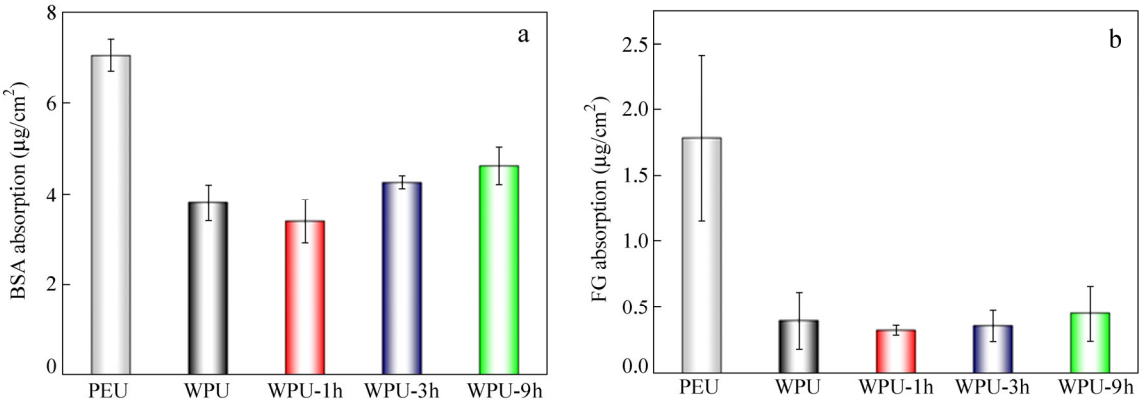

Fig. 3 (a) BSA adsorption and (b) FG adsorption of PEU, WPU and WPU/heparin samples (Error bars represent means ± standard deviation for $n = 3$.)

On the other hand, platelet adhesion on the coating surfaces and platelet activation were also observed using SEM. The SEM images of platelet adhesion and activation of different samples are shown in Fig. 4. For PEU (the front two pictures of Fig. 4), a large number of platelets adhere and aggregate on the PEU surface. Some platelets even show irregular shapes and grow up to the pseudopodium, which indicates the high activation of platelets on the surface. In the experimental series, barely any platelets are observed on the surfaces of samples. As discussed above, this high anti-adhesion property should result from the low Fg protein adsorption and good surface hydrophilicity. Moreover, comparing the microstructures of blend coatings, quite distinguishing surfaces are observed. The surface of WPU looks like smooth and hard, while the surface of WPU-1h containing a few scrobiculus shows a little rougher than WPU. Apparently, the surface of WPU-3h exhibits serious rough with

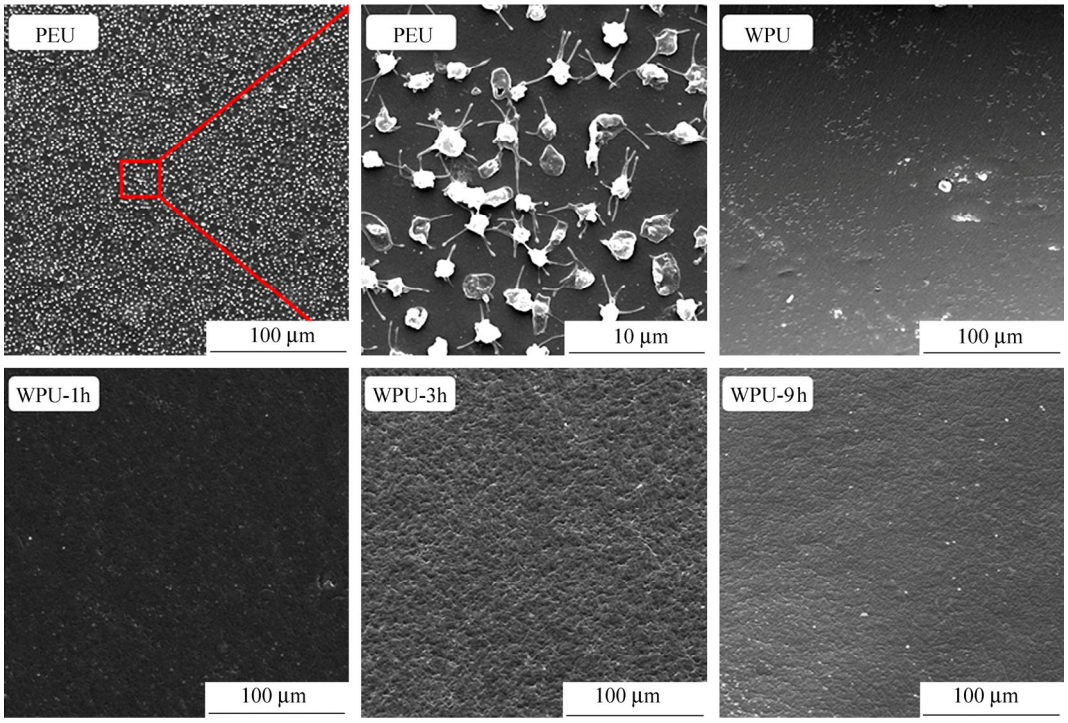

Fig. 4 SEM images of PEU, WPU, and WPU/heparin samples for evaluating platelet adhesion

a large amount of scrobiculus. At last, the surface of WPU-9h also contains many scrobiculus but smoother than that of WPU-3h. As mentioned above, the different microstructures of blend coatings might be one of the reasons for the results of WCAs.

As the material makes contact with blood, the membranes of red blood cells may become damaged, leading to the release of hemoglobin and other internal components. Therefore, hemolysis testing is primarily carried out to investigate the extent of hemoglobin loss. In general, blood-contacting materials with less than 5% hemolysis are considered to be nontoxic[28]. In the present study, WPU exhibits the highest ratio of hemolysis at 1.4%, as shown in Fig. 5, which is substantially less than the 5% cutoff point. The hemolysis rate tends to decrease with increasing the content of heparin. The results demonstrate that all the WPU/heparin blend coating systems possess excellent hemocompatibility.

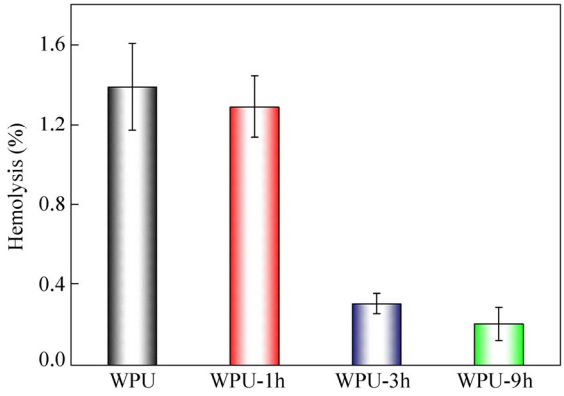

Fig. 5 Hemolysis of WPU and WPU/heparin coating (Error bars represent means ± standard deviation for n = 3.)

Simultaneously, cell viability is evaluated by incubating the L929 cells on the surfaces of different samples for 72 h to investigate the cytotoxicity. As shown in Fig. 6, cell viabilities of all samples exceeded 90%, with cytotoxicity grade 1, after culturing for 1 d or 3 d. Meanwhile, the degradation products of the WPU and biodegradable polyurethanes with similar structures have shown no cytotoxicity *in vivo* in our previous research[21]. Thus, the blend coatings possess the potential for controlled release of heparin *in vivo*.

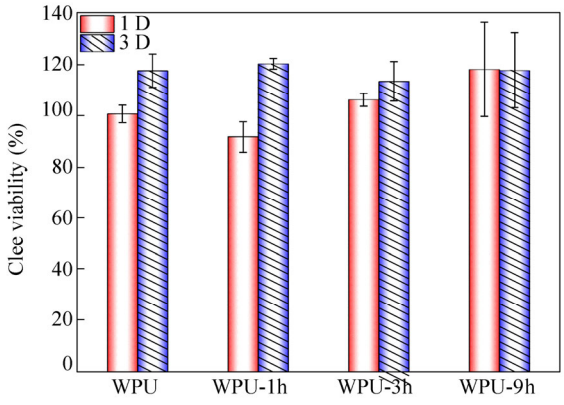

Fig. 6 Cell viability of WPU and WPU/heparin coating surfaces for culturing 1 day and 3 days

Heparin Release and Antithrombogenicity of WPU/Heparin

Figure 7 shows the accumulated release amount of heparin over time. There is no remarkably initial burst release of heparin for WPU-1h and WPU-9h in the first 50 h. Especially, after 4 weeks of releasing, 36.5% and 40.2% of heparin is only released from WPU-1h and WPU-9h, respectively. The long-term release behavior of heparin

makes the blend coatings can be used for medium-time application. Unfortunately, WPU-3h releases 70% of its heparin within one day and 90% within 4 weeks, the release rate is much higher than that of the other samples. The release behavior of heparin also certainly depends on the microstructure of the blend coatings. As shown in Fig. 4, the surface of WPU-3h is the roughest of all the samples containing largest scrobiculus. Thus, the heparin is fast released into the solution. For WPU-1h and WPU-9h, the surface roughness of WPU-9h is a little higher than that of WPU-1h, so that the release rate of heparin for WPU-9h is faster than that for WPU-1h. Doubtlessly, this interesting phenomenon may be related with many other factors that still need further investigation.

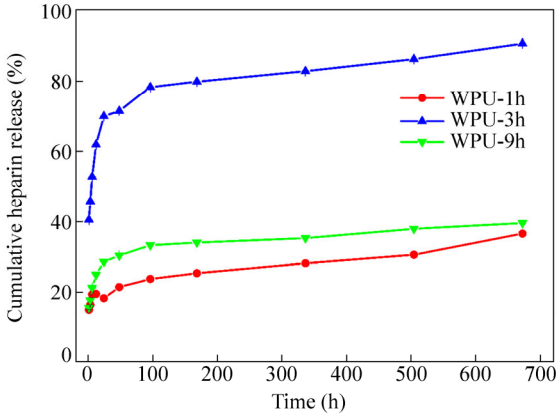

Fig. 7 Heparin release of WPU-1h, WPU-3h, and WPU-9h blend coatings

APTT and TT are used to characterize antithrombogenicity of WPU/heparin coatings. The APTT and TT of rabbit blood measured are approximately 25.95 s and 28.70 s, as shown in Table 2. Although WPU shows excellent resistance to protein adsorption and platelet adhesion, it still does not satisfy the requirements of anticoagulant properties, of which APTT and TT are extremely close to those of rabbit blood (Table 2). In contrast, the APTTs and TTs for WPU-1h, WPU-3h, and WPU-9h markedly increase, as shown in Table 2 as "◊". This means that the clotting time is above the max setting value of the CA-50 automatic blood coagulation analyzer. When a cloud point cannot be found within 10 min, the system outputs the result "no coagulation." The results indicate that the WPU/heparin blend coating system has a prominent anticoagulant effect. Again, the coating samples are immersed in PBS for 30 days, and then the APTT (APTT-30d) and TT (TT-30d) are tested. The APTT and TT are only 43.10 s and 31.50 s, respectively, for WPU-1h. The data show that the heparin content on the surface of WPU is not sufficient to maintain its anticoagulant activity. For WPU-3h, the APTT is only 55.10 s and TT shows no coagulation. Since over 90% of the heparin is released from WPU-3h (Fig. 7), the anticoagulant activity reduces. However, WPU-9h can maintain excellent anticoagulant activity after 30 days of immersion in PBS (Table 2). The long-term anticoagulation makes this system have the potential to be applied in devices designed for medium-time use.

Table 2. APTT and TT of WPU and WPU/heparin coatings

Samples	APTT (s)	TT (s)	APTT-30d [a] (s)	TT-30d [b] (s)
Blood	25.95 ± 0.21	28.70 ± 0.28	–	–
WPU	30.40 ± 0.57	25.80 ± 0.42	30.10 ± 0.56	27.35 ± 0.35
WPU-1h	◊	◊	43.10 ± 0.14	31.50 ± 0.71
WPU-3h	◊	◊	55.10 ± 0.84	◊
WPU-9h	◊	◊	◊	◊

[a] APTT-30d refers to the APTT of the coating sample immersed in PBS for 30 days; [b] TT-30d refers to the TT of the coating sample immersed in PBS for 30 days; ◊ refers to no coagulation.

CONCLUSIONS

A novel waterborne polyurethane/heparin release coating system was developed to enhance hemocompatibility of biomedical materials. The amount of heparin in the coatings could be controlled by adjusting the concentration of heparin solution mixed with the synthetic waterborne polyurethane emulsions, and heparin could be facilely dispersed into polyurethane coatings, as verified by ATR-FTIR and water contact angle measurements. These WPU/heparin blend coatings displayed various release profiles of heparin in PBS, and showed no cytotoxicity, low protein adsorption and hemolysis, no platelet adhesion, and excellent anticoagulant effects. Even the excellent anticoagulant activity of high heparin content blend coatings could be maintained over one month. Considering all these results, the controlled heparin release waterborne polyurethane coating systems hold great promise as coatings to reduce thrombin formation for blood contact biomaterials.

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