Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(10):707-713

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Polyhemoglobin-rPA, a novel reteplase modification form for *in vivo* circulation time extension

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ABSTRACT

Reteplase (r-PA), as a plasminogen activator, has shown thrombolytic efficacy for thrombosis diseases. However, due to its short circulation time, double-bolus intravenous injection is required to maintain blood drug level. Improper dosing would bring about serious bleeding risk. Here we report a Polyhemoglobin-rPA preparation by introducing polymerized bovine hemoglobin (PolyHb) to crosslink r-PA with bifunctional reagent glutaraldehyde. Modified r-PA is more stable and possesses longer circulation time after intravenous injection. Results show that after intermolecular polymerization with hemoglobin, circulation half life span of r-PA was increased more than 65%. In vivo study demonstrated that, PolyHb-rPA significantly attenuated the severity of mice hemiparalysis and effectively recanalize rat embolism model. PolyHb-rPA could lead to r-PA administration dosage reduction, sustaining blood drug level, and bleeding risk decrease, which shows promise for its potential role as a protective therapeutic agent in clinical situations of myocardial infarction, cerebral thrombosis and other cardiovascular disease.

Keywords: Polyhemoglobin-rPA, reteplase, polymerization, circulation time, thrombolysis

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INTRODUCTION

Reteplase (Retavase, r-PA) is presently the preferential plasminogen activator agent for the treatment of myocardial infarction, cerebral thrombosis and other cardiovascular disease [1, 2]. As a third-generation recombinant form of Tissue plasminogen activator (t-PA), the plasminogen activator precipitates thrombolysis by catalyzing the cleavage of endogenous plasminogen to generate plasmin. r-PA is superior to t-PA for the former showed more extensive clot permeation, greater retention and lysis[3].

However, the half-life span of reteplase was less than 20 min, and the administration of r-PA was confined to perform as a double-bolus (30 min apart) intravenous injection in order to maintain blood drug level and reduce bleeding risk [3]. Improper dosing would cause serious bleeding problem. Here we report a PolyHb-rPA preparation by introducing bovine hemoglobin (Hb) to crosslink r-PA with bifunctional reagent glutaraldehyde. Modified r-PA is more stable and possesses longer circulation time after intravenous injection. Bovine hemoglobin, as the supporter macromolecule of r-PA, has little immunogenicity between biological species. Even in the polymerized form (PolyHb), there is no increase in antibody titers using the very severe test of Freud's adjuvant [(4)]. Furthermore, bovine Hb does not require 2,3-diphosphate glyceric acid to adjust oxygen-Hb dissociation, and P_{50} values of polymerized bovine Hb is very close to natural human hemoglobin [4, 5]. The size of PolyHb-rPA complex is

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hundreds times smaller than natural red blood cells. So, PolyHb-rPA can easily pass through narrow blood vessel at early thrombosis and provide oxygen supply to the ischemic tissue after embolization [6, 7]. Due to half-life span extension of r-PA after polymerization, the possibility of a lower dosage administration arises. And the risk of bleeding would be further reduced.

EXPERIMENTAL SECTION

Reteplase (Retavase, r-PA) (0.5 IU/mg) was purchased from Boehringer Mannheim Corporation. Glutaraldehyde (25%), lysine (monohydrochloride, SigmaUltra >99%, S2288 substrate, and pentobarbital sodium were obtained from Sigma-Aldrich Chemical Co. All other chemicals and reagents used were of analytical grade.

Experimental animals

Wistar rats (250-275 g), Kunming Mice (18-20 g) were supplied by Experimental Animal Center of Shandong University. The rats and mice were fasted but allowed free access to water 12 h prior to experiment. The environment was maintained at 22°C with a 12 h light and dark cycle. The animal care and use were in accordance with guidelines of Shandong University.

Stroma-free Hemoglobin (SFHb) Preparation

Fresh bovine blood with heparin as an anticoagulant was centrifuged at 2000×*g* for 30 min at 4°C. The plasma supernatant and the upper layer of the red blood cell pellet that contains the buffy coat were removed. The sedimented red blood cells were washed four times with sterile, ice-cold 0.9% NaCl. Red blood cells were then suspended in 2 packed-cell volumes of potassium phosphate, 12.5 mM, pH 7.4, and mixed thoroughly. After 30 min for lysis to occur, the stroma lipid and in suspension was removed with 1/2 volumes of ice-cold reagent-grade toluene two times. Cellular debris was separated by centrifugation at 15000×*g* for 2 h at 4°C. The solution was aliquoted and stored at -80°C [6].

Preparation of polyHb-rPA

Polymerization reactions began with 10.0 g/dL hemoglobin and variety of r-PA (20-200 IU)in 10.0 mM potassium phosphate buffer containing 50 mg/mL sucrose and 0.5% Tween-80, pH 7.0. Prior to the start of crosslinking, 1.3 M of lysine was added at a molar ratio of 10:1 lysine/hemoglobin. Crosslinking reaction was started with the addition of glutaraldehyde (0.5 M) at molar ratio of 17:1 glutaraldehyde/hemoglobin. Glutaraldehyde was added in four equal aliquots over a period of 15 min. After 4 h of crosslinking with constant stirring under aerobic conditions at 4°C, the reaction was stopped with the addition of excess lysine at a molar ratio of 100:1 lysine/hemoglobin. Solutions were centrifuged at 15000×*g* for 1 h. Then the supernatants were dialyzed at 4°C against Ringer's lactate solution overnight and passed through a sterile 0.45 mm micron filter [4, 7].

Determination of Reteplase (Retavase, r-PA) activity

Chromogenic assay with some modifications was used to determine the r-PA activity. Chromogenic assays of rPA were performed using synthetic substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-dehydrochloride (S2288, Sigma). Free form of rPA and polyHb-rPA were added to the wells of a microtiter plate with trypsin inhibitor. An aqueous solution of S2288 was added at 2 mM in a reaction buffer containing 100 mM Tris, 100 mM NaCl, 0.02% sodium azide. Color development was monitored at 405 nm using a plate reader (Biotek) [8].

Determination of plasma circulation half-time of r-PA in rats

Male Wistar rats were first anaesthetized with intraperitoneal injection of sodium pentobarbital (65 mg/kg). Polyethylene cannulae were inserted and secured distal to the superficial epigastric branches in the femoral arteries and veins. And then free form of rPA and polyHb-rPA samples were injected into the vein catheter using a syringe with injection flow rate at about 0.5 mL/min. After injection of thrombolysis samples, the blood samples were taken as desired and centrifuged at 1000×*g* for 10 min. Supernatants were collected for r-PA activity measurements. When monitoring procedures were completed, catheters were removed, the vessels were ligated, and local skin was sutured [6].

Murine embolism model establishment

To assess biologic activity in vivo, we developed murine model for embolism. Briefly, Collegen was soaked, homogenized, and diluted to 1.0 mg/mL, then mixed with 40 μg/mL adrenaline. The mixture was further blended with various concentrations of poly-rPA and was injected into tail vein of male Kunming mice. The administration volume is 0.1 mL/10g (Weight). Mice mortality rate was monitored in 5 min of administration. And the number of recovery from hemiparalysis was monitored in 15 min of administration.

Rat embolism model establishment

Male Wistar rat embolism model was constructed through subcutaneous injection of carrageenin (10.0 mg/kg) into

foot plantar of posterior limb. The tip of the tail skin color was monitored to determined thrombosis conditions (Room temperature: 22 ℃, relative humidity: 40%). When the length of thrombus reached to 4.0 cm, variety of concentrations of PolyHb-rPA was intravenous administrated immediately through dorsal vein. Observation: Recanalization rate (%) and length of thrombus (cm) were determined by color change of the rat tail skin in 6 h of intravenous injection. Tail thrombosis conditions were further analyzed by pathological observation.

Statistical analysis

Statistical analysis of differences between groups was performed by using the exact probability method. Average thrombus lengths comparison between groups after the treatment was examined by using unpaired t-test. $P<0.05$ was considered statistically significant. $P<0.01$ was considered statistically extremely significant.

TABLE 1. PREVENTION OF POLYHB-RPA AGAINST EMBOLISM FORMATION INDUCED BY COLLEGEN-ADRENALINE

Dosage（IU/kg）	Number of mice	Mice mortality number in 5 min	Recovery number from hemiparalysis in 15 min	Recovery rate from hemiparalysis in 15 min（%）
0.2	20	4	9	45
0.4	20	3	12	60
0.8	20	0	17	85
Solvent control	20	5	0	0

TABLE 2. THROMBOLYSIS OF POLYHB-RPA ON RATS TAIL THROMBOSIS

Dosage（IU/kg）	Number of rats for thrombosis	recanalized rats number from thrombosis	Recanalization rate（%）	Length of thrombuses（cm）
0.2	10	4	40	1.34±0.85**
0.4	10	4	40	1.06±0.97**
0.8	10	7	70	0.51±0.47**
Solvent control	10	0	0	5.8±0.63

P<0.05;P<0.01*

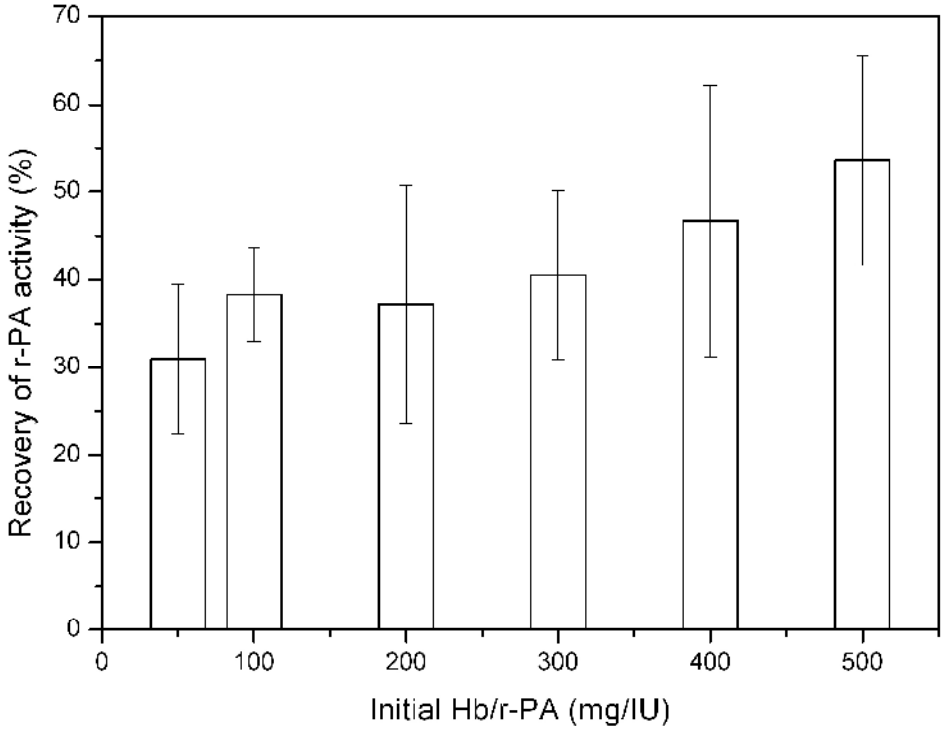

Fig. 1. r-PA activity retained following polymerization with stroma-free hemoglobin

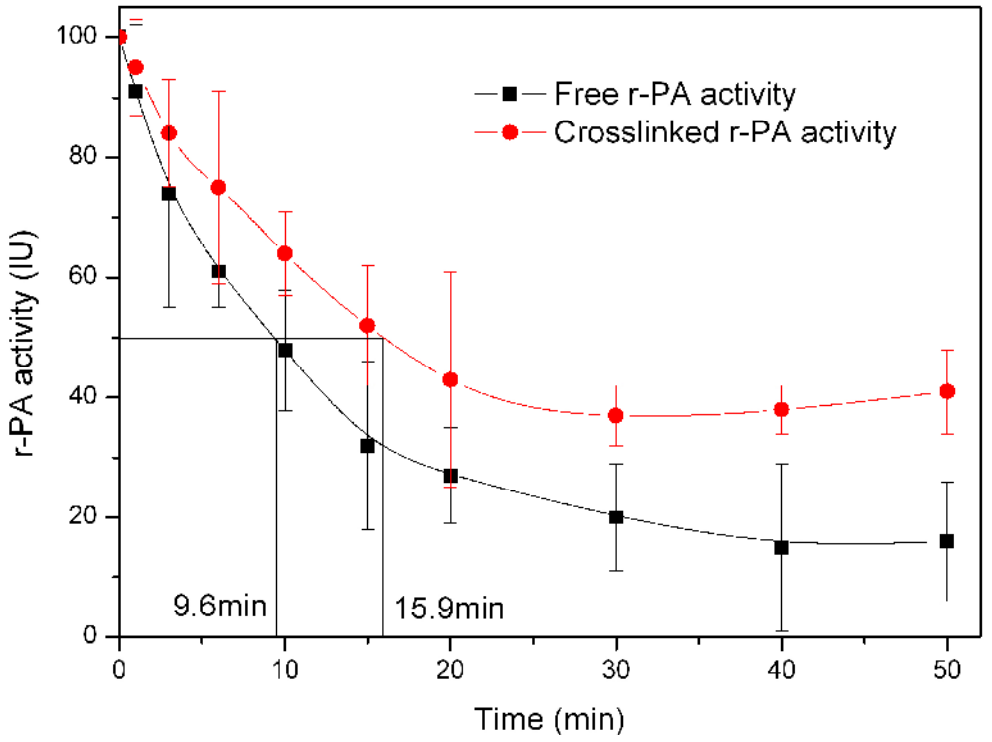

Fig. 2. Plasma circulation half-time of r-PA in rats. Free form of r-PA or crosslinked form of r-PA (PolyHb-rPA) was injected intravenously into anesthetized Male Wistar rats. Blood samples were collected every 1-10 min. The activities of r-PA (units) remaining in the plasma were measured by chromogenic assay
Administration dosage: 3 IU r-PA/kg, 300 mg Hb/kg.
Polymerization condition: Hb/r-PA=100/1, final glutaraldehyde concentration: 2.5%.

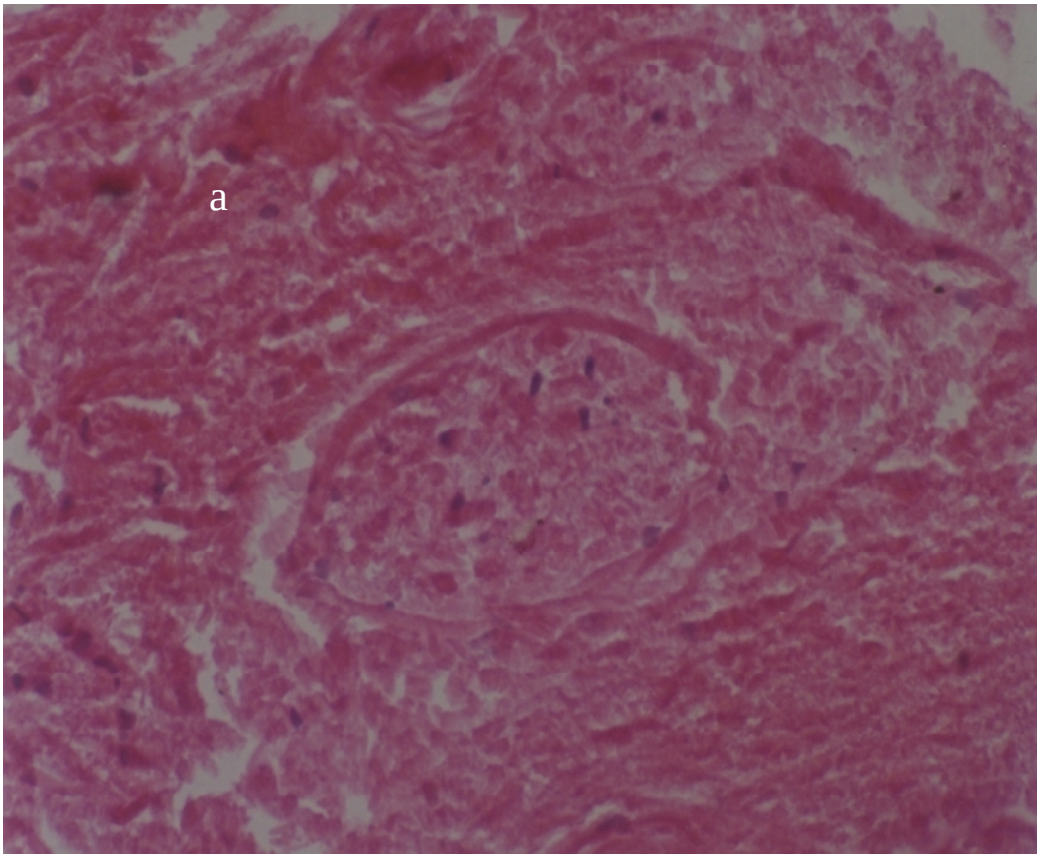

Fig. 3a. Pathological section graph of rats tail thrombosis induced by carrageenin(Vessel was completely blocked) (×400)

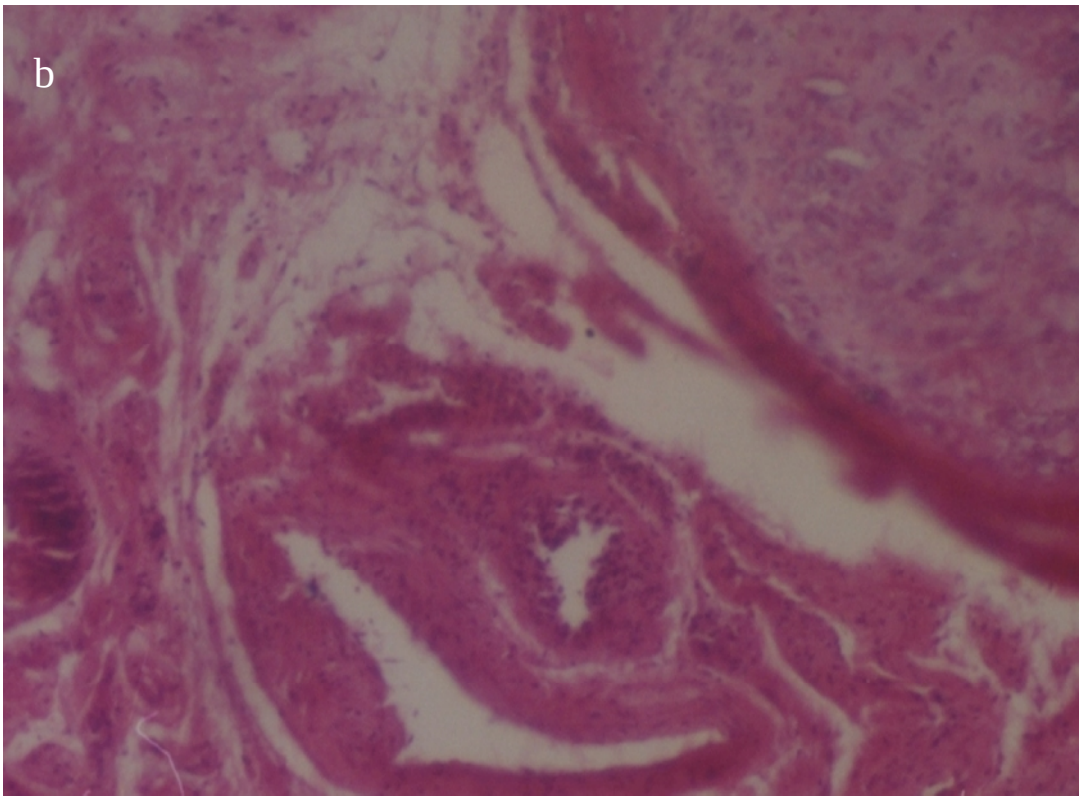

Fig. 3b. Pathological section graph of rats tail vein recanalization from thrombosis (Vessel wall became thicker and the quantity of endotheliocytes increased) (×400)

RESULTS

r-PA ctivity Retained Following Polymerization

As shown in Fig. 1, Increasing amounts of r-PA were added into stroma-free hemoglobin solution before polymerization. The ratio of Hb to r-PA varied from 50:1 to 500:1(mg/IU). After cross-linking with glutaradehyde (molar ratio 17:1 glut:Hb), 30-55% of the r-PA original activity was recovered. Moreover, the recovery rate was increased along with growth of Hb/r-PA ratio.

Plasma circulation half time of PolyHb-rPA in rats.

Fig. 2 demonstrated the increased circulation time of the crosslinked r-PA in rats. Free form r-PA was cleared from the circulation in rats with a half life ($t_{1/2}$) of about 9.6 min, whereas in the crosslinked form the circulation half life of r-PA was extended to 15.9 min. After intermolecular polymerization with hemoglobin, circulation half life of r-PA was increased more than 65%.

Prevention of PolyHb-rPA against embolism formation induced by collegen-adrenaline

As shown in Table 1, collegen-adrenaline effectively induced mice thrombosis in our embolism model. Injected mice displayed obvious hemiparalysis features. 25% of injected mice died in 5 min of after collegen-adrenaline treatment. In contrast with solvent control group, number of mice mortality remarkably decreased in PolyHb-rPA administration group. Recovery rate from hemiparalysis in 15 min reached to 85% after 0.8 U/kg of PolyHb-rPA was injected. And recovery rate showed apparent linear relationship with poly-rPA administration dosage.

Thrombolysis of PolyHb-rPA on rats embolism model

As Table 2 displays, carrageenin effectively induced rats tail thrombosis in our embolism model (Fig. 3a). None of solvent control group was recanalized without PolyHb-rPA administration. On the other hand, thrombolysis occurred immediately after PolyHb-rPA treatment. Tail thrombosis was completely recanalized in 2-6 h (Fig. 3b). And recanalization rate showed remarkable linear relationship with PolyHb-rPA administration dosage. Recanalization rate from thrombosis was 70% after 0.8 U/kg administration of PolyHb-rPA. Even for the non-recanalization models, lengths of thrombuses were significantly reduced (Table 2).

DISCUSSION

Reteplase (r-PA) has shown thrombolytic efficacy for peripheral vessel occlusions, acute ischemic stroke, occluded catheters or grafts, and massive pulmonary embolism[9]. In large randomized clinical trials of patients with ST-segment elevation myocardial infarction (STEMI), r-PA was superior to alteplase for coronary artery patency. There was reduced incidence of some cardiac events with r-PA versus streptokinase. But no significant difference appeared between r-PA, alteplase and streptokinase recipients patients on 35-day mortality rate and incidence of intracranial bleeding. Moreover, there was a greater incidence of hemorrhagic stroke for r-PA agents [10].

Incidence of bleeding complications associated with r-PA treatment appeared to be similar to that associated with other thrombolytic agents. Controlled-release introduction is able to effectively reduce the amount of r-PA administration, and better maintain blood drug level, thereby efficiently reduce bleeding risk. Here we have employed the principle of nanobiotechnology to assemble r-PA and Hb molecules into nanodimenion PolyHb-rPA using glutaraldehyde so as to elongate circulation life span of r-PA. The glutaraldehyde method has been developed independently by other groups for clinical trials [11]. Gould's group has carried out Phase III clinical trials on 171 patients showing that polyHb can successfully replace extensive blood loss in trauma surgery by maintaining the Hb level at the 8 to 10 g/dL needed for safe surgery with no reported side effects [(11)]. In North America, polyhemoglobin has already been approved for compassionate uses in patients, and in South Africa and Russia, this polyHb has been approved for routine use in patients [7, 12].

In our study, r-PA was crosslinked with hemoglobin by glutaraldehyde, and 30-55% of the r-PA original activity was retained after crosslinking reaction (Fig. 1). Crosslinking the enzyme to polyhemoglobin is important because otherwise, free r-PA is removed rapidly from the circulation with a half-time of less than 10 min (Fig. 2). In the form of PolyHb-rPA, these enzymes circulate with a half-time more comparable to PolyHb which is about 16 min in rats (Fig. 2), corresponding to 28 min in human. Results demonstrate that half life span of r-PA was effectively improved more than 65% after intermolecular polymerization with hemoglobin.

We have also carried out studies on prevention of PolyHb-rPA against embolism formation and thrombolysis in both murine embolism model and rat embolism model. PolyHb-rPA significantly attenuated the severity of mice hemiparalysis induced by collegen-adrenaline. For rat tail thrombosis induced by carrageenin, recanalization rate from thrombosis was 70% after 0.8 U/kg administration of PolyHb-rPA in 2-6 h. Even for the non-recanalization models, lengths of thrombuses were obviously reduced (Table 2).

The elongation of rPA circulation half life span using PolyHb-rPA probably leads to r-PA dosage reduction, sustaining blood drug level, and bleeding risk decrease. In addition, PolyHb-rPA efficient attenuation of thrombosis injuries shows promise for its potential role as a protective therapeutic agent in clinical situations of myocardial infarction, cerebral thrombosis and other cardiovascular disease.

Acknowledgments
This work was supported by the National Science and Technology Major Projects of New Drugs (2013ZX09103003-002), General Program Grant from the National Natural Science Foundation of China (Grant No. 31370090, 31070046), Program Grant from Natural Science Foundation of Shandong Province (Grant No. ZR2010CM002), and 5150 High Level Talent Project (B01692012006).

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