Original Paper

Poly (Ethylene Glycol)-*Block*-Brush Poly (L-Lysine) Copolymer as an Efficient Nanocarrier for Human Hepatocyte Growth Factor with Enhanced Bioavailability and Anti-Ischemia Reperfusion Injury Efficacy

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Key Words

Poly (ethylene glycol)-*b*-brush poly (L-lysine) copolymer • PEG-*b*-P(ELG-*g*-PLL) • Human hepatocyte growth factor • Ischemia/reperfusion injury • Different organs

Abstract

Background/Aims: The aim of this study was to assess the effect of human hepatocyte growth factor (hHGF)-loaded poly (ethylene glycol)-*b*-brush poly (L-lysine) (PEG-*b*-P(ELG-*g*-PLL)) copolymer on ischemia/reperfusion (I/R) injury to different organs. **Methods:** The isoelectric point (pI) of hHGF is 5.5, and hHGF combined with PEG-*b*-P(ELG-*g*-PLL) copolymer via electrostatic interactions at pH 7.4. The synthesized PEG-*b*-P(ELG-*g*-PLL) copolymer was analyzed using ¹H nuclear magnetic resonance (¹H NMR) and gel permeation chromatography (GPC). The hHGF/PEG-*b*-P(ELG-*g*-PLL) complex was evaluated using a nanoparticle size instrument and transmission electron microscopy (TEM). In addition, *vivo* performance of hHGF/PEG-*b*-P(ELG-*g*-PLL) complex was evaluated using plasma hHGF concentration and different organs ischemia reperfusion injury in rats. **Results:** An *in vitro* investigation showed that PEG-*b*-P(ELG-*g*-PLL) could serve as a potential hHGF nanocarrier with efficient encapsulation and sustained release. An additional *in vivo* investigation revealed that the hHGF/PEG-*b*-P(ELG-*g*-PLL) complex could prolong increases in plasma hHGF concentration and protect different organs (the brain, heart and kidney) against I/R injury. **Conclusion:** Poly (ethylene glycol)-*block*-brush poly (L-lysine) copolymer as an efficient nanocarrier for human hepatocyte growth factor with enhanced bioavailability and anti-ischemia reperfusion injury efficacy.

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Introduction

Human hepatocyte growth factor (hHGF), initially identified and cloned as a potent mitogen for hepatocytes, was shown to have mitogenic, antiapoptotic, angiogenic, and antifibrotic effects in multiple cell types, especially epithelial and endothelial cells [1-4]. Recent research has shown that hHGF can attenuate brain, heart and kidney ischemia/reperfusion (I/R) injury [5-7]. However, the half-life of hHGF is very short, and hHGF that enters the body is metabolized and rapidly cleared from tissues [8]. In addition, the biological activities of hHGF cannot be anticipated when the protein is injected in solution form because of its rapid excretion from the injection site by diffusion [9]. Thus, one possible method for enhancing the in vivo activity of hHGF is to control the release of hHGF over a prolonged time period by incorporating it into a copolymer nanocarrier.

In our previous study, we reported PEG-b-P(ELG-g-PLL) as a potential protein nanocarrier to prolong the in vivo half-life of a protein [10]. In the present study, because the isoelectric point (pI) of hHGF was 5.5, hHGF combined with PEG-b-P(ELG-g-PLL) copolymer through electrostatic interactions at pH 7.4. The encapsulated hHGF demonstrated sustained release, which facilitated the investigation of the protective action of hHGF. Thus, hHGF-loading capacity and in vitro release were measured. Via in vivo research, we sought to evaluate the sustained pharmacological function of hHGF/PEG-b-P(ELG-g-PLL) for protecting different organs against I/R injury in rats, using free hHGF as a control.

Materials and Methods

Materials

Sprague-Dawley (SD) rats (160-200 g) were purchased by Jiaxing University Medical College, Jiaxing, China. All reagents and drugs were supplied from Sigma.

Synthesis and cytotoxicity of block copolymer PEG-b-P(ELG-g-PLL)

Detailed methods for synthesizing and analyzing the cytotoxicity of the block copolymer PEG-b-P(ELG-g-PLL) are provided in our prior work [10].

Cytotoxicity of the block copolymer PEG-b-P(ELG-g-PLL)

As described in our previous article [10], EC9706 cells were cultured in a 96-well plate with 5×10^3 cells per well and incubated for 24 h. The original medium was removed, and MTT solution was added to each well. During incubation, MTT levels decreased in viable cells, leading to the precipitation of insoluble formazan crystals. The minimal essential medium was removed, and ethanol-dimethyl sulfoxide solution was added to each well. After the dark blue crystals of MTT formazan were completely solubilized (15-20 min), the absorbance of each well was measured at 570 nm using a multimode reader. A well without cells that was incubated with the MTT solution was used as the blank reference. PEG-b-P(ELG-g-PLL) was used as a positive cytotoxicity control. Cells cultivated in a well were used as a negative cytotoxicity control. Values were expressed as optical densities (ODs). The obtained absorbance values were subtracted from the absorbance of the ethanol-dimethyl sulfoxide blank and presented as percentages relative to the control, as detailed in equation 1:

$$(\%) \text{ Cell viability} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{cells}} - \text{OD}_{\text{blank}}) \times 100 \quad (1)$$

Encapsulation and in vitro release of hHGF in PEG-b-P(ELG-g-PLL)

Detailed methods can be found in our previous work [10].

Blood hHGF concentration evaluation

A total of 30 SD rats were administered a dose of 1 mg/kg hHGF or hHGF/PEG-b-P(ELG-g-PLL) by abdominal subcutaneous injection. Blood (0.2 mL) was extracted and transferred into heparinized tubes at specified time intervals. The blood was immediately separated by centrifugation ($10000 \times g$ for 20 min at 4°C) and stored at -80°C for the following analyses. The concentration of hHGF and hHGF/PEG b-P(ELG-g-PLL) in rat blood was measured by enzyme-linked immunosorbent assay (ELISA) following previous methods [11].

Animal model

(a) A total of 50 SD rats were assigned to five groups (ten rats in each group): 1) Sham group; 2) brain ischemia/reperfusion injury (I/R) group: I/R was achieved by clamping the arteria carotis communis for 2 h followed by 24 h of reperfusion; 3) hHGF group: hHGF was administered once per day at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure; 4) hHGF/PEG-b-P(ELG₅₀-g-PLL₃) group: hHGF/PEG-b-P(ELG₅₀-g-PLL₃) complexes were administered once at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure; and 5) hHGF/PEG-b-P(ELG₅₀-g-PLL₆) group: hHGF/PEG-b-P(ELG₅₀-g-PLL₆) complexes were administered once at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure. (b) A total of 40 SD rats were assigned to four groups (ten rats in each group): 1) heart ischemia/reperfusion injury (I/R) group: the left anterior descending coronary artery was occluded for 45 min followed by 24 h of reperfusion; 2) hHGF group: hHGF was administered once per day at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure; 3) hHGF/PEG-b-P(ELG₅₀-g-PLL₃) group: hHGF/PEG-b-P(ELG₅₀-g-PLL₃) complexes were administered once at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure; and 4) hHGF/PEG-b-P(ELG₅₀-g-PLL₆) group: hHGF/PEG-b-P(ELG₅₀-g-PLL₆) complexes were administered once at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure. (c) A total of 40 SD rats were assigned to four groups (ten rats in each group): 1) kidney ischemia/reperfusion injury (I/R) group: I/R was achieved by clamping the renal artery and vein for 45 min followed by 24 h of reperfusion; 2) hHGF group: hHGF was administered once per day at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure; 3) hHGF/PEG-b-P(ELG₅₀-g-PLL₃) group: hHGF/PEG-b-P(ELG₅₀-g-PLL₃) complexes were administered once at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure; and 4) hHGF/PEG-b-P(ELG₅₀-g-PLL₆) group: hHGF/PEG-b-P(ELG₅₀-g-PLL₆) complexes were administered once at a dose of 1 mg/kg by abdominal subcutaneous injection 7 days prior to the I/R procedure. Drugs were administered 7 days prior to I/R induction based on our *in vitro* release and hHGF plasma concentration results.

Injury to different organs

The methods used have been described previously [12]. Briefly, after I/R, the different organs were removed from the rats, and the organ blood was cleaned. The injury was evaluated by high-speed camera imaging.

Brain water content, SOD activity and MDA level on brain I/R injury

After I/R, a portion of the rat brain was removed to weigh the wet brain, and then the brain was baked for 48 h at 80°C to obtain the weight of the dry brain [13]. The brain water content (%) was calculated as the (weight of the wet brain) - (weight of the dry brain) × 100. A part of the brain was collected after reperfusion for 24 h and centrifuged at 3600 × g for 20 min to harvest the supernatant. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) level were measured with the xanthine oxidase and thiobarbituric acid methods, respectively [14]. The absorbance was measured at 550 and 532 nm. The levels of lipid peroxides are expressed as units (U) of SOD/mg protein and μmol of MDA/g protein.

BUN level, Scr level, SOD activity and MDA level on renal I/R injury

After I/R, blood samples were collected from the abdominal aorta and centrifuged at 3600 × g for 20 min to harvest the sera. The left kidneys of the animals were removed, homogenized in ice-cold normal saline, adjusted to 10% homogenate, and then centrifuged at 3600 × g for 20 min to harvest the supernatant. The blood urea nitrogen (BUN) and serum creatinine (Scr) levels were examined with the o-phthalaldehyde and picric acid methods, respectively [15]. The SOD activity and MDA level were measured with the xanthine oxidase and thiobarbituric acid method, respectively [14]. The absorbance was measured at 550 and 532 nm. The levels of lipid peroxides were expressed as U of SOD/mg protein and nmol of MDA/mg protein.

Serum CK and LDH activities on heart I/R injury

After I/R, the blood samples were collected from the abdominal aorta and centrifuged at 3600 × g for 20 min to harvest the sera. The blood creatinine kinase (CK) and lactate dehydrogenase (LDH) activities were examined via the previously described method [16].

Fig. 1. Synthesis of the linear-brush copolymer of PEG-*b*-P(ELG-*g*-PLL). Abbreviations: PEG-NH₂, poly(ethylene glycol) amine; PEG-*b*-PBLG, poly(ethylene glycol)-*b*-poly(γ-benzyl l-glutamate); DMF, N,N-dimethylformamide; 2-HP, 2-hydroxypyridine; PEG-*b*-PELG, poly(ethylene glycol)-*b*-poly(ethylenediamine l-glutamate); PEG-*b*-P(ELG-*g*-PZLL), poly(ethylene glycol)-*b*-(poly(ethylenediamine l-glutamate)-*g*-poly(ε-benzyloxycarbonyl-l-lysine)); PEG-*b*-P(ELG-*g*-PLL), poly(ethylene glycol)-*b*-(poly(ethylenediamine l-glutamate)-*g*-poly(l-lysine)).

Statistical analysis

All data are expressed as the mean values $\pm$ the standard deviation (SD). An analysis of variance (ANOVA) and Student's *t* test were used to evaluate statistical significance. A value of less than 0.01 ($P < 0.01$) was used to establish statistical significance.

Results

Synthesis and characterization of PEG-*b*-P(ELG-*g*-PLL)

The PEG-*b*-P(ELG-*g*-PLL) consisted of a linear PEG block and a brush-like PLL block (Figure 1). In our study, the molecular weight of the PEG block was 5000 Da, and the polymerization degree of the PELG brush backbone was 50; in addition, the polymerization degrees of the PLL brush side chains were 3 and 6. The synthesis was performed in a four-step procedure as shown in Figure 1. The ¹H-nuclear magnetic resonance (NMR) spectra of PEG-*b*-P(ELG-*g*-PLL) are shown in Figure 2A. The gel permeation chromatograms (GPCs) of PEG-*b*-P(ELG-*g*-PLL) are shown in Figure 2B. M_n (kDa)/¹H NMR and M_n (kDa)/GPC refer to our previous work [10].

Cell viability

The cytotoxicities of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆) on EC9706 cells were measured within 24 h of culturing, and the results are shown in Figure 3. The block copolymer showed high cell viability even under concentrations as high as 500 mg/L. Cell viabilities were (100 $\pm$ 1.34)% and (100 $\pm$ 1.11)% in 0 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (95 $\pm$ 1.78)% and (93 $\pm$ 1.26)% in 10 mg/L of PEG-

Fig. 2. ^1H NMR spectra and GPC of PEG-*b*-P(ELG-*g*-PLL). Notes: (A) ^1H NMR spectra of PEG-*b*-P(ELG-*g*-PLL) in D_2O ; (B) GPC of PEG-*b*-P(ELG-*g*-PLL). Abbreviations: ^1H NMR, ^1H -nuclear magnetic resonance; GPC, gel permeation chromatogram.

b-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (93±2.32)% and (91±1.34)% in 50 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (92±2.56)% and (90±2.22)% in 100 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (90±3.01)% and (88±1.56)% in 125 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (89±0.89)% and (87±1.13)% in 200 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (87±1.11)% and (85±1.44)% in 300 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; (86±1.34)% and (83±2.45)% in 400 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively; and (85±2.01)% and (81±1.17)% in 500 mg/L of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively.

Fig. 3. Cellular viability of EC9706 cells cultured with different concentrations of PEG-*b*-P(ELG-*g*-PLL).

Loading capacity of hHGF in PEG-*b*-P(ELG-*g*-PLL)

hHGF could be efficiently entrapped by PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆) under physiological pH via the electrostatic interactions between the positively charged PLL and the negatively charged hHGF. The loading capacity of hHGF was determined to be 8.21% in PEG-*b*-P(ELG₅₀-*g*-PLL₃), and the loading capacity of hHGF was determined to be 9.78% in PEG-*b*-P(ELG₅₀-*g*-PLL₆) (Table 1).

Characterization of the hHGF/PEG-*b*-P(ELG-*g*-PLL) complex

The hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) complexes were observed using transmission electron microscopy (TEM), and a representative image is shown in Figure 4A. The hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) complexes also adopted a spherical structure, and the diameters were approximately 51 and 67 nm, respectively (Table 1). The hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) complexes were further characterized with a granulometer. The average particle size distributions of PEG-*b*-P(ELG₅₀-*g*-PLL₃), PEG-*b*-P(ELG₅₀-*g*-PLL₆) and hHGF-loaded

Table 1. Particle size, TEM, and hHGF-loading capacity of PEG-*b*-P(ELG-*g*-PLL). Abbreviations: TEM, transmission electron microscopy; PEG-*b*-P(ELG-*g*-PLL), poly(ethylene glycol)-*b*-(poly(ethylenediamine l-glutamate)-*g*-poly(l-lysine)); NA, not applicable

Sample	Particle size (nm)	TEM	Loading capacity (%)
PEG-block-P(ELG50-graft-PLL3)	6	NA	NA
PEG-block-P(ELG50-graft-PLL6)	10	NA	NA
hHGF/PEG-block-P(ELG50-graft-PLL3)	16	51	8.21
hHGF/PEG-block-P(ELG50-graft-PLL6)	21	67	9.78

PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆) are shown in Figure 4B and Table 1. The average diameters of PEG-*b*-P(ELG₅₀-*g*-PLL₃), PEG-*b*-P(ELG₅₀-*g*-PLL₆), hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃), and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) were approximately 6, 10, 16, and 21 nm, respectively.

In vitro release of hHGF from PEG-*b*-P(ELG-*g*-PLL)

The release of hHGF from PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆) was measured via dialysis (MWCO, 100 kDa) at 37°C with 5 mL of hHGF-loaded PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-*g*-PLL₆) against a 0.02 mol/L phosphate buffer (PB) solution (pH 7.4). The cumulative release profile of PEG-*b*-P(ELG₅₀-*g*-PLL₃) and PEG-*b*-P(ELG₅₀-

Fig. 4. Characterization of the PEG-*b*-P(ELG-*g*-PLL) and hHGF/PEG-*b*-P(ELG-*g*-PLL) complexes. Notes: (A) A TEM image of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) complexes (a) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) complexes (b); (B) The diameter of PEG-*b*-P(ELG₅₀-*g*-PLL₃) (a) in PB, diameter of PEG-*b*-P(ELG₅₀-*g*-PLL₆) (b) in PB, diameter of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) complexes (c) in PB and diameter of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) complexes (d) in PB. Abbreviations: TEM, transmission electron microscopy; PB, phosphate buffer.

g-PLL₆)-encapsulated hHGF is shown in Figure 5. After 2 h, approximately 28.94% and 19.83% of the hHGF was released from hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆), respectively, which is indicative of an initial burst release of hHGF. Approximately 69.16% and 57.51% of the hHGF was released after 7 days, which is indicative of biphasic release profiles from hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆).

Serum hHGF concentration evaluation

As shown in Figure 6, the concentration of hHGF in the plasma of SD rats following a single subcutaneous injection in the hHGF group at a dose of 1 mg/kg rapidly reached a C_{max} of 534 ng/mL at a T_{max} of 40 min and then declined to baseline values within 3 h of administration. The plasma hHGF concentration in SD rats following a single subcutaneous injection in the hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) group at a dose of 1 mg/kg reached a C_{max} of 74.65 ng/mL at a T_{max} of 3 h and then declined to baseline values within 7 d of administration. The plasma hHGF concentration in SD rats following a single subcutaneous injection in the hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) group at a dose of 1 mg/kg reached C_{max} of 65.34 ng/mL at a T_{max} of 6 h and then declined to baseline values within 3 d of administration. These results showed that the release of hHGF from hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) was sustained in comparison to the results obtained in the hHGF group.

Evaluation of injury to different organs

As shown in Figure 7, the injuries to the brain, heart and kidney in the I/R groups were higher than those in the Sham group, as assessed via high-speed camera imaging. The injuries to the brain, heart and kidney in the hHGF, hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) groups were less than those in the I/R group, as assessed via high-speed camera imaging. In particular, the hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) group showed significantly less injury than the I/R group based on high-speed camera imaging.

Brain water content, SOD activity and MDA level following brain I/R injury

As shown in Figure 8, the brain water content in the I/R group was higher than that in the Sham group ($P < 0.01$). The brain water content in the Sham group reached $18.17 \pm 3.18\%$, whereas the brain water content in the I/R group reached $75.29 \pm 5.05\%$. Administration of hHGF reduced the brain water content to $62.17 \pm 2.89\%$ ($P < 0.01$). In addition, administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) also reduced the brain

Fig. 5. Cumulative release profile of insulin from hHGF/PEG-*b*-P(ELG-*g*-PLL) complexes.

Fig. 6. Serum hHGF concentration in SD rats.

Fig. 7. Organ damage following I/R injury measured with high-speed camera imaging. Notes: The brain damage in the brain I/R injury group was greater than that in the Sham group. Administration of hHGF decreased the brain damage compared with that of the brain I/R injury group. Administration of hHGF/PEG-b-

P(ELG₅₀-g-PLL₆) significantly decreased the brain damage compared with that of the brain I/R injury group. Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) further significantly decreased the brain damage compa-

red with that of the brain I/R injury group. The damage to the heart in the heart I/R injury group was greater than that in the Sham group. Administration of hHGF decreased the heart damage compared with that of the heart I/R injury group. Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₆) significantly decreased the heart damage compared with that of the heart I/R injury group. Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) further significantly decreased the heart damage compared with that of the heart I/R injury group. The kidney damage in the kidney I/R injury group was greater than that in the Sham group. Administration of hHGF decreased the kidney damage compared with that of the kidney I/R injury group. Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₆) significantly decreased the kidney damage compared with that of the kidney I/R injury group. Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) further significantly decreased the kidney damage compared with that of the kidney I/R injury group.

water content to 43.27±3.01% and 50.08±4.05%, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly reduced the brain water content ($P<0.01$) (Figure 8A). The SOD activity in the I/R group was less than that in the Sham group ($P<0.01$). The SOD activity in the Sham group reached 154.25±21.17 U/mg; however, the SOD activity in the I/R group reached 72.31±15.39 U/mg. Administration of hHGF increased the SOD activity to 93.85±19.41 U/mg ($P<0.01$). In addition, administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) also increased the SOD activity to 131.18±23.28 U/mg and 112.08±18.85 U/mg, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly increased the SOD activity ($P<0.01$) (Figure 8B). The MDA level in the I/R group was higher than that in the Sham group ($P<0.01$). The MDA level in the Sham group reached 3.15±1.89 µmol/g, while the MDA level in the I/R group reached 12.05±2.01 µmol/g. Administration of hHGF increased the MDA level to 8.87±2.25 µmol/g ($P<0.01$). In addition, administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) also increased the MDA level to 6.18±1.54 µmol/g and 7.74±2.18 µmol/g, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly decreased the MDA level ($P<0.01$) (Figure 8C).

BUN level, Scr level, SOD activity and MDA level following renal I/R injury

As shown in Figure 8, the BUN level in the I/R group was higher than that in the Sham group ($P<0.01$). The BUN level in the Sham group reached 5.04±1.89 mmol/L, while the BUN level in the I/R group reached 24.52±2.38 mmol/L. Administration of hHGF reduced the BUN level to 16.34±3.05 mmol/L ($P<0.01$). In addition, administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) also reduced the BUN level to 12.16±2.45 mmol/L and 14.36±3.14 mmol/L, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly reduced the BUN level ($P<0.01$) (Figure 8D). The Scr level in the I/R group was higher than that in the Sham group ($P<0.01$). The Scr level in the Sham group reached 7.32±2.16 µmol/L, whereas the Scr level in the I/R group reached 26.16±3.28 µmol/L. Administration of hHGF reduced the Scr level to 19.05±1.89 µmol/L ($P<0.01$). In addition, administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) also reduced the Scr level to 15.32±3.06 µmol/L and 17.23±3.18 µmol/L, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly reduced the Scr level ($P<0.01$) (Figure 8E). The SOD activity in the I/R group was less than that in the Sham group ($P<0.01$). The SOD activity in the Sham group reached 121.5±2.08 U/mg, while the SOD activity in the I/R group reached 51.17±1.75 U/mg. Administration of hHGF increased the SOD activity to 78.31±2.17 U/mg ($P<0.01$). In addition, administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) also increased the SOD activity to 108.3±3.01 U/mg and 89.15±2.14 U/mg, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly increased the SOD activity ($P<0.01$) (Figure 8F). The MDA level in the I/R group was higher than that in the Sham group ($P<0.01$). The MDA level in the Sham group reached 23.4±3.2 nmol/mg, while the MDA level in the I/R group reached 70.5±5.6 nmol/mg. Administration of hHGF increased the MDA level to 58.8±6.3 nmol/mg ($P<0.01$). In addition, administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) also increased the MDA level to 42.9±2.8 nmol/mg and 50.3±4.1 nmol/mg, respectively, while hHGF/PEG-b-P(ELG₅₀-g-PLL₃) significantly decreased the MDA level ($P<0.01$) (Figure 8G).

Fig. 8. Brain water content, SOD activity, MDA level, serum BUN, serum Scr, serum CK activity and LDH activity following I/R injury. Notes: (A) Brain water content: The brain water content in the brain I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the brain water content compared with that of the brain I/R injury group ($P < 0.01$). Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₆) significantly decreased the brain water content compared with that of the brain I/R injury group ($P < 0.01$). Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) further significantly decreased the brain water content compared with that of the brain I/R injury group ($P < 0.01$); (B) SOD activity: The SOD activity in the brain I/R injury group was less than that in the Sham group ($P < 0.01$). Administration of hHGF increased the SOD activity compared with that of the brain I/R injury group ($P < 0.01$). Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₆) significantly increased the SOD activity compared with that of the brain I/R injury group ($P < 0.01$). Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) further significantly increased the SOD activity compared with that of the brain I/R injury group ($P < 0.01$); (C) MDA level: The MDA level in the brain I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the MDA level compared with that of the brain I/R injury group ($P < 0.01$). Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₆) significantly decreased the MDA level compared with that of the brain I/R injury group ($P < 0.01$). Administration of hHGF/PEG-b-P(ELG₅₀-g-PLL₃) further significantly decreased the MDA level compared with that of the brain I/R injury group ($P < 0.01$); (D) Serum BUN: The BUN level in the kidney I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the BUN level

compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) significantly decreased the BUN level compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) further significantly decreased the BUN level compared with that of the kidney I/R injury group ($P < 0.01$); (E) Serum Scr: The Scr level in the kidney I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the Scr level compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) significantly decreased the Scr level compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) further significantly decreased the Scr level compared with that of the kidney I/R injury group ($P < 0.01$); (F) SOD activity: The SOD activity in the kidney I/R injury group was less than that in the Sham group ($P < 0.01$). Administration of hHGF increased the SOD activity compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) significantly increased the SOD activity compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) further significantly increased the SOD activity compared with that of the kidney I/R injury group ($P < 0.01$); (G) MDA level: The MDA level in the kidney I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the MDA level compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) significantly decreased the MDA level compared with that of the kidney I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) further significantly decreased the MDA level compared with that of the kidney I/R injury group ($P < 0.01$); (H) Serum CK: The CK activity in the heart I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the CK activity compared with that of the heart I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) significantly decreased the CK activity compared with that of the heart I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) further significantly decreased the CK activity compared with that of the heart I/R injury group ($P < 0.01$); (I) Serum LDH: The LDH activity in the heart I/R injury group was higher than that in the Sham group ($P < 0.01$). Administration of hHGF decreased the LDH activity compared with that of the heart I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) significantly decreased the LDH activity compared with that of the heart I/R injury group ($P < 0.01$). Administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) further significantly decreased the LDH activity compared with that of the heart I/R injury group ($P < 0.01$). * $P < 0.01$ vs Sham, # $P < 0.01$ vs I/R, ## $P < 0.01$ vs I/R, ### $P < 0.01$ vs I/R. Abbreviations: SOD, superoxide dismutase; I/R, ischemia/reperfusion; MDA, methane dicarboxylic aldehyde; BUN, blood urea nitrogen; Scr, serum creatinine; CK, creatine kinase; LDH, lactate dehydrogenase.

Serum CK and LDH activities following heart I/R injury

As shown in Figure 8, the CK activity in the I/R group was higher than that in the Sham group ($P < 0.01$). The CK activity in the Sham group reached $1,132.08 \pm 145.6$ U/L, while the CK activity in the I/R group reached $3,203.16 \pm 118.5$ U/L. Administration of hHGF decreased the CK activity to $2,409.54 \pm 127.9$ U/L ($P < 0.01$). In addition, administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) also decreased the CK activity to $1,689.43 \pm 109.8$ U/L and $1,998.06 \pm 131.2$ U/L, respectively, while hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) significantly decreased the CK activity ($P < 0.01$) (Figure 8H). The LDH activity in the I/R group was higher than that in the Sham group ($P < 0.01$). The LDH activity in the Sham group reached $1,687.16 \pm 138.4$ U/L, whereas the LDH activity in the I/R group reached $3,405.04 \pm 181.3$ U/L. Administration of hHGF decreased the LDH activity to $2,717.83 \pm 154.6$ U/L ($P < 0.01$). In addition, administration of hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) and hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₆) also decreased the LDH activity to $2,006.78 \pm 127.8$ U/L and $2,345.67 \pm 118.9$ U/L, respectively, while hHGF/PEG-*b*-P(ELG₅₀-*g*-PLL₃) significantly decreased the LDH activity ($P < 0.01$) (Figure 8I).

Discussion

In the present study, we evaluated whether hHGF could attenuate I/R injury and if its actions could be enhanced by hHGF-loaded PEG-*b*-P(ELG-*g*-PLL). Our results showed that

abdominal subcutaneous injection of hHGF alone had a limited effect on I/R injury due to the short half-life of hHGF. Specifically, we observed that high numbers of hHGF injections (hHGF was administered once per day by abdominal subcutaneous injection starting 7 days prior to the I/R procedure) could reduce I/R injury in the hHGF group; however, a single abdominal subcutaneous injection of hHGF-loaded PEG-b-P(ELG-g-PLL) 7 days prior to the I/R procedure remarkably resulted in reduced I/R injury relative to hHGF alone and decreased injection times. These findings indicated that the use of PEG-b-P(ELG-g-PLL) is warranted for hHGF therapy for I/R injury and improved the half-time of hHGF. Drugs were administered 7 days prior to I/R induction given the in vitro release and hHGF plasma concentration results presented in Figures 5 and 6.

The potential for PEG-b-P(ELG-g-PLL) to be used as a protein nanocarrier was previously reported by our group [10]. The block copolymer was composed of a linear PEG block and a brush-like PLL block. At pH 7.4, the positively charged PLL brush block and the negatively charged hHGF (pI=5.5) combined to form a polyion complex through electrostatic interactions. The encapsulated hHGF showed sustained release, which facilitated further investigation into the protective effect of hHGF. Thus, our study measured the hHGF-loading capacity and in vitro release of these complexes. With respect to in vitro release, we found that hHGF release from both hHGF/PEG-b-P(ELG₅₀-g-PLL₃) and hHGF/PEG-b-P(ELG₅₀-g-PLL₆) could be sustained for 7 days but that hHGF release from hHGF/PEG-b-P(ELG₅₀-g-PLL₃) was significantly greater than hHGF release from hHGF/PEG-b-P(ELG₅₀-g-PLL₆). However, hHGF-loading capacity was lower for PEG-b-P(ELG₅₀-g-PLL₃) than for PEG-b-P(ELG₅₀-g-PLL₆). A possible reason for these phenomena is that at pH 7.4, the high amino density of PEG-b-P(ELG₅₀-g-PLL₆) led to strong positive charges that strengthened the connection between hHGF and PEG-b-P(ELG₅₀-g-PLL₆) by fostering strong electrostatic interactions, thereby improving hHGF-loading capacity but increasing the difficulty of hHGF release from hHGF/PEG-b-P(ELG₅₀-g-PLL₆). In in vivo research, we found that blood concentrations of hHGF could be sustained for 7 days in the hHGF/PEG-b-P(ELG₅₀-g-PLL₃) group and 3 days in the hHGF/PEG-b-P(ELG₅₀-g-PLL₆) group. There were contradictions between the in vivo and in vitro findings; these contradictions may be attributable to the complex environment and the high amino density of PEG-b-P(ELG₅₀-g-PLL₆), which caused hHGF to be difficult to release from hHGF/PEG-b-P(ELG₅₀-g-PLL₆) in vivo. In addition, this was the first study to show that hHGF and hHGF/PEG-b-P(ELG-g-PLL) were both capable of attenuating I/R injury (brain, heart and kidney).

Previous studies have demonstrated that I/R injury is a major concern in brain, heart and kidney surgeries and an important pathophysiological phenomenon in clinical medicine [17-29]. Although the pathogenesis of I/R damage has not been well characterized, pretreatment with drugs or transient ischemia prior to I/R can reduce tissue injury; however, ischemic preconditioning is difficult to perform in clinical practice. Therefore, it is necessary to search for and study drugs that can effectively prevent and cure I/R injury. The gradual transition from animal experiment to clinical application has been a major task for basic and clinical workers. Currently, many drugs have been shown to experimentally modify pathophysiological conditions [30-32]. hHGF, also known as scatter factor, has been isolated and purified from rat platelets [33]. However, in many investigations, hHGF decreased brain I/R injury via an ERK-dependent pathway, reduced heart I/R injury via the regulation of cell apoptosis and alleviated kidney I/R injury via the inhibition of apoptotic cell death [5-7]. In this study, we found that in rats, both hHGF and hHGF/PEG-b-P(ELG-g-PLL) protected the brain and kidney against I/R injury by decreasing oxidative stress damage and protected the heart against I/R injury by reducing CK and LDH release. In particular, I/R injury was markedly decreased in hHGF/PEG-b-P(ELG-g-PLL)-treated rats compared with control rats.

Conclusion

the present study showed that hHGF and hHGF/PEG-b-P(ELG-g-PLL) both prevented brain, heart and kidney damage after I/R injury in an animal model. Indeed, hHGF may play an

important role in clinical situations associated with brain, heart and kidney dysfunction after I/R injury. More importantly, the hHGF nanocarrier PEG-*b*-P(ELG-*g*-PLL) may significantly improve hHGF bioactivity and protect against I/R injury.

Disclosure Statement

All of the authors declare no competing interests.

Acknowledgments

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