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Research paper

Poly(ethylene glycol)-Radix Ophiopogonis polysaccharide conjugates: Preparation, characterization, pharmacokinetics and in vitro bioactivity

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ABSTRACT

Radix Ophiopogonis polysaccharide (ROP), a natural graminan-type fructan with Mw of ~5 kDa, had been found to have an excellent anti-myocardial ischemic activity. However, its rapid renal excretion following administration remarkably limits its efficacy and clinical use, which makes necessary the development of an effective delivery system. In this article, the feasibility of PEGylation to solve this problem was examined. A moderate coupling reaction between the hydroxyl-activated ROP and the amino-terminated mPEG was chosen to PEGylate ROP. Five different mPEG-ROP conjugates (with mPEG of molecular mass 2, 5 or 20 kDa) were prepared, purified, characterized and evaluated in pharmacokinetics and in vitro bioactivity. Results showed that only when the apparent molecular weight of the conjugate approached to a certain value, would its plasma elimination reduce abruptly. In general, the conjugation caused the reduction in the bioactivity of ROP; however, well-preserved bioactivity was observed when the grafting degree of the conjugate was lower. Among the five conjugates studied, the one with an average 1.3 mPEG (20 kDa) residues per single ROP was found to be satisfactory both in plasma retention and in bioactivity. It had a 47.4-fold increased elimination half-life and preserved approximately 74% of the bioactivity of ROP; moreover, the decrease in bioactivity is not significant. These findings demonstrate that PEGylation would be a promising approach for improving the clinical efficacy of ROP by prolonged retention in plasma.

1. Introduction

Polysaccharides have been generating considerable interests due to their plentiful bioactivity and low toxicity. To date, hundreds of polysaccharides from a variety of sources such as animals, plant cell walls and fungal cells have been discovered. Carbohydrates are an essential part of every cell surface and are crucial in cell surface recognition and information transfer, which makes researches on carbohydrate-based drugs become an increasingly active area in drug discovery, especially with a rather recent development in our understanding of fundamental glycobiology [1]. Radix Ophiopogonis polysaccharide (ROP), a natural graminan-type fructan with a weight average molecular weight of 4800 Da, a polydispersity index of 1.41, a backbone composed of Fruf (2 → 1) and a branch of Fruf (2 → 6) Fruf (2 → per average 2.8 of main chain residues [2] (Fig. 1a), had been found to have the anti-myocardial ischemic activity by a variety of animal and cultured cell models [3–5]. By in vivo animal models, it was found that ROP could protect against myocardial ischemic damage caused by isoprenaline in rats [3,4] and prevent myocardiocyte death induced by coronary artery ligation in rats [5]. By in vitro animal model, it was found that ROP could alleviate ischemia/reperfusion injury in isolated guinea pig myocardia [4]. By cultured cell models, it was found that ROP could protect myocardial cells and human microvascular endothelial cells (HMEC-1) from damage induced by oxygen and glucose deprivation, promote the tube formation and migration of HMEC-1, up-regulate the mRNAs and proteins of sphingosine kinase 1 and S1P receptor 1 and induce the expression of basic fibroblast growth factor and the phosphorylation of Akt and ERK in HMEC-1 [5].

Abbreviations: ROP, Radix Ophiopogonis polysaccharide; mPEG, poly(ethylene glycol) monomethyl ether; FITC, Fluorescein isothiocyanate; ${}_iP_{jk}$-R, mPEG-grafted ROP with an average *i* mPEG (*j* kDa) residues per single ROP; DMAP, 4-*N*,*N*-(dimethylamino) pyridine; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; bFGF, basic fibroblast growth factor; HMEC-1, human microvascular endothelial cells; HPGPC, high-performance gel permeation chromatography; OGD, oxygen and glucose deprivation.

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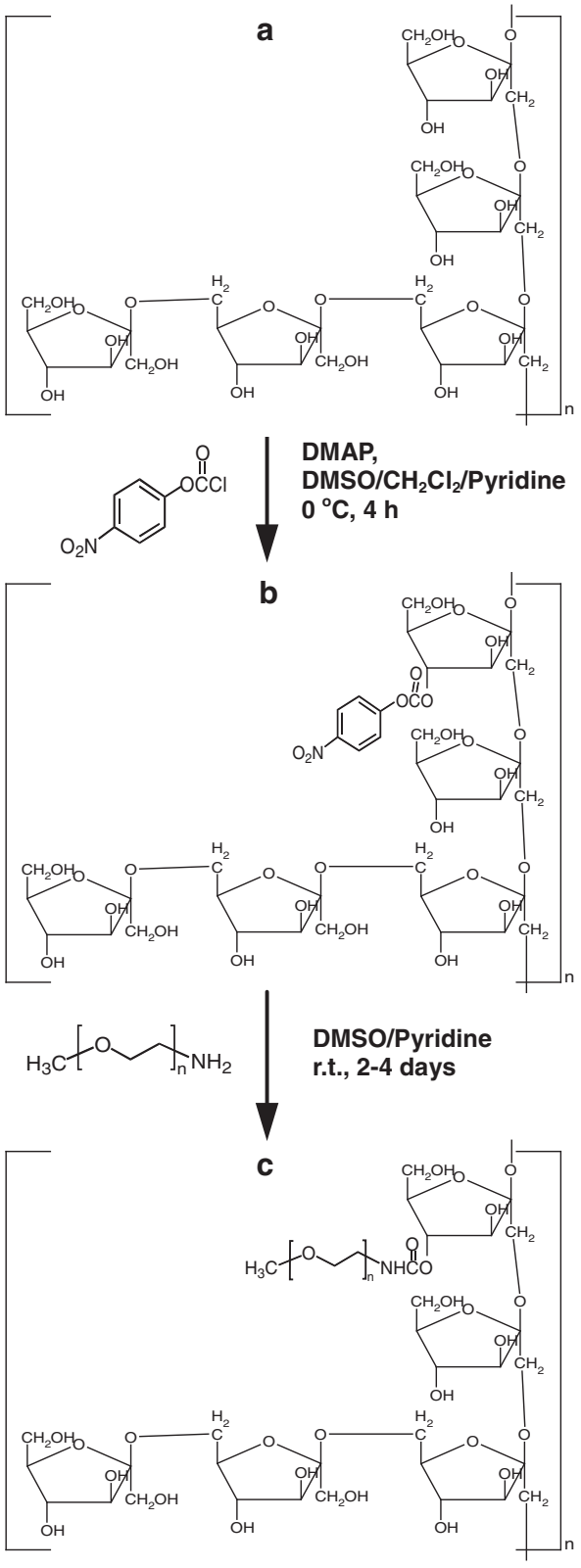

Fig. 1. Synthetic route for PEGylation of ROP. (a) ROP, (b) hydroxyl-activated ROP and (c) PEGylated ROP.

However, due to its unsuitable hydrodynamic size (~2 nm), which is larger than the intercellular space of intestinal epithelium (~1.5 nm) and much smaller than the sieving threshold of the glomerular capillary wall (~10 nm) [6], as well as its high hydrophilicity and low protein binding, ROP was poorly absorbed after oral administration, while following i.v. administration, it was very rapidly excreted by kidney [7,8]. These undesirable pharmacokinetic properties limit to a large extent its efficacy and clinical use. Many carbohydrate-based drugs such as phosphosulfomannan and its analogues [9], heparin and its mimetics [10,11] and dermatan sulfate [12] also encounter such problems, necessitating the development of an effective delivery system to improve their pharmacological action and also to reduce the administration frequency for patients.

PEGylation, the process of covalent attachment of polyethylene glycol (PEG) polymer chains to a therapeutic molecule, is now established as the method of choice for improving the pharmacokinetics and pharmacodynamics of parenteral agents. It has been successfully applied to small molecule drugs, oligonucleotides, polypeptides, proteins and long-circulating colloidal drug delivery systems [13,14]; however, although there are some reports on the application of PEGylated polysaccharides as drug carrier [15,16] or surfactant [17] and recently on the technology of glycoPEGylation for the site-directed PEGylation of polypeptides and protein [18,19], there are few if any reports on the application of PEGylation in carbohydrate-based drugs. In this study, five different PEG-ROP conjugates (with PEG of molecular mass 2, 5 or 20 kDa) were prepared, purified, characterized and evaluated in pharmacokinetics and in vitro bioactivity. The feasibility of PEGylation of carbohydrate-based drugs was tested on ROP for the first time.

2. Materials and methods

2.1. Materials, cells and animals

ROP was prepared by extraction from the tube root of *Ophiopogon japonicus* (Cixi, Zhejiang province, China) with water, followed by ethanol precipitation, chromatographic purification using DEAE Sepharose Fast Flow and Sephadex G-25 columns (Pharmacia, Uppsala, Sweden) in tandem and finally lyophilization. ROP was dried in a vacuum oven at 60 °C for 8 h before use. Linear amino-terminated poly(ethylene glycol) methyl ethers (mPEG-NH_2) were purchased from Jenkem technology Co., Ltd. (Beijing, China). *p*-nitrophenyl chloroformate and 4-*N*,*N*-(dimethylamino) pyridine (DMAP) were purchased from Fluka (Buchs, Germany). Fluorescein isothiocyanate (FITC) was purchased from Sigma (St. Louis, MO, USA). Extra dry dimethyl sulfoxide (DMSO) was purchased from Acros Organics (Geel, Belgium). Dichloromethane (CH_2Cl_2) and pyridine from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) were dried over CaH_2 and KOH, respectively, and distilled prior to use. The cell culture medium MCDB131, fetal bovine serum (FBS) and all other cell culture supplements were purchased from GIBCO-BRL (Grand Island, NY, USA). Basic fibroblast growth factor (bFGF) was obtained from Biosource (Owensboro, KY, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA). All other chemicals were of reagent grade and purchased from commercial sources.

Human microvascular endothelial cells (HMEC-1) were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. Male Sprague–Dawley rats, approximately 8–9 weeks old at dosing (300–360 g), were supplied by Lab Animal Center of Shanghai University of Traditional Chinese Medicine, Shanghai, China. They were kept in an environmentally controlled breeding room for 6–7 days before starting the experiments and fed with standard laboratory food and water ad libitum. The Animal Ethical Experimentation Committee of Shanghai University of TCM, according to the requirements of the National Act

on the Use of Experimental Animals (PR China), approved all procedures of the animal experiments.

2.2. Preparation of mPEG-ROP conjugates

In order to reduce the effect of reaction on the structure of ROP and, in turn, its bioactivity, mPEG-ROP conjugates were synthesized through a moderate coupling reaction between the hydroxyl-activated ROP and the amino-terminated mPEG (Fig. 1).

Hydroxyl-activated ROP was prepared by reaction with *p*-nitrophenyl chloroformate catalyzed by a small amount of DMAP. In detail, ROP (1 g, 0.21 mmol), *p*-nitrophenyl chloroformate (1 or 2 g; 5.0 or 10 mmol) and DMAP (100 mg) were dissolved in 40 ml of a mixture of DMSO, CH_2Cl_2 and pyridine (2:1:1, v/v/v) and kept at 0 °C for 4 h. After reaction, the product was precipitated in 360 ml of a cold diethyl ether/ethanol (1:1, v/v) mixture and filtered. The final product was washed at least nine times with the precipitation reagent and dried in vacuo for 24 h. The yield was approximately 90%. The *p*-nitrophenyl carbonate content of each activated ROP was determined spectrometrically by the concentration of *p*-nitrophenolate liberated from the activated ROP in 0.1 M NaOH (λ_{max} = 402 nm, ε_{max} = 18,400 $M^{-1}\cdot cm^{-1}$).

The activated ROP and 2-, 5- or 20-kDa mPEG-NH_2 were dissolved in a mixture of DMSO/pyridine (1:1, v/v). The reaction mixture was stirred for 2–4 days at room temperature and then added to a cold diethyl ether/ethanol mixture with stirring. The white precipitate was collected, washed with an excess of the precipitation reagent and dried in vacuo. The product was dissolved in a 0.01 M NaOH aqueous solution to hydrolyze the residual *p*-nitrophenyl groups on the conjugate, followed by dialysis and lyophilization. Optimal conditions for the synthesis of five different mPEG-ROP conjugates were listed in Table 1.

2.3. Characterization of mPEG-ROP conjugates

PEGylation was affirmed by high-performance gel permeation chromatography (HPGPC) coupled with the anthrone-sulfuric acid colorimetry. The apparent molecular weights and mPEG-grafting numbers of the conjugates were determined by HPGPC. The HPGPC system consisted of an Agilent 1100 liquid chromatograph and a PL-ELS 1000 detector (Polymer Laboratories Ltd., Shropshire, UK). A Shodex OHpak SB-803 HQ gel-filtration column (300 × 8.0 mm) (Tokyo, Japan) was used with the eluting double distilled water at a flow rate of 0.6 ml/min. The chromatographic procedures were performed at 25 °C. PEG standards for GPC (approximately 2.01, 6.55, 17.9, 42.7 and 118.0 kDa) (Fluka, Buchs, Germany) were used to calibrate the system. Colorimetry was performed as follows: to a portion of each aqueous sample, four times in volume of 2 mg/ml fresh anthrone-sulfuric acid reagent was added. After vortexing, the mixture was cooled in an ice bath for 30 min, and then, the absorbance was immediately determined at 625 nm in a 752 N UV–Vis spectrophotometer (Shanghai precision & scientific instrument Co., Ltd., Shanghai, China).

2.4. Protection effects of mPEG-ROP conjugates on HMEC-1 cells under oxygen and glucose deprivation (OGD)

HMEC-1 cells were maintained in the MCDB131 medium containing 10% FBS (v/v), 2 mM L-Glutamine, 5 ng/ml epidermal growth factor (EGF) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a 5% CO_2 incubator. For OGD experiment, HMEC-1 cells were cultured in 96-well culture plates (1.5×10^4 cells/well) for 24 h, then transferred into a temperature-controlled (37° ± 1 °C) anaerobic chamber (BioSpherix, NY, USA) containing a gas mixture of 5% CO_2 and 95% N_2 and incubated in the glucose-free Hank's balanced salt solution (116 mmol NaCl, 5.4 mmol KCl, 0.8 mmol $MgSO_4$, 1 mmol NaH_2PO_4, 0.9 mmol $CaCl_2$, 10 mg phenol red, pH 7.4). The oxygen levels in the chamber were near complete anoxia (<0.8% oxygen). Cells were maintained in the chamber under OGD conditions with different treatment for 8 h to induce injury. The control cell cultures were maintained in the standard culture medium for the same time period in a 5% CO_2 incubator. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method according to the manufacturer's instructions (Dingguo Company, Beijing, China).

2.5. Promotion of the formation of microvessel tubes by mPEG-ROP conjugates

Culture plates (96 well) were coated with 50 μl Matrigel and incubated at 37 °C for 45 min to allow the Matrigel to solidify. HMEC-1 cells were plated at a density of 1×10^4 cells/well with test substances and incubated at 37 °C for 16 h. The cells were photographed using the Olympus digital camera. Tube formation was quantified by measuring the length of capillary structures in pixels using NIH ImageJ software. Branching points were counted manually. Five randomly selected fields of view were photographed in each well and averaged to determine the value for each sample.

2.6. Labeling of mPEG-ROP conjugates with FITC

Labeling of PEG-ROP conjugates with FITC was carried out according to the method used to label ROP [8]. Briefly, the conjugate (~1 g) was dissolved in DMSO (10 ml) containing a few drops of pyridine. FITC (0.1 g) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 30 min at 95 °C. After the reaction, nine volumes of the cold diethyl ether/ethanol mixture were added to the reaction mixture. The resulting precipitates were recovered by filtration and washed with the precipitation

Table 1
Optimal conditions for the synthesis of mPEG-ROP conjugates.

Conjugates	Reagents	Molar ratio	Time (h)	Precipitation reagent (vol. ratio)	Yield (%)
C-1	Activated ROP[a] 2-kDa mPEG-NH_2	1:1	48	Ethanol/ether (1:8)	45
C-2	Activated ROP[b] 2-kDa mPEG-NH_2	1:5	72	Ethanol/ether (1:8)	48
C-3	Activated ROP[a] 5-kDa mPEG-NH_2	1:1	60	Ethanol/ether (1:3)	80
C-4	Activated ROP[b] 5-kDa mPEG-NH_2	1:8	96	Ethanol/ether (1:3)	85
C-5	Activated ROP[b] 20-kDa mPEG-NH_2	1:2	96	Ethanol/ether (1:3)	88

[a] Degree of activation: 13%.
[b] Degree of activation: 24%.

reagent until free FITC and the other excess reagents were fully removed. Finally, they were dried in vacuo.

2.7. HPGPC-FD assay of FITC-labeled mPEG-ROP conjugates

The assay system consisted of an Agilent 1200 series HPLC with a fluorescence detector set at λ_{ex} 495 nm and λ_{em} 515 nm. Samples were separated by HPGPC using an 8.0 × 300 mm Shodex OHpak SB-803 HQ column. The eluent was 0.1 M phosphate buffer (pH 7.4) delivered at a flow rate of 0.5 ml/min.

2.8. Pharmacokinetics of FITC-labeled mPEG-ROP conjugates in rats

Rats were dosed with 50 mg/kg FITC-labeled ROP or each mPEG-ROP conjugate (50 mg/ml in sterilized physiological saline) i.v. via the femoral vein. Blood samples (approximately 350 μl) were obtained from orbital sinus with heparin-containing capillaries at the specified time points after administration and then immediately centrifuged at 3000 rpm for 10 min. The separated plasma was frozen at −20 °C until assay was performed.

To a 50-μl portion of each plasma sample, 20 μl 1 M perchloric acid was added. The mixture was vortexed and then centrifuged at 10,000 rpm for 2 min to precipitate the denatured plasma proteins. The supernatant was neutralized by the addition of 15 μl 1 M NaOH and then assayed by the HPGPC-FD method described above.

2.9. Data analysis

Data were expressed as means ± standard deviation (SD). Statistical analyses were assessed using the Student's *t*-test. Statistically significant differences were indicated by P values of < 0.05. The DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society) was used to calculate pharmacokinetic parameters such as AUC (area under the curve), MRT (mean residence time), $t_{1/2}$ (terminal phase half-life), V_{ss} (total volume of distribution) and CL (systemic clearance) by non-compartmental analysis.

3. Results

3.1. Characterization of mPEG-ROP conjugates and FITC-labeled conjugates

Five mPEG-grafted ROPs were successfully synthesized through a coupling reaction between the hydroxyl-activated ROP and the amino-terminated mPEG and characterized by HPGPC coupled with the carbohydrate-specific anthrone-sulfuric acid colorimetry. Results showed that the PEGylated products eluted as a slightly fronting or tailing peak, or as a main peak with a small shoulder peak (Fig. 2), indicating that the heterogeneously PEGylated conjugates were obtained. The apparent molecular weights and grafting numbers for the conjugates were calculated and listed in Table 2. PEGylation was confirmed by several pieces of evidence, including (1) the disappearance of the HPGPC peak corresponding to ROP and the notably onward shift of that corresponding to mPEG after conjugation reaction and (2) the strong chromogenic reaction between the anthrone-sulfuric acid reagent and the eluate corresponding to the HPGPC peak for each conjugate.

In order to study their pharmacokinetics, the conjugates were pre-labeled with FITC. After FITC labeling, no remarkable differences in the elution position and the peak shape of the conjugates were observed, indicating no alteration in the length of the conjugate chain during reaction. The FITC label assumes negative charge at physiologic pH. However, since the degree of substitution is very low (less than 0.5 mol FITC per mol each conjugate), pharmacokinetic properties of FITC-labeled conjugates are expected to be similar to those of unlabeled conjugates.

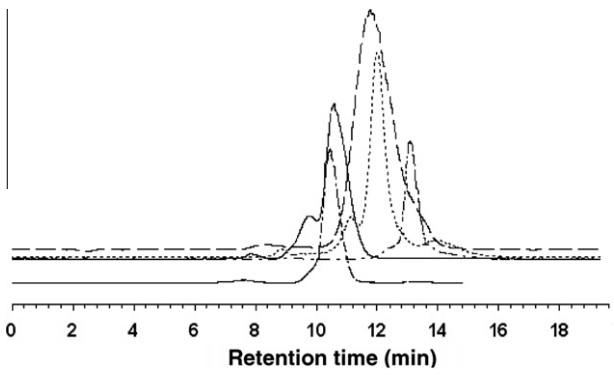

Fig. 2. HPGPC chromatograms of mPEG-ROP conjugates. From left to right in terms of retention time are the conjugates C-5, C-4, C-2, C-3 and C-1.

3.2. Bioactivity of mPEG-ROP conjugates

The cytoprotection and tube formation promotion activities of each mPEG-ROP conjugate were evaluated and compared with those of ROP using HMEC-1 cells. Under OGD conditions, the conjugates C-1 ($_{0.8}P_{2k}$-R), C-3 ($_{1.1}P_{5k}$-R) and C-5 ($_{1.3}P_{20k}$-R), just like ROP, could effectively preserve the cell viability (Fig. 3). Moreover, there were no significant differences between their effects, and approximately 67–85% of the cytoprotection activity of ROP was

Table 2
Apparent molecular weights and grafting degrees of mPEG-ROP conjugates.

Conjugates	Apparent M_w (kDa)	Grafting degree[a]	Nominal M_w of mPEG (kDa)	Designation
C-1	3.8	0.8	2	$_{0.8}P_{2k}$-R
C-2	10.4	4.0	2	$_{4.0}P_{2k}$-R
C-3	8.3	1.1	5	$_{1.1}P_{5k}$-R
C-4	31.2	5.4	5	$_{5.4}P_{5k}$-R
C-5	34.2	1.3	20	$_{1.3}P_{20k}$-R

[a] The determined apparent molecular weights for ROP and 2-, 5- and 20-kDa mPEGs were 2.2, 2.1, 5.4 and 24 kDa, respectively.

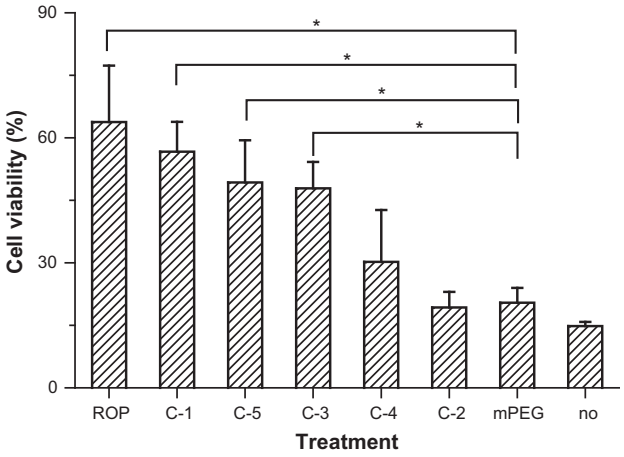

Fig. 3. Protection effects of ROP and mPEG-ROP conjugates on HMEC-1 cells under OGD. HMEC-1 cells were treated with 4 mM of ROP, each PEG-ROP conjugate or mPEG under OGD for 8 h and then assessed for viability using the MTT method as described in Section 2. The viability of cells incubated under the normal conditions was defined as 100%. No treatment group under ODG was set as a negative control, which was denoted as "no" in the figure. Data are means ± SD for triplicate experiments (*$P < 0.01$).

preserved. Although the conjugates C-2 ($_{4.0}P_{2k}$-R) and C-4 ($_{5.4}P_{5k}$-R) as well as mPEG alone also showed some cytoprotection effect, there were no significant differences in cell viability between them and the no treatment group. The significant difference between the activities of mPEG and C-1, C-3 or C-5 clearly indicated that the contribution of mPEG to the activity of the conjugates was, if any, little.

The initial phase of angiogenesis involves the organization of individual endothelial cells into a three-dimensional tube-like structure. In this study, tube-like structures appeared on Matrigel after cell culture for 16 h. In addition to markedly increasing microvessel tube length when compared with the no treatment group (Fig. 4A and C), the conjugates C-1, C-3 and C-5 also significantly increased tube branching points (Fig. 4B and C); however, PEGylation caused a decrease of approximately 20–55% in the potency. Both the activities of ROP and the three conjugates were weaker than those of bFGF (10 ng/ml); furthermore, the differences between the activities of the conjugates and bFGF were significant ($P < 0.05$ or $P < 0.01$). Similar to the result of the OGD experiment, mPEG alone also showed some activity of tube formation promotion; however, the significant difference between the actions of mPEG and C-1, C-3 or C-5 still existed.

In general, the conjugation caused the reduction in the bioactivity of ROP; however, well-preserved bioactivity was observed when the grafting degree of the conjugate was lower.

3.3. Pharmacokinetics of mPEG-ROP conjugates

Effects of PEGylation on pharmacokinetic properties of ROP were evaluated in rats following i.v. administration. Blood plasma concentration–time profiles for the FITC-labeled ROP and conjugates are shown in Fig. 5, and the main pharmacokinetic parameters are summarized in Table 3. Results showed that only when the apparent molecular weight of the conjugate approached to a certain value, would its plasma elimination reduce abruptly. With the apparent molecular weight of the conjugate increasing up to 10.4 kDa, the elimination half-life of the conjugate increased only ~2.1 times. However, for the conjugates C-4 and C-5, whose apparent molecular weights were 31.2 and 34.2 kDa, respectively, their elimination half-lives increased 99.6 and 47.4 times, respectively. Intriguingly, even having a slightly smaller apparent molecular weight, C-4 ($_{5.4}P_{5k}$-R) displayed a 2.1-fold longer elimination half-life than C-5 ($_{1.3}P_{20k}$-R). With few high molecular mass PEG chains rather than a higher number of low molecular mass ones, C-5 would display a more rod-like conformation and have higher flexibility and deformability in blood than C-4, which

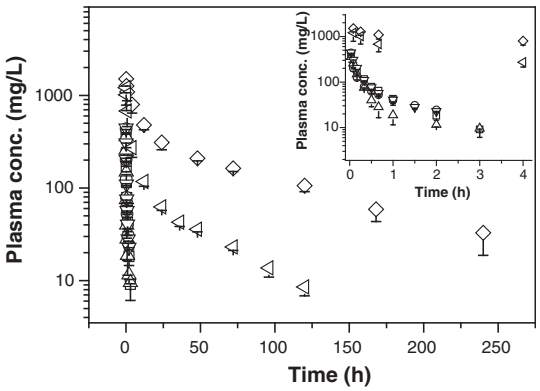

Fig. 5. Plasma FITC-labeled ROP (open squares), mPEG-ROP conjugate C-1 (open circles), C-2 (open up-triangles), C-3 (open down-triangles), C-4 (open diamonds) or C-5 (open left-triangles) level versus time profile following intravenous administration at 50 mg/kg in rats. Each data point represents the mean ± SD for triplicate experiments.

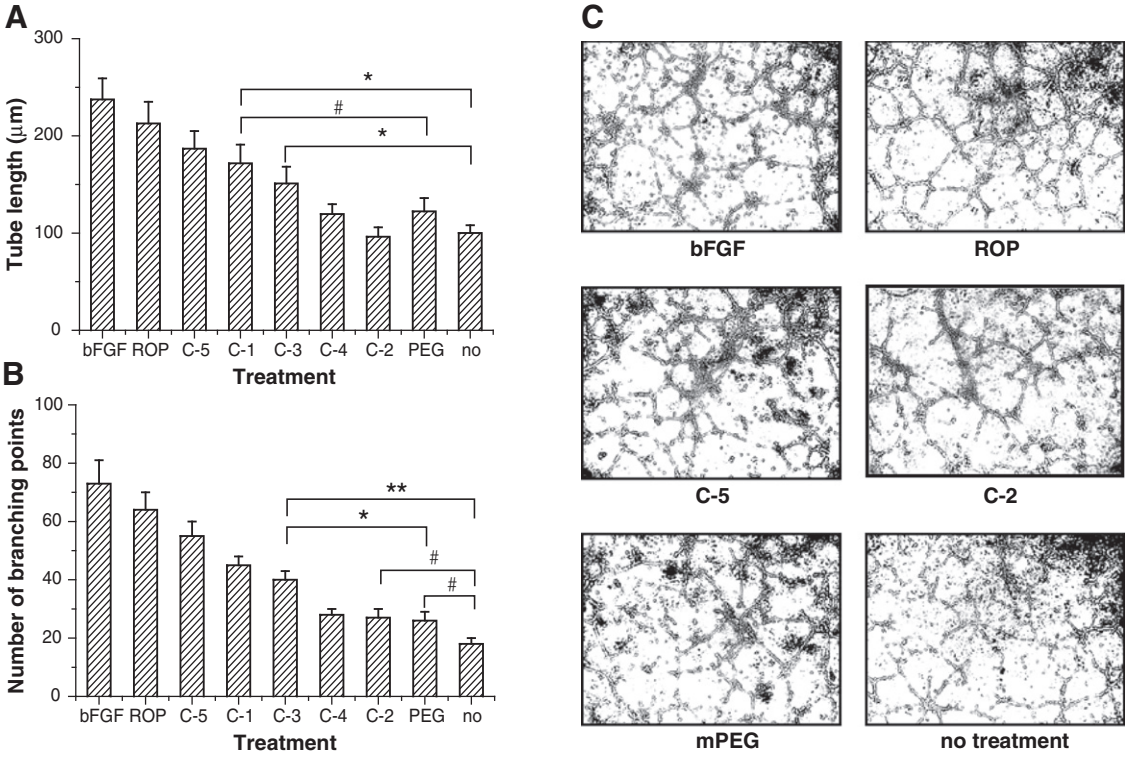

Fig. 4. Promotion of the formation of microvessel tubes by ROP and mPEG-ROP conjugates. Tube formation was determined at 16 h after plating as described in Section 2. A and B, Statistical analysis of tube length (A) and branching points (B) of the HMEC-1 cells treated with 10 ng/ml of bFGF or 20 nM of ROP, each PEG-ROP conjugate or mPEG. Data are means ± SD for triplicate experiments ($^{**}P < 0.001$, $^{*}P < 0.01$ and $^{\#}P < 0.05$). C, Representative micrographs of tube formation of HMEC-1 cells treated with bFGF, ROP, mPEG, C-2 or C-5.

Table 3
Pharmacokinetic parameters following i.v. administration of 50 mg/kg FITC-labeled ROP or mPEG-ROP conjugates in rats (means ± SD, $n = 3$).

Treatment	Pharmacokinetic parameter[a]				
	$AUC_{0-\infty}$ (g h/l)	$MRT_{0-\infty}$ (h)	$t_{1/2}$ (h)	V_{ss} (ml/kg)	CL (ml/h per kg)
ROP	0.170 ± 0.024	0.826 ± 0.031	0.724 ± 0.004	314 ± 46	300 ± 44
C-1	0.179 ± 0.012	1.50 ± 0.07	1.39 ± 0.08	564 ± 71	280 ± 18
C-2	0.140 ± 0.019	1.86 ± 0.14	1.47 ± 0.16	570 ± 92	325 ± 28
C-3	0.176 ± 0.009	0.814 ± 0.068	0.791 ± 0.072	324 ± 14	285 ± 14
C-4	41.9 ± 5.8	85.6 ± 17.3	72.1 ± 16.7	123 ± 13	1.21 ± 0.18
C-5	7.82 ± 0.84	31.8 ± ± 5.4	34.3 ± 6.3	319 ± 65	6.33 ± 1.15

[a] Calculated by non-compartmental moment analysis assuming first-order elimination. $t_{1/2}$ is calculated from the elimination rate constant (λ_Z) using the formula $t_{1/2} = 0.693/\lambda_Z$.

allowed for its much easier glomerular filtration especially when their molecular weights are both around the glomerular filtration threshold (~30 kDa for PEG [20,21]). This coincides well with the conclusion obtained by Arendshorst and Navar, who demonstrated that glomerular filtration of macromolecules was related not only to their size and charge but also to shape and rigidity [22]. In addition, C-4 displayed a much smaller distribution volume than ROP and the other conjugates studied, suggesting its significantly reduced distribution outside of the vascular compartment.

4. Discussion

Due to the lack of anomeric proton in the structure of the fructan ROP, the ^{1}H NMR method used to determine the graft number of PEGylated dextrans [15] was inapplicable for PEGylated ROPs. The very few carbamate bonds in the mPEG-ROP conjugates also make the confirmation of PEGylation by ^{1}H NMR or FTIR impractical. Thus, in this study, the carbohydrate-specific anthrone-sulfuric acid colorimetry was used in combination with HPGPC to successfully confirm the conjugation between ROP and mPEG. However, if there is free mPEG in conjugates, the graft degree would be overestimated when it was calculated according to this assay. Furthermore, the degree of overestimation will depend not only on the amount of free mPEG in conjugates, but also on the effect of conjugation on the chromogenic reaction, both of which are nearly impossible to be accurately determined. For example, the calculated graft degree for C-5 was approximately 2.1 by this assay, which deviates far from the GPC result (approximately 1.3) and should be incredible when the 2:1 M ratio of mPEG to ROP in reaction is also taken into account. Therefore, the colorimetry is quite a useful qualitative method for the confirmation of conjugation rather than a quantitative one for the determination of grafting degree. In addition, due to the lack of direct and specific microassay methods, a fully validated HPGPC-FD method, which utilizes the FITC pre-labeling and the high-performance gel permeation chromatography to obtain the high analytical sensitivity and specificity, respectively, was established to study the pharmacokinetics of mPEG-ROP conjugates.

Presently, there are many biological drugs, either marketed or developing, having similar properties to ROP, which are hydrophilic, smaller in volume than the glomerular filtration threshold and, as a result, rapid renal clearance after injection administration. Solutions in common use to solve this problem and to enhance drug distribution in target tissues with the enhanced permeability and retention (EPR) effect include (1) loading of drugs in various long-circulating colloidal drug delivery systems and (2) chemical modification of drugs, which can be subdivided into hydrophobic and polymeric modification [23,24]. Compared with the method one, polymeric conjugation can often achieve a prolonged blood residence, an increased resistance to degradation and decreased side-effects. Besides, the linkage between polymer and drug can be designed to control where and when the drug is released; thus, patient compliance is generally improved.

As to polymeric modification, the first vital factor needing to be considered in the design is the polymeric type. Although a wide variety of polymers have been used in this field, either from natural or from synthetic sources, only few have won great success. Among them, PEG emerged as the most potential one, which has already led to nearly ten marketed products such as PEG-filgrastim, PEG-interferon, PEG-asparaginase, PEG-adenosine deaminase, PEG-anti-VEGF aptamer and PEG-erythropoietin due to its unique advantages: (1) the lack of immunogenicity, antigenicity and toxicity; (2) its high solubility in water and in many organic solvents; (3) the high hydration and flexibility of the chain; and (4) its approval by the FDA for human use in injectable formulations [13,14]. However, it is also important to recognize that PEG itself may possess antigenic and immunogenic properties. The much more frequent emergence of antibodies-against-PEG positive sera now than before may reflect increasing exposure to PEG and PEG-containing compounds due to their widespread use in agrochemicals (pesticides, herbicides and fertilizers), processed food products, cosmetics and pharmaceuticals, which has led to increasingly extensive and intensive attention on their potential impact on the effectiveness of treatment with PEGylated therapeutics [25]. To better exploit the potential of PEG modification, several strategies have been developed: (1) using site-selective conjugation; (2) using few high molecular weight PEG chains than a higher number of low molecular ones; and (3) performing PEGylation under gentle conditions.

In practice, the first strategy is feasible for proteins [26,27] but is difficult or even impossible for carbohydrates due to both the limited understanding of their structure–activity relationship and the lack of reactivity-specific functional groups. The second strategy should be used with caution in terms of the fact that the possibility of accumulation of PEG in the body will increase sharply after reaching a threshold value (~30 kDa) due to both the limited renal excretion and in vivo chemical and biological degradation of PEG [20,21]. Therefore, 2-, 5- and 20-kDa mPEGs were considered as the suitable testing forms to prepare the PEGylated ROP. It was found in this study that the desirable pharmacokinetic properties could be achieved when approximately 5.4 5-kDa or 1.3 20-kDa mPEGs were conjugated onto a ROP molecule.

As to the PEGylation process for the fructan ROP, methods reported for the PEGylation of dextran [15,16,28–30] might be used for reference. All the methods more or less change the structure of the carbohydrate modified, being expected to have varying degrees of effects on its bioactivity. The fewer changes occur, the better the method should be. Thus, methods that cause charged conjugates [16] or require highly alkaline conditions for the coupling [30] should be avoided to be used for the PEGylation of carbohydrate-based drugs. In this study, the hydroxyls of ROP were reversibly activated using *p*-nitrophenyl chloroformate. After PEGylation, the residual *p*-nitrophenyl groups on the conjugate were hydrolyzed in a weakly alkaline aqueous solution, which ensures the least influence on the carbohydrate structure. This, together with

a proper choice of the molecular mass and shape of mPEG, makes the synthesis of conjugates with well-preserved bioactivity possible. In addition, a stable linkage between the PEG moiety and the drug is rather important to ensure that PEG-induced pharmacokinetic changes are maintained. One of the main reasons for the absent in vitro/in vivo bioactivity correlation for PEGylated drugs is the premature release of drug from the conjugate. In the present study, a stable carbamate linkage was formed between ROP and mPEG. Its stability was confirmed by the detection of plasma samples obtained in the pharmacokinetic study, which showed that no detectable degradations were found during the study. Therefore, good in vitro/in vivo activity correlation for PEG-ROP conjugates may exist. In addition, no matter which method was adopted, all chemicals and materials used should be anhydrous and the process should be carried out in a low humidity condition due to the high sensitivity to moisture of the reactions involved.

Presently, polymeric modification of drugs has been considered as a tool to enhance the drug delivery to pathologic tissues having the EPR effect, such as tumors and ischemic or inflammatory tissues. One of the most prominent characteristics of EPR effect is its size-dependent nature [31–33], which would make polymeric drugs penetrate the leaky capillary endothelium and diffuse in the extracellular space much more effectively than larger sized colloidal drug delivery systems. Due to the EPR effect caused by ischemia, PEG-PE micelles (7–20 nm) and long-circulating liposomes (120–150 nm) were confirmed being able to effectively accumulated in ischemic myocardia [33,34]. In our previous study, it was found that the accumulation of ROP (~2 nm) in hearts of experimental myocardial ischemic rats was ~2.2-fold higher than that in normal rat hearts [35], indicating that the EPR effect caused by ischemia can surpass the negative effect of decrease in blood flow on drug distribution during ischemia. However, the utilization of EPR effect is to a large extent limited by the rapid renal excretion of ROP, making the increase yet far from ideal. Therefore, PEG-ROP conjugates with small sizes (tens of nm), markedly prolonged blood retention time and well-preserved bioactivity, just like the conjugate C-5 presented in the study, would show further increased accumulation and retention in ischemic myocardia and, in turn, increased efficacy. In contrast, like PEG-doxorubicin conjugates [36], the decreased blood vessel permeability of conjugates in normal myocardia may become the rate-limiting factor in distribution, leading to their decreased accumulation in normal heart tissues.

In conclusion, the effects of PEGylation on the bioactivity and pharmacokinetics of ROP mainly depend on the grafting degree and the molecular weight of the conjugate, respectively. With proper design, PEG-ROP conjugates that are bioactive and long circulating in blood would be obtained. The improvement in systemic exposure of ROP by PEGylation may translate into clinical benefits and therefore represent an attractive opportunity for further development.

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