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The heparin-like activities of negatively charged derivatives of low-molecular-weight polymannuronate and polyguluronate

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ABSTRACT

A series of low-molecular-weight polymannuronate (LPM) and polyguluronate (LPG) polyanionic derivatives, including LPM/LPG phosphate (LPMP/LPGP), LPM/LPG H-phosphonate (LPMHP/LPGHP) and LPM/LPG sulfate (LPMS/LPGS), were prepared as heparinoids by chemical modification of LPM and LPG. The structures and characteristics of LPM, LPG and their derivatives were elucidated based on high performance gel permeation chromatography (HPGPC), fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance spectroscopy (NMR) and polyacrylamide gel electrophoresis (PAGE). In order to test the heparin-like activities of these derivatives and to reveal the activities affected by substituent groups and PM/PG polysaccharide backbones, the anticoagulant activities and FGF/FGFR1c signaling activation abilities were evaluated *in vitro*. The results showed that sulfate group was the best substituent group to improve the heparin-like activities of LPM/LPG compared with the other two anionic groups. The results also showed that sulfated derivative based on PG structure had better activities than that based on PM structure.

1. Introduction

Heparin, a member of glycosaminoglycans (GAGs), is well known for its anticoagulant activity, and has been used as an anticoagulant drug for more than 70 years in clinical practice (Rabenstein, 2002; Thulesius, 2006). Heparin also plays important roles in inhibition of complement activation (Girardi, Redecha, & Salmon, 2004; Oberkersch, Attorresi, & Calabrese, 2010), inhibition of tumor growth (Smiley, Henry, & Wong, 2006; Varki & Varki, 2002), inhibition of viral invasion (Lin et al., 2002; Seki et al., 2012), regulation of angiogenesis (Chiodelli, Bugatti, Urbinati, & Rusnati, 2015), etc. It is becoming clear that the biological functions of heparin are mediated by the heparin-protein interactions (Capila & Linhardt, 2002). Heparin-binding proteins typically contain large numbers of basic residues, such as lysine, arginine, and in some cases histidine. Because of the high negative charge densities, heparin is a polyelectrolyte, which can bind with the cationic sites of proteins to affect changes in conformation, thus modulate the functions of hundreds of proteins (Zhang, 2010).

The advancement in understanding of heparin-protein interactions has led to design novel highly specific therapeutic agents and biomaterials. For example, Borgenström et al. (2003) semi-synthesized a series of sulfated derivatives based on *Escherichia coli* K5 polysaccharides as modulators of FGFs/FGFRs signaling. Ran et al. (2012) synthesized a heparin-like macromolecule containing functional groups of $-SO_3H$, $-COOH$, and $-OH$, and this macromolecule can be blended into PES membrane for blood purification. However, all heparin analogs designed so far are modified by sulfate groups. It is still unknown if sulfate groups are required for biological activity or other polyanionic derivatives containing different anionic groups, such as phosphate or H-phosphonate, could also be used to make heparin analogs.

Alginate, a natural anionic polysaccharide, is composed of $1 \rightarrow 4$ linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) in the forms of M-blocks, G-blocks and interspersed MG-blocks (Panikkar

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& Brasch, 1996). It is the only polysaccharide, which naturally contains a carboxyl group in each constituent residue. Due to the heparin-like structure, sulfated alginate has drawn much attention as heparin analog (Huang, Du, & Yang, 2003). In China, propylene glycol alginate sodium sulfate (PSS), which is a sulfated derivative of alginate, has been used as a heparin-like drug for anticoagulation for 30 years (Lin et al., 2007; Zeng et al., 2014). We have recently reported the heparin-like anticoagulant, antithrombotic and FGF/FGFR signaling activation activities of low-molecular-weight PSS (Wu et al., 2016; Xin et al., 2016). The M and G in alginate are epimers and possess different structural and physicochemical characteristics (Atkins, Nieduszynski, Mackie, Parker, & Smolko, 1973a; Atkins, Nieduszynski, Mackie, Parker, & Smolko, 1973b). It has been reported that the sulfated M and G blocks have different bioactivities (Arlov, Aachmann, Sundan, Espevik, & Skjåk-Bræk, 2014). Therefore, it is important to study the biological activities of polymannuronate (PM) and polyguluronate (PG) side-by side when novel derivatives are made.

In this paper, we firstly prepared low-molecular-weight polymannuronate (LPM) and polyguluronate (LPG) from alginate, then semi-synthesized a series of polyanionic derivatives containing $-PO_3^{2-}$, $-PO_2H^-$ and $-SO_3^-$ groups. Anticoagulant activities and FGFs/FGFRs signaling activation abilities were detected *in vitro*. The effect of substituent groups and PM/PG basic structures of the novel alginate derivatives on the two heparin-like biological activities were revealed and discussed.

2. Materials and methods

2.1. Materials

Low-molecular-weight alginate (prepared by partial hydrolysis of alginate, M/G ratio was 1.64:1, and the weight averaged molecular weight was 13.4 kDa) was provided by Chia Tai Haier Pharmaceutical Co., Ltd. (Qingdao, China). Activated partial thromboplastin time (APTT) reagent, prothrombin time (PT) reagent and thrombin time (TT) reagent were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Heparin sodium (Hep) was purchased from Tianjin Biochem Pharmaceutical Co., Ltd. (Tianjin, China). Low molecular weight heparin (LMWH) was purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, China). Sheep plasma was purchased from Jiulong Biological Product Co., Ltd. (Zhengzhou, China). Fibroblast growth factors (FGF-1, -2, -7, -8, -9 and -10) and G418 were purchased from Goldbio. Fetal bovine serum (FBS) was purchased from Gibco. RPMI 1640 medium, penicillin and streptomycin were purchased from Jinuo Bio-pharmaceutical Tech. Co., Ltd. (Hangzhou, China). Resazurin, β-mercaptoethanol, and hyaluronic acid (HA) were purchased from Sigma (St. Louis, US).

2.2. Preparation of LPM and LPG

LPM and LPG were obtained after the crude PM and PG, separated via pH fractionation of alginate (Haug, Larsen, & Smidsroed, 1966), were hydrolyzed under high temperature and high pressure conditions. Briefly, 50 g of low-molecular-weight alginate was dissolved in 1000 mL of 2% Na_2CO_3 solution. The solution was adjusted to pH 2.85 using dilute hydrochloric acid, and then centrifugated to afford crude PM (the supernatant) and PG (the precipitate).

The crude PM was directly hydrolyzed at 110 °C for 20 min in an autoclave, and then adjusted to pH 2.80. The mixture was centrifugated, and LPM was obtained after the resulting supernatant was desalinated using a nanofiltration membrane with molecular weight cut off (MWCO) of 200–300 Da, and spray-dried.

The crude PG was resuspended in 1000 mL of distilled water, and adjusted to pH 2.85. The mixture was hydrolyzed at 120 °C for 1 h, and then adjusted to pH 2.90. The mixture was centrifugated, and the resulting precipitate was redissolved in 2% Na_2CO_3 followed by desalinating and spray drying to afford LPG.

2.3. Preparation of negatively charged derivatives of LPM and LPG

The negatively charged derivatives of LPM and LPG were prepared according to our previously reported methods (Li et al., 2013).

The phosphoric acid/urea method was used to prepare LPM/LPG phosphates (LPMP/LPGP). Briefly, 1 g of LPM/LPG, 18 g of urea and 7 mL of H_3PO_4 (85%) were added to 80 mL N,N-dimethyl formamide (DMF), and the mixture was kept at 130 °C for 4 h with stirring. The resulting precipitate was collected and washed with ethanol, and then dissolved in 50 mL of distilled water. The solution was adjusted to pH 9–10 followed by being dialyzed, rotary evaporated and freeze-dried to afford LPMP/LPGP.

LPM/LPG H-phosphonates (LPMHP/LPGHP) were prepared according to the phosphorous acid/urea method in that the experimental procedure was similar to the preparation of LPMP/LPGP except that 8.3 g of phosphorous acid was used as phosphorylation reagent in the reaction.

LPM/LPG sulfates (LPMS/LPGS) were synthesized according to the chlorosulfonic acid/formamide method. Briefly, 1 g of LPM/LPG was added to sulfation reagent (2.68 mL of chlorosulfonic acid was slowly added to 16 mL of formamide), and the mixture was stirred for 3 h at 65 °C. The reaction mixture was cooled and precipitated with ethanol. The resulting precipitate was collected and washed with ethanol before being dissolved in 50 mL of distilled water. The water solution was then adjusted to pH 9–10. LPMS/LPGS was obtained after the solution was dialyzed, rotary evaporated and freeze-dried.

2.4. Analytical methods

The phosphorus content (P%) was determined by a modified molybdenum blue method (Harris & Popat, 1954), and sulfate content (S%) was analyzed via the barium chloride/gelatin method (Kawai, Seno, & Anno, 1966). The weight average molecular weight (Mw) was determined via high performance gel permeation chromatography (HPGPC) on an Agilent 1100 instrument using our previously reported method (Li et al., 2013). Fourier transform infrared (FT-IR) spectroscopy was performed with a Nicolet Nexus 470 FT-IR spectrometer using the KBr pellet technique. The ^{13}C nuclear magnetic resonance (^{13}C NMR) spectra were collected with JNM-ECP600 spectrometer, and recorded with acetone as an internal standard (δ CH_3 = 31.45 ppm). Polyacrylamide gel electrophoresis (PAGE) analysis was carried out on a mini-protean tetra electrophoresis system from Bio-Rad Laboratories (Hercules, CA) according to the method by Lu, Zhao, Yu, Wang, & Xu (2002), and the gel was visualized and photographed in an UV-light chamber.

2.5. Anticoagulant activity

The anticoagulant activities of LPM, LPG and their derivatives were determined based on APTT, PT and TT assay using an ACL TOP automatic coagulation analyzer (Instrumentation Laboratory Co. USA). Before the tests, 590 μL of sheep plasma was mixed with 10 μL of tested samples (1.5 mg/mL in 0.9% NaCl solution), heparin sodium (0.1875 mg/mL in 0.9% NaCl solution), LMWH (0.75 mg/mL in 0.9% NaCl solution) or normal saline (0.9% NaCl solution), respectively.

For APTT assay, 50 μL of the sample-plasma mixture was added to 50 μL of APTT reagent, and incubated at 37 °C for 2 min. 50 μL of pre-warmed (at 37 °C) calcium chloride solution (0.025 mol/L) was then added, and the time to clot formation was measured. For PT assay, 50 μL of the sample-plasma mixture was incubated at 37 °C