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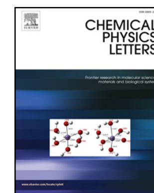

Research paper

Depolymerization of heparin by dielectric barrier discharge: Effect of operating modes and anticoagulant potential analysis of low-molecular-weight products

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HIGHLIGHTS

- DBD treatment result in uniform oligo heparin product.
- Any desirable molecular size of oligo heparin could be obtained by adjusting the DBD parameters.
- Although the anticoagulant potent declined, the digested heparin keeps good anticoagulant potent.

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ABSTRACT

Low-molecular-weight heparin and ultra-low-molecular-weight heparin are widely used as anticoagulant and antithrombotic drugs. An atmospheric pressure dielectric-barrier discharge procedure was developed to depolymerize heparin to low- or ultra-low-molecular-weight fragments. Dielectric-barrier discharge treatment of heparin resulted in several well-separated and uniform fragments. The structural character of digested fragments below 3,000 Da was elucidated. Expected digestion products such as sulfated disaccharide, sulfated tetrasaccharide, and pentasaccharides with alternatingly-linked N-acetyl glucosamine and iduronic acid were detected. The anticoagulant potential of the digested heparin was tested. The results indicated that dielectric-barrier discharge treatment can produce various molecular weight (MW) heparin fragments and maintain anticoagulant potency.

1. Introduction

Heparin is a highly-sulfated glycosaminoglycan polymer which is comprised of β-1-4-linked hexuronic acid and glucosamine residues [1]. It is generally obtained from animal tissues such as porcine intestinal mucosa and purified to remove contaminants [2]. Heparin may have direct anticancer benefits owing to reported effects on tumor growth, angiogenesis, and metastasis, besides the better-known anticoagulant and anti-inflammatory properties [3,4]. However, like other naturally-occurring polysaccharides, unfractionated heparin has a highly complex, heterogeneous composition including many branching chains [5]. Further, contamination by unfractionated heparin has been reported in association with bleeding complications, negative effects on bone metabolism, and risk of heparin-induced thrombocytopenia (HIT) [5–7]. Thorough processing is essential for the usage of heparin-based drugs.

Low-molecular-weight heparin (LMWH) shows reduced binding to plasma proteins which makes it more predictable pharmacokinetically, increases half-life, and reduces risk of HIT and bone loss in comparison to unfractionated heparin. The depolymerization of heparin to LMWH provides an alternative means for venous thromboembolism prevention in cancer treatment and it is widely-used in clinical practice as an anticoagulant and antithrombotic drug [3].

Multiple heparin depolymerization methods have been developed and applied in LMWH production including β-eliminative cleavage of benzyl esters within heparin by alkaline treatment, deaminative cleavage with nitrous acid, β-eliminative cleavage by heparinase, and isoamyl nitrite depolymerization [3]. However, most of these reagents used during heparin depolymerization result in negative environmental impacts and the accumulation of toxic substances in the final product. Although the application of heparinase makes it environmentally

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friendly, the low efficiency and high cost remain unresolved.

A promising alternative to these methods is plasma treatment. Plasma is partially-ionized gas and produces a reactive environment where a variety of energetic species (charged and excited particles, reactive neutrals, and UV photons) are formed mainly from collisions of energetic electrons with heavy particles (atoms or molecules) [8]. The primary active components of uncharged plasma acting during water treatment are high energy electrons, active free radicals (·H, ·O, ·OH, etc.), reactive molecules (O_3, H_2O_2, etc.), UV photons, and shock waves [9]. All of these stress factors are proposed to play roles in treatment with aqueous solutions, but a direct effect of plasma-generated species and chemical sputtering seem to be the predominant ones [10]. Due to the moderate strength and high energy, the non-equilibrium plasma discharges (energetic electrons, but cold, heavy particles) offer an entirely new approach for polysaccharide depolymerization. Performing a plasma treatment could provide a means of producing a uniformly-digested polysaccharide product efficiently, at low cost, and in an environmentally-friendly manner.

In our present study, dielectric-barrier discharge (DBD) was employed to generate plasma to digest heparin in aqueous solution. The anticoagulant activities of the digested heparin treated by DBD were tested. Factors (i.e. input voltages, treatment time, substrate concentration, electrode gap length, and solvents used to dissolve the heparin) affecting the degradation of heparin were investigated. Degradation products smaller than 3,000 Da were characterized using HPLC-PAD-MS^n analysis. To the best of our knowledge, the utilization of plasma for the degradation of heparin has not been reported before. Therefore, we propose a new efficient method for LMWH production by applying plasma as a preferred alternative to traditional chemical lysis methods.

2. Materials and methods

2.1. Materials and chemicals

Heparin sodium was purchased from BBI Life Sciences (Shanghai, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was acquired from Sinopharm Chemical Reagent Co. (Beijing, China). Acetonitrile (Fisher, Pittsburgh, PA, USA) was of HPLC grade and ultrapure water was used for HPLC-MS analysis. Trifluoroacetic acid (TFA), monosaccharide standards and dextran standards were purchased from Pharmacia Co. (Uppsala, Sweden). All chemicals used in this study were of analytical reagent grade or better.

2.2. Degradation by DBD

A DBD operated to atmospheric pressure has been used for the degradation of heparin sodium. The experiments were carried out in a 500 W atmospheric pressure DBD instrument (CTP-2000 K, Nanjing Suman Plasma Technology Co., Ltd., Nanjing, China) with a frequency of 1 to 100 kHz and maximal voltage output of 30 kV. The experimental apparatus mainly included a plasma generator, a dielectric barrier plasma reactor, and a cylindrical quartz reaction chamber (12 mm height and 6 cm inner diameter). The dielectric-barrier plasma reactor consists of top and bottom electrodes with the cylindrical quartz reaction chamber in between. Plasma treatments were performed in the cylindrical quartz reaction chamber which surrounds the sample. Heparin sodium was dissolved in distilled water and transferred to the reaction chamber for DBD degradation. The effects of treatment time (3–9 min), input voltage (60–90 V), sample concentration (1–7 mg/mL), electrode gap (12–32 mm), and solvent (water, 0.5% sodium chloride, and 0.5% sodium sulfate, w/v) on degradation were investigated.

2.3. HPGPC analysis

The degradation effect and MW were monitored by high-performance gel permeation chromatography (HPGPC), which was performed on a liquid chromatography system Waters e2695 (Waters Co., Milford, MA, USA) equipped with a 7.8 × 300 mm TSK-gel G4000PWXL column (Tosoh Bioscience, Tokyo, Japan), and a 2414 Refractive Index Detector (Waters Co., Milford, MA, USA). 20 µL of sample solution (1 mg/mL) was injected in each run using distilled water as the mobile phase at a flow rate of 0.2 mL/min. The column was calibrated using dextran standards (1×10^3 Da, 1.2×10^3 Da, 5×10^3 Da, 4.7×10^4 Da, 5×10^4 Da, and 6.1×10^4 Da).

2.4. Sulfated polysaccharide content analysis

The degradation products of heparin were collected, combined, and centrifuged in an ultrafiltration centrifuge tube (UFC901024, Millipore Co., Bedford, MA, USA) to separate by MW. Ultrafiltration membranes with MW cut-offs of 1 kDa, 3 kDa, and 10 kDa were used to separate the degradation products. Therefore, we separated the heparin degradation products into 1–3 kDa, 3–10 kDa, and > 10 kDa MW range fractions. The sulfated polysaccharide content of the three heparin fractions was determined via dimethylmethylene blue assay as described by Farndale, Buttle, and Barrett [11]. Briefly, 10.7 mg of dimethylmethylene blue was dissolved in 1 L distilled water and adjusted to pH 3.3 with formic acid. This reagent was mixed with 2 M Tris at a 10:1 ratio of dimethylmethylene reagent to Tris buffer to obtain the color reagent. The sulfated heparin standards and sample solutions were mixed with 4 mL color reagent and left in the dark for 15 min, then measured with a spectrophotometer at 525 nm wavelength in an Infinite M200 microplate reader (Tecan, Switzerland). A sulfated heparin standard curve was created and applied to determine the sulfated polysaccharide content of the degraded heparin fractions.

2.5. FTIR spectroscopic analysis

The Fourier transform infrared (FTIR) spectra of samples was determined by a Specord 75 IR spectrometer (Carl Zeiss, Jena, Germany) with KBr pellets.

2.6. PMP derivatization

The DBD-treated heparin products were combined, centrifuged in an ultrafiltration centrifuge tube (UFC901024, Millipore Co., Bedford, MA, USA) equipped with a 3 kDa cut-off membrane, concentrated, lyophilized, and then derivatized by PMP according to Wang et al. [12]. All samples were converted to their PMP derivatives for HPLC-PAD-MS^n analysis.

2.7. HPLC-PAD-MS^n analysis

The HPLC-PAD-MS^n analysis was performed on an LXQ linear ion trap mass spectrometer equipped with an electrospray ion source and photodiode array detector (PAD) controlled by Xcalibur software (Thermo Fisher Scientific, Basel, Switzerland). A Silgreen ODS C18 (250 × 4.6 mm, 5 µm) column was used for this analysis. The ESI-MS settings and the elution requirement were performed as described by Wang et al. [12].

2.8. Anticoagulant activity analysis

The activated partial thromboplastin time (APTT) test, thrombin time (TT) test, and the prothrombin time (PT) test were carried out to determine the anticoagulant activity *in vitro* according to Zhao et al. [13]. Briefly, human blood was obtained from healthy donors from Dalian Central Hospital, Liaoning Province, China. Then the blood was mixed with sodium citrate solution (3.8%, w/v) at a ratio of 9:1 (v/v). The mixture was centrifuged at 3,000 ×g for 10 min to isolate the plasma for further usage. Heparin fractions were dissolved in saline

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