Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

# Effects of linear and branched polyethylene glycol on PEGylation of recombinant hirudin: Reaction kinetics and *in vitro* and *in vivo* bioactivities

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ARTICLE INFO

*Keywords:*
Recombinant hirudin
PEGylation
Branched mPEG
Reaction kinetics
*In vitro* anticoagulant activity
*In vivo* pharmacological efficacy

ABSTRACT

To improve the therapy efficacy of recombinant hirudin variant-2 (HV2), its PEGylation was investigated using linear mPEG-succinimidyl carbonate (mPEG-SC) and branched $mPEG_2$-*N*-hydroxysuccinimide ($mPEG_2$-NHS). The reaction mixtures of PEGylation were analyzed by RP-HPLC and the mono-PEG-HV2 products were purified by anion exchange chromatography (IEC). Effects of linear and branched PEG on the hydrolysis kinetics of the PEG reagent, the PEGylation kinetics of HV2 and the *in vitro* and *in vivo* bioactivity of mono-PEG-HV2 were investigated. The RP-HPLC and IEC analyses showed that linear and branched PEG-HV2 with identical molecular weight had different chromatographic behaviors. The reaction kinetics showed that branched $mPEG_2$-NHS displayed higher hydrolysis rate but lower PEGylation rates than linear mPEG-SC. Consequently, HV2 conjugated with $mPEG_2$-NHS required a greater molar ratio of PEG to HV2 than that of mPEG-SC to achieve the identically desired yield of mono-PEG-HV2. The *in vitro* and *in vivo* bioactivities of mono-PEG-HV2 showed that branched PEG-HV2 had higher therapeutic efficacy than linear PEG-HV2 with identical molecular weight. The *in vivo* bioactivity of mono-B-PEG40k-HV2 (mono-PEG-HV2 derived from 40 kDa branched $mPEG_2$-NHS) had a markedly longer duration in rabbits than did unmodified HV2, which showed its potential to be developed as a candidate antithrombotic drug.

## 1. Introduction

Hirudin is a 7-kDa single-chain polypeptide of 65 amino acids that is the most potent known thrombin inhibitor [1]. Recently, recombinant lepirudin and desirudin and the hirudin analogue bivalirudin have been approved by the FDA as anticoagulants. As with many other therapeutic proteins and peptides, recombinant hirudin has a short plasma half-life, as well as adverse side effects, such as bleeding and immunogenicity [2,3]. These disadvantages limit recombinant hirudin's clinical application. PEGylation, a well-known and effective strategy to overcome such disadvantages of proteins and peptides, has led to 12 marketed drugs [4]. Common site-specific PEGylation may be not suitable for recombinant hirudin due to its structure [5], which has already been discussed in our previous report [6]. Thus, most of the reported PEG-recombinant hirudin conjugates have been random PEGylation at lysine residues. PEGylation can be affected by the properties of the protein, the reaction conditions as well as the properties of the PEG reagent (*e.g.*, structure, size and linker chemistry of PEG moiety) [7–11]. Random PEGylation of proteins such as recombinant hirudin should therefore be carefully optimized to improve the homogeneities and the retained bioactivities of mono-PEGylated protein conjugates. Both linear and branched structures of PEG reagents have been used. Compared with linear PEG, branched PEG has many advantages [11–17]: (1) branched PEG displays higher steric hindrance, which could prevent the PEG from coupling to the protein at the bioactive and buried sites, and thus improving the retained bioactivity of mono-PEG-protein conjugates and the selectivity of PEGylation by reducing the formation of multi-PEG-protein conjugates; and (2) branched PEG has a greater shielding effect on a protein surface, thus resulting in higher proteolytic resistance and lower immunogenicity of mono-PEG-protein conjugates.

In previously published reports, PEGylation of recombinant hirudin has been achieved using mPEG-SPA [18,19] and mPEG-SC [6,20–22]. Moreover, some reported PEG-recombinant hirudin conjugates showed higher *in vivo* pharmacological efficacy compared to the unmodified

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http://dx.doi.org/10.1016/j.procbio.2017.08.004
Received 30 March 2017; Received in revised form 12 July 2017; Accepted 4 August 2017

recombinant hirudin [6,19]. Both mPEG-SPA and mPEG-SC are, however, linear structure PEGs. So far, no report on PEGylation of recombinant hirudin with branched PEG has been published. Because many other proteins conjugated with branched PEG showed higher therapy efficacy than proteins conjugated with linear PEG [11–17], recombinant hirudin conjugated with branched PEG is worth investigating to further improve its therapy efficacy.

In this study, recombinant hirudin variant-2 (HV2) was conjugated with 20 and 40 kDa branched $mPEG_2$-*N*-hydroxysuccinimide ($mPEG_2$-NHS). The hydrolysis kinetics of $mPEG_2$-NHS and its PEGylation kinetics with HV2 were investigated. Reaction conditions (molar ratio of PEG to HV2 and reaction time) were optimized to obtain mono-PEG-HV2 at a desirable yield. The PEGylation reaction mixture was purified by anion exchange chromatography and characterized by SDS-PAGE and RP-HPLC. The *in vitro* anticoagulant activity and *in vivo* pharmacological efficacy of mono-PEG-HV2 were characterized. PEGylation of HV2 with linear mPEG-succinimidyl carbonate (mPEG-SC) was performed as a control to compare the characteristics of PEGylation of HV2 with branched $mPEG_2$-NHS.

## 2. Materials and methods

### 2.1. Materials

Recombinant hirudin variant-2 (HV2) (> 95% HPLC pure, Fig. 1A) was from Chongqing Kerun Biomedical R & D Co., Ltd. (Chongqing, China). Linear mPEG-succinimidyl carbonate (mPEG-SC, MW = 20 kDa, Fig. 1B) and branched $mPEG_2$-*N*-hydroxysuccinimide ($mPEG_2$-NHS, MW = 20 and 40 kDa, Fig. 1C) were from Beijing Kaizheng Biotech Development Co., Ltd. (Beijing, China). Thrombin (Sigma-T4648), fibrinogen (Sigma-F8630), acetonitrile (HPLC grade), trifluoroacetic acid (TFA) and other chemicals of analytical grade were from Sigma-Aldrich (St. Louis, USA). LiChrospher 100 RP-18 column (250 mm × 4.6 mm, 5 μm) was from Merck (Darmstadt, Germany). A 5 mL HiTrap Q HP column was from GE Healthcare (Piscataway, New Jersey, USA). Thrombin time (TT) assay kits were from Nanjing Jiancheng Biotechnology Co. Ltd. (Nanjing, China).

### 2.2. Animals

Male New Zealand White rabbits (2.0 ± 0.2 kg) were from the Experimental Animal Center of Dalian Medical University (Dalian, China, quality certificate number: SCXK (Liao) 2008-0002). Animal care and handling were according to the Animal Guidelines of Dalian Medical University and approved by the Ethics Committee of Dalian Medical University.

### 2.3. Hydrolysis of mPEG-SC and $mPEG_2$-NHS

mPEG-SC and $mPEG_2$-NHS (Fig. 1D and E) hydrolysis kinetics were measured as our previously reported [21]. Briefly, the desired concentration of mPEG-SC (MW = 20 kDa) or $mPEG_2$-NHS (MW = 20 or 40 kDa) was incubated in a 20 mM sodium phosphate buffer solution, pH 8.0, at 25 °C. The reaction was monitored at an absorbance of 260 nm using a Jasco V-560 UV/VIS spectrophotometer (JASCO Co., Ltd., Tokyo, Japan) at 25 °C for up to 120 min.

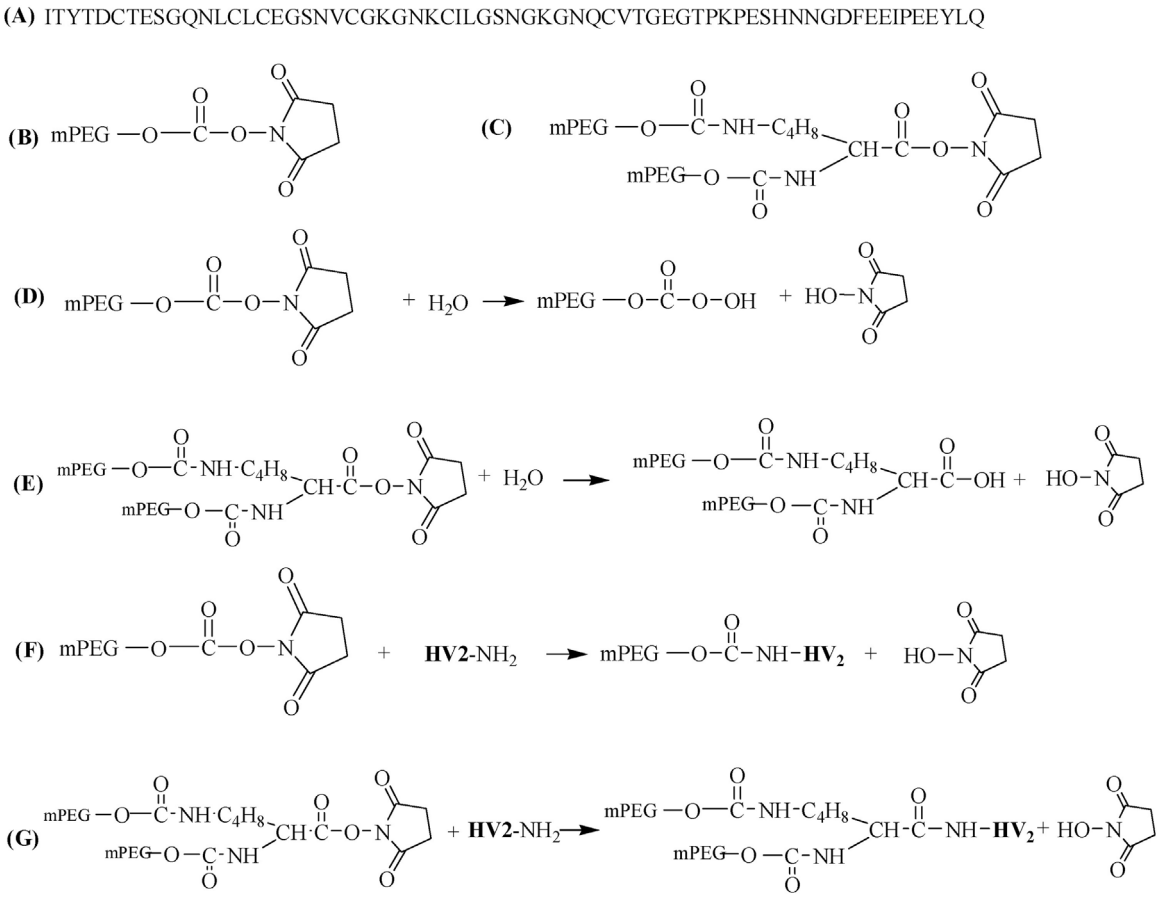


**Fig. 1. (A)** Amino acid sequence of recombinant hirudin variant-2 (HV2); **(B)** Structure of linear mPEG-succinimidyl carbonate (mPEG-SC); **(C)** Structure of branched $mPEG_2$-*N*-hydroxysuccinimide ($mPEG_2$-NHS); **(D)** Hydrolysis of mPEG-SC; **(E)** Hydrolysis of $mPEG_2$-NHS; **(F)** Simplified PEGylation of HV2 with mPEG-SC; **(G)** Simplified PEGylation of HV2 with $mPEG_2$-NHS.