Journal of Controlled Release 159 (2012) 353–361

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

Selective conjugation of poly(2-ethyl 2-oxazoline) to granulocyte colony stimulating factor

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ARTICLE INFO

Article history:
Received 12 December 2011
Accepted 26 February 2012
Available online 3 March 2012

Keywords:
Polymer therapeutics
Polyoxazoline
Protein pharmaceuticals
Granulocyte colony stimulating factor
Transglutaminase
Reductive alkylation

ABSTRACT

Poly(2-ethyl 2-oxazoline) (PEOZ) is a water-soluble, stable and biocompatible polymer that was prepared in a linear form for the conjugation of protein biomolecules. Polymers of molecular weights ranging from 5 to 20 kDa, with an aldehyde or an amine functional terminal group, were synthesized with narrow polydispersities. To assess the suitability of the polymer for therapeutic application, granulocyte colony stimulating factor (G-CSF) was used as a model protein for PEOZ conjugation. Two coupling strategies were employed, namely the chemical N-terminal reductive amination and the enzymatic transglutaminase (TGase) mediated glutamine conjugation. The secondary structure of the protein, measured by circular dichroism, was maintained upon PEOZylation and the stability of conjugates toward aggregation at 37 °C was improved compared to G-CSF. The potency of PEOZ-G-CSF mono-conjugates was tested in vitro by cell proliferation assays and in vivo by studying the effects on white blood cell and neutrophil count increases in normal rats. The results have shown that PEOZ is suitable for protein conjugation by both chemical and enzymatic methods and that the conjugates of G-CSF retained high biological activity, both in vitro and in vivo.

1. Introduction

The biopharmaceutical drug portfolio contains a significant number of naturally occurring and recombinant proteins. These biomolecules have been developed as drugs to treat several diseases such as neutropenia, anemia, hepatitis, hemophilia, cancer and Crohn's disease. However, protein drugs might suffer from several limitations, such as short blood circulation with poor in vivo residence time, proteolysis, immunogenicity, chemical and physical instability. Protein modification with biocompatible polymer is a well-known method to improve the pharmacological properties and the stability of such drugs. The polymer conjugation reduces proteolytic digestion and slows the rate of renal glomerular filtration by providing a camouflage around the protein and producing an increase of its hydrodynamic size. This shield also protects the drug from possible protein antigen-antibody like reactions. So far, PEG is the most used polymer in this field and has achieved wide success in protein drug development as demonstrated by the several approved products [1,2]. It has been covalently attached to many proteins among them granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO), interferon α (IFN), and human growth hormone (hGH) [3]. PEG is biocompatible, non-toxic, safe, widely used as excipient in several drug formulation, body-care products and food. Nevertheless, some recent reports have shown the presence of specific antibodies against PEG in the serum of patients treated with PEG-asparaginase [4] and PEG-uricase [5] that yielded a loss of therapeutic efficacy. However, it has to be highlighted that in these cases the conjugated proteins were of heterologous origin and very immunogenic and to date it is unclear to what extent the anti-PEG response is related to the nature of the conjugated protein. Another controversy is related to the possible kidney cell vacuolization observed in animals following repeated administrations of PEG conjugates, as demonstrated with TNF-α binding protein, leptin [6], and hemoglobin [7,8]. Also in this case, it is important to note that this vacuolation was not correlated with any renal dysfunction and was reversible after 2 months. Furthermore it has been reported only with certain conjugates administered at high doses, and not with PEG alone. Another constrain for the use of PEG in protein conjugation is the high number of patents issued on claims of composition, methods and application that can hamper the ability to introduce new PEGylated therapies into the clinic. On these bases, companies and research groups have gone to seek and test

Abbreviations: POZ, poly(alkyl-2-oxazoline); PEOZ, poly(2-ethyl 2-oxazoline); PEG, poly(ethylene glycol); HPLC, high performance liquid chromatography; RP, reverse-phase; SEC, size exclusion chromatography; IEC, ionic exchange chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time of flight, G-CSF, human granulocyte colony stimulating factor; TGase, transglutaminase.

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doi:10.1016/j.jconrel.2012.02.025

alternative polymers as hyaluronic acid [9], polysialic acid [10,11], hydroxyethyl starch [12], poly(2-methacryloyloxyethyl phosphorylcholine) [13], polyvinylpyrrolidone [14], poly(acryloil-morpholine) [15] and dextrin [16,17].

Polyoxazoline (POZ) is a synthetic water-soluble polymer used in food container products and limited dermatological formulations. Previous reports have shown that it has stealth like properties and can be used in biomedical applications when grafted to phospholipids [18]. The chemistry, the general properties and the applications of POZ have been described in recent papers [19,20]. Recently, we have made pharmaceutical grade poly(2-ethyl 2-oxazoline) (PEOZ) with molecular weights ranging between 500 and 40,000 Da and with low polydispersity indices of less than 1.10. The polymer was accurately characterized by several techniques that demonstrated its high solubility, hydrodynamic volumes comparable but slightly lower than PEG, batch to batch reproducibility, low polydispersity values, and good yields of reaction. Furthermore, after conjugation with model enzymes, as uricase, catalase and ribonuclease, we found that the resulting conjugates retained high activity [21].

In this paper we studied human recombinant granulocyte colony stimulating factor (G-CSF), the sequence reported in Fig. 1, because of its important therapeutic interest. G-CSF is a hematopoietic cytokines [22–26] that regulates the growth and differentiation of hematopoietic progenitor cells to functionally activate the formation of mature neutrophils. G-CSF, marketed by Amgen as Neupogen, has been widely used to treat neutropenia that is often induced by myelosuppressive chemotherapy [27,28]. Unfortunately, it is rapidly cleared from the body by a combination of fast renal and receptor mediated neutrophil eliminations and as a result, it must be injected repeatedly or in a continuous infusion in order to achieve sufficiently elevated neutrophil and mobilized progenitor stem cell levels in the peripheral blood [29]. Neulasta is the next generation of G-CSF, and has a 20-kDa PEG group attached to the N terminus of the protein. It exhibits comparable clinical benefits as G-CSF, but due to its long half-life in the serum, only needs to be dosed once per chemotherapy cycle, as opposed to repeated daily administration [30]. Neulasta was prepared using a site-selective conjugation of PEG-aldehyde 20 kDa to the N-terminus of the protein exploiting the lower *p*Ka of α-amino group when compared to that of the ε-amine lysines [30,31]. In other reports site-specific G-CSF PEGylation was carried out at cysteine 18, by performing the coupling under reversible unfolding conditions [32], or at glutamine (Gln) 135 by exploiting a transglutaminase (TGase)-mediated conjugation [33]. In the latter, the highly selective conjugation at Gln 135 was achieved although the protein sequence contains a total of 17 Gln(s) [34]. The high flexibility of the peptide chain embedding Gln 135 protein allows for the recognition of the enzyme only at this site [35].

In this paper PEOZ is proposed as carrier of the pharmaceutical protein G-CSF. A detailed description and comparison is made between two PEOZ 20 kDa-G-CSF conjugates. The polymer was conjugated at the N-terminal methionine by a reductive amination using a PEOZ aldehyde, and at the Gln 135 by a TGase mediated conjugation of a PEOZ amine. The physicochemical properties, aggregation potential and the in vitro and in vivo potencies of each compound were studied.

2. Experimental procedures

2.1. Materials

Poly(2-ethyl 2-oxazoline) 5, 10 and 20 kDa polymers were synthesized by the chemistry group at Serina Therapeutics as previously reported [21]. Granulocyte colony stimulating factor (G-CSF) was obtained from Biocon Ltd., Bangalore, India. Activa RM, which contains 1% of the enzyme transglutaminase (TGase), was purchased from Ajinomoto Food Ingredient, LLC. N-hydroxysuccinimide (NHS) anddicyclohexylcarbodiimide (DCC) were from Acros Chemicals. Trifluoroacetic acid (TFA), 2,4,6-trinitrobenzene sulfonic acid (TNBS), methyl trifluoromethylsulfonate, ethylenediamine, cadaverine, hexanediamine, sodium dodecyl sulfate (SDS), polyacrylamide powder, coomassie brilliant blue and acetonitrile (ACN) were purchased from Sigma-Aldrich. All other organic solvents for synthesis and analysis were from EMD Chemicals.

2.2. Analytical methods

A Gilson high performance liquid chromatography (HPLC) system with a Jasco UV-875 ultraviolet detector was used. Reverse-phase chromatography (RP–HPLC) was used to monitor the conjugation reactions and to determine purity of PEOZ-G-CSF conjugates, the analytes were separated on an Agilent C18 column (4.6 × 250 mm, 5 μm), settling the detection at 280 nm. The separation was achieved by a linear gradient of 40–70% acetonitrile containing 0.05% TFA (eluent B) over a 25 min period, followed by an isocratic wash at 80% of eluent B and at a flow rate of 1 mL/min. Eluent A was water containing 0.05% TFA. Strong-cation exchange column (IEC–HPLC) was used to check the presence of the eventual positional isomers of purified PEOZ-G-CSF conjugates, the analytes were separated on a TSK-gel, SP-5PW (7.5 mm × 7.5 cm, 10 μm) column and detected at 280 nm. The column was pre-equilibrated with 10 mM sodium phosphate buffer pH 4.7 at a flow rate of 1 mL/min. The bound protein derivative was eluted using a NaCl gradient (0.01 to 0.1 M) over 90 min. Size exclusion chromatography (SEC) was exploited to monitor the conjugation reactions of PEOZ-N^{ter}-G-CSF and to study the aggregation propensity of conjugates, a Phenomenex BioSep SEC-4000 column (7.6 mm × 300 mm, 5 μm) was used. The mobile phase was 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0 and the flow rate was 0.5 mL/min. The effluent from the column was monitored at 280 nm.

2.3. Preparation of aldehydes of PEOZ 10 kDa and PEOZ 20 kDa

The synthesis of terminal carboxylic acid polyethyloxazoline acid (PEOZ-COOH) and the corresponding NHS esters has been described elsewhere [21]. In this preparation, 3-amino-1,2-propanediol (15 mmol) was first dissolved in 0.1 M boric acid buffer (pH 8) and the pH adjusted to 9. The PEOZ-NHS ester (0.05 mmol of 20 kDa or 0.1 mmol of 10 kDa) was added to this solution and mixed vigorously for 3 h at room temperature. The solution pH was then adjusted to pH 6.8 by the addition of 1 N HCl and sodium chloride was added to partially saturate the solution. Purified water was added to the aqueous

MTPLGPASSL P**Q**SFLLKCLE **Q**VRKI**Q**GDGA
AL**Q**EKLCATY KLCHPEELVL LGHSLGIPWA
PLSSCPS**Q**AL **Q**LAGCLS**Q**LH SGLFLY**Q**GLL
QALEGISPEL GPTLDTL**Q**LD VADFATTIW**Q**
QMEELGMAPA L**Q**PT**Q**GAMPA FASAF**Q**RRAG
GVLVASHL**Q**S FLEVSYRVLR HLA**Q**P

Fig. 1. Sequence representation of human granulocyte colony stimulating factor. In bold the glutamines, potential sites of TGase conjugation.