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Research Article

The influence of polyethylene glycol structure on the conjugation of recombinant human interferon α2b overproduced using synthetic gene in *E.coli*

Heni Rachmawati, Prima L. Febrina, Ratih A. Ningrum, Debbie S.Retnoningrum

Research Group of Pharmaceutics, School of Pharmacy, Bandung Institute of Technology, Ganesha 10 Bandung 40132, Indonesia

ABSTRACT

Interferon α2b (IFNα2b) is a cytokine which has antiviral, antiproliveration and immunomudulator activities. IFN α2b has been used as a drug for treatment of hepatitis B and C infections. However, the use of this protein is limited, as rapidly cleared by the kidney. Approaches have been made to prevent this fast renal clearance such as increasing the molecular weight of protein using inert compounds like polyethylene glycol (PEG). The aim of this research was to study the influence of PEG structure on the interferon alpha 2b pegylation. rhIFNα2b was obtained from *Eschericia coli* BL21 containing synthetic gene for rhIFNα2b. rhIFNα2b was pegylated with two different form of PEGs, i.e linear PEG-carbonyldiimidazole (PEG-CI) and branched, trimethyl succinimidyl polyethylene glycol ($TMS(PEG)_{12}$). PEG-CI was activated and characterized with Dragendorf reagent and infra red spectrophotometry. rhIFNα2b was pegylated using molar ratio 1:100 and 1:300 (protein:PEG). Characterization was performed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). PEG 2000 was successfully activated with CDI. rhIFNα2b was only successfully conjugated with $TMS(PEG)_{12}$ resulting in polyform of pegylated proteins with 1, 3, and 5 molecules PEG attached. The structure of PEG molecule influenced the pegylation of interferon. Linear PEG activated with carbonyldiimidazole showed less reactive to interferon than branced PEG.

Keywords: recombinant human IFNα2b; pegylation; PEG-carbonylimidazole; trimethyl succinimidyl PEG

INTRODUCTION

Human IFN2b (hIFNα2b) is a non-glycosylated polypeptide that belongs to cytokine family (Valente et al., 2004). It is an important therapeutic protein since it has been used in various terminal disease treatments. The hIFNα2b is used to treat hepatitis B and C and several types of cancer, i.e. hairy cell leukemia, malignant melanoma, follicular lymphoma, condylomata acuminata, AIDS-related Kaposi's sarcoma, and in combination with retinoids, hIFNα2b induces regression in advanced squamous carcinomas of the skin and cervix. It is also reported to inhibit vascular and endothelial cell proliferation (Neves et al., 2004; Srivastava et al., 2005; Rabhi-Essafi et al., 2007).

Recently, therapeutic peptides and proteins have gained interest for human use due to rapid establishment in the discovery of these molecules, they are potent drugs which are well known of mechanism of actions to treat the diseases, improvement in the pharmaceutical formulation to have better biological responses. However, some limitations are faced when they are used in the clinic. *In vivo* responses like rapid elimination by the kidney, antibody recognition, enzymatic degradation and fagocytosis process all of them caused the proteins lack of use in therapy. Several strategies have been offered to overcome those clinical barriers. These are including manipulation both the molecular structure as well as the delivery system. Glycosilation and pegylation are the most common ways to improve the *in vivo* stability of the proteins (Abuchowski et al, 1977).

Polyethylene glycol (PEG) is the most interesting polymer for protein modification for many pharmaceutical and biotechnical applications. The protection action of PEG molecule through covalent attachment to the protein moiety are numerous and include shielding of antigenic as well as immune system recognition, shielding the uptake by the reticuloendothelial system (RES), and preventing rapid clearance of glomerular filtration by increasing the molecular weight and modification of the charge of the proteins.

The modification of macro molecules like protein through covalent binding with polyethylene glycol (PEG), an inert polymer, and is aimed to improve the potential use associated with some biopharmaceuticals is termed as pegylation. PEGylation of interferon has proven of improving the treatment of several chronic diseases, including hepatitis C (Roberts et al, 2002.

* Corresponding Author
Email: h_rachmawati@fa.itb.ac.id
Contact: +62-22-2504852
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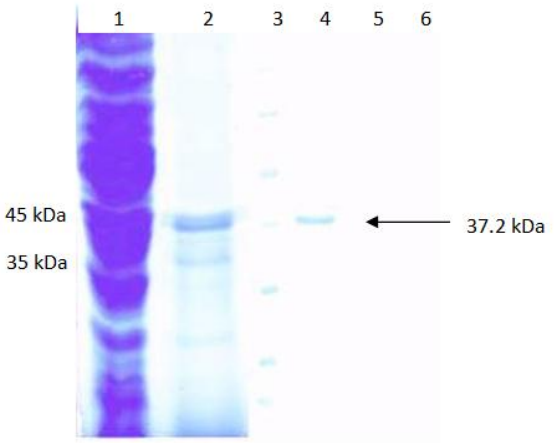

Figure 1: SDS-PAGE analysis of rhIFNα2b overproduced in *E.coli*. (1) protein total *E.coli* pET32b rhIFNα2b; (2) refolding of protein total *E.coli* pET32b rhIFNα2b; (3) protein marker; (4) rhIFNα2b fraction 1; (5) rhIFNα2b fraction 2; (6) rhIFNα2b fraction 3

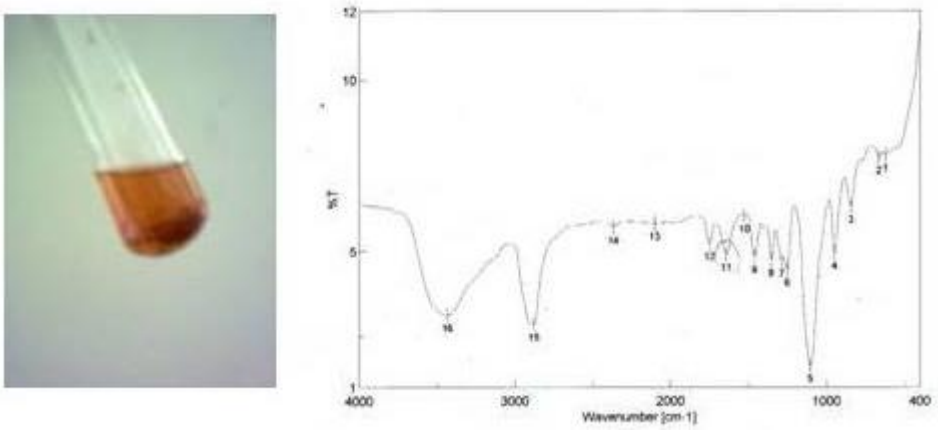

Figure 2: Dragendorf-positive reaction of PEG2000-CDI showing red-orange precipitate (a) and FTIR spectrum of PEG2000-CDI (b).

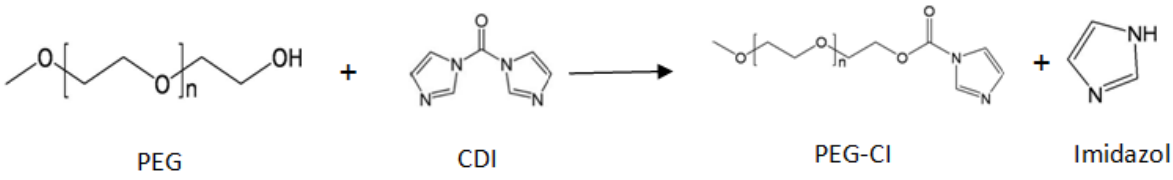

Figure 3: Scheme of activation reaction of linear PEG molecule using carbonyl di imidazole (CDI)

As PEG is a non reactive molecule, the early step to couple PEG to a molecule, it is absolutely required to activate the PEG by preparing a derivative of the PEG having reactive functional group at one or both termini (Roberts et al, 2002). Consideration on the functional group is based on the type of available reactive group on the molecule that will be coupled to the PEG. In case of macromolecules like proteins, common reactive groups are amino acids i.e. including lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid (Roberts et al, 2002).

This paper described the influence of molecular structure of PEGs on the effectivity of interferon pegylation. The chemical procedures and the conditions of pegylation reaction to achieve PEGylation of interferon in particular the molar ratio of protein:PEG was studied. Sodium Dodecy Sulphate Polyacrilamide Electrophoresis was used to analyze the influence of PEGs structure both on the reactivity and degree of interferon pegylation.

MATERIALS AND METHODS

Materials

Bacterial strains, plasmids, and culture media

As previously reported in our work (Retnoningrum et al, 2010), E. coli strain JM109,DH5α, Top10 and BL21 were used for cloning and gene expression of our interferon. pGEM-T (Promega) and pET32b (Novagene) were employed for gene cloning and expression, re-

spectively. Luria Bertani (LB) broth and agar containing 100 µg/ml of ampicillin and or 25µg/ml of tetracyclin, 50 mM of isopropyl β- d-1-thiogalactopyranoside (IPTG) for induction were applied for bacterial growth, selection of transformants and protein overproduction.

Chemicals for conjugation

$TMS(PEG)_{12}$ (Pierce), dioksan, 1,1'carbonyl diimidazole "CDI" (Merck), polyethylene glycol 2000 (Sigma), Phenylmethyl Sulfonyl Fluoride "PMSF" (Merck. Other materials used in this study are either proanalytic grade or pharmacopoeia quality.

Methods

Protein overproduction and purification

Overproduction of rhIFN-α2b was performed according to our previous work (Retnoningrum et al, 2010). Briefly, rhIFN-α2b was overproduced in *E. coli* pET32bhIFNα2b as fusion protein with thioredoxin 6xHis-tagged at its amino (N) terminus. Total proteins were obtained by quick lysis and sonication. For quick lysis procedure, cell pellet from 1.5 mL of culture was harvested by centrifugation and lyzed by loading buffer [Tris–HCl 1MpH 6.8, 0.1% (w/v) bromophenol blue, 10% (v/v) glyserol, 2% (w/v) sodium dodecyl sulphate and 100mM dithiothreitol]. The rhIFNα2b fusion protein was purified from total proteins obtained from sonication and inclusion bodies (IB) after renaturation by binding buffer containing 6M urea. The affinity purification was done according to manufacturer (Novagen).

Activation of PEG-carbonylimidazole (PEG-CDI)

Activation of PEG was performed according to Rajagopalan with small modification (Rajagopalan et al, 1985). Briefly, PEG activation was done by reacting the CDI (648.6 mg) and PEG 2000 (400 mg) in 4 mL dioxan. The solution of PEG-CDI was then incubated for 4 h at 37°C in the orbital shaker with speed of 160 rpm. Unreacted CDI was removed using dialysis technique with spectrafor membrane (cut off 1000). The dialysis for each 1 mL sample was done for 10 h in 1L aquadest. The medium was changed each 2 h. Pure activated-PEG was then characterized with Dragendorf reagent, UV/Vis spectrophotometry, FT-IR spectrophotometry, and melting point.

Interferon pegylation

Pegylation of interferon was performed using 2 different molecular structures of PEG:PEG 2000 (linear PEG) and $TMS(PEG)_{12}$ (branched PEG). Conjugation with PEG 2000 was carried out with molar ratio of rhIFNα2b:PEG-Cl 1:100 and 1:300 in boric buffer pH 8.5. Reaction was allowed at 4°C for 48 h under stirring condition at 60 rpm. While, conjugation with $TMS(PEG)_{12}$ was performed according to the manufacture protocol, using similar ratio as PEG 2000. PEG-rhIFNα2b conjugates were characterized using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Unpegylated rhIFNα2b was used as a standard.

RESULT AND DISCUSSION

Overproduction and purification of rhIFN¸2b fusion protein

rhIFNα2b was produced as a fusion protein of 37.2 kDa (figure 1), confirming our previous report (Retnoningrum et al, 2011). Based on separation process using 15%SDS-PAGE, total protein obtained has size of 37 kDa which is corresponding to the theoretical size of hIFNα2b fusion protein (Fig. 1, lane 2,4,5) that was only present with IPTG induction.

Pegylation interferon with a linear PEG

The first step process prior to conjugation reaction between PEG and protein, the PEG must be activated to have reactive group on the terminal end. As described previously, the target functional group on the protein molecule can be lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid (Roberts et al, 2002). In this report, pegylation was performed on purified interferon. Using PEG 2000 as an inert molecule, CDI was used to activate the PEG. Positive in red-orange precipitate indicates the present of primary amine group in Dragendorf reaction (Figure 2a). FTIR spectrum shows peaks at 1751 cm^{-1} and 1527 cm^{-1} which corresponding to carbonylimidazole groups: C=O/C=C and C=N, respectively (Figure 2b). Carbonylimidazole is a first generation linker for pegylation resulting in the conjugation via acylation and react preferentially with lysine residues to form a carbamate linkage (Roberts et al, 2002).

The molecule target of protein coupling is reactive amino acids such as lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid (Ramon et al., 2005). The most typical route for protein pegylation is to activate the PEG with functional groups suitable for binding with lysine and N-terminal amino acid groups. Lysine is one of the most target amino acids in proteins. In reactions between electrophilically activated PEG and nucleophilic amino acids, it is typical that several amines are replaced (Ramon et al., 2005).

Hydroxyl groups, typically anhydrides, chlorides, chloroformates and carbonates are groups in which the first generation of PEG derivatives are commonly react to. These techniques lack the ability to produce pure monofunctional PEG derivatives of high molecular weight.

As seen in figure 5, both molar ratios seems did not produce pegylated protein. This may due to instability of activated PEG with carbonyldiimidazole undergoing hydrolytic degradation during the reaction. In addition,

Figure 4: Pegylation of protein using PEG2000-CDI

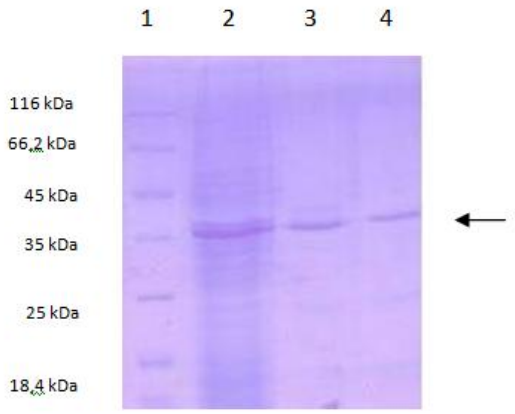

Figure 5: SDS-PAGE analysis showing the product of PEG2000-IFN with different molar ratio of PEG:protein. (1) protein marker; (2) rhIFNα2b; (3) PEG-IFN 1:100; (4) PEG-IFN 1:300

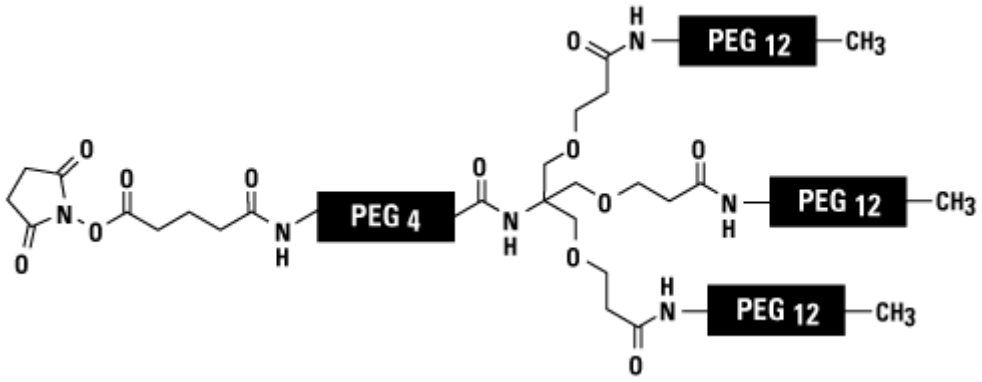

Figure 6: The molecular structure of trimethyl succinimidyl polietilenglikol (TMS(PEG)$_{12}$)

Figure 7: The scheme of pegylation of protein using TMS(PEG)$_{12}$

imidazole group is less reactive thus higher amount of PEG-CDI is required to allow the pegylation undergone.

In the conjugation reaction using first generation of PEG derivatives, the mild condition must be applied as the reaction was involved labile molecules. As PEG is soluble both in water and organic solvents, it makes PEG is suitable for end group derivatization and chemical conjugation to biomolecules under mild physiological conditions. In the case of protein, the most reactive groups contributed in the coupling reaction are the alpha or epsilon amino groups of lysine (Roberts et al, 2002). Seems, first generation PEG chemistry is inefficient for interferon conjugation although the incubation time was prolonged. Generally, the slower the reaction the more specific the reagent reacts to certain amino acid groups of the protein. But, we did not observe this phenomenon by using first generation PEG linker for interferon.

Pegylation of interferon with a branched PEG

Trimethyl succinimidyl polietilenglikol (TMS(PEG)$_{12}$), a second generation PEG linker, was used as a branched PEG to modify the interferon. In addition to the linear structure of the PEG molecule, branched structures have proven useful for protein and peptide modification. TMS(PEG)$_{12}$ was the reaction product of PEG with N-hydroxysuccinimide (NHS) (Figure 6). The ester NHS group is the reactive group to attach PEG forming a stable amide bond with primary amine group of protein (Figure 7).

To overcome the problems encounterd by using first generation of PEG, unstable linkages, side reactions and lack of selectivity in substitution, the second-generation PEGylation has been designed. Electrophilic PEGs conjugation to amino acid residues on proteins is highly influenced by the nucleophilicity of each amino acid residue. In addition, the pH of the protein solution must be near or above the residue's p*K* to lead the nucleophilic attack. Whole protein structure including the position of amino acid sequence determines the reactivity of each residue (Harris and Herati, 1993).

According to Ramon, the target pegylation of IFNα2b are amino acid group of lysines, α-amino group at N-terminal cysteins, nitrogen imidazolil of histidines, and hydroxyl group of serines, threonines and tyrosines (Ramon et al., 2005). Using coanalytical methods of high performance cation exchange chromatography, peptide mapping, amino acids sequencing and mass spectrophotometry, Bailon et al. found that 4 main target sites of IFNα2a for pegylation were lysine^{31}, lysine^{121}, lysine^{131} and lysine^{134} (Bailon et al., 2001).

As shown in figure 8, pegylation of interferon using TMS-PEG with the molar ratio of protein:PEG 1:100 and 1:300 produced 3 bands with different molecular weight and intensity. The molecular weight of each product was calculated by simply plotting the migration length to calibration curve of log protein marker MW versus migration length. The equation of y = -0.133x + 2.114 was obtained, thus the molecular weight of the protein: unpegylated, pegylated a, b, and c is 37.04, 39.44, 44.24, and 49.12 respectively. This increase indicates the addition of PEG molecule to the protein as 1, 3 and 5 molecules respectively. Thus, by using branched PEG, multiple products of PEG-interferon was observed and seems to be influenced by the molar ratio between PEG and protein as can be seen on the intensity of the bands. Using higher molar of PEG, the conjugation reaction was more extensive. Since monoproduct is preffered than polyform, it is important to consider the correct molar ratio of protein:PEG.

Using second generation PEG linker like (TMS(PEG)$_{12}$, active esters of PEG carboxylic acids are the most used acylating agents for protein modification (Zalipsky and Barany, 1986). Active esters react with primary amines near physiological conditions to form stable amides as shown in figure 8.

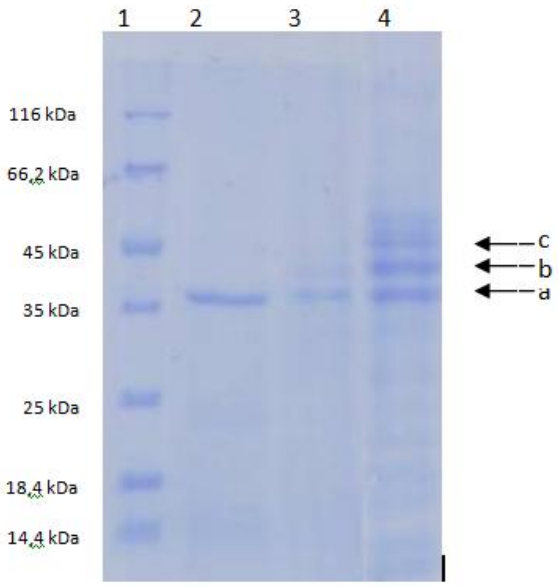

Figure 8: SDS-PAGE analysis showing the pegylated product of rhIFNα2b withTMS-PEG using different molar ratio of protein:PEG. (1) protein marker; (2) unpegylated rhIFNα2b; (3) pegylated rhIFNα2b with molar ratio of protein:PEG 1:100; (4) molar ratio 1:300

CONCLUSION

The molecular structure of polyethylene glycol determines the successful protein pegylation. Carbonyldiimidazole as first generation of linker shows less effective for interferon pegylation and more susceptible for hydrolytic degradation during conjugation reaction. Up to molar ratio of interferon:PEG 1:300, the pegylated product was not obtained, while using similar ratio but with branched PEG (TMS(PEG)$_{12}$) interferon was successfully pegylated but with polyproducts. This phenomenon suggests that branched PEG (TMS(PEG)$_{12}$) is more reactive to attact interferon alpha 2b but optimization of molar ratio must be controlled to obtain monopegylated form.

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