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Site-Specific PEGylation of Recombinant Immunotoxin DAB$_{389}$IL-2: Structural and Functional Assessment

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ABSTRACT

Background: DAB$_{389}$IL-2 is considered a fusion immunotoxin and used for the treatment of Cutaneous T Cell Lymphoma (CTCL). It is composed of two distinct portions; the catalytic domain of diphtheria toxin and IL-2. Because of DAB$_{389}$IL-2 free cysteine residue (Cys 513 in IL-2 part), it is prone to unwanted intramolecular and intermolecular disulfide bonds formation and aggregation problems. Aggregation is considered as the most common physical instability. PEGylation is a practical approach to increase the stability and half-life of therapeutic proteins.

Materials and Methods: In this study, the PEGylation of recombinant DAB$_{389}$IL-2 was performed by mPEG-vinylsulfone, through partial denaturation condition at 4° C for 24 h. To confirm the PEGylation reaction, SDS-PAGE and Dynamic Light Scattering (DLS) was used. The structure of DAB$_{389}$IL-2 and its PEGylated immunotoxin form were analyzed using the Circular Dichroism (CD) and fluorescence methods. Also, the K562 cells line were treated with various concentrations of DAB$_{389}$IL-2 and conjugated form. In the following, the nuclease activities of DAB$_{389}$IL-2 and PEGylated form were determined.

Results: The SDS-PAGE result confirmed the site-specific PEGylation of DAB$_{389}$IL-2. Spectroscopy results exhibited that the PEGylation does not affect the protein original structure. Also, the cytotoxicity assay and nuclease activity test confirmed that PEGylated protein induces death in K562 cells line and DNA degradation, respectively.

Conclusion: PEGylated immunotoxin DAB$_{389}$IL-2 has a proper structure and function; thus, PEGylated immunotoxin requires more study because of its unique properties.

Introduction

In the late 1980s, the number of recombinant protein drugs in the market was only 3, but in 2010, it increased to about 20 antibodies and more than 150 confirmed products. The development of recombinant DNA technology and the increased human knowledge about protein structure and the factors that influence the stability of protein have played an important role in this progress [1-3]. There are several strategies and advances for stabilizing the protein, including 1. use of compounds such as osmolytes, buffers, surfactant, cyclodextrins, and metal ions, 2. freeze-drying (lyophi-

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lization), 3. site-directed mutagenesis, and 4. chemical modification. PEGylation is one of the most important techniques of chemical modification [4]. Poly (Ethylene Glycol) (PEG) is a polyether compound with many applications, from industry to medicine [5].

PEG is a hydrophilic and nontoxic molecule in two forms of linear and branched. This polymer attaches to macromolecules by covalent bonds and alters their properties. Among macromolecules, peptides, and proteins, especially therapeutic peptides, are the most suitable substrate for PEGylation. Conjugated protein is not recognized by the immune system and is protected against the destruction of proteases. Also, the conjugated protein has a bigger size in comparison to non-conjugated protein, thus reducing renal filtration.

The PEGylated proteins, now on the market (Adagen®, Oncospar®, PEG-intron®, Somavert®, Mircera®), are the first products of PEG-protein therapeutics [6, 7]. To attach PEG to macromolecules such as proteins, hydroxyl groups at the ends of PEG should be activated by activator compounds to allow PEG binding the active group in surface of protein. The selection of the appropriate and reactive group for PEG derivatives is according to the functional group on the proteins that paired with PEG [8]. For proteins, reactive amino acids contain lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group, and C-terminal carboxylic acid. The most common form of PEG to modify the proteins is methoxy-containing structure (mPEG), which has only one hydroxy group and prevents PEG molecules from cross-linking.

In the PEGylation process, the covalent bond is formed between electrophilically activated PEG and nucleophilic amino acids of the protein. In the first-generation of PEGylation, the most usual reactive groups are the α- or ε-amino groups of lysine. The first-generation chemistry usually had defects such as PEG impurities, unstable linkage, and lack of heterogeneity of products. Random PEG binding to proteins reduces the bioactivity of proteins and leads to heterogeneous products. Therefore, the technique used to PEGylation of the proteins in a specific site (site-specific PEGylation) is important. PEGylation in the N-terminal amino group, cysteine PEGylation, and enzymatic PEGylation with transglutaminase are the most common sites for site-specific PEGylation [7, 9-12]. The molecular weight of the PEG and the position of PEG on protein and chemistry for PEGylation have remarkable importance.

PEGylation of free cysteine residues is one of the most important site-specific PEGylation methods because cysteine amino acids in proteins are fewer than lysine amino acids. In the absence of free cysteine in the structure of a protein, one or more free cysteine amino acids can be inserted using genetic engineering methods. The PEG derivatives that react with the thiol cysteine group include PEG-maleimide, vinylsulfone, iodoacetamide, and orthopyridyl disulfide. Each of these PEG derivatives has its advantages and disadvantages. DAB_{389}IL-2 (ONTAK®) is a recombinant fusion protein which was approved by the Food and Drugs Administration [13-16].

It is used for the treatment of cutaneous T cell lymphoma. DAB_{389}IL-2 contains two distinct portions: Catalytic domain of Diphtheria Toxin (DT) and IL-2 [17-19]. DT and IL-2 are considered as cell-killing and cell-binding, respectively [20]. Thus the immunotoxin exclusively enters tumor cells that have over-expression of IL-2 receptors. In cancer cells, DT exerts its cytotoxic role after ADP-ribosylation of Elongation Factor 2 (EF-2) and eventually, inhibition of protein synthesis and cell death [21]. Also, the DT-moiety of immunotoxin has a nuclease activity by which it can induce cellular death through DNA degradation [19, 22].

DAB_{389}IL-2 with 521 amino acids has a highly reactive free cysteine residue in the IL-2 portion along with two disulfide bonds in 202-187 and 446-493 that play an important role in its toxicity and function. Incorrect formation of disulfide bonds in immunotoxin and formation of the intramolecular disulfide bond between free cysteines in IL-2 part are key factors for protein aggregation during production and purification. In this study, PEGylation is used to increase stability through the reduction in aggregation matter.

Thus, considering the benefits of the second-generation PEGylation, the free cysteine residue was chosen for site-specific PEGylation with mPEG-vinylsulfone, (20 kDa). In this study, we expressed DT_{389}IL-2 in E. coli BL21 (DE3) and after refolding the recombinant protein, it was partially denatured, and PEGylation of immunotoxin was carried out [23]. After PEGylation, the mixture reaction was separated by ion exchange chromatography and the conjugated fractions were pooled for further structural and functional assessment [24-26].

Materials and Methods

General materials

The pET21a plasmid was purchased from Novagen. PEG-VS (mPEG-vinylsulfone, 20 kDa) was purchased from Jenkem company. Also, Q Sepharose Fast Flow was supplied from GE Healthcare Life Sciences. IPTG (isopropyl-ß-D-thiogalactoside) from Roche, agarose from Fluka. Luria-Bertani, Tris, $CaCl_2$, Ethylenediaminetetraacetic Acid (EDTA), Sodium Dodecylsulfate (SDS), and acrylamide were purchased from Merck Company. 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT), urea, and other chemicals were purchased from Sigma. RPMI medium, Fetal Bovine Serum (FBS), and penicillin were purchased from Gibco. The K562 cells line supplied from the National Cell Bank of Iran.

Expression and refolding of recombinant protein

The pET21a plasmid was transformed into competent E. coli BL21 (DE3). Recombinant protein expression was induced at OD600 of 0.7 with 1 mM IPTG at 37° C for 4 h. After that, the bacterial cells were harvested by centrifugation at 7000 rpm for 5 min. These cells were disrupted by sonication in a buffer, including 50 mM Tris-base, 8% sucrose, 8 mM EDTA, 3% Triton X- 100, and 1 mM PMSF (pH 8) and centrifuged at 10000 rpm for 25 min at 4° C. The isolated inclusion bodies were washed three times in washing buffer to remove Triton X- 100. These inclusion bodies were solubilized in a buffer containing 2 M urea, 25 mM cysteine, and 1 mM EDTA, pH 12; the final concentration of the recombinant protein was 7-8 mg/mL.

Then, the suspension was kept at 25° C for 20 min. Afterward, the suspension was centrifuged at 20335 RCF for 15 min at 4° C. The clear supernatant was filtered through 0.45 µm filter. The soluble protein was diluted 40 times with a refolding buffer containing 0.2 M urea, 0.1% Tween 20 (pH 8.2) up the final concentration of the recombinant protein to 0.2 mg/mL [27, 28]. The refolding of recombinant protein continued for 18 h at 4° C. Then the buffer of refolded protein was exchanged with 100 mM phosphate (pH 7.8) and concentrated up to 1 mg/mL.

PEGylation and purification

The refolded immunotoxin (1 mg/mL) was dissolved in 5 M urea containing 50 mM Tris-base (pH 8) to obtain a partially denatured structure. Then mPEG-VS (20 kDa) was added at10-fold molar excess with respect to the protein. The PEGylation was carried out at 4° C overnight. The reaction mixture was loaded on anion exchange chromatography for purification of the conjugates.

The column was equilibrated with 5 M urea containing 50 mM Tris-base at 4° C. Afterward, 2 mL of reaction mixture applied to the column at 1 mL/min through Q Sepharose Fast Flow from (GE Healthcare Life Sciences). The unreacted mPEG washed from column and PEGylated proteins were eluted by using a linear salt gradient from 0-1 M NaCl. SDS-PAGE was used to determine the purity of PEGylated bands. The purified fraction was pooled, and the buffer was substituted with phosphate buffer to omit NaCl using a Filtron concentrator and an Mr 50000 cut-off. To estimate the yield of PEGylation, we employed the Core Laboratory Image Quantification Software (CLIQS).

Spectroscopy analysis of PEGylated and non-PEGylated immunotoxin

Circular Dichroism (CD) was carried out using the CD spectrometer model 215 (AVIV instrument INC). The Far-UV CD spectra of PEGylated recombinant protein and non-modified protein at 0.2 mg/mL concentration were recorded in the range of 190-260 nm with a spectral resolution of 1 nm. Quartz cell with a path length of 10 mm was used, and all measurements were carried out at 25° C. Also, fluorescence spectroscopy of PEGylated and non-PEGylated immunotoxin were performed with a Hitachi MPF-4 fluorescence spectrophotometer at 25° C and the monochromatic slits were set at 5 nm. For PEGylated and original protein at a final concentration of 0.1 mg/mL, a wavelength ranges of 300-400 nm was used after excitation at 295 nm. Zeta potential values of PEGylated and non-PEGylated immunotoxin were measured by photon correlation spectroscopy (Brookhaven instrument Ltd, USA). The 0.1 mg/mL sample was measured at a temperature of 25° C (pH 8) [29].

Biological activity of PEGylated immunotoxin

The K562 cells line have a moderate affinity for IL-2 receptors containing β and γ subunits. This cell line was purchased from the National Cell Bank of Iran and maintained in RPMI 1640 supplemented with 10% FBS, 50 IU/mL penicillin, and 50 µg/mL streptomycin. The cells were seeded in triplicate at a density of 1.2× 104 cells/well in 96-well plate, including 200 µL complete medium. After 24 h of incubation at 37° C in 5% CO_2 atmosphere, different concentrations of conjugated and

non-conjugated immunotoxin (10^{-6} to 10^{-10} M) were added and incubated for an additional 48 h. Then, the MTT assay was carried out following the Mosmann method.

Nuclease activity

To evaluate the proper function of PEGylated immunotoxin, we assessed its nuclease activity. For this purpose, 1 µL of PEGylated and non-PEGylated recombinant proteins with the final concentration of 0.3 mg/mL dissolved in 10 mM Tris-HCL (pH 7.5) was added to 5 µL of reaction buffer, including 10 mM Tris-base, 2.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$ (pH 7.6). The reactions were started by adding 4 µL of DNA plasmid (pET21a) with the final concentration of 500 ng. The reaction mixture was kept at 25° C for 4 h. Then the mixture was analyzed by 1% agarose gel electrophoresis in TAE buffer.

Results

Expression, purification, and refolding of $DAB_{389}IL-2$

The recombinant protein was expressed successfully in E. coli BL21 (DE3). The extracted inclusion bodies were washed in a washing buffer. The washed inclusion bodies were in solubilization buffer containing 2 M urea, 25 mM cysteine, and 1 mM EDTA (pH 12). The denatured protein was refolded 40 times by rapid dilution into a refolding buffer. The expression of immunotoxin was confirmed by using 10% polyacrylamide SDS-PAGE gel electrophoresis (Figure 1A).The SDS- PAGE indicated a protein band with a molecular weight of 58 kDa corresponding to the production of recombinant protein in an inclusion body form, and the purity of the inclusion body after repeated washing.

PEGylation and purification of mono-PEGylated $DAB_{389}IL-2$

To access the free thiol group in the IL-2 part in the original form, we studied PEGylation under partially denaturing conditions (Figure 1B). So, we used urea as a denaturant. Afterward, we used ion exchange chromatography for purification of mono-PEGylated conjugated from unreacted protein. The fractions of chromatography were collected and then analyzed by 10% polyacrylamide SDS-PAGE gel electrophoresis (Figure

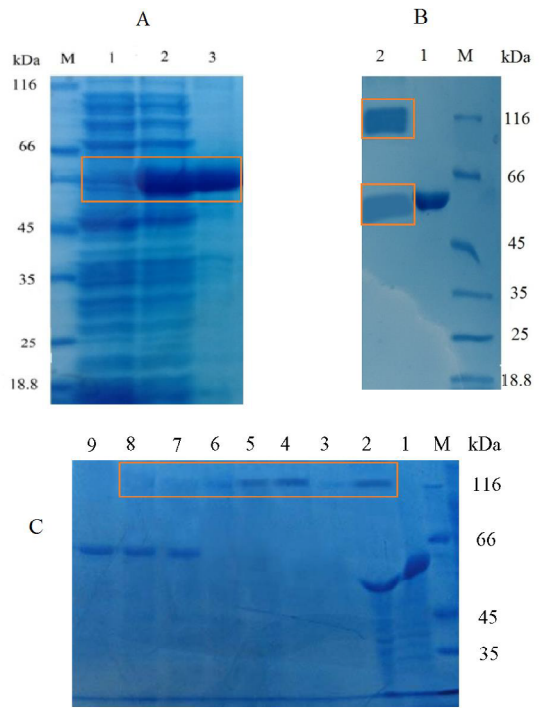

Figure 1. PEGylation under partially denaturing conditions

A. Expression analysis, M. The molecular-weight size marker (kDa), 1. Expression of bacteria before induction, 2. Expression after induction, 3. Inclusion bodies after three times washing; B. SDS-PAGE of PEGylated immunotoxin $DAB_{389}IL-2$ reaction mixture. M. molecular-weight size marker, 1. $DAB_{389}IL-2$ before PEGylation, 2. reaction mixture after 24 h of PEGylation; C. SDS-PAGE of PEGylation of $DAB_{389}IL-2$ and assessment of the purification procedure. Lane M indicates a molecular-weight size marker. Lanes 1 and 2 show the band of the recombinant $DAB_{389}IL-2$ before and after the PEGylation, respectively. Lanes 3-9 correspond to eluted fraction (with 0 to 1 M of NaCl) of the PEGylated mixture of immunotoxin

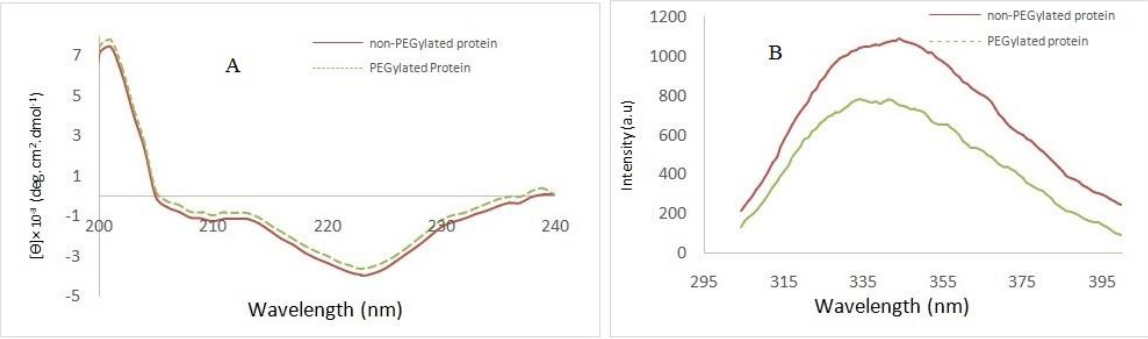

Figure 2. non-PEGylated and PEGylated protein spectra

(A) Circular dichroism profile of recombinant DAB$_{389}$IL-2 and non-PEGylated immunotoxin DAB$_{389}$IL-2. The concentration of DAB$_{389}$IL-2 and PEGylated DAB$_{389}$IL-2 were 0.2 mg/mL, (B) Structural analysis of DAB$_{389}$IL-2, and PEGylated DAB$_{389}$IL-2 by intrinsic fluorescence spectroscopy. Concentration of DAB$_{389}$IL-2 and PEGylated DAB$_{389}$IL-2 were 0.1 mg/mL.

1C). The PEGylation yield and the highly purified protein fraction were estimated 42% and 99%, respectively, with the CLIQS software.

Assessment of DAB$_{389}$IL-2 Structure and Zeta potential

Circular dichroism was performed for the second-structure analysis of the recombinant protein in the original and PEGylated form. The CD-spectra exhibited three distinct peaks. For both samples, peaks were assigned to the alpha-helix conformation of the protein (Figure 2 A). Comparisons of non-PEGylated and PEGylated protein spectra showed a little change in their fluorescence properties. Reduction in the intensity of the fluorescence emission of the PEGylated protein compared with its original form indicates the movement of aromatic roots from the non-polar environment to the polar environment.

Because of the insignificant changes in the emission of intrinsic fluorescence, we can expect no remarkable

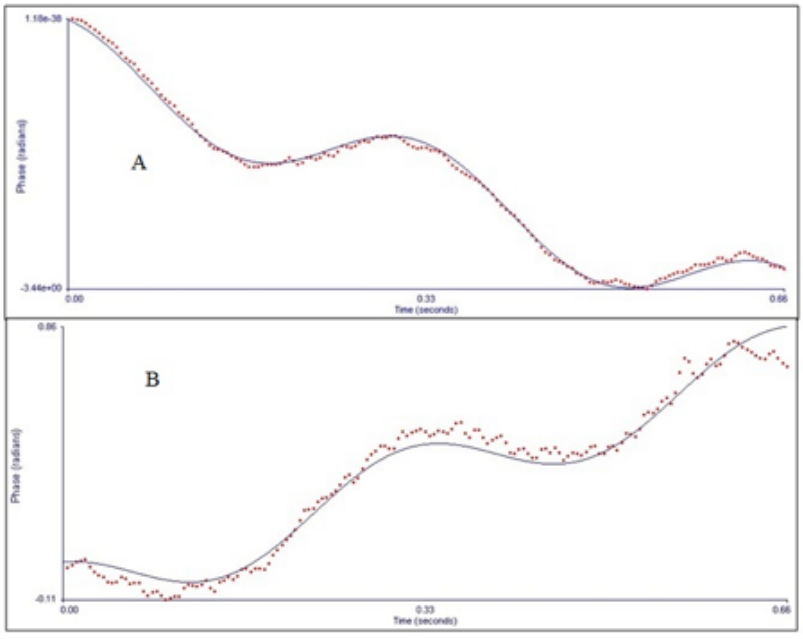

Figure 3. Zeta potential of non-PEGylated and PEGylated recombinant protein DAB$_{389}$IL-2

A. non-PEGylated recombinant protein DAB$_{389}$IL-2; B. PEGylated recombinant protein DAB$_{389}$IL-2DAB$_{389}$IL-2.

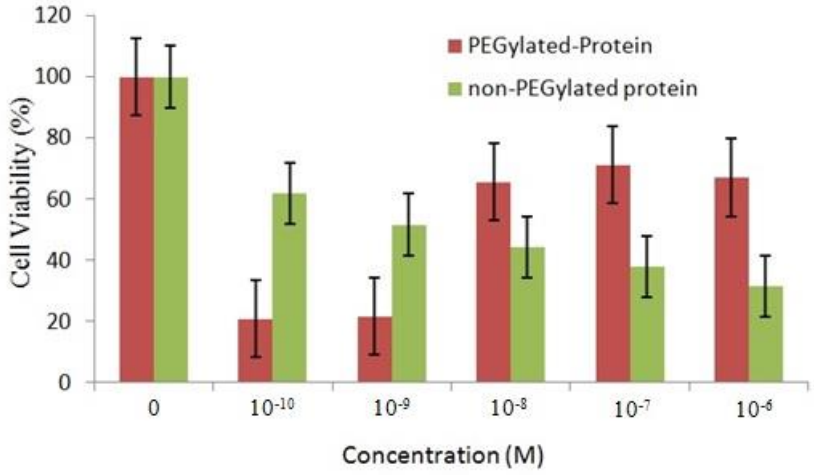

Figure 4. The effect of various concentrations of the recombinant $DAB_{389}IL-2$ and PEGylated recombinant $DAB_{389}IL-2$ on the cell viability in K562 cells

change in the second structure of the PEGylated form (Figure 2B). Zeta potential values of PEGylated and non-PEGylated immunotoxins were -2 mV and -12 mV, respectively. Based on the results, the PEGylated protein showed a decrease in the negative surface charge compared to the non-PEGylated protein. In other words, this reduction is due to the surface coating of protein by PEG molecules. Thus, the PEGylated form has a lower zeta potential. By coating the surface of the protein and increasing the hydrodynamic volume of the protein, PEG molecules reduce the electrophoretic mobility of the PEGylated protein, which ultimately leads to a reduction in the peculiarity of the zeta potential (Figure 3).

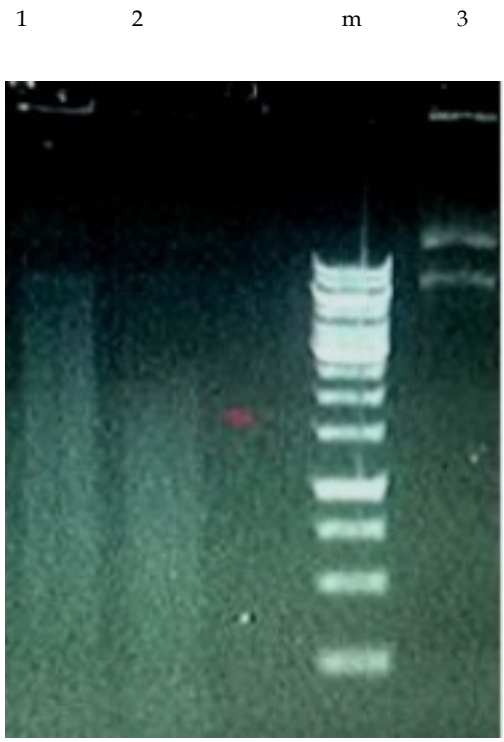

Figure 5. Agarose 1% gel electrophoresis showing the nuclease activity of recombinant protein $DAB_{389}IL-2$ and PEGylated $DAB_{389}IL-2$. Lane M. DNA size marker1 Kb, lane 1. pET21a incubated with non-PEGylated fusion protein for 4 h at 25 °C, lane 2. pET21a incubated with PEGylated fusion protein for 4 h at 25 °C. Lane 3. pET21a incubated with unfolded fusion protein for 4 h at 25 °C

Cytotoxicity assay

The K562 cells line were used to assay the cytotoxicity of native form and PEGylated form of immunotoxin. The IC_{50} values after 48 h of exposure of non-PEGylated and PEGylated $DAB_{389}IL-2$ to the K562 cells line were obtained 2.45×10^{-9} M and 1.1×10^{-10} M, respectively (Figure 4). These results suggest the induction of apoptosis. It is one of the ways that the fusion toxin can induce cell death [30].

Nuclease activity

According to Figure 5, nuclease activities of conjugated and non-conjugated proteins show fragmentation of DNA into smear form. But in lane 3, which is assigned to denatured protein, no changes were observed. These data emphasized that DT-moiety of both forms of $DAB_{389}IL-2$ obtained its proper structure and has an accurate DNase activity.

Discussion

Today, the most common challenges regarding recombinant therapeutic proteins are low solubility, aggregation, proteolytic degradation, and short half-life. Aggregation is considered as the most common physical instability of proteins occurring during manufacture and storage. Several strategies have transpired to augment the virtues of protein [31]. The most successful strategy is PEGylation, which refers to the conjugation of one or more PEG molecules to macromolecules. $DAB_{389}IL-2$ has 5 cysteine in its structure. The free Cys 513 in the IL-2 portion is not accessible in the wild-type folding, but it becomes accessible when it is denatured reversibly. The other cysteine involved in two disulfide bridges are (Cys 187-Cys 202) in the diphtheria toxin portion and (Cys 493-Cys 446) in the IL-2 part.

In this research, $DAB_{389}IL-2$ was PEGylated at the free cysteine position with mPEG-vinylsulfone, (20 kDa) to reduce the aggregation kinetic rate, and omit the free thiol Cys 513 resulting in the formation of covalent aggregates. So, the plasmid pET21a with the $DAB_{389}IL-2$ gene was transferred to the E. coli strain BL21 (DE3) competent cells, and after expression of protein in an inclusion body form, these inclusion bodies were extracted and washed. The washed inclusion bodies were solubilized and refolded. The refolded $DAB_{389}IL-2$ was used for PEGylation.

For this purpose, we applied the PEGylation of $DAB_{389}IL-2$ at the position of Cys 513 using thiol-reactive PEGs. The electrostatic attack of PEGs to amino acid residues generally depends on the nucleophilic property of these residues. The nucleophilic attack occurs only when the pH of buffer in which the protein is dissolved, be close to or higher than pKa of amino acid. Accordingly, the pH used for PEGylation of $DAB_{389}IL-2$ was 8 because at this pH, the likelihood of PEG binding to cysteine roots increases. PEGylated proteins need to maintain their original structures to exert their proper function, and profound changes in protein structure result in a significant reduction in their activity.

Overall, the CD and fluorescence spectroscopy results indicated that the PEGylation does not affect the protein's original secondary and tertiary structures. Also, both conjugated $DAB_{389}IL-2$ and non-conjugated form kept their high cytotoxic activity, indicating that disulfide bonds have been formed correctly. Besides, the conjugated form of immunotoxin with low concentration has an intact structure and remains functional with a decrease in its IC_{50}. Hence, this form of immunotoxin with low concentration could be a suitable alternative for a high concentration of drug and can be a topic for further studies and use in treatments. However, this conjugated protein requires more investigation due to its unique properties.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article.

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Authors contribution's

Writing and spectroscopy analysis: All authors; Purification, PEGylation, analysis: Maryam Ghodrati Siahmazgi; Revision: Mohammad Ali Nasiri Khalili

Conflict of interest

The authors declare no conflict of interest.

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References

[1] Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: An update. Pharma Res. 2010; 27(4):544-75. [DOI:10.1007/s11095-009-0045-6] [PMID]

[2] Baynes BM, Wang DI, Trout BL. Role of arginine in the stabilization of proteins against aggregation. Biochemistry. 2005; 44(12):4919-25. [DOI:10.1021/bi047528r] [PMID]

[3] Gervais D. Protein deamidation in biopharmaceutical manufacture: Understanding, control and impact. J Chem Technol Biotechnol. 2016; 91(3):569-75. [DOI:10.1002/jctb.4850]

[4] Roberts M, Bentley M, Harris J. Chemistry for peptide and protein PEGylation. Adv Drug Deliv rev. 2012; 64:116-27. [DOI:10.1016/j.addr.2012.09.025]

[5] Kolate A, Baradia D, Patil S, Vhora I, Kore G, Misra A. PEG- A versatile conjugating ligand for drugs and drug delivery systems. J Controll Release. 2014; 192:67-81. [DOI:10.1016/j.jconrel.2014.06.046] [PMID]

[6] Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov. 2003; 2(3):214-21. [DOI:10.1038/nrd1033] [PMID]

[7] Alconcel SNS, Bass AS, Maynard HD. FDA-approved poly(ethylene glycol)-protein conjugate drugs. Polymer Chem. 2011; 7:1442-8. [DOI:10.1039/c1py00034a]

[8] Veronese FM. Peptide and protein PEGylation: A review of problems and solutions. Biomaterials. 2001; 22(5):405-17. [DOI:10.1016/S0142-9612(00)00193-9]

[9] Fontana A, Spolaore B, Mero A, Veronese FM. Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase. Adv Drug Deliv Rev. 2008; 60(1):13-28. [DOI:10.1016/j.addr.2007.06.015] [PMID]

[10] Sato H, Suzuki C, Kawasaki S. Enzymatic procedure for site-specific pegylation of proteins. Adv Drug Deliv Rev. 2002; 54(4):487-504. [DOI:10.1016/S0169-409X(02)00024-8]

[11] Moosmann A, Blath J, Lindner R, Müller E, Böttinger H. Aldehyde PEGylation kinetics: A standard protein versus a pharmaceutically relevant single chain variable fragment. Bioconjugate chem. 2011; 22(8):1545-58. [DOI:10.1021/bc200090x] [PMID]

[12] Cazalis CS, Haller CA, Cargo LS, Chaikof EL. C-Terminal site-specific PEGylation of a truncated thrombomodulin mutant with retention of full bioactivity. Bioconjug Chem. 2004; 15(5):1005-9. [DOI:10.1021/bc049903y] [PMID]

[13] Strebhardt K, Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. Nat Rev Cancer. 2008; 8(6):473-80. [DOI:10.1038/nrc2394] [PMID]

[14] Madhumathi J, Verma RS. Therapeutic targets and recent advances in protein immunotoxins. Cur Opinion Microbiol. 2012; 15(3):300-9. [DOI:10.1016/j.mib.2012.05.006] [PMID]

[15] Potala S, Verma RS. Modified DT-IL2 fusion toxin targeting uniquely IL2Rα expressing leukemia cell lines-Construction and characterization. J Biotechnol. 2010; 148(2-3):147-55. [DOI:10.1016/j.jbiotec.2010.04.006] [PMID]

[16] Babavalian E, Zeinoddini M, Saeedinia A, Mohammadi R, Xodadadi N. Design of a recombinant immunotoxin against the human granulocyte-colony stimulating factor receptor. Mol Biol report. 2019; 46(1):1093-7. [DOI:10.1007/s11033-018-4567-z] [PMID]

[17] Bagha ME, Zeinoddini M, Saeedinia A, Xodadadi N. Overexpression and purification of diphtheria fusion toxin: DA-B_{389}IL-2. J Bionanoscience. 2018; 12(5):671-6. [DOI:10.1166/jbns.2018.1575]

[18] Bayat S, Zeinoddini M, Azizi A, Khalili MN. Co-Solvents effects on the stability of recombinant immunotoxin denileukin diftitox: Structure and function assessment. Iran J Sci Technol, Trans A: Sci. 2019; 43:1-7. [DOI:10.1007/s40995-019-00676-7]

[19] Zarkar N, Khalili MA, Khodadadi S, Zeinoddini M, Ahmadpour F. Expression and purification of soluble and functional fusion protein DAB_{389}IL-2 into the E. coli strain Rosetta-gami (DE3). Biotechnol appl biochem. 2019. [DOI:10.1002/bab.1833] [PMID]

[20] Moghaddas M, Zeinoddini M, Saeedinia A, Bayat S. Structural and functional assessment of diphtheria fusion toxin: DT_{389}GCSF. J Bionanoscience. 2018; 12(2):240-4. [DOI:10.1166/jbns.2018.1517]

[21] Zarkar N, Khalili MA, Ahmadpour F, Khodadadi S, Zeinoddini M. In silico and in vitro evaluation of deamidation effects on the stability of the fusion toxin DAB_{389}IL-2. Cur Proteom. 2019; 16(4):307-13. [DOI:10.2174/1570164616666190131150033]

[22] Lee JW, Nakamura LT, Chang MP, Wisnieski BJ. Mechanistic aspects of the deoxyribonuclease activity of diphtheria toxin. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2005; 1747(1):121-31. [DOI:10.1016/j.bbapap.2004.10.003] [PMID]

[23] Veronese FM, Mero A, Caboi F, Sergi M, Marongiu C, Pasut G. Site-specific Pegylation of G-CSF by reversible denaturation. Bioconjug Chem. 2007; 18(6):1824-30. [DOI:10.1021/bc070123+] [PMID]

[24] Pabst TM, Buckley JJ, Ramasubramanyan N, Hunter AK. Comparison of strong anion-exchangers for the purification of a PEGylated protein. J Chromatography. 2007; 1147(2):172-82. [DOI:10.1016/j.chroma.2007.02.051] [PMID]

[25] Cisneros-Ruiz M, Mayolo-Deloisa K, Przybycien TM, Rito-Palomares M. Separation of PEGylated from unmodified ribonuclease a using sepharose media. Separation and Purification Technol. 2009; 65(1):105-9. [DOI:10.1016/j.seppur.2008.10.038]

[26] Abe M, Akbarzaderaleh P, Hamachi M, Yoshimoto N, Yamamoto S. Interaction mechanism of mono-PEGylated proteins in electrostatic interaction chromatography. Biotechnol J. 2010; 5(5):477-83. [DOI:10.1002/biot.201000013] [PMID]

[27] Rao DVK, Narasu ML, Rao AKSB. A purification method for improving the process yield and quality of recombinant human granulocyte colony-stimulating factor expressed in Escherichia coli and its characterization. Biotechnol Appl Biochem. 2008; 50(2):77-87. [DOI:10.1042/BA20070130] [PMID]

[28] Siahmazgi MG, Khalili MA, Zeinoddini M, Ahmadpour F, Khodadadi S. Purification and characterization of DT 389 GCSF fusion protein: A unique immunotoxin against the human granulocyte-colony stimulating factor receptor. Int J Pept Res Ther. 2019; 1-8.

[29] Kouchakzadeh H, Shojaosadati SA, Maghsoudi A, Farahani EV. Optimization of PEGylation conditions for BSA nanoparticles using response surface methodology. Aaps Pharmscitech.

2010; 11(3):1206-11. [DOI:10.1208/s12249-010-9487-8] [PMID] [PMCID]

[30] Horita H, Frankel AE, Thorburn A. Acute myeloid leukemia-targeted toxin activates both apoptotic and necroptotic death mechanisms. PLoS One. 2008; 3(12):e3909. [DOI:10.1371/journal.pone.0003909] [PMID] [PMCID]

[31] Shahbazi M, Zahedy ET, Kiumarsi S, Soltanabad MH, Azar SS, Amini H. Determination of pegfilgrastim aggregates by size-exclusion high-performance liquid chromatography on a methacrylate-based column. Biologicals. 2017; 46:153-8. [DOI:10.1016/j.biologicals.2017.02.003] [PMID]

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