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SNIPER SHOT PEGylation: TGase mediated site specific conjugation of PEG to proteins

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ABSTRACT

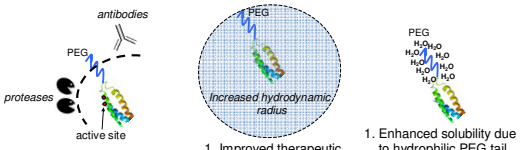
The commercially avaliable recombinant protein drugs often cause immune reactions in the body which reduces its efficiency. Protein drugs can be PEGylated, (attachment of polyethylene glycol) to overcome this problem. PEGylation increases bioavaliability by reduced immune reactions and decreased renal clearnace [1]. So far the traditional approaches for PEGylation involve harsh reaction conditions which provides a heterogenous product (PEG attached randomly to different sites) along with the formation of several byproducts. Due to heterogenousity, the PEGylated protein drug faces challenge for the FDA approval. Therfore, there is an immense need to develop an approach which could generate a homogenous PEGylated protein drug.

The transglutaminase (TGase) is an enzyme which catalyses specifically the formation of a covalent bond (-CONH-) between the glutamine residue and the amine group of the lysine. This TGase reaction can be engineered by substituting the lysine with primary amines, which results into the formation of similar covalent bond between the glutamine and the primary amine [2]. We utilized this specificity of TGase by using primary PEG-amines for conjugation with the glutamine present in the model protein (apomyoglobin). The reaction conditions were optimised in order to get a mono conjugated PEGylated derivative. The site of conjugation was determined by affinity purification of the modified peptides and characterized by the ESI Q-TOF mass spectrometer. Therefore, we were able to develop a site specifically PEGylated apomyoglobin along with no byproducts, which eventually reduced the derivative purification steps as compared with the traditional PEGylation approaches. This strategy was further implemented on commercial pharmaceutical proteins.

Recombinant protein drugs have several limitations:

1. Susceptibility to degradation by proteases,
2. Rapid kidney clearance, and
3. Propensity to generate immunogenic reactions.

Most promising solution ➡ **Site specific covalent attachment of poly(ethylene glycol) (PEG) on the surface of proteins**



- Stability to proteolytic digestion,
- Non-immunogenic,

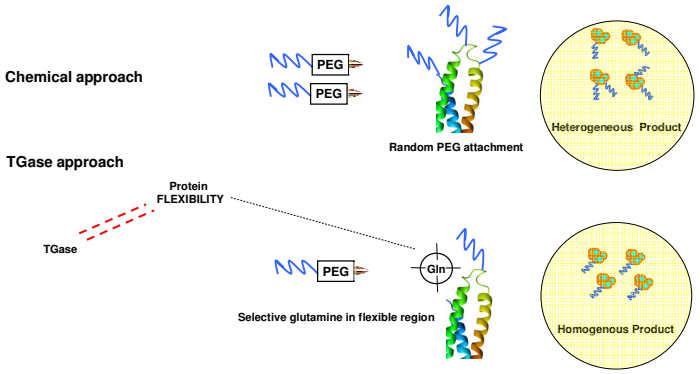
1. Improved therapeutic efficacy
2. Longer half-life than native proteins

1. Enhanced solubility due to hydrophilic PEG tail
2. Non-aggregating,

But, PEG conjugation at a specific site is *CHALLENGING*

Therefore, **We focused on the transglutaminase (TGase) approach for site specific conjugation of PEG on model protein (apomyoglobin).**

Chemical approach Vs TGase approach



TGase attacks only the glutamine residues in the flexible region of protein..

Efficiency

A] N terminal chemical PEGylation **B] TGase mediated PEGylation**

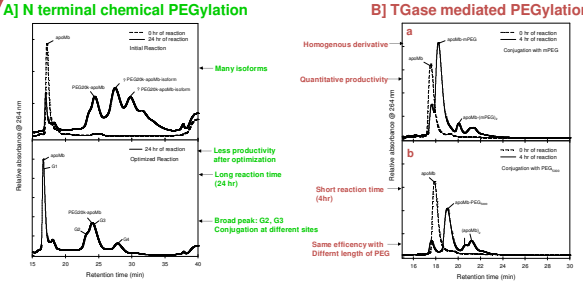


Fig. 1. RP-HPLC elution profile for N terminal chemical PEGylation & TGase mediated PEGylation of apoMb. with PEG20k-CHO. (A) N terminal chemical PEGylation of apoMb with 20 kDa PEG-aldehyde (PEG_{20k}): comparison between the initial reaction and optimized reaction. (B) TGase mediated PEGylation of apoMb with a) mono disperse BOC-PEG-amine (mPEG) & b) poly disperse 5 kDa PEG-amine (PEG_{5000}) All RP-HPLC analyses was performed using a C4 (Phenomenex) RP-HPLC column, at the flow rate of 0.8 ml/min, with a gradient from 40 to 50% of ACN in 25 min.

Characterization of site(s) of conjugation by ESI-Q-TOF mass spectrometer

Conjugation at the N terminal apoMb[1-16] peptide

Conjugation at the level of Glutamine 91

Rapid characterization using Biotin-PEG-amine

Apomyoglobin. + Biotin-PEG-amine. —TGase→ Apomyoglobin-PEG-Biotin —Avidin column Purification→ Mass spectrometric analysis

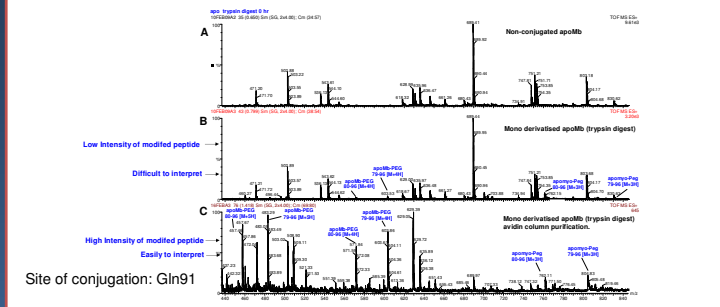


Fig. 2. The region of the mass spectrum between 440 and 840 *m/z* is reported in order to show the derivatization with PEG of peptide 79-96 of apoMb. (A) Trypsin digest of non conjugated apoMb. (B) Trypsin digest of monoderivatised apoMb. (C) Trypsin digest of monoderivatised apoMb after avidin column purification.

In spite of 6 glutamines in apomyoglobin sequence. ➡ The conjugation occurred only on glutamine residue present in the **flexible** region

Implementation on target pharmaceutical proteins

- The site specific PEGylation of a few valuable pharmaceutical proteins already in the clinical practice was achieved using the TGase approach.
- The sites of conjugation by TGase were easily identified using biotin-PEG-amine coupled to MS analysis.
- Only one or few sites of derivatization were observed in the conjugated proteins (data not shown due to confidentiality of the work).
- The glutamine residues that are derivatised are only those located in flexible regions of the proteins.

CONCLUSIONS

- **TGase mediated PEGylation using** Polydisperse PEG-NH_2, & biotin-PEG-NH_2 ➡ specific modification of only the selective Gln residue
- Use of **(Biotin-PEG-NH_2)** for affinity enrichment of modified peptides ➡ Rapid accurate identification of the sites of TGase-mediated PEGylation by mass spectrometry.
- Modified Gln residues of the investigated pharmaceutical proteins ➡ **located in disordered regions of the proteins**

Hence, the sites of PEGylation in the target proteins could be predicted from the structure and dynamics as given by the X-ray or NMR data.

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