

Site directed Pegylation: Role of pH and temperature, to increase the productivity of insulin oligomer conjugate

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Abstract

An oral alternative to insulin has remained elusive for over ninety years and there are still multiple challenges to be addressed in this regard. Here in our work, we present a conjugation of an oral insulin precursor and an oligomer with increased binding affinity. During the conjugation of oral insulin precursor and oligomer the insulin oligomer conjugate is formed by the oligomer attaching to Amino group (NH_3^+) at ϵ -carbon of lysine at B29 and free N-terminal of leader peptide followed by protease cleavage to remove the leader and linker peptide. To increase the binding affinity of oligomer towards Amino group (NH_3^+) at ϵ -carbon of lysine at B29 the concentration of oligomer to precursor ratio is varied at different conditions like pH and temperature which yields 70% conversion to monoconjugated precursor and confirmed by protein mass fingerprinting in LC-MS. Due to high pKa value of lysine than glycine the affinity of oligomer shifts towards the Amino group (NH_3^+) at ϵ -carbon at specific temperature and pH. This change in the conjugation reaction parameters is expected to be useful for later purification steps in the synthesis of an oral alternative for insulin.

Keywords: Mono-conjugated Precursor, Oral-insulin precursor, Oligomer, ϵ -carbon

Introduction

As of 2014, the number of people effected with diabetes was reported to be 387 million which amounts to one person effected for every twelve people in the world. Even more alarming is the fact that 46.3% of the population remain undiagnosed. In addition this number is expected to rise to 592 million by 2039.⁽¹⁾ The gold standard for control of diabetes remains injectable insulin. However efforts have been ongoing to produce an oral alternative.

Insulin is a biomolecule that consists of two chains and 51 amino acids. Chain A consists of 21 amino acids and chain B is composed of 30 amino acids. Amino acids all contain the same backbone, which has both an acidic and a basic group. The factors that influence stability, solubility, and susceptibility to proteolysis include their ionic charge, size, and structure.⁽²⁾ Each amino acid has a functional group attached to the backbone. These functional groups can be positive, negative, neutral, or polar in nature.⁽³⁾ The backbone and functional groups give a protein its overall charge. At a pH below the protein's pI, a protein will carry a net positive charge while above its pI, it will carry a net negative charge. Protein structure and reactivity are reliant on the specific properties of the constituent amino acids. Particularly relevant for the understanding of PEGylated activity, though, is the effect of buffer on the structure, both geometric and electronic, of amino acids or pH and temperature effects the pKa of Lysine⁽⁴⁾ (Fig. 1) in precursor.

Especially the amino acids side chains have a strong influence on how the protein behaves in buffer. Peptides and proteins protonation equilibrium is strongly influenced by its surrounding media.⁽⁵⁾ Due to amphoteric nature of amino acids pKa plays a decisive role in PEGylation. The pKa of lysine (Fig. 1) (side chain which has to be attached to oligomer) at B29 is 10.54.⁽⁶⁾

For lysine (Fig. 1), the butylamine side chain is responsible for its basic reaction in aqueous solution.⁽⁷⁾

Oral insulin precursor N-terminal-{Chain-A}----Linker----{Chain-B} (Scheme 1) where G is the leader sequence and R is the linker peptide between chain A and chain B. The inclusion bodies (oral insulin precursor crystals) are subjected to conjugation with active oligomer to form insulin oligomer conjugate. The propensity of binding the oligomer towards the precursor is purely contingent on the pKa of the amino acids. The two sites present in the precursor one is the free amino terminus of leader peptide and lysine (Fig. 1) in B-chain at 29th position. In order to produce the activated oral insulin the precursor is subjected to protease treatment to remove the leader and linker peptide. The oligomer attaching to the free N-terminus of leader peptide will be removed by protease digestion in order to decrease the propensity of oligomer binding towards the leader or increasing the propensity towards the Amino group (NH_3^+) at ϵ -carbon of lysine (Fig. 1) at B29.

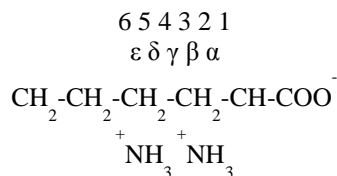


Fig. 1: Structure of Lysine

Materials and Method

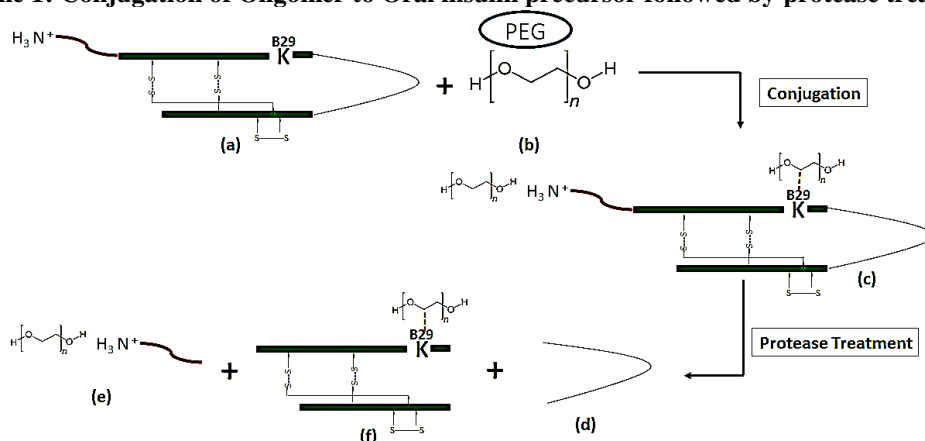
Oral-Insulin precursor: The Oral-Insulin precursor (OI-P) is secreted by *P. pastoris* into the culture medium. The broth is centrifuged and cells are separated from the supernatant and the Inclusion bodies are captured by cation exchange chromatography. Crystallization of the Oral-Insulin precursor OI-P, removes any impurities carried through from the fermentation broth. A pure crystalline precursor also helps in reducing cost in the subsequent conjugation step and increases the efficiency of the reaction. The crystalline form can be frozen and stored which would be significantly stable for multiple days when stored at -20°C .

Oral-Insulin is produced by a reaction wherein OI-P, is first conjugated at B29 Lysine using an activated oligomer to give OI-P-oligomer conjugate. The OI-P-

oligomer conjugate is subjected to protease treatment, where the linker-peptide (RDADDR) and the leader sequence (GAVR) are cleaved to get the active insulin-oligomer conjugate. The two proteases used are trypsin and carboxypeptidase B. As the LysB29 is blocked by the oligomer, the probability of trypsin cleavage at LysB29 is minimum.

But the other probable sites for trypsin cleavage are c-terminus end of B-22 (Arg), C-1 (Arg) and C-6 (Arg). A 2nd protease (carboxypeptidase B) treatment is done to remove the free basic (Arg) amino acid from the B chain, where one extra Arg is attached with the B-30(Thr) to get final product oral insulin. The two protease treatment (**Scheme 1**) is carried out in a single operation at optimum reaction condition where yield was maximized with minimum product related impurities generated.

Scheme 1: Conjugation of Oligomer to Oral insulin precursor followed by protease treatment



a: Oral insulin Precursor (with leader and connecting peptide), b: Polyethylene glycol, c: Intermediate after conjugation (Di-conjugated Precursor), d & e: Connector and leader peptides removed by protease treatment, f: activated Oral Insulin

MS(PEG)*n*- (Thermo Fisher Scientific): MS(PEG)*n* reagents permit simple and effective modification of proteins and other molecules that have primary amines. Modification fallouts in the addition of polyethylene glycol (PEG) spacers (PEGylation) with terminal methyl groups.

Typical PEGylation reagents contain heterogeneous mixtures of different PEG chain lengths; however, Pierce PEGylation Reagents are homogeneous compounds of defined molecular weight and spacer arm length, providing precision in optimizing modification applications.

N-Hydroxysuccinimide (NHS) ester which were the most popular type of reactive group used for protein modification was used. In pH 7-9 buffers, NHS-ester reagents react efficiently with primary amino groups ($-\text{NH}_2$) by nucleophilic attack, forming amide bonds and releasing the NHS. Proteins typically have many sites for labelling, including the primary amine in the side chain of each lysine (K) residue and the N-terminus of each polypeptide. The MS(PEG)*n* reagents are readily soluble in water or organic solvents such as ACN, DMSO, methylene chloride and DMF.⁽⁸⁾

Boric Acid: Fischer scientific, **Sodium Hydroxide** - Sigma-Aldrich, **Acetonitrile** - JT-Baker.

150mM Borate buffer was used to dissolve the intact oral insulin precursor. 1N Sodium Hydroxide (NaOH) was used to adjust the pH and the oligomer was dissolved in Acetonitrile, (JT-Baker, as received).

Conjugation Reaction: The conjugation reaction is optimized by varying different concentrations (12, 15, 18mg/ml respectively) of oligomer by keeping the OI-P at optimized concentration of 30mg/ml. The oligomer dissolved in Acetonitrile, (JT-Baker, as received) and precursor in borate buffer (150mM, pH-10.2) added in the ratio of 1:1 for the three different concentrations of oligomer at RT and CR respectively. The mixture was allowed to stand for one hour. The reaction was stopped by using TFA. For one set of reaction the pH was maintained at >10.2 using 1N NaOH.

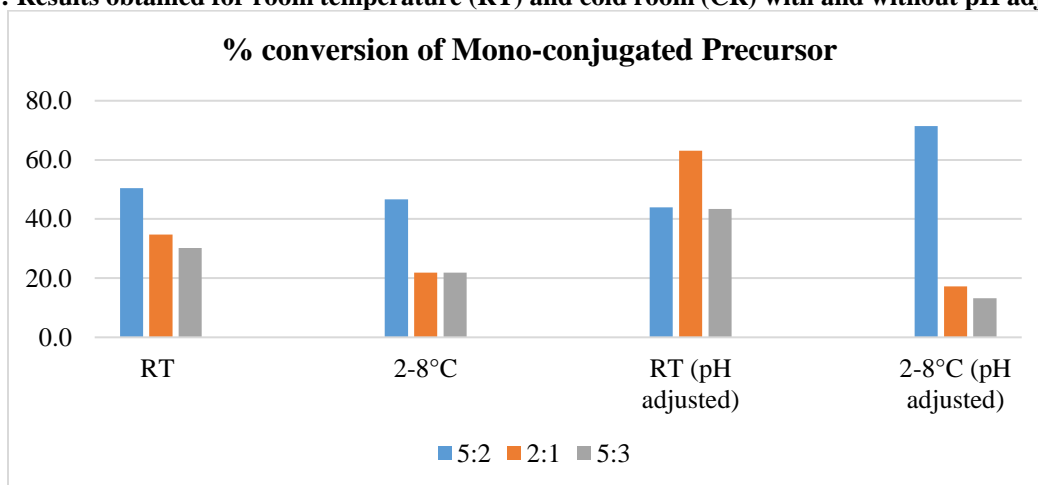
Results and Discussion

Based on the reaction conducted the results obtained for room temperature (RT) and cold room (CR) with and without pH adjustments are shown in Chart 1. In both cases full conversion of precursor to MCP or diconjugated precursor (DCP) was observed, with increase in concentration resulting in a decrease of MCP. The concentration of oligomer-precursor ratio is varied at different concentrations (5:2, 2:1, 5:3) as shown in Chart 1. The optimum concentration was found to be 5:2 oligomer-precursor ratio.

By keeping the precursor concentration constant the oligomer ratio has been changed and checked for the conversion of mono conjugated precursor. At room temperature yield was observed as 63.1% as shown in Chart 1 with adjustment of pH with NaOH.

Chart 1 represents graphically the results obtained in terms of the percentage of MCP produced. With pH adjustment in cold room with 5:2 (Precursor: Oligomer) ratio showed maximum conversion (71.4%).

Chart 1: Results obtained for room temperature (RT) and cold room (CR) with and without pH adjustments



Reaction with pH adjustment: One set of reactions were carried out with pH maintained at above 10.2. The results are shown in Fig. 2. As seen in Fig. 2a, the MCP generated was highest at the concentration of 15mg/ml. At a higher concentration (18mg/ml) the amount of DCP generated increased, whereas at a concentration below (12mg/ml) unreacted precursor was still observed with lower concentration of MCP generated. In contrast, as seen in Fig. 2b, a change in the temperature at which the reaction is conducted results in a decrease of MCP generated with increase in concentration.

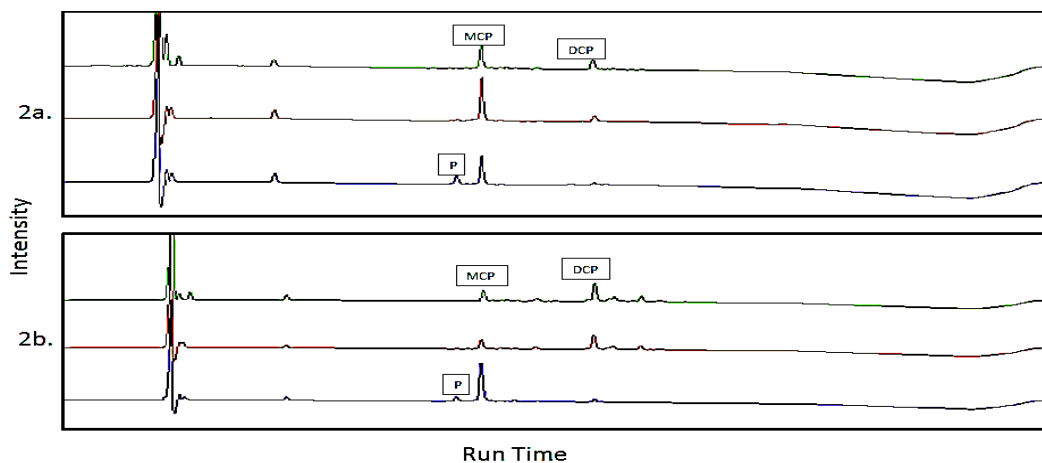


Fig. 2: a) Conjugation at room Temperature (pH>10.2) (1) Precursor +12mg/ml oligomer (2) precursor +15mg/ml oligomer (3) precursor +18mg/ml oligomer, b) Conjugation at Cold room (pH>10.2) (1) Precursor +12mg/ml oligomer (2) precursor +15mg/ml oligomer (3) precursor +18mg/ml oligomer

Based on the results obtained both the reaction temperature as well as pH seem to play an important role in directing the synthesis of mono conjugated precursor at B29 Lysine (Fig. 1). One of the possible reasons for the preferential attachment for the oligomer to B29 is the pKa of the lysine (Fig. 1). In addition to the pKa of Lysine another possible explanation (Fig. 2b) for the increase MCP observed is the increased activity of the functional group at 2-8°C.

Reaction Without pH adjustment: In order to confirm the effect of the pH on the reaction a complimentary set of experiments was conducted. As shown in Fig. 3a; at room temperature the utilization of precursor is complete however the conversion to DCP remains the same at different concentrations and does not decrease. The likely explanation is the propensity of the oligomer to attach to the free amino terminus of the precursor in the absence of pH control. Further confirmation of the significance of pH to the conjugation is highlighted by the similar levels of DCP at 2-8°C (Fig. 3a).

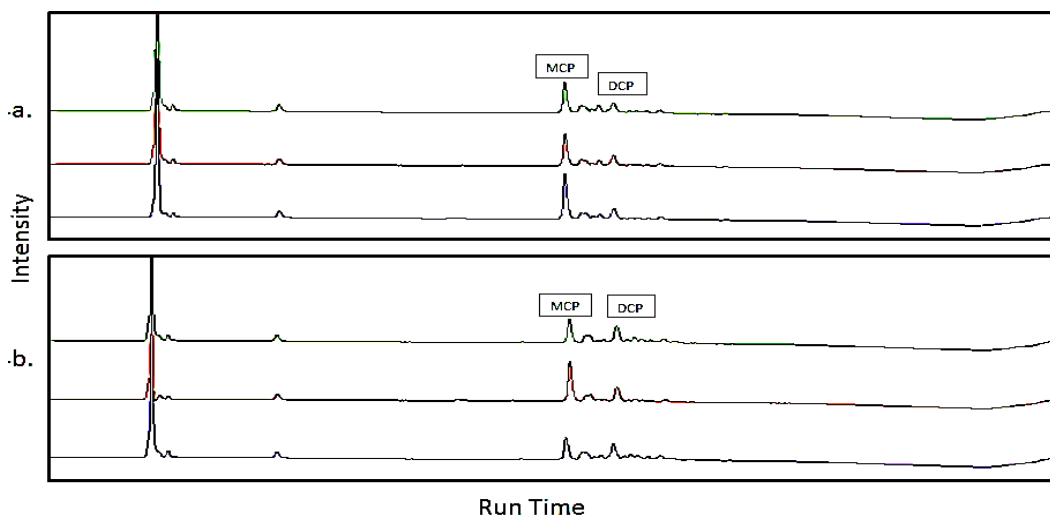


Fig. 3 a): Conjugation at Room temperature (pH-9.1) (1) Precursor +12mg/ml oligomer (2) precursor +15mg/ml oligomer (3) precursor +18mg/ml oligomer b): Conjugation at Cold room (pH-8.9) (1) Precursor +12mg/ml oligomer (2) precursor +15mg/ml oligomer (3) precursor +18mg/ml oligomer

MS-MS Sequence Confirmation of oligomer attachment to B29 Lysine: The reaction mixture was subjected to PMF analysis (PEGylated Insulin) and subsequent MS-MS analysis had been done to find the site of attachment based on b and y ions sequence conformation.

Fig. 4 illustrates the modification in the precursor with the oligomer attached at b7 ion (Lysine). This increase of 218Da at b7 is not observed in the control fragment. Further confirmation of the attachment to lysine is the change in mass of y4 ion in the fragment with oligomer attached.

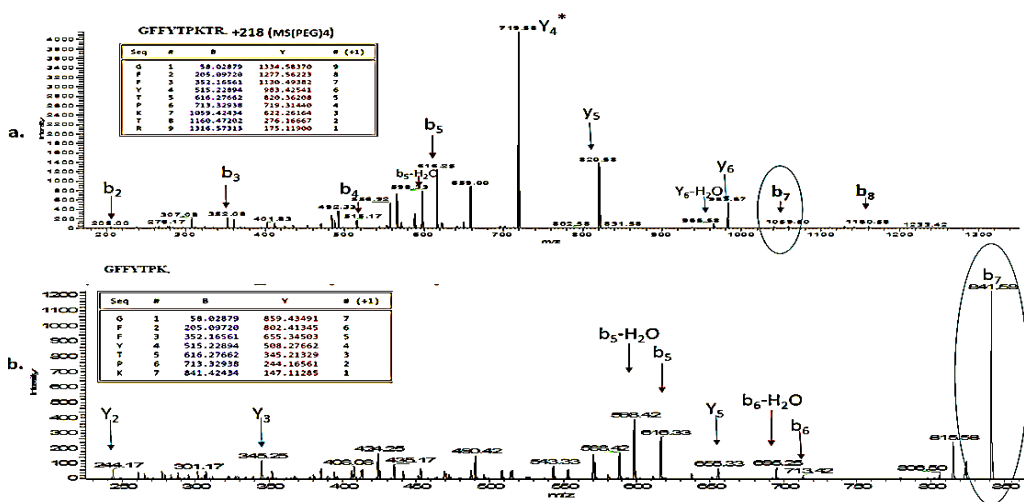


Fig. 4: B and Y ions confirming the site-specific attachment of oligomer to B29

Structural elucidation of PEGylated insulin using circular dichroism: The Conjugated Insulin was compared with recombinant human insulin in order to confirm its similarity in secondary structure. The sample was subjected to a temperature ramp from 5°C to 40°C (5°C Interval) to further understanding effect of temperature on secondary structure. As shown in Figure.5 & 6 both conjugated insulin and recombinant Human Insulin exhibits similar secondary structure. The data helps to confirm that conjugation does not affect the folding of the protein. It may be possible to infer that the conjugation does not affect functionality either.

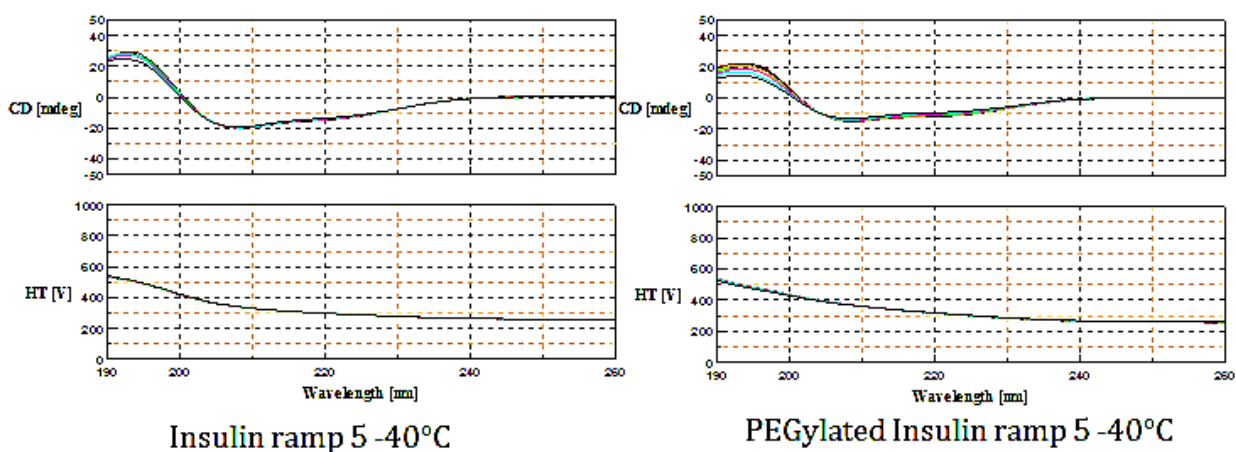


Fig. 5: Recombinant Human & conjugated Insulin ramping study at 5-40°C

Conclusion

By varying the temperature and pH at different concentrations of the oligomer the results presented in the paper indicates the importance of controlling the reaction temperature and pH in order to direct the conjugation of oligomer to a desired site within the protein of choice. Further, it might be beneficial to alter the substrate for conjugation by modifying its terminal end.

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