Strategic Optimization of C-Terminal Directed PEGylation: Impact on the Hydrodynamic and Structural Properties of Trastuzumab

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Availability Of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Tabriz University of Medical Sciences, (Ethics Code :IR.TBZMED.VCR.REC.1401.282)

Consent for Publication

Not applicable.

Competing Interests

There are neither ethical nor financial conflicts of interest involved in the manuscript. The manuscript was not submitted for publication elsewhere.

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Abstract:

Background: PEGylation of therapeutic antibodies generally decreases immunogenicity, extends systemic circulation, and reduces clearance. Trastuzumab, a monoclonal antibody targeting HER2, is typically administered with a loading dose of 4 mg/kg followed by weekly maintenance doses of 2 mg/kg, with a half-life varying from 10 to 18 days depending on the patient population. In general, site-specific PEGylation has been reported to improve pharmacokinetic behavior, product homogeneity, and analytical tractability in antibody modification.

Objectives: This study aimed to explore and optimize a C-terminal PEGylation approach for trastuzumab using amin-PEG (5 kDa) and to examine its potential effects on the antibody's physicochemical and hydrodynamic properties.

Methods: PEGylation reactions were performed at pH 3.5 and 7.5 with a PEG: protein molar ratio of 11.2 over a range of reaction times. PEGylated trastuzumab was separated from the unmodified antibody using size-exclusion chromatography (SEC) and subsequently analyzed by SEC, cation-exchange chromatography (CEX), and SDS-PAGE. The physicochemical properties were compared with those of native trastuzumab.

Results: At pH 3.5, PEGylation primarily occurred on the light chain of trastuzumab, whereas at pH 7.5, both light and heavy chains were modified, with a preference for the heavy chain. The hydrodynamic radius (R_h) increased from 4.357 nm for native trastuzumab to 4.519 nm for mono-PEGylated and 4.600 nm for di-PEGylated forms. SDS-PAGE analysis revealed shifts in the apparent molecular weight of PEGylated fragments, likely due to the interaction of PEG moieties with SDS and the increased hydrodynamic radius resulting from conjugation.

Conclusion: Our findings suggest that trastuzumab can be PEGylated at its putative C-terminal site, leading to distinct physicochemical changes. This study provides guidance for future methodological developments and optimizations regarding the site-directed PEGylation of monoclonal antibodies.

Keywords:

Monoclonal antibody (mAb), Trastuzumab, amin-PEG 5 kDa, Putative C-terminal site specific PEGylation, C-terminal modification, Hydrodynamic radius, Trastuzumab PEGylation

Introduction

Monoclonal antibodies are engineered to target specific aspects of cancer cells. Since its 1998 approval, trastuzumab has been crucial for treating HER2-positive breast cancer. It binds to the HER2 protein to block signaling and reduce cell growth in these cancers.[1,2] Monoclonal antibodies, complex eukaryotic proteins, are expensive and require large injections for clinical efficacy and treatment, may not be cost-effective, and have numerous side effects. In addition to extending the half-life of monoclonal antibodies, modifying their physicochemical structure may also decrease the number of prescriptions and the amount of medication required.[3,4] Site-specific PEGylation which refers to the targeted attachment of polyethylene glycol (PEG) chains to specific sites on proteins, such as monoclonal antibodies (mAbs), can be utilized to increase the therapeutic properties of mAbs by improving their pharmacokinetics and reducing immunogenicity through a physicochemical change.[5] Polyethylene glycol (PEG) is an FDA-approved, non-toxic, and non-immunogenic molecule first studied by Davis and Obuchowski. PEG is a carrier and active component in biopharmaceutical formulations. Adagen, the first medicine based on PEGylation, was developed in 1990. [6] Protein PEGylation offers numerous benefits, including reducing renal excretion, protecting amino acid sequences, protecting metabolic enzymes, improving solubility in organic solvents, supporting drug dissolution, minimizing opsonization, reducing protein accumulation, and improving the structural stability of PEGylated proteins. It also supports the dissolution of water-insoluble drugs, minimizes protein accumulation, and enhances stability during drug formulation and blood circulation.[7-9]

PEGylation technology in pharmaceutics is now available for clinical use, with numerous studies conducted to examine various types of PEGylation reactions. Generally, two types of specific and nonspecific PEGylation reactions are used for the production of PEGylated proteins, and most PEGylated drugs are produced using nonspecific methods on the market. However, currently, the technology of PEGylation of biopharmaceuticals is more focused on specific PEGylation.[10] One of the important biopharmaceutical medications with C-terminal site-specific PEGylation is Cimzia (certolizumab pegol), which is utilized to treat Crohn's disease. In 2015, Chan and colleagues studied the impact of PEGylation (with a 40kDa linear PEG) on trastuzumab pharmacokinetics in rats. They found a five-fold decrease in HER2 affinity for PEGylated trastuzumab, possibly due to its random nature.[11] In 2016, a study by Selis and colleagues examined the effects of PEGylation on trastuzumab fragments. The antibody was digested with pepsin and papain, producing Trast-F(ab')2, which was reduced and PEGylated to form Trast Fab'-

Cys-PEG. The study found that PEG increases the half-life of trastuzumab fragments but reduces biological activity. [12]

The properties of PEGylated protein are significantly influenced by the PEG molecule, leading to a larger molecular size or hydrodynamic volume. The molecular weight of proteins and PEG-protein conjugates can be determined using methods like size exclusion chromatography (SEC), electrophoretic methods (SDS-PAGE, Native-PAGE), light scattering, and mass spectrometry.[13,14] Size exclusion chromatography (SEC) is used to separate PEGylated proteins due to the increase in molecular weight caused by PEGylation, but it has limitations, like not separating positional isomers, poor resolution, low throughput, and high cost.[15] For purification of PEGylated monoclonal antibodies, like trastuzumab, SEC is often combined with Ion exchange chromatography (IEX) for product purity and removal of unreacted PEG in large-scale production. Hydrophobic interaction chromatography (HIC) is not widely used due to poor resolution and binding of unreacted PEG reagents.[16] Ion exchange chromatography, divided into anion exchange chromatography (AEX) and cation exchange chromatography (CEX), is used for purifying pegylated mAbs.[17] CEX is preferred for separation of PEGylated proteins, allowing one-step purification from non-PEGylated proteins, further PEGylated molecules, and unreacted PEG.[18,19] The ion exchange chromatography method is the only method capable of identifying positional isomers of PEGylated proteins. PegIntron® and Pegasys® are products with 15 different positional PEG isoforms and four main isoforms, respectively, which can be identified using IEX.[20,21]

It should be mentioned that monoclonal antibodies have a complex structure and are sensitive to changes, and the desired specific PEGylation requires careful optimization of the conditions. In addition, the purification of PEGylated antibodies according to the synthesis is a challenge and requires considerable research. This article focuses on the C-terminal-directed PEGylation of trastuzumab and how it affects the physicochemical properties of the PEGylated protein, such as hydrodynamic radius and apparent molecular weight, surface charge. Investigating the impact of PEGylation on the therapeutic efficacy of trastuzumab can provide valuable insights into the optimization of monoclonal antibody therapies to enhance patient outcomes.

Method

1. **PEGylation of Trastuzumab's C-terminal Region**

- **Conducting the trastuzumab-specific PEGylation with PEG-NH2 at pH 3.5 and pH 7.5, followed by sample analysis with the SDS-PAGE assay:**

C-terminal-directed PEGylation of trastuzumab was performed using amine-reactive PEG-NH_2 at two different pH conditions (3.5 and 7.5) to evaluate pH-dependent modification. A molar ratio of 11.2:1 (PEG to trastuzumab) was selected based on preliminary assessments to provide efficient PEGylation while preserving protein integrity.

The conjugation process was initiated by dissolving 64 mg of DCC in 2.4 mL of 50 mM phosphate buffer (pH 5.9) under gentle stirring for approximately 30 min to promote carbodiimide-mediated activation. Subsequently, 16 mg of purified trastuzumab was introduced into the activation medium, and the mixture was maintained at 20 °C under gentle agitation overnight to allow the formation of activated carboxyl intermediates.

Separately, 6 mg of mPEG-NH_2 (5 kDa), pre-dissolved in 4 mL of 50 mM phosphate buffer (pH 8.25), was added to the activated antibody solution to achieve an approximate PEG-to-protein molar ratio of [١١.2:1]. This step was defined as the onset of the conjugation reaction (t = 0). The resulting mixture was then incubated at 4 °C for reaction times ranging from 1 to 24 h .For comparative experiments conducted at pH 3.5, 16 mg of trastuzumab and 6 mg of mPEG-amine was dissolved in phosphate buffer (pH 3.5), and the final reaction volume was adjusted to 6.4 mL.

The reaction mixture was gently stirred and incubated at 4°C for 1 to 24 hours. samples of the reaction mixtures were taken every one hour between 1 and 6 hours, as well as at 8, 10, 22, and 24 hours. A solution of pure trastuzumab (1 mg/mL) was prepared in PBS and loaded separately into a lane of the SDS-PAGE gel as a control. PEGylation under varied conditions was compared to native molecular weight pattern using this control.

SDS-PAGE was performed under reducing conditions using a discontinuous gel system consisting of a 7% stacking gel and a 10% separating gel. Samples were mixed with sample buffer at a 3:1 (v/v) ratio, and 10 µL of the mixture was loaded into each well.

- **Silver nitrate staining of SDS-PAGE gel:**

The gel was fixed in a solution of 50% methanol, 10% acetic acid, followed by three washes with a 50% ethanol solution. Then it was briefly exposed to a hypo solution containing 0.002 g/100 ml sodium thiosulfate for exactly 1 minute, washed three times with water each for 30 seconds, and then immersed in a silver nitrate solution for 30 minutes. To visualize native and PEGylated light chains as well as heavy chains, the gel was developed in a solution of sodium carbonate, a hypotonic solution, and just 50 microliters of formaldehyde. The staining reaction was terminated by the addition of 100 mL of 5% acetic acid. At the end, the gel was thoroughly washed with distilled water, scanned, and documented for further analysis.

2. Analysis of PEGylated Trastuzumab by Size Exclusion Chromatography:

PEGylated trastuzumab samples were analyzed using an Agilent HPLC system equipped with a TSKgel SuperSW mAb HTP column (4.6 mm × 15 cm, 4 μm particle size; Tosoh Bioscience). The column was equilibrated with 200 mM phosphate buffer containing 200 mM NaCl (pH 6.5). The flow rate was set to 0.25 mL/min.

This column was selected for its high resolution and throughput, allowing efficient separation of monomeric, dimeric, and higher-order species of trastuzumab. Trastuzumab was separated efficiently on this column owing to its high resolution and throughput. The sample volume and chromatographic conditions were optimized for maximum performance. We used a flow rate of 0.25 mL/min, a mobile phase of 200 mM phosphate buffer with 200 mM NaCl at pH 6.5, a column temperature of 25°C, an injection volume of 20 μL, and a total run time of 40 minutes. Chromatographic data were collected at 280 nm and analyzed using Agilent ChemStation software.[22,23]

3. Analysis of PEGylated Trastuzumab by cation exchange chromatography

Cation exchange chromatography was performed using the same Agilent HPLC system equipped with a ProPac™ WCX-10 weak cation exchange column (4.0 mm × 250 mm, 10 μm particle size; Thermo Fisher Scientific). Separation was achieved using a salt gradient elution using following

buffer solutions: Buffer A: 20 mM MES, 20 mM NaCl, pH 5.6, and Buffer B: 20 mM MES, 500 mM NaCl, pH 5.6.

The flow rate was set to 0.5 mL/min. Chromatographic profiles of PEGylated trastuzumab and its charge variants of native trastuzumab were monitored at 280 nm and processed using Agilent software.

4. Data Analysis with Conan Fee's Equations:

Hydrodynamic Radius Calculation: Conan Fee's equations [1, 2, 3] were utilized to calculate the hydrodynamic radius. The native molecular weight was compared with the molecular weight after the addition of PEG.[24,25] The hydrodynamic radius of a protein (Rh) has been calculated to determine how PEGylation affects its size and diffusion properties. A key aspect of PEG conjugation is understanding how the apparent molecular size of the protein changes in solution, which has an impact on its pharmacokinetic properties, biodistribution, and size-based analysis methods such as size exclusion chromatography (SEC).

1. $R_h=(0.82\pm0.02)M^{1/3}_r$
2. $R_{h,PEG}=0.1912M_r^{0.559}$
3. $R_{h,PEGprot}=\frac{A}{6}+\frac{2}{3A}R^2_{h,PEG}+\frac{1}{3}R_{h,PEG}$

$A=[108R^3_{h,prot}+8R^3_{h,PEG}+12(81R^6_{h,prot}+12R^3_{h,prot})^{\frac{1}{2}}]^{1/3}$

Result

To investigate protein PEGylation, a series of reactions were performed and subsequently analyzed using SDS-PAGE electrophoresis. The results, as depicted in Figures 1 to 3, showcase distinct patterns in each gel, providing valuable insights into the PEGylation process.

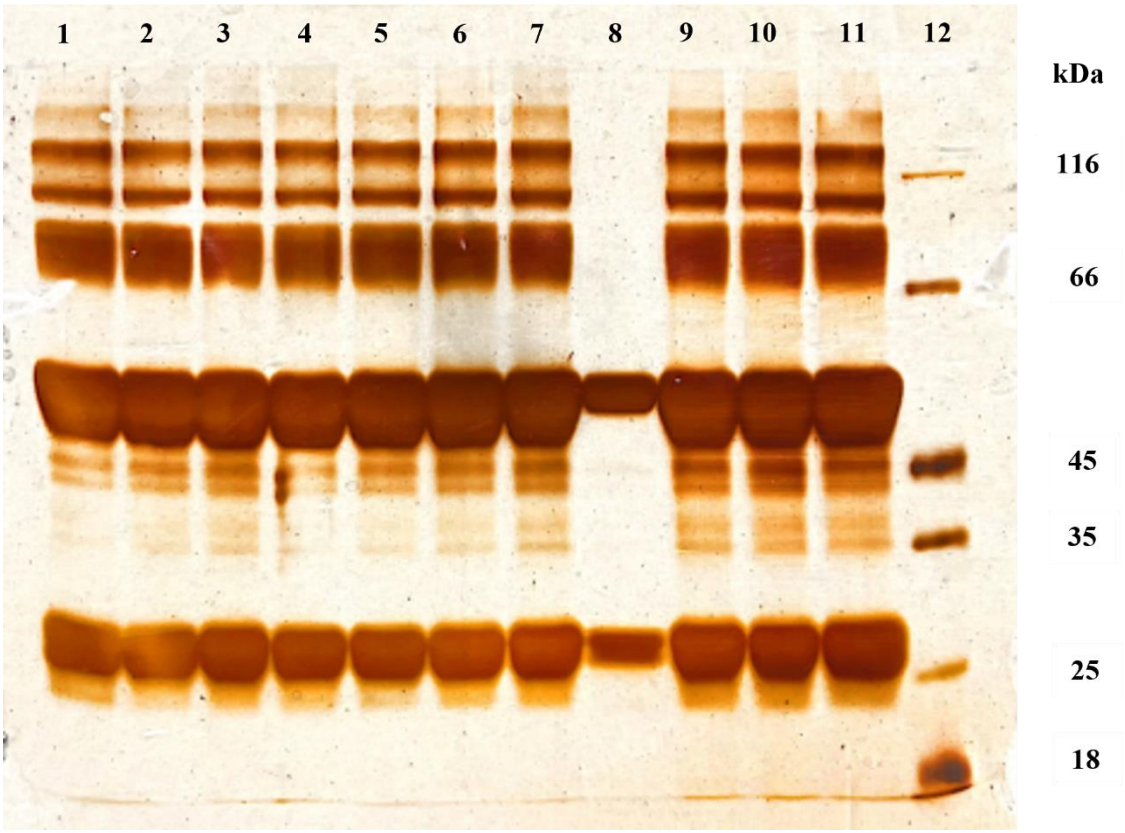


Figure 1: SDS-PAGE analysis of the PEGylation reaction of trastuzumab with amin-PEG 5 kDa on acrylamide gel 10 %, (pH:7.5, molar ratio: 11.2). In all lanes 20 microliter sample is loaded: reaction mixture after 24 hours: lane 1, reaction mixture after 22hours: (lane 2), reaction mixture after 10 hours: (lane 3), reaction mixture after 8- hours: (lane 4), reaction mixture after 6 hours (lane 5), reaction mixture after 5 hours (lane 6), reaction mixture after 4 hours: (lane 7), Trastuzumab standard: (lane 8), reaction mixture after 3 hours: (lane 9), reaction mixture after 2 hours: (lane 10), reaction mixture after 1 hours: (lane 11), ladder: (lane12); Proteins were visualized by silver nitrate staining.

Figure 1 depicts the results of PEGylation reaction at pH 7.5 over a time frame of 1 to 24 hours. A control sample of untreated trastuzumab was utilized for comparison. The molecular weights were determined using a molecular weight marker ladder ranging from 18 to 116 kDa.

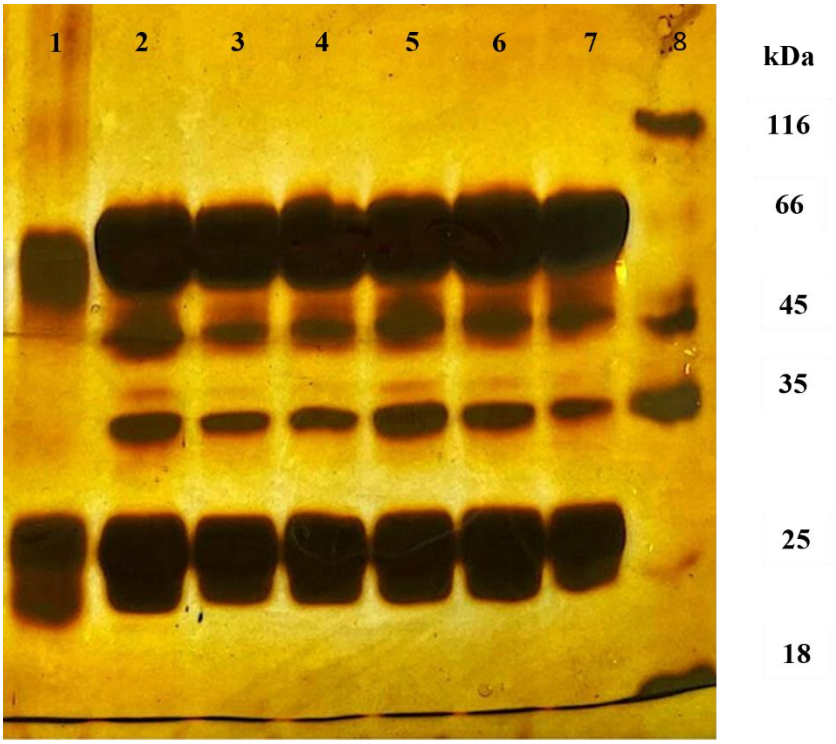


Figure 2: SDS-PAGE analysis of the PEGylation reaction of trastuzumab with amin-PEG 5 kDa on a 10% acrylamide gel (pH: 3.5, molar ratio: 11.2). In all lanes, 20 microliters of sample were loaded: trastuzumab standard (lane 1), reaction mixture after 24 hours (lane 2), reaction mixture after 12 hours (lane 3), reaction mixture after 8 hours (lane 4), reaction mixture after 6 hours (lane 5), reaction mixture after 3 hours (lane 6), reaction mixture after 1 hour (lane 7), and ladder (lane 8). Proteins were visualized by silver nitrate staining.

Figure 2 displays the outcomes of the PEGylation reaction at a pH of 3.5 and the same molar ratio over time frame of 1 to 24 hours. The PEGylation condition depicted in Figures 1 and 2 involves a molar ratio of 11.2 (PEG: protein) and a temperature of 4°C, while the pH is adjusted to 7.3. These 2 figures reveal two leading bands in the weight range of 25 kDa and 50 kDa. In Figure 1, a set of bands in the weight range of 100 to 115 kDa can be seen at all reaction time points. In Figure 2, a set of bands in the weight range of 25 to 50 kDa can also be observed.

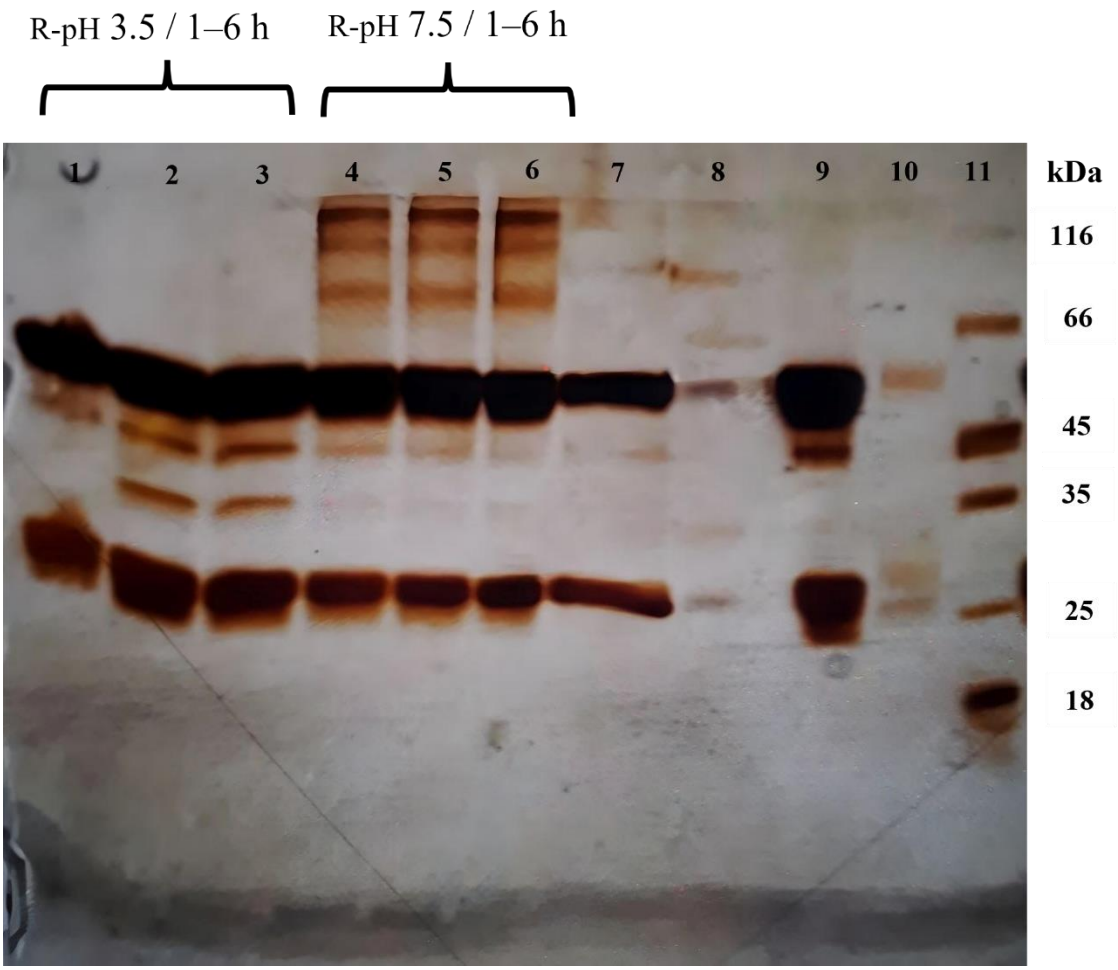


Figure 3: SDS-PAGE analysis of the PEGylation reaction of trastuzumab with amin-PEG 5 kDa on acrylamide gel 10 %, (molar ratio: 11.2, pH:3.5 & 7.5). In all lanes 20 microliter sample is loaded, reaction mixture with pH of 3.5 after 1 to 6 hours (lane1 to 3), reaction mixture with pH of 7.5 after 1 to 6 hours (lane4 to 6), Control – pure trastuzumab solution (lane 7), PEGylated-trastuzumab SEC fraction (pH 7.5) (lane 8), trastuzumab commercial formulation (lane 9), PEGylated- trastuzumab SEC fraction (pH 3.5) (lane 10), ladder (lane 11), Proteins were visualized by silver nitrate staining.

Moving to Figure 3, a depiction of the outcomes of the PEGylation reaction within a time frame of 1 to 6 hours at both pH levels of 3.5 and 7.5 is reported. These outcomes are then compared to the results showcased in Figures 1 and 2, which were obtained under distinct experimental

conditions. The PEGylation condition illustrated in this figure was performed at a PEG-to-protein molar ratio of 11.2:1. The reaction was conducted at a temperature of 4°C.

In Figure 3, Lanes 1-3 and 4-6 depict time-course PEGylation reactions at pH 3.5 and 7.5, respectively, showing the progression of PEGylation over 1, 3, and 6 hours. Lane 7 represents the untreated trastuzumab solution as control. Lanes 8 and 10 represent SEC-separated PEGylated trastuzumab at pH 7.5 and 3.5, respectively. Lane 9 depicts the trastuzumab commercial formulation (AryoTrust). Lane 11 displays the molecular weight marker.

Investigating the hydrodynamic radius (R_h) of the PEGylated protein and the native protein by using the Conan Fee equations revealed a noticeable disparity (Table 1). PEGylation leads to a 24.2% increase in the hydrodynamic radius of di-PEGylated trastuzumab, compared to native trastuzumab, as shown in the table. Similarly, mono-PEGylated trastuzumab exhibits a 16.2% increase in its hydrodynamic radius compared to the native form. Furthermore, the difference between the hydrodynamic radius of di-PEGylated trastuzumab and mono-PEGylated trastuzumab is approximately 8%. Similarly, the hydrodynamic radius of di-PEGylated heavy chain and native heavy chain in the case of trastuzumab undergoes a 25.5% increase, while the hydrodynamic radius of mono-PEGylated heavy chain and native heavy chain increases by around 16.5%. The difference in hydrodynamic radius between di-PEGylated and mono-PEGylated trastuzumab is approximately 9%. Furthermore, the hydrodynamic radius of the light chain experiences a 25.5% increase in the di-PEGylated form in comparison to the native form and a 14.7% increase in the mono-PEGylated form. The difference in radius between di-PEGylated and mono-PEGylated trastuzumab is 10.5%.

By substituting the acquired R_hs into equation 2, which represents the hydrodynamic radius of the native protein, the molecular weight of PEGylated proteins was once again determined. This was done by assuming that these PEGylated proteins are equivalent to native proteins with a hydrodynamic radius that has been calculated.

Table 1. Hydrodynamic radius of various forms of pegylated and native trastuzumab using Conan fee's equation

Protein	**Molecular weight**	**Rh (nm)**	**Mw (Rh)**
PEG (native)	5	0.69	-
Trastuzumab (native)	150	4.357	-
Heavy chain (native)	50	3.02	-
Light chain (native)	25	2.39	-
PEGylated Trastuzumab (Mono-PEGylated)	155	4.519	**167.37**
PEGylated Heavy Chain (Mono-PEGylated)	55	3.185	**58.6**
PEGylated Light Chain (Mono-PEGylated)	30	2.557	**32.46**
PEGylated Trastuzumab (Di -Di-PEGylated)	160	4.599	**176.42**
PEGylated Heavy chain (Di -Di-PEGylated)	60	3.275	**63.71**
PEGylated Light Chain (Di-PEGylated)	35	2.642	**33.45**

Figures 4 and 5 demonstrate the size exclusion chromatogram of the reaction mixture after PEGylation of trastuzumab at pH 7.5 (Figure 4) and pH 3.5 (Figure 5) in comparison to the control (unmodified) trastuzumab. The control trastuzumab displayed a singular, clear peak aligning with its estimated molecular weight of approximately 149 kDa. The chromatograms of the reaction mixture at both pH levels exhibited an extra peak eluting before the principal trastuzumab peak.

Under the selected conditions, SEC chromatograms were obtained for the PEGylation reactions performed at pH 7.5 and pH 3.5. Representative chromatograms, demonstrating the separation profiles, are shown in Figures 4 and 5. Fractions corresponding to the PEGylated trastuzumab species—which eluted earlier than the native antibody due to their increased hydrodynamic volume—were collected based on these elution profiles. The collected fractions were subsequently concentrated by ultrafiltration for further characterization. Protein concentration was determined by Nanodrop spectrophotometry, and the processed samples were used for further characterization by SDS-PAGE.

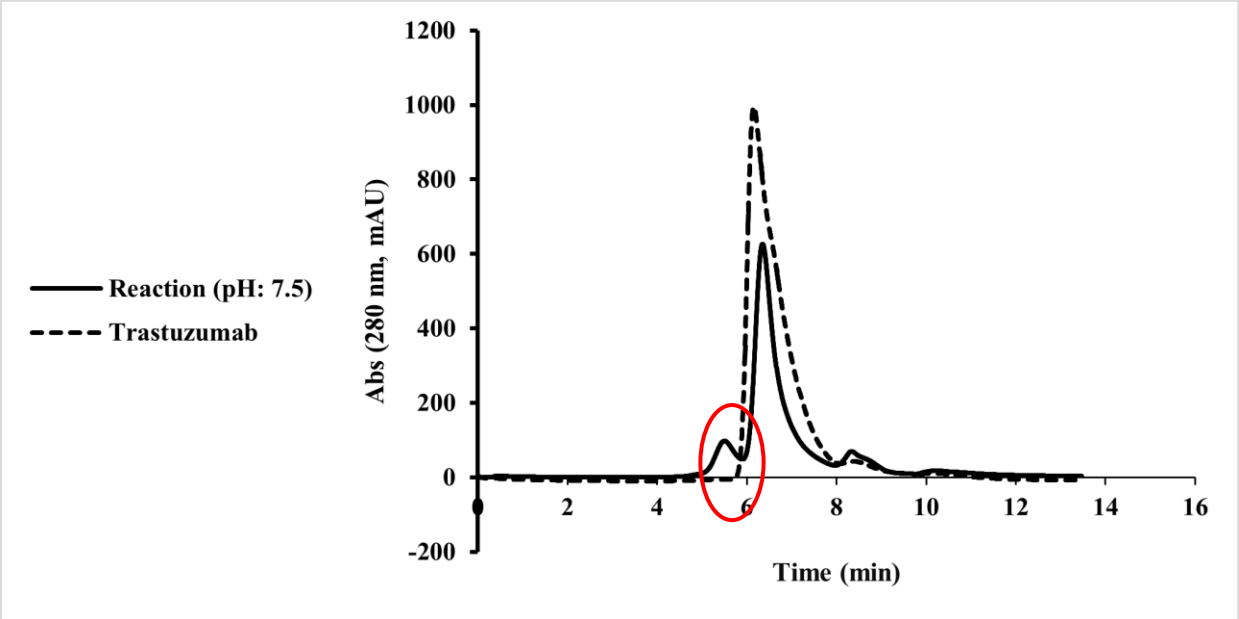


Figure 4: Size Exclusion Chromatogram of pure native trastuzumab solution (2.5mg/ml) and PEGylation reaction at pH 7.5.

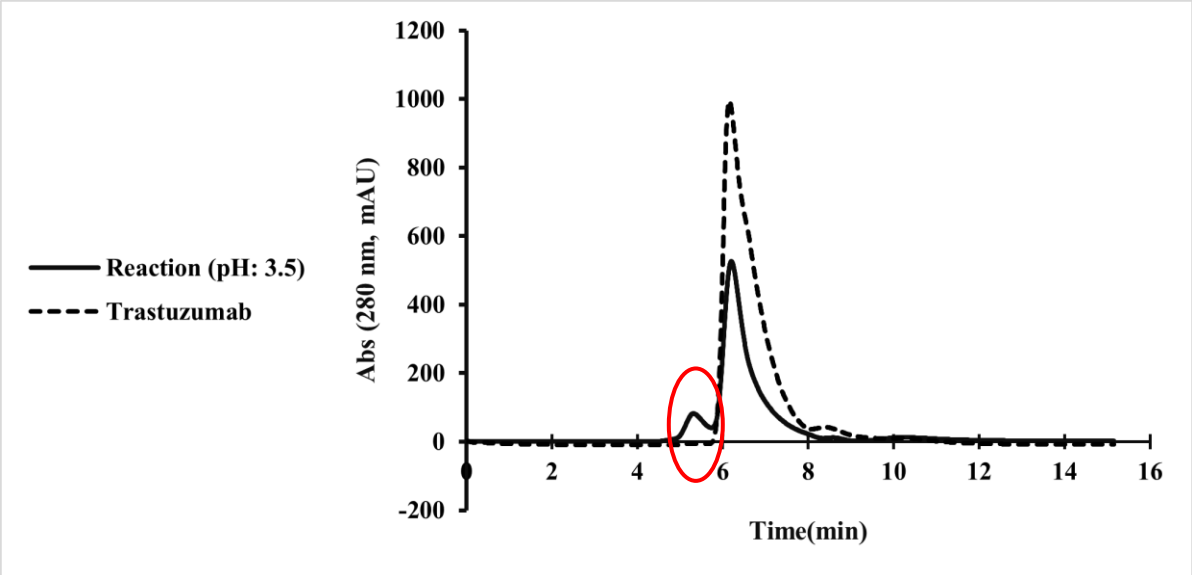


Figure 5: Size Exclusion Chromatogram of native trastuzumab solution (2.5mg/ml) and PEGylation reaction at pH 3.5.

Nanodrop spectrophotometric analysis was performed to determine the protein concentration of PEGylated fractions collected from SEC before and after Amicon filtration at pH 7.5 and pH 3.5. The measured concentrations are summarized in Table 4.3. Minor peaks below the 1% reporting threshold (~19–22% total area) were excluded from the table.

Figure 6 represents the cation exchange chromatograms (Absorbance at 280 nm vs. Time) of the reaction mixture following PEGylation of trastuzumab at pH 7.5, compared to the native trastuzumab solution, using a pH 5.6 separation buffer system composed of Buffer A (20 mM MES, 20 mM NaCl, pH 5.6) and Buffer B (20 mM MES, 500 mM NaCl, pH 5.6), control-trastuzumab eluted as a major peak at approximately 11–12 minutes under a linear salt gradient. In contrast, the chromatogram of the PEGylation reaction mixture at pH 7.5 showed a distinct elution profile. At approximately 9-10 minutes, a significant peak eluted earlier than the control peak. Some species show a reduced retention time compared to the main peak corresponding to unmodified trastuzumab, suggesting the presence of modified species.

Figure 7 shows cation exchange chromatograms (Absorbance at 280 nm vs. Time) of reaction mixtures with pegylated trastuzumab at pH 3.5 and un-pegylated trastuzumab, using a separation buffer system pH of 5.6. After approximately 11 minutes, control trastuzumab eluted in a single peak with a maximum absorbance. On the other hand, the chromatogram of the PEGylation reaction mixture at pH 3.5 showed a distinct pattern. Approximately 9-10 minutes, a new, larger peak on the leading edge of the peak corresponding to unmodified trastuzumab in the reaction mixture that eluted just before the main control peak.

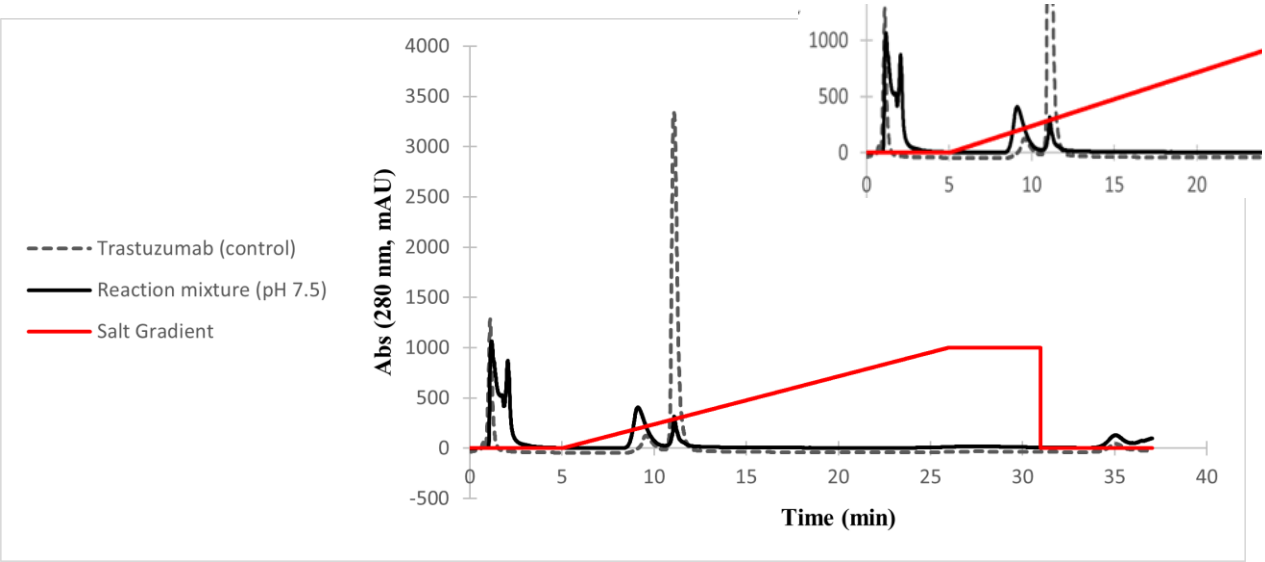


Figure 6: Cation exchange chromatography of pure native trastuzumab and PEGylation reaction at pH 7.5.

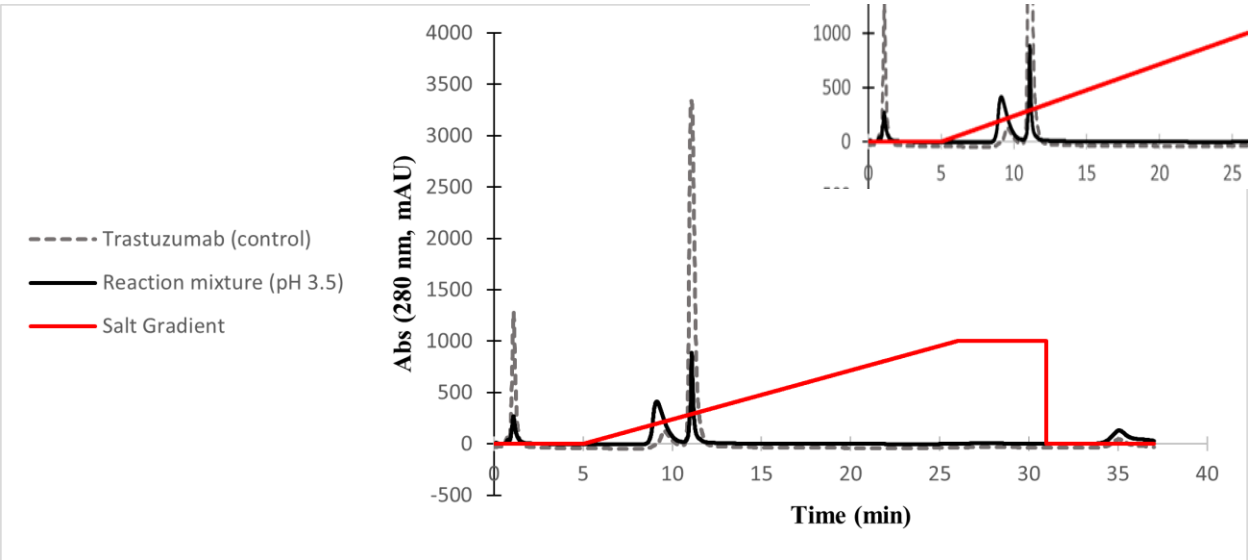


Figure 7: Cation exchange chromatography of pure native trastuzumab and PEGylation reaction at pH 3.5.

Discussion

Trastuzumab (Herceptin®) is a monoclonal antibody (mAb) used to treat HER2-positive cancers, including breast cancer and gastric cancer, as well as first-line therapy in metastatic disease. It also stimulates the immune system to kill cells with a high level of HER2, a process known as antibody-dependent cellular cytotoxicity (ADCC). In addition, trastuzumab inhibits the MAPK and PI3K/Akt pathways, resulting in a reduction in cell growth and proliferation. Considering the growing use of this medication in various cancer types, enhancing the efficiency and physicochemical properties of trastuzumab can be highly useful. [26,27]

Polyethylene glycol (PEG) is a water-soluble polymer commonly used in protein and drug conjugation. PEG conjugation can improve water solubility and protein stability, reducing its immunogenicity. [11] One of the techniques utilized in PEGylation is commonly known as C-terminal PEGylation. Trastuzumab possesses a total of four C-terminal domains, which consist of two light chains and two heavy chains. To achieve specific PEG attachment to the C-terminal of the antibody chains, a specific procedure needs to be followed.[28] This study aims to PEGylate trastuzumab by employing a C-terminal-directed approach through the covalent conjugation of a commercially available methoxy-PEG5kD-amine to the carboxyl-terminal (-COOH) region of the light chains present in this particular monoclonal antibody.

The methoxy PEG-amine with a low molecular weight was applied to minimize potential steric interference with the active site of the antibody. SDS-PAGE analysis was subsequently employed to evaluate the progress of the PEGylation reaction and to investigate the distribution and apparent molecular weight of the resulting conjugate species. As PEG can interact with SDS in the gel during the electrophoresis analysis, resulting in slower movement, PEGylated moieties show a molecular weight higher than that expected for a protein with a molecular weight equal to the sum of the molecular weights of the protein and the attached PEG chain.[25,29]

This study aimed to obtain C-terminal-directed PEGylation of trastuzumab at the light chain's C-terminal. There are several PEGylation sites on the trastuzumab molecule, which make this modification challenging. Trastuzumab contains four C-terminal residues (two on the heavy chains and two on the light chains) and several free carboxyl groups on its side chains, particularly the negatively charged aspartic acid and glutamic acid. These additional carboxyl groups present competing reaction sites for the PEG-amine reagent. Therefore, a strategy was designed to direct

the PEGylation reaction first towards the C-terminals and subsequently to achieve selectivity for the light chain C-terminal cysteine. To achieve selectivity, we targeted the light chain C-terminal cysteine, using its unique reactivity. Data from Drug Bank and ProtParam confirmed the presence and accessibility of this residue.

The rationale for targeting the light chain C-terminal cysteine was based on the information gathered from the Drug Bank and the ProtParam tool (ExPASy). The resources provided by these sources confirmed that the C-terminal residue of the light chain is cysteine (C), and the C-terminal residue of the heavy chain is lysine (K). Additionally, there were other potential active sites for this reaction, including the carboxyl groups of the acidic amino acids (aspartic acid and glutamic acid). As part of determining the ideal pH for PEGylation, the pKa values of these key amino acids (cysteine, lysine, aspartic acid, and glutamic acid) are important.

To enable amide bond formation between amin-PEG and the carboxyl groups of trastuzumab, activation of the carboxyl groups is required. DCC (N, N′-dicyclohexylcarbodiimide) was employed to activate these groups via the formation of reactive O-acylisourea intermediates. These intermediates subsequently facilitate nucleophilic attack by the amine group of PEG, leading to the desired amide bond. Following this activation step, trastuzumab and amine-terminated PEG were combined under various PEGylation conditions (including variations in pH, reaction time, and PEG-to-protein molar ratio) at ambient temperature.

Certain structural features of the antibody C-terminal regions may partly contribute to the conjugation pattern observed in this study. The amino acid at the C-terminus of the heavy chain is typically lysine, whereas the light chain terminates with cysteine. In contrast, Asp and Glu side chains, which are the primary acidic residues capable of carrying negative charges at near-physiological pH, are present as carboxylates.

According to the Henderson–Hassel belch equation,

$pH = pKa + \log([A^-]/[HA])$,

carboxyl groups—including the terminal carboxyl groups as well as those of Asp and Glu residues—are expected to exist predominantly in their deprotonated (COO^-) form at approximately

pH 6. However, differences in local pKa values and structural accessibility may influence their effective reactivity. In particular, C-terminal carboxyl groups are often more solvent-accessible due to their location at the ends of the polypeptide chains. In contrast, Asp and Glu side chains in the compact IgG structure are often partially buried and involved in hydrogen-bonding or salt-bridge networks. Such interactions may limit their mobility and reduce their effective accessibility for carbodiimide-mediated activation.

Under these conditions, the combination of favorable ionization and higher spatial accessibility makes the C-terminal carboxyl groups more likely to participate in carbodiimide-mediated activation. Although carbodiimides undergo partial hydrolysis in aqueous environments, classical studies have shown that particularly under mildly acidic condition (pH 5.5-6), the formation of O-acylisourea intermediates generally proceeds faster than hydrolysis to DCU. [30-32]

In this context, DCC primarily participates during the early activation phase of the reaction. Even though a fraction of DCC converts to DCU upon contact with the aqueous buffer, transient activated intermediates can still form during the initial stages of the reaction, enabling subsequent coupling with amine-terminated PEG. [33,34] After this initial period, the remaining DCC is largely converted to DCU, while the transient O-acylisourea intermediates may remain sufficiently reactive toward nucleophilic attack by amine-terminated PEG.

Consistent with this mechanism, incubation of the activated antibody with amine-terminated PEG resulted in detectable shifts in SDS-PAGE, SEC, and CEX profiles, suggesting the formation of PEGylated species. These analytical observations suggest that carbodiimide-mediated activation occurred under the controlled reaction conditions used in this study. Taken together, these findings suggest that, under the reaction conditions employed here, terminal carboxyl groups may preferentially participate in the activation step. This behavior could plausibly contribute to the enhanced reactivity observed at these sites despite the presence of multiple competing carboxyl groups. Nevertheless, definitive confirmation of the specific conjugation sites would require further structural analyses, such as peptide mapping and mass spectrometry.

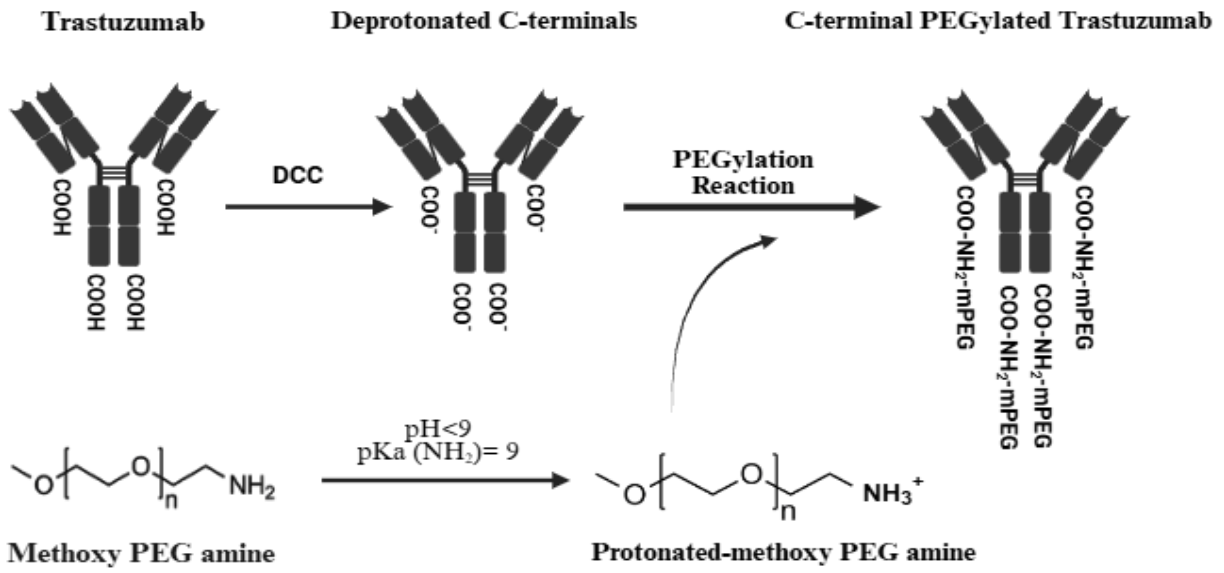


Figure 8: Schematic representation of trastuzumab PEGylation. The carboxylic acid groups of trastuzumab are first activated by DCC to form an intermediate (deprotonated C-terminal trastuzumab), followed by nucleophilic attack of NH2-PEG, resulting in the formation of a stable amide bond and C-terminal-directed trastuzumab-PEG conjugates.

Lysine presents three characteristic pKa values corresponding to its α-carboxyl (~2.2), α-amino (~9.0), and ε-amino side chain (~10.5) groups. In the context of carbodiimide-mediated PEGylation, the relevant functional group is the terminal α-carboxyl group located at the C-terminus of the polypeptide chain. Efficient activation of this group requires that it be present in its deprotonated form (COO^-), which occurs at pH values above its pKa (~2.0–2.2).

Although cysteine possesses a reactive thiol group (pKa ~8.3), this functionality does not participate in carbodiimide-mediated activation, because DCC exclusively targets carboxyl groups. Therefore, for C-terminal cysteine, only the α-carboxyl group (pKa ~1.7) is relevant. At pH 7–8 this group is fully deprotonated and behaves similarly to the terminal carboxyl group of C-terminal lysine in terms of availability for carbodiimide activation.

Based on these pKa values, PEGylation reactions were performed in the neutral to mildly basic range (pH 7–8), where terminal carboxyl groups are fully deprotonated and available for

carbodiimide activation. At this pH, the primary amine of amin-PEG also remains sufficiently nucleophilic to attack the activated O-acylisourea intermediate. Although the side-chain carboxyl groups of Asp and Glu residues are likewise largely deprotonated in this pH range, their participation in coupling reactions may be influenced by steric accessibility and structural constraints within the IgG molecule. This pH range is frequently used for amidation reactions, ensuring the optimal reactivity of the amin-PEG group.

According to the results represented in Figure 1, the reaction was carried out at different hours under optimized conditions (Molar ratio 11.2, pH=7.55). (Optimization data are not shown.) In this figure, bands corresponding to the control-trastuzumab can be observed, showing a heavy chain with an approximate molecular weight of 50 kDa and a light chain with an approximate molecular weight of 25 kDa. The bands in the upper region that are attributed to proteins of higher molecular weights are conceivably associated with pegylated fragments. Within the region linked with pegylated proteins, three sub-bands can be seen above 50 kDa and between 66 kDa and 130 kDa, and a series of other sub-bands can be seen between 25 kDa and 50 kDa. These bands are potentially associated with C-terminal PEGylation products based on our predictions and the activation conditions involving the -COOH group by DCC.

The bands observed between 50 and 70 kDa, which migrate above the native heavy-chain band (~50 kDa), are consistent with the expected mobility shifts often observed following PEG conjugation. The presence of these additional bands may reflect a degree of sample heterogeneity after modification. Furthermore, additional faint bands observed around ~100 kDa may represent incompletely reduced species or minor antibody aggregation. Since SDS-PAGE alone does not allow for definitive structural assignment, these features were interpreted cautiously as potentially related to the modification process and/or minor aggregation.Previous studies have reported that antibody fragments may form multimeric species under certain analytical conditions, particularly in chromatographic analyses such as size-exclusion HPLC.[35]

The bands observed in the 25–50 kDa region migrate above the native light-chain band (~25 kDa), which is consistent with the formation of PEG-modified light chain species. While it is well-established that PEGylated proteins frequently exhibit anomalous electrophoretic mobility due to PEG-induced hydrodynamic effects, these observations align with the expected molecular weight shift for a mono-PEGylated light chain. Furthermore, when interpreted within the framework of

our C-terminal-directed reaction strategy, and supported by orthogonal data from SEC, CEX, and mechanistic analysis of the reaction chemistry, these results provide strong evidence supporting the proposed preferential modification of the light chain.

Under the optimized reaction conditions (DCC activation at pH 7.5), PEG conjugation was observed on both heavy and light chains, suggesting that the reaction was not completely restricted to a single chain. This implies that further optimization may be required to improve the selectivity of the conjugation process. The reduction in the intensity of native heavy- and light-chain bands, together with the appearance of higher-molecular-weight species, is consistent with the formation of PEG-modified products. To improve the yield and selectivity of the C-terminal-directed PEGylation strategy, the effect of pH on the reaction was investigated. The relationship between pH and the pKa of carboxyl groups plays an important role in carbodiimide-mediated coupling reactions. At pH 7.5, deprotonation of carboxyl groups is favored, resulting in negatively charged carboxylate ions ($–COO^-$) that can participate more readily in activation and conjugation reactions.

Consistent with this chemical rationale, the experimental results suggested that PEGylation preferentially occurred at the light chain C-terminus under the selected reaction conditions. The C-terminal cysteine of the light chain requires deprotonation of its carboxyl group to serve as a reactive site for amidation with amin-PEG. Since the pKa of the cysteine carboxyl group is approximately 1.8, a pH above this value enables deprotonation and reactivity.

Cysteine also contains a thiol (–SH) group that may undergo oxidation under alkaline conditions; therefore, highly alkaline conditions were avoided. As a result, pH 7–8 was chosen to maximize carboxyl deprotonation while minimizing thiol oxidation. Both light and heavy chains were PEGylated under these conditions, suggested that residues other than the C-terminal cysteine, including lysine residues in the heavy chain, may also participate in PEGylation reactions.

To further interpret these observations, the ionization states of relevant amino acid residues were analyzed across different pH values. Based on the pKa values of the amino acids (Table 2) and the Henderson–Hassel Balch equation, the ratio of non-protonated to protonated amino acids at each pH was calculated to determine the pH range most favorable for selective PEGylation.

Table 2. Different pKa values of amino acids (lysine, cysteine, glutamic acid, aspartic acid)

Amino acid	**pK$_a$** (C_α-COOH)	**pK$_a$** (C_α-NH^{3+})	**pK$_a$** (side chain)
Lysin	2.18	8.95	10.53
Cysteine	1.71	10.78	8.33
Glutamic acid	2.19	9.67	4.25
Aspartic acid	2.09	9.82	3.86

Considering the calculations, at pH = 3.5, the ratio of the presence of the leaving group in the C-terminal light chain area [COO^-] /[COOH] is about 62, which is a very suitable environment for the desired PEGylation reaction.

At pH 3.5, the calculated ratio of [COO^-]/[COOH] for the C-terminal carboxyl group of the heavy chain is approximately 0.21, indicating a predominantly protonated state. Under these acidic conditions, the availability of reactive carboxylate groups in certain regions of the antibody may be limited. Figure 2 illustrates the reaction between trastuzumab and amin-PEG at pH 3.5. Several bands are observed between 25 and 50 kDa, migrating above the native light-chain band (~25 kDa), while no prominent bands are detected above 50 kDa. In contrast, the calculated [COO^-]/[COOH] ratios for the side-chain carboxyl groups of aspartic acid and glutamic acid at pH 3.5 are approximately 0.44 and 0.78, respectively. Although these values indicate partial deprotonation, the proportion of reactive carboxylate groups remains relatively limited under acidic conditions. Consequently, these residues may be less favorable sites for carbodiimide-mediated coupling reactions. Taken together, these observations suggest that PEG conjugation under acidic conditions may preferentially generate species associated with the light chain. However, SDS-PAGE analysis alone does not allow definitive identification of the PEGylation site or confirmation of C-terminal modification.

In Figure 3, the SDS-PAGE analysis of trastuzumab PEGylation shows a clear pH-dependent modification pattern. Two distinct bands are seen in lane 7 of the untreated pure control,

representing the heavy chain (50 kDa) and the light chain (25 kDa) of trastuzumab. Lane 9 shows the marketed trastuzumab formulation without PEGylation as a control. To highlight the difference between pure trastuzumab and the formulated drug product, it was included as a control. There is a striking pH-dependent PEGylation pattern observed in the time-course reaction (lanes 1–3 and 4–6). At pH 3.5 (lanes 1–3), a more pronounced shift in the light chain band toward higher molecular weights is evident, which may be indicative of preferential modification associated with the light chain. In contrast, at pH 7.5 (lanes 4–6), a progressive shift of the heavy chain band toward higher molecular weights is observed, consistent with the formation of PEGylated products.

Several sub-bands are also seen in the higher molecular-weight range of the light chain, supports heterogeneity in PEG conjugation and the presence of multiple PEG attachment states. It is likely that the C-terminal carboxyl group of the lysine residue at pH 7.5, according to the Henderson–Hasselback equation, becomes more available for carbodiimide-mediated activation, which may contribute to heavy chain PEGylation. As a consequence of DCC activation, other COOH groups on monoclonal antibodies can also be PEGylated. At pH 7.5, the observed shift in the heavy chain band is consistent with PEG conjugation occurring predominantly in the heavy chain, with several sub-bands which may be indicative of possible additional modification.

It is noteworthy that the heavy chain of trastuzumab contains a C-terminal lysine residue (K447). The presence of this residue provides a terminal carboxyl group that may become reactive under carbodiimide-mediated coupling conditions. Therefore, PEG conjugation observed in the heavy chain region at pH 7.5 could be consistent with modification occurring near the C-terminal region.[35]

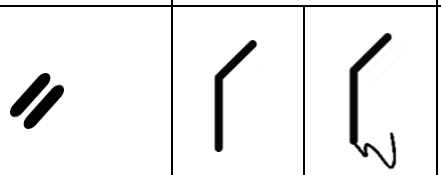
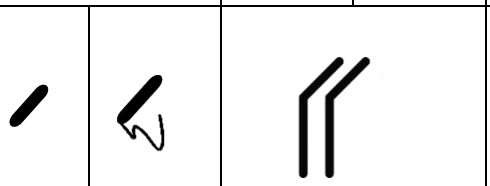
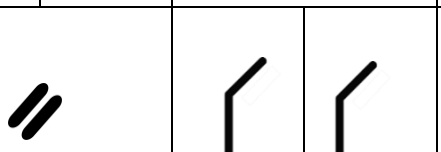
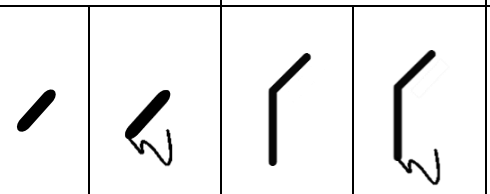
At pH 7.5, DCC facilitates non-specific PEGylation at the C-terminus of the antibody molecule, but this COO^- group can be present in both heavy and light chains and certain amino acid side chains. At pH 3.5, without DCC, the percentage of reactive COO^- groups around the cysteine residue is significantly higher than lysine and other amino acids. This is visible in Figures 2 and 3, where only bands corresponding to the PEGylated light chain are observed at pH 3.5. This strategy is validated by a distinct shift in the light chain band at pH 3.5 (lanes 1-3) and in the purified fraction (lane 10), indicating preferential cysteine-mediated PEGylation. The light chain band exhibits a more pronounced shift than the heavy chain.

In addition, from both pH 3.5 and pH 7.5 reactions, the Fractions corresponding to the PEGylated protein (the first fraction based on SEC principles) were collected, concentrated, and analyzed by SDS-PAGE. The SEC-purified fractions (lanes 8 and 10) further support these results. The SEC fraction at pH 3.5 (lane 10) shows a significant shift in the light chain band, consistent with preferential modification associated with the light chain. On the other hand, a noticeable shift in the heavy chain band is observed in the SEC fraction at pH 7.5 (lane 8), suggesting predominant modification of the heavy chain under these conditions.

It is important to recognize the limitations of SDS-PAGE in the analysis of PEGylated proteins. PEGylated proteins often exhibit anomalous electrophoretic migration due to altered SDS binding and increased hydrodynamic radius. In the present study, SDS-PAGE findings were interpreted alongside complementary SEC and CEX data to provide a preliminary physicochemical assessment of PEG conjugation; however, these methods are not sufficient for definitive assignment of the modification site.

Given that PEGylation can yield a heterogeneous mixture of conjugate species with varying charge properties, the reaction mixtures were analyzed by cation exchange chromatography (CEX). This complementary analysis was performed to assess the charge-related heterogeneity and to evaluate the distribution of the modified antibody species under different pH conditions.

Table 3: Summarizing the molecular properties of un-PEGylated, mono-PEGylated, and di-PEGylated trastuzumab species. The antibody is composed of heavy chains (HC ~50 kDa) and light chains (LC ~25 kDa). PEG molecules (5 kDa) are represented by a curve (∿). The nomenclature indicates the putative number of PEG molecules attached to the heavy chain HC and LC. Reduction with 2-Mercaptoethanol (2ME) yields separated HC and LC. The predicted molecular weight (Mw) increases by ~5 kDa per PEG. The hydrodynamic molecular weight (Mw-Rh), reflecting size in solution, also increases with PEGylation. Schematic representations illustrate the different PEG attachment patterns.

Structure after reduction with 2ME	Schematic structure	Mw (Rh)	Mw	Structure name
		**150**	**150**	Un-PEGylated trastuzumab
		**167.37**	**155**	Mono-PEGylated Trastuzumab (1HC-1PEG)
		**167.37**	**155**	Mono-PEGylated Trastuzumab (1LC-1PEG)
		**176.42**	**160**	Di-PEGylated Trastuzumab (2HC-2PEG)
		**176.42**	**160**	Di-PEGylated Trastuzumab (2LC-2PEG)
		**176.42**	**160**	Di-PEGylated Trastuzumab (1HC-1PEG, 1LC-1PEG)

As shown in Figures 4 and 5, size-exclusion chromatography (SEC) results at pH 7.5 and pH 3.5 were fully consistent with the reducing SDS-PAGE patterns (Figures 1 and 2), consistent with PEG conjugation under both conditions. On both SEC chromatograms, a minor peak eluting slightly before the main trastuzumab peak was observed. Importantly, the retention-time shift of this peak relative to the native antibody is very small and consistent with conjugation of a low-molecular-weight PEG (e.g., 5 kDa). Such a minimal shift is consistent with the limited

increase in molecular weight and can distinguish the PEGylated species from high molecular weight aggregates, which typically elute substantially earlier. The SDS-PAGE results under reducing conditions further support these observations. At pH 7.5 (Figure 1), distinct bands corresponding to PEGylated light and heavy chains were observed, with a greater degree of modification in the heavy chain. At pH 3.5 (Figure 2), the pattern shifted, showing a more pronounced PEGylation of the light chain, which may suggest a pH-dependent difference in conjugation behavior. Together, these results demonstrate that PEG attachment occurred on both chains, but with differences in extent depending on the reaction conditions. Overall, SEC performed under non-reducing conditions allowed assessment of the intact molecular weight of PEGylated trastuzumab in the reaction mixtures. In both pH settings (Figures 4 and 5), the appearance of higher-molecular-weight species supports the successful formation of PEG-antibody conjugates. According to a Tosoh application note, mono-/di-PEGylated Fab-arms (with PEG 30kDa) can be separated from native species by using a SEC column, exhibiting higher hydrodynamic radius (earlier elution times). The method allows aggregate profiling and PEGylation efficiency to be quantified. In comparison to natives, the PEGylated mAbs eluted earlier, and variations became more pronounced as PEG Mw increased.[36,37] In another study conducted in 2019, PEGylation of GD2-specific scFv fragments with maleimide-activated PEG increased the hydrodynamic radius and reduced renal clearance. SDS-PAGE/Western blotting, paired with reducing SDS-PAGE, is used to distinguish covalent PEGylation from non-covalent aggregation.[38]

The SEC profile revealed both native and PEGylated trastuzumab species, with native trastuzumab representing the major fraction. This result is consistent with the selectivity-oriented reaction conditions employed in this study, which aimed to promote controlled and preferential PEGylation while minimizing extensive non-specific modification. Thus, the relatively low level of PEGylated species should be considered in the context of the study objective, which was to assess the physicochemical changes induced by PEG conjugation using SEC, CEX, and SDS-PAGE, rather than to optimize the process for maximum preparative yield.

The CEX data from Figure 6, where the PEGylation reaction is carried out at pH 7.5 and the separation is carried out at pH 5.6, show that PEG conjugation significantly affects the charge properties of trastuzumab. At pH 7.5, the positively charged lysine residue at the C-terminus of

the heavy chain undergoes efficient modification, resulting in earlier elution than standard trastuzumab. Neutralizing the positive charge with the attached PEG, combined with potential electrostatic shielding, reduces the affinity of PEGylated species for the negatively charged CEX column, resulting in earlier elution. PEGylation at pH 7.5 alters the antibody's overall charge profile by reducing the main peak corresponding to unmodified trastuzumab.

In the CEX analysis of the trastuzumab PEGylation reaction performed at pH 3.5 (Figure 7), the earlier eluting peak was consistent with the presence of modified species with a reduced positive charge, pointing toward a different pattern or site of modification at lower pH values. Consistently, SDS-PAGE data at pH 3.5 suggested a preference for PEGylation at the light chain. The earlier elution observed in CEX at pH 5.6 is likely due to electrostatic shielding by the PEG molecule attached near the C-terminus, where the negatively charged carboxyl group of the C-terminal cysteine may limit the interaction between the positively charged regions of trastuzumab and the CEX resin. Cation-exchange chromatography is facilitated by electrostatic interactions between positively charged domains of trastuzumab and the negatively charged moieties of the resin. PEGylation can reduce these interactions in two ways. steric hindrance from PEG chains prevents the resin’s functional groups from accessing the protein surface. Moreover, the hydrophilic and partially anionic properties of PEG can locally modify the electrostatics surrounding the protein, reducing the net positive surface charge available for binding. As a result, PEGylated antibodies typically display weaker binding and retention compared to their native counterparts.[39-43]

Cation exchange chromatography is frequently employed in monoclonal antibody purification due to its ability to resolve charge variants and aggregates. The performance of CEX is further optimized by combining high-throughput process development (HTPD) with design-of-experiment strategies. According to a 2015 study, this approach can enhance dynamic binding capacity and aggregate clearance, while reducing development timelines and enabling robust scale-up for monoclonal antibodies.[44]

In the case of PEGylated antibodies, PEG conjugation can alter the electrostatic properties of the molecule due to charge shielding, which reduces the accessibility of surface charges and consequently affects binding interactions with cation exchange resins. Previous studies have demonstrated that buffer pH plays a critical role in counteracting this effect, thereby influencing

the resolution of charge variants in PEGylated proteins. These findings underline how CEX, when combined with innovative optimization strategies, can provide both high-resolution separation and process efficiency in therapeutic antibody production.[45]

AEX can, however, be used in some studies to distinguish PEGylated mAb subpopulations based on charge-shielding effects. Anti-Sialoadhesin mAbs (SER-4, 3D6) modified with 5–20 kDa PEG exhibited salt gradient elution shifts on Mono Q columns, with higher PEG loading reducing anion exchange binding affinity.[46]

Several engineered cysteine residues were PEGylated in the Cys1-LMB-2 immunotoxin. As a result of anion exchange chromatography, conjugation success was confirmed while preserving antigen-binding activity, demonstrating the utility of CEX as a method for characterizing site-specific modifications.[46,47] Additionally, the other study in 2022 explored optimizing the cation/anion exchange membrane chromatography process for monoclonal antibody charge variant separation. By tuning static and dynamic parameters, researchers achieved 71% recovery of the target mAb and reduced acidic variants. The optimized process cleared host cell proteins below detection limits, demonstrating scalability for industrial applications.[48]

These examples collectively demonstrate that both CEX and AEX are sensitive to the physicochemical alterations introduced by PEGylation. Accordingly, characterization of the present PEGylated trastuzumab construct should account for changes in apparent molecular size, surface charge distribution, and chromatographic behavior. As highlighted in the review literature, monoclonal antibody purification and characterization should be guided by the physicochemical properties of the product and the intended quality attributes. This is particularly relevant in the context of the present PEGylated trastuzumab construct, where PEG conjugation may influence apparent molecular size, surface characteristics, and chromatographic behavior. Accordingly, the combined use of orthogonal analytical methods provides a more comprehensive assessment of product heterogeneity and integrity.[49]

This study provides essential chemical feasibility data regarding a site-selective PEGylation strategy, which forms the basis for subsequent biological and structural investigations. The primary objective of the present work was to establish and characterize a preferential conjugation

approach, representing a necessary first step in ensuring structural control before undertaking more extensive functional analyses.

Preliminary biological evaluations, including MTT cell-viability assays and flow-cytometric binding analyses, have already been performed to assess the functional behavior of the PEGylated conjugate. To maintain a clear focus on the chemical characterization of the PEGylation process, detailed biological findings will be presented separately in a forthcoming publication. The physicochemical results reported here establish a robust foundation for the more comprehensive biological investigations currently in progress for PEGylated trastuzumab.

Conclusion

This study aimed to optimize the C-terminal PEGylation conditions of trastuzumab using activated PEG, mPEG-NH2, including the molar ratio, the molecular weight, the reaction time, and the pH value. Size exclusion chromatography was used to separate the PEGylated trastuzumab, and SDS-PAGE and cation exchange chromatography were used to analyze its physicochemical properties. As a result of PEGylation, a new conjugate, PEGylated trastuzumab, was formed, which had a higher apparent molecular weight and an increased hydrodynamic radius than the native antibody, resulting from the PEG chain's structural and conformational alteration. In addition, based on this study, the PEGylation reaction with a PEG to protein molar ratio of 11.2 at pH 3.5 is more inclined towards C-terminal light chain, and at pH 7.5, PEGylation occurs in C-terminal of both light chain and heavy chain, with the preference to heavy chain. Furthermore, the CEX chromatogram results suggest that PEGylation at the C-terminus of trastuzumab with this PEG may lead to a slight reduction in the protein’s positive surface charge.

This study presents preliminary experiments aimed at obtaining a C-terminally directed PEGylated form of trastuzumab. The results provide methodological insights that may support future optimization strategies for site-selective antibody PEGylation. Although the precise PEGylation site was not confirmed by mass spectrometry, the combined SDS-PAGE and SEC data, together with the reactivity of accessible carboxyl groups, suggest preferential modification associated with the light chain. Under the experimental conditions employed, detectable PEGylated conjugates were successfully generated.

Overall, this study provides initial physicochemical evidence of PEG conjugation of full mAb and demonstrates that reaction conditions influence the observed PEGylation patterns of trastuzumab.

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