

N-Terminal Site-Specific PEGylation and Analytical-Scale Purification of PEG Lysozyme

Agilent 1260 Infinity Bio-Inert Quaternary LC with Agilent BioHPLC Columns

Application Note

Biotherapeutics & Biosimilars

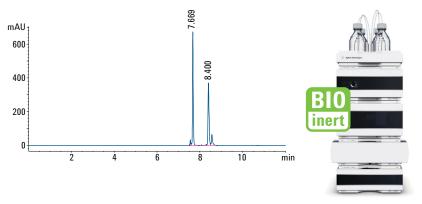
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Abstract

PEGylation is the process of covalent attachment of polyethylene glycol polymer chains to another molecule, normally a drug or therapeutic protein. PEGylation is routinely achieved by incubation of a reactive derivative of PEG with the target macromolecule. In this study, hen egg white lysozyme was used as a model protein for PEGylation using N-terminal specific mPEG propionaldehyde (PEG aldehyde) in presence of cyanoborohydrate. A method for the analytical-scale purification of PEG lysozyme was developed using an Agilent 1260 Infinity Bio-inert LC System and an Agilent Poroshell 120 SB-C18 column. Analytical-scale Fraction Collector with peak-based fraction collection was employed to collect the purified PEG lysozyme. The fractions were then re-analyzed by RP HPLC and Size Exclusion Chromatography (SEC) using an Agilent Bio SEC 3 column to demonstrate the homogeneity of the purified PEG conjugate. The results indicated that lysozyme was PEGylated in a site-specific manner and the purified PEG lysozyme was homogenous.





Introduction

Many therapeutic proteins have been conjugated with a water-soluble synthetic polymer, polyethylene glycol (PEG), to enhance their pharmacological activities. Conjugation of PEG (PEGylation) to various proteins not only increases their half-life in the blood stream, but also significantly reduces their immunogenicity. In particular, the prolonged circulation of PEGylated proteins reduces the necessity of multiple injections to patients. A wide range of therapeutic proteins have been PEGylated:

- Recently, site specific PEGylation of proteins has been attempted using a special class of functionalized PEG derivatives under specific conjugation conditions.
- Purification methods for PEG protein conjugates are currently dominated by ion exchange and size exclusion chromatography.

Other methods in common use for protein separations including hydrophobic interaction chromatography, affinity chromatography, and membrane separations, are rarely used in PEGylated protein purification schemes.

This Application Note demonstrates the suitability of the Agilent 1260 Infinity Bio-inert Quaternary LC System for the analytical-scale purification of PEG-lysozyme using RP HPLC employing peak-based fraction collection. This system enables the chromatographer to perform highly accurate peak-based fraction collection in a completely metal-free environment. Additionally, we also demonstrate the suitability of the Agilent 1260 Infinity Bio-inert Quaternary LC System for the re-analysis of the fractions collected to show the homogeneity of the purified PEG lysozyme using RP HPLC and SEC methods.

Experimental

Instrumentation

A completely bio-inert Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60-mm Max-Light high sensitivity flow cell (G4212B option 33)

 Agilent 1260 Infinity Bio-inert Analytical-scale Fraction Collector (G5664A)

Columns

- Agilent Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 μm column (p/n 683975-902)
- Agilent Bio SEC-3, 300Å,
 7.8 × 300 mm packed with 3-μm particles (p/n 5190-2511)

Software

OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.05

RP HPLC and SEC parmeters

Chromatographic parameters for RP HPLC and SEC using Agilent 1260 Infinity Bio-inert LC System are shown in Table 1.

Table 1. Chromatographic parameters used for RP HPLC and SEC.

Parameters	RP HPLC	SEC
Mobile phase	Mobile phase A (Water + 0.1 % TFA) Mobile phase B (Acetonitrile + 0.09 % TFA)	150 mM sodium phosphate (pH = 7.0) containing 150 mM sodium chloride
Flow rate	1 mL/min	0.8 mL/min
Gradient	At 0 minutes \rightarrow 5 % B At 12 minutes \rightarrow 100 % B	Isocratic
Injection volume	1 μL	2 μL
Thermostat autosampler	5 °C	5 °C
Temperature TCC	50 °C	Ambient
DAD	214 and 280 nm	214 and 280 nm
Peak width	20 Hz	20 Hz
Fraction collection mode	Peak based fraction trigger	Not applicable

Reagents, samples, and materials

Hen egg-white lysozyme (E.C 3.2.1.17) was purchased from Sigma (St. Louis, MO). Methoxypolyethylene glycol-propionaldehyde 20,000 Da from Creative PEG works. All chemicals and solvents used were HPLC grade and highly purified water from Milli Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile was of 'gradient grade' and purchased from Lab-Scan (Bangkok, Thailand).

PEGylation of lysozyme with mPEG-propionaldehyde

Different molar ratios of mPEG-propionaldehyde were used to examine their effects on the degree of lysozyme modification and 1:7 molar ratio of lysozyme: mPEG-propionaldehyde was found to be optimum. A 10 mg/mL solution of lysozyme was prepared in 50 mM sodium phosphate buffer, pH 7.0 and stoichiometric amounts of lysozyme: mPEG-propionaldehyde was added at 1:7 (w/w) ratios. The mixtures were incubated at 4 °C overnight containing 20 mM sodium cyanoborohydrate. Aliquots (10 µL) of sample were taken and chromatographed by RP HPLC to monitor for purity.

Separation and purification of modified lysozyme

The reaction mixture containing lysozyme and modified lysozyme was separated using an Agilent Infinity Bio-inert Quaternary LC System and an Agilent Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm analytical column. A 1-µL solution of mobile phase A was injected as blank, followed by lysozyme-PEG reaction mixture onto the Poroshell 120 SB-C18 analytical column, which was previously equilibrated with Solvent A at a flow rate of 1 mL/min. The separation of lysozyme and PEG lysozyme was achieved using a linear gradient

of 0–100 % buffer B in 12 minutes. Peak based fraction trigger mode was employed to collect the purified fractions. For the re-analysis, 10 μ L of the collected fractions were re-injected using the same chromatographic parameters as described in Table 1.

Size Exclusion Chromatography (SEC)

To determine the homogeneity of the purified fractions, SEC was carried out using an Agilent 1260 Infinity Quaternary Bio-inert LC and an Agilent Bio SEC-3, 300Å, 7.8×300 mm, $3 \mu m$ column. Table 1 shows the chromatographic parameters for Size Exclusion Chromatography.

Results and Discussion

Separation and purification

The elution profile of the lysozyme and PEG-lysozyme reaction mixture on the Poroshell column is shown in Figure 1, demonstrating excellent separation of lysozyme in 12 minutes. Lysozyme was eluted at 7.6 minutes RT (Figure 1A) and the reaction mixture had an additional peak at 8.4 minutes RT (Figure 1B) indicating PEG-lysozyme.

Peak-based fraction collection mode was employed to purify the molecules for further analysis. Figure 2 shows lysozyme and PEG lysozyme collected in F10, F11, and G2 wells of the fraction collection plate respectively.

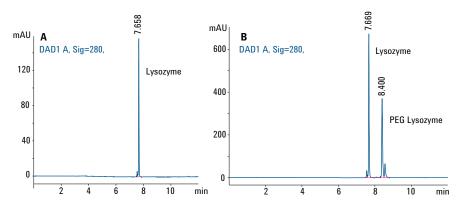


Figure 1. RP-HPLC chromatography of Lysozyme and PEG-lysozyme reaction mixture on an Agilent Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm column. (A) Lysozyme, (B) Lysozyme-PEG reaction mixture.

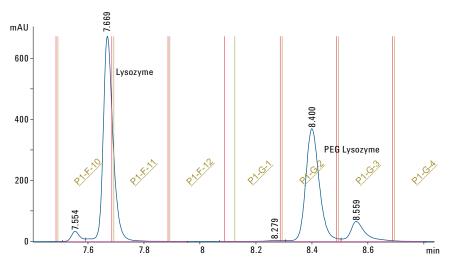


Figure 2. Analytical scale preparative RP HPLC of lysozyme-PEG reaction mixture. Fraction collection windows are marked.

Re-analysis of the fraction was carried out by RP HPLC on the Poroshell analytical column to check for its purity. Figure 3 shows the RP HPLC profile of the purified fractions. Lysozyme eluted at 7.6 minutes, as expected. However, an additional small peak corresponding to the RT of modified lysozyme was also observed. (Figure 3A). PEGylated lysozyme eluted as a homogenous peak at 8.3 minutes (Figure 3B).

Monitoring the degree of PEGylation is important to achieve a balance between retention of bioactivity, stability, and immunogenicity of PEGylated proteins. SEC HPLC technique is routinely used for monitoring PEGylation reactions and purification. Lysozyme, reaction mixture, and the purified fractions obtained from the analytical-scale method were tested using a Bio SEC-3 column. Lysozyme eluted at 13.62 minutes (Figure 4A),

whereas the reaction mixture had three peaks, the peak corresponding to 10.30 minutes represented PEG-lysozyme, and the one at 8.854 minutes represented to some nonspecific PEGylation (Figure 4B). In addition, when the purified fractions were re-analyzed by SEC HPLC, it showed a single symmetrical peak at 10.3 minutes, indicating the purified fractions were homogenous.

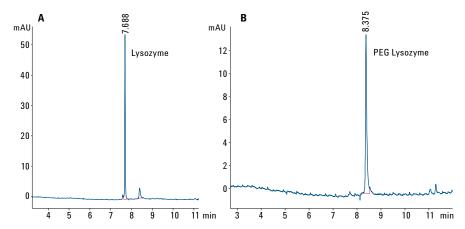


Figure 3. Re-analysis of fractions isolated from analytical separation of lysozyme-PEG reaction mixture. (A) Fraction F10 and F11, (B) Fraction G2.

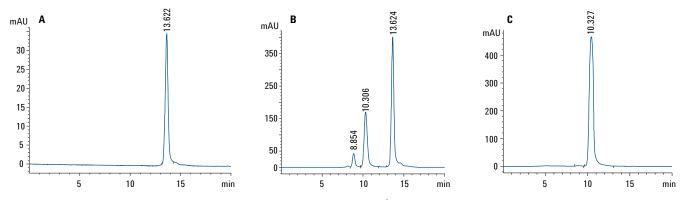


Figure 4. SEC profile of lysozyme PEGylation and purification on an Agilent Bio SEC-3, 300Å, 7.8 × 300 mm, 3 μm column, (A) Native Lysozyme, (B) Reaction mixture, and (C) Purified PEG lysozyme.

Conclusion

PEG-proteins as well as other polymer-conjugates present new challenges with regard to both their preparation and purification. This Application Note demonstrated that PEG-propionaldehyde derivates could be conjugated to the N-terminus of lysozyme in a site-specific manner. A method for the analytical-scale separation and purification of PEG-lysozyme was developed using an Agilent 1260 Infinity Bio-Inert LC System and an Agilent Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm column. Automated peak-based fraction collection facilitated the workflow enormously. A simple and sensitive SEC method for monitoring the protein PEGylation reaction and purification was developed using an Agilent Bio SEC-3, 300Å column. The SEC HPLC analysis was useful in monitoring the PEG reaction and indicated that the purified PEG-lysozyme was homogenous. The bio-inertness and corrosion resistance of the instrument coupled with simple and reproducible method make this solution particularly suitable for the biopharma process development and process monitoring.

References

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- O.B. Kinstler, et al. "Characterization and stability of N-terminally PEGylated rhG-CSF," Pharm. Res., 13:996–1002 (1996).

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