Semi-Preparative Purification and Characterization of Lysozyme Modified with Poly Ethylene Glycol (PEG)

The Agilent 1260 Infinity Bio-Inert Quaternary LC System, Agilent 2100 BioAnalyzer, Agilent 6530 Accurate-Mass Q-TOF LC/MS and Agilent Cary 60 UV-Vis

Application Note

Biotherapeutics & Biosimilars

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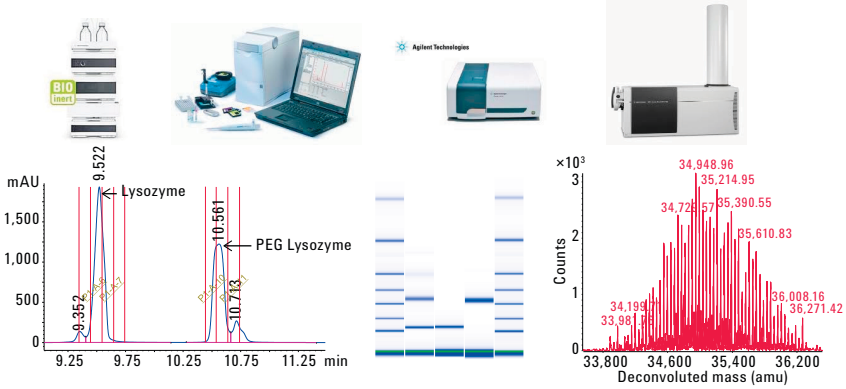
Abstract

This Application Note used hen egg white lysozyme as a model protein for purification and biophysical characterization of PEGylated proteins. PEGylation to the N-terminus of lysozyme in a site-specific manner was achieved using PEG-propionaldehyde derivatives with a molecular weight of Mw 20,000. A semi-preparative RP-HPLC method for the purification of PEGylated lysozyme was developed using an Agilent 1260 Infinity Bio-inert LC System and an Agilent ZORBAX Semi-Preparative 300 SB-C18 column. An analytical-scale fraction collector with peak-based fraction collection was used to collect the purified PEG lysozyme. The PEG lysozyme was characterized for its purity using the Agilent 2100 Bio-analyzer, intact mass was determined using an Agilent 1290 Infinity LC System coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS. The biological activity of lysozyme and its conjugate were determined by a turbidometric method using an Agilent Cary 60 spectrophotometer and *Micrococcus lysodecticus* cells as substrate.



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Introduction

Chromatographic methods are used to both purify and characterize PEGylated proteins. The most common chromatographic method for purifying PEGylated proteins is ion exchange chromatography (IEC). This method has been successfully used to separate several different monoPEGylated proteins. Size exclusion chromatography (SEC) can be used to purify a PEGylated sample as well as to determine the extent of PEGylation. Other methods are used for protein separations, including hydrophobic interaction chromatography, whereas affinity chromatography and membrane separations are rarely used in PEGylated protein purification schemes[1]. Biophysical characterization provides essential information about the structural, thermal, kinetic, and functional stability, which are crucial in the further development steps[2]. While PEG characteristically prolongs the plasma circulating time of a drug, this often comes at the expense of reduced binding affinity for the target receptor or enzyme activity resulting in a trade-off between the degree of PEGylation and its biological activity. This Application Note demonstrates Agilent Solutions for the purification and characterization of PEGylated proteins.

Pegylation of lysozyme with mPEG-propionaldehyde

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. A 50 µL amount of the lysozyme-PEG reaction mixture was taken and purified by semi-preparative RP HPLC.

Reagents, samples, and materials

- Mobile phase A (water + 0.1 % TFA)
- Mobile phase B (acetonitrile + 0.09 % TFA)

Hen egg-white lysozyme (E.C 3.2.1.17) and *Micrococcus lysodeikticus* cells were 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).

Equipment and Instruments

An 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 1290 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 ZORBAX Semi-Preparative 300 SB-C18, 9.4 × 250 mm, 5 µm column (p/n 880975-202)
- Agilent Poroshell 120 SB-C18 column, 4.6 × 150 mm, 2.7 µm (p/n 683975-902)

Semi-preparative RP HPLC chromatographic parameters

Table 1 shows the chromatographic parameters for semi-preparative RP HPLC using an Agilent 1260 Bio-inert LC System.

Table 1. Chromatographic parameters used for semi-preparative RP HPLC.

Parameters	Semi preparative RP HPLC
Flow rate	3 mL/min
Gradient	At 0 minutes to 5 % B At 12 minutes to 100 % B
Injection volume	50 µL
Thermostat autosampler	5 °C
Temperature TCC	50 °C
DAD	214 and 280 nm, Ref 360 ± 60
Peak width	20 Hz
Fraction collection mode	Peak based fraction trigger

Bioanalyzer and P250 assay

Purity check was done on the Agilent 2100 Bioanalyzer with the Agilent Protein 250 kit[3].

Spectrophotometer

Agilent Cary 60 UV-Vis Spectrophotometer (G6860AA)

Software

- OpenLAB CDS ChemStation Edition for LC & LC MS Systems, Rev. C.01.05
- Agilent MassHunter Qualitative Analysis software B.06
- Agilent MassHunter BioConfirm software B.06
- Agilent WinUV version 5.0

Enzyme assay: lytic activity with *M. lysodeikticus*

The enzymatic activity of lysozyme and its conjugate was determined by measuring turbidity changes in *M. lysodeikticus* bacterial cell suspensions (0.5 mg/mL) in 50 mM of pH 7.0 phosphate buffer using an Agilent Cary 60 UV-Vis Spectrophotometer. Absorbance of the suspension was measured at 450 nm, and a decrease in absorbance of 0.001 was defined as one unit of lysozyme activity[4].

LC MS conditions

For this study, the Agilent 1290 Infinity LC System was coupled with the Agilent 6530 Accurate-Mass Q-TOF LC/MS platform. Post column addition of amine solution (1 % TEA in 50:50 acetonitrile/water) was infused with syringe pump continuously with steady flow rate of 0–10 µL/min during the LC/MS runs using a tee junction.

LC parameters	
Agilent 1290 Infinity LC System	
Column	Agilent Poroshell 120 SB-C18 column, 4.6 × 150 mm, 2.7 µm
Mobile phase A	Water + 0.1 % formic acid
Mobile phase B	Acetonitrile + 0.09 % formic acid
Gradient	At 0 minutes → 5 % B At 12 minutes → 100 % B
Flow rate	0.2 mL/min from an Agilent 1200 Series Binary Pump
Column temperature	40 °C
Stop time	15 minutes
The QTOF MS parameters	
Positive ion mode	ESI (profile)
Drying gas temperature	350 °C
Drying gas flow	6 L/min (nitrogen)
Nebulizer	40 psi
Capillary voltage	3,800 V
Fragmentor voltage	300 V for the PEGylated protein and 250 V for the PEG alone
Skimmer voltage	65 V
Oct RF Vpp	750 V
Acquistion parameters	
MS mode	Data were acquired on 1 GHz, MS only mode, mass range 300–10,000 *m/z*. Finally, the data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis software and Agilent MassHunter BioConfirm software.

Results and Discussion

Preparative and analytical methods for protein purification and analysis require two different instrument setups. Traditionally, they are done on two instruments specifically designed for these purposes. With the 1260 Infinity Bio-inert Quaternary LC system, we have shown that both tasks can be done on a single instrument. Method development was done on an analytical scale HPLC (data not shown) followed by a semi-preparative scale for purification of the fractions.

Separation and purification of modified lysozyme

A 50 µL solution of mobile phase A was injected as blank, followed by a lysozyme-PEG reaction mixture on the semi-preparative column, which was previously equilibrated with buffer A at a flow rate of 3 mL/min. The separation of lysozyme and PEG lysozyme was achieved using a linear gradient of 0–100 % buffer B in 12 minutes. Figure 1 shows the chromatogram for the semi-preparative run. Lysozyme eluted at 9.5 minutes (Peak 1) and PEGylated lysozyme eluted at 10.5 minutes (Peak 2) respectively. Fractionation was done using peak-based fraction collection into to a 96-well microtitre plate. Peak samples were pooled and re-analyzed for purity using an Agilent 2100 Bioanalyzer.

Bioanalyzer analysis

SDS-PAGE electrophoresis technique is routinely used for monitoring PEGylation reactions and purification. The 2100 Bioanalyzer analysis of the eluates (Peak 1 and Peak 2) were carried out on a high sensitivity Protein 250 kit. Inspection of the gel-like image of reaction mixture (Lane 2) and the eluate Peak 2 (Lane 4) clearly shows the absence of a lysozyme band, demonstrating the fraction to be predominantly PEG lysozyme (Figure 2). In order to confirm these findings, LC/MS analysis was carried out.

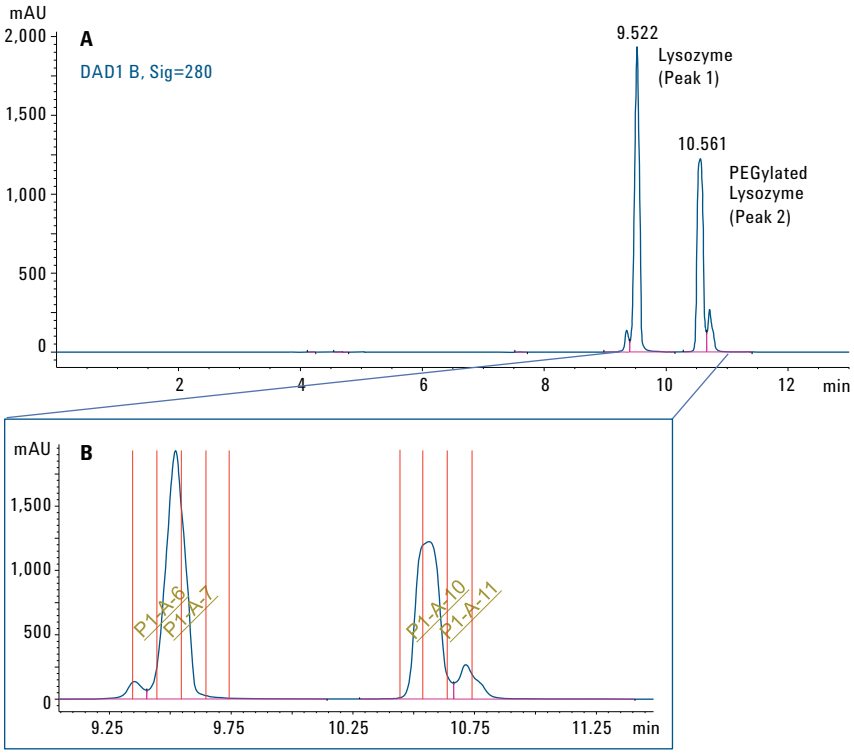

Figure 1. A) Semi-Preparative RP-HPLC of PEG lysozyme reaction mixture on an Agilent ZORBAX Semi-Preparative 300 SB-C18, 9.4 × 250 mm, 5 µm column. B) Zoom-in showing fraction collection.

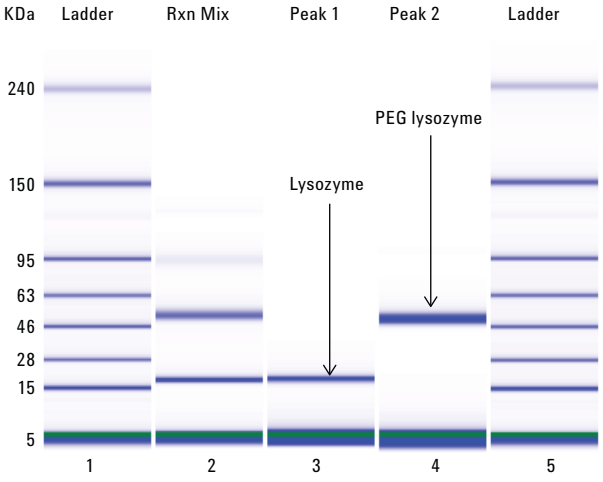

Figure 2. Agilent 2100 Bioanalyzer analysis of lysozyme at various stages of purification.

LC/MS analysis

The intact molecular weight of lysozyme and mPEG-propionaldehyde was measured to be 14,305.41 Da and ~ 20,000 Da respectively (Figure 3). Heterogeneity in PEG masses with different ethylene glycol units is seen in the deconvoluted spectrum. Post-column addition of TEA leads to well distributed charge-state of mPEG propionaldehyde resulting in a charge state groups in a wide *m/z* range. After PEGylation on N-terminus of lysozyme with 20 kDa PEG, a shift in mass of 14 kDa to 34 KDa is observed in the deconvoluted spectrum of the PEG lysozyme (Figure 4).

The LC/MS analysis confirmed that the eluate (Peak 2) from semi-preparative column was monoPEGylated in a site-specific manner, that is, addition of 20 kDa PEG onto the N-terminal lysozyme.

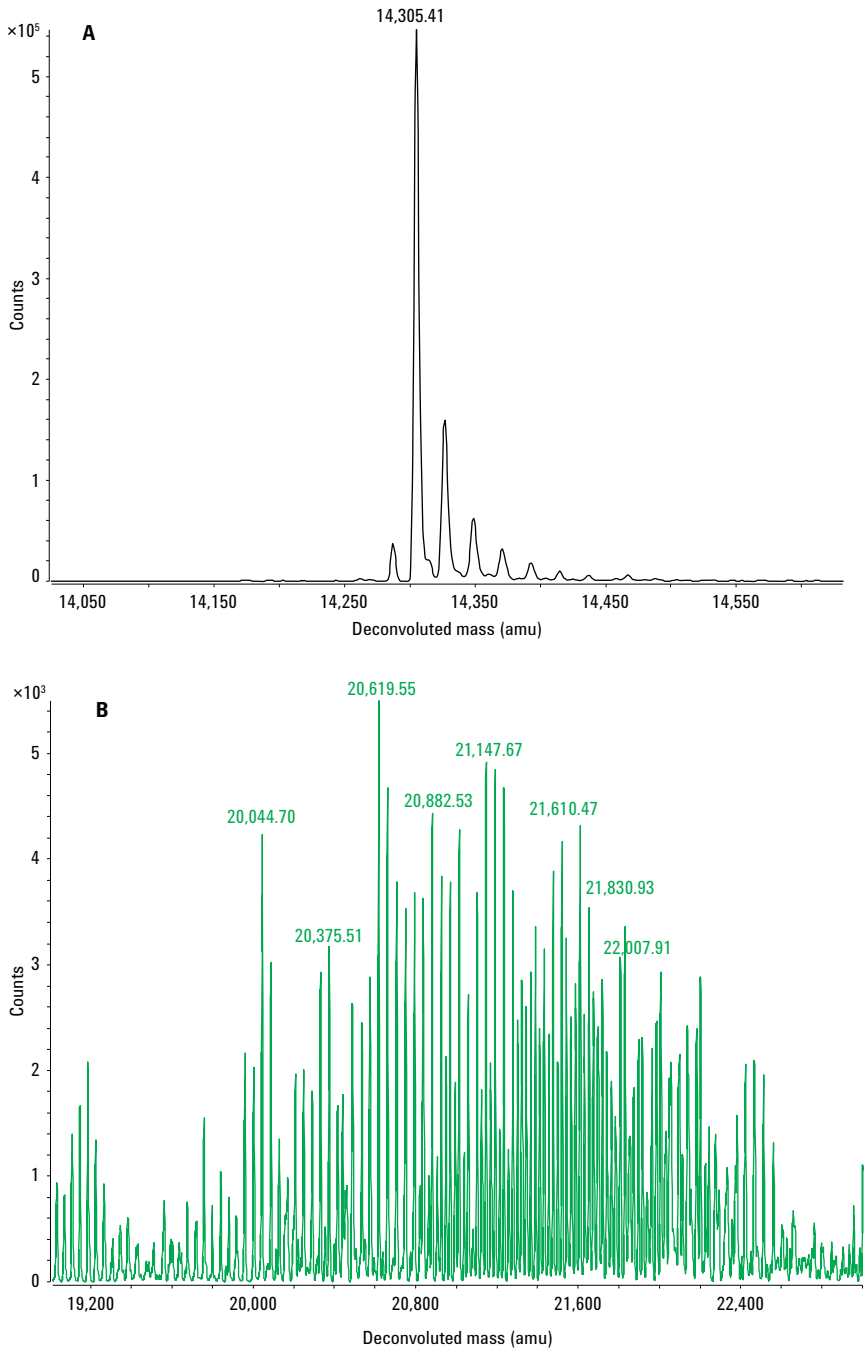

Figure 3. Deconvoluted mass spec profile of (A) Lysozyme and (B) mPEG propionaldehyde (20 KDa).

Enzyme assay

An approximately 50 % loss in activity was observed when PEGylated with a 20 kDa PEG attachment at the N-terminus as compared to unmodified lysozyme (Figure 5). Although the *M. lysodecticus* activity of 34 kDa monoPEG-lysozyme is reduced compared to unconjugated lysozyme, it is more than offset by other advantages of PEGylation such as the enhancements to circulating serum protein concentrations, increased serum protein half-life, and decreased antibody binding.

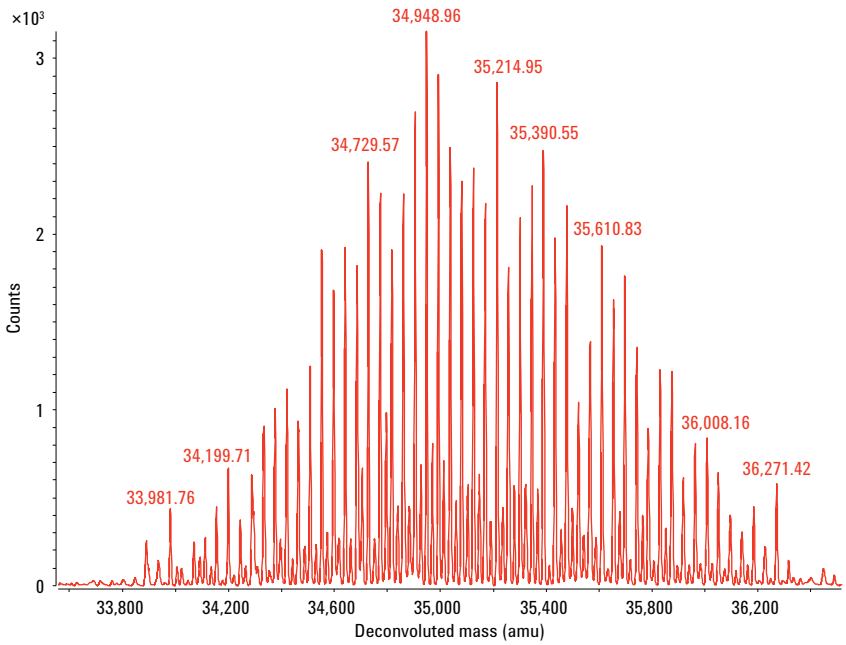

Figure 4. Deconvoluted mass spec profile of purified PEGylated lysozyme.

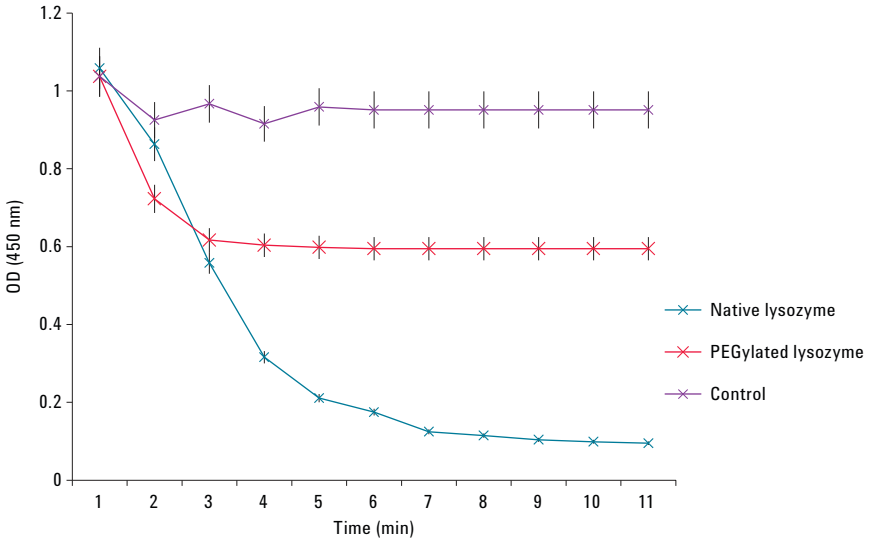

Figure 5. Cell killing assay of lysozyme and PEGylated lysozyme using *Micrococcus lysodecticus* (Assay done in triplicates).

Conclusion

This Application Note showcases several Agilent tools for the purification and characterization of PEGylated proteins. We first used the Agilent 1260 Infinity Bio-inert Quarternary LC System and an Agilent ZORBAX Semi-Preparative 300 SB-C18 column to develop a simple method for the purification of PEG lysozyme. The bio-inertness and corrosion resistance of the instrument coupled with a simple and reproducible method make this solution particularly suitable for biopharmaceutical process development and process monitoring. Secondly, the Agilent 2100 Bioanalyzer with Agilent Protein 250 assay provided high resolution analysis of PEGylated protein, allowing efficient optimization of reaction conditions as well as fast monitoring of protein purification. An LC/MS method with the post-column addition of amine was developed to analyze PEG and PEGylated proteins. This method is useful for studying the PEGylation process during various stages of therapeutic drug development. Finally, we also demonstrated the capability of an Agilent Cary 60 UV-Vis spectrophotometer for accurate and reproducible measurements of enzyme activity.

References

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