Evaluating Separations of PEGylated Proteins using Gel Filtration Chromatography

Vita Knudson, Tividar Farcas, and Michael McGinley
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

The use of polyethylene glycols (PEG) is widespread in the pharmaceutical industry as a method to improve the pharmacokinetic qualities of protein and peptide therapeutics. However, such modifications also increase the heterogeneity of protein structures and generate difficulties in purifying such modified species away from the native protein. As PEGylation exhibits the majority of its effect on the size of protein and peptides, gel filtration chromatography is an excellent method for separating proteins by their degree of PEGylation. Efforts were undertaken to evaluate using gel filtration as a method for purifying PEGylated proteins from their unmodified precursors. Several different proteins and PEGylation chemistries were evaluated on BioSep SEC 2000 HPLC columns. Results show that retention of PEGylated species is directly related to the size of the PEG reagent used; proteins modified with larger PEG moieties generally demonstrate greater resolution from unmodified proteins than small modifications. Such results show the utility of GFC using BioSep 2000 as a means of characterizing and purifying PEGylated proteins.

Introduction

A major disadvantage in the use of proteins and especially peptides as therapeutic drugs has been the short half-life that many of these drugs demonstrate in vivo. Small proteins and peptides are rapidly removed from a patient's circulatory system by dialysis thru the kidneys; many studies have found that by adding carbohydrate or other groups the half-life of a protein in serum can be dramatically increased[1]. The common practice in the development of protein and peptide therapeutics is to attach polyethylene glycol (PEG) groups to a protein to increase its serum half-life[2]. This is due in part to the increase in overall molecular size that the PEG group contributes to the protein resulting in a reduced loss of protein thru dialysis in the kidneys. While the addition of such PEG groups to a protein or peptide improves its usefulness as a therapeutic drug, the addition also complicates both the characterization and purification of such PEG / protein conjugates away from the "non-PEGylated" protein species[3].

The purification of PEGylated species is complicated by the polar nature of the PEG modifying group and a limited number of separation methods to purify a PEGylated protein from its unmodified precursor. Ion exchange has limited utility for PEGylated proteins as the net charge change between species is limited; however, reversed phase does provide good separation of PEGylated proteins from their unmodified precursors. Optimization of reversed phase methods have been covered in Phenomenex Technical notes TN-1046 and TN-1034. While reversed phase is a good solution for isolating PEGylated proteins based on degree and site of PEGylation, gel filtration chromatography (GFC) is also a separation solution that has several unique advantages. Gel filtration is performed under physiological conditions; this may be an advantage for cases where protein stability might be a concern. GFC separates PEGylated proteins based on degree of modification, and thus is advantageous where the site of modification is not a concern. In this technical note several different proteins and PEG forms were reacted and purified on HPLC using gel filtration with BioSep™ GFC 2000; representative examples of each purification are shown.

Materials and Methods

Analyses were performed using a HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD detector. Various HPLC columns were used for evaluations including BioSep-SEC-S® 2000 columns 300 x 7.8 mm (Phenomenex, Torrance, CA, USA). Native proteins were purchased from Sigma Chemicals (St. Louis, MO, USA) and PEGylation reagents to modify proteins were obtained from Jenkem Technology (Beijing, China). Solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Proteins PEGylation was performed using two PEG N-hydroxysuccinimide (NHS) esters derivatives including Methoxy PEG Suiccinimidyl Carboxy Methyl Ester, MW 20 kDa (M-SCM-20K) and Methyl-PEO12-NHS Ester; Methoxy PEG Propionaldehyde, MW 20 kDa (M-ALD-20K), and Y-shape PEG Aldehyde, MW 40 kDa (Y-ALD-40K). Native proteins were dissolved in phosphate buffer pH 7.8, M-SCM-20K were dissolved in dry water-miscible DMSO; proteins PEGylation reaction was done with 6 fold molar excess of M-SCM-20K. Another set of native proteins and PEG substances (M-ALD-20K and Y-ALD-40K) were dissolved in phosphate buffer pH 6.5 with 20 mM of sodium cyanoborohydrate; the reaction was done with 8 fold molar excess of M-SCM-20K. The reaction mixture is incubated in an ice bucket for up to two hours (different time-points were taken for some experiments). Reaction mixture is quenched with an equal volume of 50 mM Tris / 1 % TFA (pH~2).

For most of the HPLC runs, 50 mM of phosphate buffer pH 6.8 was used for mobile phase. Flow rate for the HPLC analysis was 1 mL/min, protein separation was monitored at 214 nm, and loading amount of intact protein was 13 µg and PEGylated protein 6-10 µg.

Results and Discussion

Proteins were PEGylated to demonstrate the different behavior that PEGylated proteins have to their unmodified counterparts when separated on chromatographic media. In this technical note the focus is on demonstrating examples of PEGylated protein separations on a gel filtration column (BioSep 2000 in all of the shown examples).

Various PEG derivatives with different PEG chain length, shape, and molecular weight were chosen to monitor heterogeneity and complexity of PEGylated proteins / peptides. Two N-terminal PEGylation reagents were chosen, Methoxy PEG aldehyde, MW 20 kDa (PEG3) and Y-shape 40 kDa PEG aldehyde (PEG4) which undergo reductive amination reactions with primary amines in presence of cyanoborohydride pH 6.5 [to take advantage of the lower pKa of the N-terminal amine (pKa~8) compared to amino acid side chains, such conditions result in selective modification of the N-terminus].[4] While in principle such reagents should only react stoichiometrically with reacted proteins, concurrent studies[5] show that under the conditions tested the proteins were modified on multiple sites. Another amine PEG reagent, 12-mer Methoxy PEG NHS-ester, MW 1 kDa (PEG2) reacts efficiently with primary amino groups (-NH2) at higher 7.8 pH forming amide bonds at each lysine (K) residue as well as the N-terminal amine.

APPLICATIONS

A typical example of a GFC separation on a BioSep 2000 column of a PEGylated protein from its unmodified precursor is shown in **Figure 1**. Note the beta lactoglobulin PEGylated with the 40 kDa PEG elutes much earlier than the unmodified protein and both analytes are easily separated from each other. Several timepoints are shown that give some information regarding the reaction kinetics ; from the results it appears that a small amount of the protein PEGylates quickly (upon addition of the reagent) but that it can take overnight for a majority of the beta lactoglobulin to react. Indeed, it appears that there is still a small amount of the unmodified protein still present in the overnight reacted timepoint. This phenomenon demonstrates why most PEGylation reactions necessitate purification afterwards to remove unreacted species.

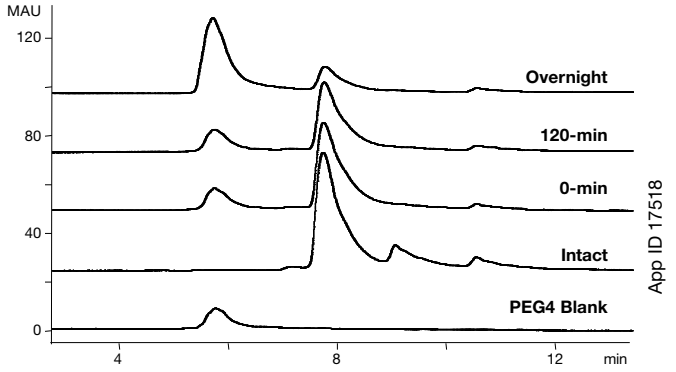

Figure 1.
BioSep-SEC-S 2000 column for separation of PEGylated beta-Lactoglobulin at different time-points with N-Terminus 40 kDa Y-shape Methoxy PEG aldehyde (PEG 4). The GFC separation of final PEGylated product shows excellent separation of the two species. Note that in the overnight sample there still some unreacted proteins; however, GFC using BioSep 2000 easily removes the unreacted species.

While GFC using BioSep 2000 column is very useful when a large PEG moiety is used as was shown in **Figure 1**, the results are a bit less encouraging when a smaller PEG modifier as is shown with several proteins in **Figure 2**. Note that most of the PEGylated proteins run are only slightly resolved from their unmodified counterparts. Because GFC separates based on size differences, when a small PEG is used (in this case a 1 kDa was used {PEG2}) the size difference between the PEGylated and unmodified protein may be insufficient for complete resolution. While each separation is unique, a general rule of a 10 % difference in molecular weight is usually suggested to get any possibility of a feasible separation using GFC. This size difference limitation is not a concern with reversed phase chromatography and strongly indicates its use for applications with small PEG modifiers. In addition, the inability to separate PEGylated species by their site of PEGylation using GFC can be both a limitation if one wishes to isolate a specific species, or can an advantage if one only wants to isolate PEGylated forms based on their degree of modification.

An excellent example of the use of GFC using the BioSep 2000 HPLC column for separation of PEGylated proteins from unmodified proteins is shown in **Figure 3**. In this example Ribonuclease A is reacted with a 20 kDa PEG that is designed to modify only the N-terminus of a protein. The presence of several molecular weight species in the overnight timepoint suggests that some of the PEGylated protein present may contain more than one PEGylation. While such results are not definitive without MS verification, this result is a good example of how gel filtration chromatography can be used to determine the degree of modification that is occurring during a PEGylation reaction.

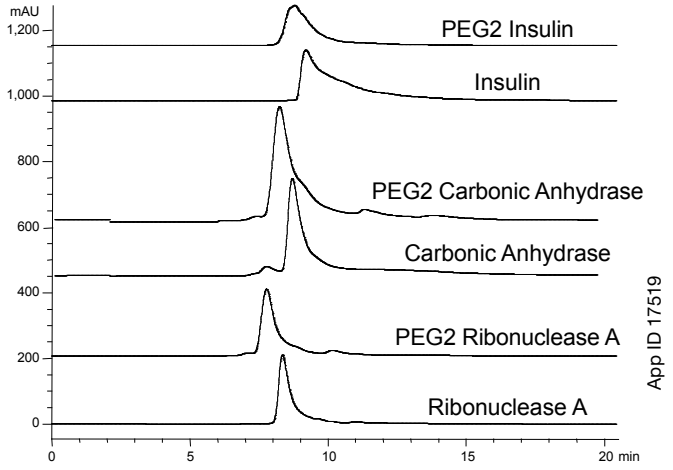

Figure 2.
GFC run of PEGylated and native proteins run on a Phenomenex Biosep 2000 column using a small (1 kDa) PEG. Note the only slight shift due to PEGylation (PEG1). Such minimal size separation makes GFC only useful when the PEGylated species reflects at least a 10 % increase in molecular weight over the unmodified protein. For such circumstances reversed phase chromatography is the better purification technique.

APPLICATIONS

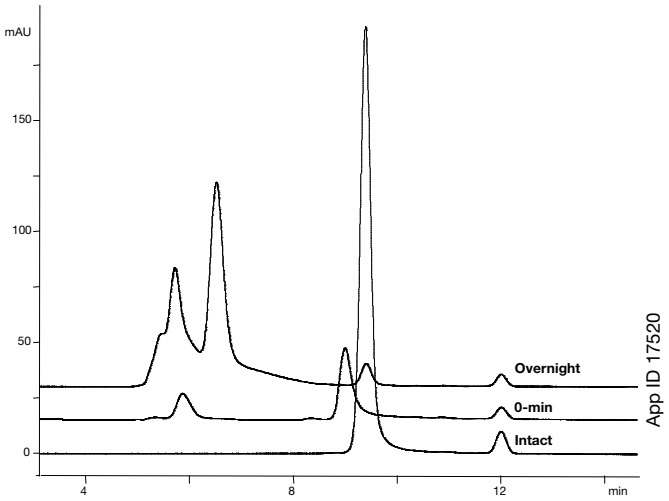

Figure 3.
GFC separation of N-Terminus PEGylated Ribonuclease A with 20 kDa Methoxy PEG aldehyde (PEG 3). Note the excellent separation of the PEGylated species from the unreacted protein using the BioSep 2000 column. Note that several different molecular weight species in the overnight timepoint. Such molecular weight heterogeneity suggest that significant amounts of protein with multiple PEGylations despite the reaction being "tailored" for only N-terminal modification.

Conclusions

These experiments show how gel filtration chromatography is a useful technique for isolating PEGylated from unmodified proteins; such an application is useful as a purification step after a PEGylation reaction. In addition, GFC is also useful for determining the degree of modification that has occurred during a PEGylation reaction, and is performed under native conditions which can be important for proteins with marginal stability. Key to using GFC for PEGylation applications is to take into account the size of the PEG moiety compared to the protein being modified; GFC can only separate species with a minimum 10 % difference in size. If smaller PEG groups are used or if isolating by site of modification is desired, then reversed phase chromatography is the indicated method.

References

1. Sinclair, A.M., Elliott, S., Glyco engineering: the effect of glycosylation on the properties of therapeutic proteins J. Pharm Sci. 2005 Aug, 94(8) 1626-35
2. Harris, J.M., Martin, N.E., Modi, M., Pegylation: a novel process for modifying pharmacokinetics Clin. Pharmacokinet. 40 539-51
3. Monkarsh, S.P. et al Positional Isomers of monopegylated interferon alpha-2a: isolation, characterization, and biological activity Anal. Biochem 1997 247(2) 434-40
4. Fee, C.J., VanAlstine, J.M. Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins Bioconjug. Chem. 2004 15(6) 1304-13
5. Na, D.H., Deluca, P.P., PEGylation of Octreotide: I. Separation of Positional Isomers and Stability Against Acylation by Poly (D,L-lactide-co-glycolide) Pharma. Res. 2005 22(5) 736-42

Order Information

Part No.	Description		Unit
00H-2145-K0	BioSep-SEC-S, 2000	300 x 7.8 mm	ea
00H-2146-K0	BioSep-SEC-S, 3000	300 x 7.8 mm	ea
00H-2147-K0	BioSep-SEC-S, 4000	300 x 7.8 mm	ea

APPLICATIONS

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: +31 (0)30-2418700
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: 4810 6265
dkinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

All other countries:
Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
info@phenomenex.com

www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country, contact Phenomenex USA, International Department at international@phenomenex.com.

TN63170708