SYNTHESIS AND PURIFICATION OF

PEGYLATED PROTEINS

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PROTEINS

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Abstract

PEGylation, referring to the covalent attachment of poly(ethylene glycol) or PEG to protein, has become the most established technology for improving pharmacokinetic behavior of native proteins, especially the prolongation of circulation half-life *in vivo*. This thesis focuses on the synthesis and purification of PEGylated proteins.

The conventional way to synthesize PEGylated proteins is in liquid phase batch reaction, which usually causes the formation of significant amount and high diversity of by-products (i.e. di-, tri-, and/or higher-PEGylated forms of a protein). Many chemical and physical ways have been explored to increase the specificity of mono-PEGylated protein. Chemical ways involve manipulation of operating conditions towards site-specific PEGylation. Understanding reaction kinetics is helpful in optimizing conversion and specificity of mono-PEGylation. In this thesis, the PEGylation reaction kinetics between a model protein and PEG NHS ester under various operating conditions was investigated.

In the physical perspective, the key point is to gain degree of control on reactant addition instead of one-time addition as in liquid phase batch reaction. Herein, two novel reactor systems were developed. One is solid phase PEGylation bioreactor, bringing free protein to react with immobilized PEG on a membrane surface; the other is Hollow-fiber Membrane Reactor (HMR), distributing PEG into the fiber lumen (where protein is flowing) through the pores on the fiber wall. Greatly improved conversion and specificity of mono-PEGylated protein were observed in both systems, compared to liquid phase batch reactor.

An effective and efficient purification technique is very essential because purification step accounts for a significant portion of total cost. In this thesis, the use of hydrophobic interaction chromatography with environment-responsive microporous membranes was examined for the fractionation of different PEGylated proteins. The capability of this technique was demonstrated by obtaining mono-PEGylated protein in a pure form and observing well-resolved chromatographic peaks for different PEGylated proteins.

Acknowledgements

I would like to take this opportunity to thank all those who supported me during my Ph.D. study at McMaster University.

Foremost, my deepest gratitude goes to my supervisor Prof. Raja Ghosh for his continuous support and guidance. His motivation, enthusiasm, and insightful thoughts in research strongly influenced me, and made my life and study enjoyable. He taught me many skills in paper writing, oral presentations and data analysis. I really appreciate the opportunities Prof. Ghosh provided for me to share my research in conferences. Without his guidance and persistent help this dissertation would not have been possible.

I would also like to acknowledge the rest of my supervisory committee members Dr. Ravi Selvaganapathy and Dr. James Dickson for their constructive comments and valuable advice on my research work. Besides, I want to express my appreciation to Dr. Prashant Mhaskar for his insightful comments and suggestions on our collaborative research work.

My appreciation also goes to Brandon Corbett from Dr. Prashant Mhaskar's group who worked with me on our collaborative work for enriching my knowledge of modeling, Brian Macdonald who is working with Brandon Corbett as a summer student and is responsible for carrying out experiments for our collaborative project, and William Wittbold from Wyatt Technology who contributed in the analysis of mono-PEGylated protein sample using dynamic light scattering (DLS) and size exclusion chromatography combined with multi-angle light scattering (SEC-MALS) for one of my project. In addition, I would like to express my great gratitude to all my colleagues from

Bioseparations Engineering group, Dr. Deqiang Yu, Dr. Lu Wang, Dr. Mukesh Mayani, Mah Zheng Kor, Seungmi Yoo, Si Pan, Rahul Sadavarte, Evan Mah, Pedram Madadkar, and Yusra Shahidy, for their generous support and warm encouragement. I want to offer my special thanks to a project student Mochao Zhao for her hard working and great contributions she made for one of my project.

I would like to acknowledge the funding support from Natural Sciences and Engineering Research Council Canada. Without this support, it would not be possible for me to carry out so many fantastic experiments.

I am deeply grateful to the staffs in Chemical Engineering department, Ms. Kathy Goodram, Ms. Lynn Falkiner, and Ms. Cathie Roberts for their administrative assistance, Mr. Paul Gatt, Mr. Dan Wright, Mr. Doug Keller, and Ms. Justyna Derkach for their technical support. Moreover, I would like to thank my dear friends Dan Chen, Meng Li, Jingyun Wang, Lina Liu, and Songtao Yang, and etc. for their kind help in my life.

Last but certainly not the least, I would like to thank my family members my parents, younger brother and my husband, for their constant love and spiritual support throughout my life.

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Tables in chapters 2, 3, 5 and 6 are as they appear in published paper, submitted manuscript, or manuscript with pending submission.

Nomenclature

BSA   Bovine serum albumin

DLS   Dynamic light scattering

EGF   Epidermal growth factor

E1   First eluted peak

E2   Second eluted peak

FT   Flow through

G-CSF   Granulocyte colony stimulating factor

HSA   Human serum albumin

HIC   Hydrophobic interaction chromatography

HIMC   Hydrophobic interaction membrane chromatography

HMR   Hollow-fiber membrane reactor

HPLC   High pressure liquid chromatography

IEC   Ion exchange chromatography

kDa   Kilo Dalton

LCST   Lower critical solution temperature

MALS   Multi-angle light scattering

MDa   Mega Dalton

MWCO   Molecular weight cut-off

NHS   N-hydroxysuccinimide

PEG   Poly(ethylene glycol)

PVDF   Polyvinylidene fluoride

SDS-PAGE   Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC   Size exclusion chromatography

SERC   Size-exclusion reaction chromatography

C_{app} Overall apparent concentration of lysozyme in HMR (kg/m^3)

C_{lys} Feed concentration of lysozyme in HMR (kg/m^3)

C_{PEG} Feed concentration of PEG in HMR (kg/m^3)

D_t Translational diffusion coefficient (m^2/s)

k Boltzmann's constant (m^2 kg s^{-2} K^{-1})

K_{av} Distribution coefficient

M Molecular weight of PEG adduct (kDa)

Q_{lys} Flow rate of lysozyme solution (m^3/s)

Q_{PEG} Flow rate of PEG reagent (m^3/s)

R_{app}	Overall apparent molar ratio of PEG to protein in HMR
R_h	Hydrodynamic radius (nm)
R_{hP}	Hydrodynamic radius of a protein being PEGylated (nm)
R_{hPEG}	Hydrodynamic radius of PEG adduct (nm)
T	Temperature (°C)
V_e	Elution volume of a macromolecule (mL)
V_o	Column void volume (mL)
V_t	Total bed volume (mL)
τ_i	Residence time of lysozyme in HMR based on inlet flow rate (s)
τ_{lm}	Log-mean residence time of lysozyme in HMR (s)
τ_o	Residence time of lysozyme based on the outlet flow rate (s)
η	Solvent viscosity (Pa·s)

Preface

This Ph.D. thesis is organized in a sandwich style based on the following published, submitted and prepared articles:

1. Yu D., Shang X., Ghosh R. 2010. Fractionation of different PEGylated forms of a protein by chromatography using environment-responsive membranes. J. Chromatgr. A 1217:5595-5601. This article was made into Chapter 2.

2. Shang X., Wittbold W., Ghosh R. 2013. Purification and analysis of mono-PEGylated human serum albumin by hydrophobic interaction membrane chromatography. In press in J. Sep. Sci. Manuscript # JSSC. 20130511. R2. This article was made into Chapter 3.

3. Investigation of effects of pH and molar ratio of PEG: protein on PEGylation kinetics and conversion and specificity of mono-PEGylation using PEG NHS ester. Pending submission to either American Institute of Chemical Engineers Journal or Chemical Engineering Science Journal. This article was made into Chapter 4.

4. Shang X., Yu D., Ghosh R. 2011. Integrated solid-phase synthesis and purification of PEGylated protein. Biomacromolecules 12:2772-2779. This article was made into Chapter 5.

5. Shang X., Ghosh R. 2013. Membrane reactor for continuous and selective protein mono-PEGylation. Accepted by J. Membr. Sci. on 22 Sep 2013. Manuscript # JMS-13-947R1. This article was made into Chapter 6.

The article made into Chapter 2 was prepared by Dr. Raja Ghosh. All the other articles were prepared by Xiaojiao Shang. Dr. Ghosh provided guidance in research direction and

project planning, and also helped in manuscript preparation. Dr. Deqiang Yu conducted partial experiments for the project described in Chapter 2. He was also involved in the start-up stage of the project covered in Chapter 5. All experiments for Chapter 5 were conducted by Xiaojiao Shang. Mr. William Wittbold analyzed mono-PEGylated HSA using DLS and SEC-MALS at Wyatt Technology for the project in Chapter 3. Ms. Mochao Zhao worked in Bioseparations Engineering lab as a project student and helped run experiments under the supervision of Xiaojiao Shang for the project included in Chapter 4. The work reported in this dissertation was undertaken from September 2009 to June 2013.

Chapter 1

Introduction and literature review

1.1 Overview of PEGylation

The huge potential of regulatory polypeptides used for treatment of various diseases has been revealed (Walsh, 2004). However, the amount of such polypeptides produced and recovered by direct extraction from native biological sources was far from sufficient to meet clinical demand (Walsh, 2004; Ryu *et al.*, 2012). Thanks to the development of genetic engineering and hybridoma technology in the 1970s (Jackson *et al.*, 1972), this hurdle was overcome, and the first product produced by the two technologies -recombinant human insulin was born and approved in the United States in 1982 (Brogden and Heel, 1987;

Johnson, 1983). After then, hundreds of such products had gained marketing approval (Walsh, 2002). These products are named as first-generation protein biopharmaceuticals, displaying an identical amino acid sequence to a native human protein and replacing or increasing levels of that protein *in vivo* (Szymkowski, 2005).

Although the first-generation protein biopharmaceuticals have continued to be approved and used for decades (Szymkowski, 2005; Walsh, 2004), they have limitations such as relatively short *in vivo* circulation time, poor solubility, physicochemical and proteolytic instability, and immunogenicity (Jevsevar *et al.*, 2010). In order to get the desired pharmacokinetic properties, the first-generation protein biopharmaceuticals have been engineered during the last decade in different ways, such as amino acid sequence manipulation for reducing immunogenicity and proteolytic cleavage (Carey, 1996; Mateo *et al.*, 2000), genetic fusion to immunoglobulins (Lyczak and Morrison, 1994; Walsh, 2004), use of drug delivery vehicles for protection and slow release (Cohen *et al.*, 1991; Jevsevar *et al.*, 2010), and post-production modification by conjugating to natural or synthetic polymers (Abuchowski *et al.*, 1977; Jevsevar *et al.*, 2010; Walsh, 2004). The resultant products are the so-called second-generation biopharmaceuticals.

Post-production modification undoubtedly brings one of the most important benefits for patients - prolongation of circulation half-life, and thus greatly reduces administration frequency (Jevsevar *et al.*, 2010). This approach enables a chemical group covalently attached to the protein's backbone through various strategies such as glycosylation (Styslinger *et al.*, 2012), acylation (Farazi *et al.*, 2001), and PEGylation (Abuchowski *et al.*, 1977), just to name the most known.

PEGylation, the most successful strategy among the post-production modification techniques, refers to the covalent conjugation of poly(ethylene glycol) (or PEG) to protein. It was first described by Abuchowski *et al.* in the 1970s. They conducted conjugations of PEG to bovine liver catalase (Abuchowski and van Es *et al.*, 1977) and bovine serum albumin (Abuchowski and McCoy *et al.*, 1977). It was observed that the attachment of PEG rendered non-immunogenic and *in vivo* long-circulating proteins that still maintained activities. Since then, many researchers have started working on PEGylation and explored more benefits that PEGylation can bring to a native protein. PEG is a chemically stable, neutral, hydrophilic, non-immunogenic, and flexible polymer (Bailey *et al.*, 1967). Its hydrophilic property makes each monomer unit coordinated with 6 to 7 water molecules (Harris, 1991; Pasut and Veronese, 2012). As illustrated in Figure 1, PEGylation creates a hydrophilic shell composed of PEG and its abundant coordinated water for a protein molecule, resulting in an increased hydrodynamic size, and thus prolongs its *in vivo* half-life (Abuchowski *et al.*, 1977; An *et al.*, 2007). The administration of PEGylated protein drugs became less frequent due to the increased *in vivo* circulating time, and thus patient convenience was greatly enhanced (Fung *et al.*, 1997). The non-immunogenic PEG shields the antigenic sites on a protein, resulting in a decrease in immunogenicity (Abuchowski *et al.*, 1977; An *et al.*, 2007, Basu *et al.*, 2006). The hydrophilic PEG shell stabilizes the protein molecules and thus reduces aggregation (Basu *et al.*, 2006; Hinds *et al.*, 2000).
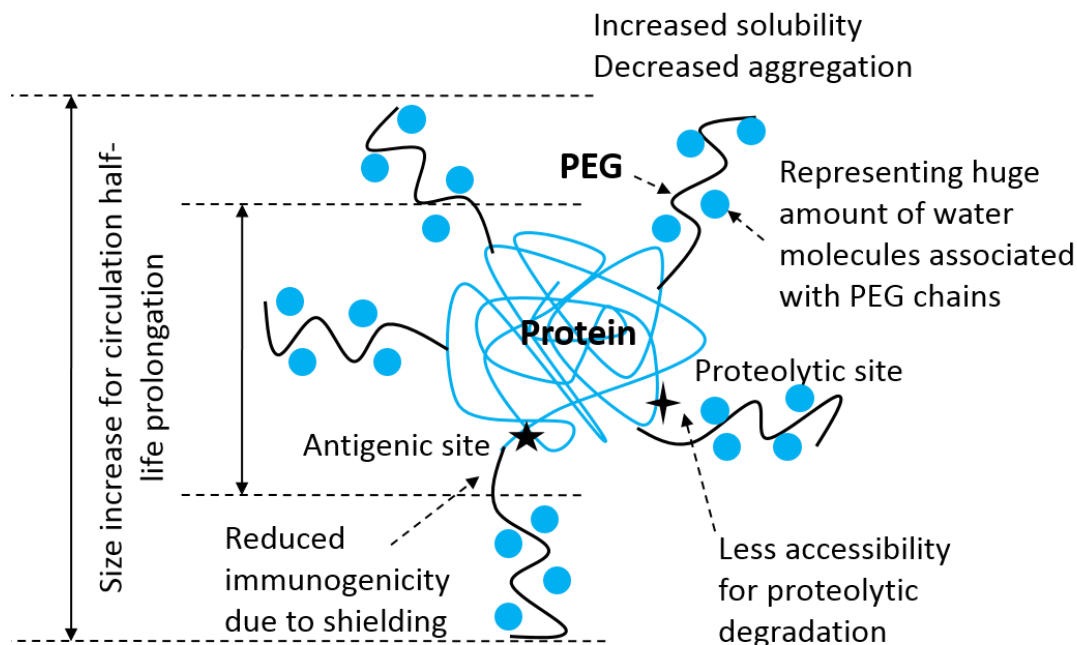
Figure 1. Cartoon figure representing a PEG-protein conjugate and showing the main advantages of PEGylation including increased circulation half-life, reduced immunogenicity, and reduced proteolytic degradation.

There have been eleven FDA-approved PEGylated products on the market until now since the first appearance of Adagen® (PEG-adenosine Deaminase, Enzon Pharmaceuticals) for the treatment of severe combined immunodeficiency disease (SCID) in 1990 (Levy *et al.*, 1988). Four blockbuster drugs out of the eleven include: 1) PEG-INTRON® (PEG-interferon-α2b, Schering-Plough, approved 2001) (Alconcel *et al.*, 2011) for the treatment of chronic hepatitis C; 2) PEGASYS® (PEG-interferon-α2a, Hoffmann-La Roche, approved 2002) (Alconcel *et al.*, 2011) for the treatment of chronic hepatitis C; 3) Neulasta® (PEG-G-CSF, Amgen, approved 2002) (Alconcel *et al.*, 2011) for the management of febrile neutropenia; 4) Mircera® (PEG-erytropoietin, Hoffmann-La Roche, approved 2007) (Alconcel *et al.*, 2011) for the treatment of renal anemia associated with chronic kidney disease. Other PEGylated products include: 5) Oncaspar® (PEG-asparaginase, Enzon,

approved 1994) (Alconcel *et al.*, 2011) for the treatment of acute lymphoblastic leukemia; 6) Somavert[®] (PEG-human growth hormone mutein antagonist, Pfizer, approved 2003) (Alconcel *et al.*, 2011) for the treatment of acromegaly; 7) Cimzia[®] (PEG-anti-TNF Fab', UCB Pharma) (Alconcel *et al.*, 2011) for the treatment of Crohn's disease (approved 2008) and rheumatoid arthritis (approved 2009); 8) Krystexxa[®] (PEG-uricase, Savient, approved 2010) (Alconcel *et al.*, 2011) for the treatment of chronic gout; 9) Omontys[®] (PEGinesatide, Affymax and Takeda, approved 2012) (Bennett *et al.*, 2012), and 10) one more non-protein PEGylated product - Macugen[TM] (PEG-anti-VEGF aptamer, OSI Pharmaceuticals, approved 2004) (Ng *et al.*, 2006) for the treatment of ocular vascular disease.

The pharmacokinetic properties of the above PEGylated products are significantly improved by PEGylation especially in terms of *in vivo* half-life, when compared to the corresponding native forms. For example, Neulasta[®] (PEG-G-CSF) has a greatly increased circulation time (42 h) relative to Neupogen[®] (3.5-3.8 h) which is the unmodified G-CSF (Alconcel *et al.*, 2011). This enables once-per-chemotherapy-cycle administration of Neulasta[®] which is as effective as daily administration of Neupogen[®] up to two weeks per chemotherapy cycle (Jevsevar *et al.*, 2010). Unfortunately, there is often bioactivity loss accompanied with protein PEGylation (Veronese, 2001). For example, PEGASYS[®] (PEG-interferon-α2a) retains only 7% of the antiviral activity of the unmodified Interferon-α2a (Bailon *et al.*, 2001). However, it still performs better *in vivo* than its native form due to the compensation by increased circulation time and stability (Bailon *et al.*, 2001).

In recent years, PEGylation of protein has become a well-established technology for the improvement of efficacy of protein biopharmaceuticals. This is thanks to the efforts made

to the development of PEGylation chemistries from random towards site-specific approaches, physical ways of executing PEGylation reactions, and downstream purification of PEGylated products. The aspects mentioned above will be addressed in the following paragraphs.

1.2 PEGylation chemistries

As mentioned before, PEG is a chemically inert polymer, so it has to be modified with functional groups to gain reactivity for the reaction with proteins (Fee and Van Alstine, 2006). The commercial PEG reagents are available in different lengths (e.g. <1kDa up to 80 kDa), shapes (e.g. linear, branched and multi-arm) and chemistries (examples of functional groups: dichlorotriazine, tresylate, succinimidyl carbonate, succinimidyl succinate, aldehyde, N-hydroxysuccinimide ester, and maleimide, just to name a few) (Jevsevar *et al.*, 2010; Roberts *et al.*, 2002). The PEG reagents for PEGylation reaction are usually methoxylated at one end and functionalized at the other end (i.e. the reactive end) (Veronese, 2001). The elimination of PEG reagents *in vivo* depends on their molecular sizes (Jevsevar *et al.*, 2010). A PEG chain smaller than 400 Da is usually metabolized by alcohol dehydrogenase; one below 20 kDa is cleared by kidney; and an even larger PEG is eliminated through the immune system (Jevsevar *et al.*, 2010). PEG is typically polydisperse, but has a relatively narrow polydispersity of no more than 1.05 up to 30 kDa products and 1.1 for higher molecular weight forms when compared with other polymers (Jevsevar *et al.*, 2010; Roberts *et al.*, 2002). Many companies are producing PEG reagents, such as Creative PEG Works (USA), JenKem (China), NOF Corporation (Japan), Polymer

Source (Canada), and SunBio (South Korea).

The functional group on a PEG chain is responsible for the covalent conjugation at the target attachment site(s) on a protein. The available sites (e.g. -NH$_2$, -COOH, -SH, and -S-S-) are involved in various PEGylation chemistries (Jevsevar *et al.*, 2010). Random PEGylation is the most frequently used approach targeting the ε-amino group (-εNH$_2$) on lysine residue (Fee and Van Alstine, 2006; Jevsevar *et al*., 2010; Reddy *et al*., 2002; Schlesinger *et al*., 2011). Figure 2 shows two examples of random PEGylation using mPEG succinimidyl carbonate and mPEG succinimidyl succinate. The former produces conjugates through acylation with a carbonate linkage formed. It is susceptible to hydrolysis with a short half-life of 20.4 min at pH 8 and 25°C (Roberts *et al.*, 2002). The latter polymer contains a second ester linkage in its backbone. This linkage becomes highly susceptible to hydrolysis after PEGylation reaction, resulting in loss of PEG moieties (Roberts *et al.*, 2002). Lysine accounts for about 10% of amino acids in a typical protein and is usually located on the protein surface (Fee and Van Alstine, 2006). Due to its abundance and ease of access for PEG, this approach usually results in a complex reaction mixture of PEG-protein conjugates having different numbers and attachment sites of the attached PEG chains (i.e. mono-, di-, tri- and high-PEGylated forms of a protein and their positional isomers) (Wong and Jameson, 1991). Mono-PEGylated form which has only one PEG chain attached on one protein molecule is usually desirable, since one PEG chain conserves the bioactivity the most as it fulfills the required improvement of pharmacokinetic property (Gaertner and Harris, 1996; Harris *et al.*, 2001). More than one PEG chains are very likely to shield the active site of the protein resulting in deactivation.

In addition, from the point of view of product characterization, homogeneity of a product is very important. Since the various PEGylated forms of a protein have very similar physicochemical properties, the downstream purification of the target form would become extremely challenging and thus consume considerable amount of time and money. In the early stages of PEGylation, several FDA-approved PEGylated products were complex mixtures of different PEGylated forms produced by Random PEGylation, such as Adagen® (mPEGdichlorotriazine), Oncaspar® (mPEGsuccinimidyl succinate), PEG-INTRON® (mPEGN-succinimidyl carbonate), PEGASYS® (mPEG N-succinimidyl carbonate), and Somavert® (mPEGsuccinimidyl succinate). The PEG reagents used for the above products are shown in the brackets following the product names.
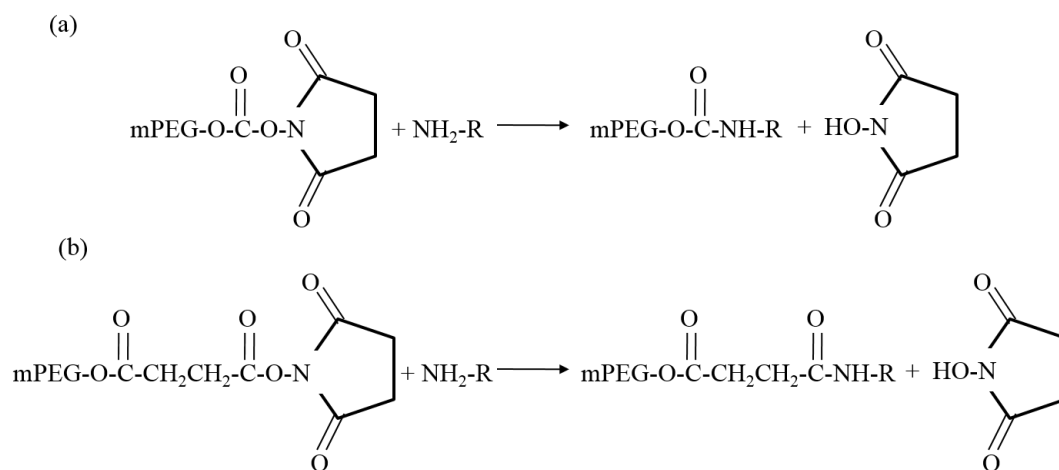
Figure 2. Examples of Random PEGylation using (a) mPEG succinimidyl carbonate and (b) mPEG succinimidyl succinate.

Nowadays, the requirements of drug approval have been raised in terms of homogeneity, i.e. a PEGylated product to be commercialized has to be thoroughly well characterized to satisfy the regulatory agencies (Pasut and Veronese, 2012). Therefore, the current research focuses on the development of site-specific chemistries to improve the specificity of

PEGylation, such as N-terminal PEGylation, cysteine-specific PEGylation, bridging PEGylation, and enzymatic PEGylation (Pasut and Veronese, 2012).

N-terminal PEGylation (Jevsevar *et al.*, 2010; Kinstler, 1998; Veronese and Mero, 2008; Veronese and Pasut, 2005) is the most common chemistry amongst the above, targeting the N-terminal α-amino group of a protein by taking advantages of lower pKa of the α-amino group (pKa 7.6-8) than the ε-amino groups (pKa 9.3-9.5) (Wong and Jameson, 1991). At acidic pH condition, lysine is protonated and consequently not reactive to PEG (Pasut and Veronese, 2012). The free α-amino group, in equilibrium with the protonated form, will be available for PEG conjugation to produce mono-PEGylated form of a protein (Pasut and Veronese, 2012). Figure 3 shows an example of this approach using PEG-propionaldehyde. The aldehyde group is coupled to primary amines to produce a Schiff base, which is then reduced to form a stable secondary amine linkage (Roberts *et al.*, 2002). This approach has been used to synthesize Neulasta[®] (mPEG aldehyde) (Kinstler *et al.*, 1996), PEGylated staphylokinase (Wang *et al.*, 2011), and PEGylated EGF (Lee *et al.*, 2003).

$$
\underset{\text{mPEG-OCH}_2\text{CH}_2\text{CH}}{\overset{\text{O}}{\overset{\|}{}}} + \text{H}_2\text{N-R} \underset{\text{acidic pH}}{\overset{\text{Condensation}}{\longleftrightarrow}} \underset{\text{mPEG-OCH}_2\text{CH}_2\text{CH}}{\overset{\text{N-R}}{\overset{\|}{}}} + \text{H}_2\text{O}
$$

$$
\underset{\text{mPEG-OCH}_2\text{CH}_2\text{CH}}{\overset{\text{N-R}}{\overset{\|}{}}} \underset{\text{acidic pH}}{\overset{\text{Reduction}}{\longleftrightarrow}} \text{mPEG-OCH}_2\text{CH}_2\text{CH}_2\text{NH-R}
$$
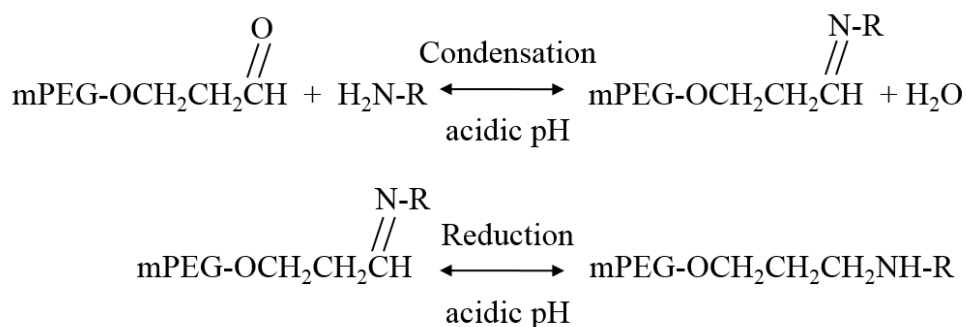
Figure 3. An example of N-terminal PEGylation reaction using PEG-propionaldehyde.

Another well-known highly specific approach - cysteine-specific PEGylation (Jevsevar *et al.*, 2010; Veronese and Mero, 2008; Veronese and Pasut, 2005) involves PEG

conjugation at thiol groups of cysteines which are seldom present in proteins (Veronese and

Pasut, 2005). This attachment site is often hidden inside the protein structure due to its high

hydrophobicity, making the access to PEG difficult (Veronese and Pasut, 2005). Fortunately,

genetic engineering enabled to introduce a cysteine residue into a protein by replacing a

non-essential amino acid (Veronese and Mero, 2008). Figure 4 shows an example of

cysteine-specific PEGylation using PEG-maleimide. This highly reactive PEG reagent can

react with thiol groups even under acidic conditions (pH 6-7) to form a stable thioether

linkage, but it is susceptible to hydrolysis and undergoes ring opening or addition of water

across the double bond (Roberts *et al.*, 2002). Cimzia$^®$ (mPEG maleimide) (Jevsevar *et al.*,

2010; Veronese and Mero, 2008) was synthesized using this approach. Other examples

include PEGylated G-CSF (Veronese *et al.*, 2007), PEGylated IFN-α2a (Rosendahl *et al.*,

2005) and PEGylated Bone morphogenetic protein-2 (Hu *et al.*, 2010).
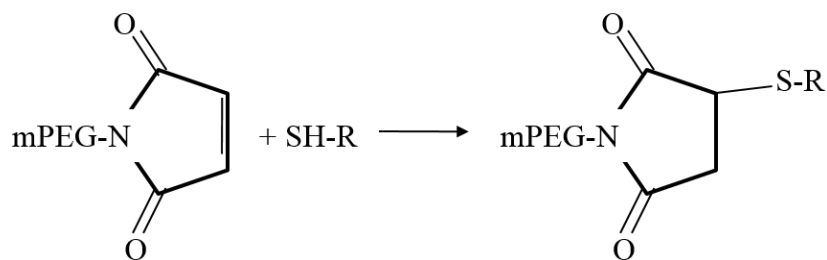
Figure 4. An example of cysteine-specific PEGylation using PEG maleimide.

In bridging PEGylation, the protein disulphide bridges are reduced to expose thiol groups,

at which the PEG conjugation takes place. This approach has been used to PEGylate

antibody fragments (Chapman, 2002). Enzymatic PEGylation utilizes a naturally occurring

enzyme - transglutaminase which recognizes glutamine as substrate to catalyze the

conjugation reaction between amino PEG and the amide group of glutamine (Sato, 2002;

Veronese and Pasut, 2005) as shown in Figure 5. This approach is still in research.

$$H_2N\text{-}(AA)_n\text{-}NH\text{-}CH\text{-}\overset{\overset{\displaystyle O}{\displaystyle \|}}{C}\text{-}NH\text{-}(AA)_n\text{-}COOH \ + \ PEG\text{-}NH_2$$

CH$_2$

CH$_2$

C

O   NH$_2$

Glutamine of protein

Transglutaminase

Ca²⁺

$$H_2N\text{-}(AA)_n\text{-}NH\text{-}CH\text{-}\overset{\overset{\displaystyle O}{\displaystyle \|}}{C}\text{-}NH\text{-}(AA)_n\text{-}COOH \ + NH_3$$

CH$_2$

CH$_2$

C

O   NH-PEG

Glutamine PEGylated protein
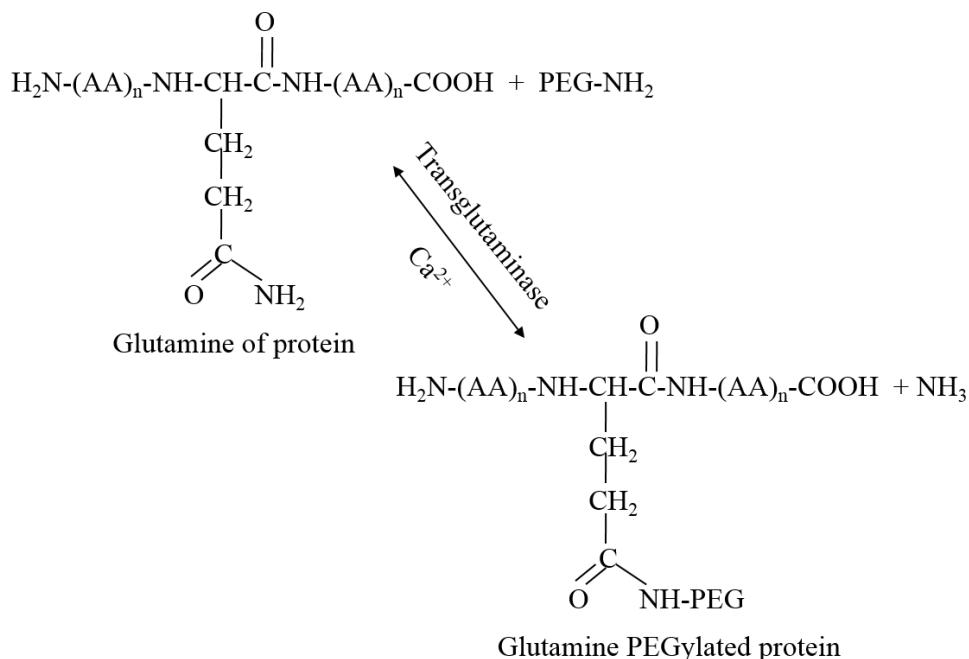
Figure 5. Enzymatic PEGylation using amino PEG.

1.3 PEGylation reactions

Protein PEGylation is usually carried out in liquid-phase batch reaction. Reactants and any other agents if required are added into the reactor together and stirred constantly. After a period of time, the reaction is quenched by adding in an appropriate quenching solution with further stirring or by pH modulation. This approach is still in use for large-scale manufacturing because of the ease of operation. However, due to continuous contact of reactants, products and by-products, a complex mixture of different PEGylated forms of a protein are usually synthesized even a site-specific chemistry (e.g. N-terminal PEGylation) is used (Dou *et al.*, 2007). The formation of by-products (i.e. di-, tri- and high-PEGylated

forms) results in an inefficient reaction process and requires costly purification procedures. In addition, the disposal of by-products which are actually impurities may cause environmental problems. Therefore, to increase the specificity of a PEGylation reaction is very essential. Specificity defines how specific the product is in a PEGylation mixture.

It has been suggested that the way of bringing PEG into contact with protein is a key point for specificity improvement (Fee and Van Alstine, 2006). Several attempts from physical point of view have been made at controlling the addition of PEG into the reactor and/or separate product from reactants as they are formed. One attempt is size-exclusion reaction chromatography (SERC) demonstrated by Fee *et al.* (Fee, 2003; Fee and Van Alstine, 2006). The working mechanism was established based on the fact that different species have different moving speeds in a SEC column depending on their molecular size. A pulse of activated PEG was injected into the column, and followed by a pulse of faster-moving protein. The two reactants were brought into contact when the latter caught up the former, forming a moving reaction zone in which PEGylation reaction occurs. Since the attached PEG appendage increases the molecular size of a native protein significantly due to the water molecules associated with it, the mono-PEGylated protein once synthesized moved out of the reaction zone faster than the two reactants such that the further conjugation with more PEG chains was avoided and in consequence the specificity was increased. Even though SERC integrates two processes - reaction and separation, the production capacity is limited due to the pulse-wise operation.

The other big category of physical manipulation for protein PEGylation is solid-phase PEGylation (Huang *et al.*, 2012; Huang *et al.*, 2012; Lee *et al.*, 2007; Monkarsh *et al.*, 1997;

Ottow *et al.*, 2011; Suo *et al.*, 2009). The two reactants were usually brought into contact by flowing PEG through an ion exchange packed bed column in which protein was only present in immobilized form on media surface (Huang *et al.*, 2012; Huang *et al.*, 2012; Lee *et al.*, 2007; Monkarsh *et al.*, 1997; Suo *et al.*, 2009). However, the operating pH has to be suitable for both immobilization and protein PEGylation. To release the pH-sensitive ion exchange media, we developed a new solid-phase bioreactor with PEG immobilized on hydrophobic membrane surface in the presence of lyotropic salt, aiming at pegylating any protein coming for contact. The detail about this system will be discussed in Chapter 5. Recently, protein was immobilized on magnetic adsorbent surface while it was flowing with the adsorbents in microfluidics channels, and subsequent PEGylation was accomplished by bringing in another PEG stream (Ottow *et al.*, 2011). The responsible mechanisms for the immobilization in the above approaches are electrostatic interaction, hydrophobic interaction, and affinity interaction, respectively. The product was finally recovered by controlled elution by lowering the interactions between it and the adsorbent. This strategy is effective in improving manufacturing convenience and product recovery due to the integration of reaction and purification processes, and it is also capable of increasing specificity of PEGylation due to steric hindrance and easy-to-access orientation of the reactant. However, the problem of limited production capacity still exists because of the batch-wise nature as SERC. Batch production consumes more time and more labor power between batches for cleaning and preparation for the next when compared to continuous mode. Products may differ in quality between batches due to variations in operation. Therefore, for matters of quality control, production efficiency and capital cost

management, continuous PEGylation is in great demand for practical and economic reasons, especially for the improvement of production capacity.

1.4 Purification of PEGylated proteins

As mentioned above, for SERC and solid-phase PEGylation strategies, the separation process is integrated with the reaction process on the same device; while liquid-phase batch reaction, resulting in the most complex reaction mixture (i.e. a mixture of unmodified protein, unreacted PEG, mono- and high-PEGylated forms), needs a separate purification device to obtain the product (i.e. mono-PEGylated form) with a reasonable purity. Some purification techniques which are about to be presented here are actually those which are integrated in the solid-phase PEGylation strategies. PEGylated proteins are commonly fractionated based on differences in electrostatic charge, molecular size, and hydrophobicity in the chromatographic methods of ion exchange chromatography (IEC) (Edwards *et al.*, 2003; Fee and Van Alstine, 2006), size-exclusion chromatography (SEC) (Fee and Van Alstine, 2006; Yang *et al.*, 2003), and hydrophobic interaction chromatography (HIC) (Cisneros-Ruiz *et al.*, 2009; Fee and Van Alstine, 2006), respectively, using packed bed columns.

IEC (Edwards *et al.*, 2003; Fee and Van Alstine, 2006), the most commonly used technique, gives the highest resolution among the three techniques for purification of mono-PEGylated protein from the liquid-phase reaction mixture (Jevsevar *et al.*, 2010). PEG is neutral, so the unreacted PEG is obtained in the flow through after a pulse of the reaction mixture is injected into an IEC column, while all other protein-containing species

are bound on the media at a proper operating condition. Due to PEG shielding effect, the charges on a protein is partially shielded by the PEG appendages. The more PEG chains attached, the lower average surface charge of the protein, and the less interaction the conjugate would have with the resin. Therefore, the order of being detached upon elution is in increasing average surface charge, i.e. high-, mono-PEGylated and unmodified forms of a protein (Edwards *et al.*, 2003; Fee and Van Alstine, 2006; Jevsevar *et al.*, 2010). However, IEC requires careful control of pH value for a specific protein. SEC is a widely used technique in history for PEGylated protein separation based on the dramatic increase in protein molecular size due to the attachment of PEG and the huge association of water molecules with PEG. It is effective in small impurities removal (e.g. small MW reagents and unmodified protein), but has limited resolution for various PEGylated forms of a protein (Jevsevar *et al.*, 2010). Low throughput and high costs are also its shortcomings (Jevsevar *et al.*, 2010). In HIC, the species in the reaction mixture are expected to be bound on the media due to hydrophobic interaction. The separation would be based on the hydrophobicity difference of the bound species. The degree of PEGylation would be the factor that makes their hydrophobicity different (Fee and Van Alstine, 2006). The potential of this technique in the application of PEGylated protein separation has been demonstrated by some researchers (Jevsevar *et al.*, 2010), but it is not widely used because of its poor resolution (Jevsevar *et al.*, 2010).

As described above, traditionally packed bed column based chromatographic techniques are very widely used in separation of PEGylated products. However, they have several major limitations in common. First of all, high pressure drop across a packed bed is usually

generated and it is very likely to keep increasing during a process due to bed consolidation and column blinding (Ghosh, 2002). Also, the slow diffusion for the transport of solute molecules to their binding sites within the pores of the media (as shown in Figure 6) results in long process time and large buffer volume (Roper and Lightfoot, 1995). In addition, the conventional polydisperse media causes radial and axial dispersion limitations (Klein, 2000; Zeng and Ruckenstein, 1999). Although newly developed monodisperse, non-porous, rigid media have overcome this problem (Hashimoto, 1991), they are generally expensive and show lower binding capacity because the binding sites are only on the surface. Moreover, the problem of high-pressure drop still remains. Some factors above make it very difficult to scale up a packed bed based chromatographic process.

To overcome the limitations associated with packed bed columns, synthetic membranes are better options as chromatographic media (Ghosh, 2002). In membrane chromatographic processes, the transport of solute molecules are dominated by convection (as shown in Figure 6), thus saving process time and buffer volume. The pressure drop is significantly reduced and the process can be operated at higher flow rates. It is relatively easy to scale up membrane chromatographic devices when compared with packed beds (Ghosh, 2002). Some of the work done for protein separation using membrane chromatography has been cited (Ghosh, 2001).
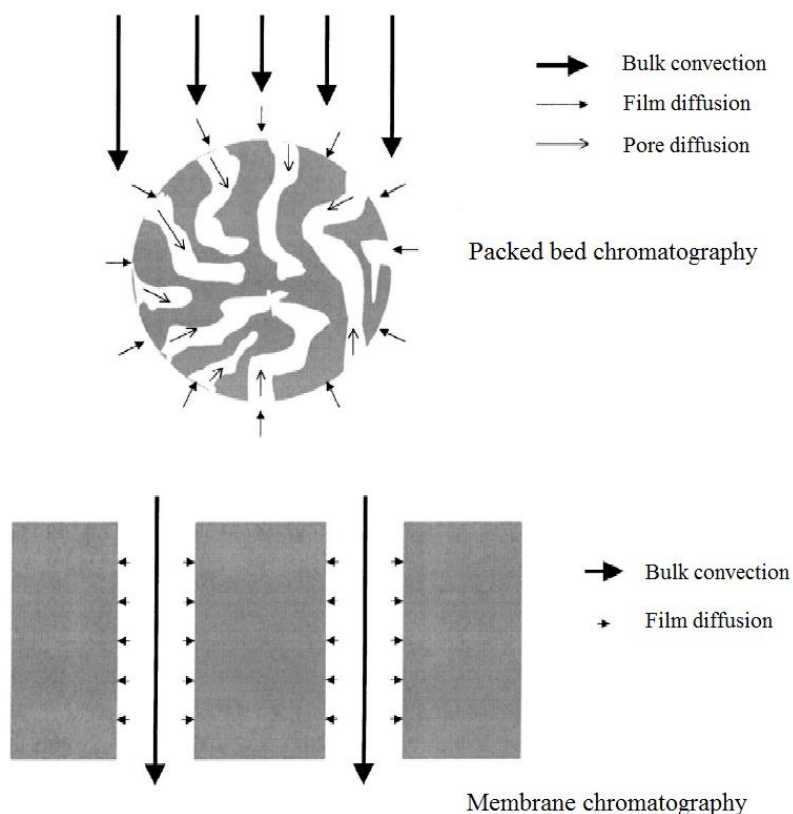
Figure 6. Solute transport in packed bed chromatography and membrane chromatography (Ghosh, 2001)

1.5 Structure of thesis

This thesis consists of seven chapters. Chapters 1 (this chapter) is an introductory chapter giving a comprehensive literature review of PEGylation in the aspects of PEGylation chemistries, PEGylation reaction, and purification of PEGylated proteins. Chapters 2 to 6 cover the accomplished projects during the last four years, spreading in the topics of fractionation of different PEGylated proteins prepared in liquid-phase batch reactor, investigation of PEGylation reaction kinetics under various operating conditions in liquid-phase batch reaction, and development of novel bioreactor systems for improving

conversion and specificity of mono-PEGylation. Conversion generally refers the portion of total protein converted to the product, and specificity defines how specific the product is in the reaction mixture. In the last chapter, all the projects are summarized, the author's contributions to the area of PEGylation are specified, and suggestions on future work are put forward.

Membrane chromatography with hydrophobic interaction has been applied successfully in the area of protein separation (Ghosh, 2001). This technique, termed as hydrophobic interaction membrane chromatography (HIMC), is reported for fractionation of different PEGylated proteins in Chapter 2. Instead of packed bed columns in conventional HIC, a stack of microporous hydrophilized PVDF membranes were used as chromatographic media. This membrane has a reversible change-over between hydrophobic and hydrophilic status depending on if lyotropic salt is present. In the presence of salt, a PEGylation reaction mixture from liquid-phase batch reactor was injected into a membrane module for fractionation of different species present in the mixture based on their hydrophobicity difference. The resolution of separation was investigated in both preparative and analytical scale. The sensitivity of this method was also examined in analytical scale. HIC with conventional packed bed column was also applied to fractionate the reaction mixture for comparison of separation capacity with HIMC.

Chapter 3 shows the application of HIMC for purification of mono-PEGylated human serum albumin (HSA). The model protein - HSA (~67 kDa) represents the medium-sized category, while lysozyme (~14.1 kDa) is the representative of small size proteins. This project was used to verify the capability of this technique for separating the PEGylated

forms of a medium-sized protein.

Undoubtedly, a robust purification technique is very important to get the product in a relatively pure form. Furthermore, understanding a PEGylation reaction is also essential. Chapter 4 introduces systematic analysis of the effects of various operating conditions and process variables on reaction kinetics, conversion and specificity of a PEGylation reaction between lysozyme and PEG-NHS ester. With such information, the PEGylation reaction could be directed towards producing mono-PEGylated protein such that by-products could be suppressed at the first place, making the subsequent purification process easier. PEGylation reactions were carried out in liquid-phase batch reaction under various operating parameters. Two crucial parameters - pH value and molar ratio of PEG: protein were tested. The HIMC technique was used to generate chromatograms for conversion and specificity estimation of different PEGylated forms of lysozyme present in the reaction mixture.

Most likely the effect of chemical manipulation on conversion and specificity of a PEGylation reaction is not straightforward, and constrains in operating conditions are normally seen, so a novel bioreactor system was developed from a physical point of view. Chapter 5 describes a reactor system for solid-phase protein PEGylation integrated with purification of PEGylated proteins by HIMC. PEG was first immobilized on the membrane surface in the presence of lyotropic salt due to hydrophobic interaction. Protein was then flowed into the membrane module to react with the immobilized PEG. Product was recovered by controlled elution. Due to steric hindrance, the specificity of mono-PEGylated protein was expected to be improved. Liquid-phase batch reactions were also carried out

for comparison.

Due to the pulse-wise nature of the above solid-phase bioreactor system, the conversion of mono-PEGylation would be limited. A continuous reactor system was developed for enhancing the conversion and specificity of PEGylation reaction. Chapter 6 introduces the hollow-fiber membrane reactor (HMR) system, in which PEG was added in a distributive way into the fiber lumen where the protein was flowing. PEGylation reaction was expected to take place when the two reactants met each other within the lumen. The reactions in HMR were executed under various operating parameters (e.g. flow rates of both reactants, residence time of protein, and molar ratio of PEG: protein in feed solutions). The compositions of the samples collected at the reactor outlet were examined. Liquid-phase batch reactions were also carried out for comparison.

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Chapter 2

Fractionation and analysis of different PEGylated forms

of a protein by hydrophobic interaction membrane

chromatography

This chapter is an edited version of the paper published in Journal of Chromatography A, 1217: 5595-5601 (2010) by Deqiang Yu, Xiaojiao Shang, and Raja Ghosh. Dr. Deqiang Yu started this project and carried out the experiments corresponding to Figures 2-4 and Table 1. Xiaojiao Shang conducted the experiments corresponding to Figures 5-7, 9 and 10. We together carried out the experiments for Figures 11 and 12.

2.1 Abstract

PEGylation of therapeutic proteins can enhance their efficacy as biopharmaceuticals through increased stability and hydrophilicity, and decreased immunogenicity. A site-specific (e.g. N-terminus) mono-PEGylated protein is frequently desirable as a product. However, other PEGylated forms such as di- and tri-PEGylated proteins are also produced as byproducts. In this paper we discuss the fractionation of PEGylated proteins by hydrophobic interaction membrane chromatography. With the model protein examined in this study (i.e. lysozyme), the apparent hydrophobicity in the presence of a lyotropic salt increased with degree of PEGylation. Based on this, unmodified lysozyme and its mono- and di- PEGylated forms could each be resolved into separate peaks. The use of membrane chromatography ensured that the fractionation was fast and hence suitable for analytical applications.

Keywords PEGylated protein; lysozyme; fractionation; hydrophobic interaction; membrane chromatography

2.2 Introduction

As discussed in Chapter 1, PEGylation is one of the post-production modification techniques to improve the pharmacokinetic properties of protein drugs. This type of chemical modification of proteins serves several important purposes: increase in biological half-life, protection from enzymatic degradation, increase in hydrophilicity, and reduction in immunogenicity.[1] N-terminus PEGylation which involves the attachment of PEG to the amino terminal end of a protein is commonly used for producing mono-PEGylated products.[2] However, even at highly optimized reaction conditions, byproducts such as di- and tri- PEGylated proteins are also produced.[3] Due to the formation of such heterogeneous mixtures, high-resolution fractionation techniques are required for both analysis and purification of PEGylated proteins.

Size exclusion chromatography[4] (or SEC) and ion exchange chromatography[5] (or IEC) are commonly used chromatographic methods for fractionation of PEGylated proteins. Some researchers have shown that hydrophobic interaction chromatography (or HIC) could potentially be used for purification of PEGylated proteins.[1, 6-9] Factors that have been suggested as being responsible for fractionation include a) the tendency of PEG to undergo phase change at high salt concentrations[7], and b) the shielding of hydrophilic portions of proteins by PEG, thus leaving the hydrophobic regions on the molecule more exposed.[1] More recently, Cisneros-Ruiz et al.[10] have discussed the separation of PEGylated and unmodified ribonuclease A using amphiphilic Sepharose HIC media. While the PEGylated proteins could collectively be separated from the unmodified protein, the mono- and di-PEGylated forms themselves could not be resolved into separate peaks.

Earlier workers have shown that in the presence of a lyotropic salt, a PEGylated protein could often be more hydrophobic than its unmodified form.[6-10] PEG is a lower critical solution temperature (or LCST) polymer i.e., it can undergo phase transition accompanied by a collapse of the polymer chain, if temperature or salt concentration is increased. It is normally very hydrophilic but becomes relatively hydrophobic upon phase change. If the PEG component of a PEGylated protein undergoes phase transition, its apparent hydrophobicity could therefore be higher than its unmodified form (unless of course, the protein itself is extremely hydrophobic). The working hypothesis of the current study is shown in Figure 1. In the absence of a lyotropic salt, i.e. when PEG is in its hydrophilic state, overall hydrophilicity would increase with the number of PEG molecules attached to a protein. In the presence of salt (at concentrations resulting in PEG phase transition), a collapsed PEG chain would be expected to form a hydrophobic appendage on the surface of the protein to which it is attached. Therefore, the greater the number of PEG chains attached to a protein, the greater would its apparent hydrophobicity be (due to higher surface coverage by collapsed PEG). This hydrophobicity difference could potentially be utilized to resolve the unmodified, mono- and di-PEGylated proteins (and indeed other PEGylated forms, if they exist in the reaction mixture) into separate peaks.

This paper discusses the fractionation of unmodified and the different PEGylated forms of lysozyme by hydrophobic interaction chromatography using microporous environment-responsive membranes. Hydrophobic interaction membrane chromatography (or HIMC) has been shown to be an effective method for carrying out analytical separation of proteins.[11] Whereas conventional HIC media is prepared by grafting hydrophobic ligands

such as phenyl and butyl on a hydrophilic support, membranes used for HIMC consist of hydrophilic polymers coated microporous membranes.[12] In the presence of lyotropic salts, the membrane surface is hydrophobic whereas in their absence the membrane is quite hydrophilic. The resolution of separated peaks using HIMC is therefore expected to be better than in conventional HIC. Membranes chromatography is also particularly suitable for carrying out fast separations. Typical superficial velocities used in conventional column chromatography of PEGylated proteins are in the 50 – 100 cm/h range[13] while superficial velocities as high as 240 cm/h have been used in preparative membrane chromatography.[14] Therefore, even higher velocities could potentially be used in analytical separation techniques involving membranes. In the current study, lysozyme was chosen as a model small biopharmaceutical protein. It was subjected to N-terminus PEGylation and the reaction mixture thus obtained was fractionated by using a stack of microporous hydrophilized PVDF membranes as chromatographic media. The fractionated proteins were analyzed by different analytical techniques such as size exclusion chromatography and gel electrophoresis, and the results obtained are discussed. The fractionation obtained using HIMC is also compared with that obtained using conventional HIC.

2.3 Materials and methods

Lysozyme (L6876), glycine (G8898), Trizma base (T1503), sodium chloride (S7653), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), ammonium sulfate (A4418), sodium cyanoborohydride (156159), barium chloride (202738), iodine (326143), hydrochloric acid (258148), 25% glutaraldehyde solution (G6257) and 70%

perchloric acid (77227) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate trihydrate (SX0255-1) and glacial acetic acid (AX0073-6) were purchased from EMD (Gibbstown, NJ, USA). Potassium iodide (74210-140) was purchased from Anachemia (Montreal, QC, Canada). mPEG-propionaldehyde 5,000 Da (P1PAL-5) was purchased from Sunbio Inc. (Anyang, South Korea). High quality purified water (18.2 MΩ cm) obtained from a Diamond^{TM} NANOpure (Barnstead, Dubuque, IA, USA) water purification unit was used to prepare all the test solutions and buffers. Hydrophilized PVDF membrane (0.22 mm; GVWP) used for membrane chromatography was purchased from Millipore (Billerica, MA, USA).

PEGylation was carried out at room temperature in small flasks with continuous stirring (using magnetic stirrers). The reaction mixture in each case consisted of 1 mg/mL lysozyme, P1PAL-5 and 10 mM sodium cyanoborohydride in 100 mM sodium acetate buffer (pH 5.0) as reaction medium. The P1PAL-5 / lysozyme molar ratio used was 4:1. PEGylation reactions were carried out for 5 h and 20 h and in each case were terminated by adding 1.0 M glycine solution to the flasks such that the final glycine concentration was 10 mM. The reaction mixtures were analyzed by SEC and then used as feed solution in the HIMC based fractionation experiments.

For preparative fractionation, discs having 18 mm diameter were cut out from the PVDF membrane sheet and 30 such discs were stacked within a custom-designed membrane module.[15] For analytical fractionation, a smaller membrane module of similar design housing within it a stack of 4 membrane discs of 8 mm diameter was used. In each case, the module was integrated with an AKTA Prime liquid chromatography system (GE

Healthcare Bio-Sciences, QC, Canada). The effluent from membrane module was continuously monitored for UV absorbance at 280 nm, pH and conductivity; the data was logged into a computer using Prime View software (GE Healthcare Bio-Sciences, QC, Canada).

The effective membrane bed volume used for preparative fractionation was 0.95 mL and these experiments were carried out at 1 mL/min mobile phase flow rate. The eluting buffer used was 20 mM sodium phosphate (pH 7.0) while binding buffer consisted of eluting buffer adjusted to various concentrations of ammonium sulfate, i.e. 0.9, 1.1, 1.3, 1.4, 1.5 and 1.7 M. The feed solutions for these experiments were prepared by mixing in 1:1 ratio, the reaction mixture and 20 mM sodium phosphate buffer (pH 7.0) containing double the ammonium sulfate concentration of the corresponding binding buffer. After injection of 2 mL of feed solution, the membrane module was washed with binding buffer until the UV absorbance reached the baseline. A 40 mL gradient from 0 to 100% eluting buffer was then used to elute the membrane bound proteins. Preparative conventional HIC was carried out with a HiTrap Butyl FF column (1mL bed volume) using the same protocol at two different ammonium sulfate concentration in the binding buffer, i.e. 1.3 and 1.4 M. The flow through and eluted peak samples collected in each experiment were analyzed by SEC and SDS-PAGE.

The effective membrane bed volume used for analytical fractionation was 0.025 mL and different flow rates were examined. The superficial velocities corresponding to the different flow rates were 60, 120, 240, 360, 480 and 600 cm/h. The eluting buffer used was 20 mM sodium phosphate (pH 7.0) while the binding buffer consisted of the eluting buffer adjusted

to 1.4 M ammonium sulfate concentration. The volume of feed sample injected was 100 µL and this consisted of a 1:1 blend of the reaction mixture and 20 mM sodium phosphate buffer (pH 7.0) containing 2.8 M ammonium sulfate. After sample injection, the membrane module was washed with binding buffer until the UV absorbance reached the baseline. A 10 mL gradient from 0 to 100% eluting buffer was then used to elute the membrane bound proteins. To test the sensitivity of the analytical technique, PEGylated lysozyme feed samples containing 50, 25, 12.5, 6.25 and 3.125 µg of total protein were fractionated.

SEC analysis was carried out using a Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences, Canada) fitted to an HPLC system (Varian, Palo Alto, CA). The mobile phase used was 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl, at a flow rate of 0.2 mL/min.

SDS-PAGE experiments were carried out according to the work of Laemmli.[16] 15% non-reducing gels were run in duplicate using a Hoefer MiniVE system (GE Healthcare Bio-Sciences, Canada). The first gel was stained with Coomassie blue dye to detect the protein bands while the second gel was stained for detecting the PEG.[17] For PEG staining, the gel was first fixed in 5% glutaraldehyde solution for 15 min at room temperature. It was then kept in 20 mL of 0.1 M perchloric acid for 15 min, followed by addition of 5 mL of 5% barium chloride solution and 2 mL of 0.1 M iodine/potassium iodide solution. After 15 min of incubation, the staining solution was replaced with water and the stained gel was photographed.

2.4 Results and discussion

Figure 2 shows the preparative HIMC chromatograms obtained with lysozyme PEGylation reaction mixture (5 h reaction) using different ammonium sulfate concentrations in the binding buffer. The salt concentration range examined here was based on initial screening experiments which showed that at ammonium sulfate concentrations greater than 1.7 M, lysozyme bound to the membrane while at concentrations lower or equal to 0.9 M, nothing present in the reaction mixture bound to the membrane stack. Unmodified lysozyme present in the injected feed was therefore expected to be obtained in the flow through peak (0 – 15 mL effluent volume) while the eluted peaks presumably contained the PEGylated proteins. At 1.7 M ammonium sulfate concentration, three eluted peaks were observed: a major peak sandwiched between two minor ones. In the 1.2 – 1.5 M salt concentration range, two eluted peaks (one major followed by a minor) were observed in each chromatogram while at 1.1 M salt concentration only one small eluted peak was observed. The flow through and the eluted peak samples (the ones indicated by arrows in Figure 2) were collected and analyzed by SEC.

Figure 3 shows the SEC chromatograms obtained with the flow through peak samples collected during preparative fractionation of PEGylated lysozyme. The chromatogram obtained using the lysozyme PEGylation reaction mixture (5 h) is also shown for comparison. The third peak (80.31 min) was due to unmodified lysozyme, this being verified by comparison with chromatogram obtained with pure lysozyme (not shown here). The identity of the molecules present in the first and second peaks was verified based on protein and PEG calibration standards using the approach proposed by Fee and Van

Alstine.[3] These were found to be di-PEGylated lysozyme (45.77 min) and mono-PEGylated lysozyme (53.62 min) respectively. Table 1 lists the retention time and distribution coefficients for the three peaks in the feed chromatogram and compares these with those obtained for unmodified and PEGylated (mono-, di- and tri-) lysozyme in a previous study[15] (where the same column, mobile phase and flow rate were used). The flow through obtained using 0.9, 1.1, 1.2 and 1.3 M ammonium sulfate concentration respectively in binding buffer contained lysozyme and decreasing amounts of mono-PEGylated lysozyme, while those obtained using higher salt concentrations contained lysozyme alone.

Figure 4 shows the SEC chromatograms obtained with the eluted peak samples (as indicated using arrows in Figure 2) collected during preparative fractionation of PEGylated lysozyme. The samples obtained at 1.1 M ammonium sulfate concentration and higher contained mono-PEGylated lysozyme while that obtained at 0.9 M ammonium sulfate concentration contained di-PEGylated lysozyme. The data shown in Figures 2, 3 and 4 provide preliminary evidence that hydrophobic interaction membrane chromatography could potentially be used to fractionate unmodified, mono- and di-PEGylated lysozyme. At 1.7 M ammonium sulfate concentration, some lysozyme bound to the membrane as evident from the first (minor) eluted peak (see Figure 2). At salt concentrations equal to or lower than 1.3 M, some mono-PEGylated lysozyme observed in the flow through (see Figure 3).Therefore, 1.4 and 1.5 M ammonium sulfate concentrations were most suitable for the fractionation. 1.4 M being the lower of the two concentrations was used in all further fractionation experiments.

Figure 5 shows the preparative HIMC chromatogram obtained with lysozyme PEGylation reaction mixture (20 h) using 1.4 M ammonium sulfate concentrations in the binding buffer. Based on the results already discussed, it may be assumed that the flow through peak (1 – 10 mL effluent volume) consisted of unmodified lysozyme while the first and second eluted peak consisted on mono- and di-PEGylated lysozyme respectively. The third peak which was not observed in the chromatograms shown in Figure 2 was presumably due to tri-PEGylated lysozyme. A 5 h reaction mixture was used for obtaining the data shown in Figure 2. Quite clearly therefore, the longer reaction time resulted in the synthesis of small amounts of tri-PEGylated lysozyme.

Figure 6 and 7 show the SDS-PAGE results obtained with samples from the preparative HIMC experiment discussed in the previous paragraph. The gel in Figure 6 was stained with Coomassie blue dye for protein staining while the one in Figure 7 was stained to visualize PEG. The flow through (lane 1) consisted of unmodified lysozyme in a highly pure form as can be seen in Figure 6. This was further verified by the absence of PEG or PEGylated proteins in lane 1 of Figure 7. The first eluted peak (lane 2) consisted of mono-PEGylated lysozyme as indicated by the strong single band in Figure 6 obtained between the 17 kDa and 26 kDa protein marker bands. Lane 2 of Figure 7 shows the mono-PEGylated lysozyme band and a thick band corresponding to the 5 kDa mPEG-propionaldehyde. This unreacted PEGylation reagent presumably bound to the membrane in the presence of salt and was co-eluted along with mono-PEGylated lysozyme. This provides clear evidence that mono-PEGylated lysozyme bound to the membrane through its PEG component. The presence of mPEG-propionaldehyde in the eluate did not affect

the HIMC chromatogram since this reagent had very low UV absorbance at 280 nm, the wavelength used for tracking unmodified and PEGylated lysozyme. The sample corresponding to the second eluted peak (lane 3) contained di-PEGylated lysozyme (between the 26 kDa and 34 kDa protein marker bands) and some mono-PEGylated lysozyme. Presumably, the fraction collected also contained some overlapped material from the first eluted peak. The bands on Figure 7 gel clearly show that the sample contained more di-PEGylated lysozyme. Figure 7 also shows that some unreacted mPEG-propionaldehyde was co-eluted. However, this was significantly lower than that observed in the first eluted peak. These results provide conclusive evidence that unmodified, mono- and di-PEGylated lysozyme could be fractionated into separate peaks using hydrophobic interaction membrane chromatography.

Figure 8 summarizes the manner in which unmodified, mono- and di-PEGylated lysozyme were fractionated using HIMC. Lysozyme being the least hydrophobic component did not bind to the membrane and was obtained in the flow-through as a pure component. Mono- and di-PEGylated lysozyme being more hydrophobic at this salt concentration, bound to the membrane. When the salt concentration was reduced in the form of a linear gradient, mono-PEGylated lysozyme (the less hydrophobic of the two) was eluted out first, followed by di-PEGylated lysozyme. This was consistent with the working hypothesis of this work.

Figure 9 shows the chromatograms obtained during fractionation of lysozyme PEGylation reaction mixture (20 h) with HiTrap Butyl FF HIC column using 1.3 and 1.4M ammonium sulfate concentrations in the binding buffer. At each salt concentration

examined, the best separation was obtained at 1mL/min flow rate using a 40mL gradient, i.e. the same as with preparative HIMC. A single broad eluted peak was observed at both salt concentrations, the shape suggesting that these were composite in nature, i.e. resulting from the elution of two or more unresolved species. The position and shape of the flow through peak changed quite significantly when the ammonium sulfate concentration in the binding buffer was decreased from 1.4 to 1.3 M. At the higher salt concentration there seems to have been some interaction between the components present in the flow through and the stationary phase and hence the peak broadened and the retention time increased.

Figure 10 shows the Coomassie blue stained gel obtained with samples from the HIC experiment carried out at 1.3M ammonium sulfate concentration. The flow through peak (lane 1) consisted of unmodified lysozyme and a small amount of mono-PEGylated lysozyme. Three samples E1, E2 and E3 were collected from the broad eluted peak as indicated by arrows in Fig. 9. E1 contained primarily mono- and di-PEGylated lysozyme and a small amount of unmodified lysozyme, E2 contained primarily mono- and di-PEGylated lysozyme and a small amount of tri-PEGylated lysozyme while E3 contained almost similar amounts of mono-, di and tri- PEGylated lysozyme. These results show that the separation of unmodified lysozyme from its PEGylated forms by conventional HIC using butyl column was not as good as that obtained by HIMC using hydrophilized PVDF membrane. Moreover, mono-, di- and tri-PEGylated lysozyme could not be resolved into separate peaks. This is consistent with the observation by Cisneros-Ruiz et al.[10] While the environment-responsive property of the HIMC media was primarily responsible for the better separation obtained with it relative to HIC, mass transfer could also potentially have

some role to play. Membranes, on account of the predominance of convective transport within them, generally give better separation of large solutes than particulate chromatographic media[18]. PEGylated proteins are significantly bulkier than proteins having similar molecular mass and would therefore have difficulty in diffusing to their binding sites in the pores present within gel beads. Such diffusional limitations do not exist within a membrane stack and the separation of PEGylated proteins is therefore expected to be better.

Figure 11 shows the chromatograms obtained during analytical fractionation of lysozyme PEGylation reaction mixture (20 h). These experiments were carried out at different flow rates, the corresponding superficial velocities being as indicated in the figure. Three eluted peaks, as observed in the preparative fractionation experiment carried out using the same feed sample were obtained at superficial velocities less or equal to 360 cm/h. At higher superficial velocities, the second and third eluted peaks merged but the first and second eluted peaks were still well resolved. These results clearly demonstrated that fast analytical fractionation of different PEGylated form of lysozyme using HIMC was feasible. At a superficial velocity of 360 cm/h, the separation took less than 5 min to complete.

Figure 12 shows the chromatograms obtained during analytical fractionation of lysozyme PEGylation reaction mixture (20 h) at 360 cm/h superficial velocity using different amounts of total protein in the injected sample. Even when 3.125 µg of total protein was injected, unmodified, mono- and di-PEGylated lysozyme were detectable and resolved as separate peaks. Thus, the method was quite sensitive and suitable to separating and detecting very small amounts of PEGylated proteins.

2.5 Conclusions

Unmodified, mono- and di-PEGylated lysozyme could be fractionated into separate peaks using hydrophobic interaction membrane chromatography. The results obtained were consistent with the working hypothesis of this work, i.e. the apparent hydrophobicity (in the presence of salt) of a di-PEGylated protein was higher than that of a mono-PEGylated protein. The ammonium sulfate concentration in the feed solution affected the binding of unmodified and PEGylated lysozyme on the membrane, the best separation being obtained at 1.4 M salt concentration. The co-elution of unreacted PEG along with the PEGylated lysozyme proved that in the presence of salt (above concentrations resulting in PEG phase change), the PEGylated proteins attached to the membrane through hydrophobic appendages consisting of collapsed PEG. Di-PEGylated lysozyme, having two PEG molecules attached to it was therefore apparently more hydrophobic than mono-PEGylated lysozyme. Analytical fractionation could be carried out at high flow rates with typical separation time being less than 5 min. As low as 3.125 µg of total proteins could be resolved into identifiable peaks.

2.6 Acknowledgement

2.7 References

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2.8 Figures

Figure 1. Working hypothesis for the fractionation of unmodified, mono- and di-PEGylated proteins using hydrophobic interaction chromatography.

Figure 2. Effect of ammonium sulfate concentration in the feed on fractionation of PEGylated lysozyme using hydrophobic interaction membrane chromatography (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack:30; disc diameter: 18 mm; flow rate: 1 mL/min; elution: 40 mL linear gradient from 0 to 100% eluting buffer; feed sample: 5 h lysozyme PEGylation reaction mixture blended with ammonium sulfate containing buffer; injected volume: 2 mL; ammonium sulfate concentrations in binding buffer: 0.9 M, 1.1 M, 1.3 M, 1.4 M, 1.5M and 1.7 M). Thick curves: UV absorbance; thin curves: conductivity.

Figure 3. SEC analysis of flow through peaks from preparative fractionation experiments that had been carried out using different ammonium sulfate concentrations in binding buffer (column: Superdex 200 10/300 GL; HPLC system: Varian; mobile phase: 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl; flow rate: 0.2 mL/min). The chromatogram obtained with the reaction mixture is also shown in the figure for comparison.

Figure 4. SEC analysis of eluted peaks (as indicated by arrows in Figure 2) from preparative fractionation experiments that had been carried out using different ammonium sulfate concentrations in binding buffer (column: Superdex 200 10/300 GL; HPLC system: Varian; mobile phase: 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl; flow rate: 0.2 mL/min). The chromatogram obtained with the reaction mixture is also shown in the figure for comparison.

Figure 5. Preparative fractionation of PEGylated lysozyme using (membrane: hydrophilized PVDF; pore size: 0.22 µm; number of membrane discs in stack: 30; disc diameter: 18 mm; flow rate: 1 mL/min; elution: 40 mL linear gradient from 0 to 100% eluting buffer; feed sample: 20 h lysozyme PEGylation reaction mixture blended with ammonium sulfate containing buffer; injected volume: 2 mL; ammonium sulfate concentration in binding buffer: 1.4 M).
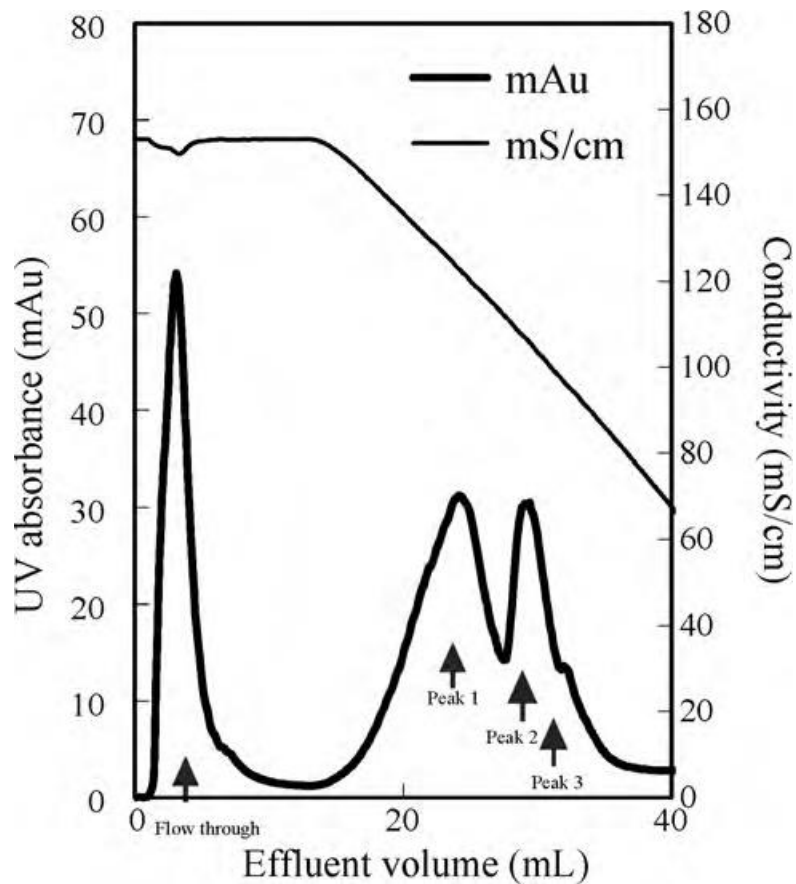
Figure 6. Coomassie blue stained gel (SDS-PAGE, 15% non-reducing) obtained with samples from preparative HIMC experiment. Lane 1: flow through peak; lane 2: first eluted peak; lane 3: second eluted peak; lane 4: third eluted peak; lane 5: protein molecular weight markers; lane 6: standard lysozyme.
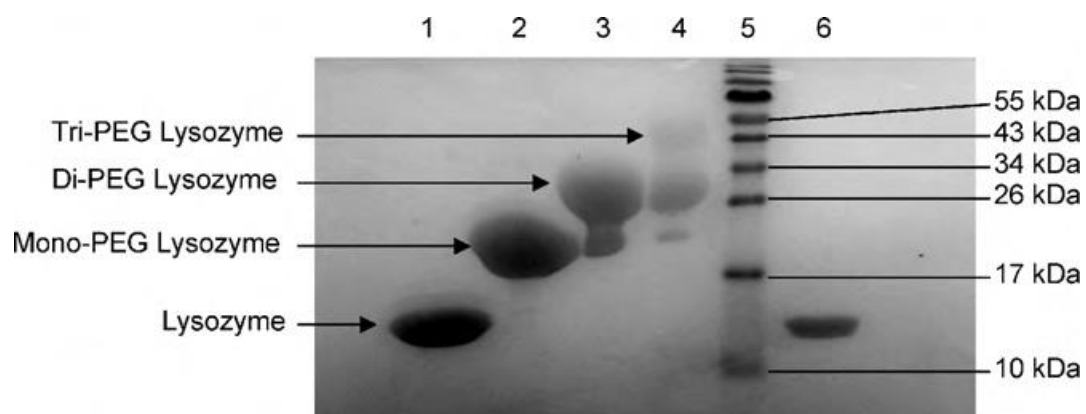
Figure 7. PEG stained gel (SDS-PAGE, 15% non-reducing) obtained with samples from preparative HIMC experiment. Lane 1: flow through peak; lane 2: first eluted peak; lane 3: second eluted peak; lane 4: protein molecular weight markers; lane 5: standard lysozyme; lane 6: PEG 5 kDa.
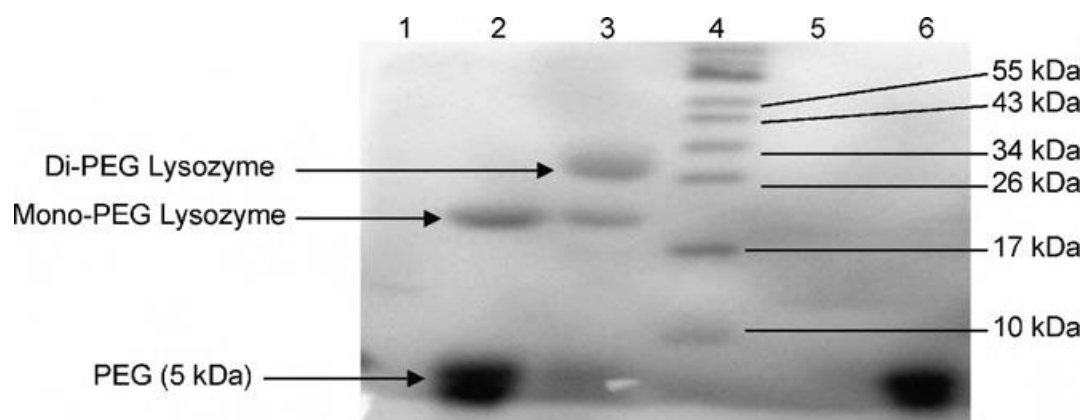
Figure 8. Mechanism of fractionation of unmodified, mono- and di-PEGylated lysozyme by HIMC.
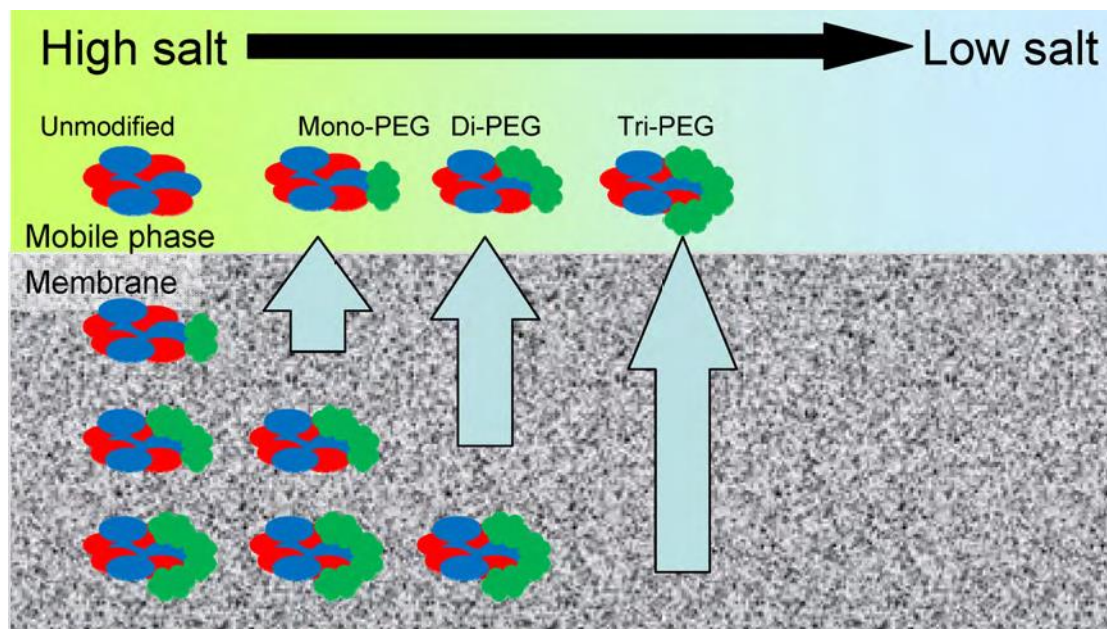
Figure 9. Preparative fractionation of PEGylated lysozyme by conventional HIC (column: HiTrap Butyl FF; bed volume: 1 mL; flow rate: 1 mL/mL; elution: 40 mL linear gradient from 0 to 100% elution buffer; feed samples: 20 h lysozyme PEGylation reaction mixture blended with ammonium sulfate containing buffer; injected volume: 2 mL; ammonium sulfate concentrations in binding buffer: 1.3 and 1.4 M).
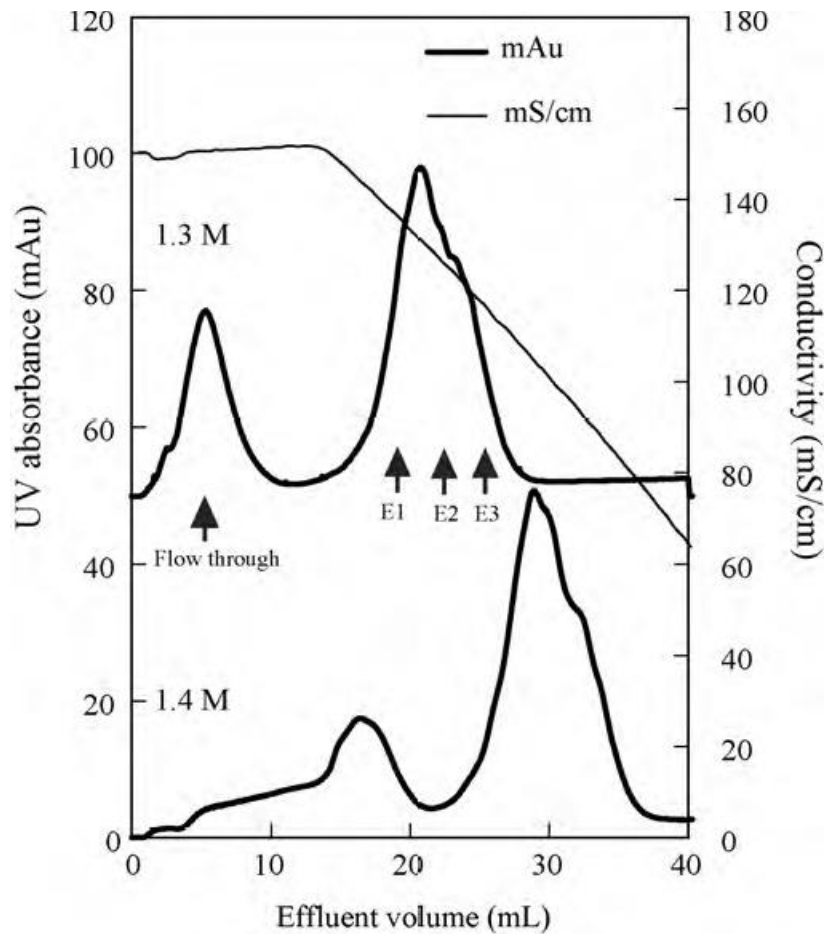
Figure 10. Coomassie blue stained gel (SDS-PAGE, 15% non-reducing) obtained with samples from preparative HIC experiment. Lane 1: flow through peak; lane 2: eluted peak sample E1; lane 3: eluted peaks sample E2; lane 4: eluted peak sample E3; lane 5; protein molecular weight makers; lane 6: standard lysozyme.
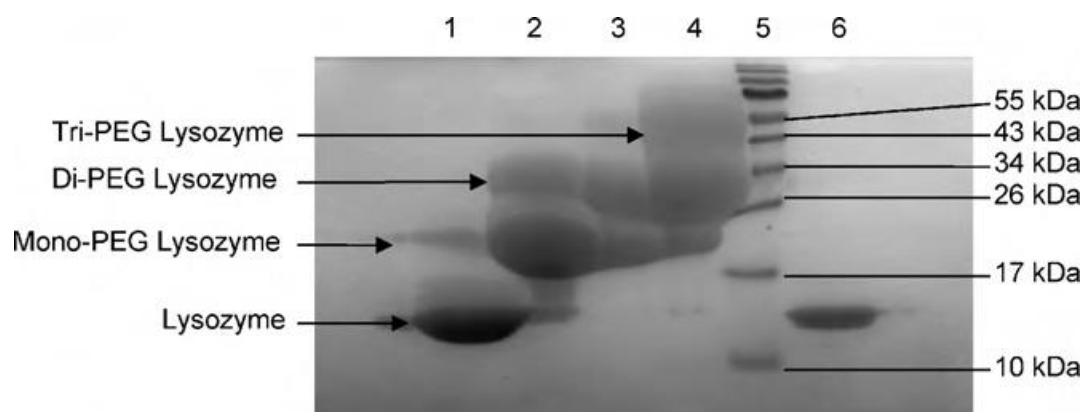
Figure 11. Effect of superficial velocity on analytical HIMC of PEGylated lysozyme (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack: 4; disc diameter: 8 mm; bed volume: 0.025 mL; superficial velocities: 60, 120, 240, 360, 480 and 600 cm/h; elution: 10 mL linear gradient from 0 to 100% eluting buffer; feed samples: 20 h lysozyme PEGylation reaction mixture blended with ammonium sulfate containing buffer: injected volume: 100 μL; ammonium sulfate concentration in binding buffer: 1.4 M).
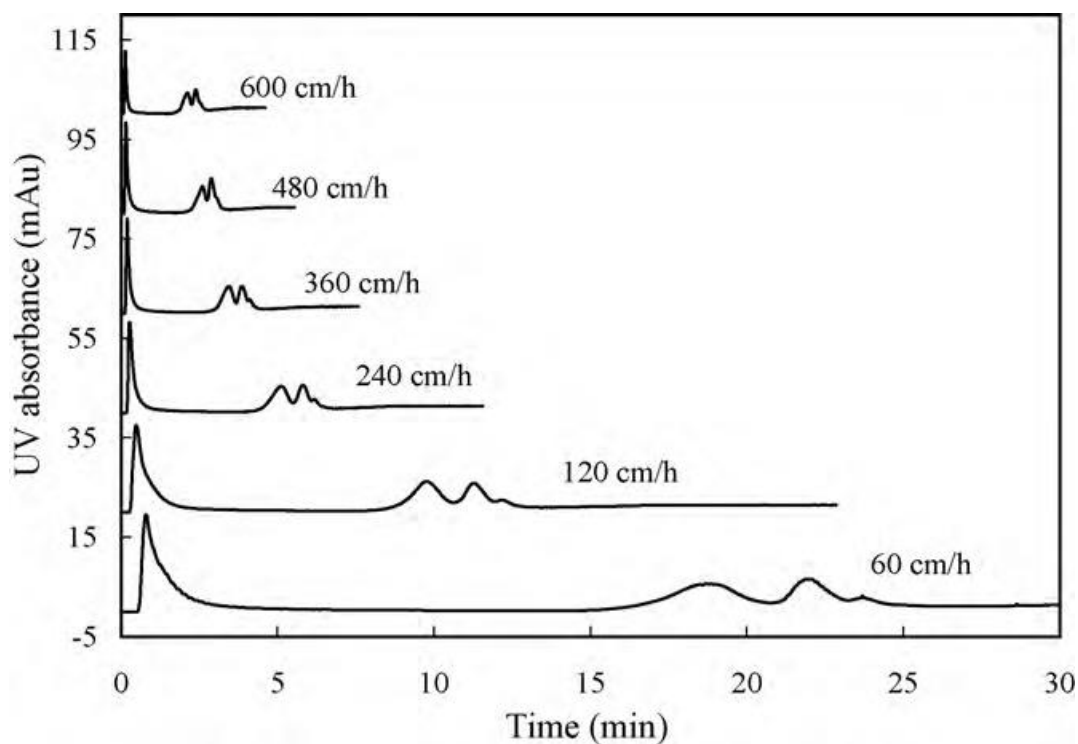
Figure 12. Sensitivity of analytical fractionation of PEGylated lysozyme (membrane: hydrophilized PVDF; pore size: 0.22 µm; number of membrane discs in stack:4; disc diameter: 8 mm; superficial velocity: 360 cm/h; elution: 10 mL linear gradient from 0 to 100% eluting buffer; feed sample: 20 h lysozyme PEGylation reaction mixture blended with ammonium sulfate containing buffer injected volume: 100 µL; ammonium sulfate concentration in binding buffer: 1.4 M; total protein amounts injected: 50 µg, 25 µg, 12.5 µg, 6.25 µg and 3.125 µg).
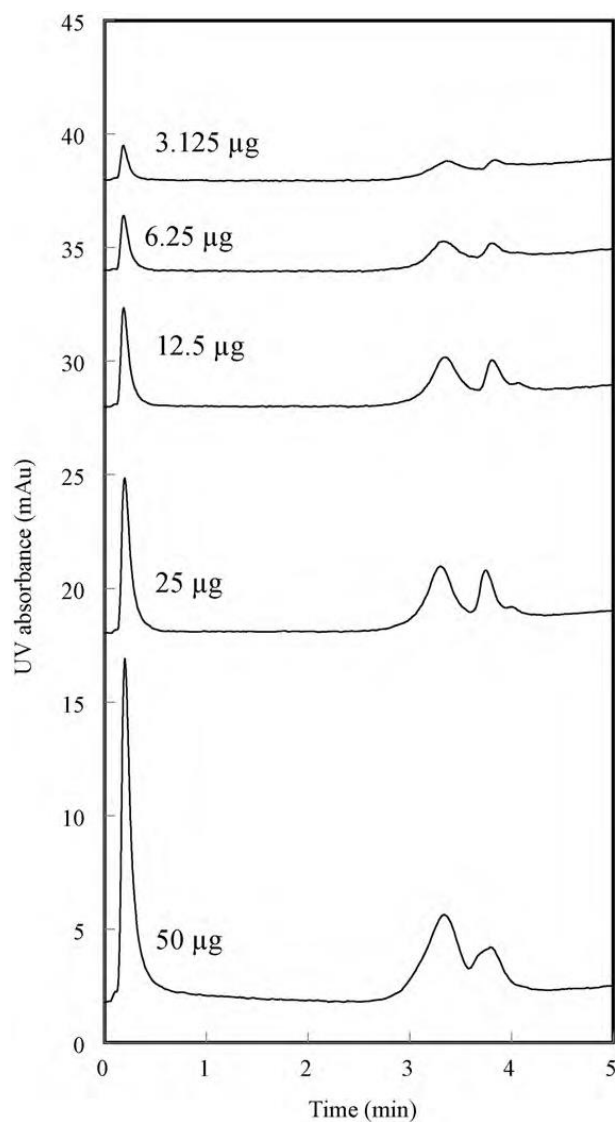
2.9 Tables

Table 1 Identification of proteins in SEC chromatogram obtained with lysozyme PEGylation reaction mixture (shown in Figure 3) based on retention time and distribution coefficient data.

Protein	Retention time (min)	Distribution coefficient (-)
Peak 1	45.77	0.0905
Peak 2	53.62	0.1896
Peak 3	80.31	0.5269
Lysozyme	79.15[a]	0.5219[a]
Mono-PEGylated lysozyme	53.58[a]	0.1891[a]
Di-PEGylated lysozyme	45.75[a]	0.0903[a]
Tri-PEGylated lysozyme	42.4[a]	0.0480[a]

[a] Data obtained from Yu and Ghosh (Yu and Ghosh, 2010)

Chapter 3

Purification and analysis of mono-PEGylated human serum albumin by hydrophobic interaction membrane chromatography

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Purification and analysis of mono-PEGylated human serum albumin by hydrophobic interaction membrane chromatography

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3.1 Abstract

We discuss the purification of mono-PEGylated human serum albumin (HSA) from a mixture of different PEGylated forms of the protein by hydrophobic interaction membrane chromatography using a stack of hydrophilized polyvinylidene fluoride membrane. The hydrophobicity difference between the fractionated species was induced by the addition of a lyotropic salt which resulted in the phase transition at ambient temperature, of PEG, hydrophilic under normal condition to a mildly hydrophobic form. Therefore, the greater the number of PEG chains attached to a protein, the greater was its apparent hydrophobicity in the presence of salt. The unmodified HSA was obtained in the flow through. Amongst the three major PEGylated forms of HSA present in the starting material (i.e. mono-, di- and tri-), mono-PEGylated HSA was eluted first. Using optimized elution gradient, the mono-PEGylated protein could be resolved from the other forms. Purified samples obtained were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, dynamic light scattering and size exclusion chromatography combined with multi-angle light scattering. All these analytical techniques indicated the presence of species having a molar mass consistent with mono-PEGylated HSA. A scaled-down version of the membrane chromatographic methods could be used for rapid and sensitive analysis of PEGylated proteins.

Keywords: membrane chromatography; PEGylated protein; purification; hydrophobic interaction; albumin

3.2 Introduction

As discussed in previous chapters, PEGylation overcomes the challenges that native protein drugs have, such as relatively short half-life [1], poor stability *in-vivo* [2], immunogenicity [3] and limited shelf-life remain. Addressing these challenges could lead to better acceptance and wider usage of such protein drugs [4], and improve the efficacy and physical properties of therapeutic proteins by covalent attachment of the synthetic polymer polyethylene glycol (PEG) [5].

Since the conventional way to synthesize PEGylated proteins in liquid-phase batch reaction always results in the synthesis of complex mixtures, even the site-specific chemistry (e.g. N-terminal PEGylation) is used [6], purification of mono-PEGylated protein becomes really essential. In addition to the by-products (i.e. di-, tri- and high-PEGylated proteins), unmodified protein, excess activated PEG and quenched PEG as well as low molecular weight impurities such as reducing agent and reaction quencher also need to be removed to obtain the purified mono-PEGylated protein.

In the last chapter, we demonstrated the use of hydrophobic interaction membrane chromatography (HIMC) using a stack of hydrophilized microporous PVDF membranes with environment-responsive property for purification of PEGylated lysozyme [7]. These membranes become hydrophobic in the presence of lyotropic salt and revert back to the original hydrophilic state when the salt is removed [8, 9]. Also, the difference in hydrophobicity of the fractionated species was induced by the addition of lyotropic salt. PEG, which is hydrophilic under normal condition, underwent salt-induced phase transition at ambient temperature to a mildly hydrophobic form [10, 11]. Consequently, the greater

the number of PEG attached to a protein, the greater was its apparent hydrophobicity i.e. under conditions at which separation was carried out, the unmodified protein was the least hydrophobic species, followed by mono-, di- and tri-PEGylated protein. Since the hydrophobicity of the membrane can be varied, the interactions between the different PEGylated proteins and the membrane surface can be manipulated. By being able to do so, difference in the degree of interaction of the different PEGylated forms with the membrane can be maximized, such that this technique not only separated the PEGylated protein from its unmodified form but was able to resolve different PEGylated forms into separate peaks, which is sometimes not feasible with conventional column based hydrophobic interaction chromatography (HIC) whose media are always hydrophobic [7, 12].

In this chapter, human serum albumin (HSA), a medium sized protein as opposed to a small protein like lysozyme was selected as a model protein to assess the wider applicability of HIMC for purification of PEGylated proteins. Liquid-phase PEGylation of HSA was first carried out, and the reaction mixtures thus obtained were fractionated using stacks of PVDF membranes, both in preparative and analytical mode. The overall objective of this study was to validate the working hypothesis for the fractionation of PEGylated proteins by HIMC as described in our previous work [7]. The objective of the analytical HIMC experiments was to see if the above approach would be used to develop membrane chromatographic methods for rapid and sensitive analysis of PEGylated HSA and PEGylated proteins in general. Samples collected during these experiments were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), dynamic light scattering (DLS) and size exclusion chromatography combined with multi-angle light

scattering (SEC–MALS). The results obtained are discussed.

3.3 Experimental

3.3.1   Materials

Human serum albumin (A1653), ammonium sulfate (A4418), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), sodium chloride (S7653), sodium cyanoborohydride (156159), hydrochloric acid (258148), Trizma base (T1503), glycine (G8898), iodine (326143), 70% perchloric acid (77227), 25% glutaraldehyde solution (G6257) and barium chloride (202738) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified human serum albumin (MW = 69 kDa, pI = 4.7, concentration = 45 g/L) used for the initial PEGylated HSA purification experiments such as elution gradient optimization was kindly donated by the Scottish National Blood Transfusion Services (SNBTS), Edinburgh, UK. mPEG-propionaldehyde (MW 10 kDa, P1PAL-10) was purchased from Sunbio Inc. (Anyang, South Korea). Potassium iodide (74210-140) was purchased from Anachemia (Montreal, QC, Canada). Sodium acetate trihydrate (SX0255-1) and glacial acetic acid (AX0073-6) were purchased from EMD (Gibbstown, NJ, USA). Hydrophilized PVDF membrane (0.22 μm; GVWP10050), nominally used for microfiltration applications, utilized for HIMC in our study was purchased from Millipore (Billerica, MA, USA). The surface treatment applied to the naturally hydrophobic PVDF membrane imparted a stimuli-responsive hydrophilic-hydrophobic property to the membrane, making it suitable for chromatographic separations [7-9]. Amicon® Ultra-4 centrifugal filters 3 kDa MWCO (UFC800324) for concentrating and desalting were

purchased from EMD Millipore Co., Billerica, MA, USA. Purified water (18.2 MΩ cm) obtained from a Diamond^{TM} NANOpure water purification unit (Barnstead, Dubuque, IA, USA) was used for preparation of all the test solutions and buffers.

3.3.2   Liquid-phase PEGylation of HSA

Liquid-phase PEGylation of HSA was carried out at room temperature for 20 h in small shaken flasks. The reactants consisted of 1 mg/ml HSA, P1PAL-10 (P1PAL-10 to HSA molar ratio being 4:1), and 10 mM sodium cyanoborohydride in 100 mM sodium acetate buffer (pH 5.0). The reaction was terminated by adding glycine solution to obtain a 100 mM final glycine concentration. The quenched reaction mixture was then processed by centrifugal filtration for concentration enhancement and removal of low molecular weight species. The processed reaction mixture was used as feed solution for the HIMC experiments.

3.3.3   Preparative HIMC experiments

Thirty membrane discs of 18 mm diameter were stacked within a custom-designed module [41], resulting in an effective bed volume of 0.95 ml. The membrane module was integrated with an AKTA Prime liquid chromatography system (GE Healthcare Bio-Sciences, QC, Canada). The binding buffer consisted of 1.25 M ammonium sulfate prepared in 20 mM sodium phosphate (pH 7.0). Elution was carried out using ammonium sulfate free buffer, i.e. 20 mM sodium phosphate (pH 7.0). The separation was carried out at 1 mL/min mobile phase flow rate using 2 mL of feed sample (containing ~1000 μg of

total protein). Various linear elution gradients i.e. 20, 40 and 60 mL from 0 to 100% salt free buffer were used to elute out bound PEGylated proteins. Samples collected during the HIMC experiments were desalted and concentrated by centrifugal ultrafiltration and were then analyzed by SDS-PAGE, DLS, and SEC-MALS.

3.3.4   Analytical HIMC experiments

Analytical HIMC experiments were carried out using two small custom-designed membrane modules having bed volumes of 0.065 and 0.025 mL (with 10 and 4 membrane discs of 8 mm diameter respectively). Experiments with 10-disc membrane module were carried out with 1.25 M ammonium sulfate in binding buffer at a flow rate of 0.8 mL/min with 100 μL of feed sample (containing ~50 μg of total protein). Step and 10 and 20 mL linear gradients were used to elute out the bound proteins. The 4-disc membrane module was used for assessing the sensitivity of the HIMC method using 1.4 M ammonium sulfate in the buffer. Samples containing 12.5 μg, 25 μg and 50 μg of total protein were injected in these tests. The information on membrane module and operating conditions of all HIMC experiments are summarized in Table 1.

3.3.5   SDS-PAGE

SDS-PAGE experiments [14] were carried out on 12.5% non-reducing gels using a Hoefer MiniVE system (80-6418-77; GE Healthcare Lab Sciences, Montreal, QC, Canada). Coomassie blue was used for gel staining to visualize the protein bands. A PEG staining protocol as described in [15] was used to observe the PEG containing components in the

gel. The Coomassie-blue-stained gel was analyzed using Image J software (freeware downloaded from http://rsbweb.nih.gov/ij/) which measures the intensity of bands to give a quantitative evaluation of the relative amounts corresponding to each band in a particular lane.

3.3.6   DLS

Batch dynamic light scattering measurements were carried out with the DynaPro NanoStar (Wyatt Technology, Santa Barbara, CA). 30 µL of each sample were held in Wyatt disposable cuvette in which 10 acquisitions of 5 second duration each were collected. Temperature was held constant at 25° C for all measurements. Analysis was performed with Wyatt's Dynamics 7 software.

3.3.7   SEC-MALS

Size-exclusion chromatography coupled with multi-angle static light scattering is a powerful tool for macromolecular characterization [16]. SEC-MALS experiments were performed using an Agilent 1200 HPLC with VWD UV detection at 280 nm (Agilent, Santa Clara, CA) attached to a Wyatt Technology WTC-030S5 300 angstrom column (Wyatt Technology, Santa Barbara, CA) in a PBS buffer, pH 7.2, at 1.0 mL/min for 30 min. Downstream static light scattering detection was performed with a DAWN HELEOS II fitted with a QELS dynamic light scattering detector (Wyatt Technology Santa Barbara CA). Concentration measurements were performed with an Optilab T-rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). Data was collected with Agilent's

ChemStation B.04.03 simultaneously with Wyatt's Astra 6 software. Samples were prepared to 1.0 mg/mL in PBS with 50 µL injection volumes. A system suitability check was performed with BSA.

3.4 Results and discussion

3.4.1   Preparative HIMC

The HSA sourced from SNBTS (see the materials and methods section) was used to generate the PEGylated protein samples used in these initial experiments. Fig. 1 shows the chromatograms obtained from experiments carried out with three elution gradients (i.e. 20, 40 and 60 mL) using 1.25 M ammonium sulfate concentration in the binding buffer. At this salt concentration, unmodified HSA almost did not bind to the membrane (as demonstrated later in Fig. 3a) and was obtained in the flow through (FT) around 10 mL effluent volume. Consistent with the expectation based on our earlier study [7], mono-PEGylated HSA was obtained in the first eluted peak (E1) followed by di- and/or tri- PEGylated forms in the second eluted peak (E2). The eluted peaks obtained with a 20 mL linear gradient overlapped slightly while peak broadening was observed with a 60 mL linear gradient. The chromatogram obtained with 40 mL linear gradient showed the best resolution of eluted peaks. The HSA used for generating the feed for the experiments described above contained up to 5% human immunoglobulin G. All subsequent PEGylated HSA separation experiments were therefore carried out using PEGylated protein mixture prepared using HSA sourced from Sigma (see materials and methods section) which was reported to have a higher purity.

Fig. 2 shows the chromatogram obtained from a preparative PEGylated HSA purification experiment carried out using the feed sample prepared with HSA sourced from Sigma. This HIMC experiment was carried out using 40 mL linear gradient elution. Consistent with the experiments discussed in the previous paragraph, a FT peak and two eluted peaks (E1 and E2) were observed. However, as the sources of HSA in the two experiments were different, slightly differences in conversion and specificity in PEGylation were observed. Based on area under the curve integration, the percentage peak areas for FT, E1 and E2 were found to be 75.6%, 20.0% and 4.4% respectively. The FT, E1 and E2 samples from this chromatogram were collected and analyzed by using SDS-PAGE. The E1 sample was also analyzed by using DLS and SEC-MALS.

3.4.2   SDS-PAGE

Duplicate SDS-PAGE gels run with samples obtained from the preparative HIMC experiment corresponding to Fig. 2 were stained with Coomassie blue dye to visualize the protein bands and with a PEG staining protocol to visualize the PEG containing components (see Figs. 3a and 3b respectively). Lanes 1 to 3 contained the protein molecular weight markers, pure P1PAL-10 and pure HSA respectively. Lane 4 contained the feed sample injected, i.e. liquid-phase HSA PEGylation reaction mixture which consisted of unreacted HSA (band corresponding to 55 kDa marker), unreacted P1PAL-10 (band between the 17 and 26 kDa markers) and its dimer (band between the 40 and 55 kDa markers in Fig. 3b) in addition to the different PEGylated forms of HSA. PEG-PEG dimers are formed by aldol condensation of PEG-aldehyde reagent [17, 18]. HSA has a molecular

weight of 67 kDa, but showed up as a band corresponding to the 55 kDa protein molecular weight marker used in this study. However, this is not unusual and there are reports in the literature of bovine serum albumin (BSA), which is quite close in molecular weight to HSA, showing an apparent molecular weight of 55 kDa on SDS-PAGE [19]. The PEGylated proteins in lane 4 consisted primarily of mono-PEGylated HSA (the relatively darker band corresponding to 75 kDa marker) and smaller amounts of di- (band corresponding to 100 kDa marker) and tri-PEGylated HSA (band corresponding to 130 kDa marker). The FT contained unreacted HSA in a pure form as evident from the single band in lane 5 of Fig. 3a, indicating that all PEG containing species present in the feed solution bound to the membrane during the binding step. This is consistent with our hypothesis that free protein (in this case HSA) would in the presence of salt, have lower apparent hydrophobicity than either PEG or the PEGylated forms of the protein. The E1 sample consisted mainly of mono-PEGylated HSA and smaller amounts of di-PEGylated HSA. E1 contained trace amounts of HSA as evident from the very faint band corresponding to the pure protein. The E2 sample (lane 7 in both figures) contained all three PEGylated forms of HSA, as evident from the bands corresponding to75 kDa, 100 kDa, and 130 kDa markers, and a trace amount of native HSA (corresponding to the 55 kDa marker). These results indicate that the mono-PEGylated form had lower apparent hydrophobicity than the di- and tri-PEGylated forms while between di- and tri-, the latter had a greater apparent hydrophobicity. Therefore, the apparent hydrophobicity of a PEGylated protein increases with degree of PEGylation. Un-complexed (presumably quenched) P1PAL-10 was co-eluted out with the PEGylated proteins as evident from its presence in both peaks while the P1PAL-10 dimer was present

in E2 only, indicating similar membrane-binding property as di-PEGylated HSA. This manner of co-elution proved that PEGylated proteins indeed bound to the membrane through their PEG component.

Image J software was used to analyze the compositions of the samples in each lane based on measurement of band intensity. The composition of E1 sample in lane 6 was estimated to be 3.9%, 82.0%, and 14.1% of unreacted HSA, mono-, and di-PEGylated HSA, respectively. E2 sample in lane 7 consisted of 2.0%, 51.3%, 20.6% and 26.0% respectively of unreacted HSA, mono-, di-, and tri-PEGylated HSA. Therefore the purity of the mono-PEGylated HSA in E1 was 82.0% and its yield in this sample was estimated to be 87.9%.

3.4.3   DLS autocorrelation functions and histogram

Batch dynamic light scattering is a sub-micron level technique which facilitates quick and easy qualitative and quantitative analysis of a sample. 30 µL of mono-PEGylated HSA was delivered to a disposable cuvette to measure the translational diffusion coefficient (D_t) from which the hydrodynamic radius (R_h) can be calculated given the Stokes-Einstein equation:

$$D_t = \frac{kT}{6\pi\eta R_h}$$

(1)

Where k is the Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity. From Figs 4a and 4b, the single decay auto-correlation function and histogram of mono-PEGylated HSA shows a relatively pure sample of 4.9 nm (2.4% percentage error). The bars corresponding to 33.6 nm (6.9% error percentage) and 717.0 nm (4.3% error

percentage) are most due to likely artifacts as they are considerably larger any species anticipated in the sample. This measurement provided a quick screen of the sample before moving on to the more time consuming SEC-MALS measurements.

3.4.4   SEC-MALS with molar mass distribution and protein conjugate analysis

Fig. 5a shows the SEC-MALS chromatogram obtained with the E1 sample (containing mainly mono-PEGylated HSA) collected from the preparative HIMC experiments. Modest resolution of the mono-PEGylated HSA from other species was achieved. Peak 1 is defined as the region from 6.2 min to 10.3 min, consisting of almost all species in the sample (i.e. mono-, di-, and/or higher PEGylated forms of HSA, and unreacted activated PEG reagents). Peak 2 is defined as the right half of the major peak region corresponding to mono-PEGylated HSA from 9 min to 9.8 min, aiming at focusing on the mono-dispersed portion of this peak. The mono-dispersion of mono-PEGylated HSA in this defined region is represented by the constant molar mass value shown in both Figs 5b and 5c. A benefit of having online light scattering detection after chromatographic separation is that we are able to accurately see the molar mass distribution, even if no baseline resolution is achieved. Fig. 5b illustrates the molar mass distribution of Peaks 1 and 2. The analysis shows that the MW of Peak 2 remains constant from 9 to 9.8 min. Prior to 9 min in Peak 1 there are a number of higher order species ranging up to approximately 10 MDa in size, which refer to di- and/or higher PEGylated forms of HSA. After 9.8 min it drops down to about 10 KDa, which is most likely due to the elution of unreacted activated PEG reagents which was eluted out after larger MW species. Fig. 5c shows the protein conjugate analysis of Peak 2,

from which the amount of protein versus PEG can be calculated. To do so, both the UV and refractive index detectors were employed. The Agilent UV detector tuned to a wavelength of 280 nm responded to the protein portion of the mono-PEGylated HSA molecule only, whereas the Optilab T-rEX refractive index detector measured the total concentration of protein and PEG present. Using a molar extinction coefficient of 0.55 (mL/(mg cm)) for HSA [20], the protein component of the complex was calculated. Using $d\eta/dc$ values of 0.185 and 0.140 mL/g for HSA and PEG respectively, the concentration of PEG was calculated:

$$[\text{mono-PEGylated HSA complex}] - [\text{HSA}_{\text{UV280}}] = [\text{PEG}] \qquad (2)$$

The data obtained by calculation are summarized in Table 2. The calculation showed the MW of HSA to be 62.2 kDa, the MW of PEG to be 14.3 kDa with the total MW of the complex being 76.5 kDa. This analysis showed a protein weight fraction of 81.3%, consistent with that expected for mono-PEGylated HSA.

3.4.5 Analytical HIMC

Fig. 6 shows the chromatograms obtained by analytical HIMC using the 10-disc membrane module. Chromatogram (a) was obtained by injecting pure HSA with 1.25 M ammonium sulfate in the binding buffer, followed by a step elution. The absence of an eluted peak indicated that HSA did not bind to the membrane at this salt concentration. The remaining three chromatograms i.e. (b), (c) and (d) were obtained by injecting HSA PEGylation reaction mixture (adjusted to 1.25 M ammonium sulfate) using linear elution gradients of 0, 10 and 20 mL respectively. Based on results obtained in the preparative

HIMC experiments, the unmodified HSA flowed through while the eluted peaks contained mono- and di-PEGylated HSA. Step elution (i.e. 0 mL elution gradient) resulted in no separation at all of the different PEGylated forms. Completely resolved eluted peaks were observed with both 10 and 20 mL linear gradients. The occurrence of unresolved peaks is a problem in both analytical and preparative chromatography. In analytical chromatography this leads to subjective peak area calculations leading to unreliable data. The results shown in Fig. 6 clearly demonstrate the suitability of using HIMC for analysis of in-process samples collected during protein PEGylation as well as for purified final product analysis.

Fig. 7 shows the chromatograms obtained from analytical HIMC experiments carried out using the 4-disc membrane module. As in the previous experiments, the flow through contained unmodified HSA while the first eluted peak contained mono-PEGylated HSA. With the 12.5 µg sample, the di-PEGylated HSA peak was barely identifiable while with the higher amount samples the second eluted peak corresponding to d-PEGylated HSA was clearly observed. These results demonstrate that HIMC could be used as a very sensitive fractionation and analytical technique for PEGylated proteins.

3.5 Conclusions

This study clearly demonstrated the role of the PEG adduct in the separation of PEGylated proteins by hydrophobic interaction membrane chromatography. The apparent hydrophobicity (i.e., in the presence of lyotropic salt) of a PEGylated protein increased with the number of PEG chains attached. This was consistent with that observed during the purification of PEGylated lysozyme using HIMC [7]. The resolution of unmodified protein

from PEGylated protein and that of the different PEGylated forms in the two studies were comparable. Moreover the salt concentration required for binding of PEGylated proteins was similar, clearly indicating the role of PEG in the adsorption process. At the solution conditions used during separation, the unmodified protein had the lowest apparent hydrophobicity followed by the mono-PEGylated form and then by the higher PEGylated forms. Based on this, mono-PEGylated HSA could be separated by HIMC from both, unmodified HSA and the higher PEGylated forms (i.e. di- and tri-). The length of the elution gradient had significant effect on the resolution of separation. The identity of the purified mono-PEGylated HSA obtained by HIMC was verified using several orthogonal separation techniques, i.e. SDS-PAGE, DLS and SEC-MALS. SDS-PAGE showed a single protein band while DLS analysis showed that the purified sample consisted of a relatively pure substance with hydrodynamic radius of 4.9 nm, consistent with that expected for mono-PEGylated HSA. SEC-MALS also indicated a molar mass consistent with mono-PEGylated HSA, with the percentage of deviation from its actual MW of 76.7 kDa being 4.3%.The sensitivity of the HIMC technique and its suitability for high-resolution analysis were also demonstrated.

3.6 Acknowledgement

3.7 References

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3.8 Tables

Table 1. Membrane modules and operating conditions for preparative and analytical HIMC

Membrane module			Operating conditions					
No. of discs	Disc diameter (mm)	Bed volume (mL)	Mobile phase			Elution gradient length (mL)	Sample loop (µL)	Total protein amount (µg)
			Eluting buffer	Binding buffer	Flow rate (mL/min)			
30	18	0.95	20 mM sodium phosphate pH 7.0	1.25 M	1	20, 40, 60	2000	1000
10	8	0.065		1.25 M	0.8	0, 10, 20	100	50
4	8	0.025		1.4 M	3	10	100	12.5, 25, 50

Table 2. Summary of calculated molecular weights obtained from molar mass distribution analysis and protein conjugate analysis of SEC-MALS.

Techniques	Molar mass distribution analysis	Protein conjugate analysis
SEC-MALS	Peak 1 MW: 87.4 kDa Peak 2 MW: 73.4 kDa	Protein MW: 62.2 kDa Modifier MW: 14.3 kDa Total MW: 76.5 kDa

3.9 Figures

Figure 1 Effect of elution gradient on the separation of PEGylated HSA by HIMC (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack: 30; disc diameter: 18 mm; bed volume: 0.95 mL; flow rate: 1 mL/min; feed sample: 20 h HSA PEGylation reaction mixture blended with ammonium sulfate containing buffer to obtain a salt concentration of 1.25 M; injected volume: 2 mL; ammonium sulfate concentration in binding buffer: 1.25 M; total protein injected: 1 mg; linear elution gradient lengths: (a) 20mL, (b) 40 mL, (c) 60 mL).
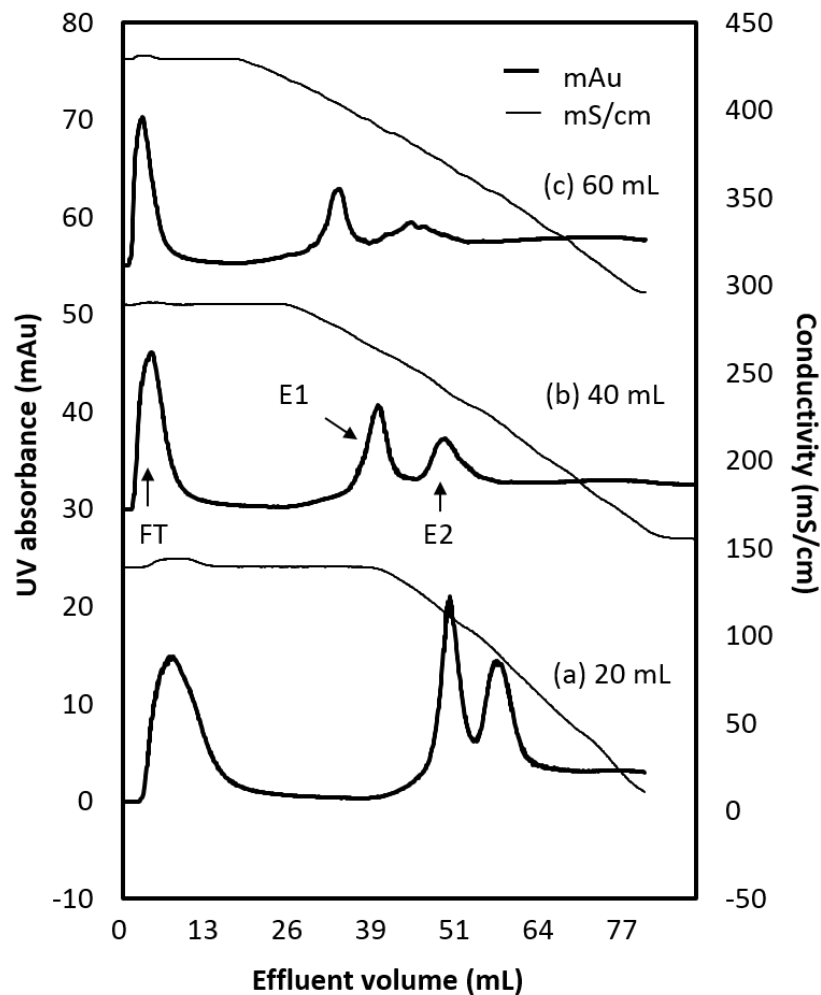
Figure 2 Preparative purification of mono-PEGylated HSA by HIMC using 40 mL elution gradient (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack: 30; disc diameter: 18 mm; bed volume: 0.95 mL; flow rate: 1 mL/min; feed sample: 20 h HSA PEGylation reaction mixture blended with ammonium sulfate containing buffer to obtain a salt concentration of 1.25 M; injected volume: 2 mL; ammonium sulfate concentration in binding buffer: 1.25 M; total protein injected: 1 mg).
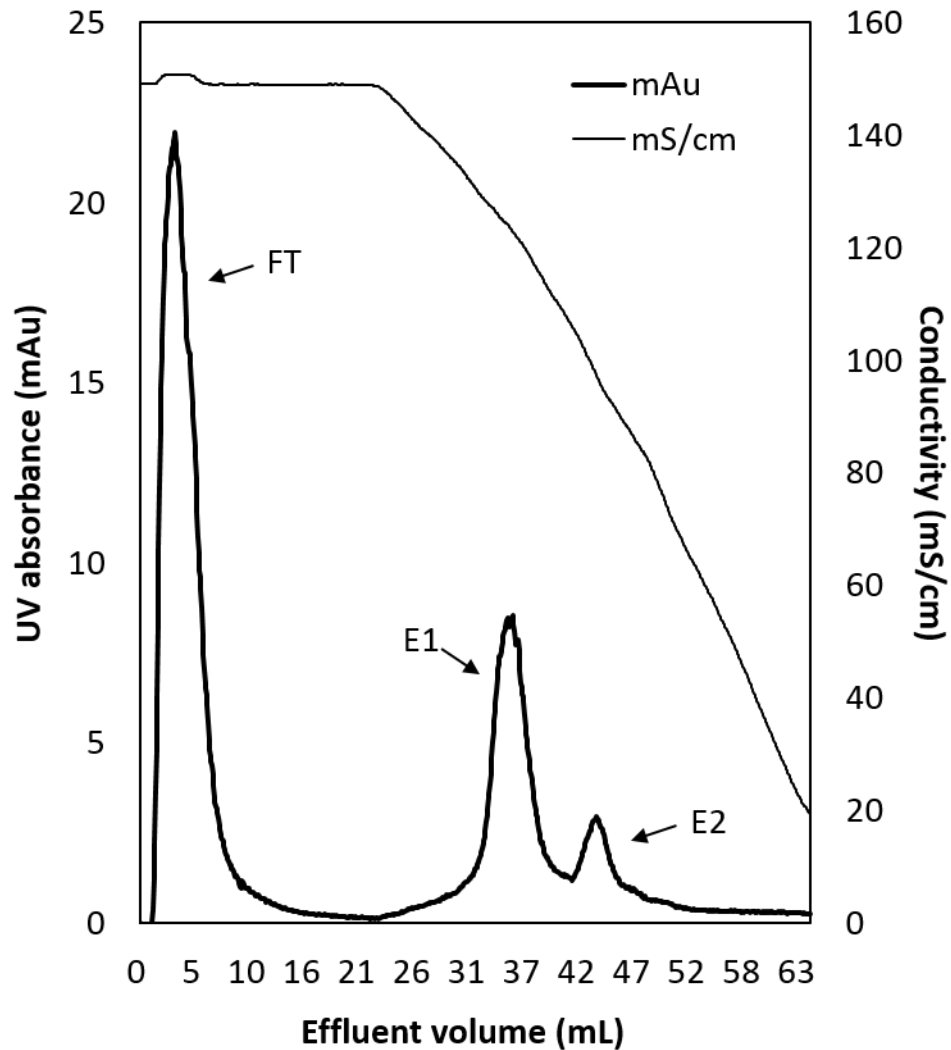
Figure 3 Coomassie blue stained (a), and PEG stained (b) gels obtained with duplicated SDS-PAGE (12.5% non-reducing) obtained with samples collected from preparative HIMC experiments with 40 mL linear elution gradient. Lane 1: protein molecular weight makers; lane 2: standard P1PAL-10 (10 kDa); lane 3: standard HSA; lane 4: liquid-phase HSA PEGylation reaction mixture; lane 5: flow through peak FT; lane 6: first eluted peak E1; lane 7: second eluted peak E2.
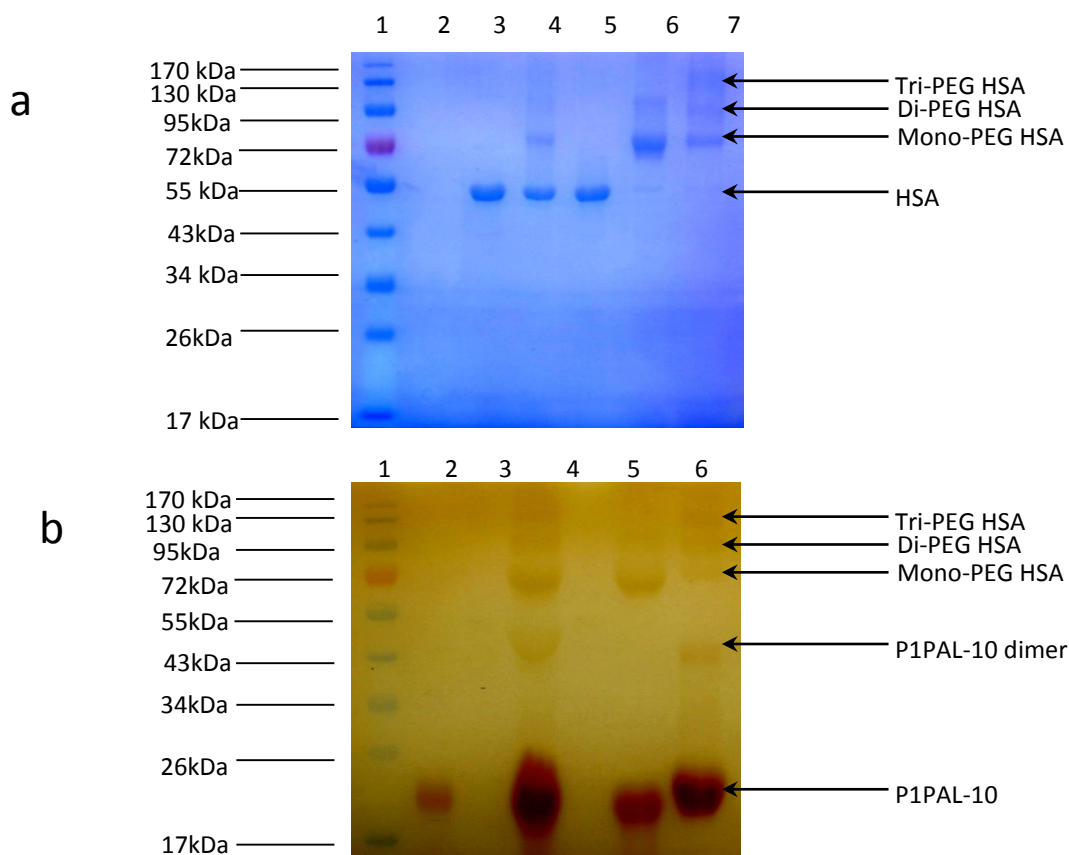
Figure 4 DLS measurements of the E1 sample (collected from preparative HIMC experiments with 40 mL linear elution gradient) at 25° C. (a) DLS Autocorrelation functions; (b) DLS Histograms. Three species with hydrodynamic radii of 4.9 nm, 33.6 nm and 717 nm respectively are shown in the histogram. The 33.6 and 717 nm species are most likely artifacts.
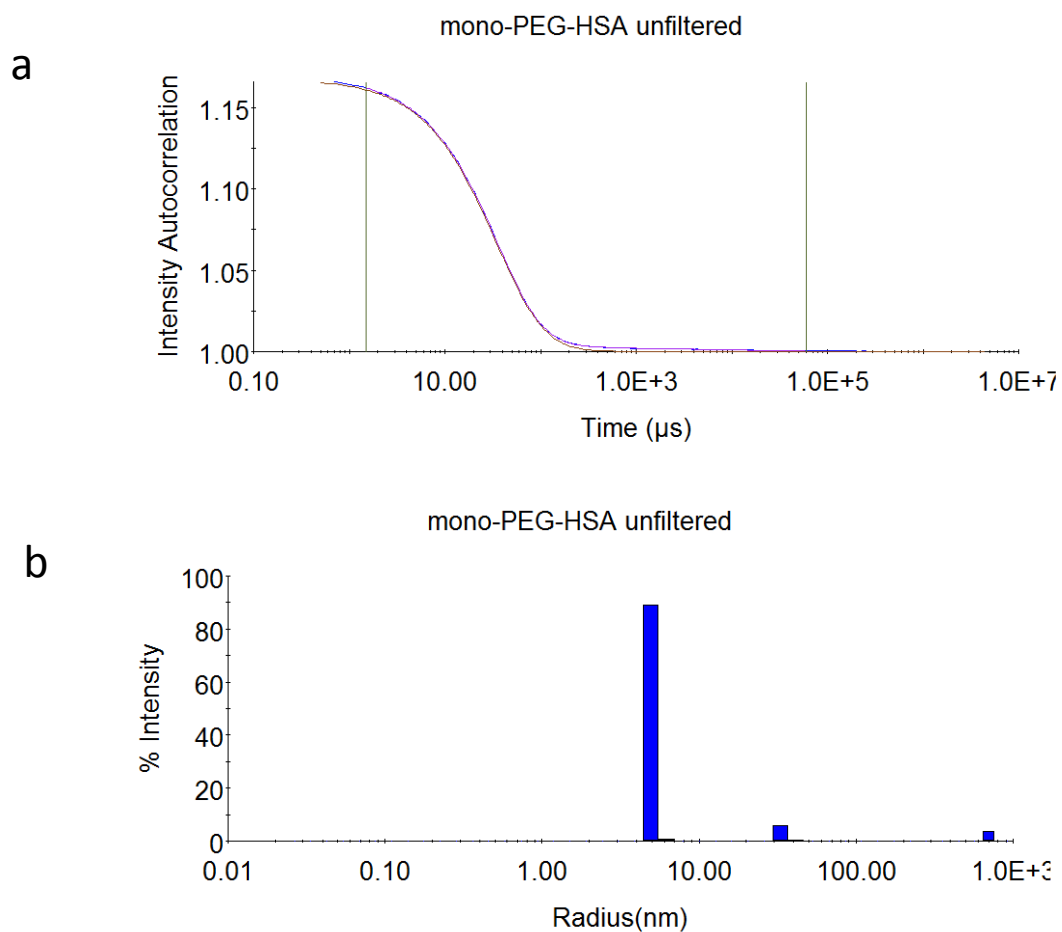
Figure 5 SEC-MALS measurements of the E1 sample (collected from preparative HIMC experiments with 40 mL linear elution gradient). (a) SEC-MALS chromatograms with defined peaks. Peak 1: 6.2 to 10.3 min; Peak 2: 9 to 9.8 min. (b) Molar mass distribution analysis. The calculated average molar mass for Peak 1 and 2 are 87.4 kDa and 73.4 kDa, respectively. (c) Protein conjugate analysis. The modifier molar mass was calculated to be 14.3 kDa, and that of protein portion was 62.2 kDa, and thus the total molar mass was 76.5 kDa.
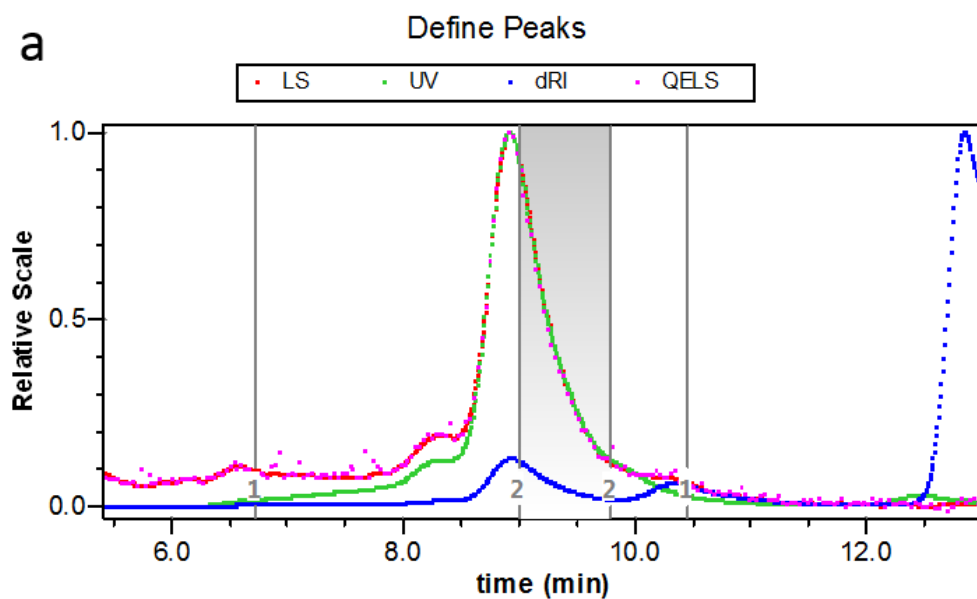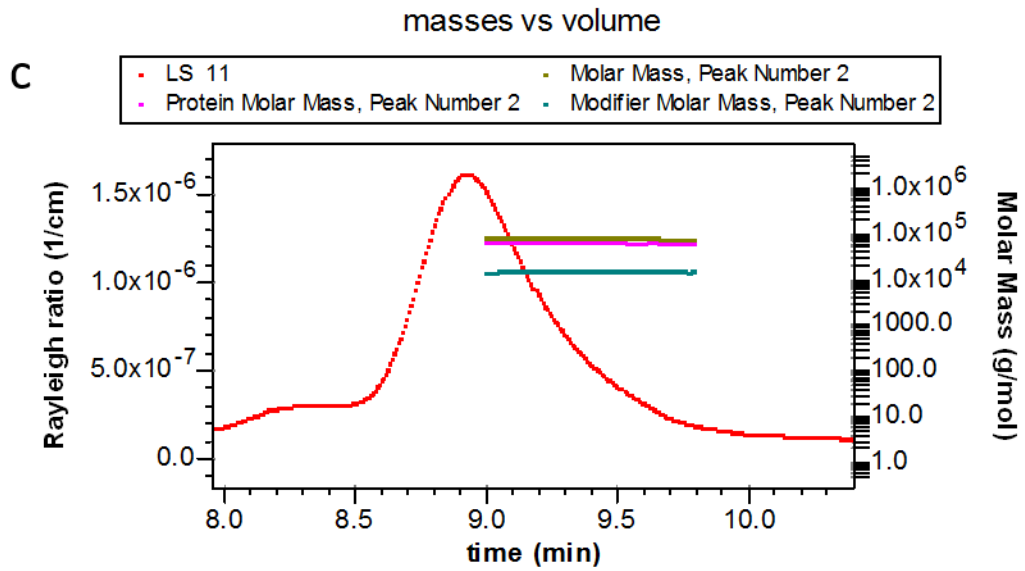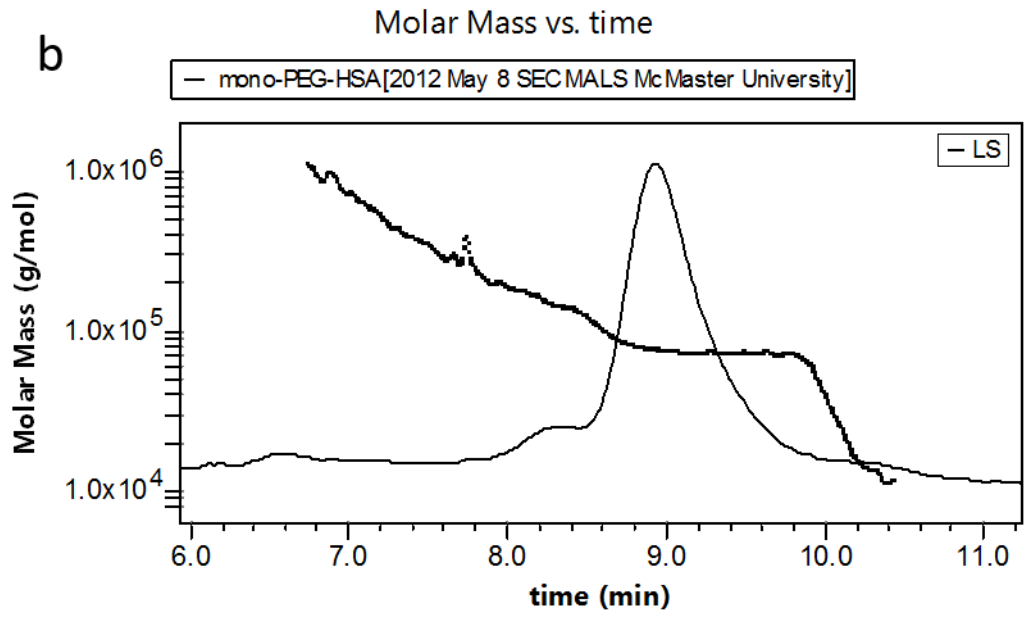
Figure 6 Analytical HIMC of HSA PEGylation reaction mixture carried out with a 10-disc membrane module (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack: 10; disc diameter: 8 mm; bed volume: 0.065 mL; flow rate: 0.8 mL/min; feed sample: 20 h HSA PEGylation reaction mixture blended with ammonium sulfate containing buffer; injected volume: 100 μL; ammonium sulfate concentration in binding buffer: 1.25 M; total protein injected: 50 μg; elution volumes with linear gradient from 0 to 100% eluting buffer: (a) Pure HSA injection with step elution; (b) 0 mL, (c) 10 mL, (d) 20 mL).
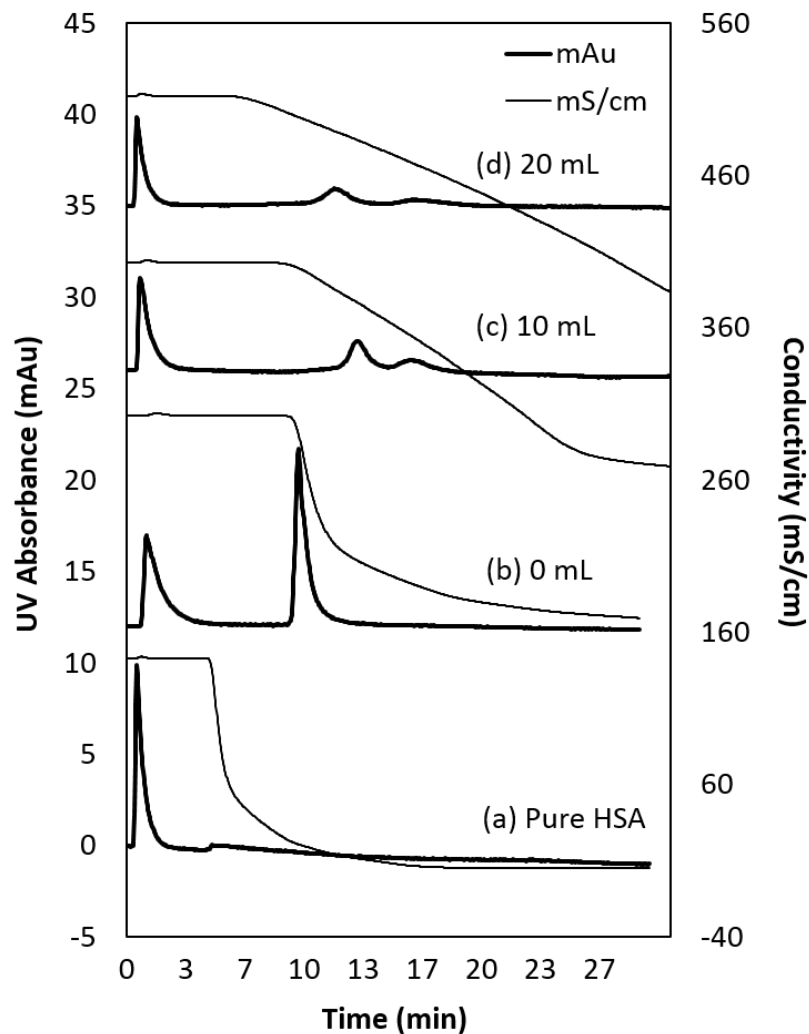
Figure 7 Analytical HIMC of the HSA PEGylation reaction mixture carried out with a 4-disc membrane module (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack: 4; disc diameter: 8 mm; bed volume: 0.025 mL; flow rate: 3 mL/min; elution volume with linear gradient from 0 to 100% eluting buffer: 10 ml; feed sample: 20 h HSA PEGylation reaction mixture blended with ammonium sulfate containing buffer; injected volume: 100 μL; ammonium sulfate concentration in binding buffer: 1.4 M; total protein amounts injected: (a) 12.5 μg, (b) 25 μg, (c) 50 μg).
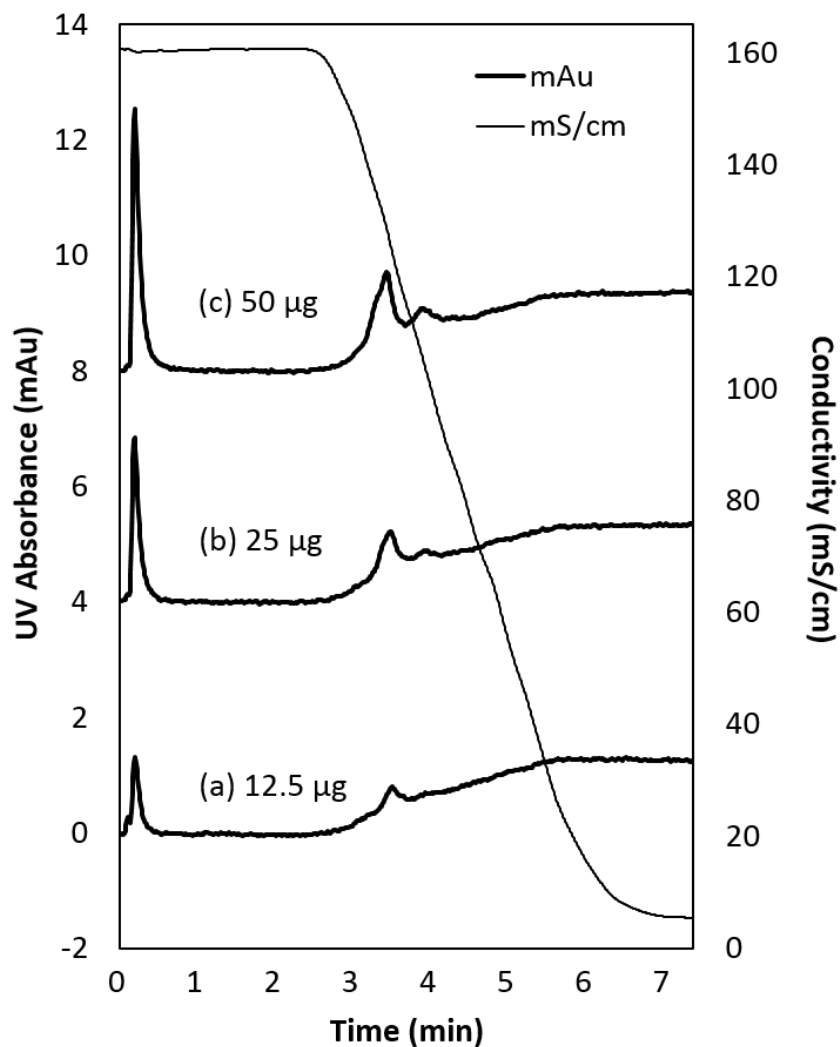
Chapter 4

Investigation of effects of pH and molar ratio on

PEGylation kinetics, conversion and specificity of

mono-PEGylation

4.1 Abstract

We studied the kinetics of a PEGylation reaction between a model protein lysozyme and PEG NHS ester. The effects of two crucial parameters – pH and molar ratio of PEG: protein on the conversion and specificity of mono-PEGylated protein were investigated. A trade-off between the two outcomes caused by an overall effect due to a combination of the two

parameters was observed. A high conversion is normally accompanied with a loss of specificity; and an increase in specificity is obtained with sacrificed conversion. The results are helpful in balancing the trade-off by controlling the two operating parameters.

4.2 Introduction

In the last two chapters, the N-terminal PEGylation chemistry with the PEG reagent functionalized with aldehyde groups is based on alkylation, which offers a stable linkage after Schiff base formation followed by sodium cyanoborohydride reduction (Roberts *et al.*, 2002). The Schiff base formation limits the reaction rate, requiring up to one day to complete the reaction (Veronese, 2001). Another chemistry using PEG reagent functionalized with active NHS ester group (i.e. N-hydroxysuccinimide ester as mentioned in Chapter 1) is known to be rapid (Nojima *et al.*, 2009), which is superior from the perspective of manufacture in industrial scale because reaction time is an important factor in determining the production rate. However, greater reactivity and shorter reaction time may result in difficult control of the reaction. Therefore, studying the parameters that affect the reaction kinetics and understanding their effects on the conversion and specificity of the reaction are very essential. The PEG reagent with NHS ester group is actually the most used acylating agents for protein PEGylation (Roberts *et al.*, 2002). However, few detailed reports on PEGylation reaction using this chemistry were seen. In this chapter, two crucial parameters (i.e. pH value and molar ratio of PEG: protein) that affect the conversion and specificity of PEGylation reaction with PEG NHS ester were examined.

As shown in Figure 1, the conjugation is based on acylation producing stable amide

linkages in the pH range of 7 to 9, and the NHS leaving group is replaced by the protein (Nojima *et al.*, 2009, Roberts *et al.*, 2002). N-terminal α-amino group (pKa 7.6-8) and ε-amino group on lysine residues (pKa 9.3-9.5) are available amino acid groups for PEG conjugation. It is known that pH value determines nucleophilicity of the nucleophiles (i.e. the amino acid groups) (Roberts *et al.*, 2002). The nucleophilic attack takes place when the operating pH is near or higher than the pKa value of the amino acid group (Roberts *et al.*, 2002). Therefore, the NHS ester group on PEG should preferentially react to the α-amino group at a pH value near or above the pKa of α-amino group and below the pKa of ε-amino group, which in turn produce mainly mono-PEGylated protein. A higher pH than the pKa of ε-amino group would facilitate the nucleophilic attack by the abundant ε-amino groups, which would theoretically results in the formation of high-PEGylated proteins. In consequence, pH would have significant influence on the reaction.

In most cases, PEG is added with an excess molar ratio to protein for the purpose of converting the relatively costly native protein as much as possible or due to the issues associated with some PEG reagents themselves (e.g. fast hydrolysis) (Fee and Van Alstine, 2006). However, it has been reported that the excess of PEG can result in the formation of diverse PEGylated proteins (Klenkler and Sheardown, 2006). That is because PEG reagents are usually designed to be mono-functional, i.e. one PEG molecule can be attached to only one protein molecule, whereas one protein molecule can react with several PEG chains at its various conjugation sites. Therefore, it could be presumed that an excess of protein would increase the specificity of mono-PEGylated form but at the cost of low conversion and wastage of expansive raw materials.

Individual effect of each parameter on the reaction is predictable from the above discussion, but the prediction for a combination of both parameters may not be straightforward. For example, a low conversion obtained at a low molar ratio of PEG: protein might be compensated by increasing pH value which can speed up the reaction; at a low pH (i.e. relatively slow reaction), a high molar ratio of PEG: protein may not lead to significant amount of high-PEGylated proteins. The situation becomes complicated at various combinations of the two parameters. In this chapter, the individual and combined effects of these two parameters on reaction kinetics and the outcomes (i.e. conversion and specificity of mono-PEGylated protein) were examined. With such information, the operating conditions would be able to be optimized to obtain satisfied conversion and specificity of mono-PEGylated protein, and in consequence to reduce cost by saving expansive raw materials and making downstream purification easier.

In this study, methoxy-PEG-$(CH_2)_5$COO-NHS (5kDa PEG equivalent) was used to PEGylate the model protein - lysozyme (MW=14100, pI=11) in liquid phase batch reaction. NHS ester reactive group is known to be very susceptible to hydrolysis (Roberts *et al.*, 2002). By increasing the distance between this group and the last PEG ether, its hydrolysis half-life could be improved dramatically (Roberts *et al.*, 2002). For example, a PEG-NHS ester with three ether groups in between has a hydrolysis half-life of 23 h, while that of a PEG-NHS ester with only one ether group in between is only 0.75 h (Harris *et al.*, 1995). Therefore, the PEG-NHS ester used in this work is inferred to have a hydrolysis half-life way longer than 23 h.

4.3 Materials and methods

4.3.1 Materials

Ammonium persulfate (A3678), 30% acylamide solution (A3699), bromophenol blue (B0126), Brilliant Blue R concentrate (B8647), glycerol (G2025), 25% glutaraldehyde solution (G6257), glycine (G8898), lysozyme (L6876), sodium dodecyl sulfate (L3771), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), sodium hydroxide (S5881), sodium chloride (S7653), Trizma base (T1503), Trizma-hydrochloride (T3253), N,N,N′,N′-tetramethylethylenediamine (T9281), DL-dithiothreitol (43817), 70% perchloric acid (77227), barium chloride (202738), hydrochloric acid (258148), and iodine (326143) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Acetic acid (1000-1) and methanol (6700-1) were purchased from Caledon Laboratories LTD., Georgetown, ON, Canada. Potassium iodide (74210-140) was purchased from Anachemia, Montreal, QC, Canada. Methoxy-PEG-$(CH_2)_5$COO-NHS (5 kDa, catalog number SUNBRIGHT ME-050HS) was purchased from NOF Corporation, Tokyo, Japan. Purified water (18.2 MΩ cm) was obtained from a SIMPLICITY 185 water purification unit (Millipore, Molsheim, France) for preparation of all test and buffer solutions. Amicon® Ultra-4 centrifugal filters (3 kDa MWCO, UFC800324) purchased from EMD Millipore Co., Billerica, MA, USA were used for concentrating and desalting samples. Hydrophilized PVDF membrane (0.22 µm, GVWP) purchased from Millipore (Billerica, MA, USA) were used for hydrophobic interaction membrane chromatography of liquid phase PEGylation mixtures.

4.3.2 Effect of pH on kinetics of PEGylation

PEGylation reactions were carried out using 3 mL reaction mixture consisting of a fixed lysozyme concentration of 1 mg/mL and a fixed molar ratio of PEG: protein of 4:1, at pH of 7, 7.5, and 8, at room temperature (22±1℃), with constant stirring. The reaction media contained 100 mM sodium phosphate and 150 mM sodium chloride. For each pH, seven reaction mixtures were prepared to carry out the reactions at seven different durations (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 24 h). The reaction mixtures were quenched by adding hydroxylamide hydrochloride solution to make its final concentration of 50 mM. All quenched reaction mixtures were processed using centrifugal filters to remove low molecular weight species and to enhance concentration. The resultant reaction mixtures were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and hydrophobic interaction membrane chromatography (HIMC). The experiments for pH 7.5 and 8 were repeated for three times.

4.3.3 Effect of molar ratio on kinetics of PEGylation

PEGylation reactions were conducted in a similar way to the above under different operating conditions. The pH was fixed at 7.5 and a molar ratio of PEG: lysozyme of 2:1 was tested for the same seven durations as above. This set of reactions was used for the comparison with the set described above which was carried out with a molar ratio of 4:1 at pH 7.5. In addition, 30 min reactions at molar ratios of 2:1 and 8:1 were carried out at three pH values, for the comparison with the 30 min reactions carried out at a molar ratio of 4:1 mentioned in the above paragraph.

4.3.4 Analysis of PEGylation reaction mixtures using SDS-PAGE

To verify the components in the reaction mixtures, SDS-PAGE experiments (Laemmli, 1970) were carried out with an equal amount of total protein (~6 μg) loaded onto 12.5% non-reducing gels by using a miniVE vertical electrophoresis system (80-6418-77) purchased from GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada. Coomassie Brilliant Blue R dye was used to stain the gel for visualization of protein-containing bands. The visualization of PEG-containing bands was made by the following protocol (Kurfurst, 1992): sequentially soaking the gel in 50 mL of 5% glutaraldehyde solution, then 20 mL of 0.1 M perchloric acid, and finally a mixture of 5 mL 5% barium chloride and 2 mL 0.1 M iodine/potassium iodide. Each soak took 15 min.

4.3.5 Separation and analysis of PEGylation reaction mixtures using HIMC

Each PEGylation reaction mixture after centrifugation was fractionated using HIMC (Yu *et al*., 2010) with a stack of hydrophilized PVDF membranes. The composition of each mixture was quantified by the software - PrimeView based on the area integration of individual chromatographic peak which corresponds to one component present in the mixture. The working principle of HIMC was described in Chapter 2. The membranes are environment-responsive, becoming hydrophobic as lyotropic salt is present while revert back to hydrophilic when the salt is removed. PEG is hydrophilic in nature, but becomes mildly hydrophobic in the presence of lyotropic salt as well. Therefore, a PEG-containing species could have hydrophobic interaction with the membrane when a lyotropic salt is

present. The fractionation of different components in a reaction mixture (i.e. unmodified protein, quenched PEG, mono-, di-, tri-, tetra- and/or high-PEGylated protein) depends on the difference in their apparent hydrophobicity (i.e. hydrophobicity in the presence of salt). A greater number of PEG chains attached to a protein results in a higher apparent hydrophobicity of the protein, i.e. unmodified protein with no PEG attached is the least hydrophobic form, and followed by mono-, di-, tri-, tetra- and/or high-PEGylated forms. As described in Chapter 2, during a HIMC experiment, unmodified lysozyme flow through the membrane stack without any interaction under the operating condition, whereas all PEGylated forms are bound on the membranes. The bound PEGylated species are eluted out in order of increasing apparent hydrophobicity by lowering the salt concentration in a gradient manner.

In this work, the eluting buffer contained 100 mM sodium phosphate at pH 7. The binding buffer with an ammonium sulfate concentration of 1.4 M was prepared in eluting buffer and adjusted to pH 7. A membrane stack having 15 membrane discs with a diameter of 18 mm was integrated with an AKTA Prime liquid chromatography system (GE Healthcare Bio-Sciences, QC, Canada). The separation was executed at 1 mL/min with 500 μL of feed sample containing about 250 μg of total protein injected. A 50 mL gradient from 0% to 100% of eluting buffer was used for elution of bound PEGylated proteins. The conversion and specificity of mono-PEGylation were calculated based on the reaction mixture composition quantified by PrimeView.

4.4 Results and discussion

The PEGylation reaction mixtures prepared at different pH values (i.e. 7, 7.5, and 8) with various durations at fixed molar ratio of PEG: lysozyme of 4:1 were analyzed using SDS-PAGE to identify the components. **Figures 2** and **3** show two example gels obtained from the reaction mixtures carried out at pH 8. They were stained with Coomassie blue and iodine to show protein- and PEG-containing components, respectively. Both figures have protein molecular weight markers in lane 1 and duplicate lanes of 3 to 9 which contained the reaction mixtures of different durations of 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 24 h, respectively. Lane 2 consisted of pure lysozyme and pure PEG 5 kDa as shown in Figures 1 and 2, respectively. Lane 3 (containing the 5-min reaction mixture) has mainly mono-PEG lysozyme (band between 15 and 25 kDa) and a very small amount of di-PEGylated lysozyme (band between 25 and 35 kDa) as shown in both figures. There are also unmodified lysozyme (band corresponding to the pure lysozyme band in lane 2) as shown in Figure 2 and quenched PEG 5 kDa (band around 10 kDa) as shown in Figure 3. The amount of di-PEG form was increased greatly from a 5-min reaction to a 15-min reaction, which can be judged by the intensity difference of the di-PEG form bands in lanes 3 and 4. Tri-PEG form (band between 55 and 70 kDa) started to arise in lane 4 (containing the 15-min reaction mixture). Lanes 5 to 9 contained faint bands of tetra-PEG lysozyme (band between 70 and 100 kDa) besides the bands of unmodified, mono-, di- and tri-PEG forms of lysozyme as well as quenched PEG. Generally speaking, the longer the reaction time, the more complex the reaction mixtures would be, in terms of the relative amount of high-PEGylated forms of lysozyme synthesized and the number of PEG chains attached. In

addition, it is shown that the reaction took place faster within 30 min, which can be seen from the obvious change, such as the shrink of unmodified lysozyme bands, the increasing intensity of mono- and di-PEG lysozyme bands from lanes 3 to 5, and the appearance of tri- and tetra-PEG forms in lanes 4 and 5 respectively. The subtle change of the bands from lanes 6 to 9 revealed that the compositions of the reaction mixtures did not vary significantly, i.e. the reactions slowed down after 30 min.

All the PEGylation reaction mixtures were analyzed using HIMC for the quantitative analysis of conversion and specificity of mono-PEGylation. **Figure 4** shows examples of chromatograms obtained from HIMC of the same PEGylation reaction mixtures described above (pH 8; molar ratio of PEG: lysozyme of 4:1; different durations as indicated in the figure). As the working principles of HIMC explained in the previous paper, we could conclude that the peak appeared at the beginning in each chromatogram contained unmodified lysozyme which flowed through the membrane stack without interactions at the operating conditions, while at the same time all the PEGylated lysozyme bound to the membrane based on hydrophobic interaction. The bound species started to be eluted out around 35 mL of effluent volume by decreasing salt concentration in a gradient manner, in order of increasing apparent hydrophobicity, i.e. mono-PEG form first, followed by di-, tri-, and tetra-PEG forms if present, as shown in the figure. The HIMC results were consistent with the SDS-PAGE results, i.e. the composition change from 5-min to 30-min reaction mixtures was significant which can be seen from the fast growth of the peaks of different PEGylated forms within 30 min, but it became less after. The quantitative analysis of conversion and specificity of mono-PEGylation and conversion of high-PEGylation were

made based on the peak area integration in each chromatogram using PrimeView software, and some of them were shown as plots in Figures 4 to 7.

Figure 5 shows the plot of mono-PEGylation conversion against reaction time obtained from HIMC experiments for the reactions carried out at three pH values and fixed molar ratio of PEG: lysozyme of 4:1. The inset at the bottom is the enlarged figure for the reactions with durations below 250 min. Initially, the conversion of mono-PEGylation increased extremely rapidly for all three pH values, and then leveled off after a certain point at which point the conversion almost reached the average value of the level-off region. However, the time spent to reach the certain point differed with respect to pH values. The higher the pH value, the shorter was it, meaning the faster was the reaction. For example, the pH 8 reaction reached a mono-PEGylation conversion factor of 0.366 at 15 min which almost approached an average (0.383) of the conversion factors at 15 min, 30 min, 1 h, 2 h, 4 h, and 24 h. The pH 7 and 7.5 reactions reached the mono-PEGylation conversion factors of 0.341 and 0.387 at 2 h and 1 h with the average values of 0.341 and 0.398, respectively. The pH 7.5 and 8 reactions have close mono-PEGylation conversion factors within the level-off region, which were higher than those of pH 7 reactions. This is because the nucleophilicity of amino acid groups were promoted by increasing the pH to near or higher than the pKa value of N-terminal α-amino group (i.e. 7.8).

Figure 6 shows the plot of mono-PEGylation specificity against time for the same reaction mixtures as above. The inset is the enlarged figure for the reactions with durations below 250 min. Generally speaking, the specificity of mono-PEGylation decreased greatly with increasing durations due to the formation of large amount of high-PEGylated

lysozyme. At the beginning of the reactions, mainly mono-PEGylated lysozyme was synthesized, resulting in high specificity factors in the range of 0.866-0.966 for 5-min reactions at all three pH; after that, the specificity factor of pH 8 reactions decreased the fastest down to 0.500 in the 1 h reaction, and then the decrease slowed down to reach a specificity factor of 0.452 in a 24 h reaction; the specificity factor reduction for pH 7.5 reactions leveled off at 4 h and reached an average of 0.565 (an average specificity factor of 4 h and 24 h reactions); the reduction was the smallest for pH 7 reactions from 0.915 (specificity factor of 5-min reaction) to 0.667 (an average specificity factor of 4-h and 24-h reactions). A lower specificity factor of mono-PEGylation means a lower proportion of mono-PEGylated lysozyme present in the reaction mixture and thus a higher proportion of high-PEGylated forms of lysozyme synthesized, i.e. pH 8 reaction mixtures contained the greatest amount of high-PEGylated lysozyme of the three. Therefore, from both Figure 5 and 6, it can be seen that not only did the mono-PEGylation take place faster at pH 8 than other pH, but also the high-PEGylation reactions.

Figure 7 and **8** show the plot of mono-PEGylation conversion and specificity against reaction time obtained from the HIMC experiments for the reactions carried out at two molar ratios of PEG: lysozyme of 2:1 and 4:1 with a fixed pH of 7.5 for various durations (i.e. 5 min, 15 min, 30 min, 1 h, 2 h, and 4 h), respectively. The one with the molar ratio of 4:1 which has been shown in Figure 4 is shown here again to demonstrate the influence of molar ratio on mono-PEGylation kinetics. The conversion factors obtained with the molar ratio of 4:1 are generally higher than those with molar ratio of 2:1, i.e. higher molar ratios resulted in greater mono-PEGylation conversion factors. As discussed in Figure 5, the

mono-PEGylation conversion profile with the molar ratio of 4:1 reached the level-off average conversion factor at 1 h, while the lower molar ratio conversion profile reached this value at 4 h eventually. It means that mono-PEGylation reaction could be accelerated to reach steady state by increasing molar ratios of PEG: protein. Although mono-PEGylation conversion factors with the molar ratio of 2:1 were lower, the corresponding specificity factors were greater. This implies that although mono-PEGylation took place more slowly at a lower molar ratio, high-PEGylation occurred even more slowly. The increasing gap between the two specificity profiles indicates that the high-PEGylation reaction rate with a high molar ratio is greater than that with a low molar ratio.

Table 1 summarizes the conversion factors of all PEGylated forms and specificity factors of mono-PEGylated form obtained from the PEGylation reactions carried out at three pH values and three molar ratios for a fixed duration (i.e. 30 min). The values in the brackets were the error percentages calculated based on the triplicate experiments done for the corresponding conditions. Some results (i.e. the conversion and specificity with the molar ratio of 4:1 at three pH values and with the molar ratio of 2:1 at pH 7.5) have been shown in previous figures. They are shown here for explanation of the combined effects of pH and molar ratio on the conversion and specificity of mono-PEGylation. An increase in molar ratio of PEG: lysozyme means a larger amount of PEG molecules are present in the reaction mixture, resulting in an increase in possibility of collisions between PEG and lysozyme molecules such that more PEGylation reactions occur. However, that if they are towards either mono-PEGylation or high-PEGylation is dependent on both pH and molar ratio. At pH 7.5, an increase in conversion and a decrease in specificity were observed throughout

the increase of molar ratios from 2:1 to 8:1. Some variations in conversion and specificity change with the increasing molar ratios were observed for pH 7 and 8. Unlike pH 7.5, pH 7 resulted in an increase in specificity as molar ratio increased from 2:1 to 4:1, and pH 8 resulted in a decrease in conversion as molar ratio increased from 4:1 to 8:1. For the former, the magnitude of increase in specificity was even less than 2% which may be due to errors in experimental operation or peak area integration, so this might not influence the conclusion to a significant extent that the mono-PEGylation specificity increases with molar ratio at a fixed pH. The latter had a big decrease in mono-PEGylation conversion factors from 0.352 to 0.315 at pH 8, so we can conclude that the more PEGylation reactions caused by molar ratio increase was towards mono-PEGylation at pH 7 and 7.5 more, but towards high-PEGylation more at pH 8. As mentioned previously, pH increase can improve the nucleophilicity of the abundant ε-amino groups, causing more formation of high-PEGylated protein. That explains why the mono-PEGylation specificity decreased as pH value increased at a fixed molar ratio. The specificity was generally higher at pH 7 and 7.5 but much lower at pH 8 no matter which molar ratio was it. A throughout increase in mono-PEGylation conversion as pH increased took place at molar ratios of 2:1 and 4:1, while an increase-to-decrease change was observed for the molar ratio of 8:1. This implies that at lower molar ratios of 2:1 and 4:1, an increase in pH has a positive effect on mono-PEGylation conversion. However, at molar ratio of 8:1, an increase from pH 7 to 7.5 can still improve the mono-PEGylation conversion, but a further increase to pH 8 exerted a negative influence on conversion. The above discussion suggests that the conversion and specificity change is due to a combination of pH and molar ratio.

4.5 Conclusions

From all the above discussion, both pH and molar ratio of PEG: protein exerted significant influence in reaction kinetics and conversion and specificity of mono-PEGylated protein. Generally speaking, pH increase accelerated the reaction by increasing the reactivity such that more protein was converted not only to mono-PEGylated form but also to by-products, which consequently resulted in decreasing specificity. A positive effect of pH on conversion was shown at lower molar ratio (i.e. 2:1 and 4:1). Once the molar ratio went up to 8:1, the pH increase exerted a negative effect on conversion. Regarding the effect of molar ratio, an increase in molar ratio made the reaction faster and resulted in increasing conversion and decreasing specificity. However, this conclusion only held well for the effect on conversion at lower pH (i.e. 7 and 7.5). The conversion became even worse by increasing the molar ratio from 4:1 to 8:1 at pH 8. Therefore, more attention has to be paid when operating at high pH (e.g. 8) and high molar ratio of PEG: protein (e.g. 8:1). The overall effect on conversion and specificity are due to a combination of both parameters.

It is worth to notice that there is always a trade-off between the conversion and specificity of mono-PEGylated protein. The conversion can be generally enhanced by increasing pH or molar ratio but accompanied with loss in specificity; while a high specificity could be achieved by lowering pH or molar ratio but sacrificing conversion. A high conversion with a low specificity requires complicated purification process to obtain reasonably pure product; while high specificity with a low conversion means the reaction is inefficient and there is wastage of expansive raw materials. For industry to decide where the balance of the trade-off should be, a comprehensive investigation on the total cost may need to be

done. To improve this situation, a novel reactor has been developed for both conversion and specificity enhancement. The control of operating parameters was based on the above information. The details will be discussed in Chapter 6.

4.6 Acknowledgement

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4.8 Figures

Figure 1. PEGylation reaction using PEG NHS esters.
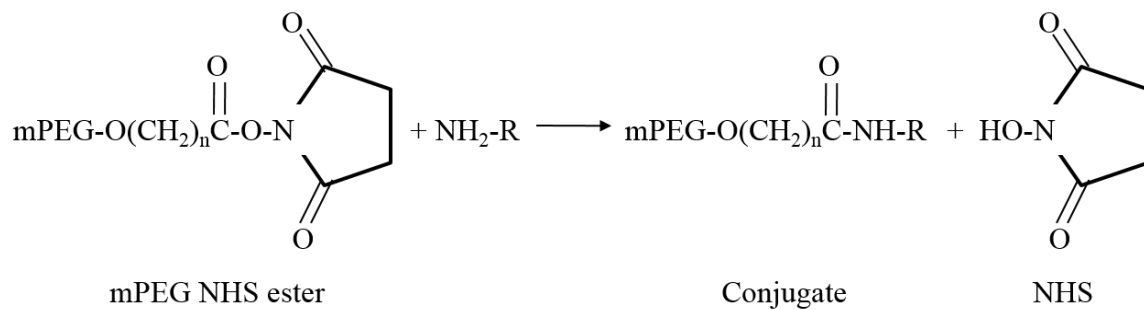
Figure 2. Coomassie blue stained gel for liquid-phase PEGylation carried out at pH 8 and molar ratio of PEG: lysozyme of 4:1 at various durations (Lane 1: Protein molecular weight marker; lane 2; standard lysozyme; lane 3: 5 min duration; lane 4: 15 min duration; lane 5: 30 min duration; lane 6: 1 h duration; lane 7: 2 h duration; lane 8: 4 h duration; lane 9: 24 h)
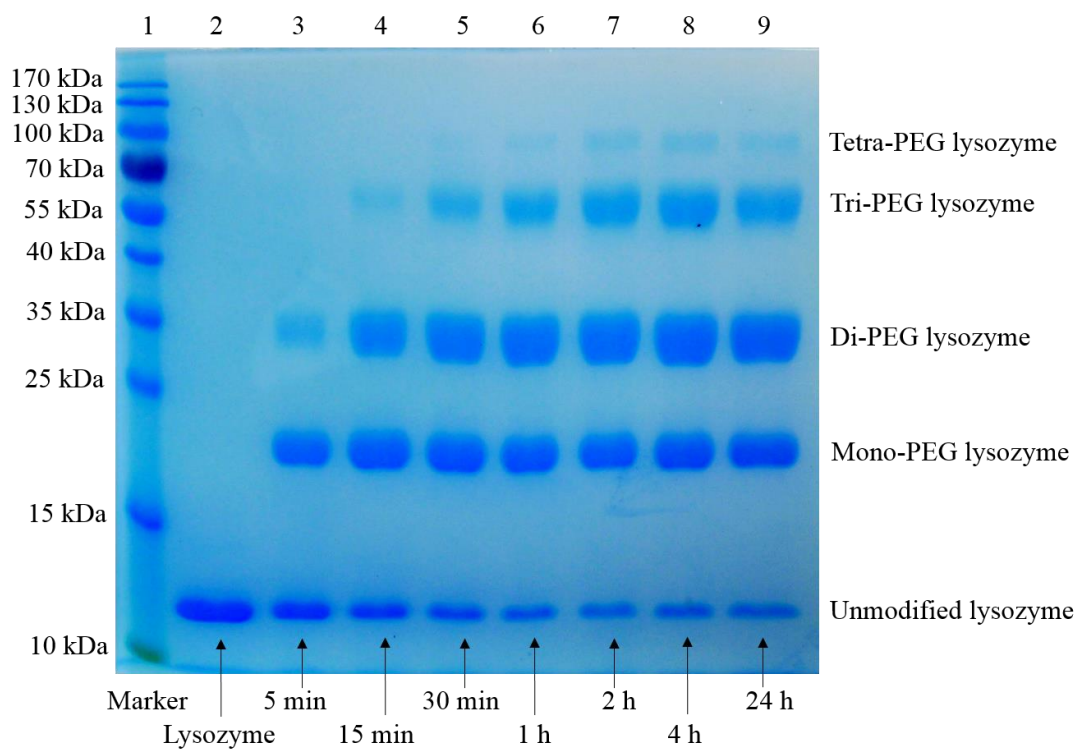
Figure 3. PEG stained gel for liquid-phase PEGylation carried out at pH 8 and molar ratio of PEG: lysozyme of 4:1 at various durations (Lane 1: protein molecular weight marker; lane 2; standard lysozyme; lane 3: 5 min duration; lane 4: 15 min duration; lane 5: 30 min duration; lane 6: 1 h duration; lane 7: 2 h duration; lane 8: 4 h duration; lane 9: 24 h)

Figure 4. HIMC analysis of liquid-phase PEGylation carried out at pH 8 and molar ratio of PEG: lysozyme of 4:1 at different durations. (membrane: hydrophilized PVDF; pore size: 0.22 μm; number of membrane discs in stack: 15; disc diameter: 18 mm; flow rate: 1 mL/min; elution: 50 mL linear gradient from 0 to 100% eluting buffer; feed sample: liquid-phase PEGylation reaction mixtures at different durations blended with ammonium sulfate containing buffer; injected volume: 0.5 mL; ammonium sulfate concentration in binding buffer: 1.4 M).

Figure 5. Effect of pH (7, 7.5 and 8) on reaction kinetics and mono-PEGylation conversion (i.e. conversion of mono-PEGylated protein) at fixed molar ratio of PEG: protein of 4:1. Reaction durations are 5, 15, 30 min, 1, 2, 4 and 24 h.

Figure 6. Effect of pH (7, 7.5 and 8) on reaction kinetics and mono-PEGylation specificity (i.e. specificity of mono-PEGylated protein) at fixed molar ratio of PEG: protein of 4:1. Reaction durations are 5, 15, 30 min, 1, 2, 4 and 24 h.

Figure 7. Effect of molar ratio of PEG: protein (2:1 and 4:1) on reaction kinetics and mono-PEGylation conversion (i.e. conversion of mono-PEGylated protein) at fixed pH 7.5. Reaction durations are 5, 15, 30 min, 1, 2 and 4 h.

Figure 8. Effect of molar ratio of PEG: protein (2:1 and 4:1) on reaction kinetics and mono-PEGylation specificity (i.e. specificity of mono-PEGylated protein) at fixed pH 7.5. Reaction durations are 5, 15, 30 min, 1, 2 and 4 h.

4.9 Tables

Table 1. Total effects of molar ratio and pH on reaction kinetics and conversion and specificity of mono-PEGylation at fixed duration of 30 min (The percentage values shown in the brackets are error ranges obtained from repeated experiments.)

pH	Molar ratio	Mono-PEGylation conversion	Di-PEGylation conversion	Tri-PEGylation conversion	Tetra-PEGylation conversion	Overall conversion	Specificity of mono-PEGylation
7	2:1	0.023	0.002	-	-	0.025	0.920
7	4:1	0.176	0.012	-	-	0.188	0.936
7	8:1	0.217	0.043	0.001	-	0.261	0.831
7.5	2:1	0.201	0.023	-	-	0.224	0.897
7.5	4:1	0.286 (±7.34%)	0.050 (±38.0%)	0.0014 (±12.1%)	-	0.337 (±12.2%)	0.852 (±4.93%)
7.5	8:1	0.427	0.244	0.041	0.001	0.713	0.599
8	2:1	0.325	0.081	0.003	-	0.409	0.795
8	4:1	0.352 (±4.07%)	0.181 (±5.64%)	0.0298 (±21.3%)	0.0020 (±3.96%)	0.564 (±8.46%)	0.633 (±11.4%)
8	8:1	0.315	0.369	0.144	0.021	0.849	0.371

Chapter 5

Integrated solid-phase synthesis and purification of

PEGylated protein

This chapter is organized based on a paper published in Biomacromolecules, 12:2772-2779 (2011) by Xiaojiao Shang, Deqiang Yu and Raja Ghosh. Dr. Deqiang Yu was involved in the start-up stage of this project. All data were generated by Xiaojiao Shang. Copyright © 2011 ACS.

Integrated Solid-Phase Synthesis and Purification of PEGylated Protein

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ABSTRACT: We describe an integrated method for solid-phase protein PEGylation and the purification of mono-PEGylated protein thus synthesized. Lysozyme was used as model protein in this study. Methoxy-polyethyleneglycol propionaldehyde (or m-PEG propionaldehyde) was first immobilized on a stack of microporous hydrophobic interaction membranes housed in a module. The membrane-bound m-PEG propionaldehyde was then contacted with lysozyme solution, which also contained sodium cyanoborohydride as a reducing agent. The PEGylated lysozyme thus synthesized remained attached to the membrane, whereas unreacted protein could easily be removed from the module. PEGylated protein was then eluted from the membrane in a partially purified form using salt-free buffer. Two separate steps were thus integrated into a single process: protein PEGylation, followed by purification of mono-PEGylated protein. This solid-phase method is likely to be suitable for PEGylating any protein because it is based on the immobilization of the activated PEG and not the protein being PEGylated.

Key:
- m-PEG propionaldehyde
- m-PEG propionaldehyde (collapsed PEG)
- Protein
- Mono-PEGylated protein (collapsed PEG)
- Mono-PEGylated protein
- HIC membrane
- Low salt buffer
- High salt buffer
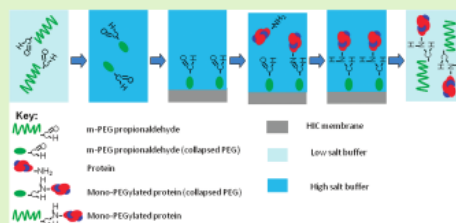
■ INTRODUCTION

PEGylation refers to the family of chemical reactions that result in the covalent attachment of polyethylene glycol (or PEG) to a protein.[1,2] PEGylation can improve certain properties of therapeutic proteins.[2,3] For instance, the in vivo half life of a protein drug could be increased by increasing its size and thereby decreasing its renal elimination rate.[2] PEG is a stable, hydrophilic, neutral, nonimmunogenic, and nontoxic polymer, which tends to form a protective layer around the protein. Other potential benefits of PEGylation include increase in solubility and stability, decrease in immunogenicity, and reduced degradation by proteolytic enzymes.[2,3]

PEG is itself not reactive and therefore needs to be modified by the addition of groups such as aldehyde to enable its attachment to proteins. Control of conjugation sites on a protein molecule and thereby the number of PEG adducts is one of the main challenges in a PEGylation reaction. A commonly used "random" PEGylation strategy is to tarzget the ε-amino groups on lysine residues present in the protein.[4] This has been used to produce numerous commercialized PEGylated biopharmaceuticals such as Adagen (PEG-adenosine deaminase),[5] PegIntron (PEG-interferon α2b),[6] and Pegasys (PEG-interferon α2a).[7] Therapeutic protein drugs are required in as homogeneous a form as possible. Because of the abundance of lysine residues present in most proteins, "random" PEGylation results in the synthesis of complex mixtures of PEGylated forms, that is, mono-, di-, tri-, and higher-PEGylated forms as well as their positional isomers.[8] Purification of the desired PEGylated form (usually the mono-PEGylated protein) from such mixtures is difficult.[9] Moreover, the synthesis of undesired PEGylated forms represents

product loss. These problems can be addressed by using more specific reactions such as N-terminal PEGylation by reductive alkylation at low pH in the presence of a reducing agent such as sodium cyanoborohydride.[10−13] Under such reaction conditions, PEGylation at the N-terminal α-amino group (pK_a 7.6 to 8.0) is favored over the ε-amino groups of lysine residues (pK_a 10.0 to 10.2). This approach has been utilized to PEGylate epidermal growth factor (EGF),[14] granulocyte colony stimulating factor (G-CSF),[15] and interferon β-1b.[16] Some of the different strategies used for protein PEGylation have been reviewed by Gauthier and Klok.[17] More recently, Gauthier and Klok[18] have described an arginine-specific method for synthesizing mono-PEGylated proteins.

PEGylated proteins are generally synthesized by liquid-phase chemical reactions where reactants, product, and byproduct remain within the same homogeneous reaction mixture until separation. Therefore, even when reductive alkylation at low pH is carried out, synthesis of some undesirable PEGylated forms is inevitable.[19] The specificity of synthesis of mono-PEGylated protein could be increased by: (a) using a large excess of protein in the reactor, (b) controlled addition of the activated PEG into the reactor, and (c) constant removal of mono-PEGylated protein as it is synthesized.[19] An elegant approach for integrating liquid-phase protein PEGylation with purification using size-exclusion reaction chromatography (SERC) has been suggested by Fee.[20]
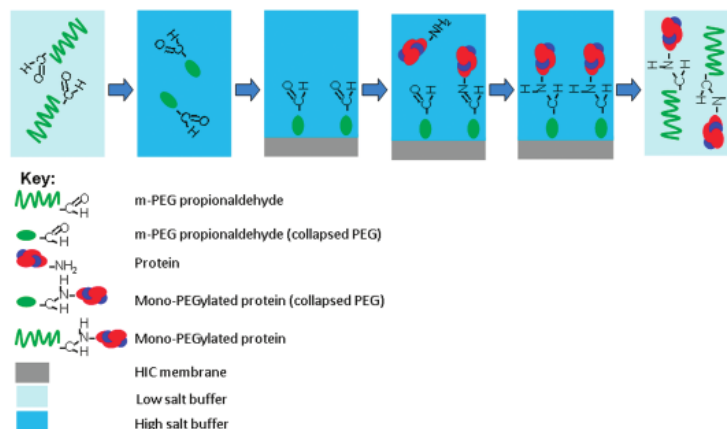
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Figure 1. Schematic diagram explaining the working principles and reactions involved in the integrated solid-phase protein PEGylation and purification method.

Alternatively, solid-phase reactions could be used for combining controlled protein PEGylation and purification.[21−26] This is carried out by immobilizing the protein on ion-exchange media held in a column, followed by flow of the activated PEG reagent through it. The PEGylated protein thus synthesized is then separated from the unreacted protein and undesirable PEGylated forms by gradient elution. This approach has been used by Monkarsh et al.[21] to produce PEGylated interferon α2a positional isomers, by Lee et al.[22,23] to PEGylate recombinant interferon α2a, by Suo et al.[24] to synthesize PEGylated human serum albumin (HSA) and staphylokinase, and by Suo et al.[26] to produce PEGylated hemoglobin. The higher site-specificity of PEGylation than in corresponding liquid-phase reactions reported in these studies has been attributed to factors such as steric hindrance.[22,23] However, proteins have a wide range of physicochemical properties. Therefore, one of the main limitations of solid-phase PEGylation based on protein immobilization is that the type of media required would be very specific to the protein being PEGylated. Moreover, the pH at which a protein is immobilized on ion-exchange media might not necessarily be suitable for PEGylation. A much better approach would therefore be to immobilize the activated PEG using a binding mechanism such as hydrophobic interaction, which is less sensitive to pH, followed by flow of protein solution through the solid phase.

In a recent paper, the fractionation by hydrophobic interaction membrane chromatography of different PEGylated forms (i.e., mono-, di-, and tri-) of model protein lysozyme has been discussed.[27] Phase transition of the PEG component of PEGylated lysozyme was carried out at room temperature using high ammonium sulfate concentration to transform it from its hydrophilic (or hydrated) form to a mildly hydrophobic (or collapsed) form.[28,29] It was observed that under such conditions PEGylated protein attached to a hydrophobic interaction membrane through its PEG component. We hypothesized that a similar approach could be used to immobilize activated PEG on a hydrophobic interaction membrane. The solid-phase PEGylation method developed based on this hypothesis is summarized in Figure 1.

Activated PEG is first converted to a mildly hydrophobic form by the addition of an appropriate lyotropic salt. It is then immobilized on hydrophobic interaction membrane, followed by contact with protein in the presence of a reducing agent. Finally, the membrane-bound PEGylated protein is eluted by decreasing salt concentration. In this study, lysozyme was used as model protein, and it was PEGylated at the N-terminus by a two-step reductive alkylation reaction using methoxy-polyethylene propionaldehyde (or m-PEG propionaldehyde). Sodium cyanoborohydride was used as reducing agent.

■ **MATERIALS AND METHODS**

Hydrophilized PVDF membrane (GVWP14250; 0.22 μm pore size) used as hydrophobic interaction membrane was purchased from Millipore, Billerica, MA. Sartobind S cation exchange membrane (94IEXS42-001, 275 μm thickness) was purchased from Sartorius, Goettingen, Germany. Lysozyme (L6876), Trizma base (T1503), glycine (G8898), sodium chloride (S7653), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), sodium cyanoborohydride (156159), ammonium sulfate (A4418), barium chloride (202738), iodine (326143), hydrochloric acid (258148), 25% glutaraldehyde solution (G6257), and 70% perchloric acid (77227) were purchased from Sigma-Aldrich, St. Louis, MO. Sodium acetate trihydrate (SX0255-1) and glacial acetic acid (AX0073-6) were purchased from EMD (Gibbstown, NJ). Potassium iodide (74210-140) was purchased from Anachemia, Montreal, QC, Canada. m-PEG propionaldehyde 5 KDa (P1PAL-5) was purchased from Sunbio, Anyang, South Korea. Purified water (18.2 MΩ cm) obtained from a DiamondTM NANOpure (Barnstead, Dubuque, IA) water purification unit was used to prepare all of the test solutions and buffers.

Liquid-phase lysozyme PEGylation was carried out using 100 mM sodium acetate buffer (pH 5.0) as reaction medium in small flasks (5 mL of reaction volume) at room temperature with constant stirring. The pH was chosen based on preliminary experiments that showed that it resulted in high selectivity of
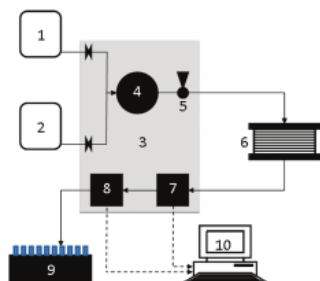
Figure 2. Experimental setup used for integrated protein PEGylation and purification (1: binding buffer reservoir, 2: eluting buffer reservoir, 3: liquid chromatography system, 4: pump, 5: sample injector, 6: membrane stack, 7: UV detector, 8: conductivity monitor, 9: fraction collector, and 10: computer for data logging).
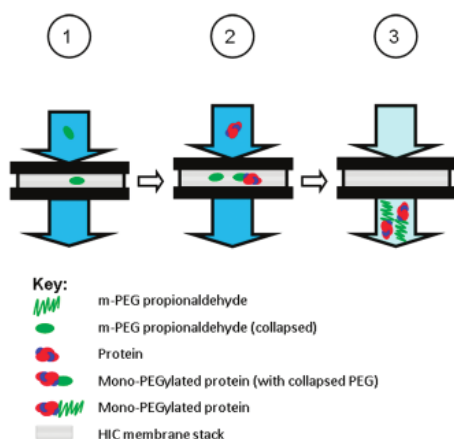
Figure 3. Sequence of events in integrated protein PEGylation and purification method (step 1: P1PAL-5 loading, step 2: lysozyme PEGylation, step 3: elution of PEGylated lysozyme).

synthesis of mono-PEGylated lysozyme, this being consistent with previous reports on protein PEGylation by reductive alkylation.[10−13] The reaction mixture consisted of 1 mg/mL lysozyme, P1PAL-5 (5:2 and 4:1 P1PAL-5/lysozyme molar ratios were examined), and 10 mM sodium cyanoborohydride. Reactions were carried out both in the presence and in the absence of 1.4 M ammonium sulfate for 50 and 100 min. Glycine solution was added to terminate the reaction, the resultant glycine concentration in the final mixture being 100 mM. The quenched reaction mixtures were desalted by centrifugal ultrafiltration (3 kDa MWCO) and analyzed by size exclusion chromatography (SEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The extent of PEGylation (expressed as percentage of PEGylation of lysozyme) in liquid-phase reaction was determined based on area under the curve data obtained by SEC analysis of samples collected from the reactor. The reaction rate was expressed as %PEGylation per unit time, which was obtained by dividing the %PEGylation value by total reaction time.

Solid-phase lysozyme PEGylation/purification was carried out using a custom-designed membrane module[30] which housed a stack of 30 hydrophilized PVDF membrane discs (18 mm diameter, 0.95 mL total bed volume). The module was integrated with an AKTA prime liquid chromatography system (GE Healthcare Biosciences, QC, Canada), as shown in Figure 2. Three types of buffers were employed in the integrated PEGylation/purification process: binding buffer (1.4 M ammonium sulfate in 100 mM sodium acetate buffer, pH 5.0), reaction buffer (10 mM sodium cyanoborohydride solution prepared in binding buffer), and eluting buffer (100 mM sodium acetate, pH 5.0). The sequence of events is summarized in Figure 3. Step 1: We injected 5 mL of P1PAL-5 solution prepared in binding buffer into the membrane module using a sample injector, followed by washing of unbound P1PAL-5 with binding buffer. Step 2: We injected 5 mL of lysozyme solution prepared in reaction buffer into the membrane module, followed by washing of excess protein with binding buffer. Step 3: Membrane-bound PEGylated lysozyme was recovered with eluting buffer using a 40 mL gradient. Steps 1 and 3 were carried out at 1.0 mL/min flow rate, whereas two different flow rates (0.1 and 1.0 mL/min) were examined for step 2. The presence of unreacted and PEGylated lysozyme in the effluent stream from the membrane module was monitored using an online UV detector, whereas the salt concentration was monitored using a conductivity meter. Samples collected during the experiment were quenched using glycine solution. These samples were desalted by centrifugal ultrafiltration and analyzed by SEC and SDS-PAGE. The extent of PEGylation in solid-phase reaction (expressed as the percentage of lysozyme injected in step 2 above that was PEGylated) was determined based on area under the curve data from the UV absorbance−volume profiles obtained during the PEGylation separation experiments. The reaction rate was expressed as %PEGylation per unit time, which was obtained by dividing the extent of PEGylation by the time required for step 2 described above.

Mono-PEGylated lysozyme obtained by the integrated solid-phase PEGylation/purification method was further polished by cation exchange chromatography using a stack of Sartobind S membrane (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The membrane stack (three discs having 20 mm diameter and 0.250 mL of total bed volume) was housed within a polycarbonate syringe filter holder (Sartorius Stedim Biotech GmbH). We used 100 mM sodium acetate buffer (pH 5.0) as binding buffer, whereas 0.5 M sodium chloride solution (prepared in purified water) was used as eluting buffer. We blended 150 μL of mono-PEGylated lysozyme obtained from the integrated process with 1850 μL of binding buffer and injected it into the membrane stack, followed by washing with 4 mL of binding buffer. The membrane-bound material was recovered using 6 mL of eluting buffer. The eluate was concentrated and desalted by centrifugal ultrafiltration prior to SDS-PAGE analysis.

SEC analysis of samples was carried out with a Superdex 75 10/300 GL column (GE Healthcare Bio-Sciences, Canada) using a Varian Star HPLC system (Varian, Palo Alto, CA). Sodium phosphate buffer (20 mM, pH 7.0) containing 150 mM NaCl was used as mobile phase at a flow rate of 0.5 mL/min. SDS-PAGE experiments were carried out
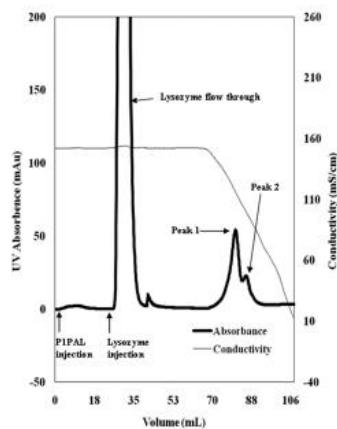
Figure 4. UV absorbance and conductivity profiles of membrane module effluent obtained during solid-phase PEGylation of lysozyme and fractionation of PEGylated lysozyme (membrane: 0.22 μm hydrophilized PVDF; number of membrane discs in stack: 30; disk diameter: 18 mm; bed volume: 0.95 mL; flow rate: 1 mL/min for loading, washing and elution, 0.1 mL/min for PEGylation; elution: 40 mL linear gradient from 0 to 100% eluting buffer; P1PAL-5: lysozyme ratio: 4:1).

Figure 5. SDS-PAGE analysis (12.5%, non-reducing) of samples obtained from experiment described in Figure 4. (a) Commassie blue stained gel. (b) PEG stained gel (lane 1: protein molecular weight markers; lane 2: standard lysozyme; lane 3: standard P1PAL-5; lane 4: P1PAL-5 flow through peak; lane 5: lysozyme flow through peak; lane 6: first eluted peak P1; lane 7: second eluted peak P2).

according to the work of Laemmli.[31] We prepared 12.5% non-reducing gels in duplicate and ran them using a Hoefer MiniVE system (GE Healthcare Bio-Sciences, Canada). One gel was stained with Coomassie blue dye to visualize the protein bands, whereas the other was stained to visualize the PEG bands.[32] For PEG staining, the gel was first kept for 15 min at room temperature in 50 mL of 5% glutaraldehyde solution, for 15 min in 20 mL of 0.1 M perchloric acid, and finally for another 15 min in a mixture prepared with 5 mL of 5% barium chloride and 2 mL of 0.1 M iodine/potassium iodide solution, following which the gel was ready to be photographed. Enzyme activity of unmodified and mono-PEGylated lysozyme obtained by both solid- and liquid-phase PEGylation was analyzed based on the rate of lysis of *Micrococcus lysodeikticus*, as described by Shugar.[33]

■ RESULTS AND DISCUSSION

Previous work on solid-phase protein PEGylation is based on immobilization of the protein on ion-exchange media, followed by contact with appropriate activated PEG reagent.[21−26] The specificity of mono-PEGylation in such reactions was found to be higher than that in equivalent liquid phase reaction. This was primarily attributed to steric hindrance; that is, solid surface-bound mono-PEGylated protein could not easily undergo further PEGylation. However, this approach is critically dependent on the PEGylation site on the media-bound protein being accessible. Therefore, the media is very specific to the protein being PEGylated as well as to the reaction conditions. Our approach of using hydrophobic interaction for immobilizing m-PEG propionaldehyde on a membrane is likely to be more versatile because there are no such restrictions. Being more hydrophilic than the collapsed PEG chain, the reactive aldehyde group on the membrane-bound m-PEG propionaldehyde is expected to face
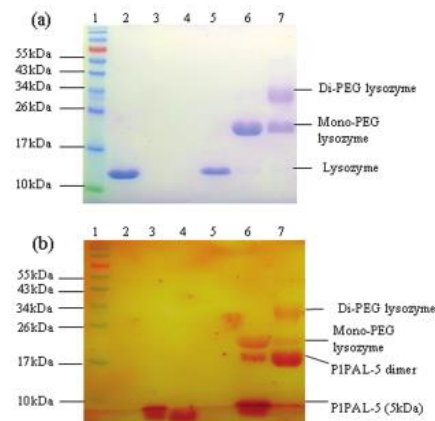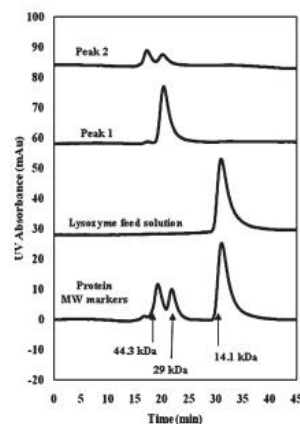
Figure 6. Chromatograms obtained during SEC analysis of samples obtained from experiment described in Figure 4 (column: Superdex 75 10/300 GL; HPLC system: Varian; mobile phase: 20 mM sodium phosphate buffer pH 7.0 containing 150 mM NaCl; flow rate: 0.5 mL/min).

outward, making it accessible for reaction with the N-terminal amino group of the protein. Figure 4 shows the UV absorbance and conductivity profiles of the membrane module effluent obtained during integrated solid-phase lysozyme PEGylation/ purification carried out using a P1PAL-5/lysozyme molar ratio of 4:1. The flow rate during the reaction phase was 0.1 mL/min, whereas during the remainder of the process the flow rate was

Table 1. Characterization of PEGylated Lysozyme

	retention volume (mL)	K_{av} (−)	R_h of PEGylated protein (Å)	R_h of PEG adduct (Å)	molecular weight of PEG adduct (Da)	type of PEGylated protein
20.1 min SEC peak	10.05	0.146	29.370	20.358	4232	mono-PEGylated lysozyme
17.24 min SEC peak	8.62	0.064	39.243	34.195	10702	di-PEGylated lysozyme
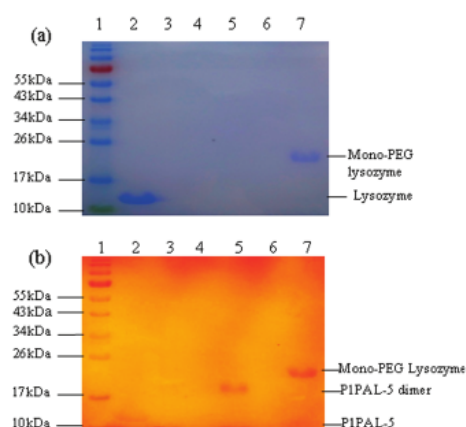
Figure 7. SDS-PAGE analysis (12.5%, non-reducing) of samples obtained during polishing of mono-PEGylated lysozyme (peak 1 from Figure 4) using cation exchanger membrane chromatography. (a) Commassie blue stained gel. (b) PEG-stained gel (lane 1: protein molecular weight markers; lane 2: standard lysozyme; lane 5: flow-through; lane 7: eluate).

1.0 mL/min. The excess unreacted lysozyme flowed through the membrane module, as indicated by the broad UV absorbance peak observed around 31 mL effluent volume. When the binding buffer was replaced with the eluting buffer over a 40 mL of linear gradient, the membrane-bound material was eluted, as evident from the two slightly overlapping peaks (peaks 1 and 2 as indicated in Figure 4). P1PAL-5 was found to have a very low absorbance at 280 nm wavelength, and preliminary experiments showed that lysozyme did not bind to the membrane stack at 1.4 M ammonium sulfate concentration. Quite clearly, therefore, the eluted peaks consisted of PEGylated lysozyme. It was reported in a previous study that mono-PEGylated proteins eluted earlier from hydrophobic interaction media than the di- and tri-PEGylated forms.[27] It may therefore be presumed that peak 1 consisted of mono-PEGylated lysozyme, whereas peak 2 consisted of the di-PEGylated form. To verify this, we collected the peak samples and analyzed them by SEC and SDS-PAGE.

Figure 5a shows the Coomassie-blue-stained SDS-PAGE gel obtained with the samples collected during the PEGylation experiment described in the previous paragraph. Figure 5b shows the corresponding PEG stained gel. Lanes 1−3 contained the protein molecular weight markers, pure lysozyme, and pure P1PAL-5, respectively. The flow through collected during P1PAL-5 loading contained P1PAL-5 in excess of the binding

Table 2. Comparison of Specific Enzyme Activity of Unmodified and Mono-PEGylated Lysozyme

	specific enzyme activity (units/mg)
unmodified lysozyme	23 174.6 (±5%)
mono-PEGylated lysozyme (liquid-phase reaction)	758.7 (±10%)
mono-PEGylated lysozyme (solid-phase reaction)	717.5 (±10%)

capacity of the membrane, as evident from the location of the band in lane 4 of Figure 5b. The flow through collected during the PEGylation reaction contained only lysozyme, as evident from the single band in lane 5 of Figure 5a. The absence of any other band in lane 5 of both Figure 5a,b clearly indicated that neither the membrane-bound P1PAL-5 nor the synthesized PEGylated lysozyme molecules desorbed from the membrane during the PEGylation reaction. Peak 1 sample contained mono-PEGylated lysozyme, as evident from the band between the 17 and 26 kDa markers in both Figure 5a,b. In addition to mono-PEGylated lysozyme, peak 1 contained some unreacted P1PAL-5 and its dimer. The formation of such PEG-PEG dimers by aldol condensation of PEG-aldehyde reagents has been reported by previous workers.[2,34] Peak 2 contained both mono- and di-PEGylated lysozyme (band between the 26 and 34 kDa markers) in addition to P1PAL-5 dimer and trace amounts of P1PAL-5. Mono-PEGylated lysozyme was present in peak 2 since this was the smaller of the two partially resolved peaks. Peaks 1 and 2 could potentially be better resolved by increasing the length of the linear gradient. The absence of lysozyme in the eluted peaks along with the presence of mono-PEGylated lysozyme in peaks 1 and di-PEGylated lysozyme in peak 2 are consistent with findings previously reported,[27] that is, in the presence of 1.4 M ammonium sulfate mono-PEGylated lysozyme was more hydrophobic than lysozyme but less hydrophobic than di-PEGylated lysozyme. The current results also support the hypothesis[27] that a PEGylated protein binds to a hydrophobic surface through its collapsed PEG chain. The co-elution of most of the unreacted P1PAL-5 along with mono-PEGylated lysozyme in peak 1 indicates that these substances have a similar affinity for the membrane. Similarly, the co-elution of most of the P1PAL-5 dimer with di-PEGylated lysozyme indicates that these substances interact with the membrane to the same extent.

Figure 6 shows the SEC chromatograms obtained with eluted peaks 1 and 2 and those obtained with pure lysozyme (31 min retention time) and protein molecular weight standards (14 kDa: 31.12 min; 29 kDa: 21.81 min; and 44.3 kDa: 19.27 min). The SEC chromatogram obtained with eluted peak 1 showed one large (20.1 min) and a very small peak (17.24 min), whereas that obtained with eluted peak 2 showed two almost equal peaks of 17.24 and 20.1 min retention time, respectively. The identity of PEGylated proteins corresponding to the 17.24 and 20.1 min SEC peaks was verified using a method originally reported by

Table 3. Comparison of Extent and Rate of Lysozyme PEGylation in Solid- and Liquid-Phase Reactions

type of reaction	media	amount of lysozyme (mg)	P1PAL-5: protein molar ratio	protein concentration in reactant (mg/mL)	reaction time (min)	flow rate (mL/min)	salt concentration (M)	PEGylation (%)	PEGylation per unit reaction time (%/min)
liquid phase	buffer	5	5:2	1 mg/mL	50		0	3.59	0.0718
liquid phase	buffer	5	5:2	1 mg/mL	100		0	5.46	0.0546
liquid phase	buffer	5	5:2	1 mg/mL	50		1.4	2.17	0.0434
liquid phase	buffer	5	5:2	1 mg/mL	100		1.4	3.17	0.0317
liquid phase	buffer	5	4:1	1 mg/mL	50		0	6.94	0.1388
liquid phase	buffer	5	4:1	1 mg/mL	100		0	8.04	0.0804
liquid phase	buffer	5	4:1	1 mg/mL	50		1.4	2.35	0.0470
liquid phase	buffer	5	4:1	1 mg/mL	100		1.4	3.38	0.0338
solid phase	PVDF	5	5:2	1 mg/mL	50	0.1	1.4	10.91	0.2182
solid phase	PVDF	5	4:1	1 mg/mL	50	0.1	1.4	13.02	0.2604
solid phase	PVDF	5	4:1	1 mg/mL	5	1.0	1.4	4.25	0.8500

Fee and Van Alstine[35] and more recently by Yu and Ghosh.[36] The distribution coefficient (K_{av}) of a macromolecule in a size exclusion column can be determined using the following equation

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)} \qquad (1)$$

where V_e is the elution volume of the macromolecule (mL), V_0 is the column void volume (mL), and V_t is the total bed volume (mL). The distribution coefficient data can then be used to determine the hydrodynamic radius (R_h) of the macromolecule (angstroms) using the universal correlation shown below, which was obtained using protein and PEG molecular weight standards[36]

$$R_h = -12 \ln(K_{av}) + 6.257 \qquad (2)$$

The hydrodynamic radius of the PEG adduct (R_{hPEG}) on a PEGylated protein can be determined using eq 3 shown below[35,36]

$$R_{hPEG} = \frac{(R_h^3 - R_{hP}^3)}{R_h^2} \qquad (3)$$

where R_{hP} is the hydrodynamic radius of the protein being PEGylated, which in the case of lysozyme is 19.81 Å. The molecular weight of the PEG adduct can then be determined using the following correlation[37]

$$M = (5.23R_{hPEG})^{1.789} \qquad (4)$$

Table 1 shows that the molecular weights of the PEG adduct on the PEGylated proteins corresponding to SEC retention times of 20.1 and 17.24 min are 4232 and 10702 Da, respectively. Because the average molecular weight of the mPEG propionaldehyde used in this study is 5000 Da, 20.1 and 17.24 min retention time correspond, respectively, to mono- and di-PEGylated lysozyme. The above results are consistent with those obtained by SDS-PAGE (Figure 5); that is, eluted peak 1 contained mainly mono- and very small amounts of di-PEGylated lysozyme, whereas eluted peak 2 contained equal amounts of these two PEGylated forms of lysozyme. Because the absorbance of PEG at 280 nm is negligible, P1PAL-5 and P1PAL-5 dimer did not show up as peaks in the SEC chromatograms. The SEC results also indicate that PEGylated proteins are bulkier than would be

expected from their molecular weights, particularly when compared with unmodified proteins. The reasons for this has been discussed by Fee and Van Alstine[35] and more recently by Yu and Ghosh;[36] whereas the hydrodynamic radius of a protein varies with molecular weight raised to the power 0.333, the corresponding factor for PEG is 0.559.

Mono-PEGylated lysozyme obtained in eluted peak 1 also contained some P1PAL-5 and P1PAL-5 dimer. These impurities were removed by cation exchange membrane chromatography. Under the operating conditions, mono-PEGylated lysozyme was positively charged and consequently bound to the cation exchange membrane stack, whereas P1PAL-5 and its dimer were neutral simply flowed through. Mono-PEGylated lysozyme was eluted in a highly pure form using sodium chloride solution, desalted, and analyzed by SDS-PAGE to demonstrate absence of P1PAL-5 and its dimer. Figure 7a,b shows the Commassie blue and PEG stained gels obtained with samples from the above experiment. The flow-through contained P1PAL-5 and its dimer as evident from the two bands in lane 5 of Figure 7b, whereas the eluate contained only mono-PEGylated lysozyme, as indicated by the single band in lane 7 of both Figure 7a,b.

Table 2 summarizes the results of lysozyme bioassay carried out using unmodified lysozyme and mono-PEGylated lysozyme obtained by liquid- and solid-phase reactions. PEGylated lysozyme showed significantly reduced enzyme activity when compared with the unmodified enzyme. Reduction in lysozyme activity could be due to a variety of factors such as steric hindrance, which in the case of a small and compact protein like lysozyme could be quite significant. However, mono-PEGylated lysozyme obtained by both liquid- and solid-phase PEGylation showed similar specific enzyme activity, clearly indicating that the solid-phase reaction did not cause any detrimental effect to mono-PEGylated lysozyme.

Table 3 compares the extent and rate of lysozyme PEGylation in liquid- and solid-phase reactions carried out for different durations at different salt concentrations. The amount of lysozyme used in each of these reactions was the same, that is, 5 mg, whereas the amount of P1PAL-5 used was varied. In liquid-phase reaction, increasing the amount of P1PAL-5 in the reaction mixture increased both the extent and the rate of lysozyme PEGylation, whereas the presence of ammonium sulfate resulted in less PEGylation. The reaction rate decreased with time, indicating that this was dependent on residual reactant concentration within the reactor. The extent and rate of lysozyme

PEGylation were both significantly higher in solid-phase reactions. This was despite the presence of ammonium sulfate, which decreased PEGylation in a liquid-phase reaction. Increasing the amount of P1PAL-5 did not improve the extent of PEGylation as significantly as in liquid-phase reactions. Increase in lysozyme solution flow rate through the membrane stack resulted in very dramatic increase in the reaction rate. The solid-phase reaction was therefore mass transport limited, that is, increase in the mass transfer coefficient of lysozyme within the membrane pores resulting from the higher flow rate led to a higher PEGylation rate. Factors likely to be responsible for the higher PEGylation extent and rate observed in solid-phase reaction are: (a) the predominance of convective mass transport in membranes as opposed to reliance on diffusion in the liquid-phase reaction and (b) the higher localized reactant concentration within the membrane and therefore the higher probability of lysozyme-P1PAL-5 collision.

The integrated solid-phase PEGylation technique described above could potentially be used to PEGylate other proteins such as hemoglobin and interferon because it relies on the immobilization of PEG and not the protein being PEGylated. However, the technique would probably not work very well for proteins, which are themselves hydrophobic because it would be difficult to resolve unmodified and PEGylated proteins. The current study is based on the use of 5 kDa activated PEG. The size of the PEG being used for PEGylation could affect the extent of immobilization, and this would need to be factored while developing the process. The reactive group on the activated PEG molecule is yet another factor that could potentially play an important role. In the current study, the aldehyde group being hydrophilic ensured its accessibility to the amino group on the protein molecule. With fewer hydrophilic reactive groups, the accessibility could very well be different.

■ CONCLUSIONS

This study provides proof-of-concept for an integrated solid-phase protein-PEGylation and purification method. The method is based on immobilization of activated PEG on a membrane by hydrophobic interaction and is therefore expected to be suitable for PEGylating any protein. The reactive aldehyde group on P1PAL-5 being more hydrophilic than its collapsed PEG portion was accessible for reaction with the amino groups on the protein. The product obtained in the eluate contained mostly mono-PEGylated lysozyme and some di-PEGylated lysozyme, whereas higher PEGylated forms of the protein were absent. The rate and extent of PEGylation were higher in the solid-phase reactions. Increasing the lysozyme flow rate during solid-phase PEGylation increased the reaction rate clearly, indicating that the overall reaction was mass-transfer-limited.

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Chapter 6

Membrane reactor for continuous and selective protein

PEGylation

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Membrane reactor for continuous and selective protein PEGylation

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6.1 Abstract

PEGylated proteins are value-added biopharmaceutical products obtained by conjugating poly (ethylene glycol) or PEG with proteins. It is difficult to simultaneously obtain both high selectivity and extent of synthesis of mono-PEGylated protein using a conventional batch reaction. (The term "selectivity" in this chapter has equivalent meaning to "specificity" used in the previous chapters.) This paper discusses a hollow-fiber membrane reactor (HMR) system suitable for addressing this issue. The HMR system was operated in a continuous mode with the protein being pumped directly into the lumen of the fiber and the PEG reagent being introduced in the hollow-fiber in a distributed manner along its length. The PEG concentration at any location within the reactor thereby maintained lower than the protein concentration, and thus the synthesis of higher PEGylated byproducts was suppressed, leading to increase in selectivity of mono-PEGylation. An additional factor that contributed towards the enhancement in selectivity was the radial concentration gradient of reactants and product within the hollow-fiber, which resulted in the diffusion of mono-PEGylated protein away from the reaction zone, thus further suppressing the synthesis of the higher PEGylated forms. The extent and selectivity of mono-PEGylation obtained with the HMR system was significantly higher than that obtained in equivalent batch reaction. Furthermore, a continuous process is much more desirable than a batch process from a manufacturing point of view.

Keywords: protein, pegylation, hollow-fiber, membrane reactor

6.2 Introduction

PEGylation refers to the covalent attachment of poly(ethylene glycol) or PEG to biomacromolecules such as proteins, and was first reported in the 1970s (Abuchowski, 1977). It has since been recognized as a promising method for improving the therapeutic efficacy of protein drugs (Ikeda and Nagasaki, 2012; Jevsevar *et al.*, 2010; Veronese and Pasut, 2005) in terms of the following aspects: prolonged in vivo half-life, owing to the increased hydrodynamic size (Abuchowski *et al.*, 1977; An *et al.*, 2007); enhanced patient convenience, thanks to the need for less frequent administration (Fung *et al.*, 1997); decreased immunogenicity, as a result of shielding of antigenic sites by non-immunogenic PEG (Abuchowski *et al*., 1977; An *et al*., 2007, Basu *et al*., 2006); and reduced protein aggregation, due to stabilization by the hydrophilic PEG component of a PEGylated protein (Basu *et al*., 2006; Hinds *et al*., 2000). Several PEGylated therapeutic proteins such as PEGASYS® (PEG-IFN-α2a, Hoffmann-La Roche, approved 2002) (Alconcel *et al*., 2011) for the treatment of hepatitis C, Neulasta® (PEG-G-CSF, Amgen, approved 2002) (Alconcel *et al*., 2011) for the management of febrile neutropenia, Cimzia® (PEG-anti-TNFα Fab', UCB Pharma) (Alconcel *et al*., 2011) for the treatment of Crohn's disease (approved 2008) and rheumatoid arthritis (approved 2009), Krystexxa® (PEG-uricase, Savient, approved 2010) (Alconcel *et al*., 2011) for the treatment of chronic gout, and Omontys® (PEGinesatide, Affymax and Takeda, approved 2012) (Bennett *et al*., 2012) have been approved by the FDA.

PEGylation is usually carried out as a homogeneous liquid-phase, batch reaction. The protein is reacted with the PEG reagent in a stirred tank reactor in the presence of other

reagents (if required). The most common approach in protein PEGylation involves the conjugation of PEG to the ε-amino groups on lysine residues in a protein (Fee and Van Alstine, 2006; Jevsevar *et al.*, 2010; Reddy *et al.*, 2002; Schlesinger *et al.*, 2011). Due to the availability of several such ε-amino groups in a typical protein, and due to the continuous contact of reactants, products and by-products in a homogeneous liquid, such reactions typically result in the synthesis of complex product mixtures containing different PEGylated forms of the protein, i.e. mono-, di-, tri- and higher-PEGylated forms, and positional isomers of these (Wong and Jameson, 1991). A mono-PEGylated protein, where one PEG molecule is attached at a specific site on a protein is usually desirable from the point of view of consistent therapeutic activity as well as for precise product characterization (Fee and Van Alstine, 2006). Purification of a mono-PEGylated protein from a mixture containing significant amount of by-products is technically challenging due to the similarities in physicochemical properties of the different PEGylated forms. Moreover, carrying out non-specific PEGylation followed by extensive purification is an extremely inefficient approach at product manufacturing, as the by-products are essentially impurities which have to be discarded, which in turn has cost and environmental implications. The current quality benchmark for new PEGylated protein drugs is very stringent in terms of specificity of product (Pasut and Veronese, 2012). The need for increasing the selectivity in protein PEGylation is therefore driven by the multiplicity of factors listed above.

Most attempts at increasing selectivity of PEGylation to obtain better defined products have come from a site-specific reaction approach (Chapman, 2002; Cong *et al.*, 2012; Hu

et al., 2010; Jevsevar *et al*., 2010; Kinstler, 1998; Kinstler, 1996; Lee *et al*., 2003; Roberts *et al*., 2002; Rosendahl, 2005; Sato, 2002; Veronese and Mero, 2008; Veronese *et al*., 2007; Veronese and Pasut, 2005; Wang *et al*., 2011). One of the most common amongst these is N-terminal PEGylation (Jevsevar *et al*., 2010; Kinstler, 1998; Veronese and Mero, 2008; Veronese and Pasut, 2005) where the conjugation of PEG molecule at the N-terminal α-amino group of a protein is carried out by utilizing its lower pKa value than those of the ε-amino groups on lysine residues. PEGylated protein drugs synthesized using this approach include Neulasta® (Kinstler *et al*., 1996), PEGylated EGF (Lee *et al*., 2003), and PEGylated staphylokinase (Wang *et al*., 2011). Cysteine-specific PEGylation (Jevsevar *et al*., 2010; Veronese and Mero, 2008; Veronese and Pasut, 2005) is another well-known approach which targets the thiol group in either a naturally present cysteine (e.g. PEGylated G-CSF (Veronese *et al*., 2007)), or in a genetically introduced cysteine residue (e.g. PEGylated IFN-α2a (Rosendahl *et al*., 2005) and PEGylated Bone morphogenetic protein-2 (Hu *et al*., 2010)). This approach was also used for site-specific PEGylation of antibody fragments which were engineered to contain one or more cysteine residues in a modified hinge region (Chapman, 2002). Cimzia® (Jevsevar *et al*., 2010; Veronese and Mero, 2008) is yet another example of a product synthesized using this approach. Other site-specific reactions include PEG conjugations to histidine affinity tags by bis-alkylation with PEG-bis-sulfones (Jevsevar *et al*., 2010; Cong *et al*., 2012), and to the amide group of glutamine through a transglutamination reaction using naturally occurring enzymes which recognize glutamine as substrate, namely transglutaminase (Sato, 2002; Veronese and Pasut, 2005). However, even with a highly specific PEGylation chemistry (e.g. N-terminal PEGylation), the

formation of significant amounts of by-products such as di- and tri-PEGylated proteins cannot be avoided (Yu *et al*., 2010; Dou *et al*., 2007).

The selectivity of a PEGylation reaction could potentially be improved by physical manipulations such as bringing reactants into contact in a controlled manner, or separating products from reactants as they are synthesized, to name just a few. While the potential benefits of such physical strategies are well known in the domain of reaction engineering, there are not many reports on their use for improving selectivity of protein PEGylation. The few attempts at physical manipulation in protein PEGylation can be broadly categorized into two groups, (a) size-exclusion reaction chromatography (or SERC) (Fee, 2003; Fee and Van Alstine, 2006), and (b) solid-phase or "on-column" PEGylation (Huang *et al*., 2012; Huang *et al*., 2012; Lee *et al*., 2007; Monkarsh *et al*., 1997; Ottow *et al*., 2011; Shang *et al*., 2011; Suo *et al*., 2009). In SERC, the two reactants, i.e. the PEG reagent and the protein, are introduced into a size-exclusion chromatography column as individual pulses in a sequential manner, such that one (the protein) catches up with the other (the PEG), on account of size difference. When this happens, they react, and the product that is formed segregates from the reactants (once again due to size difference), and each species appears at the column outlet at a different time. As the mono-PEGylated protein moves out of the reaction zone faster than any of the reactants, the selectivity of its synthesis is greatly enhanced. While SERC is an example of an elegant integrated reaction-separation process, it is limited in terms of production capacity due to its batch-wise (or pulse-wise) nature. In solid-phase PEGylation, the two reactants are contacted by sequential injection into a packed bed (Huang *et al*., 2012; Huang *et al*., 2012; Lee *et al*., 2007; Monkarsh *et al*., 1997;

Suo *et al*., 2009), or a moving bed (Ottow *et al*., 2011), or a membrane stack reactor(Shang *et al*., 2011). The reactant injected first is physically immobilized on the surface of an adsorbent, following which it is made to react with the subsequently injected reactant (Huang *et al*., 2012; Huang *et al.*, 2012; Lee *et al*., 2007; Monkarsh *et al*., 1997; Ottow *et al*., 2011; Shang *et al*., 2011; Suo *et al*., 2009). The product is then recovered from the reactor in a relatively pure form by controlled elution. Such integrated processes not only combine reaction with separation and thereby enhance manufacturing convenience and product recovery, but also result in highly selective synthesis of mono-PEGylated protein at higher reaction rates than in equivalent liquid phase reactions. A variety of factors such as steric hindrance, favorable orientation of reactant, and enhanced mass transport have been attributed to such increase in speed and selectivity of PEGylation. However, as with SERC, production capacity is restricted due to the batch-wise (or pulse-wise) nature of such processes.

   In this paper, we present a novel method for enhancing both selectivity and extent of protein mono-PEGylation. To explain the working hypothesis for this method, we begin by looking at the stoichiometric aspects of a typical PEGylation reaction. Most PEG reagents are designed to be mono-functional, and when this is the case, one PEG molecule can attach only to one protein molecule. On the other hand, one protein molecule can in theory bind to several PEG molecules through its various available conjugation sites. Therefore an excess of PEG over protein in the reaction mixture, while resulting in greater extent of protein PEGylation, would also lead to the synthesis of undesirable multi-PEGylated proteins (Bailon *et al*., 2001; Hu and Sebald, 2009; Lee *et al*., 1999; Nojima *et al*., 2008;

Wang *et al*., 2002). On the other hand, an excess of protein over PEG in the reaction mixture would increase the selectivity of mono-PEGylation, at the cost of conversion, and thereby result in wastage of valuable protein (Fee and Van Alstine, 2006; Lin *et al*., 2011). It has been suggested that the selectivity of synthesis of mono-PEGylated protein could potentially be enhanced without sacrificing the conversion by adding the PEG reagent to the protein in a controlled manner, such as in a fed-batch reactor (Fee and Van Alstine, 2006). We decided to take this one step further by developing a continuous tubular reactor within which the protein flows through the tubular channel and the PEG is added in an axially distributed manner as shown in **Figure 1**. If only a limited amount of PEG were allowed to come in at any given location within the reactor, it would be consumed by the more abundant protein present locally. This would increase the selectivity of synthesis of mono-PEGylated protein by suppression of the synthesis of higher PEGylated forms of the protein. Moreover, the manner of PEG and protein addition into the reactor would create radial concentration gradient of different species within the tube which would result in further selectivity enhancement. This later aspect has been discussed in detail in the results and discussion section. Overall, the use of this novel PEGylation reactor system could be expected to increase both selectivity and extent of mono-PEGylation, when compared with an equivalent liquid-phase batch reaction.

In order to carry out the PEGylation reaction as hypothesized above, we decided to use a hollow-fiber membrane reactor (HMR). **Figure 2** shows the schematic diagram of the hollow-fiber membrane reactor used in this study. The protein solution was pumped directly into the lumen of the hollow-fiber membrane while the PEG reagent was pushed

into the lumen, through the membrane, from the shell side, along the length of the fiber. The PEGylated protein thus synthesized, along with any unmodified protein or any unreacted PEG reagent was collected at the lumen outlet. Hollow-fiber membrane contactors have been used of a wide range of applications (Gabelman and Hwang, 1999; Rios *et al*., 2004; Wickramasinghe *et al*., 1992), including anti-solvent based crystallization of amino acids (Zarkadas and Sirkar, 2006), crystallization of proteins (Curcio *et al*., 2005), precipitation of salts (Kieffer *et al*., 2009), production of liposomes (Laouini *et al*., 2011), and direct contact membrane crystallization (Curcio *et al*., 2001), to name just a few. However, to the best of our knowledge, there are no prior reports on the use of hollow-fiber membrane contactors for protein PEGylation, or for that matter, similar chemical conjugation reactions involving macromolecules.

The model protein used in this study was lysozyme (MW=14100, pI=11) and the PEG reagent used was methoxy-PEG-$(CH_2)_5$COO-NHS (5 kDa PEG equivalent). The conjugation was based on acylation for amide linkage formation, with the protein replacing the NHS group (Kinstler, 1998). This type of PEGylation reaction is known to be rapid (Nojima *et al*., 2009) with relatively moderate selectivity of mono-PEGylated protein synthesis (Hu and Sebald, 2009; Nojima *et al*., 2009; Nojima *et al*., 2008). The effects of operating conditions and process variables such as residence time and PEG: lysozyme molar ratio on extent and selectivity of PEGylation with the HMR system were examined. These results were compared with those obtained from equivalent liquid-phase reactions.

6.3 Materials and Methods

Lysozyme (L6876), ammonium persulfate (A3678), 30% acylamide solution (A3699), bromophenol blue (B0126), Brilliant Blue R concentrate (B8647), glycerol (G2025), 25% glutaraldehyde solution (G6257), glycine (G8898), sodium dodecyl sulfate (L3771), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), sodium hydroxide (S5881), sodium chloride (S7653), Trizma base (T1503), Trizma-hydrochloride (T3253), N,N,N′,N′-tetramethyl ethylenediamine (T9281), DL-dithiothreitol (43817), 70% perchloric acid (77227), barium chloride (202738), hydrochloric acid (258148), and iodine (326143) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Potassium iodide (74210-140) was purchased from Anachemia, Montreal, QC, Canada. Methanol (6700-1) and acetic acid (1000-1) were purchased from Caledon Laboratories LTD., Georgetown, ON, Canada. Methoxy-PEG-(CH$_2$)$_5$COO-NHS (5 kDa, catalog number SUNBRIGHT ME-050HS) was purchased from NOF Corporation, Tokyo, Japan. All test solutions and buffers were prepared using purified water (18.2 MΩ cm) obtained from a SIMPLICITY 185 water purification unit (Millipore, Molsheim, France). Amicon$^®$ Ultra-4 centrifugal filters (3 kDa MWCO, UFC800324) used for concentrating and desalting samples were purchased from EMD Millipore Co., Billerica, MA, USA. MicroKros$^®$ hollow-fiber membrane module (X15S 300 04N, polysulfone, 50 kDa) was purchased from Spectrum Laboratories Inc., Rancho Dominguez, CA, USA. The module contained six hollow-fibers, each having a length of 20 cm, an inner diameter of 0.47±0.05 mm and an outer diameter of 0.6±0.08 mm. The total effective membrane area was 20 cm^2.

All PEGylation reactions were carried out at room temperature (22±1℃). The selectivity of conjugation of PEG to an amino acid residue in a protein known to be is highly dependent on the nucleophilicity of that residue (Khandare and Minko, 2006; Roberts *et al*., 2002). Nucleophilic attack only takes place at a pH value near or above the pKa value of the target amino group (Roberts *et al*., 2002). Preliminary lysozyme PEGylation experiments were carried using methoxy-PEG-$(CH_2)_5$COO-NHS at three pH values: 7.0, 7.5 and 8.0. It was found that pH 8.0 resulted in faster reaction and greater conversion, but lower selectivity of mono-PEGylation. The selectivity was higher at pH 7.0 and 7.5 but the conversion and reaction rates were drastically reduced. Therefore, pH 8.0 was selected for PEGylation experiments discussed in this paper.

Batch liquid-phase PEGylation reactions were carried out using different PEG: lysozyme molar ratios. Each reaction was carried out with 3 mL reaction volume, within small glass vials, at room temperature, with constant stirring. The reaction medium consisted of 100 mM sodium phosphate and 150 mM sodium chloride, the pH being adjusted to 8.0. For each set of operating conditions, reactions for different durations were carried out in separate vials. Glycine solution was added directly to the vials to quench the reaction, the glycine concentration in the final mixture being 50 mM. The quenched reaction mixtures were desalted and concentrated by centrifugal filtration, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Lysozyme PEGylation using the HMR system was carried out in a continuous mode with the set-up shown in **Figure 3**. The molecular weight cut-off of the hollow-fiber membrane (50 kDa) was selected to ensure that 5 kDa PEG went through the pores easily. PEG reagent

(0.5 mg/mL) and lysozyme (2 mg/mL) solutions were prepared in the same reaction medium as that used in the liquid-phase reactions. To prime the reactor system, PEG reagent solution was pre-filled into the shell side of the membrane module without pressurization, the lumen of the hollow-fibers were filled with reaction medium, and the protein solution was pumped right up to the lumen inlet. The reaction was then started by simultaneously turning on the two pumps such that protein and PEG reagent solutions were fed at constant flow rates into the lumen inlet and the shell inlet respectively. The continuous product stream obtained at the lumen outlet flowed through an UV detector and was collected as a series equal volume samples in small stirred vials containing quenching solution. The quenched samples from the HMR experiments were processed and analyzed in the same way as the batch liquid-phase reaction samples.

Feed and product samples collected from the batch and HMR experiments described above were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). 12.5% non-reducing gels were run using a miniVE vertical electrophoresis system (80-6418-77) purchased from GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada. Samples were loaded onto duplicate gels and these were stained for protein and PEG respectively. Coomassie Brilliant Blue R dye was used for observing protein-containing bands while PEG-containing bands were visualized using the following staining protocol (Kurfurst, 1992). The gel for PEG staining was first soaked in 50 mL of 5% glutaraldehyde solution for 15 min at room temperature, then in 20 mL of 0.1 M perchloric acid for 15 min, and finally in a mixture of 5 mL 5% barium chloride and 2 mL 0.1 M iodine/potassium iodide for another 15 min. A digital camera was used to photograph

the stained gels. The extent of protein PEGylation (i.e. conversion) and selectivity of synthesis were determined by densitometric scans of the Coomassie blue stained gels. Images for densitometric scans were obtained using a Bio-Imaging MiniBis Pro system (24-25-PR) purchased from DNR-Imaging Systems, Jerusalem, Israel. This system could generate 8-bit gel pictures with dark background and light bands, making these easy to analyze using Image J 1.46 (for Windows) freeware downloaded from the following NIH hosted website: (http://rsbweb.nih.gov/ij/download.html).

6.4 Results and Discussion

Batch liquid-phase PEGylation reactions are typically carried out using excess PEG reagent to maximize protein PEGylation (Bailon *et al*., 2001; Hu and Sebald, 2009; Lee *et al*., 1999; Nojima *et al*., 2008; Wang *et al*., 2002). But, this results in the synthesis of significant amounts of by-products. When the protein is present in excess, selectivity of mono-PEGylation is enhanced, but at the expense of conversion (Fee and Van Alstine, 2006; Lin *et al*., 2011). Whether such a trade-off between conversion and selectivity existed with the NHS PEGylation chemistry used in the current study was verified by carrying out batch PEGylation experiments with excess PEG reagent, and excess lysozyme respectively in the reaction mixture. **Figure 4** shows the (a) Coomassie blue, and (b) iodine stained SDS-PAGE gels obtained with samples from a batch liquid-phase reaction, carried out using a PEG: lysozyme molar ratio of 4 with an initial lysozyme concentration of 1 mg/mL. **Table 1** summarizes the conversion and selectivity data for this experiment, calculated based on Image J analysis of the Coomassie blue stained gel shown in **Figure 4**. Conversion

is defined as the fraction of lysozyme converted to any particular PEGylated form and was obtained by dividing the intensity of the relevant PEGylated protein band by the sum of intensities of all other bands (including unreacted lysozyme) in the same lane. The selectivity of mono-PEGylation was determined by dividing the intensity of the mono-PEGylated protein band by the sum of intensities of all PEGylated protein band in in the same lane. The data presented in **Figure 4** and **Table 1** show that the PEGylation reaction was very fast with a 0.364 conversion factor of lysozyme to mono-PEGylated protein being obtained within 15 min. The corresponding selectivity of mono-PEGylation was 0.583. The decrease in conversion of mono-PEGylated lysozyme after 15 min indicates that its rate of synthesis was slower than its rate of further conversion to the higher PEGylated forms. While the net conversion factor for mono-PEGylated lysozyme decreased gradually, the selectivity of mono-PEGylation decreased more significantly with time after 15 min of reaction, primarily due to increase in the amount of di-PEGylated protein.

Figure 5 shows the Coomassie blue stained gel obtained with samples from lysozyme PEGylation reactions carried out using a PEG: lysozyme molar ratio of 0.65, i.e. with an excess of protein in the reaction mixture, the initial lysozyme concentration being 1 mg/mL. The corresponding conversion and selectivity data are presented in **Table 2**. The selectivity of mono-PEGylation was consistently high, with very little di-PEGylated lysozyme and practically no higher PEGylated forms being synthesized in reaction carried out for 35 min. However, the conversion was significantly lower than in the experiments carried out using the PEG ratio of 4. Also the reaction seemed to almost die out after 30 min. The results shown in **Figures 4** and **5** and **Tables 1** and **2** clearly demonstrate the trade-off between

conversion and selectivity in the NHS PEGylation chemistry used in the current work. While the higher PEG: protein ratio gave greater conversion, the product profile obtained in such a reaction would make purification of mono-PEGylated lysozyme technically challenging and expensive. The lower PEG: lysozyme ratio resulted in higher selectivity of mono-PEGylation but a significant amount of lysozyme remained unreacted. These would either have to be salvaged for further PEGylation or simply discarded.

The HMR experiments were carried out at different operating conditions, i.e. PEG: protein molar ratio and residence time. In a homogeneous, liquid-phase batch reactor, the concentration of reactants and products within the reactor does not vary with location to any significant extent, at any given time. However, in the HMR system, reactant and product concentration gradients are generated due to the nature of the device and the manner of operation, i.e. tubular reactor with distributed PEG reagent addition. The representative concentration of lysozyme within the reactor was therefore expressed in terms of an overall apparent concentration (C_{app}) which is defined as:

$$C_{app} = C_{lys}\left(\frac{Q_{lys}}{Q_{lys} + Q_{PEG}}\right) \tag{1}$$

The representative PEG: lysozyme molar ratio was likewise expressed as an overall apparent molar ratio (R_{app}) which is defined as:

$$R_{app} = \left(\frac{C_{PEG}Q_{PEG}}{C_{lys}Q_{lys}}\right) \tag{2}$$

Defining the residence time of the HMR system is challenging as the net flow rate at the lumen outlet (which corresponds to the sum of flow rates the lysozyme and PEG reagent)

is higher than that at the lumen inlet (which is equal to the lysozyme flow rate alone). The pressure within the lumen of a hollow-fiber is higher at the inlet than at the outlet, while the pressure within the shell side is relative uniform. Therefore the transmembrane pressure could be expected to increase along the length of the lumen from the inlet to the outlet. Consequently, the PEG reagent permeation rate into the lumen would also be expected to increase along the length of the lumen. Due to this non-uniformity in permeation, the log-mean residence time (τ_{lm}), as defined in equation (3) below was used as the representative residence time for the HMR system.

$$\tau_{lm} = \frac{(\tau_i - \tau_o)}{\ln\left(\dfrac{\tau_i}{\tau_o}\right)} \tag{3}$$

Figure 6 shows the Coomassie blue stained gel obtained with samples from an HMR experiment carried out using lysozyme and PEG flow rates of 3 and 19 µL/min respectively. This combination of flow rates gave a R_{app} of 4.1, a τ_{lm} of 30 min, and a C_{app} of 0.273 mg/mL. Product samples were collected at the reactor outlet at different time intervals during the experiment. The corresponding conversion and selectivity data are shown in **Table 3**. The first two samples collected (i.e. those loaded in lanes 2 and 3) did not contain any lysozyme or PEGylated lysozyme indicating that these corresponded to the reaction buffer held within the lumen of the hollow-fibers at the start of the experiment. Once this buffer was displaced, the products and unreacted reactants appeared at the reactor outlet (see lanes 4-6). The sample collected between 40-60 min showed a mono-PEGylated lysozyme conversion factor of 0.387 and a selectivity of 0.917. The sample collected between 60-80 min showed a higher mono-PEGylated lysozyme conversion (0.472) and

almost unchanged selectivity (0.924). The composition of the sample collected between 80-100 min did not change appreciably further, indicating that the HMR system reached steady state around 60 min. Comparing results shown in **Figures 4** and **6** and **Tables 1** and **3**, it is evident that significantly higher mono-PEGylated lysozyme conversion factor and selectivity were obtained with the HMR system. The steady-state extent and selectivity of mono-PEGylation obtained with the HMR system were in the 0.454-0.472, and 0.886-0.924 ranges respectively. Moreover, higher PEGylated forms such as tri- and tetra-PEGylated lysozyme were not synthesized in the HMR experiment. As a head-to-head comparison, the product obtained after 30 min reaction time from the batch liquid-phase reactor showed a mono-PEGylated lysozyme conversion factor of 0.330, a selectivity of 0.499, and contained significant quantities of di-, tri- and tetra-PEGylated lysozyme, i.e. the extent of mono-PEGylation was about 40% higher and the selectivity of mono-PEGylation was about 81% higher with the HMR system.

Figure 7 shows the Coomassie blue stained gel obtained with samples from an HMR experiment carried out using equal lysozyme and PEG flow rates of 11 µL/min each. This resulted in a R_{app} of 0.65, a τ_{lm} of 13.7 min, and a C_{app} of 1 mg/mL. The conversion and selectivity data is shown in **Table 4**. The HMR system reached steady state around 40 min, and the steady-state mono-PEGylated protein conversion factor was in the range of 0.347-0.360, which was about 1.7 times greater than that obtained from the batch liquid-phase reactor after 15 min reaction time as shown in Table 2 (i.e. 0.132). The selectivity of mono-PEGylation was equal to 1 in both reactor systems, i.e. no higher PEGylated form of lysozyme was synthesized.

The experimental results discussed above prove the working hypothesis of this study. The distributed addition of PEG reagent along the length of the hollow fiber membrane increased both extent and selectivity of mono-PEGylation. The overall concentration distribution for lysozyme, PEG reagent and PEGylated lysozyme within a single hollow-fiber is shown **Figure 8**. While the axial concentration gradient of reactants and product within the reactor was the main contributing factor, the radial concentration gradient of the different species within the hollow-fiber as depicted in the inset of **Figure 8** also contributed towards the enhancement in extent and selectivity of mono-PEGylation. Since the PEG reagent was pushed into the lumen through the membrane, its concentration within the hollow fiber would be highest within the sheath flow immediately adjacent to inner wall of the fiber. On the other hand, the lysozyme concentration could be expected to be highest at the center-line. In this study, the HMR system was operated under laminar flow conditions with Reynolds number being less than one. Therefore, the transport of reactants and product in the radial direction within the lumen took place predominantly by diffusion. Within the hollow-fiber, lysozyme diffused in a radially outward direction, the PEG reagent diffused in a radially inward direction, and the reaction took place somewhere between the center-line and the wall of the membrane where the two diffusing reactants met. The mono-PEGylated protein thus synthesized diffused away from the reaction zone, both toward the fiber wall and towards the center-line. However, as the flow of liquid within the hollow-fiber was laminar, the velocity of the streamlines located between the reaction zone and the center-line was significantly higher than those located between the reaction zone and the fiber wall. Consequently, the flux of mono-PEGylated protein towards the center-line was

significantly higher than that towards the fiber wall. Such transport of mono-PEGylated protein away from the reaction zone also contributed significantly toward the high selectivity of mono-PEGylation, which in turn increased its conversion factor.

The results discussed in the paper clearly demonstrate the superiority of the HMR system over batch liquid-phase reactor with regards to protein PEGylation. Not only was the selectivity and extent of mono-PEGylation significantly higher with the HMR system, it could be operated in a continuous mode, which is advantageous from a product manufacturing point of view. The HMR system discussed in this paper could also potentially be used for similar chemical conjugation reactions where control over selectivity of synthesis is desirable.

6.5 Conclusions

A trade-off between selectivity and extent of synthesis of mono-PEGylated protein is typically observed in batch liquid-phase PEGylation reactions. The hollow-fiber membrane reactor (HMR) system discussed in this paper, which was operated in a continuous mode, successfully overcame this trade-off. The distributed addition of PEG reagent guaranteed that it was available in limited amounts at any location within the hollow-fiber. It was therefore consumed by the more abundant protein, thereby suppressing of the synthesis of higher PEGylated forms. Two types of concentration gradients exit within the hollow-fiber, one axial, and one radial. Both of these contribute towards enhancement in selectivity and extent of mono-PEGylation. The experimental results discussed in this paper clearly demonstrate the superiority of the HMR system over a batch liquid-phase reactor. The

extent and selectivity of mono-PEGylation was significantly higher in the HMR experiments. Operating conditions such as the R_{app} and the τ_{lm} affected both extent and selectivity of mono-PEGylation.

6.6 Acknowledgements

6.7 Nomenclature

C_{lys} = concentration of the lysozyme feed solution (kg/m^3)

C_{PEG} = concentration of the PEG reagent (kg/m^3)

Q_{lys} = flow rate of lysozyme solution (m^3/s)

Q_{PEG} = flow rate of PEG reagent (m^3/s)

τ_i = residence time based on inlet flow rate (s), i.e. total lumen volume divided by the lysozyme flow rate

τ_o = residence time based on the outlet flow rate (s), i.e. the total lumen volume divided by the sum of lysozyme and PEG reagent flow rates

6.8 References

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6.9 Tables

Table 1 Summary of conversion to different PEGylated forms and selectivity of mono-PEGylation in batch liquid phase reaction carried out using a PEG: lysozyme molar ratio of 4 (corresponding to Figure 4)

Reaction time (min)	Conversion				Selectivity of mono-PEGylation
	Mono-PEGylated	Di-PEGylated	Tri-PEGylated	Tetra-PEGylated	
5	0.276	0.112	undetectable	undetectable-	0.752
15	0.364	0.176	0.081	Undetectable	0.583
30	0.330	0.266	0.096	0.097	0.499
60	0.328	0.308	0.128	0.082	0.447

Table 2 Summary of conversion to different PEGylated forms and selectivity of mono-PEGylation in batch liquid phase reaction carried out using a PEG: lysozyme molar ratio of 0.65 (corresponding to Figure 5)

Reaction time (min)	Conversion		Selectivity of mono-PEGylation
	Mono-PEGylated	Di-PEGylated	
15	0.132	undetectable	1.000
20	0.179	undetectable	1.000
25	0.229	0.023	0.908
30	0.264	0.025	0.913
35	0.271	0.025	0.917

Table 3 Summary of conversion to different PEGylated forms and selectivity of mono-PEGylation in HMR system carried out using a R_{app} of 4.1 and a τ_{lm} of 30 min (corresponding to Figure 6)

Sampling time (min)	Conversion		Selectivity of mono-PEGylation
	Mono-PEGylated	Di-PEGylated	
0-20	undetectable	undetectable	-
20-40	undetectable	undetectable	-
40-60	0.387	0.035	0.917
60-80	0.472	0.039	0.924
80-100	0.454	0.058	0.887

Table 4 Summary of conversion to different PEGylated forms and selectivity of mono-PEGylation in HMR system carried out using a R_{app} of 0.65 and a τ_{lm} of 13.7 min (corresponding to Figure 7)

Sampling time (min)	Conversion		Selectivity of mono-PEGylation
	Mono-PEGylated	Di-PEGylated	
0-10	undetectable	undetectable	-
10-20	undetectable	undetectable	-
20-30	0.076	undetectable	1.000
30-40	0.320	undetectable	1.000
40-50	0.360	undetectable	1.000
50-60	0.347	undetectable	1.000
60-70	0.351	undetectable	1.000

6.10 Figures

Figure 1. Scheme for enhancement in selectivity of mono-PEGylation by axially distributed addition of PEG reagent to protein flowing in a tubular reactor.
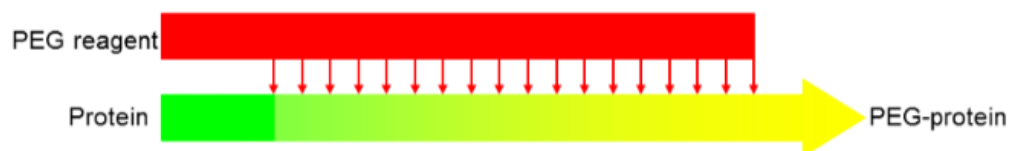

Figure 2. Schematic diagram of hollow-fiber membrane reactor for protein PEGylation.
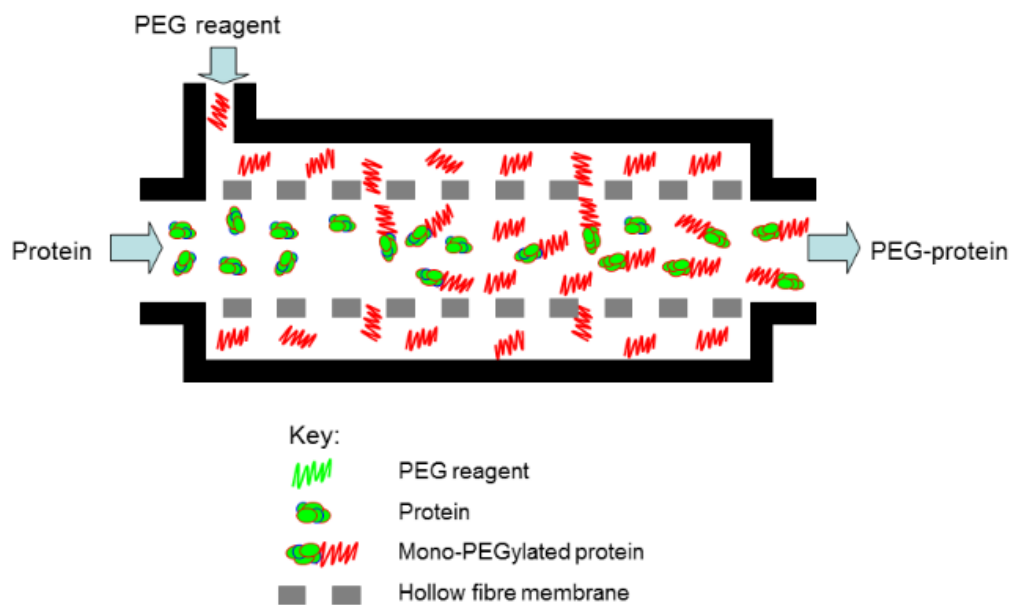
Figure 3. Experimental setup for hollow-fiber membrane reactor (1 PEG reagent reservoir, 2 pump, 3 flow meter, 4 pressure sensor, 5 protein reservoir, 6 pump, 7 flow meter, 8 pressure sensor, 9 hollow-fiber membrane reactor, 10 UV detector, and 11 sample collector).
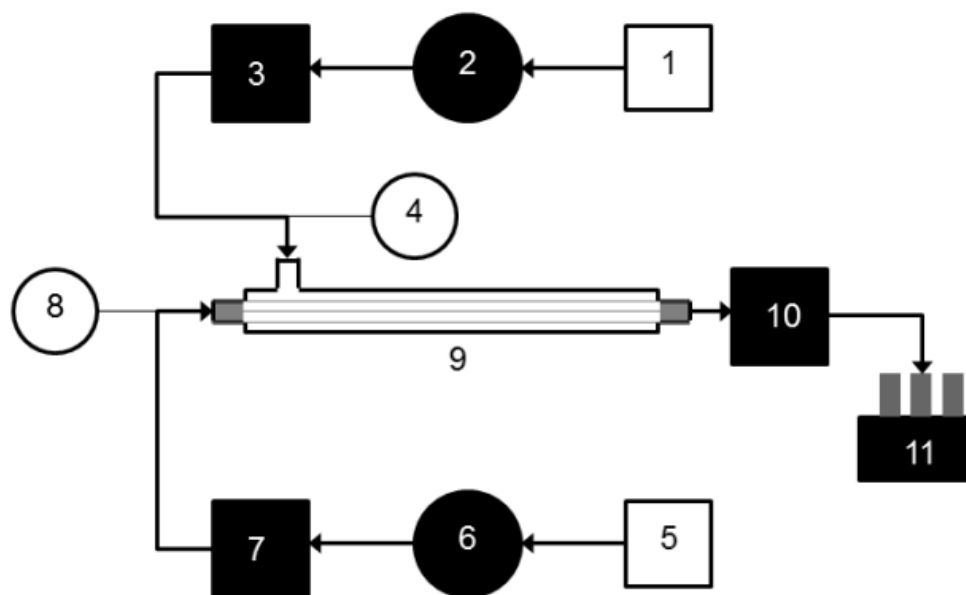
Figure 4. SDS-PAGE analysis of samples from batch liquid-phase reaction carried out using a PEG: lysozyme molar ratio of 4. (a) Coomassie blue stained gel, (b) iodine stained gel. Lane 1: protein molecular weight markers, lane 2: pure lysozyme in gel (a) and pure PEG reagent in gel (b), lanes 3-5: samples corresponding to reaction time of 5, 15, 30 and 60 min respectively.
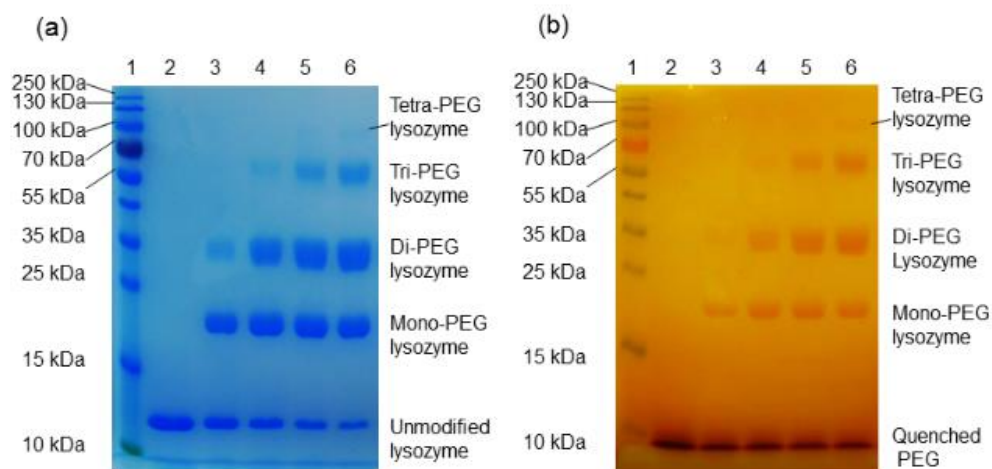
Figure 5. SDS-PAGE analysis of samples from batch liquid-phase reaction carried out using a PEG: lysozyme molar ratio of 0.65. Coomassie blue stained gel, lane 1: protein molecular weight markers, lanes 2-7: samples corresponding to reaction time of 15, 20, 25, 30 and 35 min respectively.

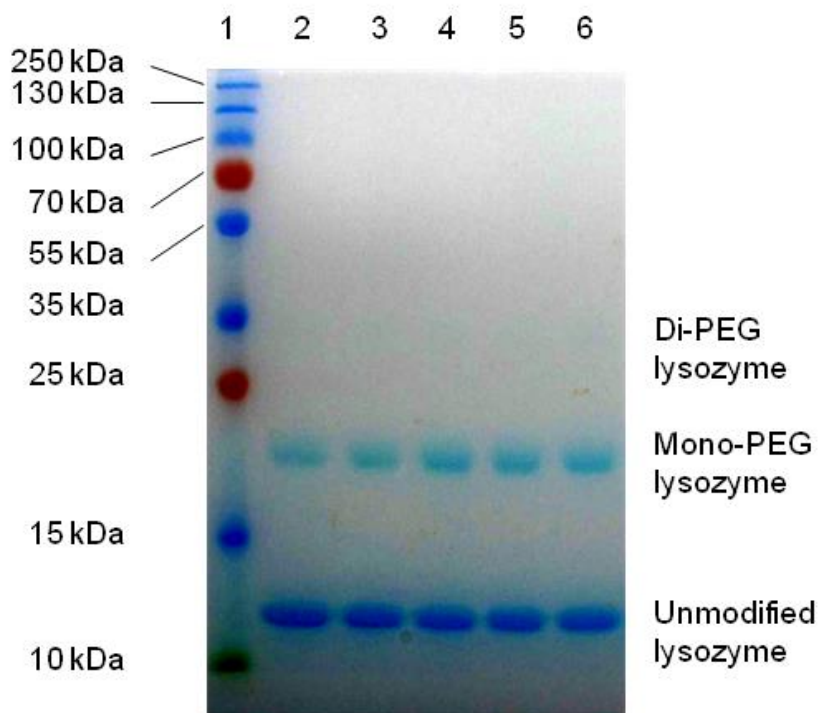Figure 6. SDS-PAGE analysis of samples from HMR experiment carried out using a R_{app} of 4.1 and a τ_{lm} of 30 min. Lane 1: protein molecular weight markers, lanes 2-6: sample collected between 0-20, 20-40, 40-60, 60-80 and 80-100 min respectively.
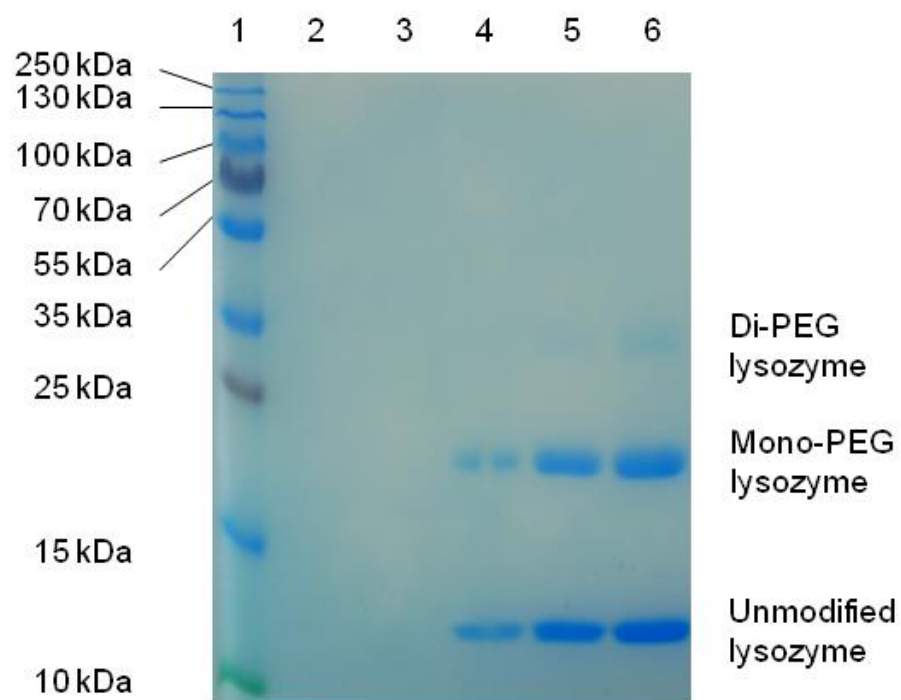
Figure 7. SDS-PAGE analysis of samples from HMR experiment carried out using a R_{app} of 0.65 and a τ_{lm} of 13.7 min. Lane 1: protein molecular weight markers, lanes 2-7: sample collected between 0-10, 10-20, 20-30, 30-40, 40-50, 50-60 and 60-70 min respectively.
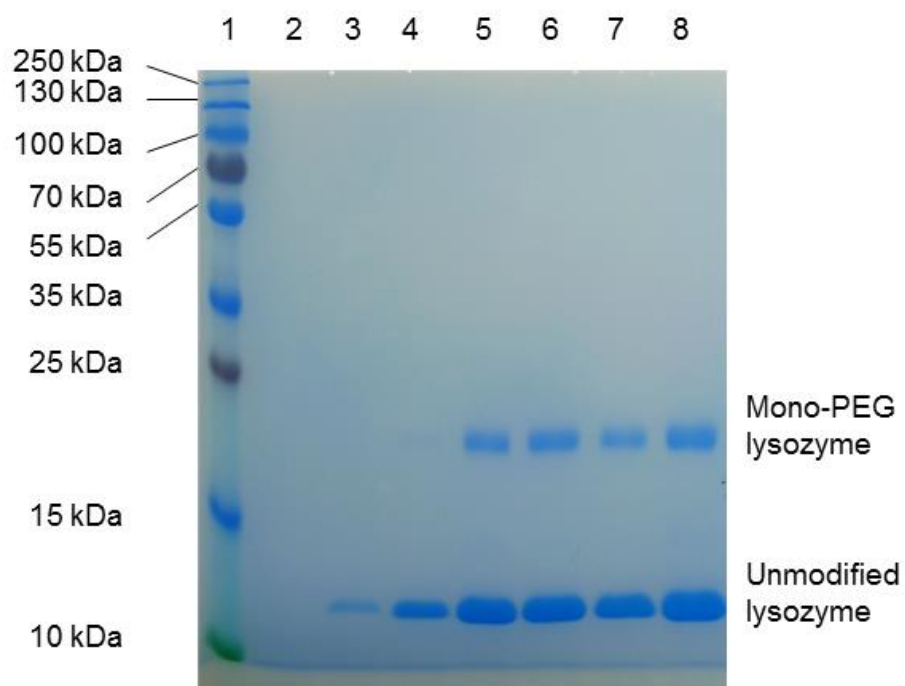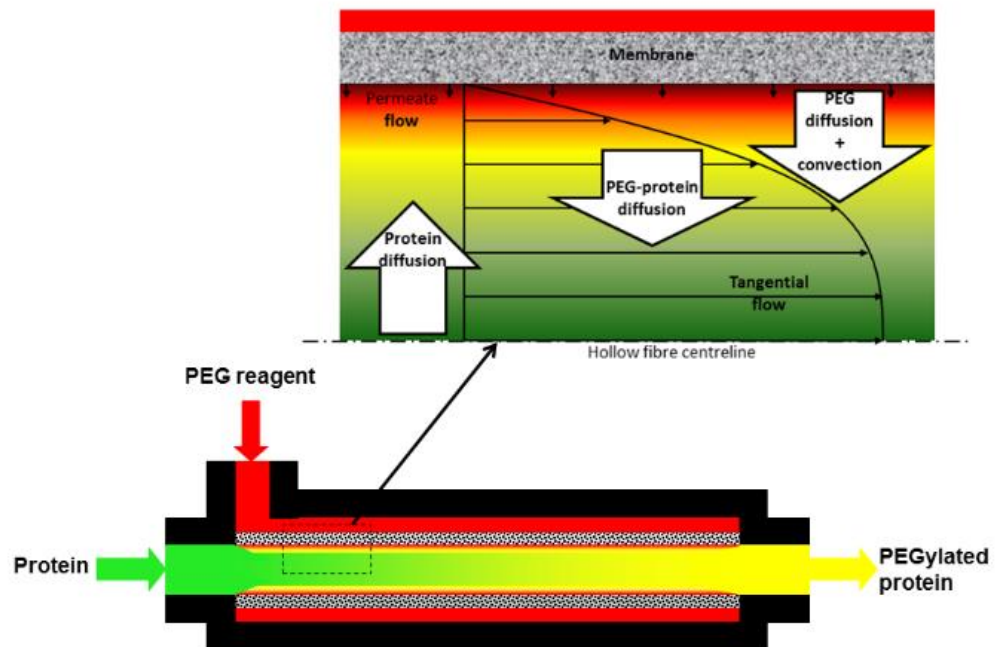
Figure 8. Axial and radial concentration gradients of reactants and product in the HMR system.

Chapter 7

Contributions and recommendations

7.1 Contributions

PEGylation has become a well-established technique which involves the improvement in the efficacy and physical properties of therapeutic proteins by covalent attachment of PEG. PEGylated proteins have longer *in vivo* half-life, enhancing patient compliance since painful injections have to be less frequently administered; and have decreased immunogenicity and increased stability due to shielding of the protein surface by the non-immunogenic and highly water-soluble PEG. PEGylated proteins are usually synthesized in liquid-phase batch reaction, which results in a complex mixture of different PEGylated forms. This makes the downstream purification of the target form challenging. Effective

ways of synthesizing mainly mono-PEGylated form and efficiently purifying the product are very crucial. This thesis has made four major contributions to the literature of synthesis and purification of PEGylated proteins and towards industrial applications. These contributions are enumerated and expanded in the following subsections.

7.1.1 Separation of PEGylated proteins

Hydrophobic interaction chromatography (HIC) was shown to have potential for separation of PEGylated proteins by some researchers, but it has not been widely used due to its poor resolution of separation. It is a packed bed column based chromatographic method, which has some limitations such as high pressure drop and slow diffusion as discussed in Chapter 1. Hydrophobic interaction membrane chromatography (HIMC) which uses environment-responsive membranes as chromatographic media overcame the limitations of HIC and improved the resolution of separation. This technique provides a way of resolving different PEGylated proteins into separate peaks and obtaining the mono-PEGylated protein in a pure form. The resolution as good as this has never been seen by using conventional HIC. This is because the environment-responsive property of the membrane media enhances the hydrophobicity difference between different PEGylated forms. Since the conventional HIC media is always hydrophobic, the hydrophobicity difference induced is not large enough for different PEGylated forms to be resolved into separate peaks. In addition, HIMC offers a sensitive and fast analytical separation technique which is able to detect a PEGylation reaction mixture containing only micro grams of total protein and resolve the different PEGylated proteins present in the mixture into separate

peaks in less than 5 min. The feasibility of using this technique for the fractionation of different PEGylated forms of small- and medium-sized protein were both demonstrated.

7.1.2 PEGylation chemistry

There have been many site-specific PEGylation chemistries available for producing mainly mono-PEGylated protein. N-terminal PEGylation using PEG NHS ester is one of the commonly used chemistries, targeting N-terminal α-amino group. The study of the effects of two operating parameters on reaction kinetics, conversion and specificity of mono-PEGylated protein discussed in Chapter 4 provided a guide on controlling the parameters to get desired outcomes. By using such information, a kinetic model for this chemistry could be built for outcome optimization and theoretical predictions at various conditions, which can be validated by experimental observations. Detailed reports of PEGylation reactions using this chemistry are rare, so this study enriched the literature of this area.

7.1.3 Integrated synthesis and purification of PEGylated proteins

Solid-phase PEGylation has the idea of immobilizing one reactant on a solid media surface and having the other come to react with it. Majority of the mechanisms used are based on ion exchange. By carefully controlling the pH value, the protein could be immobilized on the media surface. The pH value is also required to be suitable for PEGylation reaction. In some cases, the pH value may not be able to be chosen to satisfy both requirements. The solid-phase PEGylation method (discussed in Chapter 5) by HIMC

using environment-responsive membranes as the media overcomes the limitations. It has been concluded in Chapter 2 that PEGylated proteins are bound on the membrane through their PEG appendages. Therefore, PEG is immobilized on the membrane instead of protein in this method such that there would be less concern about the pH condition. This method has the potential of PEGylating any protein that comes into contact with the immobilized PEG. Due to steric hindrance, favorable access to protein and enhanced mass transport, this also provides a method to enhance the conversion and specificity of mono-PEGylation when compared to an equivalent liquid-phase reaction.

7.1.4 Continuous PEGylation reactor

Due to the pulse-wise nature of the reactor above, the conversion of mono-PEGylated protein is unable to meet the demand. Chapter 6 describes a Hollow-fiber Membrane Reactor (HMR) for continuous and selective protein PEGylation. This reactor offers an effective way of producing mainly mono-PEGylated protein with a very decent conversion when compared to an equivalent liquid-phase batch reaction. In this reactor, PEG is added in a distributive way to avoid constant contact of reactants and the laminar flow profile helps in suppressing the formation of high-PEGylated proteins by segregating the product from reactants. Reports of continuous PEGylation reactors have never been seen and it was reported for the first time. This work not only enriches the literature of PEGylation reactors, but also has huge potential in the applications of industrial production of PEGylated products.

7.2 Recommendations for future work

The research reported in this thesis opens up new pathways for future work. The HIMC for both solid-phase synthesis and purification has been demonstrated. It is also a fast and sensitive way for analysis of compositions of a PEGylation reaction mixture. It might be useful to precisely and quickly check the extent of PEGylation reactions for quality control purpose in industrial manufacturing of PEGylated proteins. The HMR system results in highly selective protein PEGylation with decent conversions when compared to liquid-phase PEGylation reactor. This clearly indicates that this system holds enormous promise in synthesis of PEGylated products, and it is strongly suggested that a fundamental study of this system in terms of pressure profile, concentration profile, and etc. should be performed. Scale-up information would be extremely useful from a manufacturing perspective. In addition, this system shows huge potential in the use for other types of chemical conjugation reactions where obtaining both high specificity and conversion is challenging or chemical reactions where by-product synthesis needs to be suppressed. Inspired by the manipulation of small amount liquid and the laminar flow regime in the HMR system, microfluidic technique which gives precise control of fluid seems to be promising in the application of PEGylating proteins. The application of this technique in synthesizing PEGylated proteins is definitely an interesting topic worth investing.

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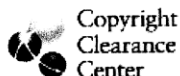
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